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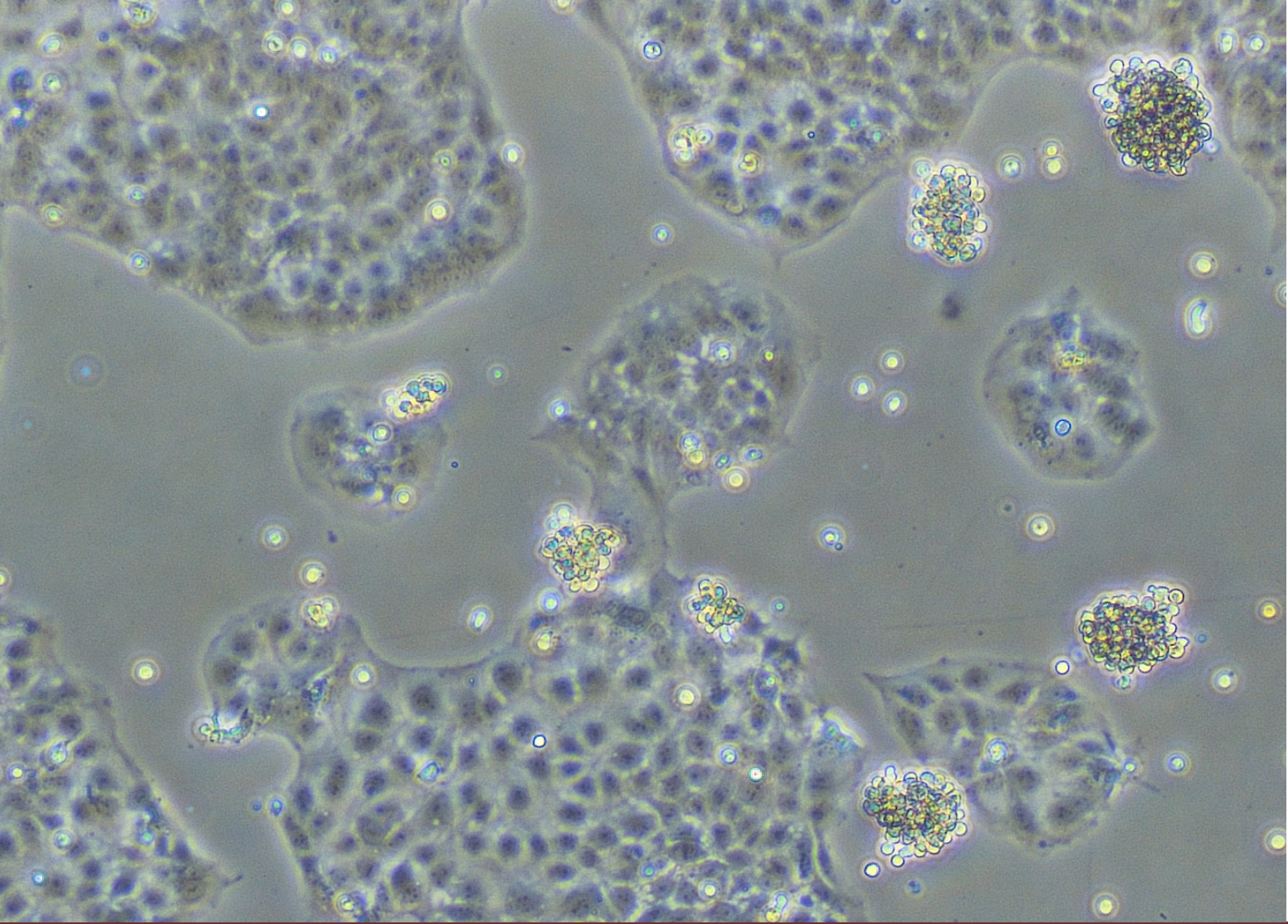
Role of HLA-G in shaping Natural Killer cell biology in cancer

Ester Arroba Hidalgo

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Role of HLA-G in shaping Natural Killer cell biology in cancer

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Barcelona, 2020

Role of HLA-G in shaping Natural Killer cell biology in cancer

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“Para mí, no hay emoción comparable a la que produce la actividad creadora, tanto en la ciencia como en el arte, literatura u otras ocupaciones del intelecto humano. Mi mensaje, dirigido sobre todo a la juventud, es que si sienten inclinación por la ciencia, la sigan, pues no dejará de proporcionarles satisfacciones inigualables. Ciertamente es, que abundan los momentos de desaliento y frustración, pero esos se olvidan pronto, mientras que las satisfacciones no se olvidan jamás”

Severo Ochoa

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ABREVIATIONS

List of abbreviations used

IS – Immune system

DC- Dendritic cell

NK – Natural killer

MHC- Major histocompatibility complex

APC – Antigen presenting cell

TCR – T cell receptor

HLA-G- Human leukocyte antigen G

Ped- Preimplantation embryo development

EVT- (Extravillous trophoblasts)

IFN γ - Interferon gamma

TNF – Tumor necrosis factor

IRSE – Interferon-stimulated response element

LCR – Locus control region

LINE1 – Long interspersed element

MPRA - (massively parallel reporter assay)

MTX - Methotrexate (MTX)

miRNAs - microRNAs

ITIMs - immunoreceptor tyrosine-based inhibitory receptor motifs

SHP-1 (Src-homology 2 domain-containing tyrosine phosphatase 1)

MSC - mesenchymal stromal cells

IL-10 - Interleukin-10

PBMCs – Peripheral blood mononuclear cells

GM-CSF – Granulocyte macrophage colony-stimulating factor

G-CSF - Granulocyte colony-stimulating factor

KIR – Killer-cell-immunoglobulin-like

LIR – Immunoglobulin- like receptor

ADCC – Antibody-dependent cell-mediated cytotoxicity

HSC- Hemopoietic stem cells

CLP – Common lymphoid progenitors

MCM4 – Minichromosome maintenance 4

CMP – Common myeloid progenitors

LIF - (leukemia inhibitory factor)

u NK – Uterine NK

d NK- Decidual NK

CARs – Chimeric antigen receptor

T regs - Regulatory T cells

pb – NK - Peripheral blood NK

KO – Knock out

GFP – Green fluorescent protein

ELISA - Enzyme linked immunosorbent assay

CM - conditioned media

TBS-T - Tris-buffered saline -Tween

RT - Room temperature

O/N - Overnight

TCGA- The cancer genome atlas

IHC – Immunohistochemistry

DAB – Diaminobenzidine tetrahydrochloride

KLH – Keyhole limpet hemocyanin

BSA – Bovine serum albumin

FC – Flow cytometry

WT - Wild-type

CC- Coculture

h-GZMB - human-granzyme B

h-PRF1 - human-perforin1

FC- Fold change

Rec-HLA-G – Recombinant HLA-G

mAb - Monoclonal Ab

INTRODUCTION

1. Cancer and immune system

The primary job of the immune system (IS) is to defend our body from the attack of pathogens and prevent infections. Moreover, the immune system plays an important role in protecting us from cancer.

In broad terms, we can divide the immune system into innate immune system and adaptive immune system. The innate immune system, is evolutionarily older and shared across all animals. The adaptive immune system (also known as acquired immune system) is a much more recent in evolution. It consists of defences that must be primed over time to become fully active. Both systems cooperate to protect us.

Regarding the innate immunity, it is our first line of defence and consists of mechanical, chemical and cellular defences. If we focus on cellular defences, we will find cells such as macrophages, neutrophils, dendritic cells and natural killer (NK) cells. Those cells have several jobs including, the phagocytosis of pathogens and cellular debris and the release of inflammatory molecules called cytokines that will alert the body of the presence of danger and recruit immune cells to the site of infection. Unlike the innate immune system, which is present from birth, the adaptive immune system takes time to develop. It is characterized by three unique features: specificity, diversity and memory. The cells that perform the adaptive immune defence are specialized white blood cells called lymphocytes. There are two main types of lymphocytes B cells and T cells.

The innate and adaptive immunity cooperate to defend the body against infections and cancer. When we have a wound in our body, appears the inflammation, which is a direct effect of the accumulation of immune cells and fluid at the site of injury. Inflammation plays an important role in cancer as well. Like wounds, developing tumors cause tissue damage that

triggers an inflammatory response. But, unfortunately, inflammation can also play a negative role in cancer, since inflammation that is prolonged very long in time (also known as chronic inflammation), promote the development of some cancers by creating an environment that damages DNA and favours metastasis. This relationship between chronic inflammation and tumor development has led scientists to describe tumors as “wounds that do not heal.”

Another role of the innate immune system is to activate the acquired immune system. In a context of cancer or pathogen infection, the cell from the innate immune system will present on its surface an endogenous or exogenous peptide fragment derived from the antigen. This antigen will be exposed in the cell surface bound into the groove of a protein called the major histocompatibility complex (MHC) in a process called antigen presentation. There are two types of MHC molecules which differ in the behaviour of the antigens: MHC class I molecules (MHC-I) bind peptides from the cell cytosol, while peptides generated in the endocytic vesicles after internalization are bound to MHC class II (MHC-II). MHC-I can be expressed by all the cell types in the body whereas MHC-II is expressed by antigen presenting cells (APCs). The MHC-antigen complex is then recognized by a corresponding T cell receptor (TCR) on a T cell, the T cell will recognize their target by the binding of the TCR to the MHC-antigen complex. Importantly, CD4+ Tcell will specifically recognize MHC-II and CD8+ Tcells recognize MHC-I. The best APCs in the body are dendritic cells, but macrophages and B cells can also do this work. In this way, APCs are the link between the two arms of the immune system, innate and adaptive (1).

1.2 Major histocompatibility complex class I (MHC I)

MHC class I molecules are transmembrane proteins that consist of a single α -chain associated with β_2 -microglobulin. They are composed by $\alpha 1$, $\alpha 2$, and $\alpha 3$ domains that create a globular protein in which a β -pleated sheet forms the floor of the peptide-binding groove and is bounded by two helical regions that form the sides of the cleft. An important characteristic is that the size of the peptide that can be accommodated in the groove is restricted, typically 9 amino acids length, since the groove is closed at both ends

Most nucleated cells express MHC class I molecules, although the amount on the cell surface varies a lot among cell types and conditions. MHC class I glycoproteins present antigens of endogenous origin to cognate TCRs of CD8⁺ T cells. Endogenous peptides derive from degradation of intracellular proteins, including therefore viral or tumor antigens in infected or transformed cells, through the proteasome. Because MHC class I molecules present peptides derived from cytosolic proteins, this pathway is often called cytosolic or endogenous pathway and it is critical to discriminate between self and non-self.

MHC class I molecules are highly polymorphic. Each allomorph of MHC class I binds a distinct subset of peptides because of structural differences between the peptide-binding grooves. The peptide sequences that a particular MHC class I molecule can bind are determined by the shape, depth, and charge of the groove and the binding pockets. Because most individuals express six different MHC class I alleles (two each of HLA-A, HLA-B, and HLA-C), a diversity of pathogen-derived peptide sequences can be presented to T lymphocytes. Besides classical MHC class I (or MHC class Ia), there are non-classical MHC class I (or MHC class Ib) which are

phylogenetically heterogeneous. The characteristics that define MHC class Ib molecules are their limited polymorphism and restricted expression pattern. In humans, these encompass three genes only: *HLA-E*, *HLA-F*, and *HLA-G* (2).

2. Fundamental aspects of HLA-G biology

2.1 HLA-G characteristics

HLA-G histocompatibility antigen, class I, G, also known as human leukocyte antigen G (HLA-G), is a protein that in humans is encoded by the *HLA-G* gene. It is a non-classical MHC class I molecule, and its main characteristics are the very low amount of polymorphism and the highly tissue-restricted expression.

The *HLA-G* gene was first described in the late 80's as a structural homologue of a murine Qa gene (3). It maps on the short arm of chromosome 6 at region 6p21.3 within the class I gene cluster of major histocompatibility complex (MHC). The non-classical MHC class I genes, also comprises *HLA-E* and *HLA-F*. In the same region are found classical HLA-class I genes, *HLA-A*, *HLA-B*, *HLA-C*. *HLA-G* sequence exhibits structure and sequence similarity (86%) with the consensus sequence of classical *HLA-class I* genes. The main difference resides in the presence of a stop codon in exon 6 which generates a shorter HLA-G protein compared with classical HLA-class I molecules (4). The coding sequence has a length of 338 amino acids. HLA-G-like locus, assigned MHC-G, also exists in new (Platyrrhini) and old (Catarrhini) world nonhuman primates that separated about 38 million years ago (5).

The HLA-G protein has classic HLA class I-like structure. It consists of a heavy chain and a light chain (β_2 -microglobulin). The heavy chain is

approximately 45 kDa and its gene contains 8 exons. Exon 1 encodes for the leader peptide, exons 2 and 3 encode for the $\alpha 1$ and $\alpha 2$ domains, exon 4 encodes the $\alpha 3$ domain, exon 5 encodes the transmembrane region, and exon 6 encodes the cytoplasmic tail. Exon 7 and 8 are not translated due to the stop codon present in exon 6 (Figure 1) (6).

HLA-G orthologues are not found outside the primate order, although the murine class Ib MHC molecule Qa-2, product of the preimplantation embryo development (Ped) gene, was described as a functional homolog of HLA-G, the homology is not complete since they just share 78% of their amino acid sequence (7). This lack of a clear murine homolog makes translational research studies more complicated due to the inability of using mice models.

HLA-G can generate by alternative splicing only seven alternative mRNAs encoding four membrane bound (HLA-G1 to HLA-G4) and three secreted isoforms (HLA-G5 to HLA-G7) (Figure 2). The HLA-G1 molecule and its soluble counterpart HLA-G5 (sometimes also named as “complete” HLA-G isoforms) are those that have been studied the most and are also the most abundant (6).

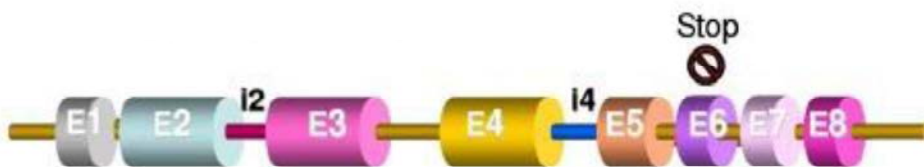


Figure 1. HLA-G primary transcript (6).

It is now well known that HLA-G is structurally much more complex than that, as it can be presented as monomers, homo- and possible hetero-multimers, ubiquitinated proteins, free in biological fluids, or as part of exosomes. Dimerization of HLA-G occurs through the creation of disulphide bonds between two unique cysteine residues at positions 42 and 147 of HLA-G heavy chains. Those interactions can also generate HLA-G homotrimers (Figure3). HLA-G1 homodimers are joined head-to-tail by Cys42-Cys42 disulphide bonds, this makes an oblique orientation that exposes the receptor binding sites of the $\alpha3$ domain and makes them more accessible. The homodimers have been shown to be expressed at the surface of trophoblasts *in vitro*.

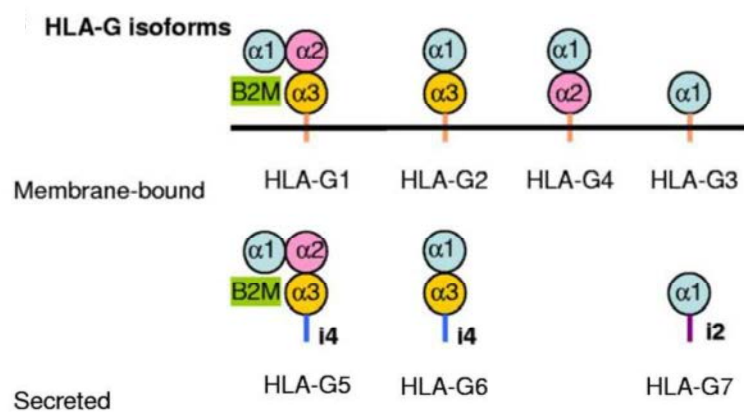


Figure 2. HLA-G isoforms generated due to alternative splicing (6).

The first crystal structure of HLA-G was that of the HLA-G1 isoform in the monomeric form (7). This HLA-G1 monomer globally resembles classical HLA-class I molecules, but differs at the level of its peptide-binding groove formed by the $\alpha1$ and $\alpha2$ domains of the heavy chain (a key structural element to the activating functions of HLA molecules) and its $\alpha3$ domain

which bind correceptors (a key structural element to the inhibitory functions of HLA-class I molecules)(7). Indeed, the peptide-binding groove of HLA-G resembles more that of HLA-E than that of classical HLA-class I molecules.

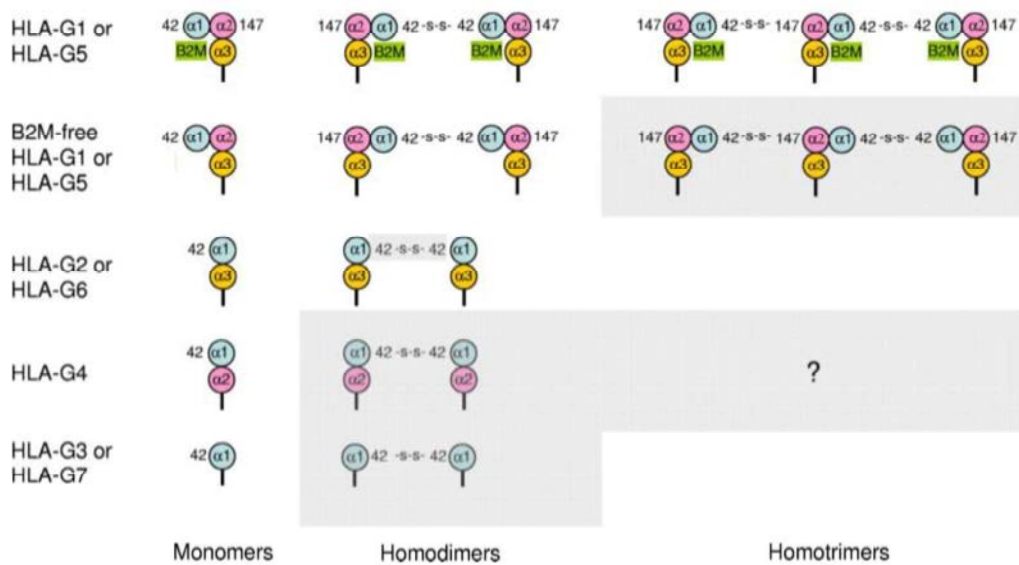


Figure 3. Homomultimeric structures of HLA-G (6).

Monomers of other HLA-G isoforms have not been crystallized, and thus, their structure is speculative. It is clear that β 2m-associated HLA-G1 and HLA-G5 present peptides, whether the other isoforms do or not is a question that remains opened. HLA-G4 is the only truncated isoform that may present peptides, since it possesses both α 1 and α 2 domains and therefore is able to form peptide-binding groove. Whether this structure can still present peptides in the absence of α 3 domain and β 2m is unknown.

The physiological expression of HLA-G is restricted to fetal tissues, mainly to the extra villous trophoblasts (EVT), where it plays an important role in immune tolerance during pregnancy. It can also be expressed in immune-

privileged organs, including the cornea, thymus, pancreatic islets, endothelial cell precursors, and erythroblasts. But apart from this physiological expression, it is well described that HLA-G expression is augmented in tumors (its relevance will be described in Section 5). Moreover, HLA-G expression has also been associated with other pathological conditions such as viral infections (8), recurrent miscarriage (9), pregnancy outcome and complications (10), autoimmune diseases (11), transplantation (12-13), and inflammatory diseases (14).

2.2. Regulation of HLA-G expression

The expression of HLA-G at the different sites depends on the combination of transcription factors, miRNAs, and environmental factors. Moreover, its expression might be induced by certain molecules very important in the pregnancy context such as progesterone.

The regulation of the HLA-G as many other features, is distinct from other HLA class I molecules. Despite its similarity with other MHC class I gene promoters, the classical promoter of *HLA-G* is defective, it has mostly non-functional regulatory elements. In particular, the *HLA-G* promoter Enhancer A does not bind the p65 subunit of NF- κ B, thus being unresponsive to tumor necrosis factor (TNF- α), the interferon-stimulated response element (IRSE) is partially deleted and consequently unresponsive to Interferon gamma (IFN γ) and the SXY module contains defective X2 and Y elements. (Figure 4)

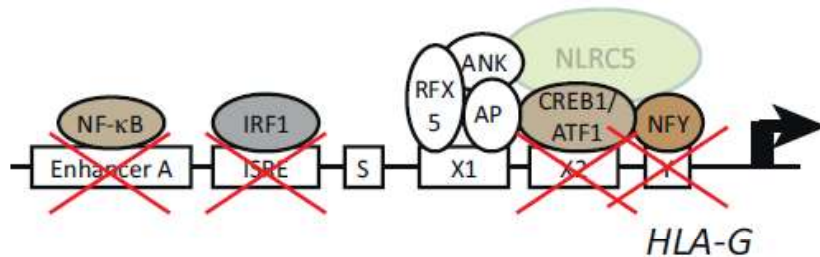


Figure 4. Schematic representation of the promoter region of HLA-G showing its defective regions (16).

A transgenic mice model demonstrated that the expression of HLA-G in EVT_s, was basically dependent on a 250 bp DNA fragment located 1.1 kb upstream of the *HLA-G* promoter. This region, which is absent from classical MHC class I, may act as a putative locus control region (LCR) (15) and is critical to control tissue-specific HLA-G expression. *In vitro* studies found that the LCR sequence bind transcription factor complexes specific from trophoblasts, however their identity reminds to be determined. Functional CREB/ATF binding sites have been identified within the LCR (16-17).

Moreover, it has also been described a potential negative regulator that overlaps the long interspersed element (LINE1) located 4 kb upstream of the *HLA-G* promoter. The presence of this silencer element may explain the limited expression of HLA-G compared with other class I genes. Massively parallel reporter assay (MPRA) led to the discovery of a trophoblast-specific enhancer of HLA-G expression located 12kb upstream of HLA-G: Enhancer L. CRISPR/Cas9 mediated deletion of this enhancer resulted in ablation of HLA-G expression in JEG3 cells and in primary human trophoblasts isolated from placenta, suggesting that it is absolutely required for HLA-G expression. In addition, transcriptional regulators that mediate its actions have been described, those are CEBP β , GATA2 and

GATA3 that can regulate trophoblast-specific HLA-G expression. Importantly, these three factors have been implicated in murine placental development and trophoblast-specific gene regulation, making them strong candidates for being transcriptional regulators of HLA-G expression in human trophoblasts (18). Mechanistically, Enhancer L loops into the core promoter of *HLA-G* upon association with CEBP β and GATA 2/3, activating HLA-G expression, however, how this looping is prevented in EVT expressing HLA-G remains still unknown (Figure 5).

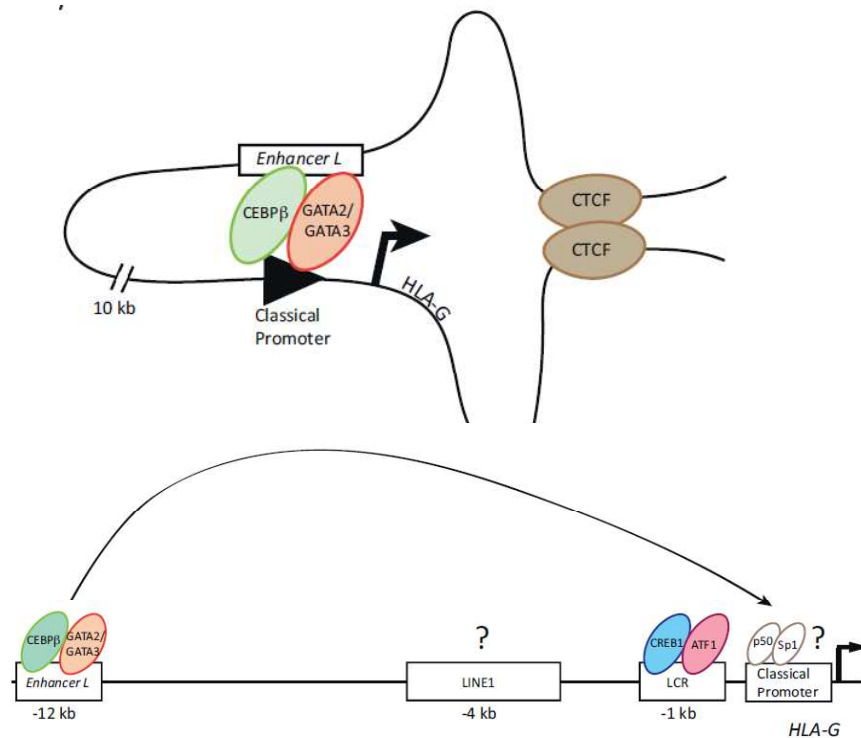


Figure 5. (a) Long-range chromatin interactions between Enhancer L and the *HLA-G* classical promoter. (b) Schematic representation of the main regulatory elements upstream of *HLA-G*. All distances in thousands of base pairs (kb). The curved arrow represents chromatin looping, the bent arrow shows the transcription start site (16).

Interestingly, the presence of Enhancer L is also unique to two of the great apes (chimpanzees and gorillas) and may have been introduced by retrotransposon insertion in co-occurrence with the acquisition of placental HLA-G expression. The only great ape lacking the Enhancer L is the orangutan, however its *HLA-G* classical promoter has functional X2 and Y motifs.

Some factors secreted locally in placenta and tumors can modulate HLA-G transcription and protein expression, but the molecular mechanisms underlying this regulation need still to be elucidated in most of the cases. In this way, growth factors and anti-inflammatory or proinflammatory cytokines are capable to upregulate HLA-G gene expression (19). Moreover, hormones such as glucocorticoids, β -estradiol, progesterone and prolactin which are very important in pregnancy regulation are also involved in HLA-G regulation (20). Also, the influence of leptin has also been investigated since it is produced by the placenta and suspected to exert important functions during pregnancy (21). In addition to this, the epigenetic control of genes through DNA methylation and histone modifications is also a critical mechanism in transcriptional regulation since it determines the accessibility and recruitment of regulatory factors to the DNA. In addition, hypoxia is a microenvironmental important factor for placentation, and it has also been described to increase *HLA-G* regulation (22-23). Some drugs such as methotrexate (MTX) used for rheumatoid arthritis may also induce HLA-G production (24).

Regarding the posttranscriptional regulation of HLA-G, we have to highlight the importance of microRNAs (miRNAs) as potential key regulators. Due to a premature stop codon, *HLA-G* gene presents a relatively large 3'UTR sequence which constitutes a key feature for its

regulation. This long 3'UTR region is important for mRNA stability, polyadenylation signals and importantly the targeting of specific miRNA. Mainly one family of miRNA which includes microRNA-148a (miR-148a), microRNA-148b (miR-148b), and microRNA-152 (miR-152) has been proven without any doubt, to target the 3' UTR region of HLA-G and to downregulate its expression with functional consequences. Another miRNA, miR-133a, was reported to regulate HLA-G by targeting its 3'UTR region. This miRNA reduces HLA-G expression in recurrent spontaneous abortion (25). In contrast, in primary colorectal cancer specimens, in which the levels of HLA-G are high, miR-133a was found significantly downregulated (26).

2.3 HLA-G receptors

HLA-G induces tolerance by inhibiting different immune-competent cells. Its inhibitory effect is mediated by binding of both soluble and membrane-bound HLA-G to inhibitory receptors (27-28). Four receptors for HLA-G have been described: ILT2, ILT4, KIR2DL4 and CD8 (Figure 5).

ILT2 (also known CD85j and LILRB1) is expressed by B cells, monocytes, dendritic cells, some T cells and some NK cells. ILT4 (also known as CD85d and LILRB2) is only expressed by monocytes and dendritic cells. KIR2DL4 (also known as CD158d) is expressed by NK cells and some T cells (6). CD8 is expressed by cytotoxic T cells as well as some NK cells.

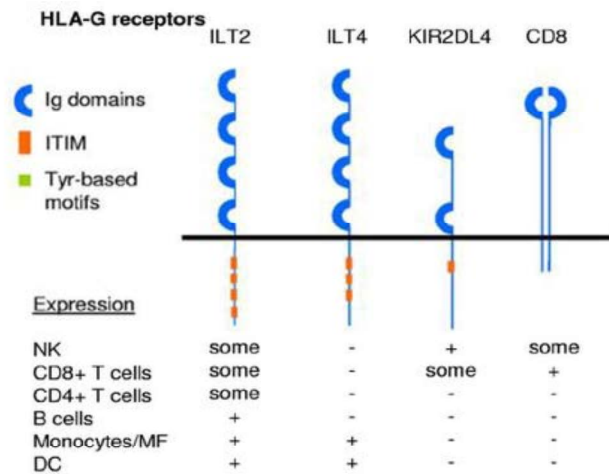


Figure 5. HLA-G receptors (6)

ILT2 and ILT4 are inhibitory receptors and contain immunoreceptor tyrosine-based inhibitory receptor motifs (ITIMs) in their cytoplasmic tails that inhibit cellular responses by recruiting phosphatases such as SHP-1 (Src-homology 2 domain-containing tyrosine phosphatase 1). ILT2 and ILT4 have four tandem Ig-like domains in the extracellular region, and the two N-terminal Ig-like domains (D1–D2) are responsible for MHC I recognition. ILT2 and ILT4 can bind a broad range of MHC I, however, a more hydrophobic $\alpha 3$ domain, makes HLA-G the ligand of highest affinity for ILT-2 and ILT-4. Both receptors have higher overall affinity to dimers than to monomers, due to significant avidity effects, however, they do not recognize the same configurations, ILT2 binds preferentially HLA-G associated with β_2 -microglobulin, whereas ILT4 also recognizes HLA-G free heavy chains (29).

Despite ILT-2 and ILT-4, KIR2DL4 has HLA-G as sole ligand. KIR2DL4 first described as inhibitory receptor is however capable of sending both inhibitory and activating signals. It is expressed by NK cells present in the decidua but undetectable at the cell surface of primary resting NK cells from the peripheral blood. Therefore, data concerning KIR2DL4 interaction with HLA-G may be relevant only for decidual NK cells in the context of pregnancy, and not for peripheral NK cells in any other context. However, KIR2DL4 remains a receptor whose interaction with HLA-G and its consequences need still to be further investigated (30).

As other HLA-I, HLA-G can also bind the T-cell co-receptor, CD8. In particular, CD8 binding to MHC I is important for proper CD8+ T-cell activation. Through binding to CD8, soluble HLA-G molecules are involved in a signal-transduction pathway leading to a Fas–FasL-mediated CD8+ cell apoptosis (31). The binding of CD8 to HLA-class I molecules happens mainly through their HLA α 3 domain, which ILT2 and ILT4 bind as well.

Moreover, it is important to mention that, peptides derived from HLA-G promoter region are presented by HLA-E and bind to the CD94/NKG2 receptor that is predominantly expressed on the surface of NK cells. The affinity of both CD94/NKG2A (inhibitory receptor) and CD94/NKG2C (activating receptor) for HLA-E is the highest when it bind the leader peptide derived from HLA-G compared with any classic HLA-I molecule bound. This would represent an indirect way for HLA-G to exert its inhibitory functions.

2.4 HLA-G functions

HLA-G, membrane-bound or soluble strongly binds its receptors on immune cells and blocks their effect. The main function of HLA-G is immune inhibition, it might be induced in physiological conditions such as pregnancy or in pathological conditions such as cancer and also in organ transplantation. This blocking may therefore be beneficial because when expressed by a fetus or in a transplant it protects them from rejection or induces better graft acceptance, but it might also be deleterious because when expressed by a tumor, it protects it from antitumor immunity and results in tumor progression. The way in which HLA-G can exert its immune-related functions might be direct or indirect as it will be explained in detail in Section 3.

Most of the functions described for HLA-G are immune functions, although some non-immune functions have also been described as it will be detailed below. First, HLA-G is expressed in unfertilized oocytes and in blastocysts in the trophoblast cells that give rise to trophoblast. Thus, it is possible that HLA-G is involved in the very early stages of pregnancy, independently of its immune functions. HLA-G expression or HLA-G soluble levels is been correlated with higher embryo cleavage rate and implantation rate. However, if this function is immunology-independent is not proven (32-33). Secondly, HLA-G is expressed by hematopoietic precursors belonging to the erythroid lineage, in all embryonic and adult hematopoietic organs, from primary to final erythropoiesis. One study described HLA-G as a novel negative regulatory mechanism controlling erythropoiesis (34). Moreover, a role in osteogenesis have been described, a study published in 2013, reported that the expression of HLA-G5 by bone

marrow derived mesenchymal stromal cells (MSCs) is amplified during their differentiation into osteoblasts having a role in bone morphogenesis. This study demonstrated that HLA-G promotes osteogenesis by inhibiting the formation of osteoclastic cells (35). Finally, a role for HLA-G in vascularization has been also reported. One study focused on this issue showed that HLA-G5 inhibited vascularization using a rabbit corneal neovascularization model (36). The antiangiogenic effect of HLA-G5 did not occur through its binding to known HLA-G inhibitory receptors (described in Section 2.3), but through CD160, a GPI-anchored receptor expressed by endothelial cells. The authors hypothesized that, during early pregnancy, HLA-G5 secreted by endovascular trophoblasts might replace vascular cells of the maternal spiral arteries by inducing their apoptosis and transform them into high conductance vessels, however further experiments are needed to confirm this observation for human beings.

In summary, apart from its main immune-modulatory role, there are some other functions described for HLA-G as summarized above. However, the evidences for this non-immune related functions are less well characterized since the results are based in very few publications. Further studies are needed to expand the knowledge in those fields which still remains controversial.

2.5 Anti HLA-G antibodies

Several anti-HLA-G monoclonal antibodies have been developed, however, their specificity remains controversial, since it has been observed that some of those antibodies are likely to cross react with other closely related HLA-I molecule family (28) (Table 1).

Monoclonal antibody clone	Isoforms of HLA-G detected	Conformation of HLA-G detected	Cross-reactivity
87G	Transmembrane and soluble	Conformationally dependant	Cross reaction with HLA-I
MEM-G/9	Transmembrane	Conformationally dependant	No binding with HLA-A, B, C or E
MEM-G/1	Transmembrane and soluble	Denaturated/unfolded	No cross-reaction with recombinant HLA-A, B, C or E
4H84	Transmembrane and soluble	Denaturated/unfolded	Also binds HLA-A and HLA-E, as well as some HLA-B and HLA-C alleles
HCA2	Transmembrane and soluble	Denaturated/Unfolded	Cross reaction with HLA-I

Table 1. Monoclonal antibodies binding HLA-G. Adapted from (28).

3. HLA-G and immune system

HLA-G has tolerogenic immune functions and acts on cells of both innate and adaptive immunity. The immune inhibitory function of HLA-G can be exerted directly or indirectly (27-28). The direct immune-inhibitory functions are exerted by direct blocking of effector cells through the binding of soluble and/or membrane-bound HLA-G to its receptors (previously described in Section 2.3). The indirect immune-inhibitory functions are performed through the generation of regulatory cells, CD4+ and CD8+ T cells stimulated in the presence of HLA-G lost their capability to respond to antigenic stimulation, and differentiate into regulatory T cells capable of inhibiting the reactivity of other T cells. Additionally, activated NK and T cells may also behave as regulatory suppressor cells and inhibit immune responses through HLA-G after the acquisition from cells in their vicinity in a mechanism called trogocytosis. Another indirect immune-inhibitory function is performed through HLA-E which binds peptides from HLA-G and interact with the inhibitory receptor CD94/NKG2A resulting in inhibition of NK cells and T-cell reactivity. Moreover, HLA-G has other immune functions such as the capability to induce apoptosis of NK cells and cytotoxic T cells, to influence the expression and release of IFN γ by NK cells and the capability to induce the upregulation of inhibitory receptors including its own (37) HLA-G function on other immune cell types such as dendritic cells, B cells and neutrophils have also been described and summarized in Figure 6.

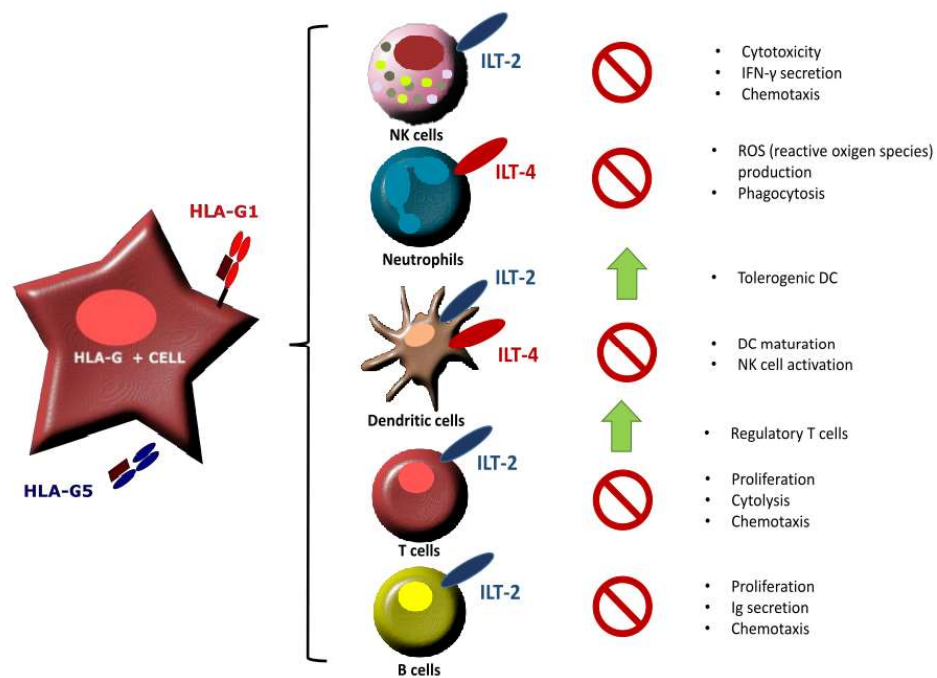


Figure 6. Immune functions of HLA-G. Adapted from (37).

3.1 HLA-G direct inhibitory functions

The direct inhibitory function of HLA-G on NK cells from the decidua was the first HLA-G function to be shown and it was soon confirmed for peripheral NK cells as well. Through the interaction with ILT2 on NK cells, the HLA-G presented by a target cell (cytotrophoblast cells, tumor cells, transfected cell lines) or secreted to the microenvironment as soluble HLA-G, inhibited NK cytolysis, thus protecting the HLA-G-expressing cell. HLA-G would confer in that way immunological tolerance to the tissues expressing or secreting it (38).

The HLA-G–ILT2 interaction was also shown to alter the NK cell IFN γ secretion, downregulate chemokine receptors and modulate chemotaxis of peripheral blood and tonsil CD56^{bright} and CD56^{dim} NK cells (39). NK cells also express KIR2DL4, another HLA-G receptor, but the HLA-G functions through it are less clear as it was described on Section 2.3.

Regarding T cells, most of their functions were shown to be inhibited by HLA-G which inhibited the alloproliferative response of CD4+ T cells and cytolytic functions of CD8+ T cells. HLA-G also induced the differentiation of CD4+ and CD8+ T cells into various subsets of regulatory cells.

If we focus on B cells, it has been published that through interaction with ILT2 receptor expressed on its surface, HLA-G inhibits the proliferation, differentiation, and antibody secretion of activated B cells originating from peripheral blood and secondary lymphoid organs, such as tonsils. The inhibitory role played by HLA-G on the allogeneic B-cell response, enabling long-term acceptance of the graft, appears to be a decisive factor in the development of new therapeutic strategies to prevent graft rejection (40). In the case of myeloid APCs, in the presence of HLA-G, they failed to stimulate T cell alloproliferative responses *in vitro*. And *in vivo*, the HLA-G–ILT4 interaction impaired DC maturation, leading to delayed skin allograft (41).

Neutrophils constitute more than half of circulating leukocytes in humans and play a major role in host defence against invading pathogens. Functional assays indicated that HLA-G inhibited the phagocytic function of neutrophils through the binding to ILT-4 expressed on the surface of neutrophils. These results reveal a unique mechanism of regulation of neutrophil functions through HLA-G and ILT4 which may have implications in inflammatory disorders (42).

3.2 HLA-G indirect inhibitory functions

The indirect immune-inhibitory functions are performed through the generation of regulatory cells. Regulatory T cells are believed to be crucial for the maintenance of normal immune homeostasis and to be key players in transplantation, cancer, autoimmune diseases, allergies, infection and immune deficiency disorders. In 2007, a subset of regulatory T cells expressing HLAG was described (43). HLA-G-positive regulatory T cells are not induced by antigenic challenge, but emerge as such from the thymus. HLA-G+ T regulatory cells are present in human peripheral blood under normal physiologic conditions. They are a discrete population of T cells that average 1.6% of CD4+ and 3.3% of CD8+ T cells. Those cells express on their surface HLA-G1 and secrete HLA-G5 and are negative for CD25 and FoxP3 (43). Induced regulatory T cells arise from the pool of peripheral T cells following antigenic stimulation, and such regulatory T cells can also be induced *in vitro*. It was demonstrated that whereas HLA-G-negative APCs induced the activation and proliferation of allogeneic T cells *in vitro*, APCs expressing cell-surface HLA-G1 induced the differentiation of regulatory T cells that were hyporesponsive to stimulation and capable of inhibiting allogeneic responses (43-44).

Apart from regulatory T cells, HLA-G can also induce regulatory myeloid cells. The first evidence of that came from the demonstration that HLA-G altered the expression of cytokines, chemokines, and chemokine receptors by myeloid APCs (44). Interleukin-10 (IL-10) upregulation is likely to be one of the most significant because IL-10 and HLA-G are known to be associated (45-46).

Another important indirect mechanism of action of HLA-G is the trogocytosis (27; 47). Trogocytosis is a rapid intercellular transfer of membrane fragments and their associated molecules, during cell to cell contact. It has been described for T cells, B cells, lymphocytes, NK cells and APCs. Trogocytosis is not a transfer of individual molecules, but the transfer of the entire membrane carrying intra- and transmembrane proteins. The molecules acquired by trogocytosis remain at the surface of their new cell host for a limited time only, but are still functional. Therefore, a cell will be able temporarily to use proteins that it does not express by itself. In particular, it have been described that membrane fragments containing HLA-G can be transferred by trogocytosis from antigen-presenting cells to T lymphocytes, from tumor cells to activated NK cells or to monocytes (48-49). Because transferred HLA-G remains functional, the cells that had acquired HLA-G gained the capability to inhibit other immune effectors through the acquired HLA-G molecules.

Moreover, apart from the acquisition of HLA-G, T lymphocytes could acquire via trogocytosis the ILT2 receptor from autologous monocytes. Through this process, T lymphocytes initially not expressing ILT2 and therefore not sensitive to HLA-G became a target cell for this molecule. These findings may explain how HLA-G can act so strongly on for example, CD4+ T cells known to only express marginally ILT2 (less than 5% of circulating CD4+ T cells express it) and gives an explanation of how cells expressing very few levels of HLA-G receptors can respond to it (48-49).

These results strongly support the fact that the expression of the HLA-G molecule is an effective and flexible immune regulatory mechanism. By the process of trogocytosis previously described, even a small proportion of cells expressing HLA-G can locally exert a considerable immune-inhibitory effect.

3.3 Interactions with HLA-E molecule and its functional implications

The last way in which HLA-G could indirectly influence the immune system is through the HLA-E molecule. The peptide derived from the leader sequence of HLA-G might be loaded into the HLA-E peptide binding groove and exert indirectly its inhibitory functions.

HLA-E is also a non-classical MHC class I molecule. Like other MHC class I molecules, the structure of HLA-E is a heterodimer consisting in a heavy chain and a light chain (β -2 microglobulin). The gene structure is very similar to the one codifying for HLA-G. It harbours 8 exons: exon 1 encodes the signal peptide, exons 2 and 3 encode the α 1 and α 2 domains, which both bind the peptide, exon 4 encodes the α 3 domain, exon 5 encodes the transmembrane domain, and exons 6 and 7 encode the cytoplasmic tail. The peptide binding groove formed within the α 1 and α 2 domains resembles of the HLA-G in structure and characteristics (See section 2.1 for more details). The mRNA of HLA-E can be virtually detected in all nucleated cells, however, its expression on the cell surface requires the presence of peptides derived from other HLA-class I molecules and the binding to the β 2-microglobulin. This make HLA-E expression extremely restricted and it has been related to cell activation (50).

The function of HLA-E is to present the bounded peptides to the NK cells through the interaction with the inhibitory receptor CD94/NKG2A for which HLA-E is its sole ligand (51) or with the activating receptor

CD94/NKG2C. Apart from the interaction with NK cells, it has been reported that HLA-E can also be recognised by the T-cell receptor expressed by CD8+ T cells and therefore play a role in the adaptive immune response (52).

It has been demonstrated that HLA-E affinity to the inhibitory NKG2A/CD94 receptor is six- fold higher than its affinity to the activating NKG2C/CD94 receptor. Moreover, it has been reported that HLA-E loaded with the peptide derived from HLA-G (VMAPRTLFL) binds NKG2X/CD94 with the highest affinity of any HLA-E/peptide combination tested so far (53). Moreover, HLA-G and HLA-E can cooperate, the expression of different isoforms of HLA-G can modulate cell surface HLA-E expression and they could also be co-expressed on different cell populations and interact to modulate the immune response (54-55).

The interaction of HLA-E with their receptors will result in the generation of an immunosuppressive microenvironment that will facilitate the escape of transformed cells from the recognition by the immune system. This have been observed in a context of tumor or viral infections. Notably, patients with tumors co-expressing HLA-G and -E display the worst clinical outcome, suggesting that the two molecules may co-operate shutting down NK cell mediated anti-tumor immune response. However, the cooperation between HLA-G and HLA-E remains controversial since several groups have demonstrated that HLA-G and -E may have different or even opposite roles in tumor progression (56). More studies need to be performed in order to have a better knowledge of these interactions and explain the mechanisms underlying this immune suppression.

4. NK cells

4.1 NK cell biology

Natural killer cells, are effector lymphocytes of the innate immune system with cytolytic functions. NK cells are known to differentiate and mature in the bone marrow, lymph nodes, spleen, tonsils, and thymus, where they then enter into the circulation. Regarding cell surface markers, they are defined as CD3- CD56+ and constitute the 10-15 % of the peripheral blood mononuclear cells (PBMCs). The role of NK cells is to give a rapid response to virus-infected cells or tumor cells. Typically, immune cells detect the MHC complex (See Section 1.1) presented on infected cell surfaces, this recognition will trigger cytokine release and will result in cell death through lysis or apoptosis. However, NK cells have the ability to recognize stressed cells in the absence of antibodies and MHC, allowing for a much faster immune reaction, hence their name. The role of NK cells is crucial for our immunity because harmful cells that are missing MHC I markers cannot be detected and destroyed by other immune cells, such as T lymphocyte cells (57).

Moreover, NK cells do not react in an invariant manner, they have the ability to adapt to their environment. NK cells can also develop a sort of antigen-specific immunologic memory. Therefore, NK cells exert sophisticated biological functions that are attributes of both innate and adaptive immunity, blurring the functional borders between these two arms of the immune response (57).

NK cells also produce an array of other cytokines, both proinflammatory and immunosuppressive, such as tumor necrosis factor- α (TNF- α) and IL-10, respectively, and growth factors such as GM-CSF (granulocyte macrophage colony-stimulating factor), G-CSF (granulocyte

colony-stimulating factor), and IL-3. NK cells also secrete many chemokines, including CCL2 (MCP-1), CCL3 (MIP1- α), CCL4 (MIP1- β), CCL5 (RANTES), XCL1 (lymphotactin), and CXCL8 (IL-8) (58). Whereas the biological function of the growth factors secreted by NK cells still needs to be clarified, their secretion of chemokines is key to their colocalization with other hematopoietic cells such as dendritic cells (DC) in areas of inflammation. Furthermore, the production of IFN γ by NK cells helps to shape T cell responses in lymph nodes, possibly by a direct interaction between naïve T cells and NK cells migrating to secondary lymphoid compartments from inflamed peripheral tissues and by an indirect effect on DC (57-58)

4.2 NK cell receptors and functions

NK cells are equipped with an array of receptors that can either stimulate NK cell reactivity (activating receptors) or inhibit NK cell reactivity (inhibitory receptors). The NK cell receptors can be divided in two types: lectine-like and immunoglobulin-like. The repertoire of activating and inhibitory receptors of NK cells is very well calibrated to ensure self-tolerance while allowing efficacy against viral infection and tumor development. In relation with HLA-G, there are the following inhibitory receptors (see Section 2.3): KIR2DL4 (which may also have activating functions), belonging to the KIR (killer-cell-immunoglobulin-like) receptors, ILT-2 belonging to the ILT or LIR (immunoglobulin-like receptor) family and CD94/NKG2A heterodimers, a C-type lectin family receptor that might bind HLA-E loaded with a peptide derived from the leader sequence of HLA-G (See section 3.2). Natural killer cell activation is determined by the balance of inhibitory and activating receptor stimulation.

NK cells can execute its cytolytic function in different ways. They can release small granules present in their cytoplasm that contain proteins such as perforin and proteases known as granzymes. These granules are released in close proximity to the target cell. Perforin will form pores in the cell membrane and kill the target cell inducing apoptosis or osmotic cell lysis. Granzymes will kill the target cell by the induction of apoptosis after caspase activation. Therefore, the release of either perforin, granzyme or both is often used as a read out of NK cell activity.

Another way is the antibody-dependent cell-mediated cytotoxicity (ADCC). This mechanism is based in the fact that infected cells are routinely opsonized with antibodies for detection by immune cells. Antibodies that bind to antigens can be recognised by CD16, a marker present on NK cells surface, this recognition will result in NK activation and the consequent release of cytolytic granules that will produce apoptosis as explained above. ADCC is the base of action of many monoclonal antibodies like cetuximab or bevalizumab used in the treatment of cancer (59). Moreover, cytokines play a crucial role in NK cell activation. They are released by cells upon viral infection, they serve as stress signal to alert the NK cell of the presence of viral pathogens in the affected area. Cytokines involved in NK activation include IL-12, IL-15, IL-18, IL-2, and CCL5. NK cells are activated in response to interferons or macrophage-derived cytokines. NK cells work to control viral infections by secreting IFN γ and TNF α . IFN γ activates macrophages for phagocytosis and lysis, and TNF α acts to promote direct NK tumor cell killing. Hence, as well as described for the perforin and granzyme, the study of TNF α and IFN γ release by NK cells is often used to evaluate their level of activation.

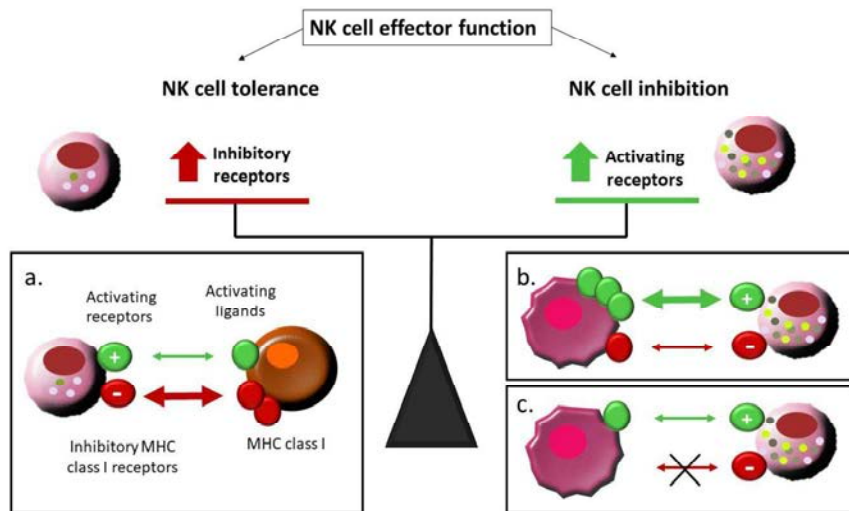


Figure 7. NK cell effector functions. NK cells distinguish between healthy cells (in orange) from cells “in distress” (in pink). (a.) Healthy cells express self-MHC class I molecules (red circles) and low amounts of stress-induced self-molecules (green circles) and are not attacked by NK cells (b.) Cells “in distress” up-regulate stress-induced self-molecules or (c.) down-regulate MHC class I molecules and are killed by NK cells. (Adapted from 57).

For NK cells to defend the body against viruses and other pathogens, they require mechanisms that enable the determination of whether a cell is infected or not. NK cell activation is determined by the balance of inhibitory and activating receptor stimulation. The inhibitory receptors will recognize MHC class I molecules, NK cells preferentially kill cells with low levels of MHC class I. This is known as “missing-self hypothesis” a term created by Klas Kärre in the 90s (60). Their revolutionary work showed that the NKs killed the cells that do not present MHC class I on their surface as considering them potentially dangerous due to this “lack of information”, in fact, one of the strategies that the tumor or viral infected cells develop is the downregulation of the MHC class I expression (61). The MHC class I receptors mediate their function by signalling through ITIMs (inhibitory) or ITAMs (activating) motifs (See section 2.3). Thus, NK cells spare healthy cells that express self-MHC class I molecules and low amounts of stress-

induced self-molecules, whereas they selectively kill target cells “in distress” that down-regulate MHC class I molecules and/or up-regulate stress-induced self-molecules such as NKG2D ligands (Fig. 7).

4.3 NK cell and CD56/CD16 surface expression

CD56 (also known as neural cell adhesion molecule or NCAM) is often considered a marker of neural lineage commitment due to its discovery site. However, CD56 expression is also found in, among others, the hematopoietic system. The expression of CD56 is associated to natural killer (NK) cells even though is not limited to them, CD56 has been detected on other lymphoid cells such as $\gamma\delta$ T cells, activated CD8⁺ T cells and dendritic cells (62).

NK cells are defined as CD3⁻ CD56⁺, however, regarding their surface abundance of CD56, they can be subdivided in CD56^{bright} and CD56^{dim}. Whereas around 90% of NK cells in peripheral blood are CD56^{dim}, CD56^{bright} NK cells are more abundant in secondary lymphoid tissues. CD56^{bright} cells have been shown to derive from CD34⁺ hemopoietic stem cells (HSC) that differentiate into common lymphoid progenitors (CLPs) and eventually into CD56^{dim} in a progressive differentiation process that might involve minichromosome maintenance 4 (MCM4). CD56^{dim} cells are also able to upregulate CD56 after activation, suggesting that a proportion of CD56^{bright} NK cells might not be precursors but activated cells (63-64). This is most accepted theory for the NK differentiation process, however, there is no consensus and it remains an unresolved issue. Some articles in the field shed doubt on this model and proposed alternatives which include that CD56^{dim} NK cells may also derive directly from CLPs and that common myeloid progenitors (CMPs) and may also be precursors for CD56^{bright} NK cells. An additional argument suggests also an ontogenic

independency of these two subsets of NK cells, because mutated GATA-2 suppresses the CD56^{bright}, but not the CD56^{dim}, population (64) (Figure 8).

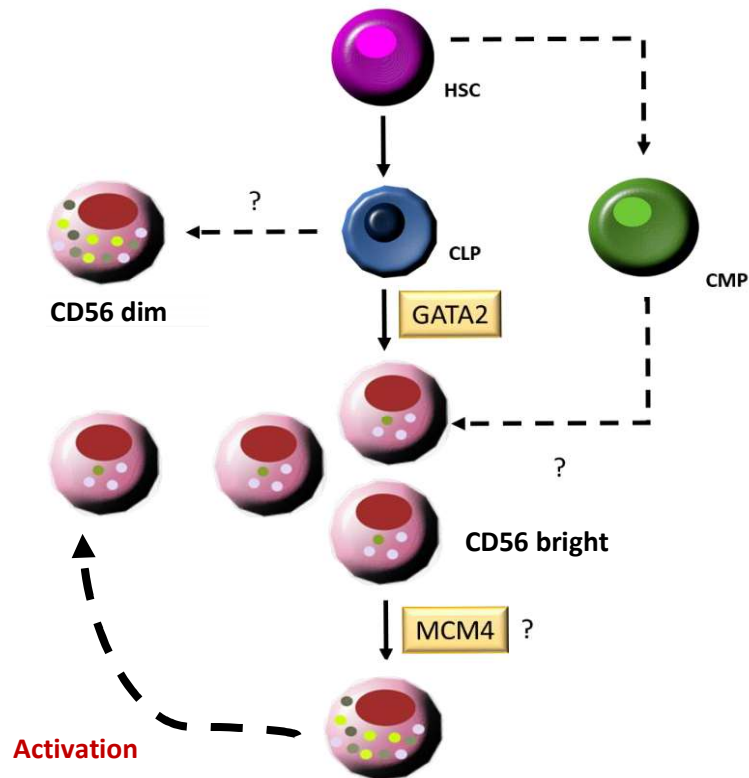


Figure 8. The ontogeny of CD56+ NK cell subsets (adapted from 64).

In regard to their functional properties, it was generally believed that CD56^{bright} are the mainly cytokine producers, while CD56^{dim} are responsible of the cytolytic activity and target cell killing, however, some studies have revealed that CD56^{dim} might also be a major source of proinflammatory cytokines and chemokines that are induced rapidly, suggesting that CD56^{dim} are important effectors not only for killing abnormal cells but also for inducing inflammatory responses and recruit other defensive cells. Moreover, CD56^{bright} NK cells also become cytotoxic after activation, under

these conditions they can be regulatory in the sense of suppressive, by analogy with regulatory T cells (65). In conclusion, the functional division of NK cell into regulatory or cytotoxic expressing higher or lower CD56 on their cell surface respectively, is not as simple as it seemed, and further studies are still needed to discern the different aspects.

Phenotypically, the CD56^{dim} NK cells are more granular than CD56^{bright} since they contain much more perforin, granzymes and cytotoxic granules in their cytoplasm. The main cytokines released by the CD56^{bright} are IFN γ , TNF- α , GM-CSF, IL-10 and IL-13 (66).

Regarding the HLA-G receptors, they are also differentially expressed in those NK subsets. The KIR and ILT2 receptors are absent from CD56^{bright} but are found on various proportions of CD56^{dim} cells, however, the expression of KIR2DL4 differs from the other KIR family members, and it is expressed exclusively in CD56^{bright}, regarding CD94/NKG2A it is expressed at higher density on CD56^{bright} NK cells (64). The chemokine receptor repertoire, it is also different between the two populations. CD56^{bright} express CCR7 and CXCR3 at much stronger density than CD56^{dim}. In contrast, CXCR1 and CX₃CR1 are exclusively expressed by CD56^{dim}. The consequences of these different repertoires of chemokine receptors and adhesion molecules are divergent migratory properties: the CD56^{bright} subset preferentially migrates to secondary lymphoid organs whereas the CD56^{dim} migrate to acute inflammatory sites (64-66).

Together with CD56, CD16 is also used to define the NK cell populations. CD16 marker, is the Fc γ receptor type III and it is essential for ADCC by NKs. In human peripheral blood, five NK cell subpopulations can be defined on the basis of the relative expression of CD16 and CD56. (1) CD56^{bright} CD16^{negative} (50–70% of CD56^{bright}), (2) CD56^{bright} CD16^{dim} (30–50%

of CD56^{bright}), (3) CD56^{dim} CD16^{negative}, (4) CD56^{dim} CD16^{bright}, and (5) CD56^{negative} CD16^{bright}. In healthy individuals, populations (3) and (5) are the minority. Whereas the role of CD56^{dim} CD16^{negative} cells is largely unknown, CD56^{negative} CD16^{bright} NK cells are functional. They are often dramatically expanded in human immunodeficiency virus infection but are hypo responsive under these conditions (Figure 9) (66).

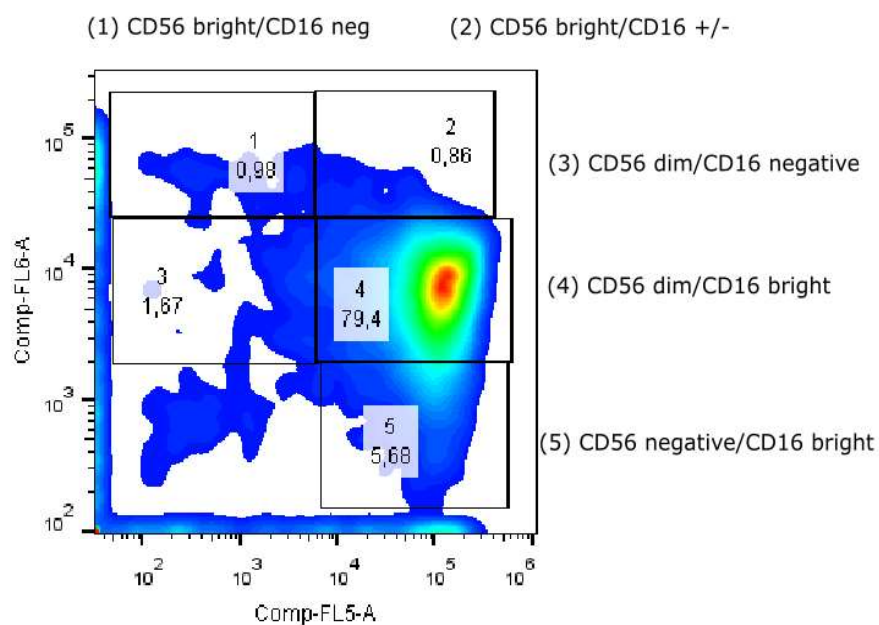


Figure 9. Natural killer (NK) cell subsets in human peripheral blood based on the expression of CD16 and CD56.

In addition, the CD56^{dim} NK cell population, can be subdivided in different subsets defined by the loss of expression of NKG2A, and the acquisition of KIRs, CD57 and CD16. CD57 might represent a marker of senescence and terminal differentiation in NK cells, CD57+ cells display a lower cytokine-induced proliferating capability compared with CD57- cells. Regarding CD16, it is acquired at late stages of peripheral blood NK cell differentiation and the amount of surface CD16 correlates with the level

of NK cell maturation. In agreement with this concept, not only CD16 but also KIRs were predominantly expressed by the CD57⁺ NK-cell subset (63;67). These data together suggests that not only NK cell can be subdivided into CD56^{bright} and CD56^{dim} but also, some intermediate populations can be defined (Figure 10).

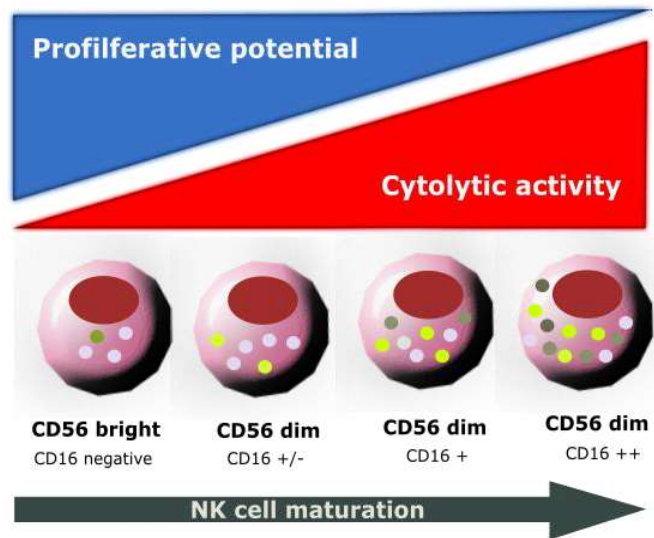


Figure 10. Model of human CD56^{dim} NK-cell differentiation (adapted from 63).

Lastly, it should be noted that there also exists a CD56^{negative} NK cell subpopulation, named CD3⁻CD4⁻CD14⁻CD19⁻CD16⁺NKp46⁺ lymphocytes. This population is rarely found in healthy individuals, however, elevated levels of CD56^{negative} NK cells are commonly found in patients with several pathological conditions, including HIV, chronic hepatitis C, human cytomegalovirus and hantavirus infections, autoimmune disorders, and following hematopoietic (stem) cell transplantation (62).

4.4 NK92 cells

NK92 is a highly cytotoxic, IL-2- dependent natural killer immortalized cell line that has features and characteristics of natural killer (NK) cells that every person has circulating in the blood. The easy and fast growing characteristic of this cell line make it widely used for basic and translational research. NK-92 cells were isolated and characterized by the laboratory of Hans Klingemann at the British Columbia Cancer Agency in Vancouver, Canada in 1992, hence their name. The cells came from a 50 year old caucasian male with rapidly progressive non-Hodgkin's lymphoma. Although several other NK cell lines have been cloned, only NK-92 cells can be expanded to larger numbers and consistently kill tumor cells. When NK-92 cells bind to a cancer or infected cell, they secrete perforin and granzymes, which will induce apoptosis in the target cells. In fact, they express high levels of molecules involved in the perforin-granzyme cytolytic pathway. NK-92 are also capable of producing cytokines that by themselves can kill cancer cells (such as TNF- α) or stimulate and expand other immune cells such as IFN γ .

NK-92 exhibits an unusual receptor expression profile, they express a relatively high number of activating receptors and few inhibitory receptors. Regarding the cell surface markers, NK92 are CD3^{negative} CD56^{bright} suggests an immature NK profile (See section 4.3). However, unlike normal NK cells, they lack the expression of CD16, this make NK-92 suitable for study direct cell-mediated cytotoxicity but not for the study of ADCC (See section 4.2).

The high cytolytic activity and broad target range combined with the ability to expand these cells, make them an ideal candidate for their use as immune effector cell. However, it is important to notice that their cytotoxic activity is only effective against target cells missing the MHC class I, as for example the K562 cell line. In addition to its use in research, several studies have suggested that NK-92 may be effective as an immunotherapeutic agent, as it will be further explained in Section 4.6 (68).

4.5 NK cells and pregnancy

A fetus can be seen as a semiallogenic graft expressing antigens from the father, however, it is not rejected by the maternal immune system, in this process of tolerance uterine decidual NK (uNKs or dNKs) cells are crucial players. Natural killer cells constitute 50–90% of lymphocytes in human uterine decidua in early pregnancy, in contrast with NK cells in peripheral blood (pNK) that comprise less than 15%. In addition, uNK cells also produce cytokines that are not produced by pNK cells such as angiogenic growth factors or leukemia inhibitory factor (LIF) (69) which support the view that dNK cells must play an important role in implantation and pregnancy.

Regarding their cell surface markers, the endometrium contains a high number of NK cells that are almost exclusively CD56^{bright} CD16^{negative}. Accounting for 20% of lymphocytes in the proliferative endometrium, they increase to 50% in the secretory phase and to 70–80% in early pregnancy decidua (66 and 70). This phenotype suggests that dNK cells are derived from CD56^{bright} pb-NK cells that are seeded in the uterus and undergo further differentiation in the decidual microenvironment (71).

The fetal cells in direct contact with the mother in the uterus are trophoblast cells, which are derived from the trophoctoderm layer surrounding the blastocyst. To ensure sufficient delivery of maternal nutrients and oxygen to the placenta, uterine blood flow need to be increased. This will be achieved by invasion of EVT's through the uterine wall in early pregnancy where this placental cells circumscribe and then destroy the muscular walls of the uterine arteries. Trophoblast invasion imply physiological changes in the uterine mucosa, a process called decidualization that is characterized by differentiation of glandular and stromal elements, as well as increased tortuosity of spiral arteries. During this process, large numbers of uNK and myelomonocytic cells and smaller numbers of T cells accumulate, particularly around invading trophoblast cells (Figure 9). The function of uNK cells in this context is to help the trophoblast cells to ensure correct arterial remodelling. This transformation depends on allorecognition of trophoblast cells by uNK cells, and this will determine a successful placentation and correct fetal development. uNK cells express a range of NK receptors that recognize adhesion molecules, stress signals and MHC class I ligands. Therefore, uNK cells will respond to a wide variety of signals, either induced by the hormonal changes of pregnancy or expressed by trophoblast cells, including allogeneic MHC molecules. In fact, the EVT cells that infiltrate deeply into the uterine wall express a unique set of MHC molecules, including HLA-E and HLA-G (70; 72-73).

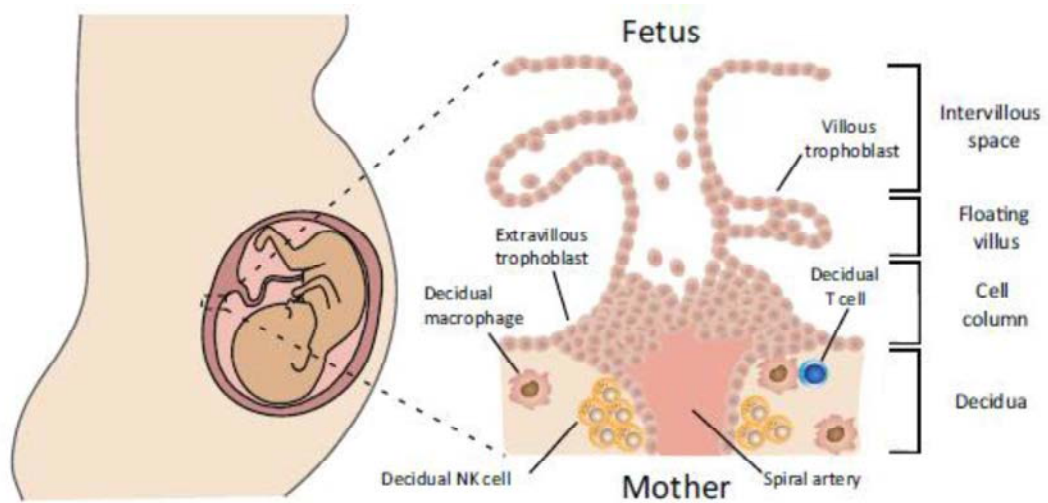


Figure 11. Maternal-fetal interface (18).

4.6 NK cells and cancer

Experimental research and clinical evidence highlighted a dual role of NK cells in the control of cancer progression and in promoting an immune-suppressive tumor microenvironment. Indeed, several mechanisms of NK cell-mediated tumor escape have been described such as: aberrant expression of activating and inhibitory receptors induced by cancer, deficient NK cell migration to tumor sites and altered NK cell effector functions. These phenomena highly contribute to tumor progression and metastasis formation (74).

As a consequence of the immune selection pressure, the tumor phenotype is continuously remodelled in a phenomenon known as “immune-editing”. This mechanism favours the development of less immunogenic tumors able to escape from effector immune cells, such as NK cells. Indeed, a common event occurring in cancer microenvironments is the alterations of antigen processing and presentation in the context of specific alleles of HLA class I molecules. As mentioned in Section 4.2, the

cancer cell tend to downregulate the MCH class I molecules and/or upregulate HLA alleles such as HLA-C, -E or -G that will bind inhibitory receptors and therefore become resistant to the NK cell attack. In addition, tumor cells can create an immune-suppressive microenvironment that down-modulates the repertoire of activating NK cell receptors and further impairs both NK cell effector-functions and trafficking to tumor sites (75).

Another important player in relation with NK cell function are the cytokines that exert potent anti-tumor functions by directly stimulating immune cells, enhancing their ability to recognize and kill cancer cells. In fact, they have been used in clinic to develop several therapeutic approaches to boost antitumor immune responses. NK cells constitutively express several cytokine receptors, whose engagement induces different immune responses including survival, development, proliferation, activation and interactions with other members of both innate and adaptive immune system. Two main cytokines that control NK cell functions and have a potential role as therapeutic agents in cancer are IL-2 and IL-15.

IL-2 is a cytokine mainly secreted by antigen-stimulated CD4⁺ T cells that maintains NK cell survival and promotes the proliferation of CD8⁺ T and NK cells. The use of recombinant IL-2 as a therapy to treat cancer has been proposed also in combination with other immune checkpoint inhibitors (76-77). However, the clinical response observed is poor since even at low doses of rIL-2 it is also induced the expansion of regulatory T cells that will in consequence control and prevent the NK cell effector-functions (78). The IL-15 cytokine shares many functions with IL-2 including the ability to induce anti-tumor responses. IL-15 mediate the development and

homeostasis of T and NK cells (79). Despite IL-2, IL-15 is highly effective and easy to use in cancer immunotherapy either alone or in combination with conventional chemotherapies and immunotherapies (80). Indeed, IL-15 can promote the proliferation and the activation of CD4+ and CD8+ T cells as well as of NK cells without any significant effect on regulatory T cells (81). Moreover, experimental findings proved that recombinant IL-15 is much better tolerated than IL-2 (82). Some clinical trials have been developed to test the safety and tolerated doses of recombinant-IL-15 in metastatic melanoma and renal cell carcinoma. The first results of this clinical trials showed that it is well tolerated and plays a role in the clearance of lung metastases in melanoma patients. Among the limitation and side effects of this cytokine, there is the induction of pro-inflammatory mediators such as IL-6 and IFN γ that can induce acute clinical toxicities (83).

Lately, it has been developed and characterized the protocols based on the expansion and infusion of NK cells as a therapy to treat patients with hematologic malignancies and solid tumors (84-87). Regarding the source of NK cells to be used, while autologous NK cells represented the first choice since they are safe and immediately available, their clinical impact against tumors is poor due to the inhibitory signal received by self-HLA molecules (88) Therefore, the latest approaches are now focusing in the infusion of allogeneic NK cells that gave much better results in term of alloreactivity against cancer cells thanks to the mismatch between HLA and KIRs (89). Since naive and resting NK cells exert a low cytotoxicity, several protocols of *in vitro* manipulation have been developed to both expand and activate freshly purified NK cells before their infusion. The majority of the procedures currently used for the expansion and activation of NK cells include their culture for 1-3 weeks with IL-2 and/or IL-15 either

in the absence or presence of a feeder layer of cells releasing other cytokines and/or expressing co-stimulatory molecules (90). Alternative approaches to expand NK cells comprised the use of other cytokines including IL-21, IL-12 and IL-18 (91-92).

Another approach using NK cell in the treatment of cancer is the use of chimeric antigen receptors (CARs)-NK cells. CAR are artificial receptors able to redirect the specificity and function of engineered immune cells. The first lymphocytes to be targeted with CAR technology have been T cells that are greatly effective in the clearance of several cancers types. However, the manufacturing of autologous CAR-T cells has several limitations as it is very expensive, requires long time for production and expansion and the prediction of its efficacy and safety is extremely difficult due to the personalized nature of the treatment. The possibility of targeting tumor-transformed cells in the absence of a prior antigen sensitization would significantly decrease or even cancel the severe toxicity of allogenic CAR-T cell therapy. Allogenic CAR-engineered NK cells certainly fulfill this last requirement and, hence, are potentially the best candidates for a clinical application on large scale (93). Indeed, unlike CAR-T cells, CAR-NK cells keep the native repertoire of NK cell receptors and therefore maintain their ability to recognize cancer cells and to perform tumor immuno-surveillance. The first attempts introducing the CAR technology on NK cells was the engineered of the NK-92 cell line (94). CAR-NK-92 cells showed a good and uniform transduction efficiency that is approximately 50% higher compared with primary NK cells (94-95). Several studies are currently investigating the impact of engineered different CAR-NK-92 cell lines to kill both hematologic and solid tumors (96). However, the clinical use of this cell line presents important disadvantages, since NK-92 cells had been first isolated from a non-hodgki

lymphoma patient, they must be irradiated before the infusion to prevent their tumorigenic potential (97). This procedure strongly affects their *in vivo* proliferation and persistence that are necessary for durable and effective anti-cancer immune responses (98). Hence, repeated infusions of CAR-NK-92 cells have been proposed to overcome these obstacles, but this increases the risk of a rapid rejection in the recipient of infused cells. Moreover, CAR-NK-92 cells generally show lower degrees of cytotoxicity and IFN γ production compared to primary NK cells and are not able to mediate ADCC as they lack CD16, as it was explained in Section 4.4.

Although great steps forward in this field have been made in the past recent years, further experimental and clinical investigation is required to develop and optimize NK cell-based anti-cancers therapies showing high efficiency and low toxicities with the final aim of transferring this gained knowledge to every day clinical practice of cancer.

5. HLA-G and maternal-fetal tolerance

During pregnancy, semi allogeneic EVT s invade the decidua and are well tolerated by the maternal immune system despite presenting paternally derived antigens. This mother-fetus immune tolerance is a critical step for the pregnancy to succeed (18).

Initially, this phenomenon was postulated to be due to systemic unresponsiveness of the mother immune cells to fetal antigens (99). However, later work described that pregnant mothers developed cytotoxicity against fetal antigens and antibody-mediated responses during gestation (100-101). These maternal immune responses to fetal antigens are controlled by regulatory T cells (Tregs) specific for fetal

antigens, as well as modulation of effector T cells and NK cells at the maternal–fetal interface.

There is a lot of evidences postulating that fetal immune tolerance is established locally at the placenta. During implantation, the EVT_s arise from the tips of anchoring villi and invade the maternal decidua, defining the boundary between mother and fetus: the maternal–fetal interface (Figure 11). The decidua harbours multiple populations of maternal immune cells, up to 40% of them are leukocytes, those immune cells extensively interact with fetal-derived trophoblasts (18).

From the leukocytes present in the decidua, NK cells constitute up to 90% of them in early pregnancy and it have been extensively described that decidual NK cells (dNK) significantly differ from peripheral-blood NK cells (pb-NK) in phenotype and function as it was described in Section 4.5.

In this process of mother-fetus immune toleration, HLA-G as emerged as a key molecule. HLA-G expression is tightly regulated and almost exclusively restricted to the EVT in the placenta (See Section 2.1 and 2.2). Additionally, reduced HLA-G levels have been found to correlate with pregnancy complications such as miscarriage, preterm birth, preeclampsia and recurrent spontaneous abortions (102).

Unlike most cells, EVT_s do not expresses HLA-A and HLA-B, and only express HLA-C and the MHC class Ib molecules HLA-E and HLA-G (103). EVT_s expressing HLA-G might direct uterine DC_s toward a tolerogenic rather than immunogenic phenotype during early pregnancy and block all possible maternal allogenic responses locally in the uterus. The consequences of the HLA-G interaction with the immune cells in the decidua are the inhibition of NK cells and CD8⁺ T cells cytotoxicity as well as the inhibition of CD4⁺ T and B cell proliferation. Apart from preventing maternal immune response against fetus, HLA-G also stimulates placental

development through secretion of proangiogenic factors by dNK cells and macrophages (104).

However, determining the mechanisms by which HLA-G induces immune tolerance at the maternal–fetal interface has been technically challenging, since murine models are not an option to investigate HLA-G function due to the lack of murine orthologue. In consequence, the experiments performed with the aim of establishing the inhibitory properties of HLA-G, were carried out using NK cells and cell lines lacking MHC class I such as LCL721.221, however, the overexpression of HLA-G in this cell line resulted in the overexpression of HLA-E (105), postulating that the NK cell inhibition by EVT could be achieved directly by HLA-G or by HLA-E loaded with HLA-G peptide it was described in Section 3.3.

However, still many questions about the implication of HLA-G in the pregnancy context are unresolved. Moreover, there are increasing evidences that HLA-G may play a role in a context other than pregnancy such as cancer, this relation between HLA-G and cancer will be described in the next Section.

6. HLA-G and cancer

6.1 HLA-G expression across tumor types

Apart from physiological conditions, the induction of HLA-G expression has been observed in various types of tumors. HLA-G has been detected in tumor lesions, where it may also facilitate immune surveillance escape in a similar way as it does in the pregnancy context. HLA-G expression has multiple effects that will promote tumor cells to escape from immune surveillance (106).

The expression of HLA-G in cancer was first demonstrated in the context of melanoma in 1998 (107), since then, HLA-G expression has been studied among several tumor types. HLA-G can be expressed at the cell surface, secreted or incorporated into tumor-derived exosomes.

HLA-G expression is frequent in endometrial carcinoma, but its expression have been described in many tumors including breast cancer (108-111), ovarian cancer (114-116), endometrial carcinoma (117-118), pancreatic adenocarcinoma (119), lung cancer (120-122), hepatocellular carcinoma (123-125), cervical cancer (126-127), glioblastoma (128), colorectal cancer (129-131), gastric cancer (132-134), and thyroid carcinoma (135-136). In conclusion, what emerges from all these studies mentioned above is that HLA-G could be potentially expressed by all tumor types.

6.2 HLA-G as a prognostic marker

HLA-G expression was found to be correlated with clinical parameters such as more advanced disease stage, tumor metastasis and poor prognosis in many tumor patients. However, although HLA-G expression in solid tumors is associated with an unfavourable outcome, no clear correlation was established in the case of haematological malignancies, thus the clinical significance of HLA-G expression in liquid tumors remains controversial (137-143). This discrepancy might be due to the fact that in malignant haematological diseases, the nature of the tumor are immune cells capable of expressing the HLA-G inhibitory receptors (144-145).

The correlation between HLA-G expression and clinical outcome was investigated comprehensively in a series of studies. Those studies revealed that HLA-G expression in ovarian cancer is associated with poor survival (113-115), in the case of breast cancer, the HLA-G expression was more frequently observed in advanced disease stage and tumor grade (108-

110). In hepatocellular carcinoma (HCC), the HLA-G expression was found to be correlated to advanced disease, short overall survival and increased tumor recurrence (122). In endometrial carcinoma, HLA-G expression was found to be associated with metastatic disease but unrelated with patient survival (116). Similar correlations were also observed in non-small cell lung cancer (119), gastric carcinomas (130) and colorectal cancer (128-129).

In summary, HLA-G might serve as a marker for prognosis and clinical outcome. However, to strengthen the implementation of HLA-G as biomarker, HLA-G expression and serum levels have to be determined in a large series of patient samples. In addition, it has still to be analysed whether HLA-G surface expression in tumor lesions is correlated with sHLA-G serum plasma levels. So far, either HLA-G expression in tumor lesions or sHLA-G in serum was determined but only very few studies have analysed both surface and secreted HLA-G expression in parallel.

6.3 HLA-G and therapeutical approaches

Since HLA-G is a potent immune-inhibitory molecule and its expression is correlated with poor prognosis, it is an attractive therapeutic target. One strategy for its clinical application could be the use of HLA-G derived peptides that may induce cytotoxic attack against HLA-G-expressing tumor cells as it was already performed experimentally in renal cancer (131). Target HLA-G-expressing cancer cells with a delivery system based on nanoparticles carrying anticancer drugs to the tumor site is another approached that have been developed (146). Given its immune inhibitory properties another important method would be to suppress its expression in tumor cells. Previous studies showed that suppression or downregulation of HLA-G by the use of interfering RNA was found to increase NK cell cytotoxicity against targets. Moreover, an attractive approach is the use of

antibodies that block HLA-G or its receptors ILT2 and/or ILT4 and therefore restoring the functions of T cells and NK cells (147). In combination with the use of blocking antibodies, the HLA-G measurement might be of special interest in clinical trials with monoclonal antibodies targeting specific receptors or targeting immunomodulatory checkpoints, since elevated levels of s-HLA-G may help predict resistance to these therapies (27).

On the basis of the above observations, an HLA-G-based immunotherapy can be achieved by employing HLA-G or HLA-G receptor antibodies to block HLA-G expression or interaction between its receptors.

OBJECTIVES

HLA-G is expressed across different types of cancer and confers poor prognosis. The aim of this thesis is to study the immunomodulation promoted by HLA-G in cancer.

The main objectives are listed below:

- Evaluate HLA-G expression across different cancer types
- Characterize cell lines physiologically expressing HLA-G
- Knock-out the expression of HLA-G in order to have a negative control
- Study the role of HLA-G in modulating the immune system, in particular the NK cells
- Characterize the modulation of NK cell activity in response to HLA-G
- Study the possibility of using anti-HLA-G antibody as a therapeutic tool

MATERIAL AND METHODS

1. Cell lines culture

All the cell lines used are derived from human, since the protein of study, HLA-G, is human and does not have a clear murine homologous. Cell lines were cultured in surface-treated plates (BD Falcon) using the appropriate culture media (Table 1). Each culture media was supplemented as described by the Manufacturer's or as suggested in previous publications (39) (Table 2). Cells were kept at 37°C in an atmosphere of 5% CO₂. Cryopreservation was performed as described in the cell line data sheet. In general, all cells were frozen in complete media with 10% DMSO (Sigma), with the exception of NK-92 cells that were frozen in 50% FBS, 40% complete growth medium and 10% DMSO, as suggested in their data sheet. For storage all cells were kept in liquid nitrogen vapour phase.

Cells were maintained in a subconfluent status and before reaching the confluence, cells were subcultivated as described by the manufacturer's protocol. For adherent cells, cell media was removed by aspiration, cells were rinsed with PBS (Labclinics) and incubated with 0.25% trypsin, 0.03% EDTA solution (Gibco, Invitrogen) at 37°C until cells detached, approximately for 5 minutes. Complete media was added to inactivate trypsin and after pellet the cells by centrifuging at 400G, cells were subsequently diluted in fresh media, mixed and pipetted in an appropriate centrifuge tube (VWR). For cells growing in suspension, they were collected by pipetting into an appropriate tube, right after, plate was washed with complete media for collect all possible remaining cells. Subsequent centrifugation was performed at 300 *g for 5 minutes (with the exception of NK-92 cells that were centrifuged at 150*g for 10 minutes as suggested in their data sheet). After centrifugation, cell media was aspirated and cells were resuspended in fresh complete media in a subcultivation ratio of 1:6 or 1:10 depending on desired confluence. In the

case of NK-92, cells were counted (Vi-CELL XR, Beckman Coulter) for a more accurate confluence control, they were seeded always in a 0.1M-0.3M cells/ml as described in their seeding protocol since they are extremely sensitive to overgrowth and media exhaustion. Moreover, as they grow in aggregates they need to be dispersed by pipetting before counting.

Cell line	Disease	Culture medium	Culture properties	Source
JEG3	Placenta Choriocarcinoma	EMEM	Adherent	ATCC
NK92	Malignant non-Hodgkin's lymphoma	Alpha MEM	Suspension	ATCC
HuPT-3	Pancreatic adenocarcinoma	EMEM	Adherent	ATCC
NCI-H2009	Lung adenocarcinoma	DMEM F-12	Adherent	ATCC
SK-OV-3	Ovary adenocarcinoma	McCoy's	Adherent	ATCC
CRL-5807	Non-small cell lung cancer	DMEM F-12	Adherent	ATCC
HEK 293T	Kidney	DMEM	Adherent	ATCC
NK cells	None	RPMI	Suspension	Blood bank*

Table 1. Characteristics of the cell lines used in the experiments. EMEM: Eagle's Minimum Essential Medium (ATCC); alpha-MEM: Alpha Minimum Essential medium; IMDM: Iscove's Modified Dulbecco's Medium (ATCC); McCoy's: McCoy's 5a Medium Modified(Gibco, Invitrogen); DMEM F-12: Dulbecco's Modified Eagle Medium(Gibco, Invitrogen); Nutrient Mixture F-12; RPMI: RPMI-1640 Medium(Gibco, Invitrogen). * Blood bank from Vall d'Hebron Hospital (Barcelona, Spain).

	FBS	P/S	Fungizone	HS	L-Glutamine	rh-IL-2
EMEM	10%	1%	1%			
Alpha-MEM	12.5%	1%		12.5%	1%	200 IU*
DMEM F12	10%	1%	1%			
McCoy's	10%	1%	1%			
DMEM	10%	1%	1%			
RPMI	10%**	1%			1%	

Table 2. Composition of the different cell culture media. FBS: Fetal bovine serum (Gibco, Invitrogen); P/S: penicillin streptomycin (Gibco, Invitrogen); HS: horse serum (Gibco, Invitrogen); rh-IL-2: recombinant human IL-2 (R&D). * IL-2 was always freshly added to the cell plate instead of adding it directly to the bottle of media since it loose activity within 7-10 days. It was added at 200 international units (IU) as recommended in the data sheet. ** In the case of NK cells the FBS was inactivated (30min at 56°C).

2. Generation of short-hairpin cell lines

JEG3 and HuP-T3 cell lines were used as a positive control for the HLA-G expression, in order to silence its expression, we performed short-hairpin cell lines. MISSION® shRNA Bacterial Glycerol Stock (SIGMA) containing pLKO.1 lentiviral vectors with interference RNA targeting HLA-G or empty vector as negative control were purchased for the generation of the short-hairpin cell lines (Figure 12). The bacteria from the glycerol stock were grown in LB with 50µg/ml Ampicillin (Sigma) in an Erlenmeyer shaker with constant shaking overnight (200 rpm, 37°C). The day after, once grown the bacterial stock, the DNA was extracted using Endo-free plasmid maxi kit (Qiagen) following the manufacturer instructions. When finished the protocol, the purified constructs were quantified by Nanodrop (Thermo Scientific).

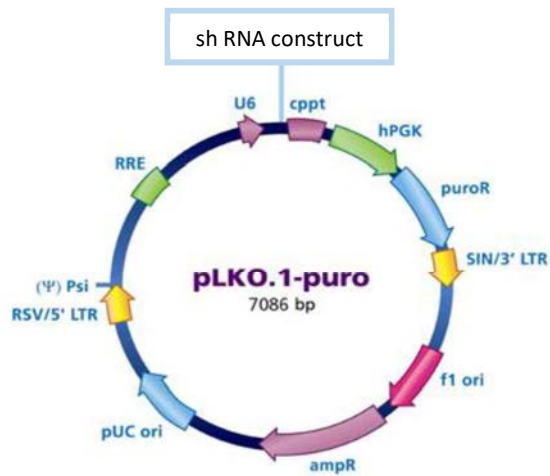


Figure 12. Map of pLKO.1 vector containing a shRNA insert with puromycin resistance purchased from SIGMA.

For the lentiviral infection, HEK 293T cells were grown until 70% of confluence and then transfected using the calcium phosphate transfection method with pMD2.G enveloping plasmid, psPAX.2 packaging plasmid, and pLKO.1-shHLA-G. 1h before the transfection, the medium was replaced adding fresh medium with 25 μ M chloroquine (Sigma). Then, the transfection was done adjusting the plasmid quantities as described in Table 3, and mixing them in a falcon:

Plasmid	μ g / plate 100mm	μ g / plate 150mm
Enveloping plasmid (pMD2.G)	3	8.75
Packaging plasmid (psPAX2)	5.5	16.25
Lentiviral of interest	8.5	25

Table 3. Plasmid quantities needed for 293T calcium phosphate transfection.

The following reagents were added on the top of plasmid mix in the order indicated in Table 4:

Reagent	μl / plate 100mm	μl / plate 150mm
TE 0,1x	250	750
H ₂ O	125	375
CaCl ₂ 2,5M	42	125

Table4. Reagents needed for 293T calcium phosphate transfection. [TE 0,1x = TRIS pH8 1mM + EDTA 0,1mM]

Mix was vortexed, and then the HBS (stock solution 2x: HEPES 100mM + NaCl 280mM + Na₂HPO₄ 1,5mM) was added drop by drop using a pipette while vortexing the mix. The final mix was immediately added on the HEK 293 cells plate slowly while moving de plate. After an O/N incubation, the medium was replaced by fresh medium (DMEM) with 5mM of sodium butyrate. 8h later, it was replaced again by fresh medium (in this case the growth medium of the target cells) without sodium butyrate and recombinant lentivirus was harvested for an additional 24h. For infection, medium containing recombinant lentivirus was filtered with 45 μm filter (Millipore), and added to the target cell line (either JEG3 or HuPT-3) with 8 $\mu\text{g}/\text{ml}$ polybrene (Sigma). After the O/N incubation, cells were washed and incubated in fresh medium. Infected cells were selected with puromycin (5 $\mu\text{g}/\text{mL}$, Sigma).

3. Generation of HLA-G CRISPR cell lines

With the use of the CRISPR/Cas9 technology, HLA-G knockout (KO) clones of JEG3 were produced. In addition, HuP-T3 cell line expressing HLA-G but in a lower percentage, was also used for the generation of HuP-T3 HLA-G KO clones.

HLA-G CRISPR/Cas9 KO Plasmid (h2) (Santa Cruz Biotechnology) was used to generate those clones (Figure 13). HLA-G CRISPR/Cas9 KO Plasmid (h2) is designed to disrupt gene expression by causing a double-strand break (DSB) in a 5' constitutive exon within the HLA-G gene. HLA-G CRISPR/Cas9 KO Plasmid (h2) consists of a pool of 3 plasmids, each encoding the Cas9 nuclease and a target-specific 20 nucleotide guide RNA (gRNA) designed for maximum knockout efficiency.

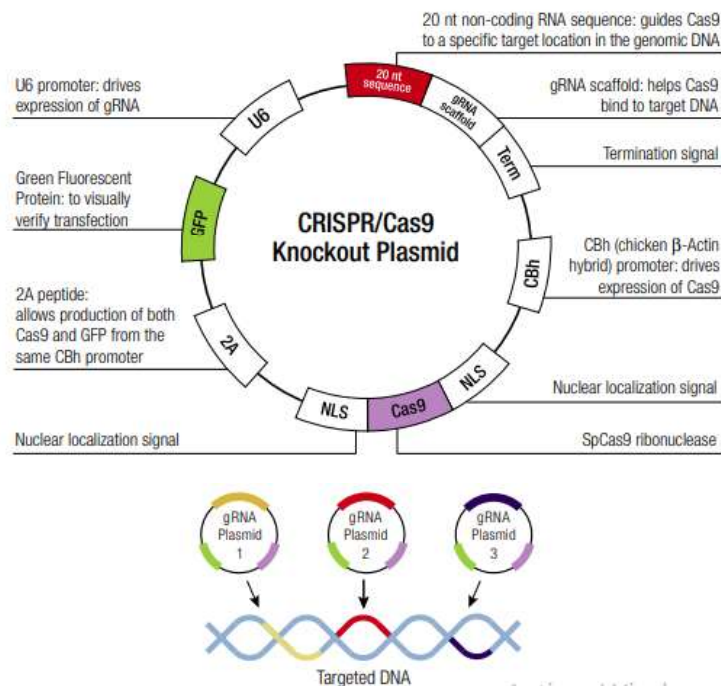


Figure 13. Structure of the HLA-G KO plasmid used (Santa Cruz Biotechnology)

Both cell lines were seeded in a 6-well tissue culture plate 24 hours prior to the transfection. 1.5×10^5 cells were seeded in 3 ml of antibiotic-free standard growth medium per well as described in the manufacturer's protocol. The cells were grown to a 40-80% confluence and the different solutions and reagents were added to the cells following the precise instructions of the manufacturer's protocol. The cell plate was incubated for 72 hours under normal culture conditions. No media replacement was performed in the first 24h. During the following hours, the media was replaced if needed. After 72h of incubation, successful transfection of CRISPR/Cas9 KO Plasmid was visually confirmed by detection of the green fluorescent protein (GFP) at the fluorescent microscope. Afterwards, positive cells were sorted due to its expression of GFP. The GFP+ cells were plated in a 96-well plate seeding only one cell per well to generate cell clones. Positive clones (those lacking HLA-G expression) that successfully grew were subcultured and when a sufficient confluent was achieved, RNA isolation (QIAGEN) from 10^5 total cells was performed to further confirm the success of the transfection by Real Time PCR (qPCR) (See Section 8). After the qPCR results, the KO clones were selected and further expanded. Subsequent check of HLA-G presence in the cell membrane by flow cytometry (Section 11) or released to the media by enzyme linked immunosorbent assay (ELISA) (See Section 10) was done to confirm the data previously obtained by qPCR.

4. NK cells isolation and culture

Buffy coats were obtained from the Blood Bank at Vall d'Hebron Hospital. The 10ml content of each buffy coat from healthy donors was emptied in a 50ml sterile tube (VWR) and immediately diluted with 10ml PBS (Labclinics), these 20ml properly mixed were added on the top of 10ml of

ficoll (Lymphosep, Biowest) in another 50 ml tube previously prepared, this addition was performed at the minimum speed possible to create a density gradient. The tubes containing the ficoll gradients (we would have one tube for each buffy coat processed) were centrifuged at 2000rpm for 25min at RT removing the centrifuge break to avoid the break of the density gradient created. After the centrifugation, a typical layer separation was obtained (Figure 14). Using a pipette, the peripheral blood mononuclear cells (PBMCs) ring was carefully transferred into a new centrifuge tube. Next, the plasma, which is essentially free of cells, was transferred into the same tube with the aim of ensuring that all PBMCs were recovered. We must avoid transferring any ficoll during this process. The cells were washed twice with PBS and centrifuged 1500rpm for 5min. Pelleted cells were resuspended in 50ml of MACS sterile buffer (PBS pH7,2 + 0,5% BSA + 2mM EDTA) and counted with the ViCELL Counter (Beckman Coulter) making a 1:10 dilution, this count would give the number of total leukocytes. Afterwards, natural killer cells were isolated using human NK cell isolation kit (Miltenyi Biotec) following Manufacturer's instructions. The isolation was performed in the autoMACS separator device (Miltenyi Biotec) and the volume of the reagents were calculated taking into account the total number of leukocyte population quantified before. The magnetic separation protocol followed was a negative isolation protocol since the beads found in the isolation kit would bind antigens not expressed by NK cells and therefore, our fraction of interest is the one that would elute from the column due to the lack of recognition by the magnetic beads. Once the isolation is finished, the cells were counted with the ViCELL Counter (Beckman Coulter). The expected fraction of NK is 10% from total PBMCs but the yield of the process might reduce those numbers.

The purity and viability of the isolated cell population was checked at the flow cytometer (FACS Navios, Beckman Coulter) using the CD56 antibody (TULY56, Invitrogen) and Fixable Yellow Dead Cell Stain Kit (Invitrogen) with the acquisition panel and protocol specific for this cell type. Data was analysed with the FlowJo V10 software. NK cells were seeded in the appropriate cell culture conditions depending on the experiment of interest.

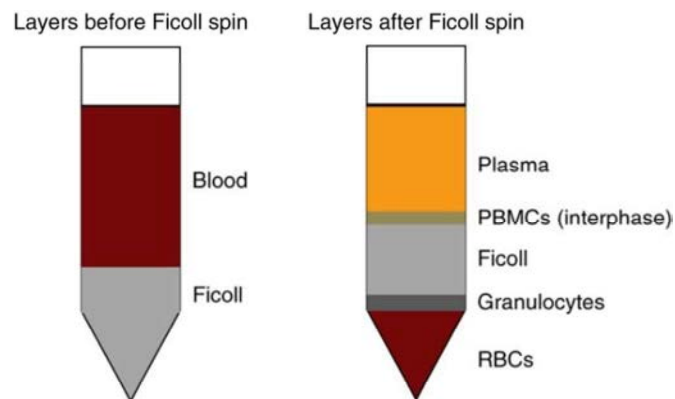


Figure 14. Ficoll gradients before and after centrifugation. RBCs: red blood cells.

5. Generation of conditioned media (CM)

Conditioned media from different cell lines was generated in this project. CM is the cell culture media from cell lines collected at a determined time. This media would have proteins and other metabolic products secreted from the cells during this time. It is widely used for research as a physiological source of metabolites, cytokines or chemokines among others. In this particular case, we generated CM from JEG3 and HuP-T3 in order to have a physiological source of secreted-HLA-G.

We detached and centrifuge the cells as described in Section 1. The cell pellet was counted with the ViCELL Counter (Beckman Coulter) and 5 million of cells were seeded in a p150 plate (BD Falcon) in a 17ml volume

of the corresponding cell media per plate and kept in the incubator for 72h. We must be precise with the time of culture in order to have reproducible results. The accurate counting and incubation time will help us in this aim. After the 72h, the cells must have reached the 90-95% confluence, it is important that they do not reach over confluence because the cells might suffer from it and may also detached from the plate. We recovered the media with a 10ml pipette and poured it to a 50ml falcon, if we have different plates with the same cell line and conditions, we could mix the CM. This volume was afterwards filtered with a 0.22 μ M filter (Merck Millipore) in order to avoid any contamination. The filtered conditioned media was aliquoted and stored at -80 $^{\circ}$ C to prevent protein degradation. One aliquot of 0.5-1ml of each of the conditions was kept in order to be quantified by ELISA (Bionova; see Section 10). The conditioned media is normally generated in the cell growth media of the cell of interest. For some experiments, we concentrated the conditioned media in order to increase the amount of any of their components, in our case, with the aim of increasing the HLA-G. We concentrated the conditioned media 4X using Amicon Ultra 4 centrifugal filter (Millipore). Those tubes have a filter device that provide fast ultrafiltration, with the capability for high concentration factors and easy concentrate recovery from dilute and complex sample matrices (Figure 15). We put a maximum volume of 4ml in the tube and centrifuge it at 4000g x 30min x 25 $^{\circ}$ C. If needed, we could increase the time of centrifuge until getting the desired concentration volume.

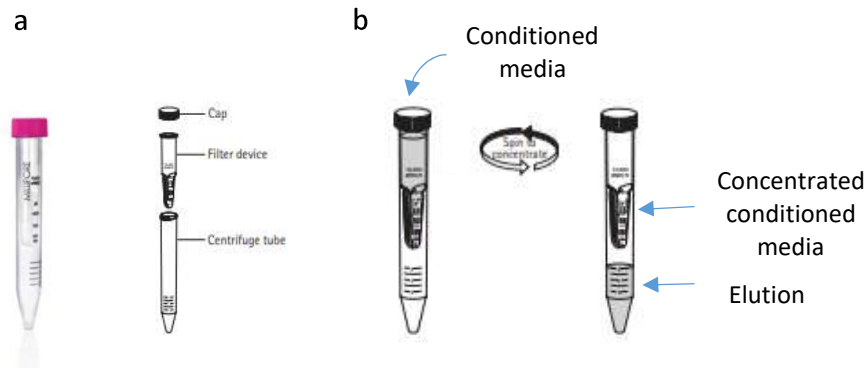


Figure 15. Concentration of conditioned media. (a). Picture showing the structure of the concentration tube showing the filter device inside it. (b). Picture representing the aspect of the tube before and after the media concentration.

6. *In vitro* cell culture experiments

NK cells (both pb-NK cells and NK92) were cultured for *in vitro* experiments. The pb-NK cells are very small (6-7 μm) and grow better in a high confluence, in fact they tend to form aggregate and grow as multicell spheres. They were seeded in a 24-well plate in a 1 million cells per well. However, the NK92 that are bigger, were seeded in a 12-well also plating 1 million cells per well, they also grow in multicell aggregates and form characteristics spheres (Figure 16).

The NK cells were resuspended in CM from JEG3 or HuP-T3 and seeded in the corresponding wells. Moreover, supplements were added to the cells to fulfil their growths needs (Table 3); These complements were dissolved in complete growth media and added in a 50 μl volume per well to complete a 750 μl volume. In addition, if some treatments were required

for the experiment, they were added in different doses as summarized in Table 4.

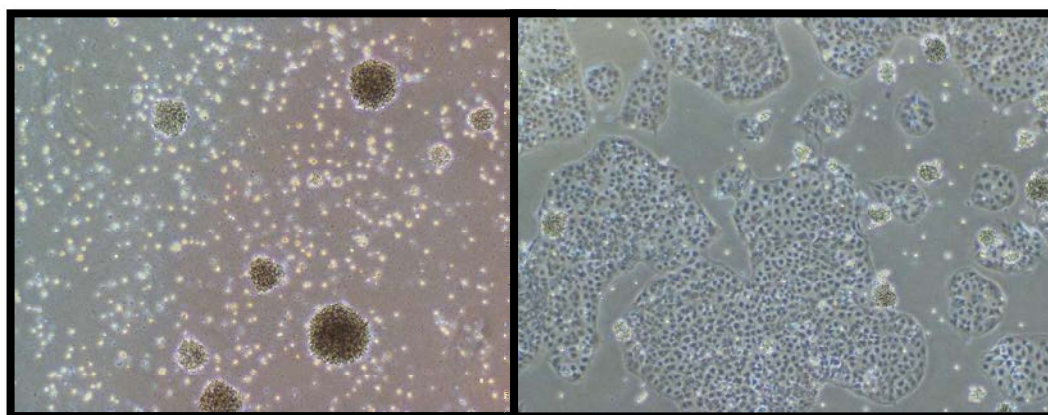


Figure 16. Cell culture pictures (10x). (Left) NK92 cells and their typical growth in multi-cell aggregates (Right) Coculture experiment showing adherent JEG3 cells and NK92 cells in suspension.

	Dose	Cells	Company
Recombinant human IL-2	200 IU	NK92 and pb-NK cells	R&D
Recombinant human IL-15	500 IU	pb-NK cells	R&D

Table 3. Supplements needed for cell culture

	Dose	Cells	Company
Recombinant human HLA-G	200 ng/ml	pb-NK and NK92	Origine
Mouse Antihuman- HLA-G	50-150ng/ml	NK92	Abyntek
Anti-HLA-G antibody	5-50 ng/ml	NK92	Thermofisher (87G)

Table 4. Treatments used for *in vitro* experiments.

7. Coculture experiments

The coculture experiments performed involved two type of cells, the target cells and the effector cells. In this case, the target cells are the JEG3 cells and the effector cells will be the cells of the immune system, in our case NK cells (pb-NK cells or NK92). Due to the fact that the target cells grow in attachment, they need to be seeded prior the effector cells. 10^5 cells/ well were seeded in a 12-well plate and kept overnight at 37°C 5% CO₂. The day after, the effector cells were counted and seeded on top of the target cells. The ratio of effector:target (E:T) used was 10:1. We kept the coculture overnight.

8. RNA extraction, retrotranscription, and Real-Time PCR

For the RNA extraction, cells were collected, centrifuged, washed with PBS and counted in the ViCELL Counter (Beckman Coulter). Then, $10^5 - 10^6$ cells, depending on the amount of cells available, were resuspended in 350µl of Trizol (QIAGEN) for subsequent RNA extraction. Cell pellet can be maintained in Trizol for 15 minutes at room temperature (RT) or kept at -80°C until the extraction is performed. This step will increase the yield of the process. RNA extraction was performed as described by the Manufacturer's protocol (miRNAeasy Mini Kit, QIAGEN). When the extraction is finished, the RNA obtained is quantified by the Nanodrop (ThermoFisher). For small samples (< 10^5 cells available) the miRNAeasy Micro Kit (QIAGEN) was used instead.

300-500ng of RNA was retrotranscribed into cDNA using iScript cDNA synthesis kit (BioRad) and the T100 Thermal Cycler (BioRad). Gene expression was determined by Real-Time PCR. To do so, the Taqman probes (Applied Biosystems) corresponding to the genes of interest, and Taqman Real-Time PCR Master Mix (Applied Biosystems) were used

following the Manufacturer's instructions. qPCR amplification protocol was developed in a 384-well white-bottom plates (BioRad) in a final volume of 10µl per well, using the CFX384 Real Time System C1000 Touch Thermal Cycler (BioRad). All samples were pipetted in triplicates to increase the accuracy of the results. The program used for amplification is described in Table 5.

RIPA buffer 1x
50 mM Tris-HCl pH 7.4
1% NP40
0,5% Na-Deoxycholate
0,1% SDS
150mM NaCl
50mM NaF
25mM β-Glicerolphosphate
0,4mM Na ₃ VO ₄
Protease inhibitors (complete mini protease inhibitor cocktail tablets, from Roche: 1 tablet/10ml)

Table 5. Program used for qPCR.

Results were expressed as fold change calculated by the $\Delta\Delta C_t$ method and normalized by the expression of an endogenous housekeeping gene (*GAPDH* or *ACT1Nb*). A value of 1 was given to the control sample.

c DNA amplification program
50° for 2 minutes
95° for 10 minutes
95° for 15 seconds
60° for 1 minutes
Go to 3 x 39 cycles

Table 6. Composition of RIPA buffer

9. Protein extraction and western blot

For the protein extraction, cells were collected, centrifuged, washed with PBS and counted in the ViCELL Counter (Beckman Coulter). Then, $10^5 - 10^6$ cells, depending on the amount of cells available, were resuspended in 70 μ l of RIPA buffer (Table 6). Cells were kept during all process at 4°C.

RIPA lysed cells were frozen at -80°C, thawed after at least 30min, centrifuged at 16000*g for 20 minutes at 4°C. The supernatant was aspirated and the whole protein extracts were quantified using the BCA protein assay kit (Pierce, Thermo Fisher Scientific) and the Tecan Infinite 200pro (TECAN). The amount of protein present on each sample was calculated by extrapolation from the standard curve used in the BCA protein assay kit. Once we have the numbers for each of the samples, the same amount of protein (ranging from 30 to 50 μ g) was mixed with loading buffer (Table 7), boiled for 5min at 100°C, and loaded in a SDS-acrylamide gel for protein separation (Table 8). Protein marker VI (Tris-Glycine 4-20%, Panreac) was loaded in the gel and used as protein weight reference. The gel was run at 100V during 90 minutes and proteins were transferred to a nitrocellulose membrane (iBlot transfer stacks, Invitrogen). The protein transfer was performed in the iBlot Dry Blotting System (Invitrogen) following the manufacturer's instructions. The program used was P0 (Table 9), suitable for protein sized between 30-150 kDa.

Loading buffer 4x
50 mM Tris-HCl pH 6.8
400mM DTT
2% SDS
10% Glycerol
0,1% Bromophenol Blue

Table 7. Loading buffer composition.

Reagents (8ml)		Acrylamide percentage						
		Reagents (20ml)	6%	8%	10%	12%	14%	
Stacking	H ₂ O	5,5ml	H ₂ O	10,6ml	9,3ml	7,9ml	6,6ml	5,2ml
	30% Acrylamide / bis solution 29:1 (BioRad)	1,3ml	30% Acrylamide / bis solution 29:1 (BioRad)	4ml	5,3ml	6,7ml	8ml	9,4ml
	1M Tris pH 6,8	1ml	1,5M Tris pH 8.8	5ml	5ml	5ml	5ml	5ml
	10% SDS	80µl	10% SDS	200µl	200µl	200µl	200µl	200µl
	10% APS (Sigma)	80µl	10% APS (Sigma)	200µl	200µl	200µl	200µl	200µl
	Temed (Sigma)	8µl	Temed (Sigma)	80µl	80µl	80µl	80µl	80µl
		Resolving	H ₂ O	10,6ml	9,3ml	7,9ml	6,6ml	5,2ml
			30% Acrylamide / bis solution 29:1 (BioRad)	4ml	5,3ml	6,7ml	8ml	9,4ml
			1,5M Tris pH 8.8	5ml	5ml	5ml	5ml	5ml
			10% SDS	200µl	200µl	200µl	200µl	200µl
			10% APS (Sigma)	200µl	200µl	200µl	200µl	200µl
			Temed (Sigma)	80µl	80µl	80µl	80µl	80µl

Table 8. Stacking and resolving SDS-acrylamide gel composition

Once the protein transfer is finished, the membrane was blocked for non-specific interactions with 5% milk or 3% BSA in Tris Buffered Saline-Tween 0,1% (TBS-T) for 30-60 minutes at RT in constant agitation. After the blocking, the membrane was incubated overnight (O/N) at 4°C in agitation with the antibody of interest (Table 10). The day after, primary antibody was rinsed for 30min (3 washes of 10 minutes) with TBS-T. Then, the membrane was incubated with the secondary HRP-conjugated antibody (GE Healthcare) for 1h at RT in constant agitation. Membrane was rinsed again with TBS-T for 30min (3 washes of 10min), and was developed with ECL (Immobilon Western, Millipore).

Program	Voltage	Running time
P0	20V for 1 minute 23V for 4 minutes 25V for 2 minutes	7 minutes

Table 9. I Blot Gel Transfer Program.

Protein detected	Clone	Dilution	Brand
HLA-G	4H-84	1:1000	Santa Cruz (sc-2199)
Actin B (HRP-conjugated)	A3854	1:50000	SIGMA
Tubulin	T9026	1:10000	SIGMA

Table 10. Antibodies used for Western Blot.

10. ELISA

We performed ELISA to quantify the soluble amount of protein secreted by cultured cells to the medium. We used the s-HLA-G ELISA kit (Antibody Exbio) for HLA-G, Quantikine ELISA kits for human-Interferon gamma (h-IFN γ) and human-Granzyme B (h-GZMB)(R&D), and human-perforin1 (h-PRF1)

ELISA	Company	Total time of the assay	Precoated plates	O/N incubation required
s-HLA-G	Antibody Exbio	20 h	Yes	Yes
h-IFN γ	R&D	24 h	No	Yes
h-GZMB	R&D	24 h	No	Yes
h-PRF1	Abcam	3 h	Yes	No

Table 11. Specific characteristics of the ELISA kits.

ELISA	Measurement weavelenght	Reference weavelenght
s-HLA-G	450 nm	630 nm
h-IFN γ	450 nm	560 nm
h-GZMB	450 nm	560 nm
h-PRF1	450 nm	630 nm

Table 12. Colorimetric reaction specification for each ELISA kit.

ELISA kit (Abcam) to quantify perforin 1 (Table 11). The protocol was performed in a 96-well clear bottom plate and following the precise instructions of the Manufacturer. In some occasions, sample dilutions were needed. Absorbance was determined by the Tecan Infinite 200pro (TECAN), colorimetric reaction was measured at the wave-length specified by each protocol (Table 12). The quantitative determination of protein levels was performed extrapolating the values from the standard curve as suggested in the Manufacturer's instructions.

FC channel	FACS NAVIOS- pb-NK/NK92 characterization				
	Fluorochrome	Protein detected	Type of staining	Company (clone)	Dilution
FL-2	PE	IFN γ	Intracellular	BD Biosciences (4SB3)	1:100
FL-5	PE/Cy7	CD16	Surface	Biolegend	1:100
FL-6	APC	CD56	Surface	Life technologies (TULY56)	1:100
FL-10	LIVE/DEAD [®] Fixable Yellow Dead Cell Stain for 405 nm excitation	Cell surface and intra-cellular amines (Viability staining)	Fixable	BD Biosciences	1:2000

Table 13. Flow cytometry panel used for HLA-G detection on cell membrane.

FC channel	FACS NAVIOS- HLA-G/Pan-HLA characterization				
	Fluorochrome	Protein detected	Type of staining	Company (clone)	Dilution
-	Non-conjugated	HLA-G	Surface	Abcam (MEM-G9)	1:100
-	Non-conjugated	Pan-HLA	Surface	Abcam (MEM-147)	1:100
FL-1	Alexa 488	Goat anti mouse- Secondary antibody	Surface	Life technologies	1:500
FL-10	LIVE/DEAD [®] Fixable Yellow Dead Cell Stain for 405 nm excitation	Cell surface and intra-cellular amines (Viability staining)	Fixable	BD Biosciences	1:2000

Table 14. Flow cytometry panel used for NK cells.

11. Flow cytometry assays

NCI-H2009, HuP-T3 and JEG3 were characterized for the presence of HLA-G in their surface membrane using flow cytometry (Table 13). Determination of pan-HLA was also studied by FC in these cells.

NK92 and pb-NK were also stained by CD56 and CD16 by FC (Table 14). In some occasions, IFN γ was also determined.

The staining protocol varies depending if it includes intracellular staining or not. The different protocol specifications are explained below.

11.1 Surface staining

Cells were collected in the appropriate centrifuge tubes. The different tubes (one for each experimental condition including duplicates/triplicates) were centrifuged at 300*g for 5 minutes and washed with PBS. The supernatants if needed were collected for subsequent experiments (ELISA) in 1.5ml Eppendorf tubes (VWR) and stored at -80°C. The cell pellets once washed with PBS, were stained for cell viability with Fixable Yellow Dead Cell Stain Kit (Invitrogen) in a 1:2000 dilution, during 30 minutes at RT. After, cells were washed with PBS, and stained for the corresponding antibodies to detect the cell surface proteins. The different conjugated or non-conjugate antibodies were added in a 1:100 dilution in PBS and incubated during 20-60 minutes at RT. In the case of conjugated antibodies, matching isotype controls for each fluorochrome were also added at this point. Afterwards, cells were washed with PBS and resuspended in 300 μ l of PBS for their acquisition with the appropriate protocol in the flow cytometry (FACS Navios, Beckman and Coulter). In the case of non-conjugated antibodies, an extra step is needed. The conjugated secondary antibody was added in a 1:500 dilution and incubated at RT for 30 minutes. Once finished, sample were washed

with PBS as explained before and ready for analysis. Each sample was acquired for 200 seconds with fixed speed (Medium) of aspiration. Data was exported and analysed with the FlowJo V software, later data was plotted with GraphPad Prism 5.0.

11.2 Intracellular staining

In case that we perform intracellular staining combined with surface staining, we followed the protocol as explained above, but with some additional steps. Once finished the surface staining, we fixed the cells for 20-60 minutes at RT protected from light using the Fixation Buffer 1X (Intracellular Fixation and Permeabilization Buffer Set, Labclinics) as described in the Manufacturer's instructions. Fixation can also be performed O/N at 4°C if needed. After fixation, we washed with previously diluted Permeabilization Buffer 1X (Intracellular Fixation and Permeabilization Buffer Set, Labclinics) and centrifuge at 400*g for 5 minutes. We diluted the antibodies of interest in the required amount of Permeabilization buffer (See Table 13) and incubated with samples for 20-60 minutes at RT protected from light; After the antibody incubation, samples were washed with permeabilization buffer and resuspended in PBS. Samples were acquired in the flow cytometry (FACS Navios, Beckman and Coulter). The acquisition and analysis was performed as explained before for the surface staining.

12. The Cancer Genome Atlas (TCGA) correlations and statistical analysis of data

Regarding the bioinformatics analysis, to build the TCGA cohort, gene expression from RNA seq (RSEM gene-normalized) for 9,174 tumors across 28 cancer types was downloaded from the Firebrowse server (version 2016_01_28). RSEM were log₂ transformed. We defined as HLA-G positive all tumor samples with an HLA-G log₂ RSEM expression higher or equal to 5. The study abbreviations used are listed in Table 15. Testicular Germ Cell Tumors (TGCT) and Thymoma (THYM) were excluded due to the fact that are immune privileged organs. Haematological tumors were also excluded because the clinical significance of HLA-G expression in liquid tumors remains controversial due to the fact that the nature of the tumor are immune cells capable of expressing the HLA-G inhibitory receptor (See Section 6.2 of the Introduction). Regarding the experimental data generated, it was analysed using GraphPad Prism 5.0 software. To compare two different experimental groups we calculated P-value (P) using Unpaired Student's T-test. *P<0.05; **P<0.01; ***P<0.001. Data is presented as mean ± SEM.

Cancer type acronym	Cancer type full name	Number of patients (RNA-seq) (n)
ACC	Adrenocortical carcinoma	78
BLCA	Bladder Urothelial Carcinoma	404
BRCA	Breast invasive carcinoma	1082
CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma	301
CHOL	Cholangiocarcinoma	36
ESCA	Esophageal carcinoma	182
GBM	Glioblastoma multiforme	163
HNSC	Head and Neck squamous cell carcinoma	515
KICH	Kidney Chromophobe	65
KIRC	Kidney renal clear cell carcinoma	515
KIRP	Kidney renal papillary cell carcinoma	285
LGG	Brain Lower Grade Glioma	514
LIHC	Liver hepatocellular carcinoma	368
LUAD	Lung adenocarcinoma	511
LUSC	Lung squamous cell carcinoma	485
MESO	Mesothelioma	87
OV	Ovarian serous cystadenocarcinoma	300
PAAD	Pancreatic adenocarcinoma	156
PCPG	Pheochromocytoma and Paraganglioma	178
PRAD	Prostate adenocarcinoma	493
READ	Rectum adenocarcinoma	599
SARC	Sarcoma	254
SKCM	Skin Cutaneous Melanoma	434
STAD	Stomach adenocarcinoma	405
THCA	Thyroid carcinoma	499
UCS	Uterine Carcinosarcoma	57
UCEC	Uterine Corpus Endometrial Carcinoma	357
UVM	Uveal Melanoma	80

Table 15. TCGA Study abbreviations for each cancer type.

13. Paraffin block generation and HLA-G Immunohistochemistry (IHC) staining

13.1. Paraffin block generation

For pellets of cells paraffin blocks generation, 3-5 150mm culture plates at 90% of confluence were rinsed with PBS and incubated with 10mM EDTA for 10min at 37°C. Medium was added to inactivate EDTA, centrifuged at 400g for 5min and transferred into a 1,5ml eppendorf (VWR) with medium. Then, cells were washed with PBS, the PBS was rinsed and the cell pellet was fixed O/N with 4% Formol. The day after, the formol was replaced with 70% ethanol. The paraffin inclusion was performed by the pathological anatomy facility at VHIO.

Tumor micro arrays (TMAs) from endometrial carcinoma were kindly provided by Dr. Matias-Guiu (Bellvitge Hospital, Barcelona).

13.2. IHC staining

IHC detection of HLA-G was performed in JEG3 WT and JEG3 HLA-G-KO cell pellet and in endometrial carcinoma TMA. The slides were heated at 65°C O/N, deparafinized in 3 consecutive incubations in xylene (Panreac) for 10min each, and progressively hydrated with 100% - 90% - 70% ethanol solutions. Antigen retrieval was performed using pH6 (115°C, 3min) Citrate Antigen Retrieval Solution (DAKO), peroxidase blocking was done with 10% H₂O₂ for 10min at RT, 3 washes were performed with TBS-Tween 0,1% (TBS-T) for 5min each at RT, and then slides were incubated in a blocking solution (2-3% BSA) for 1h at RT in constant agitation. After 3 more washes with TBS-T for 5min each at RT, primary antibody (anti human HLA-G, Bionova, clone MEM-G1) was added diluted 1/20 in the

Dako Real™ Antibody Diluent (Dako) on the top of the section in a humid chamber, and incubated O/N at 4°C or for 30min at RT. Then, 3 washes with TBS-T were performed, Dako detection systems was performed following the manufacturer's instructions, 3 more washes with TBS-T and the slices were immediately developed with freshly prepared 0,05% 3',3'-diaminobenzidine tetrahydrochloride (DAB). Finally, the slides were counterstained with Harris hematoxylin (Sigma) for 5 seconds, flushed in abundant water for 5min, progressively dehydrated in a series of ethanol solutions (70% - 90% - 100%) followed by 3 incubations in xylene solutions, and mounted with DPX mounting medium (Millipore). Before acquiring the images in a light microscope (Olympus BX61 microscope), the slides were air dry in a vacuum chamber to eliminate the xylene remains.

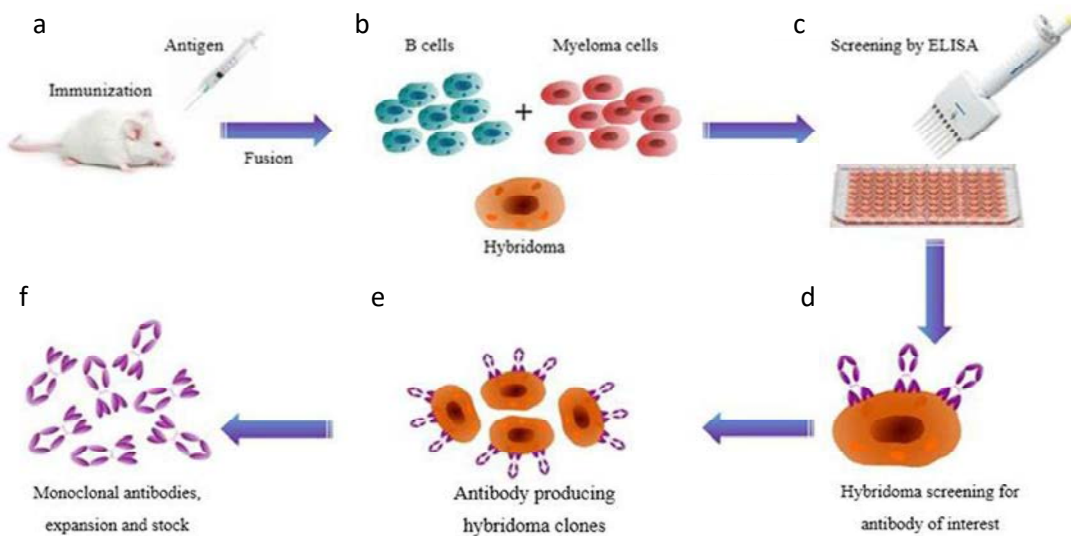


Figure 18. Antibody production steps. (a) Mice immunization, (b) fusion for hybridoma generation (c) screening by ELISA of the clones generated, (d) expansion of the positive clones previously generated (e) screening of the subclones and (f) antibodies expansion (149).

14. Production of monoclonal anti-HLA-G antibodies

The process of the anti HLA-G antibodies production undergo several steps as it is summarized in Figure 18. The total length of the process was 27 weeks and the specification of the different steps will be described below.

14.1 Mice immunization

Five balb/c nude mice and five C57BL/6 mice were immunized with the KLH peptide conjugate. Before the immunization, the sera was extracted from the mice to have the pre-immunization control. After three rounds of immunization, the sera was extracted again. Both serums were sent to us (15 µl of each sample) for the screening at Flow cytometry.

We performed the screening using JEG3 WT cells as positive binding HLA-G control and JEG3 KO cells as negative control. Moreover, for the best candidates resulting from this screening, the pre-immunization sera was also checked for an extra control of binding specificity.

The screening at FC was performed using 3×10^5 JEG3 cells resuspended in 100ul of PBS and adding 1µl of immunization sera (we got only 15µl from each mice) from the ten different mice as a putative source of antibodies. The cells were analysed at FACS Navios and compared with the commercial anti-HLA-G antibody abcam7758 (MEM-G/9 clone) as a positive control of the staining.

After this step, from the ten immunized mice, the two best candidates were selected for subsequent steps.

14.2 Fusion and screening

From the two mice selected in the previous step, the B cells were fused with melanoma cells to produce the hybridomas. The hybridomas generated were grown in 96-well plates. The supernatant was collected and analysed by indirect ELISA in a total of 20 plates.

At the end of this step, we got the hybridoma supernatant of 20 positive clones (2ml from each clone). We checked for the ability of this supernatants to detect HLA-G in the JEG3 cells as described at 13.2. In this case, the JEG3 cells were resuspended in 100µl of the hybridoma supernatant without dilution in PBS, for the detection of HLA-G.

14.3 Hybridoma subcloning

The hybridoma selected in the previous step, was further subcloned by limiting dilution. Ten subclones were generated from the initial hybridoma clone selected. The supernatant was collected (5ml) and sent to us for testing. We tested for the putative presence of anti-HLA-G following the same FC protocol explained before. One positive subclon was selected for the antibody production.

14.4 Antibody production and purification

The selected hybridoma subclone is further expanded for the antibody production. The antibody is purified and sent to us for its use as a anti-HLA-G functional blocking antibody. The final concentration obtained by the company after the purification is: 1.372 mg/ml.

RESULTS

1. Bioinformatics analysis of HLA-G expression across 28 solid tumor types (n=9403)

The study of the HLA-G expression (RNAseq data) across tumor types based on the The Cancer Genome Atlas (TCGA) data base, pointed out the cancer types expressing the highest levels of HLA-G. Across the 28 tumor (n=9403) types studied (Table 15), the Uterine Corpus Endometrial Carcinoma (UCEC, n= 357), the Kidney renal clear cell carcinoma (KIRC, n= 515) and the Kidney renal papillary cell carcinoma (KIRP, n =285), were the ones expressing the highest levels of HLA-G (Figure 1a). In addition to the UCEC, KIRC, and KIRP, the Pancreatic adenocarcinoma (PAAD, n=156) appeared as the one of the cancer types expressing the higher proportion of HLA-G positive samples (Figure 1b). As expected, the ones with the higher HLA-G expression were the ones with higher proportion of HLA-G positive samples.

Importantly, all the UCEC samples appeared to be HLA-G positive, this result might be influenced by the physiological expression of HLA-G in the uterus as it was explained in the Introduction (Section 2.2). Moreover, the high levels of HLA-G observed in the PAAD may also be related to its physiological expression in the pancreatic islets as they belong to the cohort of immune-privileged regions in the body that express HLA-G.

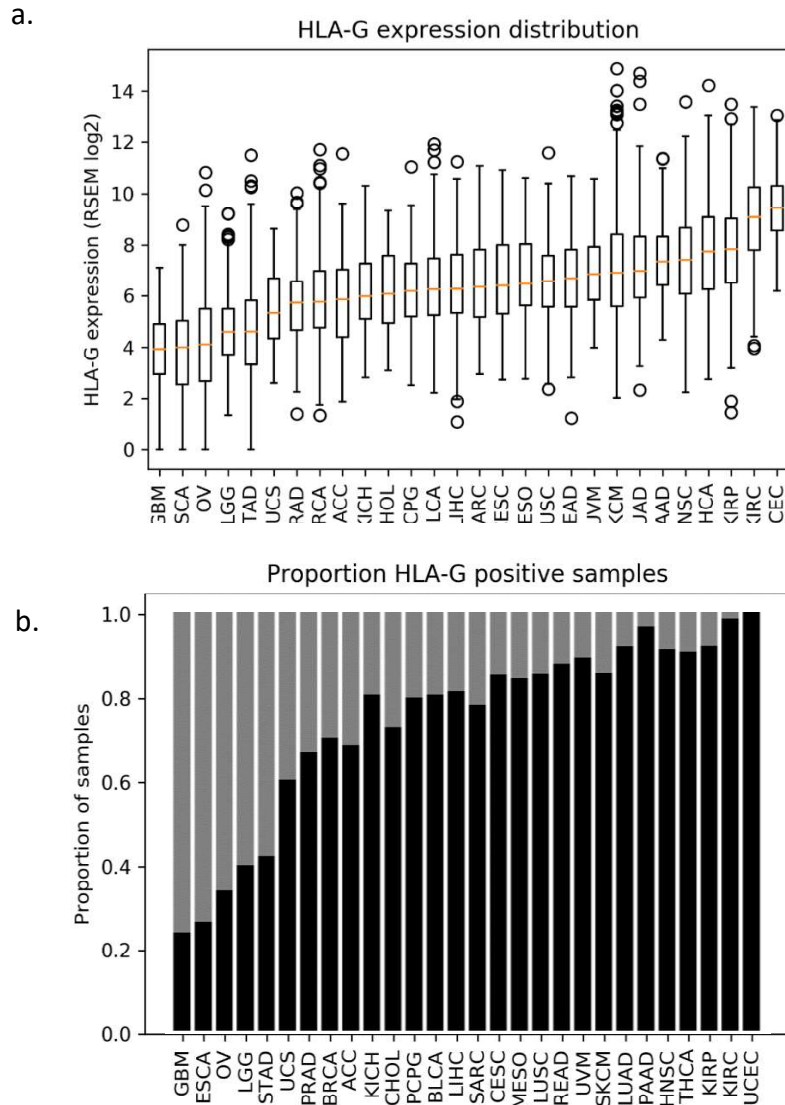


Figure 1. Bioinformatics analysis of HLA-G expression across 28 solid tumor types (n=9403). (a) Box-plots representing HLA-G expression across 28 solid tumors in The Cancer Genome Atlas (TCGA). Tumor types were sorted, from left to right, from low to high HLA-G expression. The median expression was represented in orange and the whiskers represented the first and third quartiles. (b) Stacked bar plot showing the proportion of HLA-G positive (coloured black) and negative (coloured grey) tumors across all solid tumors. A cutoff of ≥ 5 log₂ RSEM was used to consider a tumor positive, based on previous previous TCGA studies.

2. HLA-G expression in endometrial carcinoma samples

We performed IHC to detect HLA-G expression in an endometrial carcinoma TMA. We observed that as expected from the TCGA analysis previously shown, many samples expressed high HLA-G levels (Figure 2 a-c) while others expressed low HLA-G levels but were still positive (Figure 2d). Moreover, we used JEG3 WT and JEG3 KO-HLA-G cell pellet as positive and negative control of the antibody used (anti human HLA-G, Bionova, clone MEM-G1) (Figure 2 e-f).

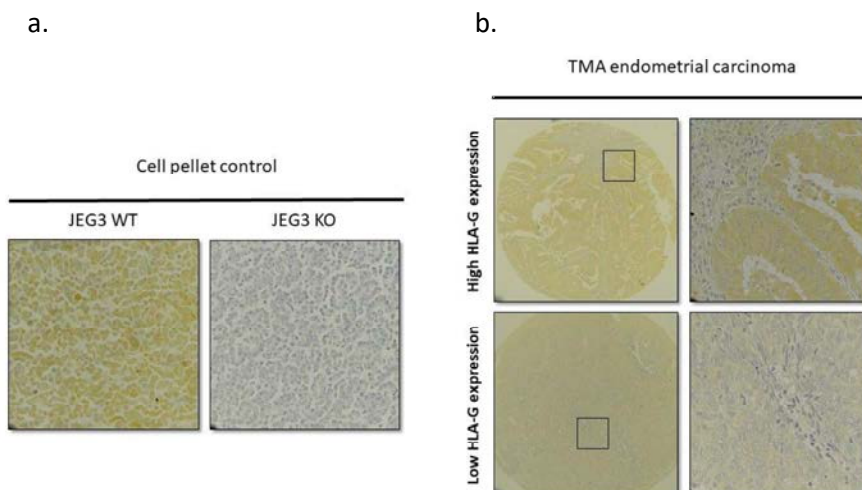


Figure 2. HLA-G IHC staining. (a) IHC of a JEG3 cell pellet JEG3 WT (left) and JEG3 HLA-G KO (right) used as control of staining (10x). (b) IHC of TMA of endometrial carcinoma showing a representative sample of high HLA-G expression and low HLA-G expression (left 4x, right 20x) (anti human HLA-G, Bionova, clone MEM-G1, 1/20 dilution).

3. *In vitro* screening of cell lines expressing HLA-G

To study the effect of HLA-G in the immune system, we performed *in vitro* experiments. To this end, we first needed to have tumor cell lines expressing HLA-G at basal conditions. In this way, we would obtain a physiological source of HLA-G expressed as well as secreted due to capability of the HLA-G molecule to be both expressed on the cell surface and secreted to the media (See Section 2.1 in the Introduction).

From the cell lines available at the cell's bank from the lab and in correlation with the TCGA results presented above, we screened the following cell lines: HuP-T3 (pancreatic adenocarcinoma), SK-OV-3 (ovary adenocarcinoma), NCI-H2009 (lung adenocarcinoma) and CRL-5816 (non-small cell lung cancer). Moreover, choriocarcinoma cell line JEG3 was used as a positive control, since high expression of HLAG has been previously described for this cell line (18).

We checked HLA-G RNA expression (Figure 3a) and at total protein level (Figure 3b). JEG3 cells were used as positive control. Among the five cell lines checked, two resulted positive for the HLA-G expression: HuP-T3 and NCI-H2009. Next, we checked HLA-G expression at cell surface by Flow cytometry (FC) (Figure 3c-d). The FC assay revealed the presence of HLA-G on the cell membrane of around 25% of the HuP-T3 cells but not in the NCI-H2009. In addition, we collected the cell supernatants and checked them by ELISA for the presence of secreted-HLA-G (Figure 3e). JEG3 cells secreted the highest amount of HLA-G doubling the levels present in the HuP-T3 supernatants, the NCI-H2009 as expected, did not secrete HLA-G therefore this cell line it was discarded for future experiments.

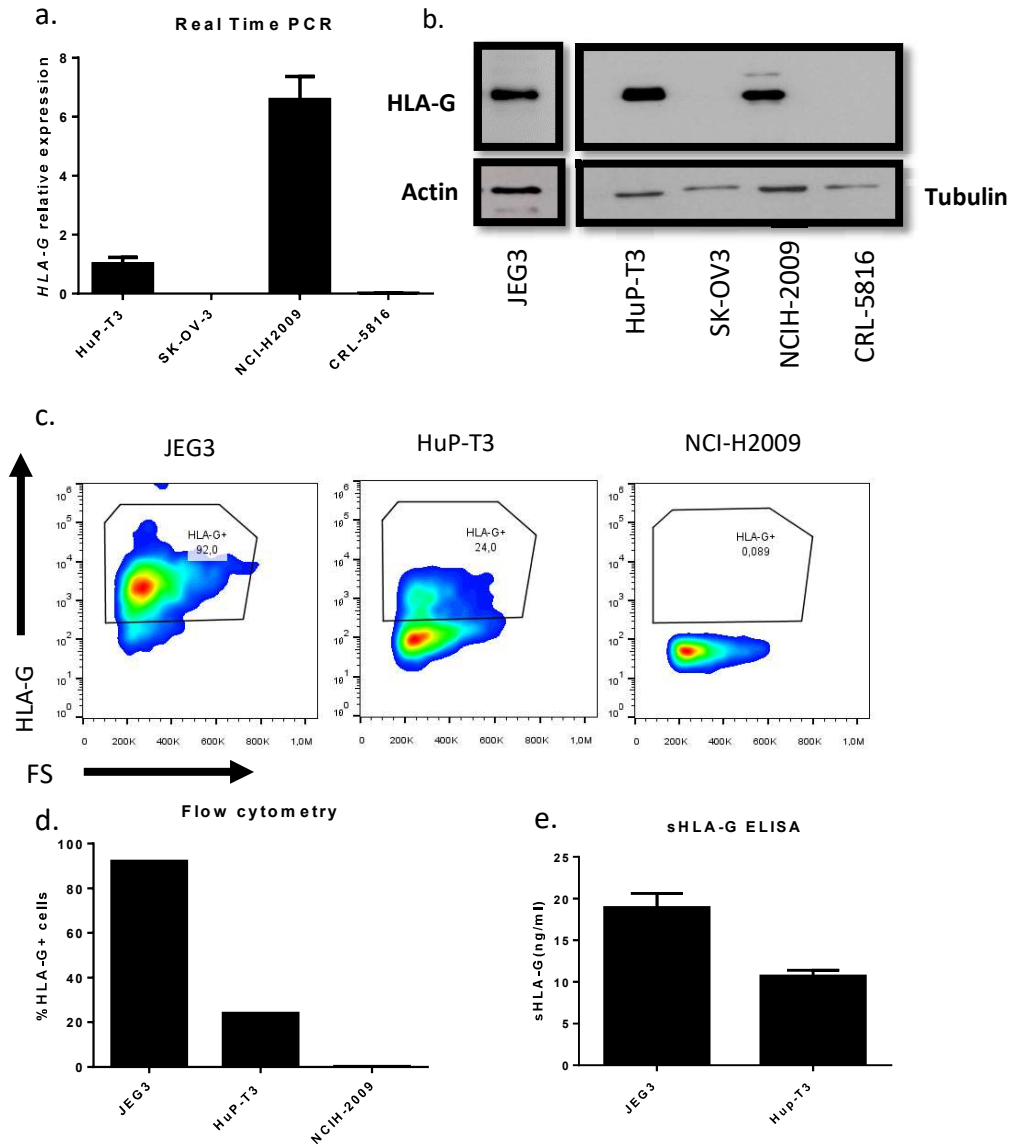


Figure 3. Screening of cell lines to check HLA-G expression. Five tumor cell lines were checked for the expression of HLA-G. (a) HLA-G expression at RNA level checked by qPCR. (b) HLA-G expression at protein level checked by western blot. (c) Cytofluorimetric analysis of HLA-G expression in HuP-T3 and NCI-H2009, JEG3 (chorioepithelioma cell line) was used as a positive control. (d) Graphical representation of the HLA-G positive cells quantified by flow cytometry. (e) Graph showing HLA-G secreted protein quantified by ELISA.

4. Generation and characterization of HLA-G KO clones

To study the effect of HLA-G in the immune system (IS) apart from the HLA-G positive cell lines previously screened and characterized, HLA-G KO cells were also required.

First, we tried to silence the HLA-G expression with short-hairpin RNA, however, the silencing was not efficient enough to completely ablate HLA-G expression, especially for the strong expression present in the JEG3 cell line (data not shown). For this reason, we performed CRISPR/Cas9 technology in the JEG3 and in the HuP-T3 cells. For each of them, KO clones were generated and screened as it was explained in Materials and Methods (See Section 2). For JEG3 cells, 14 clones successfully grew and were analysed for HLA-G by qPCR. In two of them, HLA-G was completely silenced: clone number 9 (KO-9) and clone number 12 (KO-12) (Figure 4a). To further confirm these results, the presence of HLA-G was also checked at the cell surface by FC (Figure 4c-d), in addition cell supernatants were collected and quantified by ELISA (Figure 3e). For the HuP-T3 cell line, from the 5 clones generated, one HLA-G KO clone was obtained: clone number 1 (KO-1) (Figure 3b). This KO clone was further characterized by FC (Figure 3c-3e) and ELISA (Figure 4g).

In summary, cell characterization resulted in 100% HLA-G positive JEG3 wild-type (WT) cells and HuP-T3 WT around 25% of HLA-G positive HuP-T3 wild-type cells. The CRISPR/Cas9 technology was able to completely ablate the HLA-G expression in both cell lines. Moreover, the supernatants recovered from the KO clones were negative for the HLA-G secretion. Therefore, we obtained for both cell lines, a KO counterpart completely lacking HLA-G expression (Figure 4).

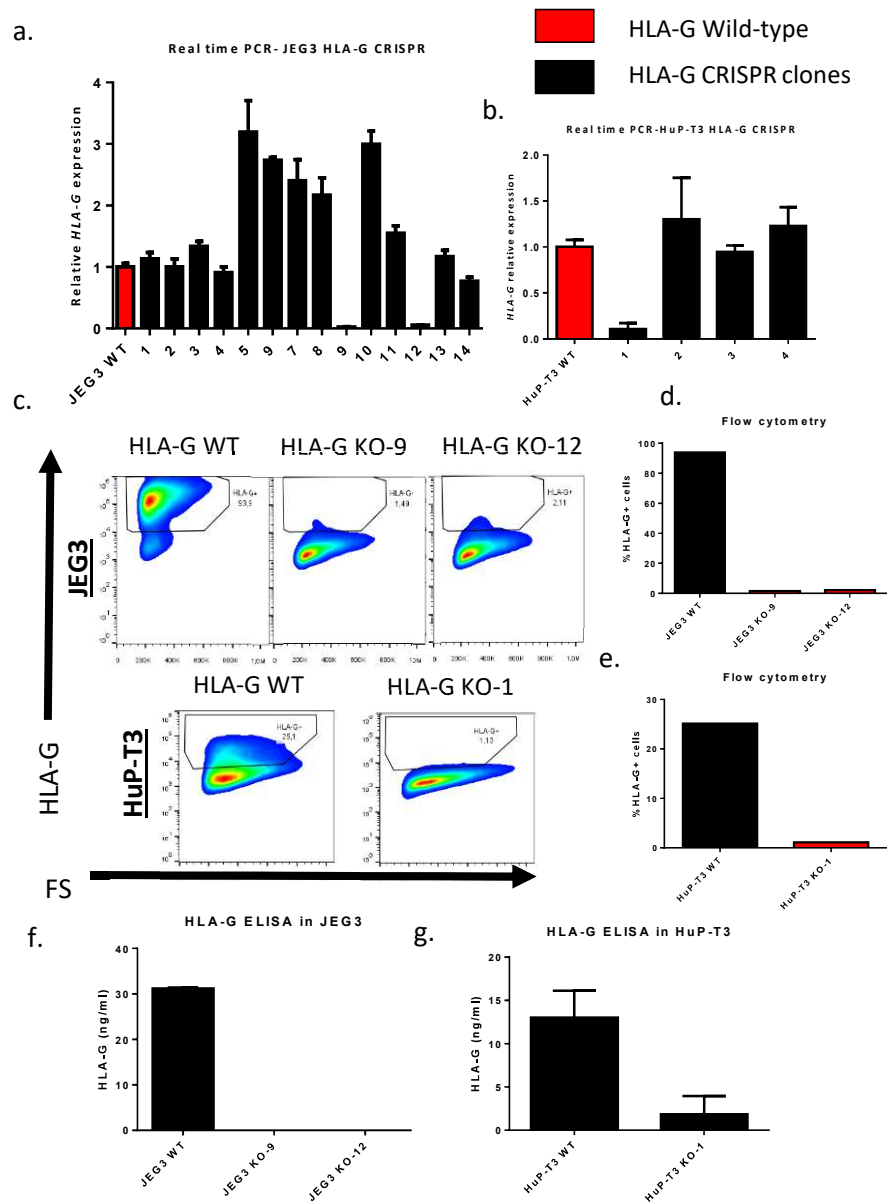


Figure 4. Characterization of HLA-G CRISPR clones. Screening of CRISPR clones for the HLA-G expression at RNA level by qPCR in (a) JEG3 and (b) HuP-T3 (c) Cytofluorimetric analysis of HLA-G expression in JEG3 and HuP-T3 comparing WT and HLA-G KO clones. Graphical representation of the HLA-G positive cells quantified by flow cytometry in JEG3 (d) and HuP-T3 (e). Secreted HLA-G quantified by ELISA in JEG3 WT and JEG3 KO clones (f) and in HuP-T3 WT and KO (g).

4.1 Characterization of the expression of HLA-G receptors in NK cells

Once we obtained the target cells (JEG3 and HuP-T3) expressing or lacking the HLA-G molecule, we also needed the effector cells: cells from the immune system able to recognize and bind to the target cells through a HLA-G specific receptor (See Section 2.3 of the Introduction)

Among all the cells in the immune system, we used natural killer (NK) cells due to its physiological relationship with HLA-G as it was explained in the Introduction (Section 4). Two different sources of NK cells were used for our experiments. On the one hand, we worked with the widely used and described NK92 cell line. On the other hand, we used NK cells isolated from peripheral blood mononuclear cells (PBMCs), named peripheral blood NK (pb-NK) cells.

First, we characterized the expression of the HLA-G receptors in both NK cell types. We checked by Real Time PCR the expression of ILT-2 and KIR2DL4 receptors (Figure 5a). Additionally, the level on their cell surface expression was also characterized by FC (Figure 5b). At RNA level we observed the expression of both receptors. For ILT-2, the expression on NK92 cell line doubled the expression of pb-NK cells. In the case of KIR2DL4, the expression was similar for both cell types. Regarding the cell membrane expression, we observed that almost 100% of the NK92 cells expressed ILT-2 in their surface, whereas it was only expressed by 25% of the pb-NK cells. This might be due to the different subpopulations present on these cells as it will be further explained in the Discussion. However, KIR2DL4 expression was almost undetectable in the cell surface of both NK cell populations, as previously reported (30).

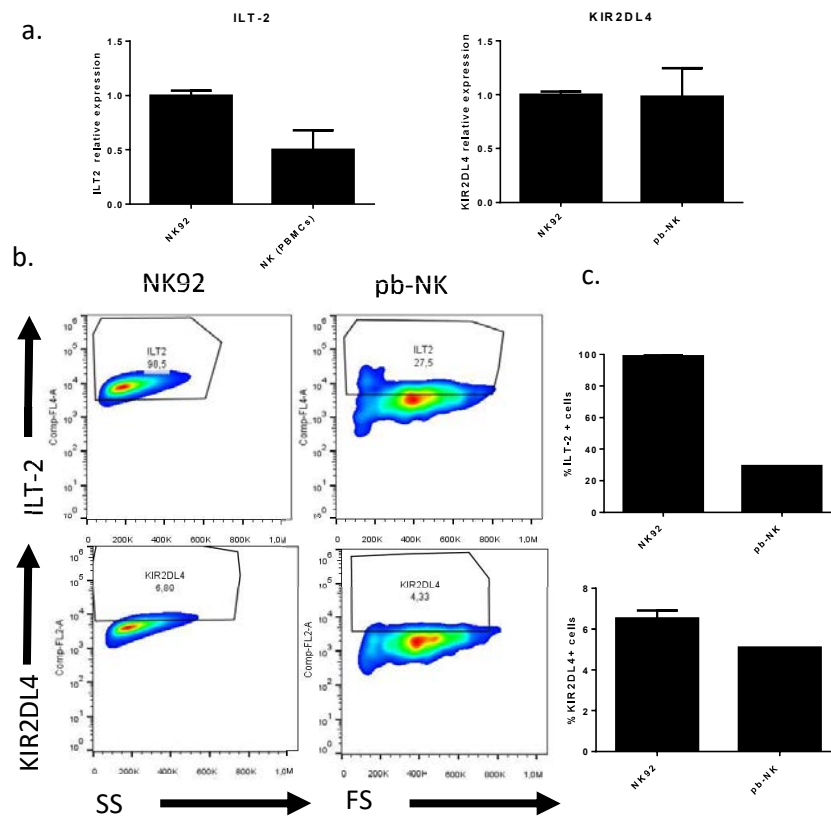


Figure 5. HLA-G receptor expression in NK92 and pb-NK cells. (a) ILT- 2 and KIR2DL4 expression checked at RNA level by quantitative PCR. (b) Cytofluorimetric analysis of ILT-2 and KIR2DL4 expression in NK92 and pb-NK cells (c) Graphical representation of the ILT-2 and KIR2DL4 positive cells quantified by FC.

At this point, we have characterized the target cells (JEG3 and HUP-T3) expressing high or medium levels of the HLA-G molecule and the HLA-G KO counterparts lacking its expression. Moreover, we have also described the effector cells (NK92 and pb-NK) regarding the levels of the HLA-G receptors. Therefore, we have the tools to study the implication of HLA-G in the regulation of the immune system. Next step was to set the best conditions for the experimental model that allowed us to understand the functional implications of HLA-G in relation with the IS in the context of cancer (Figure 6).

5. Study of HLA-E expression in HLA-G KO clones

Given the relationship between HLA-G and HLA-E previously explained (See Section 3.3 of the Introduction), we checked by flow cytometry the HLA-E expression in JEG3 and HuP-T3 cell lines in WT and KO clones. However, we did not observe any expression neither in the WT nor in the HLA-G KO (Figure 6).

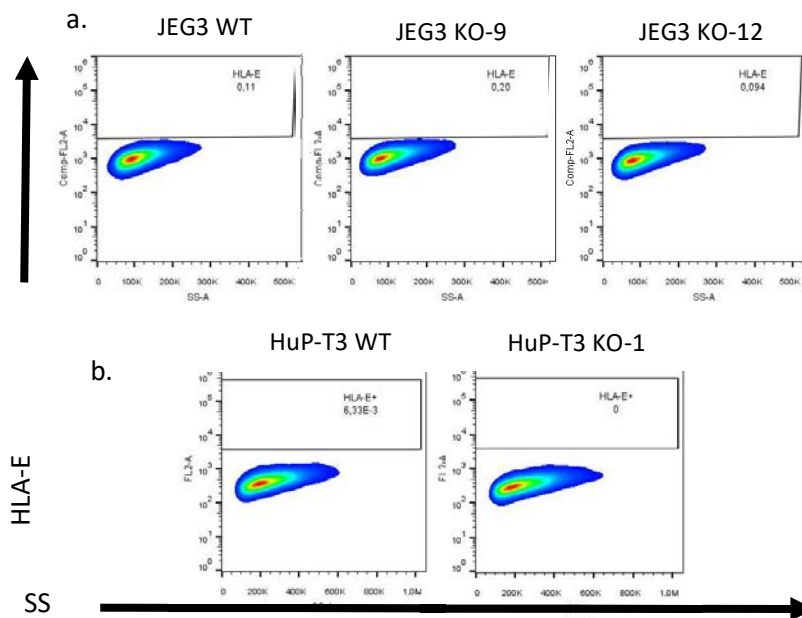


Figure 6. Cytofluorimetric analysis of HLA-E expression in JEG3 and HuP-T3 in WT and HLA-G KO clones. (a) HLA-E expression in JEG3 WT, KO-9 and KO-12. (b) HLA.E expression in HuP-T3 WT and KO-1.

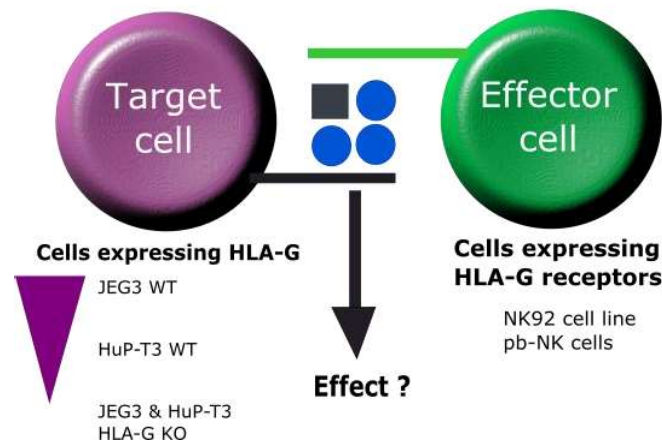


Figure 7. Diagram showing the tools for the study of the HLA-G effect in the IS. In purple the target cell harbouring the HLA-G molecule on its membrane. In green the effector cell binding the HLA-G molecule through the HLA-G receptor.

6. Modulation of CD56 expression in NK92 cells after coculture with JEG3

To evaluate the effect of HLA-G in the IS, we performed coculture (CC) experiments of JEG3 and NK92 cells. We did an overnight (O/N) coculture with an effector-target ratio of 5:1 in the presence of 200 IU of rh-IL-2. After this time, we evaluated CD56 expression in NK92 cells by flow cytometry. As shown in figure 8, CD56^{bright} population was more abundant when cultured with JEG3 WT. This result suggests a role for HLA-G in the maturation of the NK cells, since the NK cells loose the CD56 marker in their process of differentiation as it was previously described (See Section 4.3 of the Introduction).

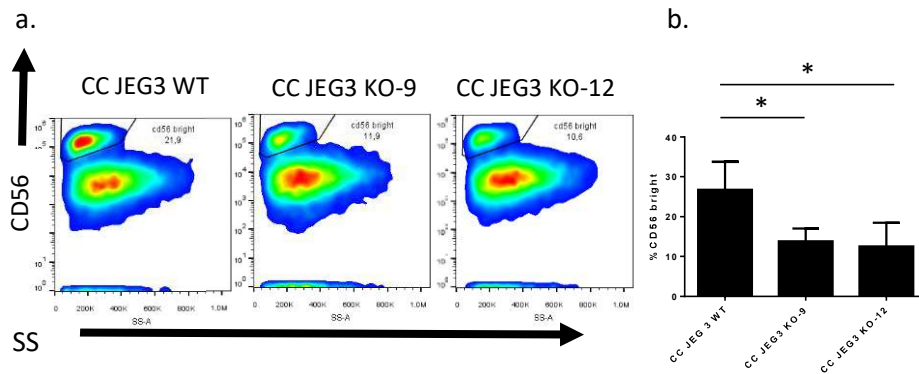


Figure 8. Flow cytometry expression of CD56 in NK92 after coculture with JEG3 cells. (a) Representative dot-plot showing CD56 expression in NK92 cells. (b) Graphical representation of the CD56 bright population quantified by flow cytometry. Three independent experiments were performed. Representative results are shown. Data are presented as mean \pm SEM.

IL-2 cytokine as described in the Introduction (Section 4.6) has an important role in NK cell modulation, in fact it promotes the CD56 bright population (77), moreover, NK92 cell line as mentioned before is IL-2 dependant (Section 4.4 of the Introduction). To establish rh-IL2 dose, we checked how different concentrations of this cytokine modulate CD56 expression in NK92. We performed CC experiments using three different doses of IL-2 (10 IU, 100 IU and 200 IU) and checked CD56 expression. We observed that the 10 IU dose was too low and the NK92 died (data not shown), the 100 IU dose had an effect in CD56 modulation, however the percentage of the CD56^{bright} NK92 cells was too low, finally, the highest dose used resulted in the best conditions of NK92 culture, so we kept on using this 200IU dose (Figure 9).

7. Changes in NK92 cell line promoted by HLA-G

After the changes observed in the abundance of CD56 on the NK92 surface when cocultured with JEG3 cells, we wanted to check whether the secreted HLA-G present in the cell culture supernatant of JEG3 WT or HuP-T3 WT was also able to promote this changes in the absence of the HLA-G anchored to the membrane. To do so, we used the conditioned media (CM) generated from JEG3 WT, KO-9 and KO-12 (Figure 10a) or HuP-T3 WT and KO-1 (Figure 10c) to treat the NK92 cells (See Section 4 in Materials and Methods). The CM generated in JEG3 WT and HuP-T3 was used as a source of HLA-G, whereas the CM from the KO clones was used as a negative control due to its absence of HLA-G. Moreover, supernatants were concentrated 4 times to increase the HLA-G amount in JEG3 CM (Figure 10b).

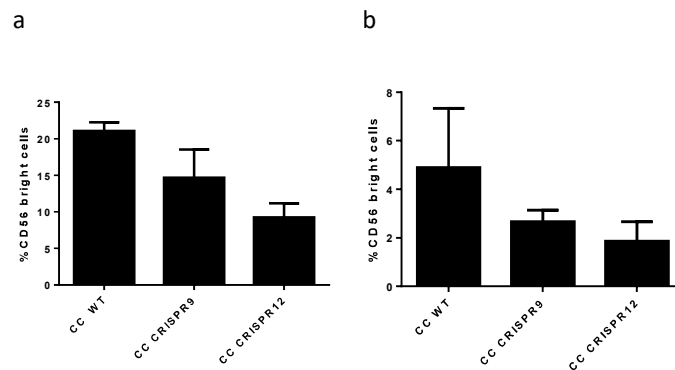


Figure 9. Graph showing the quantification of CD56 bright population in NK92 cells cocultured with JEG3 cells in the presence of different doses of rh-IL2. (a)200 IU (b) 100 IU.

7.1 Secreted HLA-G from JEG3 cells upregulates CD56 expression in NK92

After O/N culture with CM from JEG3 WT or JEG3 HLA-G KO, the expression of CD56 on the NK92 surface was modulated. It is important to highlight that in these experiments, the changes in the CD56^{bright} population were less appreciable when plotted since most of the cells were CD56^{bright}, therefore we decided to evaluate the changes on its expression by quantifying the abundance of the CD56^{dim} population instead. In summary, an increase on the CD56^{dim} population means that the CD56 surface abundance is decreased.

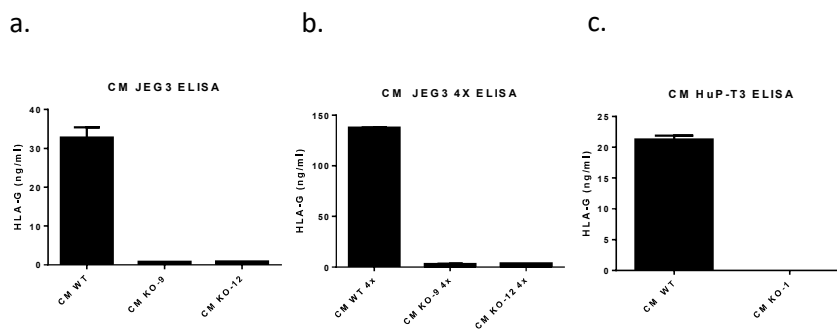


Figure 10. Secreted HLA-G amount present in the conditioned media from JEG3 WT and JEG3 HLA-G KO clones quantified by ELISA. (a) Representative ELISA showing the level of HLA-G in the conditioned media from JEG3 cells (b) in the CM after its concentration 4 times and (c) in HuP-T3 cells CM. When data obtained was below the standard curve, it was assumed as 0. Representative results are shown.

As shown in Figure 11, the CM from JEG3 WT (secreted HLA-G source), significantly upregulated CD56 expression on NK92 cells after the O/N culture. However, when cultured with the CM from JEG3 HLA-G KO and therefore in the absence of s-HLA-G, NK92 cells downregulates CD56 expression as we can observe by the increase of CD56^{dim} population. This effect was observed for both of the JEG3 KO clones.

Moreover, we wanted to check if by increasing the amount of HLA-G present in the conditioned media we were able to increase the effect on CD56 abundance. With this aim, we concentrated four times the CM with Amicon Ultra 4 centrifugal filter (Millipore) and consequently increasing four times the amount of HLA-G (Figure 10b). However, we were not able to see changes on the CD56 abundance (Figure 11c).

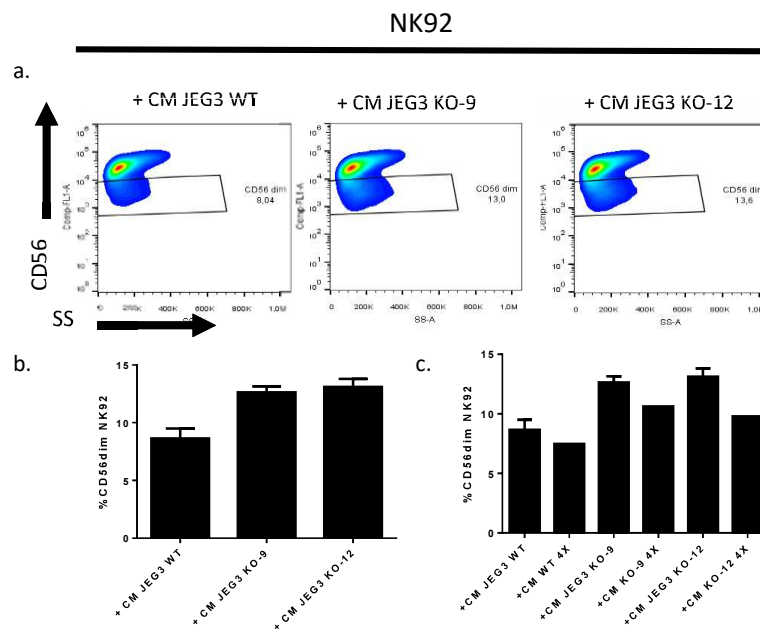


Figure 11. Flow cytometry expression of CD56 in NK92 treated overnight with conditioned media from JEG3 WT or CRISPR. (a) Representative dot plots showing the modulation of the CD56 cell surface expression in NK92 cells. (b) Graphical representation of the CD56^{dim} NK92 population quantified by flow cytometry in the presence of conditioned media and (c) in the presence of 4 times concentrated conditioned media.

Also, we wanted to test whether the addition of secreted HLA-G from a different cell line was able to promote changes in the CD56 pattern. With this aim, we added the CM generated from HuP-T3 cell line that had lower levels of the protein (Figure 10c). However, in this case we were not able to see a CD56 modulation (data not shown), this result suggests that there is a threshold for the secreted HLA-G amount that is needed to produce an effect on the NK92 cells.

7.2 The changes on the CD56 expression in NK92 cells promoted by CM from JEG3 are time dependant

We evaluated the effect of JEG3 CM on CD56 expression at different time points. We performed a time-course experiment in which we cultured NK92 cells with CM and stopped it after 18h, 40h or 64h. We evaluated the percentage of CD56^{dim} NK92 cells at the different time points. This analysis was performed by flow cytometry.

As shown in figure 12, the percentage of CD56^{dim} NK92 cells was downregulated in the presence of secreted HLA-G at the three time points checked. However, the most significant changes were observed at the 40h (figure 12b).

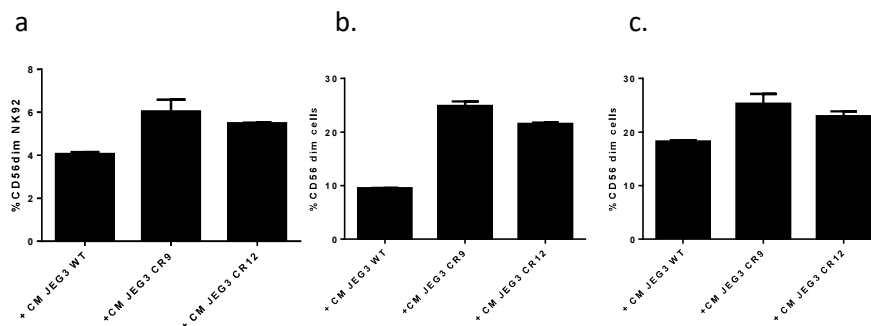


Figure 12. Flow cytometry expression of CD56 in NK92 treated at three different time points. (a) 16h (b) 40h (c) 64h.

6.3. IFN γ secretion is downregulated in NK92 cells by CM from JEG3 WT

We have observed that the CD56 expression was upregulated in the presence of JEG3 WT cells or its conditioned media in comparison with JEG3 HLA-G KO. This suggests a role for HLA-G in the regulation of NK cell maturation and activity since NK cells downregulate CD56 expression during this process.

In order to test if this could have a biological effect, we investigated the IFN γ secretion pattern since it has an essential role in the regulation of the NK cell activity.

We collected the supernatants from the NK92 cells treated with the JEG3 CM (HLA-G WT or KO) during 40h, and quantified IFN γ secretion by ELISA. We observed that the secretion pattern in NK92 cells was different when treated with CM from JEG3 WT or CRISPR. Interestingly, the level of IFN γ was significantly lower in the presence of CM from JEG3 WT (Figure 13a).

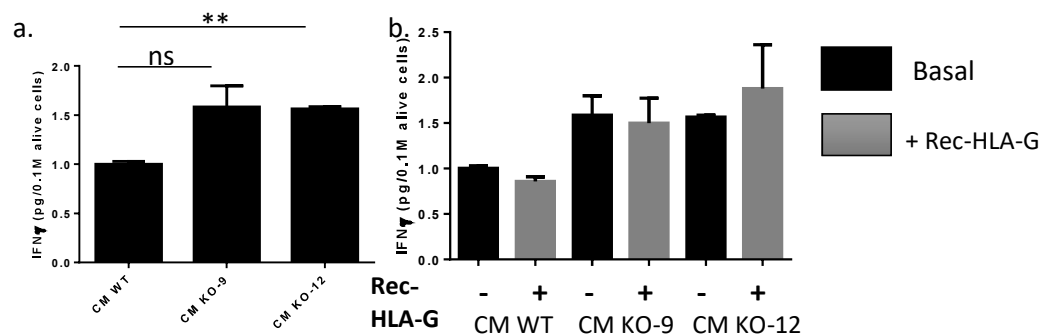


Figure 13. IFN γ secretion in NK92 quantified by ELISA. (a) IFN γ secretion by NK92 after being cultured 40h with conditioned media from JEG3 WT or HLA-G KO. (b) IFN γ secretion after 40h treatment with recombinant HLA-G (200ng/ml). Data correspond to three independent experiments and are presented as Fold Change (FC). Data are presented as mean \pm SEM.

6.4. Granzyme B and perforin secretion is downregulated in NK92 cells by CM from JEG3 WT

We have shown in the previous section that IFN γ was downregulated in the presence of JEG3 WT conditioned media compared with the addition of JEG3 HLA-G KO CM. This suggests a role for HLA-G in the regulation of NK cell activity.

In order to test if also the cytotoxicity of the NK cells was modulated by HLA-G, we investigated granzyme and perforin secreted pattern.

We collected the supernatants from the NK92 cells treated during 40h with JEG3 CM either HLA-G WT or KO, and quantified the granzyme B (GZMB) and perforine (PRF1) secretion by ELISA. We observed that the secretion amount in NK92 cells was different when treated with CM from JEG3 WT or KO. Importantly, the level of both granzyme B and perforine was significantly lower in the presence of CM from JEG3 WT (Figure 14 a-b), suggesting a role for HLA-G in the downregulation of NK cell activity given the implication of this molecules in their cytotoxic function.

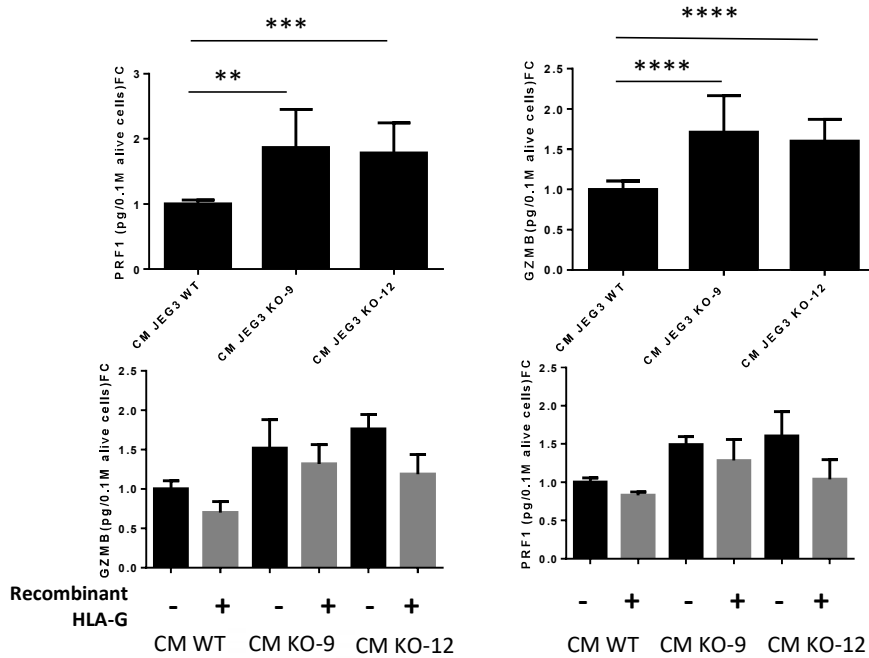


Figure 14. Granzyme B and perforin secretion in NK92 quantified by ELISA. The data is represented as fold change (FC), it was calculated as the ratio of the difference between the value of CM JEG3 KO-9 or CM JEG3 KO-12 and the value of CM JEG3 WT considered as one. (a) Granzyme B and (b) perforin secreted by NK92 after being cultured 40h with conditioned media from JEG3 WT or HLA-G KO.(c,d) Effect of the addition of recombinant-HLA-G. Data correspond to three independent experiments and are presented as Fold Change (FC). Data are presented as mean \pm SEM.

6.5 The addition of recombinant HLA-G reverts the effect in CD56 population and reverts partially the secretion pattern of NK92 cells

Furthermore, to test if the effect observed in the modulation of CD56 expression as well as in the release of IFN γ , granzyme B and perforin was due to the addition of HLA-G, we checked the effect of the recombinant HLA-G protein (Origene).

We added recombinant HLA-G (200ng/ml) to the culture and tested whether it was able to revert the effect produced by the CM from HLA-G KO clones. NK92 cells together with the CM were treated with the recombinant protein (200ng/ml) during 40h. Cells were recovered and analysed by flow cytometry and supernatants were also collected for further tests. Interestingly, we observed a down-regulation of the CD56 expression in all the conditions (Figure 15a-b). Meaning that, not only the recombinant protein was able to revert the effect in the NK92 cultured with the CM from JEG3 KO clones but also, the effect produced by the CM from JEG3 WT was increased with the addition of the recombinant HLA-G. This result suggests an additive effect of the HLA-G protein in relation with CD56 expression. Importantly, the cell viability was not significantly altered by the treatment (Figure 15c).

We evaluated if the IFN γ secretion pattern was reverted by recombinant HLA-G (200ng/ml). We performed ELISA of IFN γ in the supernatants recovered from the experiments previously described. However, in this case, we did not observe any effect of the recombinant protein in the secretion of this cytokine (Figure 13b) suggesting a more complex regulation that might involve many other factors. Also, we checked the intracellular levels of IFN γ by flow cytometry, however we found very low or undetectable amounts of it (Data not shown).

Regarding the granzyme B and perforin secretion, the addition of recombinant-HLA-G slightly reverted it in the culture with CM KO condition and increases the effect produced by the CM WT (Figure 14 c-d).

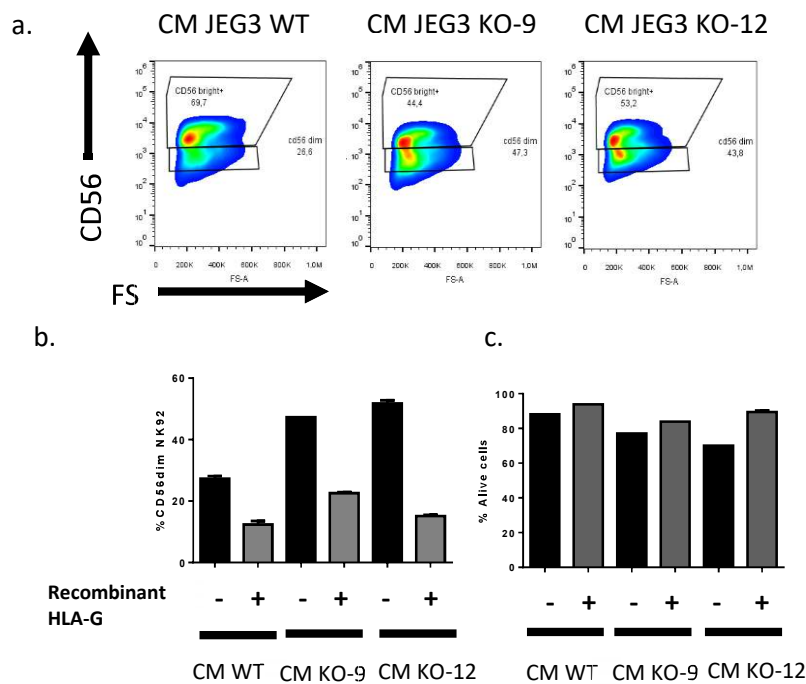


Figure 15. Modulation of CD56 expression in NK92 by the treatment with recombinant HLA-G (200ng/ml) to the JEG3 conditioned media.(a) Representative dot plots showing CD56 expression of NK92 when cultured 40h with conditioned media from JEG3 and recombinant HLA-G. (b) Graphical representation of the CD56 dim NK92 population quantified by flow cytometry. (c) Graphical representation of the percentage of alive cells for each condition.

8. Changes in peripheral blood NK cells promoted by HLA-G

Next, we evaluated the effect of HLA-G on the pb-NK cells in order to have a more physiological model. In this way, as well as for the NK92 cells, we performed flow cytometry analysis of the CD56 cell surface abundance after 40h treatment with CM from JEG3 cells.

8.1 Modulation of the CD56/CD16 population in pb-NK cells after culture with CM from JEG3 cells

In the pb-NK cells apart from CD56 we also checked CD16 marker (NK92 do not express it) that together with CD56 define the different NK subpopulations (Figure 16).

Unlike NK92, most of pb-NK cells at basal condition are CD56^{dim}, meaning that they do express lower levels of this molecule (Section 4.3 of the Introduction). Therefore, to quantify the changes on CD56, we analysed the increase of the CD56^{bright} population.

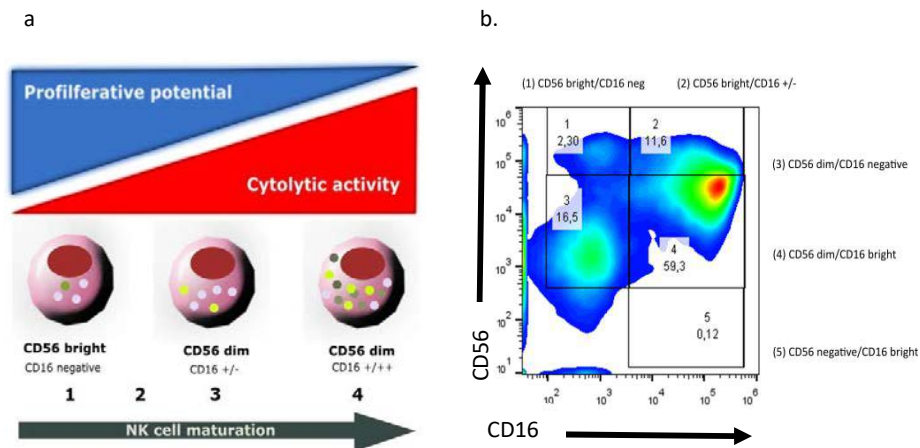


Figure 16. Peripheral blood NK cells subpopulations (a) Model of human NK cell differentiation (adapted from 63) (b) Cytofluorimetric dot plot showing the gating strategy for the different NK cell subpopulations based on the relative expression of CD56 and CD16. Population (1) has immunoregulatory activity whereas (4) is the more cytotoxic, (2) and (3) would represent intermediate states, and the role of population (5) is still unknown.

We observed that, CM from JEG3 WT cells did not produce major changes in the CD56 surface expression on pb-NK cells (Figure 17a), but when we checked CD56^{bright} population in relation with CD16 expression, we observed a slight down-modulation of the CD56^{bright} NK cells that are gated in the CD16^{dim/negative} population (Figure 17b). However, this modulation was not significant.

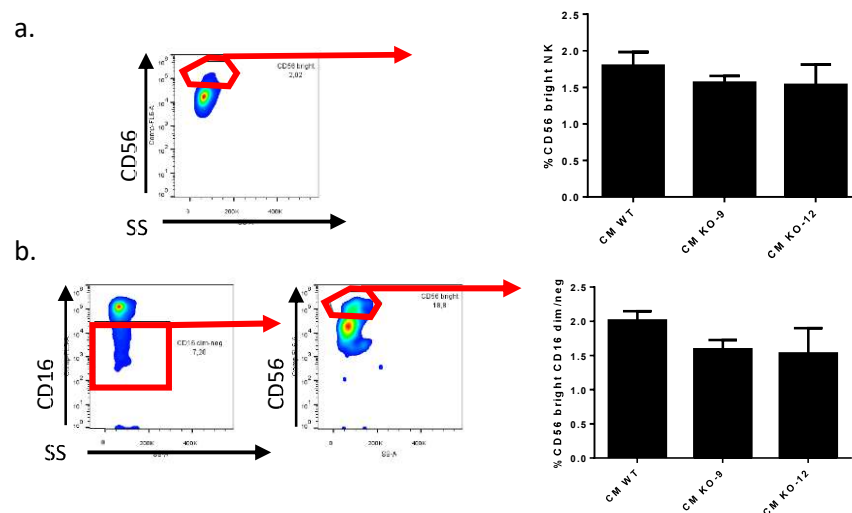


Figure 17. CD56/CD16 modulation in pb-NK cells after 40h culture with in the CM from JEG3. The data is represented as fold change (FC), it was calculated as the ratio of the difference between the value of CM JEG3 KO-9 or CM JEG3 KO-12 and the value of CM JEG3 WT considered as one. (a) Representative staining with anti-CD56 gated inside alive cells or (b) inside the CD16 dim/negative pb-NK cells (gated previously inside the alive population). Quantification is shown in the bar chart on the right. Data correspond to three independent experiments and are presented as Fold Change (FC). Representative dot plots are shown.

8.2 Recombinant HLA-G shapes the CD56/CD16 abundance in pb-NK cells

Given the slight modulation observed when culturing pb-NK cells together with CM from JEG3, we tested whether a high dose of HLA-G was able to produce a significant effect. To do so, we treated the NK cells with recombinant-HLA-G (200ng/ml, Origene) during 40h as performed previously for NK92 cells. After the treatment, we quantified the cell surface markers by flow cytometry. We plotted CD56/CD16 to define the different NK subpopulations as shown previously in Figure 17b.

In this case, we observed a significant upregulation of the CD56 expression when cultured with recombinant HLA-G (Figure 18a-b), suggesting that HLA-G promotes an immature state of pb-NK cells. Also, the CD56^{bright}/CD16^{dim/negative} population was upregulated in this conditions (Figure 18c-d).

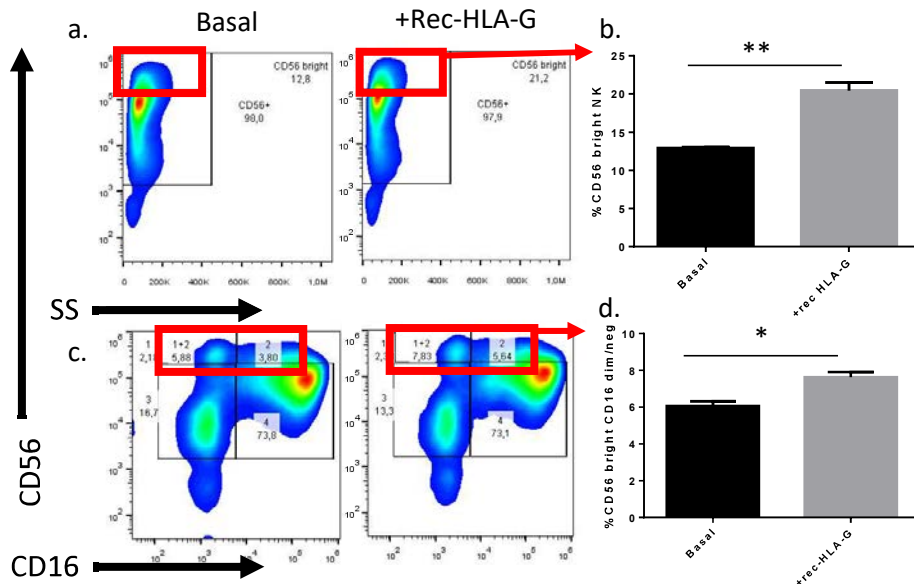


Figure 18. Flow cytometry expression of CD56/CD16 in NK cells isolated from PBMCs at basal conditions and after 40h treatment with recombinant HLA-G (200ng/ml). (a) Representative dot plots showing the CD56 expression in NK cells (b) Graphical representation of the CD56 bright NK population quantified by flow cytometry. (c) Representative dot plots showing CD56/CD16 expression in NK cells. (d) Graphical representation of CD56^{bright}/CD16^{dim/neg} population. Three independent experiments were performed. Representative results are shown. Data are presented as mean \pm SEM.

8.3 CM from JEG3 does not modulate IFN γ secretion in pb-NK cells and recombinant HLA-G has a slight effect

Next, we tested if the secreted HLA-G present in the CM from JEG3 WT apart from modulating the cells surface markers CD56 and CD16, had also an effect in the cytokine secretion.

We evaluated the effect of treating pb-NK cells with CM from JEG3 in the modulation of IFN γ . However in this case we were not able to see any effect neither by ELISA nor by Flow cytometry (Data not shown).

Moreover, we tested the effect of recombinant HLA-G treatment (200ng/ml), and we observed a slight down-modulation of IFN γ secretion after 40h treatment (Figure 19a).

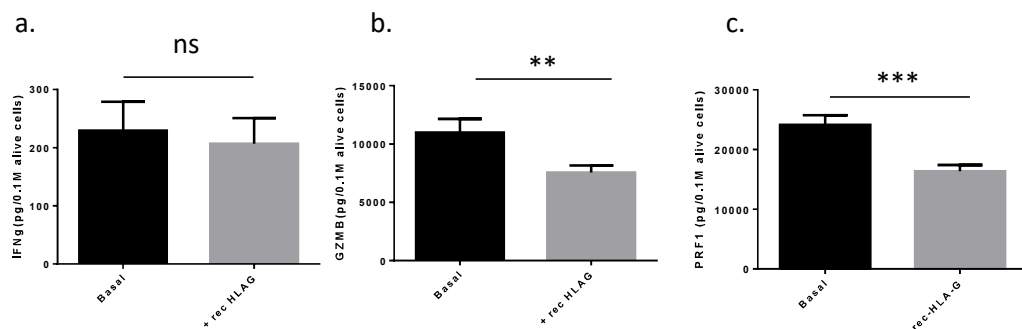


Figure 19. Cytokine secretion pattern of pb-NK cells quantified by ELISA in the presence or absence of recombinant HLA-G. (a) IFN γ (b) Granzyme B (c) Perforin. Two independent experiments were performed. Representative data is shown. Data are presented as mean \pm SEM.

This differential regulation of IFN γ in pb-NK cells compared with NK92 might be related to the differential abundance of CD56 populations and their ability to secrete IFN γ . This aspect will be deliberated in detailed in the Section 2 of the Discussion.

In addition, we evaluated the effect of HLA-G on the secretion of granzyme B and perforin in pb-NK cells. To this end, we cultured pb-NK cells in the presence or absence of recombinant HLA-G (200ng/ml) during 40h. Culture supernatants were recovered and the levels of granzyme B and perforin were quantified by ELISA.

As shown in Figure 19 b-c, recombinant HLA-G significantly down-regulated the secretion of granzyme B and perforin, suggesting a role for HLA-G in inhibiting the cytolytic activity of pb-NK cells.

9. Blocking experiments

To demonstrate unambiguously the role of HLA-G in the upregulation of CD56 and subsequent downregulation of IFN γ , granzyme B and perforin, we performed experiments in the presence of anti-HLA-G monoclonal antibody (mAb) (87G, Thermofisher) in order to block its activity.

9.1 The HLA-G blocking antibody (87G) does not revert the CD56 modulation in NK92 cells

Expression of CD56 was evaluated on NK92 cells that were treated with JEG3 CM for 40h. To test the effect of the antibodies, the CM from JEG3 WT was preincubated during 30 min with the HLA-G blocking antibody (10ug/ml, Thermofisher).

However, as shown in Figure 20(a-b) we were not able to see the reversion of the effect produced by the CM from JEG3 WT. With the same aim, we tried to block the HLA-G receptors: ILT-2 (5ug/ml) and KIR2DL4 (5ug/ml) but we did not observe reversion in the CD56 expression pattern (Figure 20c). Surprisingly, the blockade of ILT-2 resulted in a decrease in the CD56^{dim} population (data not shown).

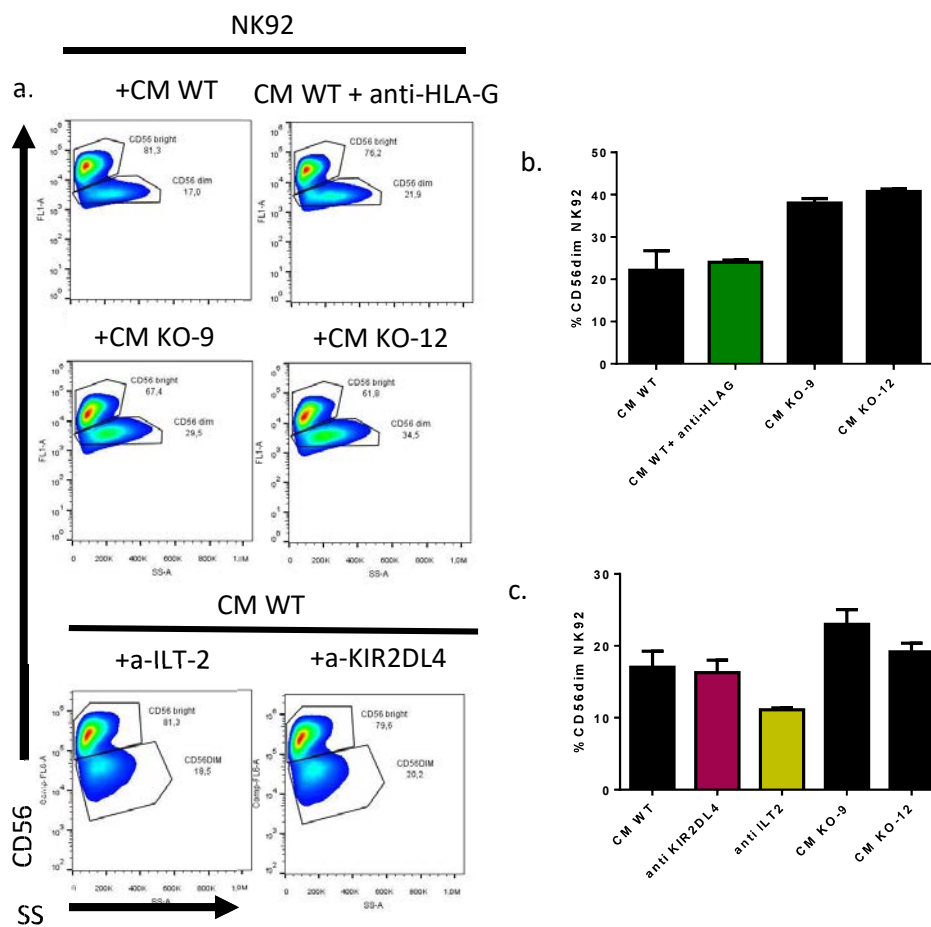


Figure 20. Blocking experiments. NK92 were treated with conditioned media from JEG3 WT, KO-9 and KO-12. The CM from JEG3 WT was pre-treated with anti-HLA-G antibody, anti-KIR2DL4 or anti-ILT-2. (a) Representative cytofluorimetric analysis of CD56 expression in NK92. CD56 bright and CD56 dim populations are gated inside total alive population. (b, c) Graphical representation of CD56 dim NK92 population in the indicated culture conditions.

There is a lot of controversy regarding the specificity of the HLA-G antibodies as it has been previously described (28). Therefore, we decided to design our own HLA-G blocking antibody with the aim of specifically block the HLA-G interaction with its receptors.

9.2 The design of a specific anti-HLA-G blocking antibody pointed out the alpha-3 domain as the best target candidate

In order to design a specific anti-HLA-G blocking antibody, we first defined the region to be recognized by the antibody among the HLA-G protein sequence. With the aim of functional blocking the effect of HLA-G, we decided to target the alpha-3 domain since it is the region that interacts with the HLA-G receptor as it have been previously described (7)

We decided to produce a monoclonal antibody because they are more specific and sensitive to target recognition and also to avoid cross-reactivity as it seems to be a major problem for the anti-HLA-G antibodies already available (28). The antibody was produced in mice.

9.3 Production of specific monoclonal anti-HLA-G antibody resulted in a positive specific subclone selection: 7H4-4

The production of the anti-HLA-G monoclonal antibodies undergo several steps as it was explained in detail in Material and Methods. We will summarize the results obtained in the four main steps: Mice immunization, fusion and hybridoma screening, hybridoma subcloning and antibody purification.

- **Immunization: One mouse were selected for subsequent steps**

First, serum was extracted pre and post-immunization steps from 10 immunized mice.

In total, twenty anti-serums were sent to us for selection (Figure 21). The JEG3 WT and CRISPR cells were used for positive and negative control of the HLA-G screening (both JEG3 KO-9 and KO-12 were used). Moreover, the commercial antibody (abcam7758, clone MEM-G9) was used as positive control for HLA-G detection.

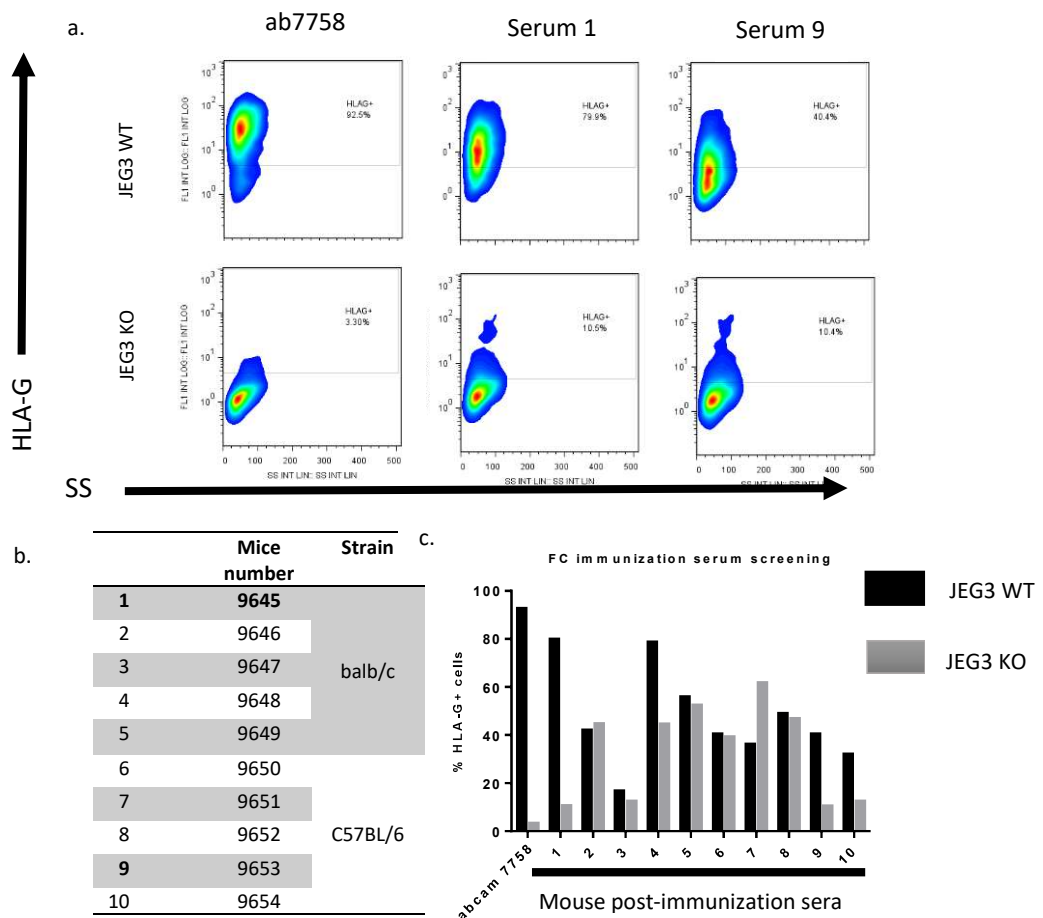


Figure 21. Flow cytometry screening of immunization serum from mice. (a) Cytofluorimetric analysis of HLA-G expression (b) Table of the different mice used in the immunization. (c) Graphical representation of the HLA-G positive cells detected by Flow cytometry. Three independent experiments were performed. Representative data is shown.

The analysis was performed by flow cytometry, and the cells were gated within the total alive cell population. The animals whose post-immunization sera resulted in the highest % of HLA-G recognition in JEG3 WT cells, were considered as good candidates. This screening step resulted in one animal selected for the following fusion step: 9645 (1) (Figure 21). Moreover, the pre-immunization sera from the two selected mice was checked as a negative control (Figure 22).

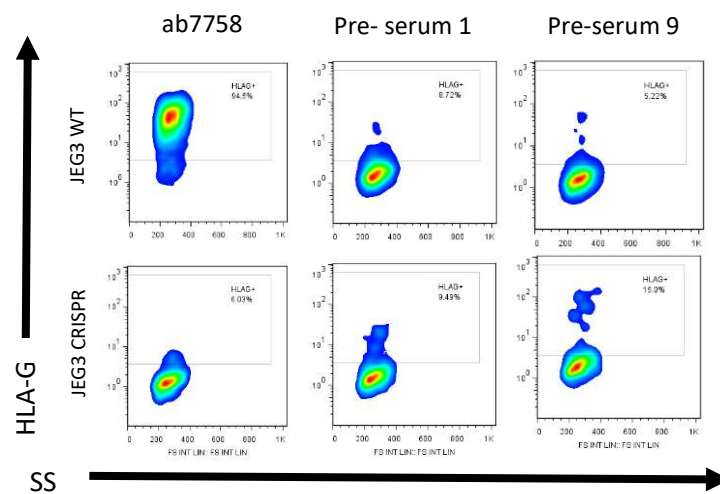


Figure 22. Cytofluorimetric analysis of HLA-G detection in JEG3 WT and JEG3 KO. Pre-immunization serum from the mice previously selected (9645 and 9653) was used as negative control to detect HLA-G in JEG3 WT and JEG3 HLA-G KO. Abcam antibody was used as positive control (ab7758). Three independent experiments were performed. Representative data is shown.

- **Hybridoma screening. One hybridoma clone was selected after the FC screening in JEG3 cells**

After the fusion step, hybridoma supernatants were collected from twenty clones generated from both mice, as well as a negative control supernatant. All the supernatants were checked for its ability to detect and bind HLA-G on the cell surface of JEG3 WT and JEG3 KO. As described in the previous point, the screening was performed by flow cytometry.

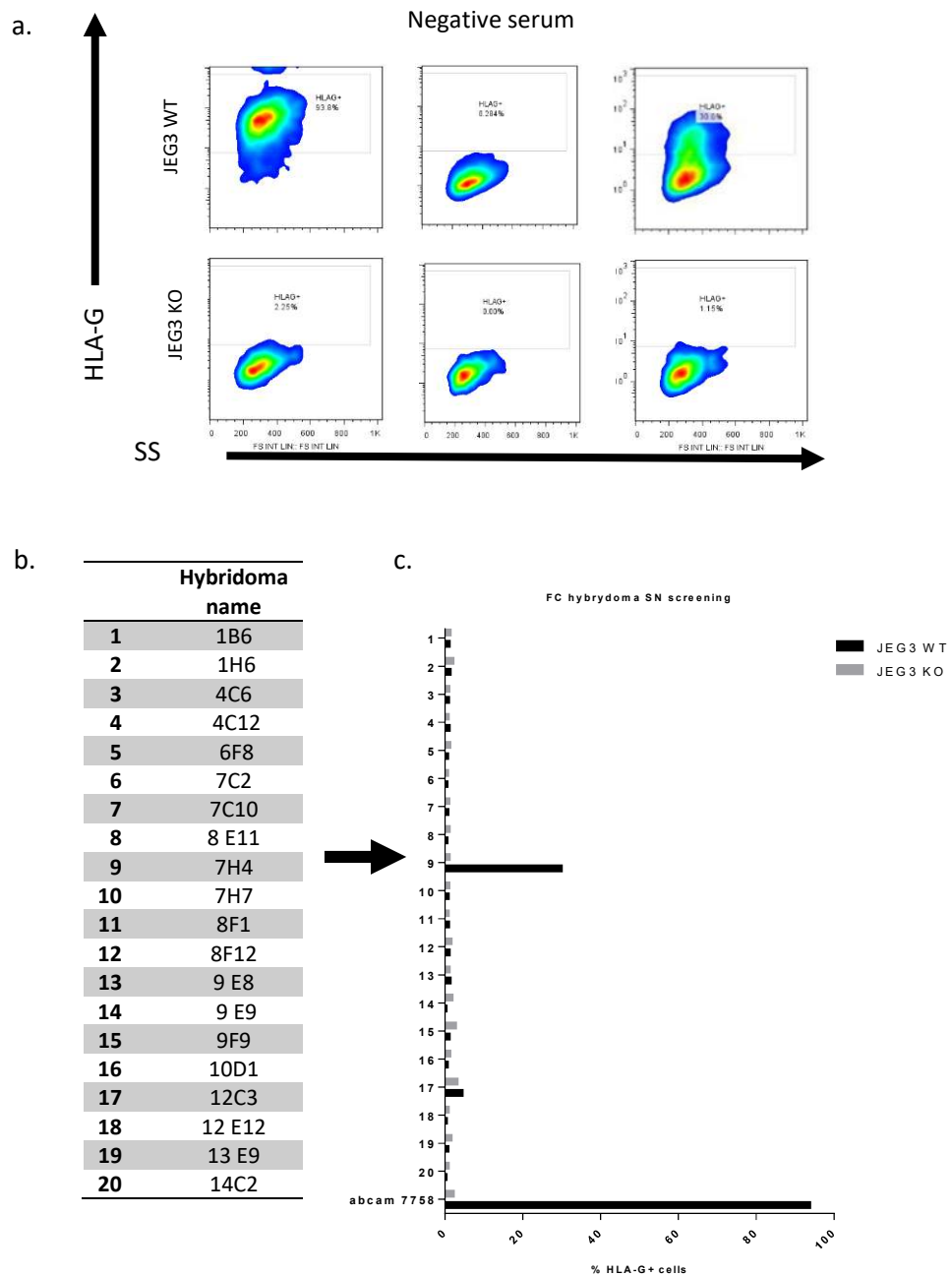


Figure 23. . Flow cytometry screening of hybridoma supernatant from 20 clones. (a) Cytofluorimetric dot plots representative image from positive control (ab7758), negative serum used as negative control and the best hybridoma SN (7H4) (B) Table showing the hybridoma clone name corresponding to each number. (c) Graphical representation of the HLA-G positive cells detected by Flow cytometry. Three independent experiments were performed. Representative data is shown.

Only one clone (7H4) showed to be positive for HLA-G detection, however the percentage of detection was just around the 30% (Figure 23).

- **Hybridoma subcloning. One hybridoma subclone was selected after FC screening in JEG3 cells.**

After the hybridization screening, ten subclons from the hybridoma selected (7H4) were generated. Those ten hybridoma subclons supernatants were checked for its ability to detect HLA-G by FC as it was explained previously. From the ten supernatants screened, none was able to detect more than 20% of positive HLA-G cells (Figure 25). One clone was selected as the best (7H4-4) detecting around 20% HLA-G positive cells.

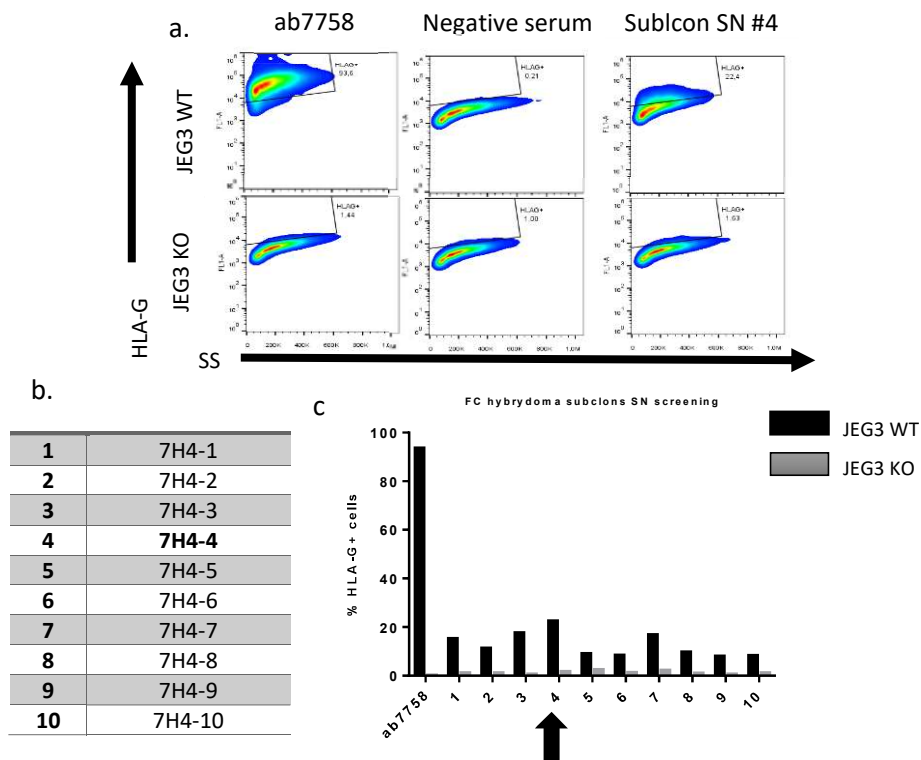


Figure 24. Flow cytometry screening hybridoma subclones supernatant. (a) Cytofluorimetric representative dot plot from positive control (ab7758), negative serum and the best hybridoma subclone SN (7H4-4) (B) Table showing the hybridoma subclone name corresponding to each number. (c) Graphical representation of the HLA-G positive cells detected by Flow cytometry. Three independent experiments were performed. Representative data is shown.

- **Antibody purification. The purified antibody does not detect the HLA-G on JEG3 cells as efficiently as the commercial antibody.**

Finally, the hybridoma subclone selected (7H4-4) was expanded for the antibody generation. Moreover the antibody was purified and a concentration of 1.37mg/ml was obtained. Their efficacy detecting HLA-G on JEG3 cells was evaluated as previously described by FC. However, the efficacy of the antibody to detect HLA-G on JEG3 WT cells was just around 10% (Figure 26). Importantly, we also checked if the purified antibody was able to detect HLA-G in the HuP-T3 cells but the percentage of positive HLA-G cells was below 5% (Data not shown). The purification was the last step of the process in the antibody production as it is summarized in Figure 26.

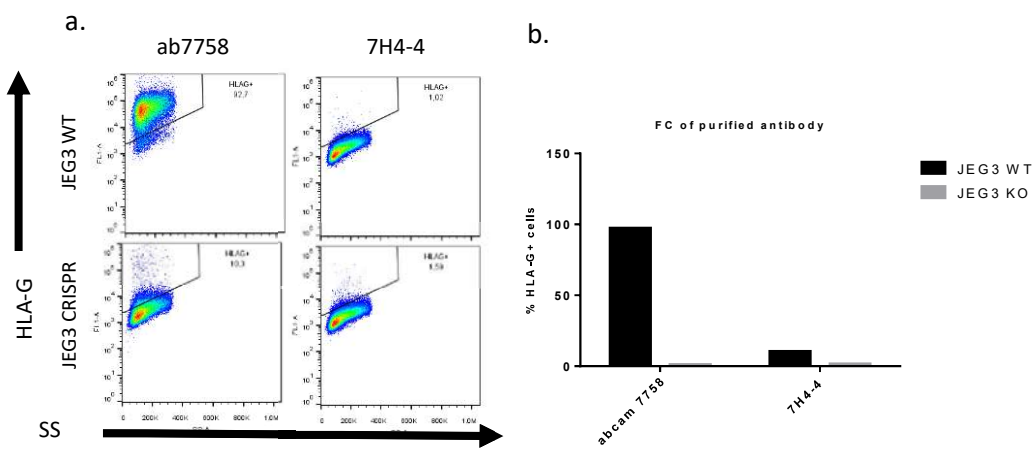


Figure 25. Flow cytometry HLA-G detection by purified anti-HLA-G (7H4-4). (a) Cytofluorimetric representative images from HLA-G positive cells detected with positive control antibody (ab7758) and purified antibody 7H4-4 (b) Graphical representation of the HLA-G positive cells detected by Flow cytometry. Two independent experiments were performed. Representative results are shown.

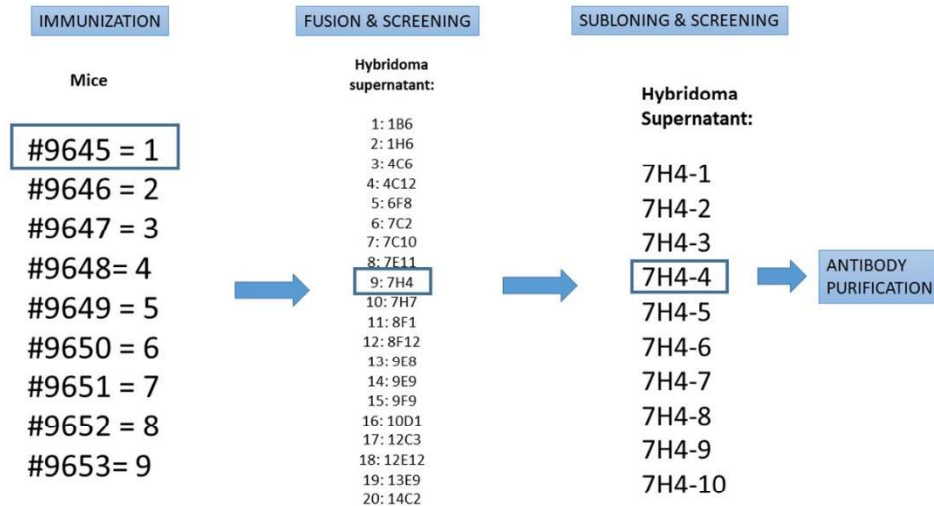


Figure 26. Scheme of the whole process performed for the anti-HLA-G antibody (7H4-H) production. Blue squares highlight mice or clones selected for the next steps. The total duration of the process was 9 months.

9.4 The 7H-4 anti-HLA-G antibody has a dose-response effect in NK92

Next, to test if the 7H4-4 antibody despite its low ability to detect HLA-G by flow cytometry, had a functional effect and was able to block HLA-G, we performed several blocking experiments.

First, we treated NK92 cells during 40h with the CM from JEG3 WT and KO (both clones were used with similar results). We preincubated during 30 minutes the CM from JEG3 WT with the 7H4-4 anti-HLA-G antibody at three different doses (10; 25 and 50ug/ml) to test the efficacy of the blocking (Figure 27).

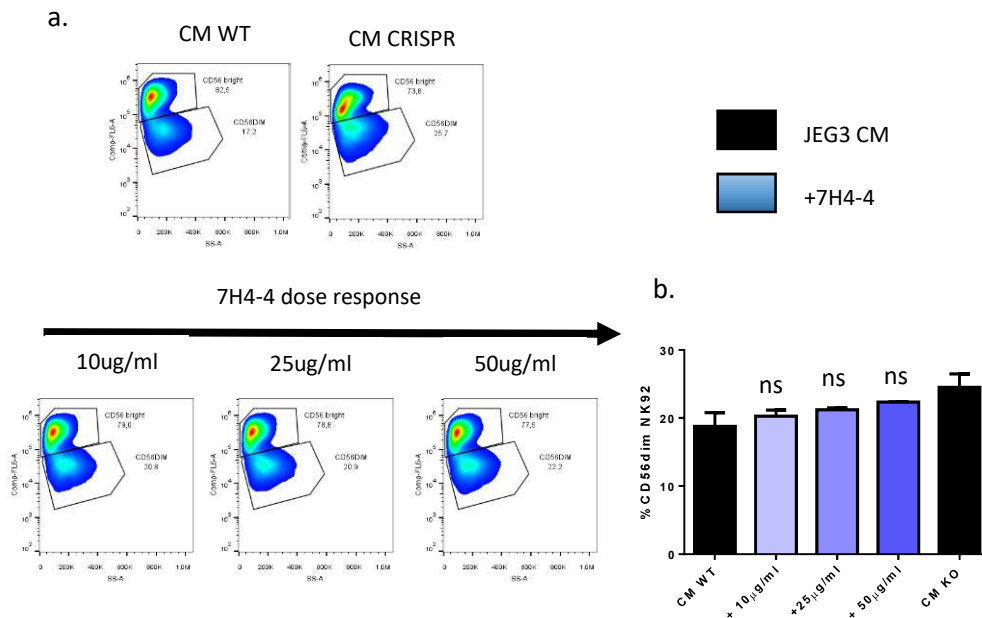


Figure 27. Dose response experiment (10ug/ml to 50ug/ml) for 7H4-4 anti HLA-G. (a) Representative cytofluorimetric analysis of CD56 expression in NK92. CD56^{bright} and CD56^{dim} populations are gated inside total alive population. (b) Graphical representation of CD56^{dim} NK92 population in the different culture conditions. Graphs coloured in blue show the condition with anti-HLA-G antibody. Black bar shows basal condition with JEG3 WT or JEG3 HLA-G KO media. Data are presented as mean \pm SEM.

After the 40h culture, we evaluated CD56 expression on NK92 cell surface by flow cytometry as performed in previous experiments. As shown in Figure 27, the antibody has a slight effect in CD56 modulation in a dose-dependent manner, however this modulation was not significant. We therefore decided to increase the antibody dose and we performed another dose-response experiment increasing the doses from 50 to 150 μ g/ml.

We observed that the NK92 CD56 dim population was increased in a dose-dependent manner when adding the anti-HLA-G antibody 7H4-4. The highest dose (150µg/ml) increased the dim population in a similar way as the CM from JEG3 HLA-G KO did (Figure 28).

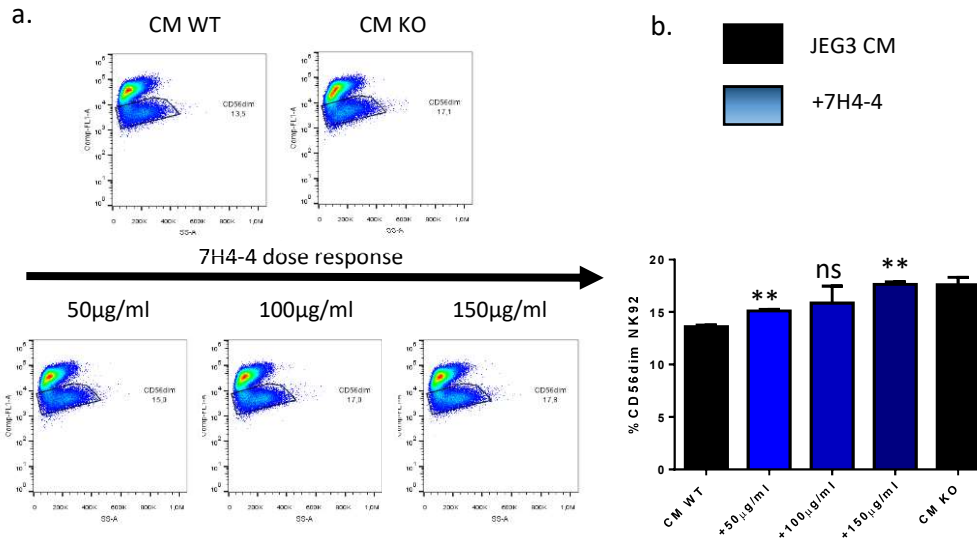


Figure 28. Dose response experiment (50, 100, 150µg/ml) for 7H4-4 anti HLA-G. (a) Representative cytofluorimetric analysis of CD56 expression in NK92 showing the CD56 dim population gated inside alive population (b) Graphical representation of %CD56 dim NK92 population in the different culture conditions. Graphs coloured in blue show the condition with anti-HLA-G antibody. Black bar shows basal condition with JEG3 WT or JEG3 KO media. Data are presented as mean ± SEM.

9.5 The 7H-4 anti-HLA-G antibody has no effect on pb-NK cells

Next, we tried to block HLA-G in the pb-NK cells using the 7H4-4 antibody (150µg/ml). As described before, we preincubated it with the CM from JEG3 WT during 30 minutes prior the culture with pb-NK cells.

We stopped the culture after 40h and analysed the cells by FC. We observed that the antibody 7H4-4 (150µg/ml) did not revert the effect produced by the conditioned media in the CD56/CD16 pattern (Figure 29).

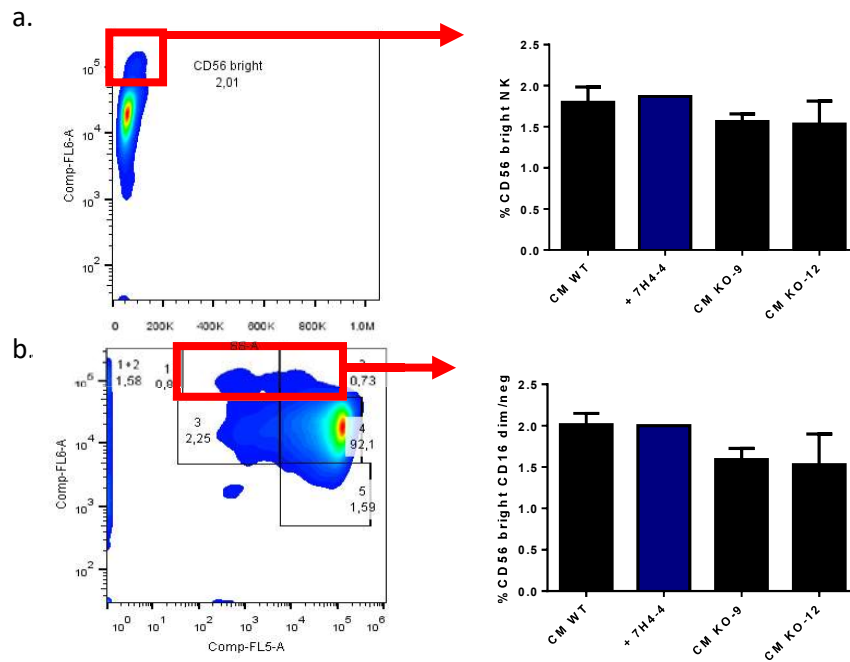


Figure 29. Modulation of CD56 and CD16 on pb-NK cells in response to JEG3 CM + antiHLA-G (7H4-4). (a) Representative dot plot showing CD56 bright population gating strategy (left) and graphical representation (right). (b) Representative CD56/CD16 dot plot (left), cells within the red squared are quantified and represented (right).

The results regarding HLA-G blocking in pb-NK cells suggest that the anti-HLA-G does not work in this cells. The particular nature of this molecule and the variability of the peripheral blood NK cells make it more complex to study than the NK92 cells. We will summarize in the discussion the different aspects that could be affecting the results previously explained, as well as future directions and possible therapeutic approaches.

DISCUSSION

1. Modulation of CD56 in response to HLA-G

In this project, we investigated the role of HLA-G in immune modulation in the context of cancer. In particular, we studied the modulation of CD56/CD16 promoted by HLA-G in NK cells. Importantly, such modulation has been observed by secreted HLA-G present in the conditioned media of JEG3 WT as well as by recombinant HLA-G treatment. Moreover, the coculture of JEG3 with NK92 cells resulted in the CD56 modulation, evidencing also a role for the HLA-G anchored to the membrane.

CD56 and CD16 expression has an important role defining the different NK cell subpopulations. NK cells downregulate CD56 and increase CD16 expression during its process of maturation, as it was described in the Introduction (See Section 4.3).

We have shown that HLA-G has an effect on the modulation of the NK cell surface markers after treating them with CM from JEG3 WT (physiological source of HLA-G) or JEG3 KO (lacking HLA-G). In particular, we observed in our experiments that HLAG present in the JEG3 WT CM promotes a less cytotoxic phenotype in NK cells evidenced by the increase of CD56^{bright} population.

When we concentrated the conditioned media to increase the amount of HLA-G, we did not observe an increase of the effect in CD56 expression as we would expect since the dose was 4 times higher. However, is conceivable that apart from concentrating the HLA-G, we also concentrate other factors that are present in this medium (growth factors, hormones,...etc.) that may interfere in our results.

We have also shown that the addition of recombinant HLA-G reverts the effect produced by the JEG3 KO CM. Moreover, the treatment with recombinant protein was also able to increase the effect produced by the JEG3 WT CM that already contained HLA-G. Those results reinforce the implication of HLA-G in the CD56 regulation and circumvent the problem observed when we concentrate the CM without being able to increase the effect produced (Figure 28).

The importance of the different CD56/CD16 abundance is its implication to define the different NK cell subpopulations that have different biological functions. Whereas the CD56^{bright} NK cell population is mainly regulatory with high proliferative potential, the CD56^{dim} NK cells are highly cytotoxic, as described in the Introduction (Section 4.3; Figure 11).

We have observed that HLA-G increases the CD56^{bright} NK cell population, suggesting a role for this molecule in shaping NK cells into a less cytotoxic phenotype as part of the immune suppressive environment promoted by HLA-G. Importantly, as shown in previous publications, in the physiological context of pregnancy, around 70-90% of the NK cells found in early pregnancy decidua are CD56^{bright} despite what is observed in the peripheral blood where just around 10% of the NK cells are CD56^{bright} (66). Since HLA-G is highly abundant in the decidua, our results might indicate a possible mechanism of CD56 regulation also in the pregnancy context.

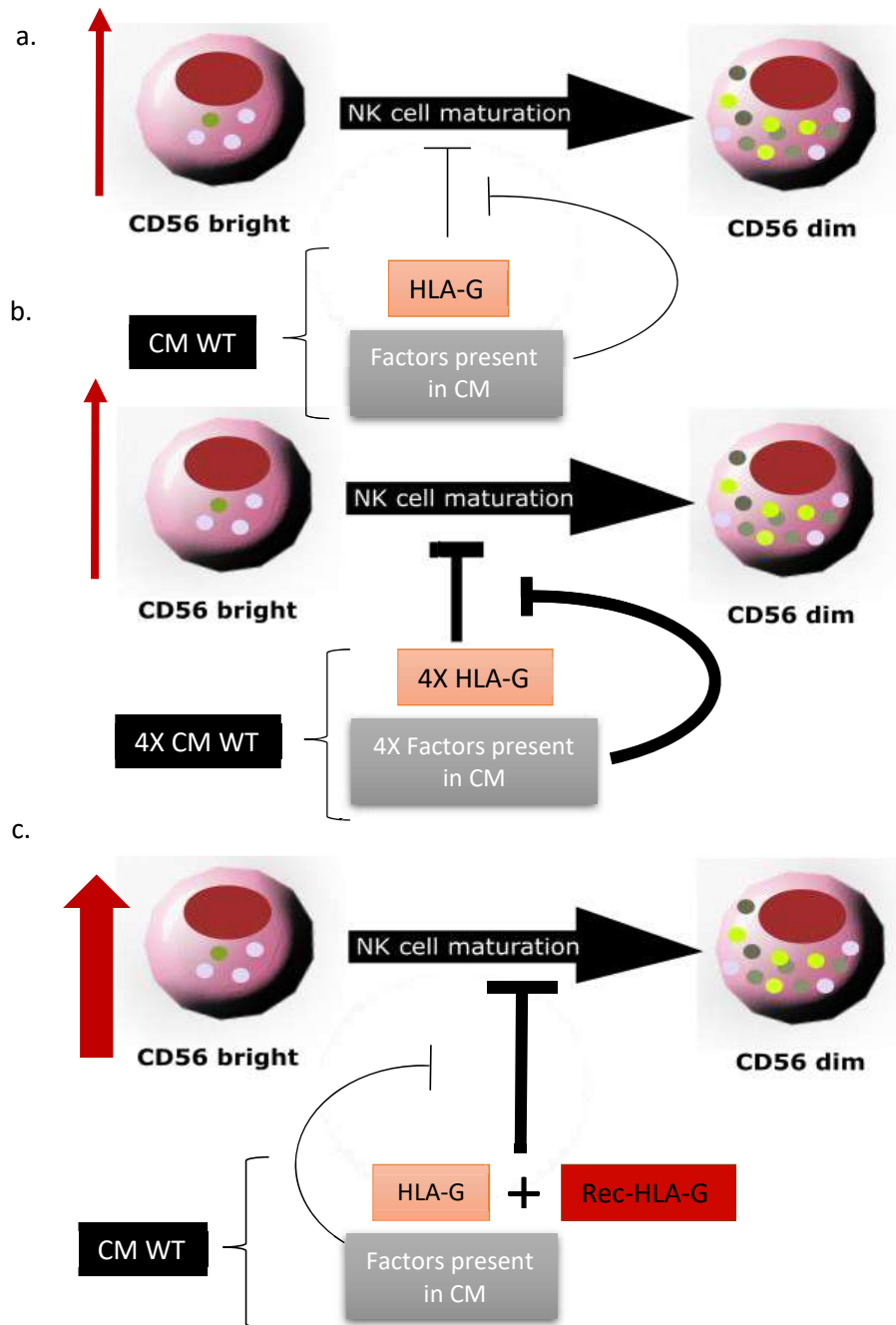


Figure 30. Diagram showing the proposed model for the CD56 regulation. Regulation by either HLA-G from the JEG3 WT CM (a) JEG3 WT CM 4X concentrated (b) and JEG3 WT CM 4X + recombinant HLA-G (c).

Importantly, the effects on the CD56/CD16 modulation reported for the NK92 and pb-NK cells were different. As it was explained in the Introduction (See Section 4.4), NK92 cells are mainly CD56^{bright} whereas the pb-NK cells are mainly CD56^{dim}. This characteristic impact on the results observed since the different NK cell subsets differ in their functions as mentioned before. NK cells function depends on the balance of activating and inhibitory signals that the cells receive through their receptors, and the relative abundance of this receptors is also different among them. NK92 cell line exhibits an unusual receptor expression profile, expressing a relatively large number of activating receptors and few inhibitory receptors. NK92 cells are therefore prone to react against other cells because they have more probabilities to be activated than inhibited. The combination of the high amount of activating receptors with the lack of most of the KIRs (they only express KIR2DL4 and in a low proportion) results in its broad cytotoxicity. This would disagree with the fact that CD56^{bright} are more regulatory than cytotoxic, so the NK92 cell line is very singular combining high cytotoxicity and high CD56 expression. Despite NK92, pb-NK cells express a balance of both activating and inhibitory receptors, therefore, they are not so predisposed to a cytotoxic behaviour. This means that pb-NK cells would need higher activating inputs to develop a cytotoxic response. In addition, the pb-NK cells are isolated from different donors, so the cell surface expression of receptors may also vary among them increasing the variability in our results.

HLA-G receptors are mainly inhibitory receptors, however the role for KIR2DL4 is ambiguous, being either an inhibitory or stimulatory receptor as described in literature (28). We checked for ILT-2 and KIR2DL4 expression in both NK cell types. We observed by flow cytometry that almost 100% of the NK92 cells expressed ILT-2 on their surface, in the literature there is a lot of discrepancy for the expression of ILT-2 in NK92 cells since some authors affirmed that it is very low (68) and other described it as relatively high (150). For the pb-NK cells, just around 30% of them expressed it. Regarding the KIR2DL4 receptor, it is mainly present in decidual NK cell subset where the HLA-G is highly expressed, importantly it is the sole ligand of this receptor, moreover, it is the only KIR member reported to be expressed in CD56^{bright} NK cells subset. However, we observed very low expression by flow cytometry in NK92 a fact that was also previously reported (68). The KIR2DL4 expression observed in pb-NK cells was very low, in agreement with the low CD56^{bright} abundance, this agrees with previous publications (64-66).

This differential expression of the HLA-G receptors, would explain the different responses observed. Almost all the NK92 cells have the ILT-2 receptor and would be able to bind HLA-G and react to this stimulus, however, just one third of the pb-NK could potentially do it. This might explain why the NK92 cells responded to a lower dose of HLA-G present in the JEG3 CM whereas in the pb-NK cells we did not observe modulations due to the treatment with CM. Importantly, ILT-2 also binds other HLA family members, however it has been previously described that it would bind HLA-G with higher affinity than any other (29; 151).

So the different basal CD56 abundance present in the NK92 vs pb- NK cells and the different receptor pattern that they express, might explain the diverse responses to HLA-G reported.

Given the results we obtained, we suggest that the HLA-G regulates the abundance of the CD56^{bright} NK cell population in the context of cancer. Moreover, we hypothesize that this regulation process might also occur at the placental-fetal interphase given the high expression of HLA-G and the extraordinary abundance of CD56^{bright} NK cells in this context as published before (66).

In conclusion, we propose that in the context of cancer the HLA-G is modulating the NK cells through a less aggressive phenotype in a parallelism of what is observed at the maternal-fetal interphase.

2. Modulation of IFN γ secretion by HLA-G in NK cells

Our results demonstrated that the conditioned media from JEG3 WT that contain secreted HLA-G down-modulates the IFN γ secretion in NK92. However, we did not obtain the same results for the recombinant HLA-G. NK derived IFN γ has proven to be fundamental in shaping adaptive immune responses. Previous studies already showed the role for HLA Class I molecules in the down-regulation of IFN γ production through the ILT-2 receptor in NK92 (150). Our data suggests a role for the HLA-G in particular, since the CM from the WT cells downregulated significantly the secretion of IFN γ in comparison with the CM from JEG3 HLA-G KO.

Also, given the abundance of the ILT-2 receptor previously explained, we could hypothesized that most likely the signalling is mediated by ILT2 rather than KIR2DL4. This would agree with the fact that we see this regulation of the IFN γ secretion when adding the conditioned media in

NK92 cells but not in the pb-NK cells, since this NK cells have much less abundance of the ILT-2 receptor and the inhibitory signal may not be strong enough to produce a downregulation of the IFN γ secretion. However, when we blocked the ILT-2 receptor, we did not observe an increase in the IFN γ secretion (data not shown), generating doubts about this hypothesis.

Nonetheless, it has been described that HLA-G can stimulate IFN γ secretion through the binding to the KIR2DL4 receptor (152). Given the low expression of this receptor observed in both NK92 and pb-NK cells, this does not have much relevance in our experiments, however, it seems to play an important role in the context of pregnancy, where the uterine NK cells are described to express high levels of KIR2DL4 (30).

Nevertheless, it is surprisingly how the HLA-G can both up- and down-regulate the IFN γ secretion, the reason for that might be the physiological context and the role behind. The binding of HLA-G to the KIR2DL4 receptor mainly expressed in the uterine NK cells would induce IFN γ production as previously reported (81), in this context the role of this cytokine is related to the remodelling of the uterine vasculature, in particular with the initiation of endometrial vascularization, a critical step in early pregnancy (153). This, contrast with the canonical antiangiogenic role described for IFN γ in many diseases including cancer (154). During pregnancy, the endometrial stromal cells produce VEGF, which contribute to endometrial neovascularization and proliferation, and the IFN γ may play an important role in regulating VEGF production by this cells (155). Moreover, it is not the only situation in which the IFN γ seems to have this role, since it have also been described the proangiogenic function in the context of neovascularization of the cornea (156). In relation with the angiogenesis

process, we also reported in the Introduction that HLA-G may play an antiangiogenic role through the binding of CD160 in the endothelial cells (36). A precise balance of this pro- and antiangiogenic signals is needed to ensure the correct development of fetal vascularity.

There is a lot of controversy regarding the IFN γ effect in pregnancy context, despite the evidences regarding its role in regulating VEGF production by endometrial cells and promoting vascularization, its precise effect on human uterine microvasculature, remains poorly understood (157).

Regarding its production by NK cells, it is well described that IFN γ is mainly produced by the CD56^{bright} NK cell subset that have a regulatory role (64). In our results, we observed more IFN γ secretion in the NK92 than in the pb-NK cells in agreement with the CD56^{bright} abundance. However, we observed an increased CD56^{bright} population when treating with CM from JEG3 WT and/or recombinant-HLA-G, meaning that even though this population is increased with the addition of HLA-G, the capacity of secreting IFN γ is highly downregulated. In fact, the NK92 cells at basal conditions secrete amounts of this cytokine that are around 5 times higher compared with the amounts secreted when cultured with CM (data not shown).

In relation with the recombinant HLA-G, it is intriguing why it is not able to revert the effect produced in the IFN γ secretion by NK92 as it did with the CD56 abundance. One possible explanation is that the different conformations that the HLA-G molecule can adopt are limited in the recombinant protein. The HLA-G is able to form homodimers as it was described in the Introduction, also it have been published that the ability to bind the ILT-2 receptor is higher in the dimeric conformation than in the

monomeric one (29). The incapacity of the recombinant protein to form dimers could explain the results observed since it would not have the same ability to bind the ILT-2 receptor, but this is a hypothesis that need to be further proved. However, regarding the effect of the CD56 surface abundance, it was modulated with the recombinant HLA-G protein, suggesting that different signalling pathways are involved in this regulation.

Surprisingly, we observed a slight down-modulation in the IFN γ secretion pattern when adding the recombinant HLA-G to the pb-NK cells. But the amount of IFN- γ secreted by this cells is really low and the differences observed are minor. Nevertheless, the secretion of granzyme B and perforine was significantly downregulated by the HLA-G recombinant protein in the pb-NK cells (as it will be discussed in Section 3). The NK cells downregulation of perforine and also IFN γ secretion mediated by HLA-G was already reported by Morandi et al (39). This effect agree with the downregulation of the cytotoxic activity that the HLA-G molecule is described to have. Despite other MHC class I molecules that are upregulated by the IFN γ , in the case of HLA-G it does not respond to it since as reported in the Introduction, the IRSE elements present in the HLA-G promotor are defective (16-17).

3. Modulation of granzyme and perforin secretion by HLA-G

Apart from the modulation of the IFN γ secretion, we have shown in our results that the conditioned media from JEG3 WT significantly downregulates the secretion of granzyme B and perforin in NK92. This fact is very important given the implication of this molecules in the cytotoxicity of NK cells.

This results correlate with the observations regarding the CD56 modulation, since as previously reported (66) the cytotoxic activity of CD56^{dim} NK cells is significantly higher than that of CD56^{bright} cells and they contain much more perforin, granzymes and cytolytic granules.

Moreover, we have studied the implication of the recombinant HLA-G in this regulation. We have observed that the recombinant HLA-G slightly reverts the effect produced by the CM, this reversion even though it is not complete, it reinforces the fact that HLA-G has a role in regulating the perforin and granzyme secretion.

Regarding the pb-NK cells, we observed that the treatment with recombinant-HLA-G downregulated strongly the granzyme B and perforine secretion, supporting the results observed with NK92. Again, this downregulation correlates with the increase of the CD56^{bright} population. In pb-NK cells, the CM was not able to induce changes in the granzyme/perforine secretion pattern maybe due to the lower HLA-G abundance previously discussed.

In conclusion, the downregulation of the granzyme and perforine secretion together with the increase of the CD56^{bright} population, would suggest a role of HLA-G in shaping NK cells into a less aggressive phenotype in the context of cancer.

4. The use of blocking antibodies to modulate HLA-G response

We used anti-HLA-G antibodies with the aim of reverting the effect produced by HLA-G. We first tried to block HLA-G effect with the commercial antibody (Thermofisher, clone 87G) without obtaining any blocking effect. Then, we tried another approach by designing and producing our own anti-HLA-G blocking antibody.

The similarity of HLA-G with other HLA members make difficult to specifically block it. In fact, the different commercial antibodies available have been reported to cross react with other HLA-I members. The 87G clone used as a blocking anti-HLA-G antibody, has been shown to cross react with other members of the HLA-I family (28, 158). Despite it, we tried to block the HLA-G effect by using this commercial antibody (Thermofisher, 87G clone) since previous studies reported its blocking efficacy (39). However, we were not able to revert the effect produced by HLA-G (Figure 20b).

Moreover, the lack of a murine orthologous of HLA-G make the translational research more complex. The use of patient derived models (patient derived xenografts, tumoroids, organotypic culture,...etc.) would be an interesting approach, however in this context the use of an specific anti-HLA-G antibody becomes an essential tool.

Given the evidences for the cross-reactivity within the available commercial antibodies and the lack of effect obtained in our experiments, and the need of this tool to perform a translational research we decided to design a novel anti-HLA-G blocking antibody.

First, we decided to target the alpha-3 region with the aim of blocking the interaction of HLA-G with the ILT-2 receptor and therefore prevent its functional activity. We choose to target the interaction with the ILT-2 receptor among the HLA-G receptors described, due to its higher abundance in NK cells but also because it is very well described the specific binding site within the HLA-G molecule. Regarding the KIR2DL4 receptor, even though it is also expressed in NK cells, little is known about how it binds the HLA-G, which made the targeting process highly complicated. There are some evidences showing the implication of the alpha-1 domain

in its binding but the specific recognition site remains still unknown (7; 158).

In particular, we targeted a region located at the amino acid position 190 within the alpha-3 domain, this sequence is responsible for the ILT-2 binding as described previously (7). However, there are some HLA-G isoforms (HLA-G3, -G4 and -G7) that lack the alpha 3 domain as it was described in the Introduction. How this isoforms are able to bind the ILT-2 receptor or if most likely they bind another unidentified receptor remains still unknown (158). One hypothesis would be the formation of heterodimers, but so far it have only been reported the homodimer formation. Importantly, the most abundant isoforms physiologically present are the HLA-G1 and -G5 that harbour all the domains and therefore will be potentially targeted by our anti-HLA-G antibody. In the particular case of the JEG3 cells, they potentially express all the isoforms as reported by checking the mRNA expression levels (160). And at protein level, the most abundant isoform present in the JEG3 cells was the complete isoform HLA-G1 (160-161).

Once decided the targeted region, we also considered the cross-reactivity of anti HLA-G antibodies with other HLAs previously reported. We first checked for the presence and abundance of Pan-HLA in the JEG3 (clone W6/32). We observed that all the JEG3 cells were positive for the Pan-HLA, meaning that they expressed HLA-A, -B and/or -C. Importantly, no differences were observed between WT and KO (Figure 31).

Moreover, the alpha-3 domain is being described to be highly conserved among the HLAs (7), therefore the HLA-G blocking antibody could potentially cross-react with other HLAs present in the JEG3 cells. To avoid this, in the process of screening for the selection of the best hybridoma clone, we used the JEG3 WT cells as positive control and JEG3 HLA-G KO cells as negative control to ensure that we selected for the clones that specifically bound HLA-G, based on a positive selection of those binding the WT cells and not the KO cells.

The process of the antibody production, resulted in the 7H4-4 anti-HLA-G antibody as the best candidate. We observed that the yield of the HLA-G recognition at flow cytometry was quite good at the beginning of the process, with 80% efficacy for the best mice immunization sera but ended up with 20% of efficacy of HLA-G+ cells detected by the hybridoma subclone supernatant as a putative source of antibodies. When we checked for the ability to recognize HLA-G by the 7H4-4 purified antibody, the results were even lower with a yield of 10% of HLA-G+ cells recognition among the JEG3 WT cells (Figure 25). We are still working in this in order to improve the ability of the antibody to recognize HLA-G and to understand the possible reasons behind the low yield previously described.

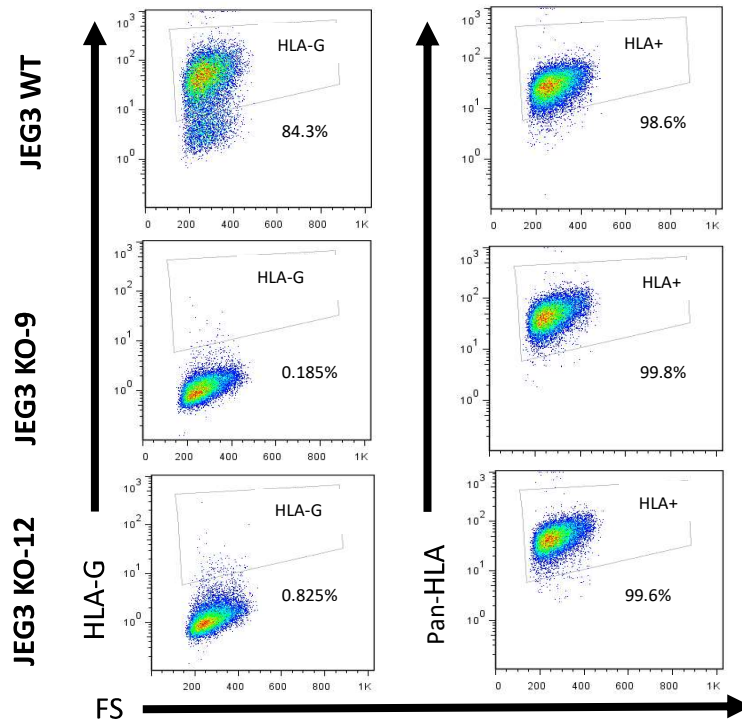


Figure 31. Flow cytometry analysis of the Pan HLA (ab9090) expression in JEG3 cells. The left side shows the expression of HLA-G and the right side the Pan HLA expression.

This hypothesis would agree with the fact that we needed a high dose of the 7H4-4 antibody to produce a blocking effect in the NK92 cells in relation with the CD56 abundance (Figure 27-28). However, we did not succeed in blocking the effect in the pb-NK cells. The high dose needed in the NK92 cells to produce an effect and the lack of response in the pb-NK cells, evidenced the fact that the antibody still needs to be improved (work in progress).

In conclusion, blocking HLA-G seems to be an attractive therapeutical approach as well as an essential translational research tool. However, the similarities with other HLAs and the HLA-G 3D conformation make it a challenging process.

Another possibility might be to block its receptor, in a similar way as it has been developed for HLA-E. Some therapeutic approaches target the HLA-E receptor: NKG2A (162), in fact, there is a monoclonal antibody that have shown promising results in cancer therapy named Monalizumab (163). However, since the ILT-2 receptor also binds other molecules it may result in undesired collateral effects. Another possibility would be the use of anti-KIR2DL4 antibodies, the use of anti-KIRs as well as the use of anti-NKG2A blocking mAbs recapitulates the condition of “missing-self” recognition and they are currently being tested in several clinical trials against hematological and solid tumors either alone or in combination with other immune therapeutic agents such as anti-PD-1 mAb (i.e nivolumab) (74). However, for the particular case of KIR2DL4 receptor, there is still a lot of controversy regarding its function and expression that need to be further investigated.

Our work have demonstrated the implication of HLA-G in the regulation of the NK cell maturation and function. This is very important given the importance of the NK cells in the context of immune-modulation in cancer. Particularly, we have described for the first time, the role of HLA-G in the modulation of the CD56 surface marker. Interestingly, HLA-G induces a NK cell phenotype that recapitulates the one present at the decidua in the pregnancy context. This parallelism between cancer and pregnancy demonstrate us that once again cancer hijacks a preexisting mechanism for its own benefit. Moreover, we have explored the possibility of reverting this effect produced in NK cells using an anti-HLA-G antibody. However we described only partial effects, highlighting the fact that further work is required to evaluate HLA-G as a potential therapeutic target.

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CONCLUSIONS

- Using JEG3 WT and JEG3 HLA-G KO we have demonstrated that in the absence of HLA-G, CD56 expression is downregulated and cytotoxic related cytokines such as IFN γ , granzyme B and perforin are upregulated in NK92 cells
- HLA-G amount secreted by HuP-T3 cells is not enough to promote changes in NK cells
- Recombinant HLA-G recapitulates the effect produced by JEG3 WT CM (physiological source of HLA-G) and reverts the effect produced by JEG3 HLA-G KO CM regarding CD56 expression in NK92 cells
- CM from JEG3 is not able to promote significant changes in pb-NK cells regardless of what was observed in NK92 cells
- Recombinant HLA-G significantly increases the CD56^{bright} population in pb-NK cells and slightly the CD56^{bright}CD16^{dim/negative}
- The cytotoxic ability of pb-NK cells is diminished by recombinant-HLA-G in pb-NK cells as suggested by the downregulation of granzyme B and perforin secretion
- The 7H4-4 anti-HLA-G antibody has a dose-response effect regarding CD56 modulation in NK92 cells but has no effect in pb-NK cells
- HLA-G is a molecule with considerable clinical interest. It has shown promising results for being used as a therapeutic target, even though the anti-HLA-G antibody still needs to be optimized for this aim (Work in progress)

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