Universidade de Aveiro Departamento de Ciências Médicas
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Estudo da expressão de CD5 e CD6 em células iNKT humanas.

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Maria de Fátima Matos Almeida Henriques Macedo, Professora Auxiliar convidada do Departamento de Ciências Médicas da Universidade de Aveiro e coorientação da Doutora Begoña Pérez-Cabezas.

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o júri

presidente

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palavras-chave Células T, células T Natural Killer invariantes (iNKT), antigénio, células apresentadoras de antigénio, CD5, CD6

resumo As células *Invariant Natural killer T* (iNKT) são um subtipo de linfócitos T que reconhecem antigénios lipídicos através da molecula de CD1d e têm um recetor de células T semi-invariante. Estas células produzem grandes quantidades de citocinas após serem ativadas. A regulação das iNKTs é crucial para controlar o seu efeito na infeção e no cancro. CD5 e CD6 são glicoproteínas transmembranares expressas na superfície das células T e que regulam a sua activação, sendo que a sua expressão em células iNKTs humanas ainda não tinha sido estudada. Neste trabalho estudou-se a expressão basal destas moléculas em iNKTs humanas e também a sua modelação após activação. O envolvimento do CD6 na indução de ativação das iNKTs pelo antigénio α-Galactosylceramide (α-GalCer) também foi estudada. Os resultados mostraram que as iNKTs presentes no sangue humano periférico expressam CD5 e CD6, sendo que em comparação com as células T convencionais, o CD5 é expresso a níveis semelhantes e o CD6 é expresso em níveis superiores. A activação de iNKTs humanas pela *Phytohaemagglutinin* (PHA) (estímulo não específico) diminuiu a expressão das moléculas CD5 e CD6 enquanto que com o antigénio prototípico α-GalCer só se verificou uma descida na expressão de CD6. O uso de células *Raji* expressando ou não o CD166, um ligando do CD6, como células apresentadoras de antigénio, não revelou ter um papel importante na indução de activação da iNKTs pelo antigénio α-GalCer.

keywords T cells, Invariant Natural Killer T cells, antigen, Antigen presenting cells, TCR, CD5, CD6

abstract Invariant Natural Killer (iNKT) cells are T lymphocytes that recognize lipid antigens presented by CD1d molecules and have a semi-invariant T cell receptor. iNKT cells produce high quantities of cytokines after antigen recognition or activation. The regulation of iNKT cell activation is crucial for its role in infection and tumor control. CD5 and CD6 are transmembrane surface glycoproteins expressed by conventional T cells that regulate the activation of these cells, but their expression on human iNKTs has not been investigated yet. Here, we studied the basal expression of both CD5 and CD6 on human iNKTs and also their modulation after activation. The involvement of CD6 in antigen induced iNKT cell activation was also addressed. Our results show that human peripheral blood iNKT cells express both CD5 and CD6, at similar and higher levels, respectively, than conventional T cells. Activation of a human iNKT cell line with the non-specific stimulus Phytohaemagglutinin (PHA) down-regulated the expression of both CD5 and CD6, whereas when the prototypic antigen α-Galactosylceramide (α-GalCer) was used only a decrease in CD6 expression was observed. The use of Raji B cells, expressing or not CD166, a ligand for CD6, as antigen presenting cells did not disclose a major role for CD6 in α-GalCer induced iNKT cell activation.

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Introduction

Immune system

The immune system is composed by different organs, cells and molecules. Its function is to protect the body from pathogens (microorganisms that can cause diseases) such as bacteria, fungi, parasites, viruses, and also to defend against cancer cells and toxins. The immune system has two lines of defense: innate immunity and adaptive immunity. Innate immunity is an antigen-independent mechanism that occurs immediately or within hours after encountering a threat. Innate immune's response involves different cells: macrophages, neutrophils, dendritic cells, mast cells, basophils, eosinophils, natural killer cells and innate lymphocytes. Adaptive is an antigen-specific mechanism with an associated lag time between exposure to the antigen and the response (Owen et al., 2009). While innate immunity does not have the capacity of memory, adaptive immunity presents it, which allows the host to assemble a more rapid and efficient response during a second exposure to the same pathogen. This can happen due to activation of T cells after antigen recognition: antigen is presented by antigen presenting cells (APC) such as dendritic cells, macrophages, B cells, fibroblasts and epithelial cells (Warrington et al., 2011) (Yatim & Lakkis 2015).

Lymphocytes

In humans, lymphocytes represents 20% to 40% of circulating white blood cells and the lymph contains 99% of the circulating lymphocytes (Haig et. al, 1999). They are one of the principal cells participating in the adaptive immune response. (Yatim & Lakkis 2015)

Lymphocytes can be subdivided into three principal populations: B lymphocytes (B cells), T lymphocytes (T cells) and natural killer cells (NK), all of them distinguished by phenotypic and functional differences. B and T cells are very similar microscopically, but they express different surface proteins, known as cluster of differentiation (CD), and an antigen-specific receptor, B cell receptor (BCR) for B cells and T cell receptor (TCR) for T cells. B and T cells express a remarkable diversity of antigen receptors. However, all the receptors present on an individual cell's surface have identical structures and therefore have identical specificities for an antigen. So, when a given lymphocyte divides to form two daughter cells, both daughters have antigen receptors with antigen specificities identical to each other and to the parental cell.

B and T cells only become activated when they encounter antigens. After contacting with the antigen, lymphocytes proliferate and differentiate into effector cells for eliminating the pathogens

(primary response) and into memory cells which stay quiescent until they reencounter the same antigen and respond then faster and more efficiently (second response).

B lymphocytes

B lymphocytes develop in the bone marrow from hematopoietic stem cells that differentiate into common lymphoid progenitors which will give rise to immature B cells (Lai & Kondo 2008). B cells distinguish themselves from other cells by the expression of surface BCR, mentioned above. The main functional characteristic of B cells is their capacity to produce antibodies. B cell's antigen-binding receptor in its native form, recognizes and binds to the antigen. Non- activated B cells circulate through lymph nodes and spleen. They can be concentrated in follicles, named follicular B cells which facilitates the differentiation of B cells into antibody secreting cells through BCR activation (Vinuesa et al., 2005). Furthermore, non-activated B cells can be concentrated in marginal zones around follicles, named MZ B cells which can be involved in T-dependent immune responses to protein antigens and also to lipid antigens once they express high levels of CD1d (Allman & Pillai 2008).

The circulating B cells may interact and be activated by T cells at extrafollicullar sites, and when activated B cells enter the follicle, proliferate, and displace resting cells. They form germinal centers and differentiate into both plasma cells that form antibody and long-lived memory B cells (Lai & Kondo 2008). These cells attracting the assistance of T helper (Th) cells which secrete cytokines that help the multiplication of the B cell and their maturation into antibody-secreting plasma cells. (Hoffman et al., 2015).

T lymphocytes

T lymphocytes maturate in the thymus and are distinguished from other immune cells because they express a unique antigen binding receptor, TCR, which only recognizes peptides or pieces from antigens bound to cell membrane proteins named major histocompatibility complex (MHC) molecules. MHC molecules are glycoproteins expressed by APCs that form complexes with the antigen exposing them to T cells, allowing their activation (Castro et al., 2015).

There are two types of T cells: helper T cells (Th) and cytotoxic T cells (Tc). They can be distinguished by the expression of CD4 and CD8 molecules on their surfaces. $CD4^+$ cells function as T helper (Th) cells and recognize antigens linked to class II MHC molecules while $CD8^+$ cells function as T cytotoxic (Tc) cells and recognize antigens in complexes with class I MHC molecules (Warrington et al. 2011)

Thymocytes pass through several steps in the thymus to become functional and mature T cells. T cell precursors travel from bone marrow to thymus via blood vessels. At this point, thymocytes are double negatives (DN) cells – do not express neither CD4 nor CD8 molecules. DN cells migrate to thymic subcapsular cortex to proliferate and generate TCRs. The ones that express TCR begin to express both CD4 and CD8 and become double positive (DP) cells. Then, thymocytes are selected accordingly to TCR reactivity to self-peptide/MHC complexes presented by cortical thymic epithelial cells (TEC). Thymocytes with a TCR that binds self-peptide/MHC complexes with high affinity are negative selected and are induced to apoptosis (to avoid autoimmunity), while those who binds self-peptide/MHC complexes with intermediate affinity are positive selected and migrate to the thymic medulla. There, mature thymocytes turns into single positive (SP) cells which express CD4 or CD8 and leaves the thymus via blood vessels. Finally, maturation will be finished in the periphery when T cells contact specifically with antigens in the secondary lymphoid tissues, like spleen and lymph nodes (Klein et al., 2014).

To become activated, CD4+ cells need to recognize a MHC-peptide complex. After that, CD4+ T cells proliferate and differentiate into T helper type I cells that produce cytokines like TNF and IFN-ϒ required for triggering immune response to intracellular pathogens or into T helper type II cells that produce mainly IL-4 and IL-5 regulating the immune response to extracellular pathogens. They can also originate regulatory T (Treg) cells which have the ability to inhibit the immune response, when necessary. CD4+ Treg are potent modulators of self-tolerance and induce autoimmune suppression.

CD8+ cells, similar to CD4+ cells, are classified as naive until encountering an antigen presented by class I MHC molecules. After that, these cells proliferate and differentiate into effector cells known as cytotoxic T lymphocytes. Its main function is to eradicate cells that compromise host integrity, for example, virus-infected cells, tumor cells and cells of a foreign tissue graft, by programming them to undergo apoptosis, protecting the host from possible threats (Andersen et al., 2006).

Natural Killer T cells

Natural Killer T (NKT) cells represent a subset of T lymphocytes that are also formed and differentiated in the thymus by positive, negative selection (Chen et al., 2015) and VDJ recombination which is a process that deletes or inserts a pair of gene segments V (variable), D (diversity), and J (joining) by DNA rearrangements occurring on the antigen binding portions of the cell receptor (variable regions) TCR α and β gene segments (Roth 2014).

NKT cells shares features with T cells and NK cells. NKT cells express NK cell markers, including NK1.1 (CD161), CD49b, Ly5.1 and NKG2D, and a TCR on their surface similarly to T cells. TCRs from this cell population are specific for lipid antigens instead of peptides, which are presented at the surface of APCs by the related MHC class I molecule CD1d (Godfrey et al., 2010).

NKT cells can be divided into two groups that differ according to their TCR expression: Type I or invariant NKT (iNKT) cells that express a semi-invariant TCR and recognize the glycolipid α-galactosylceramide (α-GalCer); type II NKT cells or diverse NKT (dNKT) cells that present variable TCRs and do not recognize α-GalCer (Pereira et al., 2017).

Invariant NKT cells

Restricted CD1d cells that express a semi-invariant TCR αβ-chain (Vα24Jα18 Vβ11 in humans and Vα14Jα18 in mice) are known as iNKT cells (figure 1) , and comprise about 0.1-0.2% of total T cells in human peripheral blood (Kumar & Terry 2014). Type I NKT cells play roles both in adaptive and in innate immunity and can be known as a bridge between these responses. As innate cells, iNKTs produce immunoregulatory cytokines that influence other immune cells (Salio et al., 2014), but they also recognize antigens through it's TCR . These cells can release generous amounts of Th1 and Th2 cytokines within minutes of TCR engagement (Salio et al. 2014) like GM-CSF, TNF-α, IFN-ϒ, IL-4, IL-10 and IL-13 which can induce the maturation of DCs into APCs (figure 1) that will in turn activate conventional T cells and consequently the immune response (Lynch et al. 2009) (Wolf et al. 2018).

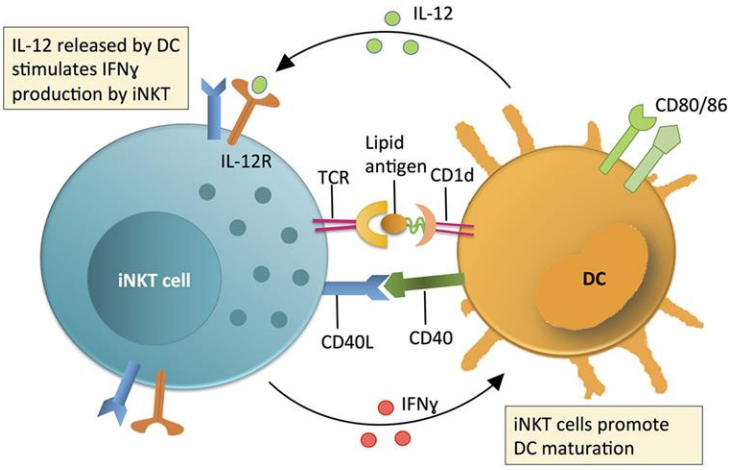

Figure 1 DC presenting lipid antigen through CD1d molecule to Invariant NKT cell. DC also can produce IL-12 which will stimulate iNKT cell IFN-ϒ production (Wolf et al. 2018).

Invariant NKT cells can also express, as conventional T cells, CD4, CD8 or to be DN for these molecules. $CD8^+$ iNKT cells produce high amounts of pro-inflammatory cytokines such as GM-CSF, TNF-α, IFN-ϒ, IL-12, and lower quantities of anti-inflammatory cytokines, like IL-4, IL-10, having a high cytolytic activity. iNKT cells expressing CD4 produce high amounts of anti-inflammatory cytokines (low cytolytic activity) providing B cell help or immunoregulatory functions (Gumperz et

al., 2002). iNKTs cells that do not express CD4 nor CD8 molecules can produce similar amounts of anti-inflammatory and pro-inflammatory cytokines promoting Th responses different than $CD4^+$ cells because they differed in many functionally relevant ways: Th cytokine profile, pattern of chemokine receptors, and integrin expression, and displayed different NK receptors on the cell surface (Lee et al., 2002) (Reilly et al. 2011).

In mice, functional subsets of iNKT cells have been identified: iNKT1, iNKT2, iNKT10 and iNKT17, being characterized by the production of the representative cytokine IFN-ϒ, IL-4, IL-10 and IL-17, respectively (Engel et al. 2016). iNKTs subsets are located preferentially in specific tissues and are present in different frequencies, which was revised in (Lee et al. 2015)(Crosby & Kronenberg 2018) and summarized in table 1.

Table 1 Presence of iNKT subsets in the different mouse tissue sites. Each "+" symbol corresponds to the relative presence of each subset in the indicated tissue. Based on (Crosby & Kronenberg 2018).

Tissue Subset	Lymph nodes	Liver	Spleen	Mesenteric lymph nodes	Thymus	Lung	Adipose tissue	Small intestine
iNKT1	+	+++++	+++	++	+++	+++	+	+++
iNKT2	+	-	+	++	+	+	-	+
iNKT10	-	-	-	-	-	-	++++	-
iNKT17	+++	-	+	+	+	+	-	+

Invariant NKT cells, mature in thymus from bone marrow-derived precursors (Pereira et al., 2017) (Salio et al. 2014); sharing the same maturation process of T cells until the phase where they are selected (figure 1). Cells that don't recognize self-peptides/MHC complexes, after TCR rearrangement, are positively selected by $CD1d^+$ DP thymocytes (Pereira et al. 2017). The maturation of these cells is divided in four stages. The first is defined as $CD24^+CD44^{lo}NK1.1^{lo}$ -stage 0. All stage 0 cells are $CD4^+$, extremely rare and non-proliferating. Then CD24 is downregulated and cells enter into stage 1 being $CD24^{lo}CD44^{lo}NK1.1^{lo}$ and start to proliferate. During proliferation and maturation, NKT cells upregulate CD44 turning into $CD24^{lo}CD44^{hi}NK1.1^{lo}$ – stage 2. Stage 3 occurs, mainly, in the periphery and is characterized by the reduction of proliferation and upregulation of NK cell markers. Transition to this last stage is CD1d dependent, suggesting that TCR/CD1d interaction could represent a checkpoint to correct TCR expression in NKT cells (Godfrey et al. 2010)(Salio et al. 2014).

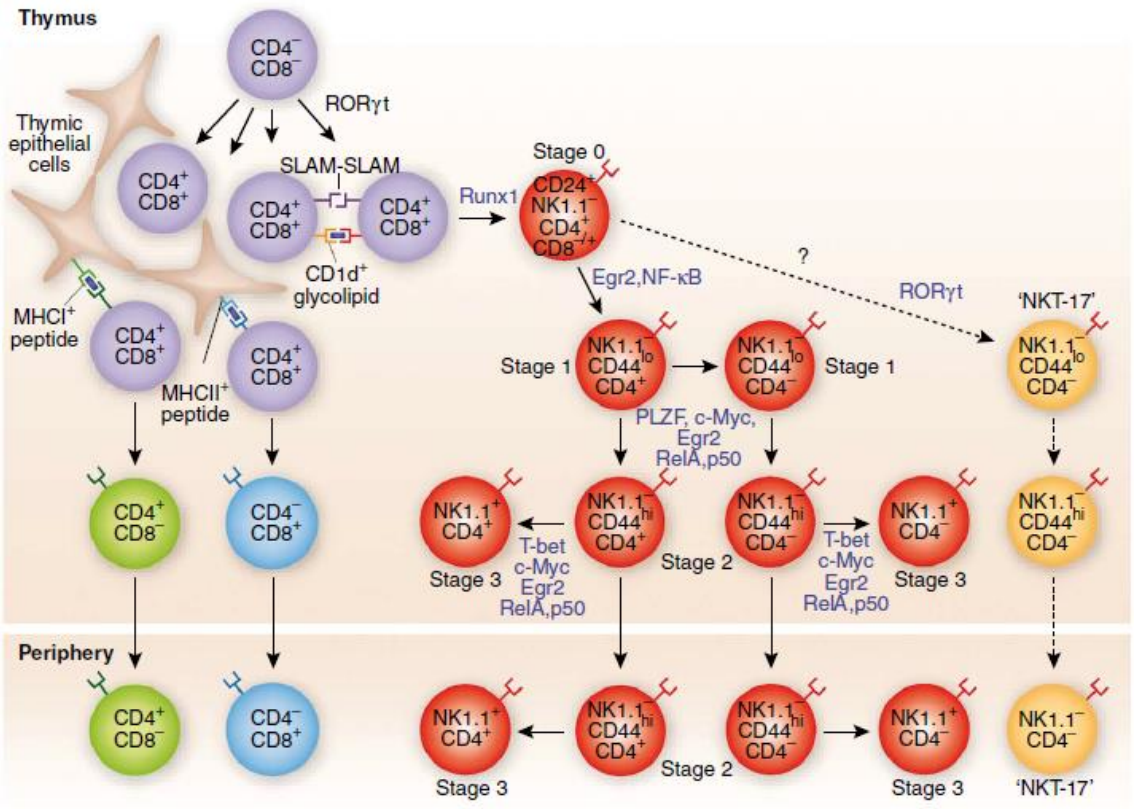

Figure 2 iNKT cells selection and differentiation in the thymus (Godfrey et al. 2010).

Invariant NKT cells activation

During infection, iNKT cells can be activated directly by recognizing microbial lipid antigen bound to CD1d, in the absence of such antigens, indirectly, by self-lipids produced by the APC in response to the pathogen or by cytokine-mediated stimulation or a combination of cytokine- and TCR-mediated stimulation.

It was proven that there are several bacteria lipids after being loaded into CD1d molecules, can stimulate iNKTs directly by TCR recognition (figure 3, left) (Mattner et al. 2005). But, stimulation of iNKTs can also be reached indirectly, by innate cytokines produced by APCs after encountering the pathogen, like IL-12, IL-18 or type I IFNs (figure 3 right) (Brigl & Brenner 2010). Bacteria such as *Salmonella typhimurium, Staphylococcus aureus* and *Mycobacterium tuberculosis* that lack agonist glycolipids have been reported to activate iNKT cells through recognition of endogenous lysosomal glycosphingolipids, presented by dendritic cells (figure 3 center) (Mattner et al. 2005). IL-12, a pro-inflammatory cytokine released by DCs after exposure to bacterial products such as LPS, is capable of stimulating iNKT cells in combination with a weak TCR-mediated signal received by recognition of CD1d-presented self-antigens (figure 3, center)

(Mattner et al. 2005). This recognition of self or altered-self antigens by the iNKT cell TCR in combination with cytokine-mediated stimulation provides a critical TCR-mediated signal for iNKT cells activation providing an immune response to a wide variety of bacterial, protozoan and viral pathogens.

In response to microbial products or infections, significant amounts of potent pro-inflammatory, IFN-ϒ-inducing cytokines (IL-12 and IL-18) are secreted by APCs. In this context, iNKT cell activation is dominantly cytokine-driven and the role for a CD1d/TCR-mediated signal seems reduced (figure 3, right) (Wang et al. 2008).

Curiously, the type of activation of iNKT cells influence their effector functions. For example, stimulation with cytokines leads to IFN-ϒ secretion and low amounts or no secretion of IL-4 while stimulation with cognate microbial antigens results in both IFN-ϒ and IL-4 secretion (Brigl & Brenner 2010).

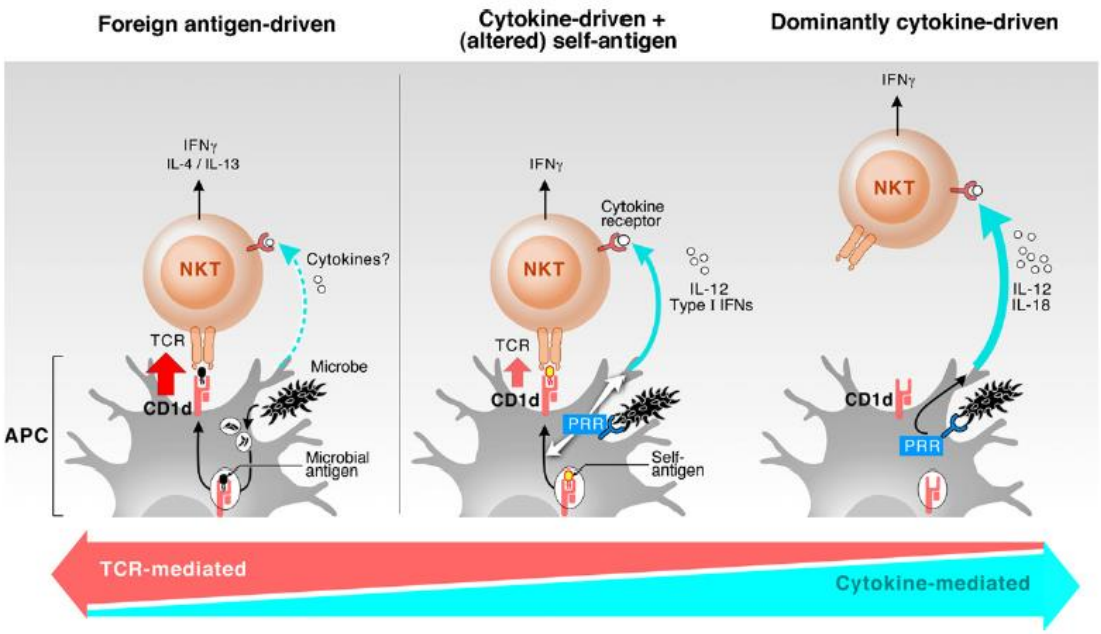

Figure 3 Different forms of iNKT cells activation. On the left: Foreign antigen-driven; on the middle cytokine and sefl-antigen driven; on the right: cytokine-driven (Brigl & Brenner 2010).

Immunotherapeutic potential of iNKT cells

In health and disease the outcome of immune responses are the function and activity of different immune cell types one of them are iNKT cells that can mediate immunosuppression and immunoregulation. iNKT cells has been considered as immunoregulators and can play an important role in the induction and prevention of autoimmune disease, inflammation and cancer (Kumar & Terry 2014).

Several studies show that iNKTs are involved in the suppression of inflammatory chronic diseases like Systemic Lupus Erythematous (SLE) (Cho et al. 2011)(Chen et al. 2015), Rheumatoid Arthritis (Gutowska-Owsiak et al., 2014) , Sjögren's syndrome (SS) (Voskuyl et al. 2001), systemic sclerosis (SSc) (Sumida et al., 1995), psoriasis (PSA) (A. M. Tobin et al., 2011), adult onset Still's disease (AOSD) (Lee et al. 2012) and Behcet's uveitis (Yu et al. 2004). The number of iNKT can be increased or decreased in the context of different diseases, which can contribute for the progression and dissemination of the disease (Kaer & Wu 2018). However, in pathological situations iNKT responses can also be harmful. In liver hepatitis, and upon increased CD1d expression, hepatic iNKT cells can accumulate and produce IL-13. $CD1d^{-/-}$ mice had an insulin-resistant phenotype, without adipose tissue inflammation, suggesting a protective role for iNKT cells in adipose tissue (Schipper et al. 2012). In patients with obesity iNKT cell response is protective leading to the secretion of IL-4 and IL-13 when modulated directly by adipocytes (Schipper et al. 2012). In obese and cancer patients the number of iNKTs in the omentum is reduced, which can be a consequence of a general and weakened immune system detrimental for the control of these diseases (Lynch et al. 2009).

Recent explosion of T cell immunotherapy in cancer incited the application of CD1d-restricted T cells in tumor patients (Lepore et al., 2018). Several clinical trials are being performed using α-GalCer, soluble CD1d-α-GalCer complexes conjugated with tumor-specific antibodies, α-GalCer conjugated with tumor-associated peptides or administration of DCs pulsed with both α-GalCer and long peptides from the tumor-associated protein (Gasser et al. 2017). These treatments induced significant improvement both in animal and in clinical settings (Lepore et al. 2018). An important therapeutic strategy is based on tumor-specific recognition by CD1-restricted T cells. As lipids are altered in tumor cells, especially glycosphingolipids, tumor-associated lipids could be specifically recognized eliciting an immune response against de tumor (Lepore et al. 2014).

Invariant Natural Killer cells defects were also associated with Lysosomal Storage Diseases (LSD) a group of rare inherited metabolic disorders resulting from defects in lysosomal function. Since lysosome is responsible for macromolecules digestion and its dysfunction leads to the accumulation of undigested or partially digested macromolecules (Pereira et al. 2017). The accumulation of non-specific glycosphingolipids within the lysosomal compartment, can cause entrapment of endogenous ligands, with consequent defective presentation of specific glycosphingolipids species and reduced loading of the CD1d ligands needed for iNKT cell selection (Pereira et al 2017). A competition could also happen between the stored lipids for CD1d binding

with a decreased probability of correct antigen presentation and iNKT selection (Pereira et al 2016; 2018).

The correlation between the frequency of iNKT cells and their capacity to be protective or not has not been well established yet, but probably parallelisms between cells frequency, their cytokine production patterns and disease onset could enhance the knowledge of different diseases (Kumar & Terry 2014).

Diverse NKT cells

Type II NKT cells (also called diverse NKT or dNKT) can be identified by the absence of invariant TCR α chain using a variable TCR αβ or ϒδ chains and their CD1d-restriction (Pereira et al. 2017). Diverse NKT cells are reactive to both glycolipids and phospholipids derived from self as well as microbes and are more abundant than type I NKT cells in humans (Dhodapkar et al., 2017).

One of the major differences in the two NKT cell subsets is in the recognition of α– vs β-anomeric linkage of a carbohydrate moiety to a lipid tail in glycolipids. For example, while type I NKT cells recognize their prototypic ligand α-GalCer, type II NKT cells are not reactive to α-GalCer. The first antigen defined for a subset of murine type II NKT cells was sulfatide, a sulfated glycolipid enriched in membranes of various tissues, such as myelin of central nervous system (CNS), pancreas, kidney and liver. Subsequently sulfatide-reactive CD1d-restricted NKT cells have been identified in humans as well (Jahng et al. 2004).

Type II NKT cell can be mainly activated via TCR signaling following recognition of lipid/CD1d complex (Dhodapkar et al., 2017).

As part of the immune system, dNKT cells play an important role in immunity maybe due to their ability to interact with other cell types and modulate their function, such as T and B cells, DCs and type I NKT cells. Type II NKT cells can inhibit pro-inflammatory functions of T cells, iNKTs and DCs, but also can have a pro-inflammatory function in gut immunity (Dhodapkar et al. 2017).

CD5 molecule

CD5 is a type I transmembrane surface glycoprotein of 67 KDa which belongs to the scavenger receptor cysteine-rich (SRCR) family (Gimferrer et al. 2003). CD5 is expressed on thymocytes, mature peripheral T cells and in the subset of B cells B1a (Gimferrer et al. 2003). Dendritic cells, a type of antigen presenting cell, can also express CD5 and those $CD5^+$ DCs are superior activators of inflammatory T cell responses (Korenfeld et al. 2017). Its expression on T cells is proportional to the avidity for self-peptide/MHC complex (Azzam et al. 1998). This molecule is composed by three

SRCR extracellular domains and a conserved cytoplasmic domain with no enzymatic activity (Bamberger et al. 2011), which includes an imperfect immunoreceptor tyrosine-based activation motif (ITAM), an immunoreceptor tyrosine-based inhibition motif (ITIM) and multiple potential tyrosine and Ser/Thr phosphorylation sites (Azzam et al. 2001). After TCR stimulation, the CD5 cytoplasmic domain becomes rapidly phosphorylated and recruits other signaling molecules like Cbl, CK2, rasGAP and SHP-1 - signaling inhibitors (Dong et al. 2016), being the protein tyrosine kinase Lck probably the main responsible for its phosphorylation (Bamberger et al. 2011).

CD5 expression can be used as a marker for post-selected thymocytes, since it's expression correlates with TCR mediated signaling during self-peptide/MHC recognition (Mier-aguilar et al., 2016). Inhibition of T cell activation and differentiation by CD5 does not diminish T cell adhesion to APCs neither affects the formation of immunological synapses (Brossard et al., 2015).

CD5 acts like a negative regulator of TCR signaling as the absence of CD5 slants the developing T cells towards uncontrolled TCR/CD3 mediated proliferation (Tarakhovsky et al. 1995). CD5 defective mice show also an increased proliferative response and improved phosphorylation of signaling effectors (Bamberger et al. 2011).

In B cells, CD5 induces IL-10 production (Garaud et al. 2016), while in T cells this molecule inhibits the production of IL-2 and IFN-Y and the proliferation and activation of these cells (Bamberger et al. 2011). When CD5 signaling is inhibited, the secretion of IL-2 increases and, as a consequence, proliferation of leukemic T cells decreases (Rosenberg 2014)(Ambak Kumar et al., 2017).

Besides being a modulator of TCR signaling, CD5 also plays a role in cell survival by controlling apoptosis. Studies performed with Tregs proved that $CD5^{-/-}$ mice undergo increased cell death and thymocytes from the same mice have increased susceptibility to apoptosis (Lozano et al. , 2009).

Casein Kinase 2 (CK2) is a serine/threonine kinase responsible for modulating cell cycle progression. CK2 is associated with the cytoplasmic tail of CD5, and upon engagement of CD5, the kinase is activated (Chander Raman et al., 1998). CD5 promotes survival through activation of CK2 (Axtell et al., 2006) that was shown to promote cell survival of lymphocytes both by directly inhibiting apoptosis or inhibiting proteins associated with apoptotic pathways, such as caspases and Bid and by activating or inducing expression of pro-survival molecules such as Bcl-2 and Bcl-xl (Ahmed et al., 2002).

CK2 associates with CD5 in thymocytes, reducing Akt phosphorylation in $CD5^{-/-}$ thymocytes in response to TCR crosslinking which could explain the increased death in these cells. Akt was

shown to play a role on thymocyte survival by induction of Bcl-2 family members (anti-apoptotic molecules), such as Bcl-XL (Lozano et al. 2009).

$CD5^{-/-}$ mice exhibited an enhanced T cell response that supports the initial idea that CD5 has an inhibitory role (Axtell et al. 2006). T cells from CK2 binding/activation-deficient mice are more susceptible to apoptosis induction (Lozano et al. 2009). In the absence of the CK2-CD5 interaction, levels of Bcl-2 decrease leading to increased cell death. On the other hand, in the presence of the CK2-CD5 interaction after TCR/CD3 stimulation, Bcl-2 levels increased suggesting that Bcl-2 could be involved in CK2- dependent thymocyte survival (Mier-aguilar et al. 2016).

CD5 could be involved on thymocyte survival (Lozano et al. 2009), and CK2 has a key role in CD5-mediated regulation of thymocyte selection and survival (Mier-aguilar et al. 2016). Also, the CD5-CK2 axis may have implications in the development of autoimmune diseases, given that the severity and incidence of experimental autoimmune encephalomyelitis (EAE; an animal model of multiple sclerosis) was dramatically reduced in CD5-CK2-binding defective mice compared with wild-type (WT) and $CD5^{-/-}$ mice (Axtell et al. 2006). Taking this into account, targeting CD5 could be therapeutically beneficial to treat inflammation, autoimmunity and perhaps leukemias without inducing immune depletion (Soldevila et al., 2011).

CD6 molecule

The immunological synapse is the interface between an APC and a T lymphocyte and is where T cell activation is initially regulated. The TCR binds to the pepide-MHC complexes expressed on the APC, and this recognition is followed by multiple interations involving membrane receptors of both cells that induce activating or repressive signals (Dustin 2014). The co-receptors CD4 or CD8, through their association with protein tyrosine kinases, are able to promote and amplify TCR-mediated signaling by inducing the phosphorilation of tyrosine residues within ITAMs present in the cytoplasmic tails of TCR-associated CD3 chains (Santos et al 2016). Similar to CD5, (figure 4) CD6 is a type I transmembrane glycoprotein with

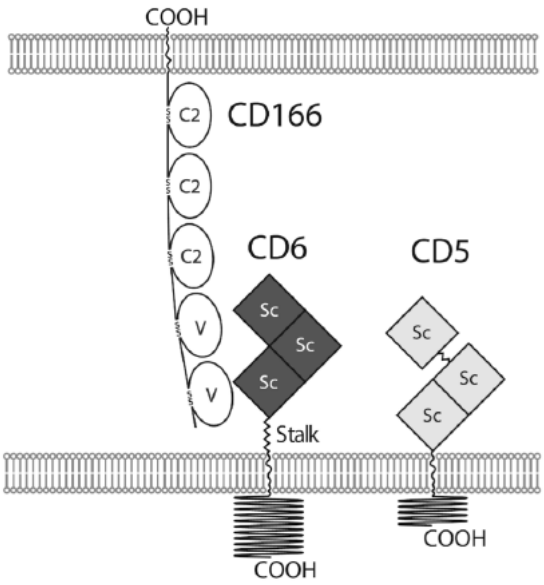

Figure 4 CD5 and CD6 molecule structure (Brown 2016).

three SRCR extracellular domains and a long cytoplasmic region, with no catalytic activity, containing tyrosine residues that are phosphorilated upon TCR stimulation (Wee et al., 1993) and is expressed on thymocytes, T cells and the B1a B cell subset (Gimferrer et al. 2003).

Studies using antibodies revealed that crosslinking CD3 or co-crossliking CD3 with CD2 or CD4 within the T cell membrane resulted in tyrosine phosphorilation of CD6 (Wee et al., 1993). Furthermore, aggregation of TCR/CD3 complex with CD6 promotes calcium signaling, IL-2 production and T cell proliferation (Oliveira et al. 2012).

CD6 can be involved on thymocyte survival and selection in mice and humans, once CD6 surface expression levels increase when DP thymocytes are selected to become SP cells, and this is correlated with both the expression of the selection marker CD69 and resistance to apoptosis (Singer et al. 2002). Moreover, CD6 expression on the surface diminished from mature thymocytes to resting peripheral blood T cells. This down-regulation probably starts before thymic emigration (Singer et al. 2002).

The main known CD6 ligand is CD166, also known as ALCAM (activated leukocyte cell adhesion molecule) belonging to the immunoglobulin cell adhesion molecule superfamily. It was recently discovered that CD318 is another ligand for CD6 (Enyindah-asonye et al. 2017) after the identification of the antigen recognized by the developed mAb 3A11 (Alonso-ramirez et al. 2010). CD6 may bind in *trans* to surface glycoproteins (such as ALCAM and 3A11), or to microbial lipopolysaccharides, and may bind in *cis* to endogenous ligands (such as CD3 and CD5), and thereby deliver a costimulatory signal (Alonso-ramirez et al. 2010).

The most membrane–proximal CD6 SRCR domain interacts with the N-terminal immunoglobulin domain of CD166. CD166 is expressed on bone marrow (BM) stromal cells, thymic epithelial cells, activated T and B cells, monocytes, dendritic cells, synovial fibroblasts, keratinocytes, and mesangial stem cells (Bowen et al. 1995). This interaction is strong enough to strengthen and stabilize T cell-APC contacts (Hassan et al., 2004).

CD6 has been targeted with antibodies in therapeutic trials to treat a number of diseases, such as multiple esclerosis, rheumatoid arthritis, psoriasis, cutaneous T cell lymphoma, achieving significant success (Pinto & Carmo 2013). Studies using cellular models have described that different CD6 antibodies may induce equivalent or divergent effects, some inhibiting and others activating T cell proliferation, and also having anti-apoptotic effects, depending on the presence or type of the acessory molecules, immunoglobulin class and on the antibody (Santos et al., 2016).

Overall, CD5 and CD6 are two transmembrane surface glycoproteins expressed by T cells and thymocytes. Through inhibition or co-stimulation, these molecules have the capacity to regulate TCR signaling and consequently control T cell proliferation and survival as well as positive and negative selection during thymocyte development.

Considering that iNKT cells are a subset of T cells that have important roles in inflammation, infection and cancer control, and only one study has reported the expression of CD5 and CD6 on iNKTs (mouse) (Tuttle et al. 2018), it would be interesting to study the expression of these molecules on human type I NKT cells.

Aims

The main objective of this work is to study CD5 and CD6 expression and function on human iNKT cells. To achieve this objective, the following specific aims were defined:

1. To study the basal expression of CD5 and CD6 on human peripheral blood iNKT cells.
2. To analyze possible alterations in the expression of CD5 and CD6 by iNKT cells after activation, using a human iNKT cell line.
3. To evaluate the involvement of CD6 in the antigen recognition by iNKT cells and their activation. For this, a human iNKT cell line and antigen presenting cells expressing or not CD166 were used.

Material and methods

Peripheral Blood Mononuclear cells isolation

Peripheral Blood Mononuclear cells (PBMCs) were isolated from Buffy coats by density gradient using HISTOPAQUE®-1077 (Sigma-Aldrich, St. Louis, MO, USA) under sterile conditions. Buffy coats were obtained from Blood Bank of *Hospital de São João* (Porto, Portugal) and diluted 1:1 in phosphate buffered saline (PBS) 1x (Invitrogen, California, USA). Diluted blood was layered over a half volume of the diluted blood of HISTOPAQUE. Tubes were then centrifuged at 400 g for 30 minutes at room temperature (RT), without break. After centrifugation, PBMCs were located in a visible ring (figure 1) that was collected and washed twice with PBS 1x by centrifugation at 350 g during 5 minutes. Afterwards, PBMCs were counted using a Neubauer chamber and Trypan blue for exclusion of dead cells and were used for flow cytometry analysis of peripheral blood iNKT cells or as APCs in iNKT cell line re-stimulations.

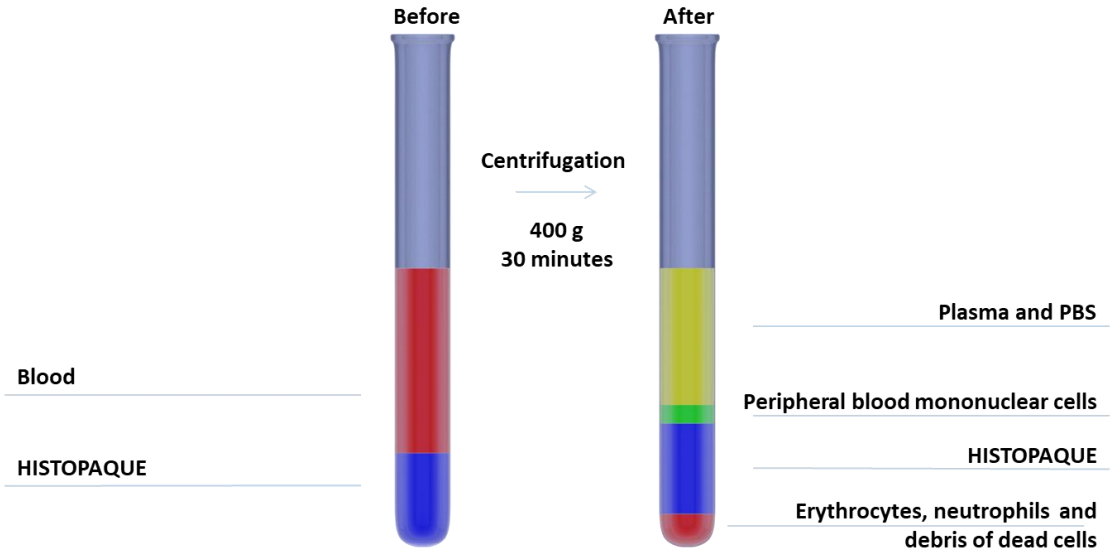

Figure 5 PBMCs isolation by density gradient using HISTOPAQUE 1077 as separation medium.

iNKT cell line culture, re-stimulation and maintenance

The iNKT $CD4^+$ cell line used in this work was generated in the laboratory as described before (Pereira et al. 2016). These cells were maintained in 24-well plates (Orange scientific, Belgium) at an approximate cell concentration of $1x10^6$ iNKT cells/mL in T cell medium composed by RPMI 1640 - GlutaMAX supplemented with 10% non essential amino acids, 10% kanamycin and 10% sodium pyruvate, all from Invitrogen (California, USA), and 5% human serum (HS) pooled from 5 AB donors obtained from *Hospital de São João* (Porto, Portugal).

This iNKT CD4+ cell line needs to be re-stimulated every 17-19 days. For this, PBMCs were used as APCs and phytohaemagglutinin (PHA) (Thermo Fisher Scientific, Waltham, MA, USA) as stimulus for cell proliferation. PBMCs were irradiated with a total of 3000 rad (Gammacell 1000, Nordion, Canada) for 5 minutes. After irradiation, PBMCs were washed by a 5 minutes centrifugation at 350 g at RT, counted using a Neubauer chamber and Trypan blue, diluted in T cell medium and, finally, $2x10^6$ irradiated PBMCs were distributed per well over iNKT cells. PHA was then added to the culture at a final concentration of 1µg/mL. T cell medium was refreshed at day 5 after re-stimulation and then each 2-3 days until the next re-stimulation.

For iNKT cell activation assays, iNKT CD4+ cell line was used between days 13 and 16 after re-stimulation.

iNKT cell line activation and monitoring of CD5 and CD6 expression

At day 0, human iNKT $CD4^+$ cell line was re-stimulated with irradiated PBMCs and with α-GalCer (1ug/mL) (Avanti polar lipids, Alabaster, AL, USA) to activate iNKTs or with PHA (1ug/mL) that was used as a positive control. These cells were collected daily (250µL), except in the day 0, and stained as described below, for flow cytometry acquisition. CD5 and CD6 Mean fluorescence intensity (MFI) were quantified in the iNKT cells gate. Cells medium was changed two times a week and cells were split to a new plate 2/3 days after re-stimulation, approximately.

iNKTs antigen presentation assays using Raji B cells as APCs

To study the involvement of CD6 in the antigen recognition by iNKT cells, Raji B cells expressing (wild type - WT) or not (knockout - KO) the CD6 ligand CD166 were used as APCs. Raji CD166 KO were produced in Prof *Alexandre do Carmo laboratory*. These cells were maintained in culture in T75 flasks using RPMI 1640-GlutaMAX medium complemented with 10% inactivated FBS (Invitrogen, California, USA), 10% non-essential amino acids, 10% kanamycin and 10% sodium pyruvate.

It is described that EBV-transformed B cell lines lose the expression of CD1d (Chung et al. 2014) and that culture with retinoic acid (RA) recover the expression of this molecule (Allan et al. 2011). To induce the expression of CD1d in Raji B cells, cells were collected, centrifuged (350 g during 5 minutes at RT) and counted in a Neubauer chamber using Trypan blue. $5x10^6$ WT and KO Raji cells were cultured, in duplicated, in 10 mL of complete media without or with 25nM of RA (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained during 3 days in these conditions for

CD1d expression induction. The EBV-transformed B cell line 447, produced in Fátima Macedo's Laboratory using B cells from a healthy individual, was used as control of CD1d expression induction. Flow cytometry was used to check if B cells increased the expression of CD1d (figure 2).

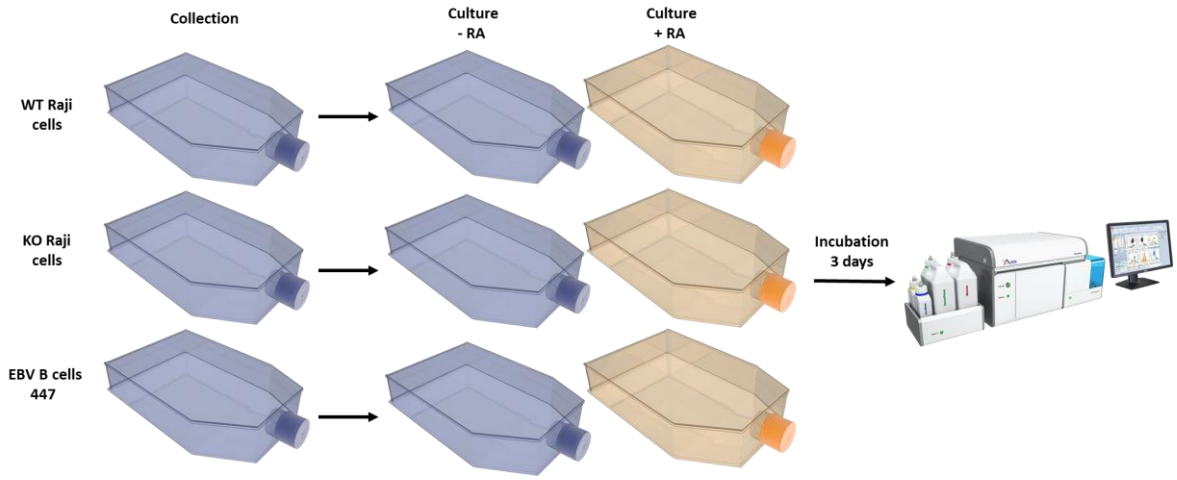

Figure 6 B cells were cultured with (+) and without (-) Retinoic acid (RA) for induction of CD1d expression that was checked by flow cytometry.

After CD1d induction, WT Raji, CD166 KO Raji, and 447 B cells were used as APCs in antigen presentation assays to study the response and activation of iNKT cells. B cells were adjusted at $1x10^6$/mL in RPMI 10% non-essential amino acids, 10% kanamycin and 10% sodium pyruvate but without FBS and plated 25000 cells/well (25 µL), in triplicate for each antigen condition, in a 384 well-plate. Then, the antigen (α-GalCer) was added in 25 µL of RPMI without FBS for 3 different final concentrations: 50, 12.5 and 3 ng/mL. PHA was used as positive control at a final concentration of 1 µg/mL and RPMI was used as negative control (figure 7). B cells were incubated with α-GalCer, PHA or media during 4 hours. Then, the iNKT $CD4^+$ cell line was collected from the culture, centrifuged, counted using a Neubauer chamber and Trypan Blue and adjusted at $1x10^6$/mL in RPMI 20% FBS. Invariant NKT cells were plated at 50000 cells/well (50 µL) and the co-culture was maintained during 40h, when supernatants were collected and frozen for GM-CSF determination by ELISA. See figure 8 for a typical plate layout of the antigen presentation assays used in these studies.

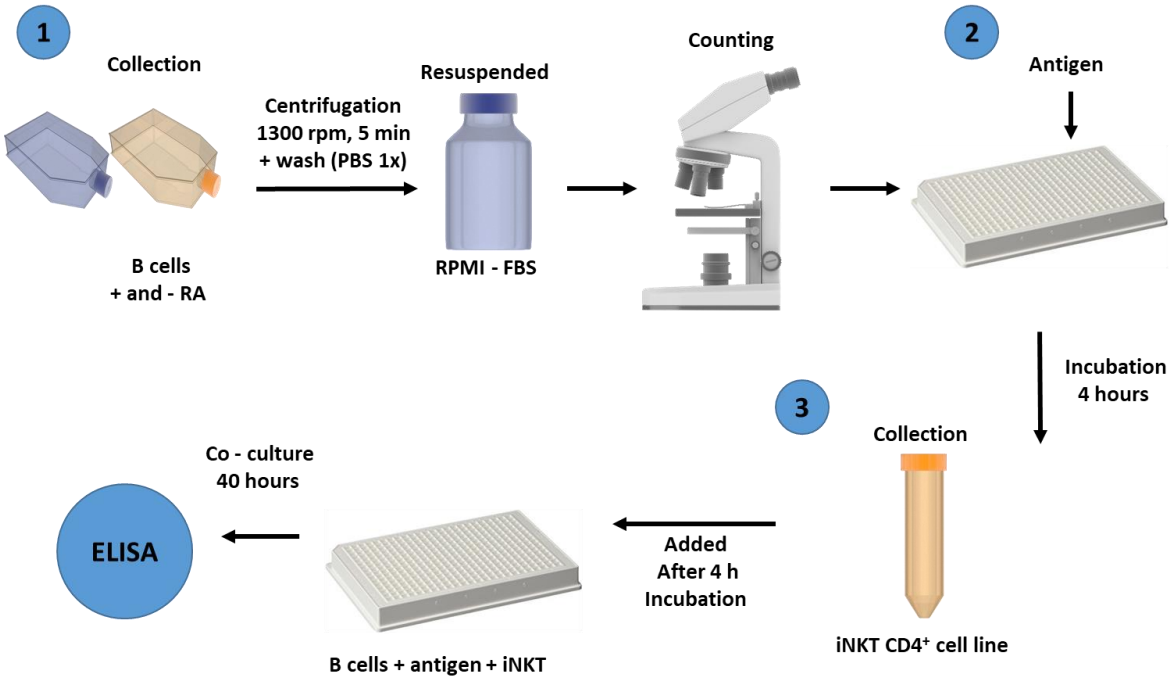

Figure 7 Antigen presentation assay. A first incubation was performed between APCs (B cells) (1) and the antigen during 4 hours (2). Then, responder cells (iNKT CD4+ cell line) were added to the culture (3). Cells were co-cultured during 40 hours. The activation of iNKT cells was measured by the quantification of released GM-CSF using an ELISA.

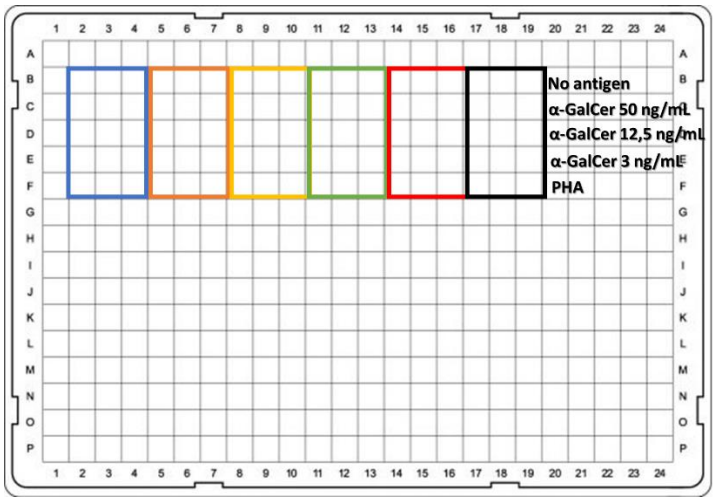

Figure 8 Antigen presentation assay plate layout. Colored squares represent the different APCs used in these studies: Blue - WT Raji cells with RA; Orange - WT Raji cells without RA; Yellow – CD166 KO Raji cells with RA; Green CD166 KO Raji cells without RA; Red – B cells 447 with RA; Black - B cells 447 without RA. The different antigen concentrations used are written in the figure: 3 different concentrations of α-GalCer were used and PHA at 1 µg/mL.

Flow cytometry

Cells for flow cytometry analysis were collected and centrifuged (350 g, 5 minutes, RT). After discarding the supernatant, cells were resuspended in 25 µL (for iNKTs) or 50 µL (for PBMCs) of fluorochrome-conjugated antibody/tetramer mixes (table 2) diluted in FACS Buffer (PBS, 0.2% BSA (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% of NaN_3 (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 4ºC during 20 minutes, in the dark (figure 9). Then, cells were washed two times with cold FACS Buffer. After that, cells were resuspended in 150 µL (for iNKTs) or 300 µL (for PBMCs) of FACS Buffer for flow cytometry acquisition. Cells were immediately acquired using 3-laser BD FACS Canto™ II flow cytometer using the BD FACS Diva™ software (both from BD Biosciences, New Jersey, USA). Flow cytometry analyses were performed using the FlowJo software V10 (FlowJo, LLC, Ashland, OR, USA).

Table 2 Fluorochrome-conjugated antibodies and CD1d tetramer used in flow cytometry analysis to evaluate CD5 and CD6 expression on iNKT cells.

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Molecule	Fluorochrome	Clone	Brand
Anti-human CD6	FITC	BL-CD6	Biolegend, San Diego, CA, USA
PBS57-loaded hCD1d tetramer	PE	-	National Institute of Health, Maryland, USA
Anti-human CD5	APC	UCHT2	Biolegend, San Diego, CA, USA
Anti-human CD3	PerCP-Cy 5.5	OKT3	eBioscience, San Diego, CA, USA
Anti-human CD4	PE-Cy7	RPA-T4	eBioscience, San Diego, CA, USA
Anti-human CD8	APC-eFluor 780	RPA-T8	eBioscience, San Diego, CA, USA

FITC – Fluorescein isothiocyanate; PerCP-Cy5.5 – Peridinin-chlorophyll proteins-Cyanine 5.5; PE – Phycoerythrin; PE-Cy7 – Phycoerythrin-Cyanine7; APC – Allophycocyanin.

Mean fluorescence intensity (MFI) of CD5 and CD6 was quantified in iNKT cells. To define the iNKT cell population, in both PBMCs and iNKT CD4+ cell line samples, the following gating strategy was used: live cells by size and complexity or granularity using forward versus side scatter (FSC vs SSC), respectively – single cells by height and area using FSC-H and FSC-A, respectively - lymphocytes by size and complexity using SSC - A and FSC- A, T lymphocytes by expression of CD3 – iNKT cells by their positive staining for the CD1d tetramer loaded with PBS57. CD4 and CD8 expression was also analyzed in iNKT cells from PBMC samples.

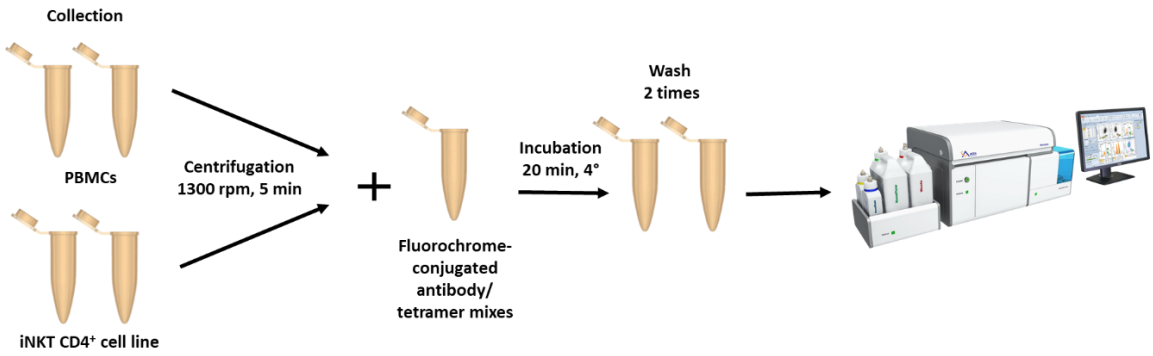

Figure 9 Scheme of flow cytometry staining and acquisition.

Quantification of GM-CSF in supernatants by Enzyme-Linked ImmunoSorbent Assay

The determination of GM-CSF concentration in the supernatants from antigen presentation assays to iNKTs was performed by ELISA (figure 10). ELISA plates (Maxisorp, Thermo Fisher Scientific, Waltham, MA, USA) were coated with 50 µL per well of purified anti-human GM-CSF (Biolegend, San Diego, CA, USA) at 1.5 µg/mL diluted in blocking buffer (PBS, 0.05% Tween 20 , 1% BSA) at 4°C, overnight (capture antibody). Then, coating solution was removed and wells were washed once by adding 300 µL of washing buffer (PBS, 0.05% Tween 20) to each well. After that, wells were blocked by adding 100 µL of blocking buffer and incubated for one hour. Afterwards, plates were washed twice and incubated with 50 µL of sample (supernatant resulting from antigen presentation assays) or 1:2 serial diluted rhGM-CSF standard (Biolegend, San Diego, CA, USA) (8 different concentrations, in duplicate, starting at 20 ng/mL). Two hours later, plates were washed three times and 50 µL of biotinylated anti-human GM-CSF antibody (Biolegend, San Diego, CA, USA) at 0.2 µg/mL, diluted in blocking buffer, were added to each well (detection antibody). One hour after, wells were washed three times and 70 µL of Streptavidin conjugated with Horseradish Peroxidase (HRP) (Invitrogen, California, USA) diluted 1:5000 in blocking buffer were added to each well for detection of the biotinylated antibody, and incubated in the dark during 1 hour. Then, the substrate solution for the HRP, – o-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, St. Louis, MO, USA), was prepared according with manufacturer's instructions. Plates were washed 3 times and 90 µL of OPD were added per well. The reaction occurred during 20-30 minutes at RT in the dark. To stop the reaction, 50 µL of 10 % H_2SO_4 (Fluka, New Jersey, USA) were added and the absorbance values were read immediately at 490nm on an ELISA plate reader (Synergy Mx microplate reader from Biotek, Winooski, VT, USA).

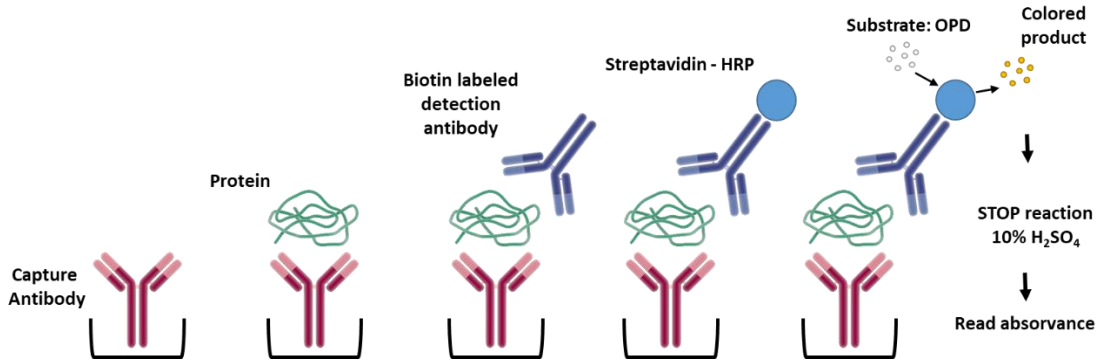

Figure 10 Scheme of the sandwich ELISA technique used in this work.

Observation of iNKTs-APCs synapses by Confocal Fluorescence Microscopy

Glass slides (Deltalab, Spain) were coated with poly-l-lysine at 0.1 mg/mL for 1 hour at RT and washed with PBS. WT Raji or B447 cells, pre-cultured during 3 days with RA to induce their expression of CD1d. were collected and incubated without or with α-GalCer ($1x10^6$ cells per condition) at 500 ng/mL during 45 or 120 minutes at 37°C in a water bath. Then, B cells and the antigen were laid over the poly-l-lysine coated slides (previously dried) and let to adhere during 30 minutes at 37°C in the incubator. After that, excess medium was removed and iNKT cells $1x10^6$/mL were added to the slides and let to adhere during 45 or 120 minutes. Excess medium was then removed and cells were fixed for 10 minutes with 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Pennsylvania, USA) at RT and washed. Afterwards, the staining was performed. For this, fixed slides were first blocked with PBS 5% BSA for 30 minutes at RT and then stained with anti-CD6 (mouse, clone MEM98) (Exbio, Czech Republic) diluted 1:50 during one hour at RT, after which the secondary antibody anti-mouse Alexa Fluor 568 (Invitrogen, California, USA) diluted 1:500 was added for 45 minutes at RT. After that, anti-CD3 (mouse, clone UCHT1) Alexa Fluor 488 (Biolegend, San Diego, CA, USA) diluted 1:100 was added and incubated for one hour at RT (slides were washed between the staining steps). Finally, DAPI diluted 1:1000 was added to slides for 5 minutes, in the dark, and washed. For sample protection and maintenance, a mounting medium, Vectashield (Vector Laboratories, California, USA) was added to the sample and a glass cover slip 18mm (Thermo Fisher Scientific, Waltham, MA, USA) was placed over the sample and sealed with nails polish. Lastly, images were acquired using confocal microscope Leica TCS SP5 (Leica microsystems, Germany).

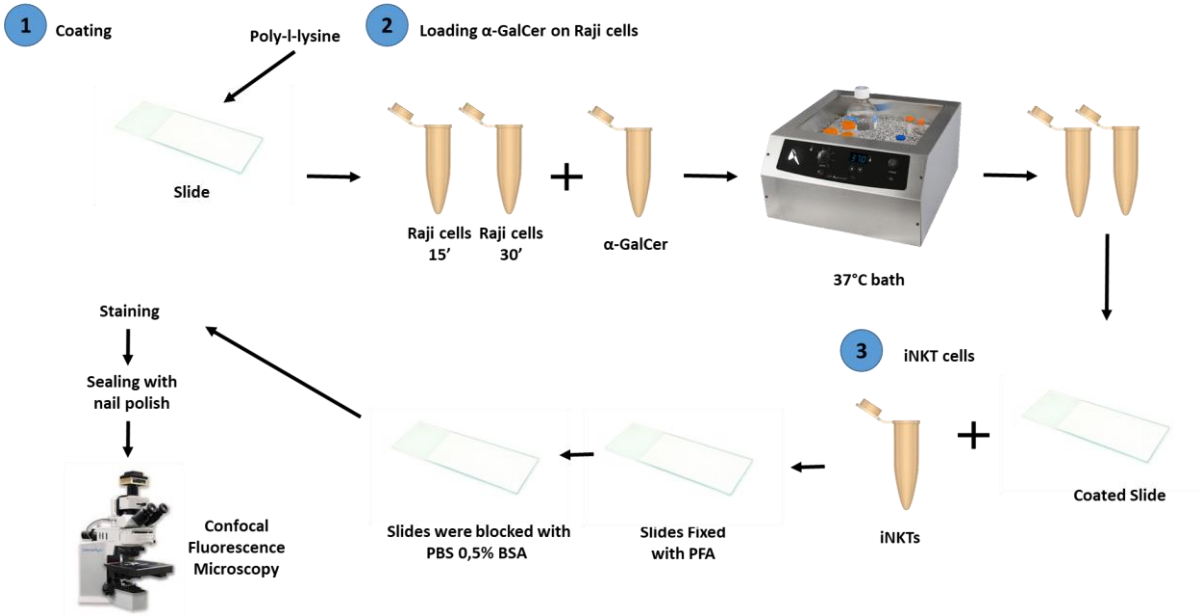

Figure 11 Scheme for sample preparation for microscopy analysis.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 from GraphPad software Inc., California, USA. Mean and standard deviation values were calculated. For comparison of two groups, the statistical significance was assessed using the Mann-Whitney U unpaired t-test for unrelated data, or the paired Wilcoxon t-test to compare two related samples. An ANOVA was used to analyze three groups among them. P-values equal or below 0.05 were considered significant.

List of solutions used in this work:

T cell medium and Re-stimulation T cell medium

- 500 mL RPMI (Invitrogen, California, USA)
- 5 mL non essential amino acids (Invitrogen, California, USA)
- 5 mL kanamycin (Invitrogen, California, USA)
- 5 mL sodium pyruvate (Invitrogen, California, USA)
- 5% HS pooled from 5 AB donors from *Hospital S. João* (Porto, Portugal)

FACS Buffer – Flow cytometry solution

- 100 mL PBS (Invitrogen, California, USA) 1x
- 0.2g of Bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA)
- 0.1g of NaN_3 (Sigma-Aldrich, St. Louis, MO, USA)

B cells medium

- 500 mL RPMI (Invitrogen, California, USA)
- 5 mL non essential amino acids (Invitrogen, California, USA)
- 5 mL kanamycin (Invitrogen, California, USA)
- 5 mL sodium pyruvate (Invitrogen, California, USA)
- 10% FBS (Sigma-Aldrich, St. Louis, MO, USA)

For Raji CD166 KO cells, Puromycin (InvivoGen, San Diego, CA, USA) was added at 2 µg/mL to promote cell selection by antibiotic resistance.

ELISA solutions

- Washing Buffer: 1000 mL of PBS, 500 µL of Tween20 (Bio-Rad, Hercules, CA, USA)
- Blocking Buffer: 100 mL of PBS, 50 µL of Tween20, 1g of BSA
- Substrate solution: Fast o-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, St. Louis, MO, USA) – 1 substrate tablet and 1 buffer tablet in 20 mL distilled water.

Results

Study of CD5 and CD6 expression on human peripheral blood iNKTs

Buffy coats from 20 different donors were obtained at the Blood bank of *Hospital de S. João*. The gender and age of each donor were always registered (Table 3).

To study the expression of CD5 and CD6 on iNKTs of human peripheral blood, PBMCs were isolated from buffy coats by density using HISTOPAQUE, as described in methods. PBMCs were collected and stained with a fluorochrome-conjugated antibody mix (Table 2) for flow cytometry acquisition. Through flow cytometry, lymphocytes were defined by the expression of CD3 ($CD3^+$ cells) and the iNKT population was identified by its positiveness for CD3 and a CD1d tetramer loaded with PBS57 (α-GalCer analogue). The gating strategy used is shown in Figure 12A. Then, the mean fluorescence intensity (MFI) of CD5 and CD6 was quantified in both total $CD3^+$ cells and iNKT populations (Table 3).

Table 3 Age and sex of blood donors, percentage of iNKT cells on total $CD3^+$ cells; CD5 and CD6 MFI on total $CD3^+$ cells and iNKT cells.

						iNKT		Total $CD3^+$	
BC	Age	Sex	% iNKT	Total events*	iNKT events*	MFI CD5	MFI CD6	MFI CD5	MFI CD6
1	31	F	0.190	422000	355	5265	1217	4759	822
2	37	F	0.080	150097	73	4349	1793	5251	1069
3	38	M	0.860	720000	3716	4374	892	3800	527
4	18	M	0.140	628000	464	2951	961	3157	753
5	27	M	0.072	886000	420	1887	495	3035	441
6	35	F	0.029	440000	73	5355	1719	4719	903
7	40	M	0.040	485000	96	3706	810	4411	531
8	42	F	0.069	230564	72	3444	1164	4020	892
9	63	F	0.039	604000	124	3503	753	3184	598
10	22	M	0.028	1030000	117	3998	2824	3406	638
11	23	F	0.120	720000	580	4840	1288	4759	892
12	33	F	0.045	792000	178	4158	1402	3623	651
13	53	F	0.085	520000	226	3644	1091	4575	719
14	59	M	0.025	499000	64	3543	885	4461	708
15	28	M	0.051	638000	231	2197	703	2222	419
16	30	F	0.038	451000	78	2934	808	3623	677
17	46	F	0.160	640000	582	1546	547	2106	445
18	47	F	0.220	609000	689	3010	872	3139	638
19	48	M	0.120	415000	228	3377	1091	3104	759
20	66	M	0.051	475000	127	1958	1118	2564	859

BC – Buffy coat, M – male; F – Female; %iNKT – percentage of iNKT cells on total $CD3^+$ cells; MFI – mean fluorescence intensity; *events acquired by flow cytometry.

Data regarding CD5 and CD6 expression by iNKTs and total CD3+ cells were compiled in Figure 12 panels B and C. Type I NKT cells from human peripheral blood expressed both CD5 and CD6 molecules. The expression of CD5 was similar between iNKT cells and total $CD3^+$ cells, since there were no statistically significant differences between the two studied populations (P value > 0.05, paired t-test). Interestingly, iNKT cells showed higher CD6 expression than total $CD3^+$ cells. This difference is statistically significant (P value = 0.0015, paired t-test figure 12C). In short, human peripheral blood iNKT cells express similar levels of CD5 and higher levels of CD6 than total $CD3^+$ cells.

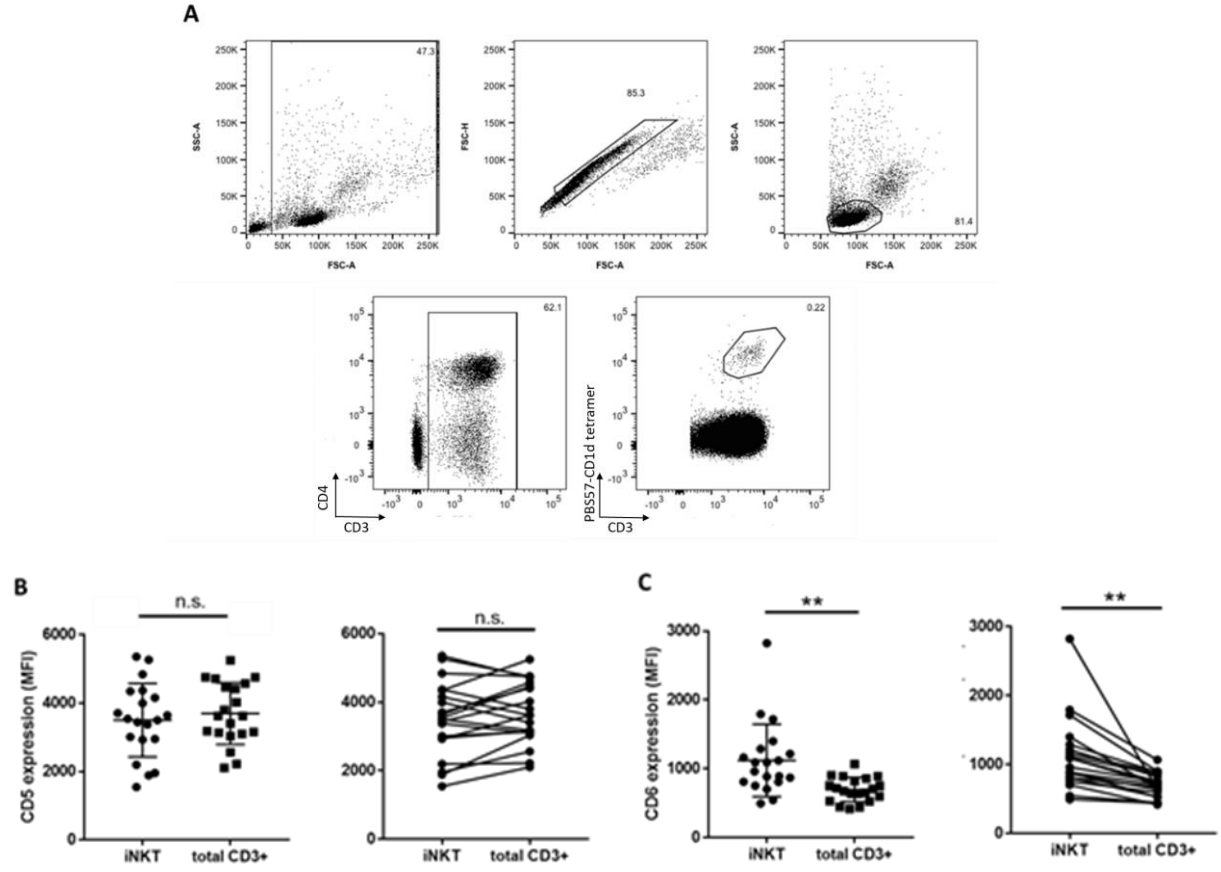

Figure 12 Human peripheral blood iNKT cells express both CD5 and CD6. A - Gating strategy. iNKT cells were defined by flow cytometry in human PBMCs from healthy donors by being CD3+ and recognizing the lipid PBS57 loaded in CD1d tetramers. Then the MFI of CD5 and CD6 was quantified in both the iNKT and the total CD3+ population. B, C – Expression of CD5 and CD6, respectively, by iNKT cells and total CD3+ T cells, indicated as MFI. Each symbol represents an individual of the 20 different donors studied. Lines from graphics on the left represents mean and standard deviation. For graphics on the right, each line links the MFI of CD5 or CD6 of iNKT cells with the MFIs of total $CD3^+$ population of the same individual. n.s. – non-significant, ** - significant with a p value ≤ 0.01; paired t-test.

The frequency of iNKT cells in human peripheral blood and their expression of CD5 and CD6 were analyzed considering the gender (Figure 13A) and age (Figure 13B) of the donors. No differences were detected in the iNKT cells frequency and in the expression of CD5 and CD6

between males and females. The expression of these molecules also seems not to be influenced by the age of the individuals, as no statiscally differences were obtained when the different age groups were compared.

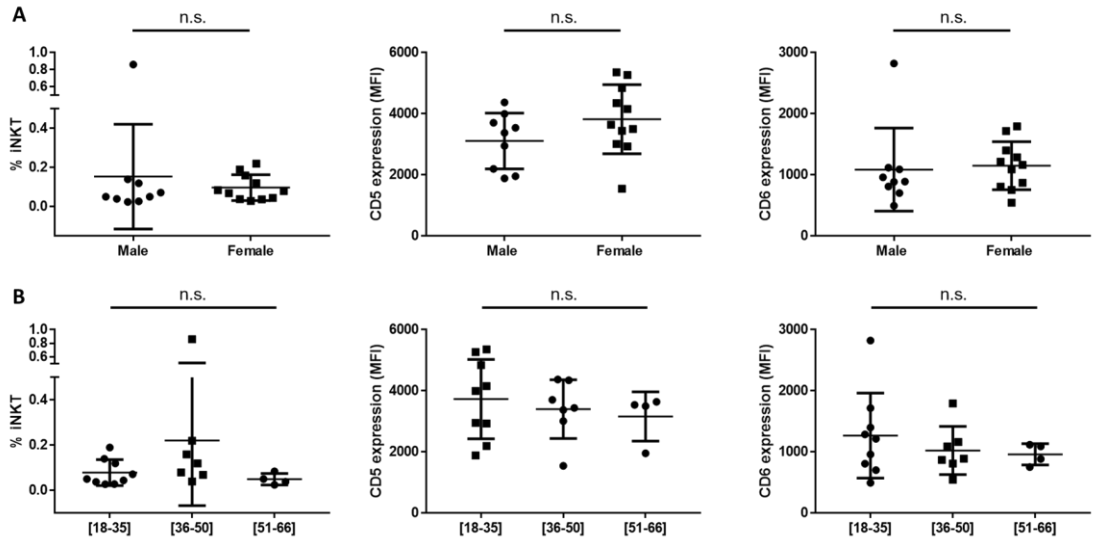

Figure 13 Gender and age do not significantly influence the frequency of iNKT cells and their CD5 and CD6 expression. A,B – Data regarding % of iNKT cells in human peripheral blood and the expression of CD5 and CD6 of these cells was classified regarding the gender (A) and the age (B) of the donors. Each symbol represents an individual of the 20 different donors studied and lines represent the mean and standard deviation of each studied population. n.s. – non-significant with a p value > 0.05; unpaired t-test (A) and one away ANOVA (B).

Study of CD5 and CD6 expression on human iNKT cells after activation

A human $CD4^+$ Type I NKT cell line was stimulated *in vitro* to study the expression of CD5 and CD6 molecules after activation over time. The stimulation was performed using irradiated heterologous PBMCs as APCs and α-GalCer or PHA as stimuli. The α-GalCer is a specific stimulus for iNKT cells and it is presented via CD1d by APCs. PHA is a plant lectin, mitogenic for T cells, that was used as a positive control. The day when iNKT cells and PBMCs were co-cultured was designated as "day 0". From day 1 to 17-19 (time to initiate a new stimulation), cells were collected daily and stained for CD5 and CD6 expression by flow cytometry. The MFI of CD5 and CD6 was quantified in the iNKT cell population (Figure 14A).

The expression of CD5 and CD6 changed over time in iNKT cells after activation (Figure 14B). The profile of CD5 expression in experiments 1 and 2 is similar between non-activated cells (open squares) and cells activated with α-GalCer (grey circles), while PHA-activated cells showed the lowest CD5 levels. In experiment 3, a decrease in the expression of CD5 was observed between day 0 and day 4 in all the conditions; while four days after re-stimulation, iNKTs

activated with α-GalCer upregulated the expression of CD5 compared with non-activated cells and iNKT cells activated with PHA showed again the lowest levels of CD5. Furthermore, activation with α-GalCer and PHA seemed to downregulate CD6 expression in experiments 1 and 2. In the third experiment, the profile of CD6 expression was similar between the non-activated cells and cells activated with α-GalCer, while PHA showed again a downregulation of CD6 expression.

The iNKT cell proliferation after PHA and α-GalCer stimulation was also monitored to analyze any relation between cell proliferation and CD5 and CD6 expression. This was made by counting the total cell numbers present in an aliquot of the culture and using the percentage of iNKT cells determined by flow cytometry (Figure 14C). Cells were analyzed 24 hours after activation.

After activation with α-GalCer or PHA, the number of iNKT cells increased, from day 3, more and faster than cells cultured with no stimulus. Perhaps the number of iNKT cells increased before but, maybe, only 3 days after re-stimulation it is possible to detect this increase due to TCR downregulation. In experiment 3, iNKT cell proliferation was low and the peak of proliferation (from day 3) was similar among cells cultured with no stimulus, with α-GalCer or PHA (cells still proliferate because they are cultured with IL-2). This may be a justification for the different results in CD5 and CD6 expression between experiment 3 and experiments 1 and 2. As previously seen, CD5 and CD6 expression seems to be downregulated when iNKT cells were activated with PHA, compared with no stimulus, between days 4 and 14 after re-stimulation which is concordant with iNKTs proliferation. Moreover, 10 days after activation, cell counting decreased, suggesting that cells started dying. This is in agreement with iNKTs CD5 and CD6 expression, that stabilize or decrease from day 10 after activation.

To conclude, the results suggest that type I NKT cells downregulate both CD5 and CD6 expression after activation.

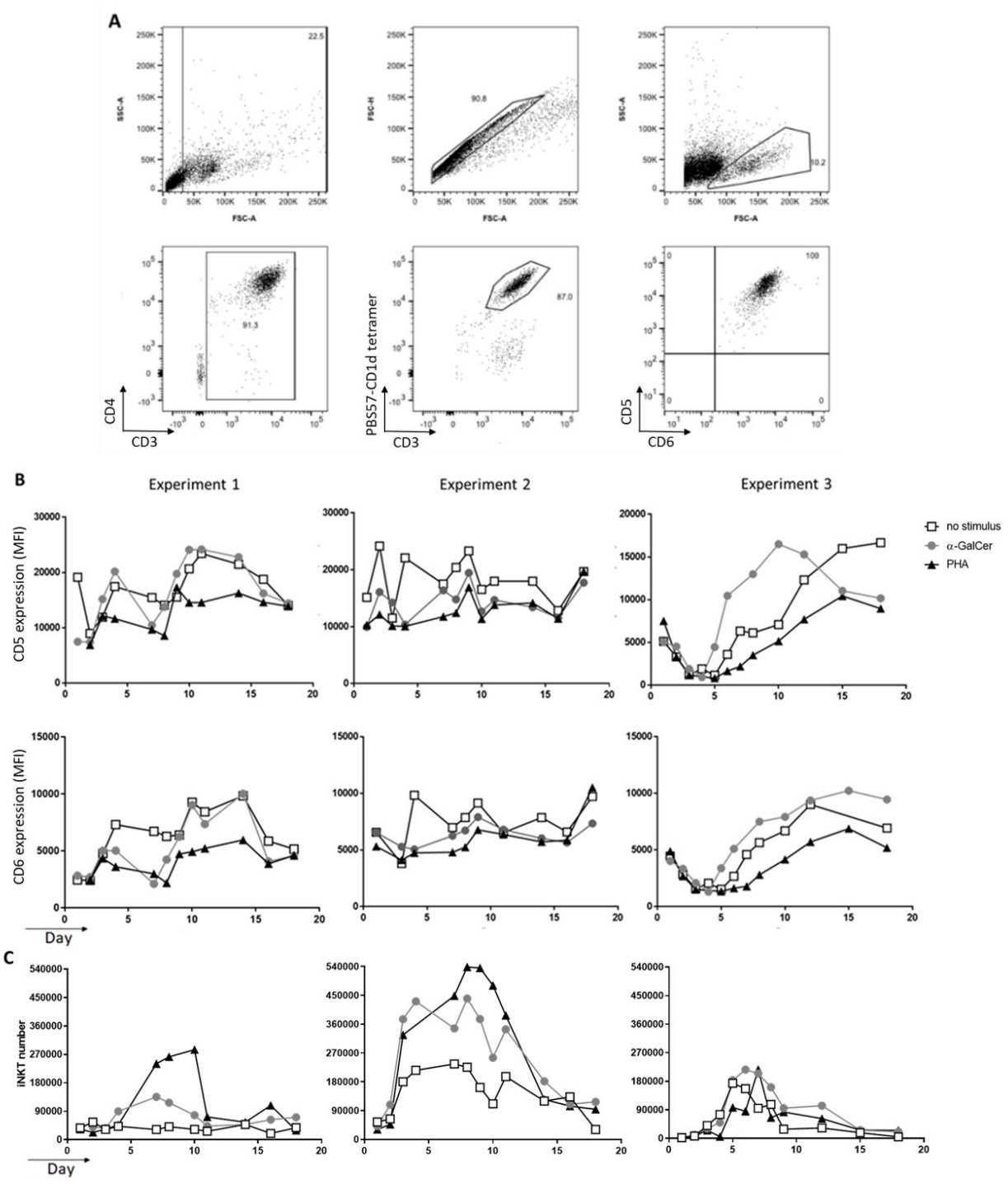

Figure 14 The T cell mitogen PHA down-regulates the expression of both CD5 and CD6 in a human CD4+ iNKT cell line after activation. A – Gating strategy. Invariant NKT cells were identified by flow cytometry using an anti-CD3 antibody and a CD1d tetramer loaded with PBS57. Then the MFI of CD5 and CD6 was quantified in the iNKT population. B – CD5 and CD6 expression levels after α-GalCer and PHA activation C – iNKT cell proliferation after α-GalCer and PHA activation, indicated by iNKT cell number in the culture aliquot. B,C Squares represent non-activated cells, circles represent cells activated with α-GalCer and triangles represent cells activated with PHA.

Study of iNKT cell activation after recognition of the antigen α-GalCer in the presence and absence of CD166, a CD6 ligand, on APCs.

Study of CD1d expression in B cell lines cultured with retinoic acid.

Three different B cell lines were used as APCs in iNKT cell activation assays: WT Raji cells, CD166 KO Raji cells; and 447 EBV control B cell line (see below). CD1d expression was induced in all B cell lines by culturing them in the presence of retinoic acid and B cells were also maintained in culture without RA. Three days after culture, B cells were collected, stained with an anti-human CD1d antibody and analyzed by flow cytometry. After defining the cell gates by side and forward scatter (Figure 15A), the MFI of CD1d was measured within this gate (Figure 15C). A parallel staining was made to confirm the lack of expression of CD166 in the Raji CD166 KO B cell line (Figure 15B).

Results concerning CD1d expression on B cell lines are presented in Figure 15C. CD1d induction worked well for the 447 B cell line. However, no increase in the expression of CD1d was observed for WT and CD166 KO Raji cells cultured with retinoic acid.

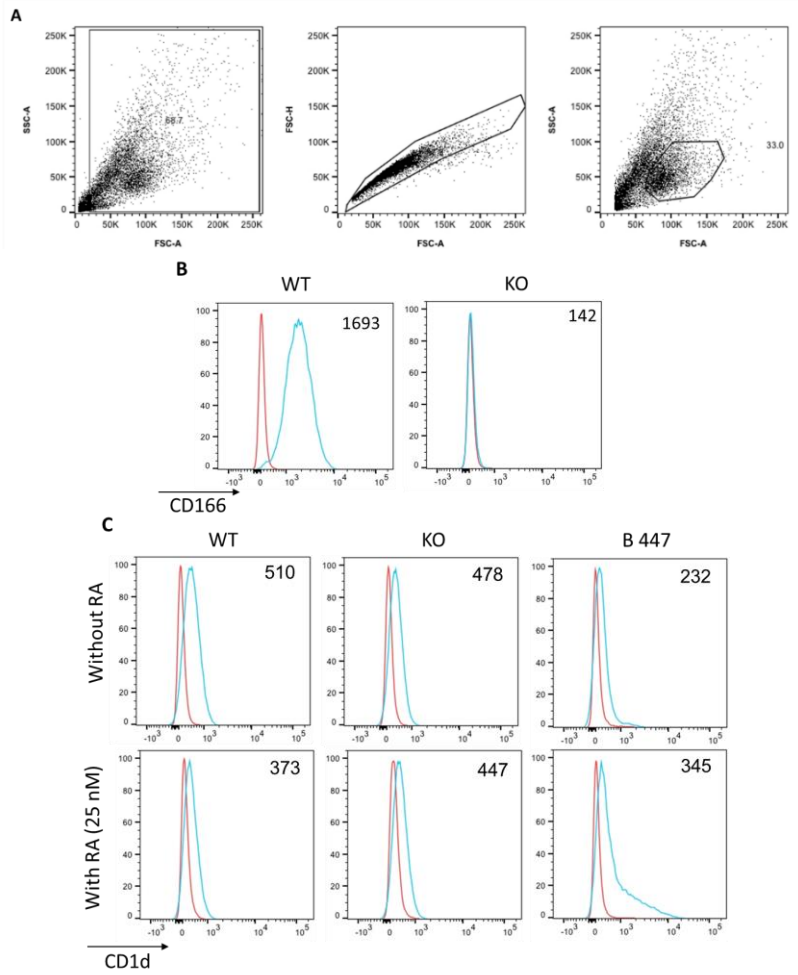

Figure 15 B447 cells, but not Raji cells, increase their CD1d expression after being cultured with RA. A – Gating strategy. Side and forward scatter were used to define the B cell line population. Then, the MFI for CD166 and CD1d was measured within this cell gate. B – CD166 expression by WT and KO Raji cells. Raji cells were cultured in the presence of RA during 3 days. After that, CD166 expression was analyzed by flow cytometry. In the histograms, red lines represent unstained cells and blue lines represent cells stained with an antibody anti-CD166. Numbers indicate the MFI for CD166 in stained cells. C- CD1d expression by B cell lines when cultured with RA during 3 days. B cells were cultured with and without RA, as described above. In the histograms, red lines represent unstained cells and blue lines represent cells stained with an antibody anti-CD1d. Numbers indicate the MFI for CD1d MFI of stained cells. CD1d expression in B cell lines cultured with and without RA from 3 independent experiments, indicated as MFI.

Study of iNKT cells activation after recognition of antigen presented by APCs expressing or not CD166

To quantify iNKT cell activation, an antigen presentation assay was performed. This assay consists on the recognition of the antigen α-GalCer that is presented by APCs (B cell lines) to the responder cells (iNKT cells). The level of iNKT cells activation was measured by the amount of GM-

CSF produced by these cells in response to the antigen, which is proportional to the level of cell activation and quantified in supernatants from B cell – iNKT cell co-cultures by ELISA.

Results from three different antigen presentation assays are shown in Figure 16. Raji WT, Raji CD166 KO and B447 cell lines were used as APCs. After α-GalCer recognition, very low levels of GM-CSF were detected when B cell lines (APCs) were cultivated without RA (white symbols), a fact that was consistent among the three experiments (Figure 16A). After being cultured with RA, all B cell lines increased their capacity to induce the production of GM-CSF by iNKT $CD4^+$ cells (black symbols, Figure 16A). The B447 cell line, which increased their CD1d expression after being cultured with RA, is the one that induces the higher degree of iNKT cell activation. WT and CD166 KO Raji cultured with RA, although they did not increase their expression of CD1d, showed an increased ability to activate iNKT cells compared to non-RA cultured counterparts. However, no differences in the activation of iNKT cells were detected when the antigen was presented by APCs expressing (Raji WT) or not (Raji KO) the CD6 ligand CD166. Moreover, no differences were observed in the activation of iNKT cells with PHA comparing the different types of APCs used in these experiments (Figure 16B).

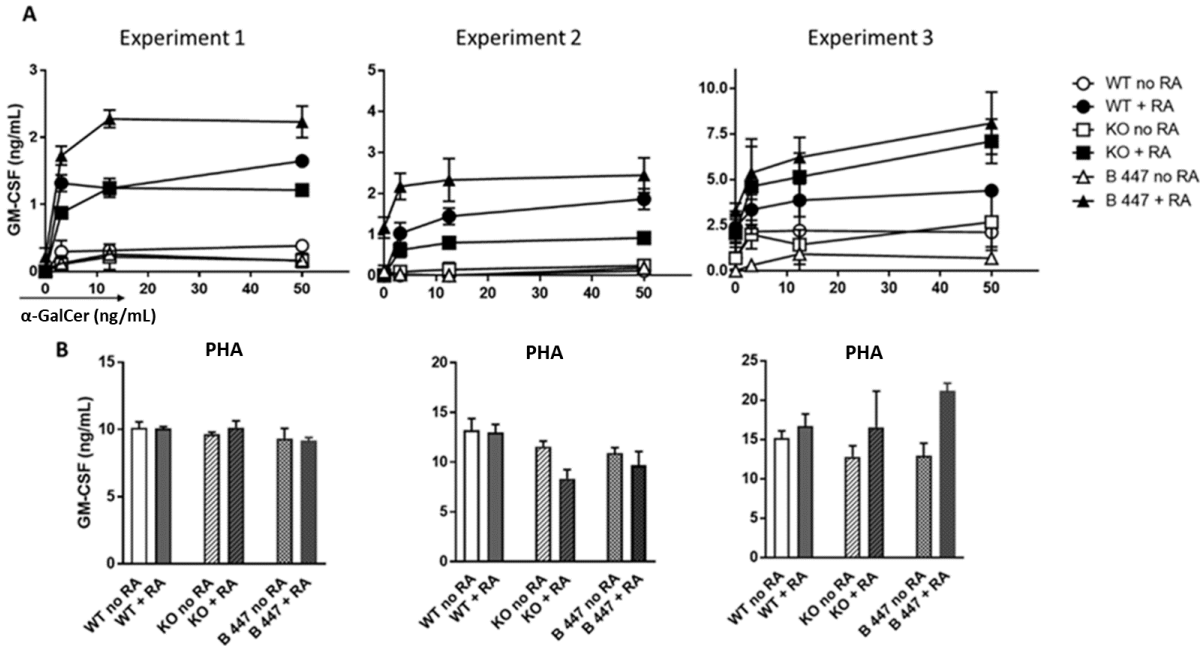

Figure 16 The absence of the CD6 ligand CD166 does not interfere in the recognition of α-GalCer by iNKT cells. A - GM-CSF production by iNKTs after recognition of α-GalCer at the indicated concentrations, presented by the B cell lines Raji WT, Raji KO CD166, B 447 cultured with (+) or not (no) with RA. B cells were incubated with the antigen α-GalCer, during 4 hours, then iNKT cells were added and cells were co-cultured during 40 hours (ratio B cell: iNKT, 1:2). GM-CSF production by iNKT after antigen recognition was quantified by ELISA. Three experiments were performed and vertical lines represents standard deviation of three replicates. Each symbol represents one B cell line, cultured without RA (filled symbols) and with RA (empty symbols). B – GM-CSF production after activation of iNKT cells by PHA. The procedure used was the same described in (A), but PHA was used as stimulus (positive control) instead of α-GalCer. Each bar

represents the mean and standard deviation of GM-CSF concentration obtained from co-cultures of iNKT with the different B cells lines used as APCs in this study. Three activation assays were made and each graph represents an independent experiment.

Study of CD6 functional role in iNKTs-APCs synapses

The study of the iNKTs-APCs synapses was done in collaboration with Rita Santos from Prof. Alexandre do Carmo group at i3S, Porto.

To study the functional role of CD6 molecule in iNKT cells, beyond the activation assay, an immunofluorescence assay was performed to see the localization of CD6 within the immunological synapse. The technique was well established for conventional T cells, and was applied and adjusted to use with iNKT cells. First, APCs (WT Raji cells or B 447 EBV cell line) were cultured with the α-GalCer, then iNKTs were added to the APC-antigen complex with a 1:2 APC:iNKT ratio. Finally, cells were fixed, blocked and stained.

Several antigen concentrations were tested and also antigen loading time. Immunological synapses were supposed to be identified with an anti-CD3 antibody, detecting CD3 at the sites of interaction between the two cells. However, CD3 seems to label the whole periphery of iNKT cells. CD6 localization is detected using an anti-CD6 antibody. In figure 17, it is possible to see the co-localization between CD3 and CD6 in panel A using B 447 cells as APCs and in panel B using WT Raji cells. Although other concentrations of α-GalCer have been tested, starting at 50 ng/mL, results are not so clear as we expected. To use this technique in future experiments, perhaps higher antigen concentrations should be tested and loading times too, to obtain better results.

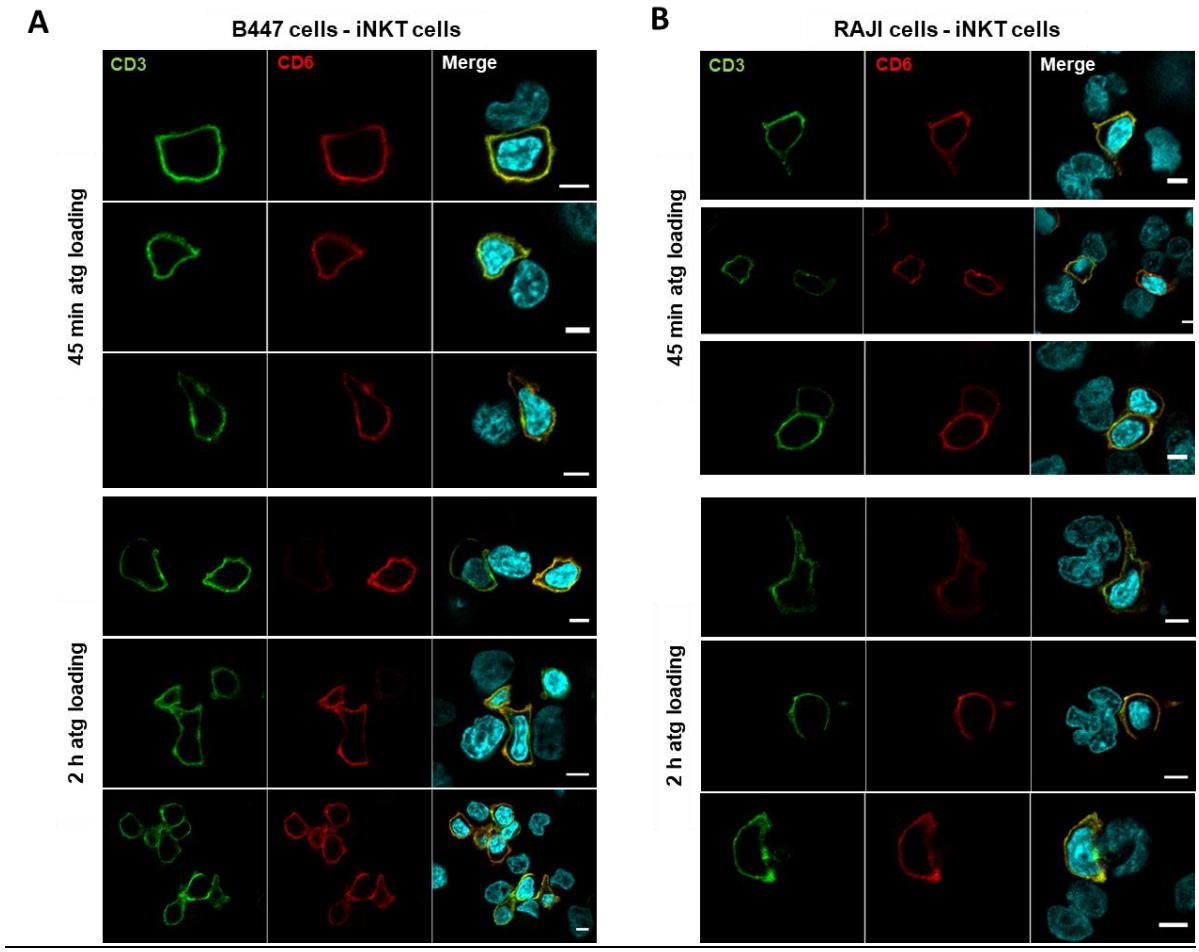

Figure 17 Confocal Fluorescence Microscopy images of co-cultures of APC, α-GalCer and iNKT cells stained with anti-CD3 and anti-CD6 antibody. Antigen (α-GalCer) at 500 ng/mL was loaded into B 447 cells (A) and in WT Raji cells (B). Two antigen loading time were used: 45 minutes present in the 3 first images and 2 hours present in the other images, after the loading time iNKTs were added, the ratio of APC:iNKTs is 1:2. Staining was done with an anti-CD3 antibody (green) and with an anti-CD6 antibody (red). The scale is 5µm.

Discussion

Invariant NKT cells, a T lymphocyte subset, are CD1d restricted cells that recognize lipid instead of protein antigens through a semi-invariant TCR. iNKTs play an important role in innate and in adaptive immunity producing immunoregulatory cytokines and recognizing antigens through its TCR. Consequently, iNKT cells have been considered as immunoregulators involved in induction and prevention of autoimmune diseases, inflammation and cancer (Kumar & Terry 2014). Moreover, the number of iNKT cells can influence the progression of autoimmune diseases (Kaer & Wu 2018).

CD5 and CD6 molecules are two surface glycoproteins expressed by conventional T cells and thymocytes, belonging to the receptor family – Scavenger Receptor cysteine-rich (SRCR). These two molecules modulate TCR signaling, which in turns controls the development and the survival of T cells (Gimferrer et al. 2003). The expression of these two molecules was not studied yet in human iNKT cells.

In this study, we aimed to investigate the expression of CD5 and CD6 molecules and the functional role of CD6 in human iNKT cells. For this, we analyzed its expression on peripheral blood from different donors (with different age, different gender) and in a $CD4^+$ human iNKT cell line, after activation, over time. Furthermore, activation assays were performed to study the functional role of CD6 in iNKTs activation, using WT and CD166, a ligand for CD6, KO Raji cell lines to evaluate the level of activation of iNKT cells in the presence and in the absence of the CD6 ligand.

CD5 and CD6 expression on human iNKT cells from peripheral blood.

Our results show that human peripheral blood iNKT cells express CD5 at similar levels as conventional T cells. It is described that CD5 expression is increased in cells undergoing activation and/or differentiation (Azzam et al. 2001). Interestingly, CD6 is also expressed by iNKTs and at higher levels than total $CD3^+$ cells.

In accordance with the results described by other authors, in this study, the percentage of iNKT comprise 0.1-0.2% of total T cells in human peripheral blood (Kumar & Terry 2014). It was described that iNKT percentage is influenced by age and sex. The percentage of iNKT cells decrease with age (Jing et al. 2007) and females tend to have more iNKTs than males (Montoya et al. 2007). Our results seem to present an higher iNKTs percentage for male donors and [36-50] age group, since no statistically significant differences were observed; therefore, these results

seem to disagree with what was expected. Furthermore, CD5 and CD6 expression tend to decrease from the younger to the older age group (figure 13), although there was no statistical significance. However, probably the number of individuals studied is not enough to significantly observe the behavior of the expression of these molecules in the healthy population. So, more experiments are needed to release a conclusion regarding the dependency of the expression of CD5 and CD6 on gender and age.

CD5 and CD6 expression on human iNKT cells after activation

Invariant NKT cells were stimulated in two ways: via CD1d using a specific lipid antigen (α-GalCer) and via TCR clustering with a non-specific stimulus (PHA). The results showed that independently of the stimuli that were added to the culture, approximately four days after activation, a trend for CD5 and CD6 downregulation was observed in the three experiments performed.

In the thymus, CD6 and CD5 molecules are upregulated during positive selection and remain expressed on mature T cells (Singer et al. 2002). Having no cytoplasmic enzymatic activity, both CD5 and CD6 contain several tyrosine residues on their cytoplasmic regions that can be phosphorylated upon TCR stimulation, recruiting other signaling molecules influencing signaling effects (Dong et al. 2016) (Hassan et al. 2006). These molecules, like SHP-1 and SLP-76 for CD5 and CD6, respectively, may be influencing its profile expression, and induce the downregulation of CD5 and CD6 after activation of iNKT cells.

PHA activated iNKT cells showed always lowest CD5 and CD6 levels than non-activated cells. It was described for conventional T cells that CD5 is an inhibitor of TCR-mediated signaling (Tarakhovsky et al. 1995). Also, in naïve T cell activation, CD5 expression is proportional to the strength of T cell-responses; consequently, high expression of CD5 identifies cells committed to activation and differentiation (Azzam et al. 2001). It would be interesting to explore this cell type differences in CD5 expression.

In the absence of extracellular engagement of CD6, CD5 and CD2, the level of cell activation decrease (Hassan et al. 2006)(Bhandoola et al. 2002)(Ohno H, Nakamura T, Yagita H, Okumura K, Taniguchi M 1991). Furthermore, during thymocyte development, CD5 mediates the downregulation of TCR signaling (Bhandoola et al. 2002). In future experiments it must be taken in consideration the expression of T cell surface proteins that can be involved in antigen-specific T cell activation coordinated by the TCR.

Induction of CD1d expression by retinoic acid on B cells.

It was described that B cell lines lose their expression of CD1d and that retinoic acid recover this CD1d expression in B cells (Allan et al. 2011). We used B cell lines as APCs to do an activation assay with iNKT cells to evaluate what is the functional role of CD6 in iNKTs antigen mediated activation. So, to induce the expression of CD1d, WT and CD166 KO Raji cell lines and a home-made EBV transformed B cell line (B 447) were cultured with and without retinoic acid before the assay. Results showed that only B 447 cell line increased CD1d expression in the presence of RA, while Raji cells did not present higher CD1d expression after the treatment.

ATRA is a ligand for retinoic acids receptor (RAR) which is generated from retinol (Szatmari et al. 2006). ATRA controls CD1 gene expression via the activation of the RAR (Allan et al. 2011). RAR function as ligand-regulated transcription factor by binding, to specific hormone response elements, RARE, in the promoter/enhancer regions of target genes. Through the activation of RAR, RA modulates the expression of a large number of genes, including CD1 gene, and their products are associated with the control of cell proliferation, differentiation, and tissue homeostasis (Mark et al. 2006). Consequently, they also regulate lipid antigen presentation (Allan et al. 2011). Is described that in EBV-infected cells, CD1d is downregulated (Chaudhry et al. 2014), B 447 cell line was transfected with EBV and also Raji cell lines derived from Burkitt's lymphoma which harbor the EBV, because of that B cells were cultured with RA. Results shows that RA worked well to B447 cells but, surprisingly Raji cells did not increased it CD1d expression. Enzymes and molecules involved in the induction of CD1d expression could be affected, maybe RAR is not activated and CD1d expression cannot increase. To overcome this limitation, in future experiments, B cell lines could be transfected with CD1d molecule, to ensure that B cells express CD1d molecule.

iNKT cells activation in the presence and absence of CD6 ligand (CD166)

When an APC presents an antigen to the iNKT cells, iNKTs respond rapidly producing cytokines, contributing to host protection. One of them is the GM-CSF. So, levels of GM-CSF production were measured providing information about iNKT cell activation, after an activation assay. The results showed that B cells cultured without RA slightly activated iNKTs, because very low levels of GM-CSF were detected using these cells as APCs, a fact that was consistent among the three different B cell types used. For B cells that were cultured with RA, the induction of GM-CSF production by iNKTs was increased. However, B 447 cell line induced the production of the

highest GM-CSF values, what agrees with the increased CD1d expression observed after the culture of these cells with RA. Furthermore, no differences were detected in the activation of iNKT cells in the presence and absence of the CD6 ligand CD166. The non-induction of CD1d expression could explain this fact. Together, these results suggested that the presence and absence of CD6 ligand did not affect or influence the activation of iNKT cells after α-GalCer recognition in the conditions tested.

Numerous conditions in the APC influence iNKT responses upon antigen recognition like transport and cellular uptake of the antigen, type of APC involved, intracellular trafficking, loading efficiency into CD1d and stability of CD1d binding. Also, different lipids may fine-tune CD1d-lipid-TCR interaction, which in turn influences the magnitude and/or quality of iNKT cell responses (Mallevaey 2012). All these factors could be manipulated to study iNKT cell responses, such as using different lipids or APCs to better understand the role of CD5 and CD6 in the iNKT cell response. Moreover, although the most studied CD6 ligand is the CD166 molecule, CD6 has other ligands that could be tested too. CD6 may bind different ligands, for example 3A11 leading to a costimulatory signal that could influence T cell signaling (Alonso-ramirez et al. 2010).

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