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CELLULAR IMMUNOTHERAPY FOR B-CELL LYMPHOMA WITH NKT-CELL AGONISTS

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Als meus pares

Que una opinió la comparteixi molta gent no és prova concloent que no sigui completament absurda

(Bertrand Russell)

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ABBREVIATIONS

4-1BBL	4-1BB ligand
α-C-GalCer	α-C-galactosylceramide
α-GalCer	α-galactosylgalceramide
APC	Antigen presenting cell
APC[1]	Allophycocyanin
ATCC	American Type Culture Collection
β-GlcCer	β-glucosylceramide
BSA	Bovin serum albumin
BTLA	B- and T-lymphocyte attenuator
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CM	Complete medium
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTLs	Cytotoxic T lymphocytes
DCs	Dendritic cells
DN	Double negative
DNA	Desoxiribonucleic acid
ER	Endoplasmatic reticulum
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
Fc	Constant fraction
FITC	Fluorescein isothiocyanate
Flt3-L	Flt3 ligand
FoxP3	Forkhead box P3
GM1	Ganglio-N-tetraosylceramide 1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Gy	Gray

ICAM	Intracellular adhesion molecule
ICOS	Inducible T-cell costimulator
Id	Idiotype
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant natural killer T cell
IP	Intraperitoneal
iTCR	Invariant T-cell receptor
iTreg	Inducible regulatory T cell
IV	Intravenous
LAG-3	Lymphocyte activation gene 3
LFA-3	Lymphocyte function-associated antigen 3
LICOS	ICOS ligand
mAbs	Monoclonal antibody
MCP-1	Monocyte chemoattractant protein-1
MDSC	Myeloid-derived suppressor cell
MFI	Mean fluorescence intensity
MICA-B	MHC class I chain-related genes A and B
MHC	Major histocompatibility complex
MIP-2	Macrophage inflammatory protein-2
MNC	Mononuclear cell
Mo-DC	Monocyte-derivated dendritic cell
NF-κB	Nuclear factor *kappa*-light-chain-enhancer of activated B cells
NK	Natural killer cell
NKT	Natural killer T cell
OX40L	OX40 ligand
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PD-1	Programmed cell death 1
PD-L	Programmed cell death ligand
PE	Phycoerythrin

PMAi	Phorbolmyristate acetate and ionomycin
PRR	Pathogen recognition receptor
Rae-1	Retinoic acid early-inducible protein 1
rmGM-CSF	Recombinant mouse GM-CSF
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SC	Subcutaneous
SCD	Sickle-cell disease
SEM	Standard error mean
TAA	Tumor associated antigen
TCR	T-cell receptor
Tet	Tetramer
TGF-β	Tumor growth factor β
Th	T helper cell
TILs	Tumor infiltrating lymphocytes
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
TNFR	Tumor necrosis factor receptor
T_R1	Periphery-induced T regulatory type 1 cell
Treg	Regulatory T cell
VISTA	V-domain Ig suppressor of T cell activation

ABSTRACT

Natural killer T (NKT) cells are a small population of lymphocytes with unique specificity for glycolipid antigens presented by non-polymorphic CD1d receptor on antigen presenting-cells (APC) (mainly dendritic cells (DCs). NKT cells play a central role in tumor immunology since they coordinate innate and adaptive immune responses. These cells can be activated with the prototypic lipid α-galactosylceramide (α-GalCer), stimulating IFN-γ production and cytokine secretion (eg, IL-12, IL-4, IL-17) that contribute to the enhancement of DC function and the induction of NK, B and T-cell activation.

In this work, we evaluated the antitumor effect of a combination of DCs and irradiated tumor cells with the NKT cell agonist α-GalCer in a mouse model of B-cell lymphoma. In addition, we analyzed the effector cells and cytokines that are involved in the antitumor immune response induced by the vaccine. We also studied for the first time the antitumoral effect of the novel NKT-cell agonist, the NKT14m antibody.

The murine 4TOO B-cell lymphoma was used as tumor model. A therapeutic vaccine was generated by mixing DCs and irradiated 4TOO tumor cells, together with the NKT-cell agonist α-GalCer. In addition, different control vaccines, including α-GalCer alone, DCs alone, DCs and tumor cells, and DCs with α-GalCer were also tested. First of all, a three-vaccination treatment was tested and the different vaccines were injected into Balb/c mice two days after tumor challenge. This study showed a 100% antitumor effect of Mix+GalCer vaccine in contrast to other treatments as DCs with α-GalCer (only 50% antitumor efficacy), α-GalCer alone (10% antitumor efficacy) and DCs alone or with tumor cells (both 0% antitumor effect). Interestingly, the NKT-cell number, analyzed by flow cytometry using the specific PE-conjugated CD1d:PBS57 loaded tetramer, showed an important decrease after the second Mix+GalCer vaccination, which was further reduced after the third dose, suggesting that recurrent administration of the vaccine induced NKT cell anergy. To solve that, a single dose of the

therapeutic vaccine was tested, showing the maximum antitumor efficacy of Mix+GalCer vaccine again. In this case, it was also observed a high increase of NKT cells in mice treated with Mix+GalCer vaccine, as well as of NK cells, in contrast with the other control groups including untreated mice, Mix treated mice and α-GalCer treated mice. Importantly, 90% of Mix+GalCer treated mice with the vaccine were resistant to a tumor rechallenge, suggesting the development of a memory immune response. In addition, the immune response was tumor-specific since all the mice were unable to reject a syngeneic A20 B-cell lymphoma. When the cytokine profile was analyzed, we observed an increase of both Th1 cytokines (IFN-γ, IL-12 and TNF-α) and Th2 cytokines (IL-4, IL-5 and IL-6), as well as IL-17, after Mix+GalCer treatment. After observing the high increment of IFN-γ in Mix+GalCer treated mice, IFN-γ secreting cells were studied. In this analysis, we observed that Mix+GalCer vaccine induced an increase of IFN-γ secreting NK, NKT and $CD4^+$/$CD8^+$ T cells in contrast to the control groups. Surprisingly, NK cells played a critical role in the antitumor effect observed after Mix+GalCer treatment since NK-cell depleted mice did not survive after treatment. In addition to the IFN-γ providing by NK cells, the presence of activating NK-cell ligands like Rae-1 in the 4TOO tumor cells could promote the direct NK-cell citotoxicity, which could be also impaired with the NK-cell depletion. Furthermore, Mix+GalCer vaccine induced the activation of B cells as specific IgG against tumor cells were found in treated mice.

As a second part of the work, a novel NKT-cell agonist, the NKT14m antibody, was evaluated for antitumoral efficacy. In this study, we observed that this antibody had a considerable antitumor effect (37% survival), which was increased with the antibody retreatment (50% antitumor efficacy). In addition, the NKT14m antibody combined with cyclophosphamide treatment further increased the antitumor efficacy of the antibody (90% survival).

A therapeutic vaccine consisting of dendritic cells, tumor cells and the NKT-cell agonist α-GalCer efficiently eradicates B-cell lymphoma in a therapeutic setting. This immune response is long-lasting, tumor-specific, and it is associated with an

expansion of NK and NKT cells and with an increase of IFN-γ secreting NK, NKT and $CD4^+/CD8^+$ T cells. In our B-cell lymphoma model, NK cells play a critical role in the antitumor effect and a humoral immune response is also induced by the treatment. In addition, the NKT14m antibody induces an effective antitumor immune response that is improved by its combination with cyclophosphamide treatment. These data support the development of immunotherapy strategies in patients with B-cell lymphoma using NKT cell agonists.

1. INTRODUCTION

1.1. General biology of the immune system

The immune system is a complex network of cells, tissues, and organs that work together to protect the body from harmful processes. These aggressions can be external like bacterial and viral infections or an internal damage such as malignant or autoimmune disorders. The recognition of these menaces involves two different but linked responses, the non-specific and the specific immune responses mediated by the innate and adaptive immune systems, respectively.

The innate immunity is characterized by the generation of a rapid and non-specific immune response. It is the first line of defense and a general protection, including physical barriers of the body (e.g. skin and mucosa), chemical barriers (e.g. secretions and enzymes), and other soluble factors (e.g. cytokines, chemokines and the complement system). It also includes innate leukocytes such as natural killer (NK) cells, mast cells, phagocytic cells like dendritic cells (DCs) and macrophages, and granulocytes including basophils, eosinophils and neutrophils (Parkin and Cohen, 2001). In contrast to the innate immunity, the adaptive immune system is composed by highly specialized cells and processes that recognize and eliminate non-self antigens in an extremely specific manner. In addition, the adaptive immunity is initially delayed in time, but it provides long-lasting protective immunity by creating an antigen-specific memory, which produces a stronger and faster immune response each time that the antigen is encountered. The most important cells to direct the adaptive immunity are B cells and $CD4^+$ and $CD8^+$ T cells. Other types of lymphocytes that can participate in the modulation of innate and adaptive immunity are natural killer T (NKT) cells and $\gamma\delta$ T cells. These lymphocytes share properties of both innate and adaptive immune cells (Dranoff, 2004) (Figure 1).

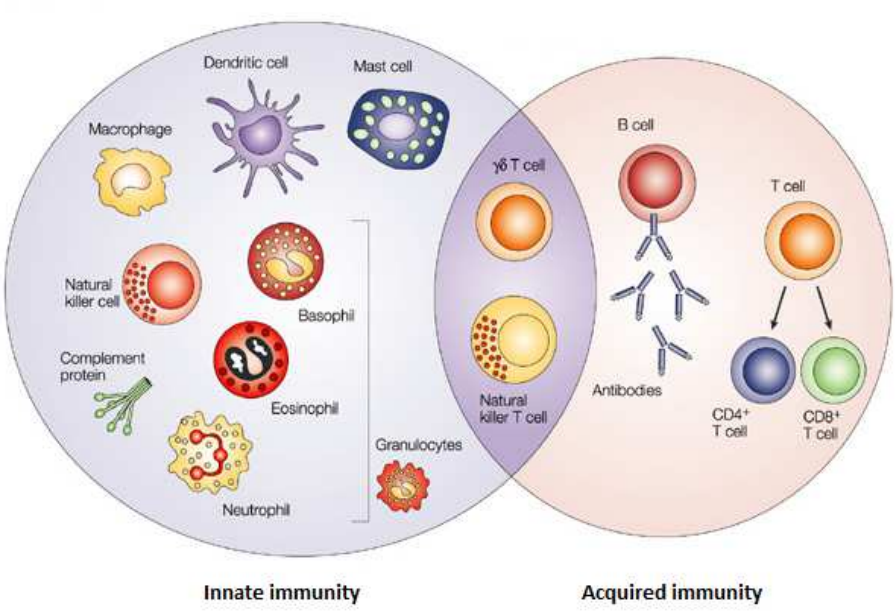

Figure 1. Components of innate and adaptive immunity. The innate immune system consists of soluble factors, such as complement proteins, and different types of cells including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells (DCs) and natural killer (NK) cells. The adaptive immune system consists in B cells, which produce the antibodies, and $CD4^+$ and $CD8^+$ T cells. Natural killer T (NKT) cells and γδ T cells are lymphocytes that share characteristics of both innate and adaptive immunity (Modified from Dranoff, 2004).

1.2. The innate immune system

The innate immune system comprises the cells and mechanisms that recognize and provide immediate responses against aggressions in a non-specific manner (Parkin and Cohen, 2001). The most important cells in the innate immune system are macrophages, DCs and NK cells. Macrophages and DCs are specialized antigen presenting cells (APCs) that play a crucial role in initiating the immune responses. These APCs are highly efficient at capturing antigens through phagocytosis and processing them into peptide fragments, which are specifically presented by the major histocompatibility complex (MHC) class I or class II molecules to T cells. Furthermore, antigen recognition by APCs causes an upregulation of costimulatory molecules and secretion of proinflammatory cytokines to modulate the immune response (Parkin and Cohen, 2001; Kenneth Murphy, 2008).

Equally important within the innate immune system are NK cells, which are classified as cytotoxic cells that are capable of lysing a variety of stressed, microbe-infected and malignant cells (Smyth, 2004). NK cells do not require specific antigen recognition to kill target cells and normally do their function prior to the induction of adaptive immunity. These cells mediate cell killing through two principal mechanisms: granule exocytosis (perforin and granzymes) and Fas-ligand (FasL)-mediated apoptosis. Their activation requires the combination of cell surface receptor recognition and pro-inflammatory cytokine signals. NK cell function is inhibited after the recognition of MHC class I by NKG2A receptor, and thus, healthy cells which express normal levels of this molecule are generally protected from NK cytotoxicity (Pegram, Andrews et al., 2010). By contrast, virus infected and tumor cells that downregulate the expression of MHC class I or increase the stress markers on their surface are susceptible to NK killing (Vivier, Ugolini et al., 2012) (Figure 2). For instance, the NK-cell receptor NKG2D can be stimulated by its ligation with retinoic acid early inducible-1 (Rae-1) proteins in mice or with the MHC class-I polypeptide-related sequence A and B (MICA and MICB, respectively) in humans. All of these molecules can be overexpressed in infected cells as well as in some malignant cell types, and their recognition by NKG2D receptor induces NK cytotoxicity (Zhou, Zhang et al.; Biassoni, Cantoni et al., 2003; Pegram, Andrews et al., 2010). Additionally, cytokines such as IL-12, IL-15 and IL-21 induce NK proliferation, and also promoting NK cell cytotoxicity and IFN-γ production, stimulating adaptive immunity (Smyth, 2004).

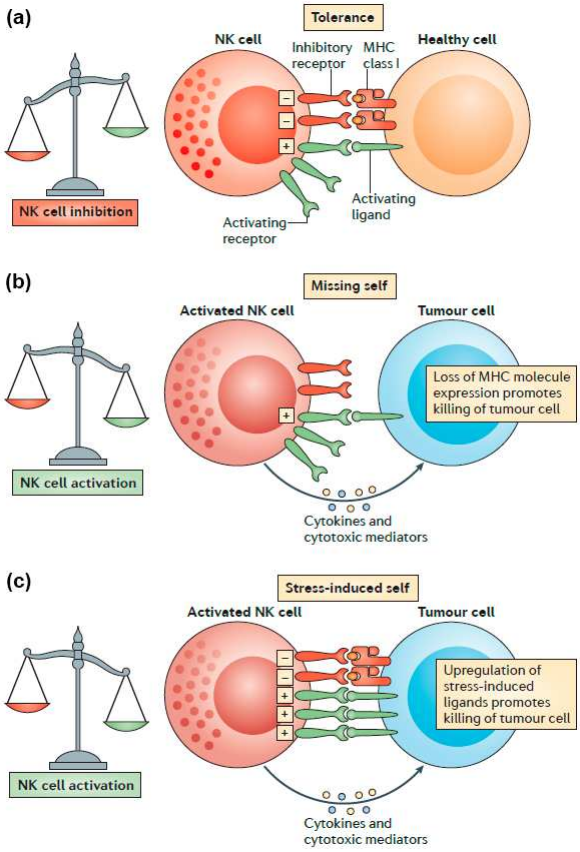

Figure 2. Recognition of tumor cells by NK cells. (a) NK cells are tolerant with healthy cells because they express normal levels of MHC class I. This receptor acts as inhibitor molecule to NK activation. **(b)** There are tumor cells that downregulate MHC class I and this lost of signal allows NK cells to kill them. **(c)** Other tumor cells that do not decrease the MHC expression can presented stress-induced ligands and also promote the NK cell activation (Vivier, Ugolini et al., 2012).

1.2.1. Dendritic cells

DCs are highly specialized APCs that only comprise 1% of the total hematopoietic cells in blood and are found mainly in skin, spleen and liver (Banchereau and Steinman, 1998; Banchereau, Briere et al., 2000). DCs arise from both myeloid and lymphoid progenitors in the bone marrow and migrate throughout the blood to tissues around the body, and also directly to peripheral lymphoid organs. Various types of DCs with differences in phenotype, function and tissue distribution indicate the coexistence of heterogeneous DC populations (Hart, 1997; Ueno, Klechevsky et al., 2007). At least two classes of dendritic cells are broadly

recognized: conventional or myeloid dendritic cells, which seem to participate most directly in antigen presentation and activation of naive T cells, and plasmacytoid dendritic cells, a distinct lineage that generate large amounts of α/β interferons, particularly in response to viral infections, but they do not seem to be as important for activating naive T cells (Kenneth Murphy, 2008).

1.2.1.1. Sources of DCs in humans and mice

DCs represent a small population in circulation and a large volume of blood is needed to obtain enough DCs for clinical use. To deal with this problem, it is possible to expand DCs *in vivo* using granulocyte-macrophage colony-stimulating factor (GM-CSF) and/or Flt-3L administration. In addition, human DCs can be generated using the $CD14^+$ monocytes from peripheral blood, which are cultured with GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994; Berger and Schultz, 2003). In mice, DCs can be obtained from bone marrow progenitors (monocyte-derivated dendritic cells or Mo-DC) cultured with GM-CSF (Inaba, Inaba et al., 1992; Lutz, Kukutsch et al., 1999).

1.2.1.2. DC phenotype

In general, conventional DCs express MHC class I, class II and CD1 receptors like CD1d, which are antigen presenting molecules, costimulatory molecules like CD80 (B7.1), CD86 (B7.2) or CD40, and adhesion molecules such as CD11c, ICAM-1 and ICAM-2. In addition, these cells are characterized by the absence of some lineage markers, for example CD3 (T lymphocytes), CD19 (B lymphocytes), CD14 (monocytes and macrophages), CD56 (NK cells) and CD66b (granulocytes) (Timmerman and Levy, 1999; Brossart, Wirths et al., 2001; Steinman and Dhodapkar, 2001). Moreover, DCs express the lymph node homing chemokine receptor type 7 (CCR7), which allows the migration of mature DCs to T cell rich areas of draining lymphoid organs after CCL19 and CCL21 ligation (Steinman,

1991; Banchereau and Steinman, 1998; Kellermann, Hudak et al., 1999) (Figure 3). In mice, the adhesion molecule CD11c is considered as the specific DC marker because it is expressed in all types of DC and is not present in other cellular lineages (Heath, Belz et al., 2004).

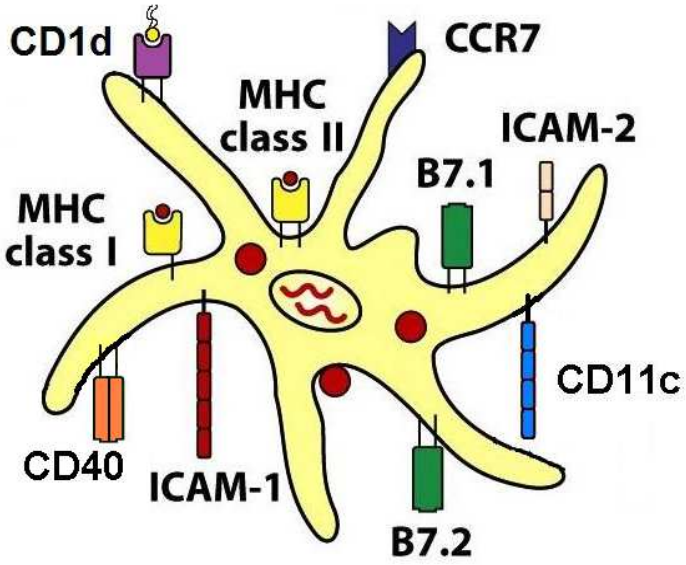

Figure 3. Phenotypic characteristics of conventional dendritic cells. Conventional DCs are primarily concerned with the activation of naive T cells and they express MHC proteins (class I and class II), CD1d receptor and other costimulatory molecules like CD40, B7.1 and B7.2. In addition, these cells express adhesion molecules such as CD11c, ICAM-1 and ICAM-2 and the chemokine receptor type 7 (CCR7) that allows the migration of DCs to T cell rich areas in lymphoid organs (Modified from Kenneth Murphy, 2008).

1.2.1.3. Antigen capture, presentation and DC maturation

DCs are differentiated from bone-marrow progenitors after GM-CSF and IL-4 stimulation. These immature DCs are highly efficient at capturing antigens and they work as immunological sensors screening the peripheral tissues for damaged cells and pathogens. In a general situation, “danger signals” are mediated by pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs), which

recognize various conserved microbial molecules called pathogen associated molecular patterns (PAMPs) (Pulendran, 2004; Akira, Uematsu et al., 2006). There are two antigen processing mechanisms by which DCs process and present peptide antigens (Figure 4):

- Endogenous pathway: intracellular antigens are processed by proteosome and the resulting peptides are moved from the cytoplasm to endoplasmatic reticulum (ER). These peptides are then loaded onto MHC class I molecules and the peptide-MHC class I complex is directed to the plasma membrane where interacts with $CD8^+$ T cells (Germain, 1995).

- Exogenous pathway: extracellular antigens are captured and internalized by DCs through phagocytosis or endocytosis. These antigens are processed by endosomes or lysosomes into different peptides that interact with MHC class II molecules. The peptide-MHC class II complex is directed to the plasma membrane of DCs to interact with $CD4^+$ T cells (Germain, 1995; Watts, 2004).

- Cross-presentation: phagocytosed antigens that are normally processed and presented by the exogenous pathway may escape to the cytosol. In this case, they become processed by the proteosome and imported to the endogenous pathway through MHC class I presentation (Albert, Sauter et al., 1998; Larsson, Fonteneau et al., 2001). This cross-presentation allows MHC class I to present exogenous antigen peptides, which is important to recognized tumor antigens and stimulate $CD8^+$ T cells in order to generate an efficient antitumor response.

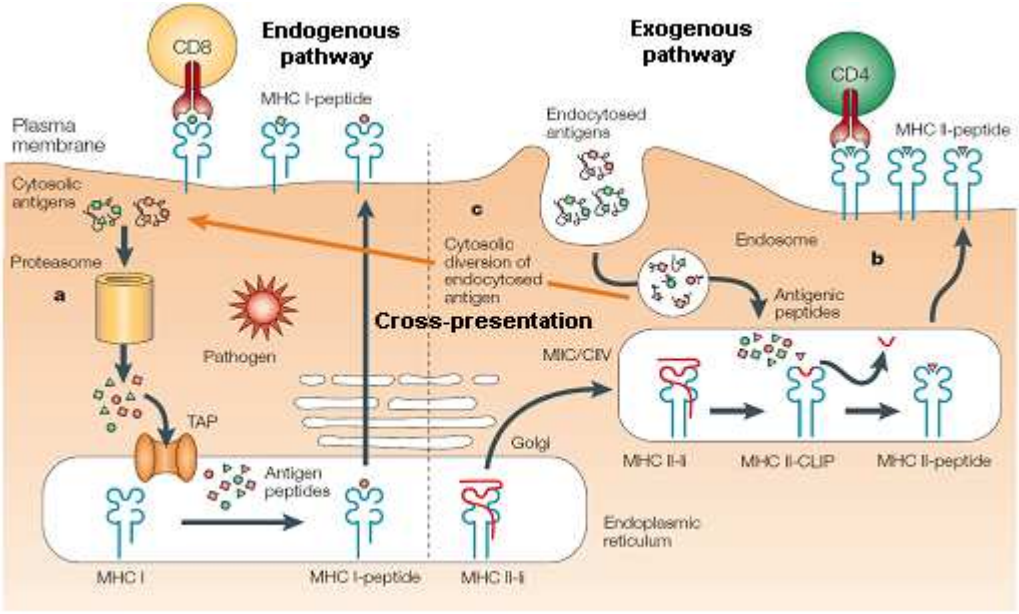

Figure 4. Molecular mechanisms for endogenous and exogenous antigen presentation. (a) Endogenous pathway processes intracellular antigens through the proteosome and the resulting peptides are actively transported into de ER by TAP proteins. Peptides are loaded onto MHC class I molecules and reach the cell surface via the secretory pathway, where they can be presented to $CD8^+$ T cells. **(b)** Exogenous pathway processes extracellular antigens that are taken up by endocytosis. These proteins are degraded inside the endosomes or lysosomes and finally antigenic peptides get into the MIIC/CIIV compartment. Here, appropiate peptides can be loaded onto activated MHC class II molecules and are presented to $CD4^+$ T cells on the cell surface. **(c)** Cross-presentation allows exogenous antigens to reach the MHC class I pathway and be presented to $CD8^+$ T cells (Modified from Heath and Carbone, 2001).

When antigen capture takes place by DCs, functional, morphological and phenotypic changes are induced (Figure 5). During this process, DCs mature and up-regulate costimulatory molecules such as CD40, CD80 and CD86 (100-fold) on their surface, as well as MHC class I and MHC class II molecules (20-fold), and produce large amounts of immunostimulatory cytokines and chemokines (Sallusto, Palermo et al., 1999; Langenkamp, Messi et al., 2000). Mature DCs are highly specialized in presenting antigens and activate T cells (Mellman and Steinman, 2001). Moreover, lymphocytes and epithelial cells also contribute to DC maturation by cell to cell interaction and cytokine secretion (Bell, Young et al., 1999). After antigen processing, DCs also upregulate CCR7 expression, leave the affected tissues and migrate to T cell rich areas of draining lymphoid organs. There, mature DCs provide T cells with pathogen-related information from the affected tissue.

The interaction between DCs and lymphocytes induces suitable antigen-specific immune responses, both humoral responses based on antibody secretion by B lymphocytes and cellular processes mediated by $CD4^+$ and $CD8^+$ T cells (Steinman, 1991; Banchereau and Steinman, 1998).

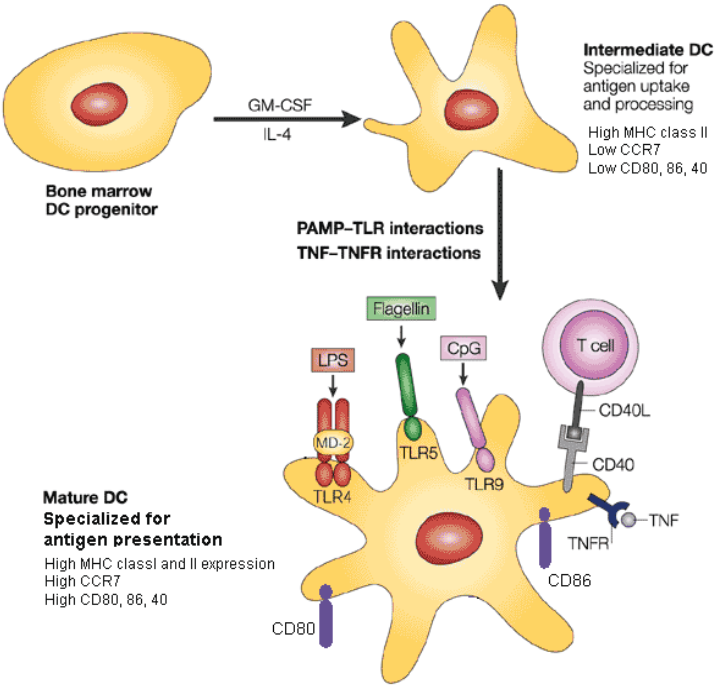

Figure 5. DC differentiation and maturation. Immature DC are differentiated from bone marrow-derivated progenitors in response to certain cytokines, of which granulocyte-macrophage colony-stimulating factor (GM-CSF) seems to be the most important, with IL-4 that can augment or modify this process. Intermediate DCs that develop and reside in peripheral tissues are specialized for antigen uptake and processing. These cells express significant amounts of MHC class II. The maturation or activation of DCs occurs in response to a broad array of signals, which can generally be divided into two categories: pathogen-associated molecular pattern molecules (PAMPs) or tumor-necrosis factor (TNF) family. These two types of signals activate DC maturation through Toll-like receptors (TLRs) and TNF receptor (TNFR) family members. DC maturation starts with the expression of homing and chemokine receptors such as CCR7 that mediate DC migration from tissues to draining lymph nodes, where DCs start to upregulate their expression of costimulatory molecules like CD80 (B7.1), CD86 (B7.2) and CD40. At this point, peptide-loaded MHC class I/II molecules are transported to the cell surface (Modified from Pardoll, 2002).

1.3. The adaptive immune system

The adaptive immunity is mediated by two different but related mechanisms: humoral and cellular immune responses. The humoral immune response is based on antibody production by the B lymphocytes which are involve in the activation of innate system control, including complement activation and opsonin promotion, which induce phagocytosis elimination (Kenneth Murphy, 2008). Moreover, B cells can modulate the adaptive immune response by cytokine production and T helper activation as they express MHC-class II and costimulatory molecules on their surface, acting as APC (Mauri and Bosma, 2012). The cellular immunity is mediated mainly by T lymphocytes, which play a crucial role in the adaptive response against foreign antigens. Naive T cells are specifically activated when the TCR strongly interacts with non-self peptide-bound to MHC. The two main T cell populations are classified based on their expression of either CD4 or CD8 glycoproteins. $CD4^+$ T cells have MHC class II-restricted TCRs and $CD8^+$ T cells are MHC class I-restricted in their antigen recognition (Parkin and Cohen, 2001).

1.3.1. Activation of T lymphocytes

Priming of naive T cells is controlled by several signals delivered by APCs. Signal 1 comprises those antigen-specific signals derived from the interaction of a specific peptide-MHC complex with the TCR. Engagement of the TCR with its peptide antigen is essential for activating naive T cells, but it is not sufficient to stimulate them to proliferate and differentiate into effector T cells (Frauwirth and Thompson, 2002). The antigen-specific clonal expansion of a naive T cell involves at least two other types of signals from the APCs (Kenneth Murphy, 2008). This two signals are divided into costimulatory signals that promote or inhibit the survival and expansion of T cells (signal 2) and the soluble components that are involved in directing T-cell differentiation into the different subsets of effector T cells (signal 3) (Figure 6).

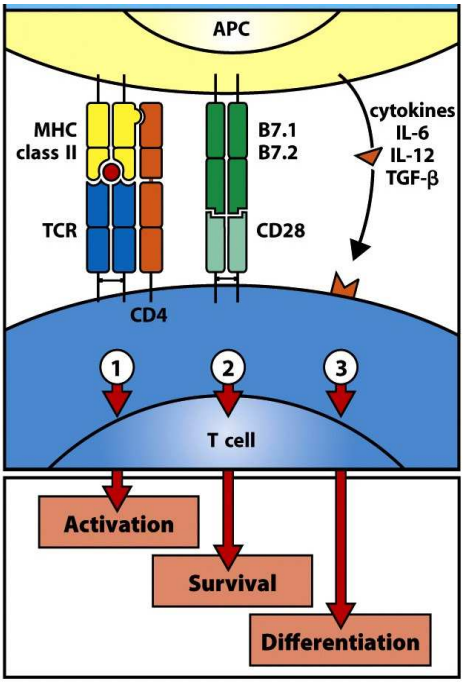

Figure 6. Three types of signals involved in the activation of naive T cells by APCs. In this example, the binding of antigen peptide-MHC class II complex and the TCR of $CD4^+$ T cell generates the signal 1 to the T cell, which warns that an antigen has been encountered. Effective activation of naive T cells requires the costimulatory signal (signal 2) that is delivered by the same APC. In this figure, CD28 on the T cell interacts with B7 molecules on the APC, which effect is the increase of survival and proliferation of the T cell that has received signal 1. Finally, depending on the the nature of the signal 3 (cytokines are commonly) effector T cells are differentiated in several subsets with different effector responses (Kenneth Murphy, 2008).

1.3.1.1. Signal 2: Costimulatory molecules

A large number of molecules have been demonstrated to mediate co-stimulation to T cells (Figure 7). The best characterized costimulatory molecules that deliver signal 2 are the B7 molecules. These homodimeric members of the immunoglobulin superfamily are found in the surface of a variety of APCs including B cells, macrophages and DCs (B7.1 and B7.2), which stimulate naive T-cell proliferation (Kenneth Murphy, 2008). The receptor for B7 molecules on T cells is CD28, yet another member of the immunoglobulin superfamily. CD28 is expressed constitutively on the surface of T cells and its co-stimulation enhances clonal expansion by cell-cycle entry, expression of IL-2 and induction of anti-apoptotic proteins (Sun, Qiu et al., 2005).

Once a naive T cell is activated, it expresses a number of different proteins in addition to CD28, which contribute to sustaining or modifying the costimulatory signals. These other costimulatory molecules generally belong to CD28 family or the tumor necrosis factor (TNF)/ TNF receptor family (Kenneth Murphy, 2008). CD28-related proteins are, for example, inducible co-stimulator (ICOS) molecule

that binds to LICOS (ligand of ICOS or B7h) on activated DCs. TNF family costimulatory molecules include CD70, CD40, 4-1BBL and OX40L. CD27 is constitutively expressed on naive T cells that binds to CD70 on DCs and delivers a potent costimulatory signal to T cells early to the activation process. Moreover, CD40 is expressed on APCs, but also on non-immune cells including endothelial cells, mast cells and epithelial cells. Its ligand CD40L is expressed on T cells after activation and their consequent binding to CD40 up-regulate CD80 and CD86 expression on APC, increase cytokine production and induce T-cell proliferation (Quezada, Jarvinen et al., 2004). The T cell molecule 4-1BB (CD137) and its ligand 4-1BBL, which is expressed on activated DCs, macrophages and B cells, are another pair of co-stimulators and, as CD40-CD40L counterparts, their interaction causes the activation of both T cell and APC (Kenneth Murphy, 2008). In addition, OX40 (CD134), and its binding partner OX40 ligand (OX40L or CD134L) on APC, are expressed on activated CD4 and CD8 T cells and their interaction promote cell proliferation and survival, augmenting the clonal expansion of effector and memory T cell populations (Croft, So et al., 2009).

In addition to positive or activating signals, negative secondary signals that downregulate or terminate T-cell responses are also important in the co-stimulation process (the so-called immune checkpoint inhibitors) (Figure 7). Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), which is a CD28-related protein and has approximately 20-fold higher affinity for B7.1 and B7.2 than CD28, is up-regulated after T cell activation and prevents positive co-stimulation, regulating the peripheral T cell tolerance (Greenwald, Freeman et al., 2005). Similar inhibitory effects are attributed to the programmed cell death-1 (PD-1) molecule, which is also induced after activation of T cells and, following its engagement with the PD-1 ligands (PD-L1 and PD-L2), results in an inhibition of T-cell proliferation (Izawa, Yamaura et al., 2007). Another inhibitory molecule is the T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), which is expressed on Th1 cells and regulates Th1 immunity as well as tolerance *in vivo*. Its ligand, Galectin-9, is presented on APCs and tumor microenvironment (Tang, Liang et al., 2013). As an inhibitory molecule, TIM-3 ligation induces T cell death

(Hastings, Anderson et al., 2009). In addition, other inhibitory proteins are expressed on T cells, such as LAG-3, VISTA and BTLA, and these novel receptors are a matter of intensive research due to their implication in exhaustion of T-cell function (Grosso, Kelleher et al., 2007; Wang, Rubinstein et al., 2011; Pasero and Olive, 2013). Thus, the ultimate fate of cellular immune responses is determined by the balance between positive and negative signals delivered by costimulatory molecules to T cells.

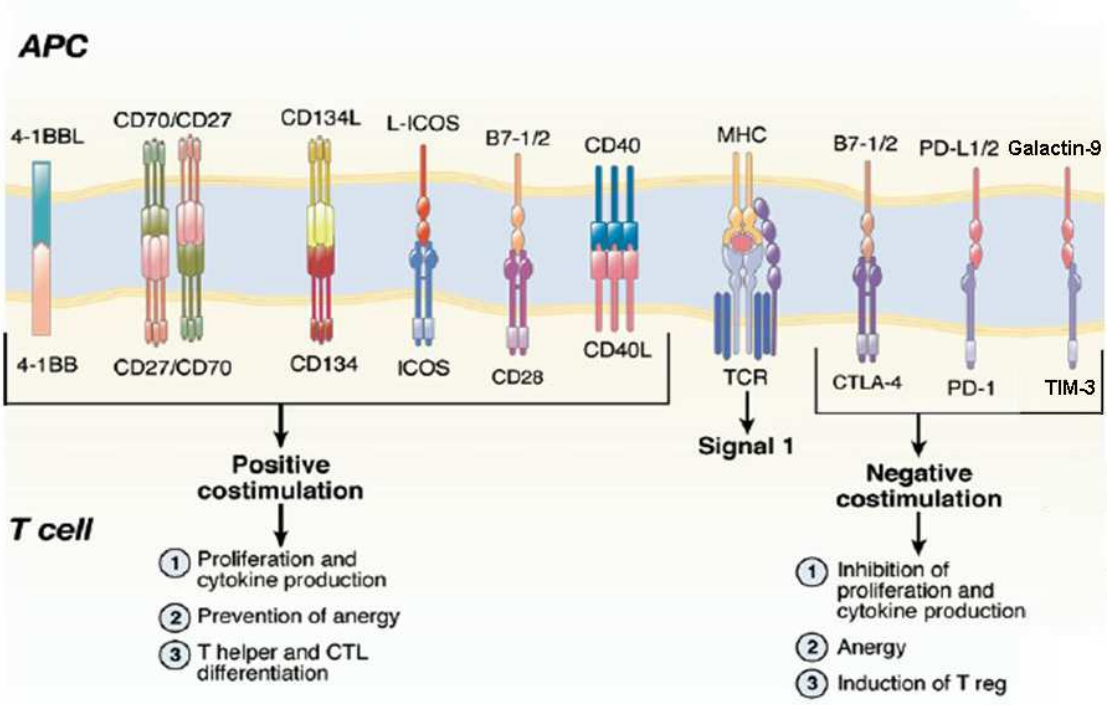

Figure 7. Positive and negative co-stimulation in T-cell activation. T-cell activation requires two principal signals: signal 1, TCR engagement with MHC-peptide complex, and signal 2, ligation of costimulatory molecules on T cells with their respective ligands on APC. The most important costimulatory molecules delivering positive co-stimulation signals are CD28 and CD40L. Under certain circumstances, ICOS, CD134 (OX40), 4-1BB and CD27/CD70 can also deliver positive T-cell stimulation. This positive signals trigger the proliferation of T cells, and cytokine production, as well as the prevention of anergy and T helper and CTL differentiation. Some costimulatory molecules, such as CTLA-4, PD-1 and TIM-3, can lead to negative T-cell signaling, resulting in decreased cell proliferation and cytokine production, cellular anergy and regulatory T (Treg) cell induction (Modified from Rosen, 2008).

1.3.1.2. Signal 3: Effector phase of adaptive immunity

a) $CD4^+$ T lymphocytes

$CD4^+$ T lymphocytes, also known as T helper (Th) cells, are the effector T cells that recognize antigen peptides presented by MHC class II molecules on DCs. Following the antigen-specific signal 1 and costimulatory signal 2, Th cells regulate the adaptive immune responses through their polarization into different functional cell subtypes (Figure 8) (Kenneth Murphy, 2008). The progeny of a naive CD4 T cell is largely decided during the initial priming and is regulated by the signals provided by local environment, particularly by the priming APC (signal 3). Classically, CD4 T lymphocytes have been classified in two different subpopulations according to cytokine secretion profile: Th1 and Th2 cells (Mosmann, Cherwinski et al., 1986; Mosmann and Coffman, 1989). CD4 T cells are differentiated to Th1 lymphocytes following IL-12 and IFN-γ stimulation and are involved in intracellular pathogen defense (intracellular bacteria, virus and protozoa). These cells produce the T cell-proliferative cytokine IL-2 and IFN-γ that, together with the secretion of IL-12 by DCs after CD40-CD40L interaction, activate the cellular immunity, maximize the killing efficacy of macrophages and induce the proliferation and differentiation of naive T cells (Bennett, Carbone et al., 1998; Ridge, Di Rosa et al., 1998; Schoenberger, Toes et al., 1998). Th cells can also be polarized to Th2 favored by IL-4 signal. Th2 lymphocytes are predominantly involved in cellular immunity against extracellular pathogens and allergy processes. These cells produce IL-4, IL-5, IL-10 and IL-13, which promote B cell proliferation, IgE production and eosinophil activation (Romagnani, 1991; Mosmann and Sad, 1996). An effector Th cell type that secreted IL-17, which is called Th17, was also recently described. CD4 T cells commit to the Th17 lineage when both IL-6 and transforming growth factor (TGF)-β are present. Th17 lymphocytes are involved in proinflammatory and autoimmune responses (Aggarwal, Ghilardi et al., 2003; Harrington, Hatton et al., 2005; Langrish, Chen et al., 2005). These cells typically produce IL-17A and IL-17F cytokines, as well as TNF-α and IL-6 in a minor proportion (Langrish, Chen et al., 2005). IL-17 induces

the expression of proinflammatory cytokines (IL-6, TNF-α), chemokines (MCP-1 y MIP-2) and metalloproteases that coordinate cellular infiltration and tissue inflammation (Kolls and Linden, 2004). Furthermore, Th17 cells have a protective role against tumors since they can trigger a strong tumor-specific $CD8^+$ T cell response and promote DC infiltration in tumor tissues (Martin-Orozco, Chung et al., 2009).

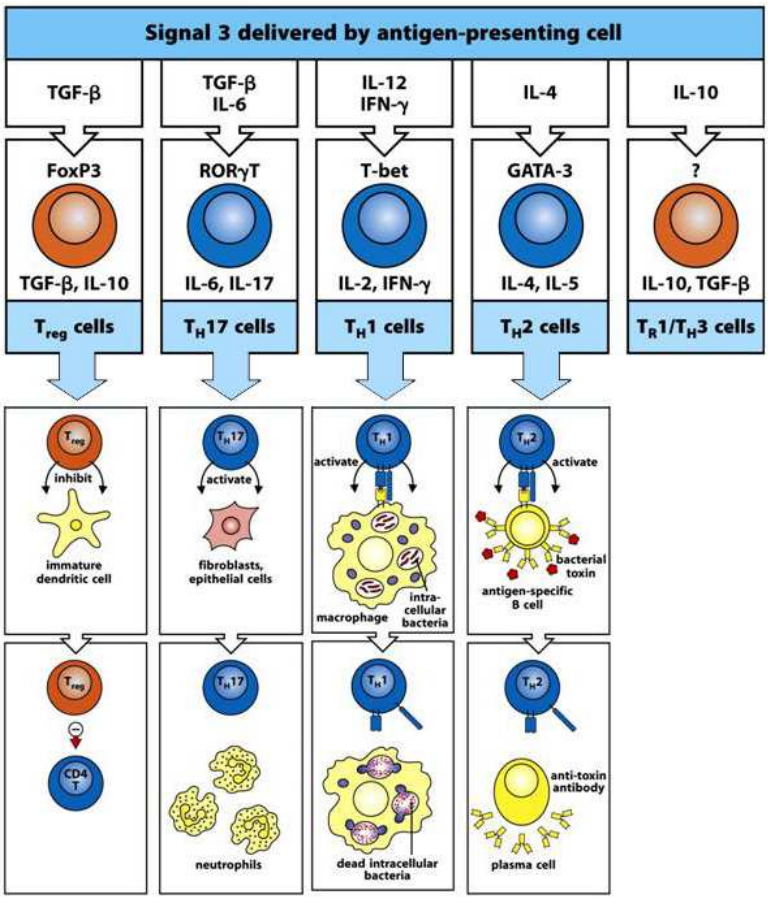

Figure 8. Signal 3 causes distinct types of effector functions in naive CD4 T cells. APCs, principally DCs, produce different cytokines or express different surface proteins that act as a signal 3 to induce the development of CD4 T lymphocytes into several effector cells. The kind of signal 3 depends on the environmental conditions, such as the exposure to various pathogens or the different states of the immune response. When TGF-β is abundant but there is a lack of IL-6, IFN-γ and IL-12, CD4 T cells polarize to Treg cells, because of the absence of pathogen. These cells are able to inhibit both innate and adaptive immune responses. Early in infection, IL-6 and TGF-β are secreted by DCs to induce Th17 cells, which secrete IL-17 and stimulate the proliferation, maturation and differentiation of neutrophils. On the other hand, DCs can produce cytokines that promote either Th1 (IFN-γ and IL-12) or Th2 (IL-4) responses, which are involve in intracellular pathogen response and antibody response, respectively. There is also the regulatory subsets T_R1 and Th3 that require IL-10 during the differentiation of CD4 T cells (Adapted from Kenneth Murphy, 2008).

Even though $CD4^+$ lymphocytes play a central role in inflammatory adaptive immunity, it is clear that they are also important for regulating and maintaining the immune balance and tolerance to self-antigens. In the last years, several $CD4^+$ T cell subsets with regulatory functions have been described. Regulatory $FoxP3^+CD4^+$CD25high T regulatory lymphocytes (Tregs), generated directly by thymic precursors under TGF-β stimulation, are crucial for the maintenance of immunological tolerance suppressing activation, proliferation and effector function of both innate and adaptive immune cells (Sakaguchi, 2000). Once activated, Tregs can mediate their effects either in a contact-dependent fashion or by secreting cytokines such as IL-10 and TGF-β. In addition, there are additional subsets of suppressive T cells called inducible Tregs (iTregs), because they are generated from periphery T cells upon stimulation by different cytokines. For instance, periphery-induced T regulatory type 1 (T_R1) cells may develop under antigen stimulation via IL-10-dependent mechanism. T_R1 cells mainly produce IL-10, suppressing antigen-specific effector T-cell responses and have been involved in the protection against autoimmunity (Groux, O'Garra et al., 1997; O'Garra and Vieira, 2004; Roncarolo, Gregori et al., 2006). There is also a population of suppressive Th3 cells that are developed under IL-10 condition. These cells mainly produce transforming growth factor-β (TGF-β) (Fukaura, Kent et al., 1996), and may suppress the action of both Th1 and Th2 cells (O'Garra and Vieira, 2004).

b) $CD8^+$ T lymphocytes

$CD8^+$ T cells are all differentiated into cytotoxic T lymphocytes (CTLs), which are characterized by CD8 co-receptor expression and peptide-MHC class I complex recognition. $CD8^+$ T cells can be activated by two different ways: APC-mediated stimulation and direct-target cell activation. The simplest is the activation by mature DCs, which have high intrinsic costimulatory activity. These cells can directly stimulate $CD8^+$ T lymphocytes to produce IL-2 that drives their own proliferation and differentiation, and this property has been exploited to generate cytotoxic T-cell responses against tumors (Bennett, Carbone et al., 1998; Ridge, Di

Rosa et al., 1998; Schoenberger, Toes et al., 1998). In addition, the priming of $CD8^+$ T cells by virus-infected antigen-presenting cells may occur in some settings, with the help of $CD4^+$ T cells (Kenneth Murphy, 2008). Upon activation, $CD8^+$ T lymphocytes actively destroy virally-infected and tumor cells through two different mechanisms that are shared by other type of cytotoxic cells, such as NK cells. The first action is the secretion of cytotoxins like perforin, which forms pores in the plasma membrane of attached cells allowing ions, water and toxins to enter into the cytoplasm, and granzymes that mediate the proteolytic activation of apoptosis on the targeted cells. The second mediator of CTL killing is the activation of Fas receptors on the target cell. Cross-linking of Fas with FasL leads to caspase-dependent apoptosis (Trapani and Smyth, 2002; Voskoboinik, Smyth et al., 2006).

1.4. Innate-like lymphocytes: NKT cells

Natural killer T (NKT) cells are a small and heterogeneous subpopulation of αβ-TCR^+ T cells that exhibits characteristics from both innate and adaptive immune cells, and play a central role in regulating immune responses by bridging the innate and adaptive immune systems (Cerundolo, Silk et al., 2009). NKT cells are rapid responders when the immune system is activated; they can activate a different number of immune cells, from NK cells (Carnaud, Lee et al., 1999) to conventional $CD4^+$ and $CD8^+$ T cells (Fujii, Shimizu et al., 2003; Hermans, Silk et al., 2003). These cells are involved in transplantation tolerance, autoimmune diseases, allergic disease and asthma, antitumor immunity, infectious diseases and inflammatory processes (Terabe and Berzofsky, 2008). Their name was based on the observation of NK cell markers in their surface, like NK1.1 in mice or CD161 in humans, not present on conventional T cells, although this is no longer a requisite for NKT cells definition (Godfrey and Kronenberg, 2004). The most important feature of these cells is that, unlike $CD4^+$ and $CD8^+$ T cells, NKT cells have an antigen-specific invariant TCR which recognizes self and foreign lipid antigens when they are presented by CD1d receptor (Figure 9). In fact, NKT cells are now defined as a CD1d-restricted T cell population.

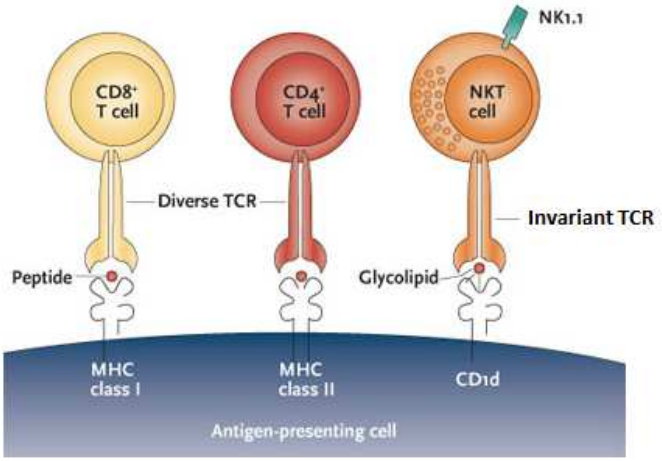

Figure 9. Principal differences between conventional $CD8^+$ and $CD4^+$ T lymphocytes and NKT cells. $CD8^+$ and $CD4^+$ T cells express diverse T-cell receptors (TCRs) to recognize peptide antigens presented by MHC class I and II molecules, respectively. In contrast, NKT cells express an invariant TCR that recognizes glycolipid antigens presented by CD1d molecules and also have NK markers in their cell surface like NK 1.1 in mice (Modified from Van Kaer, 2005).

1.4.1. CD1d: a member of a CD1 family receptor

CD1d is a conserved, non-polymorphic MHC class I-like molecule that belongs to the large family of CD1 receptor, which also includes CD1a, CD1b and CD1c. All of these receptors present lipid antigens to non-MHC-restricted T cells. There are several types of lipid antigens recognized by CD1 receptor family, from microorganism antigens to self and synthetic glicolipids. In the case of CD1d, it is constitutively expressed by hematopoietic cells that can act as APC, including dendritic cells, macrophages, granulocytes and B cells (Brossay, Jullien et al., 1997; Roark, Park et al., 1998). Accordingly, malignancies originating from such cell lineages have also been found to be CD1d-positive, as well as in some solid tumors, such as prostate cancer, breast cancer and gliomas. However, human and murine solid tumors are, generally, CD1d-negative or downregulate CD1d expression.

1.4.2. NKT cell subtypes

NKT cell population is composed of several phenotypically and functionally different subsets, and could be classified according to tissue localization, surface markers and specific TCR usage (Table 1). In mice, NKT cells represent approximately from 1% to 3% of the lymphocytes in the circulation and lymphoid organs such as spleen and bone marrow. By contrast, they are enriched in the liver, where they comprise up to 20%-30% of resident lymphocytes (Bendelac, Rivera et al., 1997). In humans, NKT cells are most frequently found in spleen and liver and also in adipose tissue and *omentum*, but the frequency of NKT cells in periphery is lower (about 0,5% of lymphocytes) and more variable than in mice (Sandberg, Bhardwaj et al., 2003).

Regarding the phenotype, the majority of NKT cells are $CD4^+$ (approximately 90% in mice) and the remainder are $CD4^-$ $CD8^-$ (commonly known as double negative or DN cells). In humans, these two subsets can also be found, but additional populations of CD8αα and CD8αβ NKT cells exist (Gadola, Dulphy et al., 2002). Human NKT cells express NKR-P1A (CD161) as a NK marker, but mouse NKT cells can be $NK1.1^+$ or negative, depending on mouse strain, which confers different functional activities (Coquet, Chakravarti et al., 2008).

Although all NKT cells are CD1d-restricted T cells, differences in TCR rearrangements allow NKT cells to be classified into two major subsets: type I and type II NKT cells (Smyth, Thia et al., 2000; Ambrosino, Terabe et al., 2007). Type I NKT cells represent the 80% of total NKT lymphocytes and express a semi-invariant TCRα chain (Vα14-Jα18 in mice, Vα24-Jα18 in humans) paired with a limited repertoire of Vβ chains (Vβ8, Vβ7 or Vβ2 in mice and Vβ11 in humans) (Terabe and Berzofsky, 2008). For this reason, these cells are also called invariant NKT cells. It is now known that these cells are involved in antitumor immunity and tumor immunosurveillance. Moreover, it is well studied that NKT cells are reactive in presence of α-galactosylceramide (α-GalCer), a potent synthetic agonist that activate and expand NKT cells and has an antitumor effect. On the other hand,

type II NKT cells recognize different glicolipid antigens from those recognized by type I NKT cells, such as sulfatide, and they do not recognize α-GalCer. In this case, TCR repertoire of type II uses the α segments from V1α to V3α, paired with Vβ8.1/Vβ8.3 (Arrenberg, Halder et al., 2010). Little is known about this NKT cell subset, but it is demonstrated that these cells have displayed immunosuppressive activity in tumor immunology.

	NKT cells	
	Type I	**Type II**
TCR Repertoire	Semi-invariant TCR: Mouse: Vα14-Jα18, Vβ2/7/8 Humans: Vα24-Jα18, Vβ11	Heterogeneous TCR repertoire using the α segments from V1α to V3α and Vβ8.1/Vβ8.3
Co-receptor	Mouse: CD4 or $CD4^-$ $CD8^-$ (DN) Humans: CD4, DN, CD8αα, CD8αβ	
Reactivity	α-Galceramide	Sulfatide
Antigen presentation molecule	CD1d	
NK receptors	Mouse: NK1.1+/- Humans: NKR-P1A (CD161)	
Localization	Timus, liver, spleen, bone marrow, lymph nodes	

Table 1. NKT cell subsets. Natural killer T (NKT) cell population encompasses several phenotypically and functionally different subpopulations classified as Type I NKT and Type II NKT cells. Type I NKT cells express an invariant TCRα chain, while type II NKT cells display a more diverse repertoire using α segments from V1α to V3α and Vβ8.1/Vβ8.3. Type I and II NKT cells share the localization and surface markers expression (CD4, CD8 and NK1.1), as well as the two groups are CD1d-restricted cells. The prototypic antigen able to activate all type I NKT cells is α-galactosylceramide (α-GalCer). Type II NKT cells recognize a greater variety of antigens, including sulfatide. (Modified from Robertson, Berzofsky et al., 2014).

1.4.3. NKT cells in tumor immunology

NKT cells can kill tumor cells directly by the recognition of CD1d-tumor lipid antigen complex in the case of $CD1d^+$ tumors (Wu, Segal et al., 2003; Haraguchi, Takahashi et al., 2006). During this process, NKT cells can also activate NK cells to help in tumor eradication (Figure 10a). The direct recognition of CD1d in some tumors has been demonstrated, for example in myelomonocytic leukemia cells that are sensitive to lysis by NKT cells. CD1d expression on human hematological malignancies has been demonstrated, but most solid tumors do not, or poorly express, CD1d. Despite of this lack of CD1d expression, these tumors can be killed as well, which indicates that NKT cells might promote tumor eradication indirectly, through cross-presentation of tumor lipids by APC and the activation of effector cells like NK cells (Figure 10b) (Vivier, Ugolini et al., 2012).

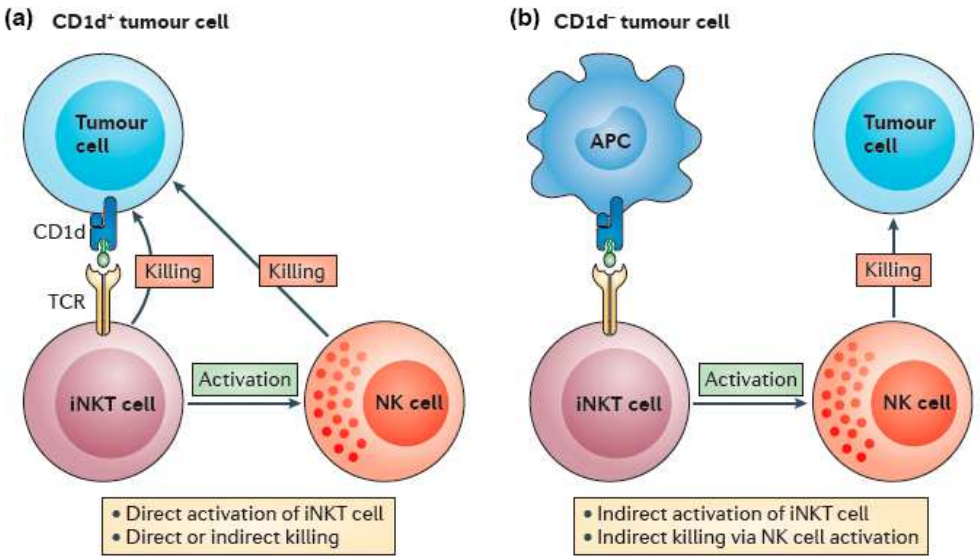

Figure 10. Direct and indirect antitumor activity of NKT cells. (a) Tumor cells which express CD1d can be recognized and eradicated by NKT cells and can be also killed by the indirect activation of NK cells through NK-cell stimulation. **(b)** $CD1d^-$ tumor cells can be eradicate by effector cells as NK cells after their activation by NKT cells, which are stimulate by lipid antigen presentation on APC (Vivier, Ugolini et al., 2012).

Regarding the indirect antitumor activity, NKT cells can be activated and expanded using pharmacological compounds that bind to CD1d molecules and are recognized by the NKT-cell TCR. The first lipid antigen to be identified as an NKT-cell activator was α-GalCer, which was found in the marine sponge *Agelas mauritanius*. In the last years, a number of α-GalCer analogues have been developed to activate NKT cells *in vivo* and *in vitro* with better biologic activities than α-GalCer, such as α-C-GalCer, OCH9, C20:2 and Threitolceramide (Cerundolo, Silk et al., 2009) (Figure 11). These synthetic compounds can induce a longer and more controlled NKT activation than α-GalCer.

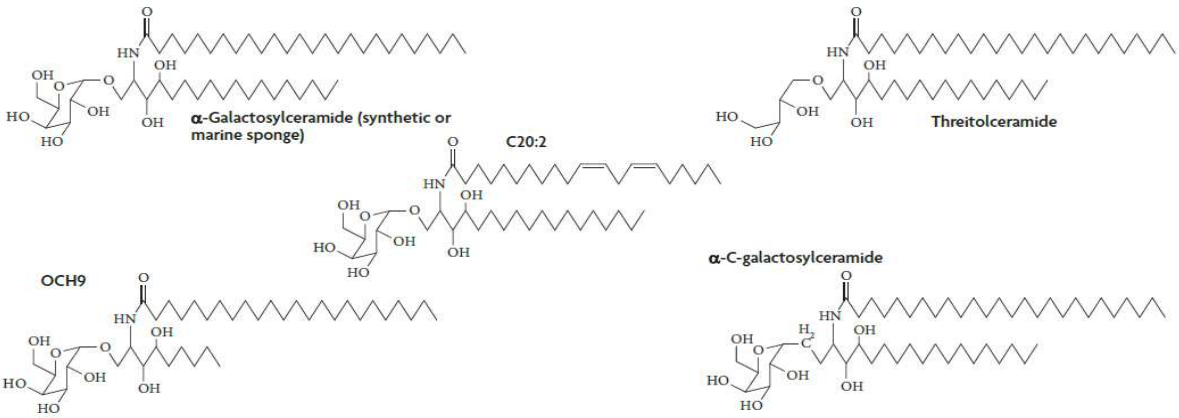

Figure 11. Synthetic ligands of NKT cells. Synthetic ligands include a-galactosylceramide, which can also be obtained from the marine sponge *Agelas mauritianus*, a-C-galactosylceramide, OCH9, CD20:2 and threitolceramide (Cerundolo, Silk et al., 2009).

Another examples of NKT-cell agonists are the novel molecules HS44 and HS161 (Figure 12), which induces a Th1-polarized immune response since it stimulates a high production of IFN-γ in contrast to low production of IL-4 (Harrak, Barra et al., 2011; Kerzerho, Yu et al., 2012). The capacity of this molecule to redirect the immune response throughout a Th1-bias is an important feature in tumor immunology.

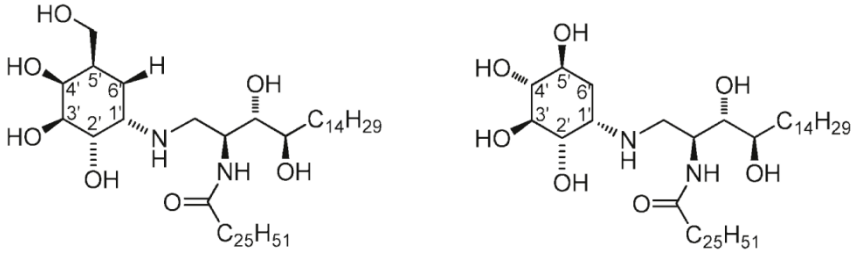

Figure 12. New synthetic NKT-cell agonists. Biological structure of HS161 and HS44, two new synthetic ligands of NKT cells (Modified from Harrak, Barra et al., 2011).

1.4.4. NKT-cell activation

Stimulation of NKT cells through recognition of the α-GalCer-CD1d complex on APC results in the rapid production of Th1 and Th2-type cytokines, such as IFN-γ and IL-4 (Kawano, Cui et al., 1997; Spada, Koezuka et al., 1998). These molecules activate T, NK and DCs (Figure 13). In addition, NKT cells specifically stimulate DCs through the CD1d-TCR complex and CD40-CD40L interaction, which induces DC maturation and IL-12 secretion (Vincent, Leslie et al., 2002). As a result of direct interaction with NKT cells, DCs can prime antigen-specific $CD4^+$ and $CD8^+$ T cells and stimulates NK cells and NKT cells to produce more IFN-γ, which has an important role in the effector functions of these cells. In addition, the activation of NK cells by NKT cells promotes NK-cell citotoxicity which helps to eradicate the tumor (Vivier et al, 2012). Invariant NKT cells also produce IL-2, which induces the proliferation of memory $CD4^+$ and $CD8^+$ T cells, and TNF-α, which enhances DC maturation, as well as a diverse range of other cytokines like IL-5, IL-6, IL-17 and IL-21. It is becoming clear that the repertoire of Th1 and Th2-type cytokines produced by NKT cells is modulated by the strength of NKT-cell TCR signaling and the type of APC presenting the NKT-cell agonist. The activation of NKT cells with strong agonists such as α-GalCer results in high levels of cytokine production because of the higher affinity for the NKT-cell TCR (Cerundolo, Silk et al., 2009).

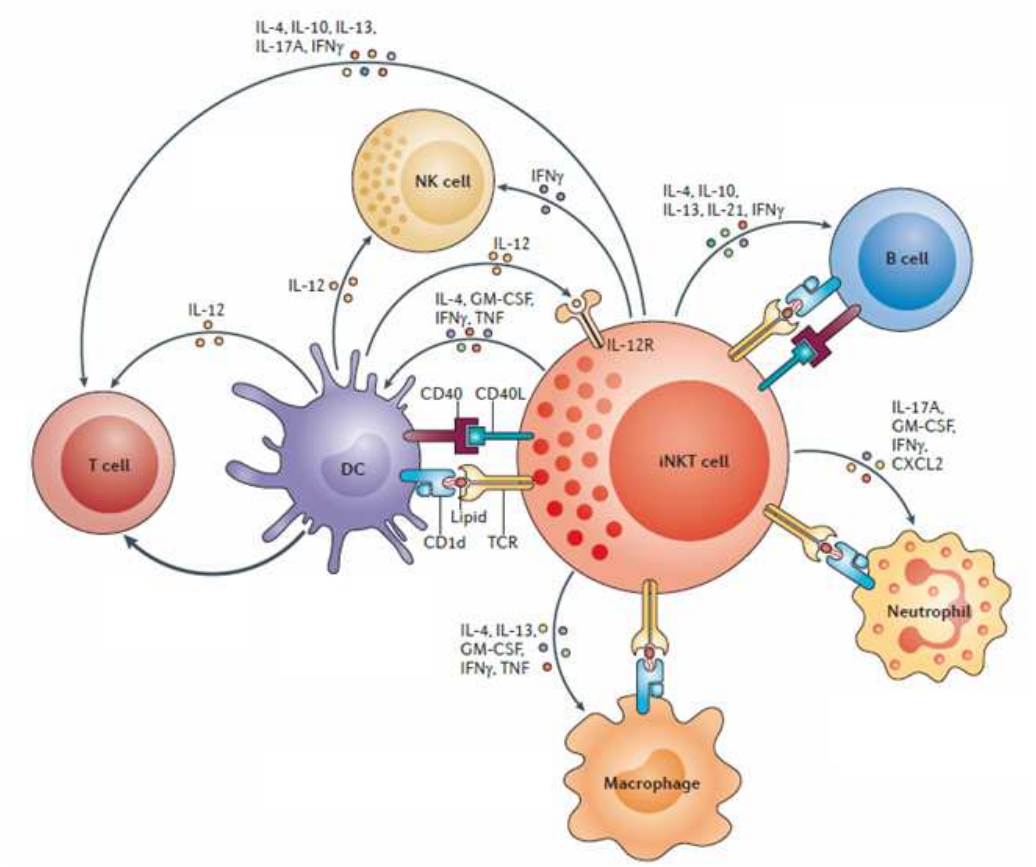

Figure 13. NKT cell activation and interaction with the other immune cells. In the absence of CD1d expression on tumor cells, NKT can be activated by CD1d-expressing APC. These activation is bidirectional, as NKT cells receive signals from APCs and DC also receive NKT-cell stimulation. These interactions can be received through cell-surface receptors like T-cell receptor recognizing glicolipid-CD1d complexes, costimulatory receptors, such as CD40, as well as through soluble mediators (cytokines like IFN-γ, IL-4 and IL-12). NKT cells promote NK-cell activation, tumor-specific T-cell proliferation and cytokine production and B-cell antibody secretion (Adapted from Brennan, Brigl et al., 2013; McEwen-Smith, Salio et al., 2015).

Recent works has also shown that NKT-cell activation causes an upregulation of CD80, CD86 and OX40 ligand by mature DCs, which is important for co-stimulating NKT cells and promoting antigen-specific $CD8^+$ T-cell responses (Zaini, Andarini et al., 2007; Taraban, Martin et al., 2008). In addition to inducing the generation of potent antigen-specific $CD4^+$ and $CD8^+$ T-cell responses, NKT cells can also induce B-cell maturation, higher antibody titers and expansion of the B-cell memory pool (Galli, Nuti et al., 2003; Galli, Pittoni et al., 2007).

The activation of NKT cells can orchestrate the function of both innate and adaptive immune systems. The fact that these innate and adaptive immune reactions occur simultaneously is important for a potent immunological response, especially for eradication of tumors, which frequently contain both MHC-negative cells (targeted by NK cells) and MHC-positive cells (targeted by $CD8^+$ T cells).

1.4.5. NKT cell identification: the use of CD1d tetramers

Human and mice NKT cells can be analyzed using the CD3 or TCRβ antibody and CD1d tetramers. The affinity of soluble monomeric CD1d/glicolipid complexes for their specific TCR partners is weak and the complexes have a very short half-life. For these reasons, soluble CD1d/glicolipid tetramers were engineered to be capable of engaging more than one copy of the TCR on the surface of NKT cell, increasing the avidity of the interaction. The CD1d tetramers are designed to include four soluble CD1d molecules linked by enzymatic biotinylation, followed by mixing of the biotinylated CD1d/glicolipid complexes with fluorescently labelled streptavidin (Figure 14). The CD1d molecules of these tetramers are loaded with the α-GalCer or its analogue PBS-57, which is more stable than α-GalCer. This reagent proved to be very effective in identifying NKT cells by flow cytometry.

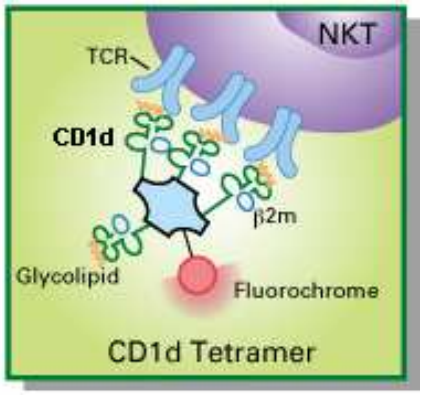

Figure 14. Structure and mechanism of CD1d tetramers. Four molecules of CD1d are bound to fluorochrome (usually Streptavidin-PE) after biotinylation and these CD1d tetramers can be loaded with a glycolipid (α-GalCer or PBS-57). They are incubated with NKT cells and interact with the invariant TCR (Extracted and modified from MBL International Corporation homepage).

1.5. Cancer immunotherapy

Over the last years, several cancer treatments were developed; basically chemotherapy and radiotherapy were the principal treatments against cancer in hospitals. But these conventional therapies have a reduced efficacy in the treatment of some solid tumors and hematological malignancies. The better knowledge of cancer immunology and molecular biology techniques may trigger the development of new cancer treatments with tumor specificity. The capacity of immune system to recognize and destroy tumor cells makes cancer immunotherapy as a good therapeutic approach to complement the conventional treatments.

1.5.1. Types of immunotherapy-based treatments in cancer

There are two principal types of immunotherapy: passive immunotherapy, which consists in patient infusion of cells, antibodies or cytokines, and active immunotherapy that promotes *in vivo* induction of the immune system of the patient. Both types of therapies can be directed against specific tumor antigens or can produce global, non-specific immune system activation.

1.5.1.1. Active immunotherapy using therapeutic cancer vaccines

Therapeutic cancer vaccines, as an active immunotherapy, are designed to stimulate patient's immune system against tumors (Drake, 2010). Stimulating the immune system, a therapeutic cancer vaccine induces an antitumor response that allows T cells to attack malignant cells and lead to improve survival. It is important to notice that therapeutic cancer vaccines generate active immune responses against an existing cancer, so their function is not preventing disease.

The ideal tumor antigen is one that is not present in normal cells but is expressed in all tumor cells. Moreover, it is important to take into account that the richest source of tumor antigens is the tumor itself. Approaches using allogeneic or generic cell lines as vaccines are widely applicable (Figure 16a). For example, irradiated tumor cells can be engineered to secrete a number of different cytokines or express costimulatory molecules. This therapeutic strategy showed a good *in vivo* protection in mice from challenge with the same tumor type. For instance, tumor cells can be transduced with a virus vector encoding immunostimulatory cytokines such as GM-CSF or IL-2, which promote DC migration and T cell proliferation, respectively. Studies in patients with advanced prostate cancer and metastasic malignant melanoma used irradiated tumor cells transduced with a retrovirus vector encoding GM-CSF resulted in 1 partial response of 21 melanoma patients, despite 11 of 16 melanoma patients presented an extensive inflammatory response with necrosis and fibrosis of tumor (Berzofsky, Terabe et al., 2004).

Because of its central role in the induction of antigen-specific immune responses, DCs constitute an interesting tool to develop antitumor vaccines (Figure 16b). DCs pulsed with tumor lysates, tumor protein extracts or synthetic peptide tumor epitopes could generate protective immunity against corresponding tumor. In the same way, transfer of nucleic acids encoding tumor antigens, costimulatory molecules or cytokines into DCs using viral vectors like recombinant adenoviruses or lentiviruses has been effective in some cases.

Furthermore, intramuscular injection of naked DNA expression plasmids, as well as antigen peptides or viral vectors, has been shown to generate antitumor immune responses (Figure 16c). These type of vaccines introduce genes encoding tumor antigens and peptides into DCs for endogenous processing and presentation to CTLs.

All of these different cancer vaccines generate a DC maturation and $CD4^+$ and $CD8^+$ T-cell activation in lymph nodes that results in a cytotoxic activity to kill tumor cells.

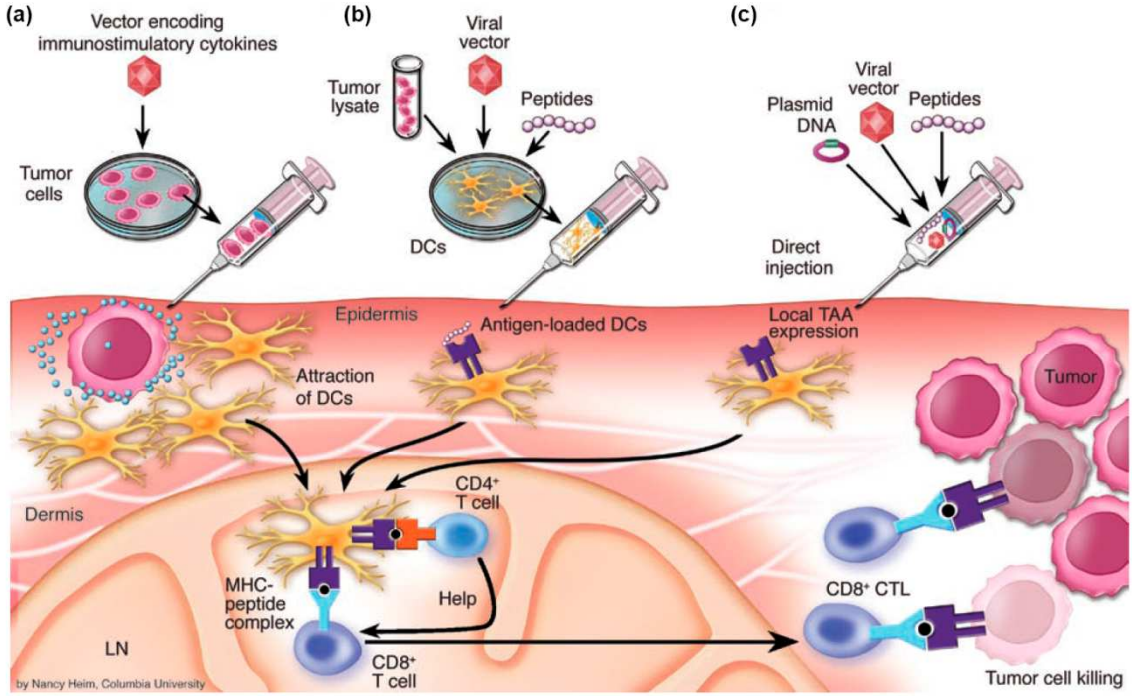

Figure 15. Approaches to antitumor vaccination. (a) Irradiated tumor cells transduced with a viral vector encoding an immunostimulatory cytokine as GM-CSF attract APCs as DCs that acquire, process and present tumor-associated antigens (TAAs) encoded by the vector through MHC complexes. **(b)** DCs can be loaded with tumor lysates and peptides encoding tumor antigens, or infected by viral vectors expressing TAA previous to infuse them into de patient. **(c)** TAAs can be locally supplied to DCs by the direct injection of plasmid DNA, peptides or viral vectors. In all of three cases, DCs migrate to secondary lymphoid tissues where they present the antigen epitopes to T cells to generate an antitumor CTL response (Berzofsky, Terabe et al., 2004).

1.5.2. Cancer immunotherapy for B-cell lymphoma

There are different types of immunotherapy against B-cell lymphoma, from specific mAb infusion to therapeutic cancer vaccines in development (Briones, 2009). Focusing on passive immunotherapy, different monoclonal antibodies have been using to treat lymphoma patients (i.e., anti-CD20, anti-CD22, anti-CD52, anti-CD40 and anti-CD30) (Table 2). All of them cause a non-specific immune response. In addition, antigen-specific T lymphocytes or idiotype (Id) specific antibodies can be used to treat B-cell lymphoma in a more specific fashion. On the other hand, active immunotherapy is a promising approach for the treatment of lymphoma. This type of therapy is based on the use of cytokines that activate the immune system in a non-specific manner, or the use of cancer vaccines, which are

developed against specific tumor antigens. There are several types of cancer vaccines in development against B-cell lymphoma, summarized in table 2.

<table>
<tr><td rowspan="6">Active immunotherapy</td><td>Unspecific</td><td colspan="2">IFN-α, IL-2, IL-12, GM-CSF</td></tr>
<tr><td rowspan="5">Specific</td><td>Vaccine</td><td>Antigen/Adjuvant</td></tr>
<tr><td>Proteins/Peptides</td><td>Idiotype (Id)</td></tr>
<tr><td>DNA in plasmids</td><td>Idiotype (Id)
Id with costimulatory genes: IL-2, IL-12, GM-CSF</td></tr>
<tr><td>Recombinant virus:
Adenovirus
Poxvirus</td><td>Idiotype (Id)
Fusion genes:
Id+GM-CSF, IL-2
Id+CD40L,OX40,B7,ICAM-1, LFA-3</td></tr>
<tr><td>Dendritic cells</td><td>Pulsed with idiotype
Pulsed with tumor lysates
Fused with tumor cells
Transduced with viral vectors encoding:
Idiotype (id)
Costimulatory molecules
Cytokines</td></tr>
<tr><td>Whole tumor cells</td><td>Transduced with viral vectors encoding:
Costimulatory molecules
Cytokines</td></tr>
<tr><td rowspan="3">Passive immunotherapy</td><td>Unspecific</td><td>Humoral</td><td>Anti-CD20
Anti-CD22
Anti-CD52
Anti-CD40
Anti-CD30</td></tr>
<tr><td rowspan="2">Specific</td><td>Cellular</td><td>Antigen-specific T lymphocytes
(TCR-modified and CAR T cells)</td></tr>
<tr><td>Humoral</td><td>Idiotype specific antibody</td></tr>
</table>

Table 2. Immunotherapy against B-cell lymphoma. (Adapted from Briones, 2009).

1.5.3. NKT cell-based cancer immunotherapy

The evidence indicating that harnessing of mouse NKT cells increases antigen-specific immune responses provides the basis for designing an effective immunotherapy to enhance immune responses against tumors through the activation of these cells. Two main NKT cell-directed therapies has been studied so far, including administration of NKT cell-activating ligands such as α-GalCer and administration of APCs pulsed with α-GalCer.

1.5.3.1. α-GalCer therapy

Activation of NKT cells by giving soluble α-GalCer *in vivo* has been shown to induce potent antitumor responses in mouse tumor models. The first studies of α-GalCer efficacy have been done using a mouse model of melanoma. In this case, soluble α-GalCer administration diminished the number of metastasis and increased mice survival (Kobayashi, Motoki et al., 1995).

The first human clinical study using α-GalCer was done in 2002 and used repeated intravenously (iv) injection of this NKT cell adjuvant at various doses in patients with solid tumors (melanoma, breast, head and neck, prostate, lung, bladder and kidney cancers). No dose-limiting toxicity was observed, suggesting the activation of NKT cells was safe and well-tolerated in humans (Giaccone, Punt et al., 2002). In addition, a significant increase of IFN-γ, IL-12 and GM-CSF was observed in the serum of some treated patients. Despite these promising observations, the injection of soluble α-GalCer leaded to NKT-cell anergy in a PD1/PD-L1-dependent manner (Cerundolo, Silk et al., 2009; McEwen-Smith, Salio et al., 2015), preventing the NKT-cell restimulation to potentiate the immune response.

1.5.3.2. α-GalCer-loaded DC vaccination

For NKT cell activation with α-GalCer, interaction of NKT cells with DCs is a key factor for the antitumor activity of α-GalCer. For this reason, vaccines consisting of α-GalCer-loaded DCs were developed. Studies with murine tumor models suggested that injection of DCs pulsed with α-GalCer induced prolonged cytokine responses as compared with injection of soluble α-GalCer. For example, Fujii and collaborators demonstrated that DCs loaded with α-GalCer was a better therapeutic strategy because the NKT cell adjuvant was directly binded to the most potent APC. This approach generated a much more prolonged immune response, with a significant expansion of IFN-γ producing NKT cells, and a reduction of melanoma metastasis in mice (Fujii, Shimizu et al., 2002).

In the first clinical trial, autologous immature DCs that had been pulsed with α-GalCer were administered intravenously into patients with metastasic solid tumors (Nieda, Okai et al., 2004). Similarly to the results observed from intravenous immunization with soluble α-GalCer, serum levels of IFN-γ and IL-12 increased after vaccination and there was also a decrease in tumor markers in the serum of two treated patients. In another clinical trial, five myeloma patients were treated with three doses of mature DC vaccine. The first one was only a dose of mature DC and the other two were in combination with α-GalCer. In this study, three patients exhibited a significant reduction of tumor markers in blood and one of them showed a stable disease (Chang, Osman et al., 2005). Moreover, nine patients with head and neck cancer were treated with immature DCs pulsed with α-GalCer and there was a significant NKT cell expansion and increase of NK activity in some of them (Uchida, Horiguchi et al., 2008).

In addition to that, it was also demonstrated that $CD1d^{+}$-tumor cells could be a good vehicle to deliver α-GalCer *in vivo*. Some studies demonstrated that this strategy induce an effective tumor immunity in mouse models of lymphoma and plasmocytoma in a prophylactic and therapeutic fashion (Liu, Idoyaga et al., 2005; Chung, Qin et al., 2007). In fact, tumor cells that serve as a vehicle are also a

source of tumor antigens that can be taken by DCs to induce tumor antigen-specific $CD4^+$ and $CD8^+$ T cells (Terabe and Berzofsky, 2008).

1.5.4. New approaches for NKT cell immunotherapy: the NKT14m antibody

Recently, the biotechnology company NKT Therapeutics has developed an anti-human invariant TCR antibody (NKTT120 mAb) that depletes NKT cells, which can be a good therapeutic approach to some diseases like allergic asthma and inflammation. To understand the role of NKT cells in preclinical models, they developed an anti-mouse invariant TCR specific monoclonal antibody (NKT14 mAb). By modifying the Fc-portion of the NKT14 depleting antibody, they generated the NKT14m agonist antibody which activates NKT cells *in vivo* in fully 52mmune-competent mice, which represents a good strategy to treat diseases such as infectious diseases and cancer. To obtain these antibodies, the invariant TCR specific antibody clone that recognizes invariant TCR of NKT cells was insert in frame with either murine wild type IgG2a Fc to obtain the depleting NKT14 mAb or an IgG2a with 4 point mutations to the Fc portion, generating the NKT-cell activator NKT14m antibody. The NKT14m agonist was not tested in any cancer model yet, but it is shown that the injection of NKT14m mAb into Balb/c mice triggers the IFN-γ production by NKT cells (Scheuplein, Lamont et al., 2015). While NKT cells can be strongly activated by agonistic NKT14m, studies about the anitumoral effect of this new reagent has not been published so far.

2. OBJECTIVES

The overall aim of this study is to evaluate the antitumor efficacy of NKT cell activation as a treatment against B-cell lymphoma.

Specific objectives:

- To evaluate the *in vivo* antitumor effect of a vaccine consisting of dendritic cells, tumor cells and the NKT-cell agonist α-GalCer in a mouse model of B-cell lymphoma.

- To analyze the mechanisms (effector cells and cytokines) involved in the antitumor immune response induced by the tested vaccine.

- To evaluate the *in vivo* antitumor efficacy of a novel NKT agonist (the NKT14m activating antibody) in a mouse model of B-cell lymphoma.

3. MATERIALS AND METHODS

3.1. Tumor cell lines

3.1.1. B-cell lymphoma line 4TOO

4TOO is a Balb/c plasmacytoma cell line expressing CD138 (Syndecan-1) and MHC class I H-2^d molecules, gently provided by Dr. M. Khuel (NCI, Bethesda, MD). Tumor cells were grown in complete medium (CM), which consists of RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-mercaptoethanol (all provided by Life Technologies, Inc.). Cells were maintained at 37ºC in 5% CO_2.

3.1.2. B-cell lymphoma line A20

A20 is a syngeneic Balb/c B-cell lymphoma expressing MHC class I and II H-2^d molecules, and was obtained from the American Type Culture Collection (ATCC). This tumor cell line was grown in CM and was maintained at 37ºC in 5% CO_2.

3.2. B-cell lymphoma mouse model

Female Balb/c mice (Charles River, France; 6-7 weeks of age) were used for *in vivo* experiments. Animals were housed at the Laboratory Animal Facility at Hospital de Santa Creu i Sant Pau (Barcelona), and maintained in controlled temperature atmosphere (range between 20-22ºC), with a light cycle of 12h light/12h dark. All experiments and care of animals were accomplished according to European Animal Care Guidelines.

To define the minimum lethal dose of tumor cells that is needed to establish the B-cell lymphoma model, different doses of 4TOO and A20 were tested *in vivo*. 4 x 10^5 cells and 5,5 x 10^5 cells from the 4TOO cell line were injected into mice

intravenously (iv), while for the A20 tumor cell line, the doses tested were 1 x 10^6 cells and 2 x 10^6 cells subcutaneously (sc). Although A20 was injected sc, both tumor cell lines disseminates to lymph nodes, spleen, liver and bone marrow.

3.3. Mix+GalCer vaccine generation

The therapeutic vaccine consists of 5x10^5 dendritic cells, as antigen presenting cells, 5x10^5 tumor cells, as antigen source, and 2µg of NKT cell agonist α-GalCer (Enzo Life Science). α-GalCer was resuspended using 0,5% Tween-20 solution (Sigma) in Phosphate Buffer Saline (PBS) and was incubated one hour at 37ºC to facilitate its dissolution. Tumor cells were irradiated (30 Gy) immediately before vaccination to arrest proliferation of cells. This vaccine was called Mix+GalCer and had been generated by mixing the three components before iv injection into mice (Fig.16).

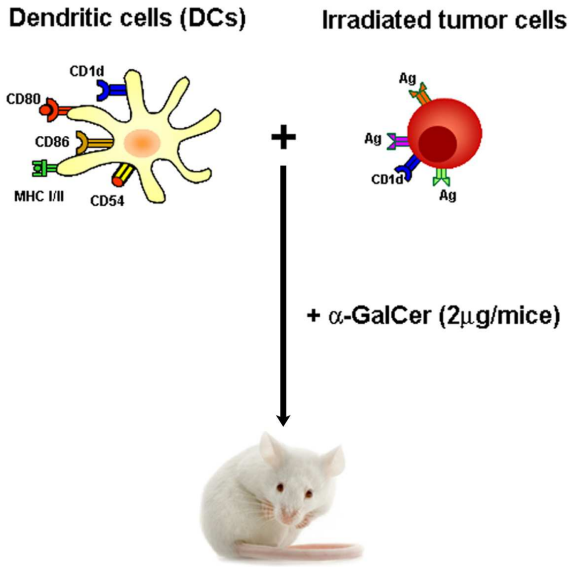

Figure 16. Therapeutic vaccine generation. DCs were mixed at 1:1 ratio with irradiated tumor cells and α-GalCer was added prior to mice injection (iv).

3.3.1. Generation of dendritic cells

Bone marrow was extracted from the femur, tibia and iliac crest bones of sacrificed mice in sterile conditions. The cellular suspension was filtered through a 70 µm cell strainer (BD Falcon, Cultek) and centrifuged 5 minutes at 1000 rpm. Erythrocytes were lysed using an Amonium Chloride Solution (Pharmlyse, Pharmingen, BD Bioscience) during three minutes in agitation. After that, cells were washed with CM, cultured in 150 mm plates (Cultek), and maintained in 25 mL of CM supplemented with 20ng/ml of recombinant mouse GM-CSF (rmGM-CSF; Peprotech), at 37ºC in 5% of CO_2. Three days later, 25 ml of CM with 20ng/ml of rmGM-CSF was added to the plate and, after two days, cells in suspension were collected and culture in a new 150mm plate with 25ml of CM supplemented with 20ng/ml of rmGM-CSF. Finally, seven days after starting the culture, all adherent and suspension dendritic cells were collected and used for the experiments.

3.4. Treatment with the Mix+GalCer vaccine

Balb/c mice (n=10/group) were challenged with 4TOO B-cell lymphoma ($4x10^5$ tumor cells/mouse in 100µl of PBS, iv) and, two days later, were treated with the vaccine. Controls and the therapeutic vaccine are described in table 3. Mice were followed for survival for 100 days after tumor challenge.

In some experiments, treated mice that were tumor-free at 100 days after treatement received a second identical lethal tumor inoculation (4TOO) or were injected with the syngeneic B-cell lymphoma A20 ($1x10^6$ cells/mouse in 100µl of PBS, sc) and followed daily for survival up to 100 days after tumor rechallenge.

	Name of vaccine	Components	Injection
Therapeutic vaccine	**MIX+GalCer**	10^6 cells with 1:1 ratio ($5x10^5$ DCs + $5x10^5$ irradiated tumor cells) + α-GalCer (2µg)	100µl of PBS, iv
Controls	**DC**	10^6 DCs	
	α-GalCer	2µg of α-GalCer	
	MIX	10^6 cells with 1:1 ratio ($5x10^5$ DCs + $5x10^5$ irradiated tumor cells)	
	DC+GalCer	10^6 DCs with 2µg of α-GalCer	

Table 3. Summary of tested vaccines. The therapeutic vaccine, Mix+GalCer, was obtained mixing $5x10^5$ DCs and $5x10^5$ irradiated tumor cells with 2µg of α-GalCer. The vaccines DC (10^6 DCs), α-GalCer (2µg/mouse), Mix ($5x10^5$ DCs and $5x10^5$ irradiated tumor cells) and DC+GalCer (10^6 DCs with 2µg of α-GalCer) were used as controls. All treatments were injected in a volume of 100 µl of PBS, iv).

3.5. NKT14m antibody treatment

Balb/c mice (n=10/group) were injected with 4TOO B-cell lymphoma ($4x10^5$ tumor cells/mouse in 100µl of PBS, iv) and, two days later, were treated with the NKT14m antibody (100µg/mice in 100µl of PBS, iv; NKT Therapeutics Inc.). In some cases, these groups were retreated with 100µg of NKT14m antibody 42 days after tumor challenge. Another group received a treatment that combined a single dose of cyclophosphamide (70mg/kg, ip) 10 days after tumor injection and a single dose of NKT14m (iv) 24h after cyclophosphamide treatment. In all cases, mice were followed for survival for 100 days after tumor challenge (Table 4).

Name of vaccine	Components	Injection
NKT14m	100 µg of antibody/mouse (Optional retreatment 100µg/mouse)	100µl of PBS, iv
Cyclophosphamide + NKT14m	Cyclophosphamide (70mg/kg) and 100 µg/mouse of NKT14m antibody 24h after cyclophosphamide treatment	100µl of PBS cyclophosphamide, ip NKT14m, iv

Table 4. Summary of NKT14m vaccines and their components.

3.6. *In vivo* depletion of T and NK cells

Mice (n=5 per group) were treated with Mix+GalCer vaccine 2 days after 4TOO tumor challenge ($4x10^5$ tumor cells/mouse in 100µl of PBS, iv). Specific groups of mice were depleted of $CD4^+$, $CD8^+$ or NK cells by intraperitoneal (ip) injection of anti-CD4 (GK1.5 clone; 150µg/mouse in 100µl of PBS), anti-CD8 (53-6.72 clone; 500µg/mouse in 100µl of PBS) or anti-asialo GM1 (10µl/mice in 90µl of PBS). These different monoclonal antibodies were injected on days -2, -1 and 1 relative to the tumor injection and then weekly for six weeks. Mice were followed for survival for 100 days.

The depleting antibodies and their administration conditions were validated by flow cytometry analysis. Two doses of each antibody were injected in Balb/c mice (n=2 per group) and after 24 hours from the second injection, they were sacrificed and splenocytes were analyzed for $CD4^+$, $CD8^+$, NKT and NK cells.

3.7. Splenocytes and liver mononuclear cells (MNC) isolation

Three days after vaccine treatment, untreated and treated mice were sacrificed and spleen and liver were extracted. These lymphoid organs were processed as follows:

a) Spleen

Spleen was disaggregated in 5 ml of CM by mechanical procedures. Splenocytes were collected, filtered through a 70µm cell strainer (BD Falcon, Cultek S.L.U.) and centrifuged 5 minutes at 1500 rpm. Erythrocytes were lysed using an ammonium chloride solution (Pharmlyse, Pharmingen, BD Bioscience) during 3 minutes in agitation. Finally, cells were centrifuged again 5 min at 1500 rpm and maintained in 10 ml of CM until use.

b) Liver

Liver was cut in several pieces of 3mm, placed into 5ml of 0,2% collagenase IV solution (Sigma-Aldrich CO) and incubated 1 hour at 37ºC. Next, supernatant was collected and diluted 1:3 with CM. Cells were centrifuged 5 minutes at 1500 rpm and liver MNC were isolated using an Optiprep gradient (Axis-Shield). After this procedure, cells were collected, diluted 1:10 with CM, centrifuged 5 minutes at 1500 rpm, and maintained in 5 ml of CM until use.

3.8. Immunophenotyping

3.8.1. Characterization of cells by flow cytometry

The phenotypic characteristics of DCs, 4TOO, NKT, NK and T cells, as well as the expression of costimulatory molecules on DCs and NK-cell ligands on 4TOO, were analyzed by flow cytometry. Cells were washed with FACS Buffer containing PBS with 1% of Bovine Serum Albumin (BSA, Sigma-Aldrich CO) and 0,1% of sodium azide (Sigma-Aldrich CO), counted and distributed in FACS tubes ($1x10^6$ cells/50µl FACS buffer for each studied marker). Isotype antibodies (table 5) and specific antibodies (table 6) were added to the corresponding tubes at 1µg of antibody (Ab)/10^6 cells, and incubated at 4ºC for 30 minutes in the dark. Finally, cells were washed with FACS Buffer during 5 minutes at 1500 rpm and the pellet was resuspended in 400µl of FACS Buffer. Data were obtained using the MACSQuant Analyzer 10 (MiltenyiBiotec) and analyzed with the FlowJo software (Tree Star).

Isotype antibodies	Clone	Company
Rat IgG2a-FITC	RTK2758	BioLegend
Hamster IgG2-PE	B81-3	BD Pharmigen
Rat IgG2a-PE	R35-95	BD Pharmigen
Rat IgG1-PE	R3-34	BD Pharmigen

Table 5. Isotype antibodies used in cell phenotyping. Rat IgG2a, Hamster IgG2 and Rat IgG1 isotype antibodies, with the corresponding fluorochrome, were used. Cell clone and company are shown. FITC: fluorescein isothiocyanate; PE: phycoerythrin.

Cells	Anti-mouse antibodies	IgGisotype	Clone	Company
Dendritic cells	IA/IE (MHC class II)-FITC	Rat Igg2a,k	2G9	BD Pharmingen
	CD11c-APC[1]	Hamster IgG	N418	Miltenyi Biotec
	CD80 (B7-1)-PE	Hamster IgG2,k	16-10A1	BD Pharmingen
	CD86 (B7-2)-PE	Rat IgG2a,k	GL1	BD Pharmingen
	CD40-PE	Rat IgG2a,k	3/23	BD Pharmingen
	CD14-PE	Rat IgG1,k	RmC5-3	BD Pharmingen
	CD1d-FITC	Rat IgG2b,k	1B1	BD Pharmingen
4TOO tumor cells	B220/CD45R-APC-Vio770	Rat IgG2a,k	RA3-6B2	Miltenyi Biotec
	CD1d-FITC	Rat IgG2b,k	1B1	BD Pharmingen
	MHC class-I (H-2Kd)-eFluor450	IgG2a	SF1-1.1.1	eBioscience
	Rae-1α/β/γ-PE-Vio770	Recombinant human IgG1	REA578	Miltenyi Biotech
NKT cells	Tet-PBS57-PE			NIH Tetramer Core Facility
	Tet-unloaded-PE			NIH Tetramer Core Facility
	TCRβ-VioBlue	Recombinant human IgG1	REA318	Miltenyi Biotec
T cells	CD4-FITC	Rat IgG2b,k	GK1.5	Miltenyi Biotec
	CD4-FITC	Rat IgG2b, k	RM4-4	eBioscience
	CD8α-VioBlue	Rat IgG2a,k	53-6.7	Miltenyi Biotec
	CD8β- APC[1]	Rat IgG2b, k	H35-17.2	eBioscience
NK cells	NKp46-PE	Rat IgG2a, k	29A1.4.9	Miltenyi Biotec
	CD3- APC[1]	Hamster IgG1	145-2C11	Miltenyi Biotec

Table 6. Specific antibodies to classify DCs, 4TOO, NKT, NK and T cells. DCs were analyzed for the MHC-II, CD11c, CD80, CD86, CD40 and CD1d antibodies. CD14 was used to verify the absence of monocytes in the DC culture. B220/CD45R and CD1d antibodies were used to study 4TOO tumor cells and MHC class-I and Rae-1α/β/γ to study NK-cell ligands in those cells. T cells were classified as $CD4^+$ or $CD8^+$. NK cells were analyzed using the specific marker NKp46 and CD3. NKT cells were analyzed using a CD1d:α-GalCer analogue (PBS-57) loaded tetramer (Tet-PBS57), and Tet-unloaded was used as a negative control. Immunoglobulin isotype, cell clone and company are shown. FITC: fluorescein isothiocyanate; PE: phycoerythrin; APC[1]: allophycocyanin.

3.8.2. Detection of IFN-γ by flow cytometry: intracellular staining

Splenocytes were obtained from spleens of untreated and treated mice three days after vaccination. To identify NKT cells, splenocytes were stained using the anti-TCRβ and Tet-PBS57 antibodies and, in the case of NK cells, the anti-CD3 and anti-NKp46 antibodies were used to identify the specific population (Table 5). To detect intracellular IFN-γ in NK and NKT cells, splenocytes were stained as follows: cells were washed with FACS Buffer 5 minutes at 1500 rpm and fixed with 1,5% formaldehyde solution in PBS during 10 minutes in the dark at room temperature. After that, splenocytes were washed with FACS Buffer and permeabilized using 0,5% Tween-20 solution in PBS (permeabilization buffer) during 15 minutes in the dark at room temperature, with agitation. Cells were resuspended in permeabilization buffer after 5 minutes of centrifugation at 1500 rpm, with IFN-γ antibody (1µg/10^6 cells) (table 7). In this case, the IFN-γ antibody was incubated 30 minutes in the dark at room temperature. Finally, cells were washed with permeabilization solution and resuspended with FACS Buffer. Data were obtained by MACSQuant Analyzer 10 and analyzed by FlowJo software.

Anti-mouse antibodies	IgG isotype	Clone	Company
IFN-γ-APC[1]	Rat IgG1,k	AN.18.17.24	MiltenyiBiotec

Table 7. Specific antibody to detect intracellular IFN-γ. Intracellular staining of IFN-γ was done using the IFN-γ-APC antibody. IgGisotype, cell clone and company are shown. APC[1]: allophycocyanin.

To analyze IFN-γ secreting $CD4^+$ and $CD8^+$ T cells, mononuclear cells obtained from untreated and treated mice were cultured in a flat bottom cell culture plate (24 wells) for 24 hours (2x10^6 cells/well in 2ml of CM) under different conditions (Table 8). Brefeldin A was added four hours before IFN-γ detection to block cytokine secretion. Splenocytes in culture were collected, counted and stained following using anti-CD4 and anti-CD8 antibodies to detect T cells (Table 5). After that, cells were washed with FACS Buffer 5 minutes at 1500 rpm. The intracellular IFN-γ staining was done as described above.

Condition	Description	Time of stimulation
No stimulus	Splenocytes plated with no stimulus	24h of culture
PMAi (positive control)	Cell with phorbolmyristate acetate and ionomycin at 5mg/ml (PMAi, both from Sigma-Aldrich)	
4TOO coculture	Cells cocultured with irradiated 4TOO tumor cells (2:1 efector/target ratio)	

Table 8. Splenocyte culture conditions to study IFN-γ production.

3.9. Serum cytokine detection

Twelve hours after vaccine treatment, blood samples of treated and untreated mice were collected using capillary collecting tubes (Microvette CB300 Z, Sarstedt). Samples were incubated 30 minutes at room temperature to allow blood to clot, and centrifuged 5 minutes at 10.000x*g*. Serum was collected carefully and stored at -80ºC until further use.

Cytokine concentration of IFN-γ, IL-12p70, IL-4, IL-5, IL-6, TNF-α, and IL-17 was analyzed using the ProcartaPlex Mouse Essential Th1/Th2 cytokine panel kit (eBioscience), by the Luminex technology. The kit consists of several types of beads with different size that allows the detection of different cytokines. Luminex quantifies cytokines by PE detection and size differentiation. The standard curve provided with the kit allows the correlation of the mean fluorescence intensity (MFI) with the cytokine concentration (in pg/ml) in the sample tested. The procedure works as follows:

a) Capture target analytes: 25µl of serum sample (analyte) were incubated with capture beads coated with target-specific antibodies for 2 hours in agitation

(500 rpm) at room temperature (Fig.17A). Plate was washed 3 times following the manufacturer instructions.

a) Detect capture analyte: The beads-analyte complexes were incubated with biotinylated analyte-specific detection antibodies for 30 minutes in agitation (500 rpm) (Fig.17B). Plate was washed 3 times as in point 1.

b) Label detection: For analyte quantification, samples were incubated with a fluorescent detection label (Streptavidin-PE) for 30 minutes in agitation (500 rpm) (Fig.17C). Plate was washed 3 times as in point 1.

c) Prepare for Luminex assay: Reading Buffer was added and plate was shacked 5 minutes at 500 rpm prior to reading on Luminex instrument.

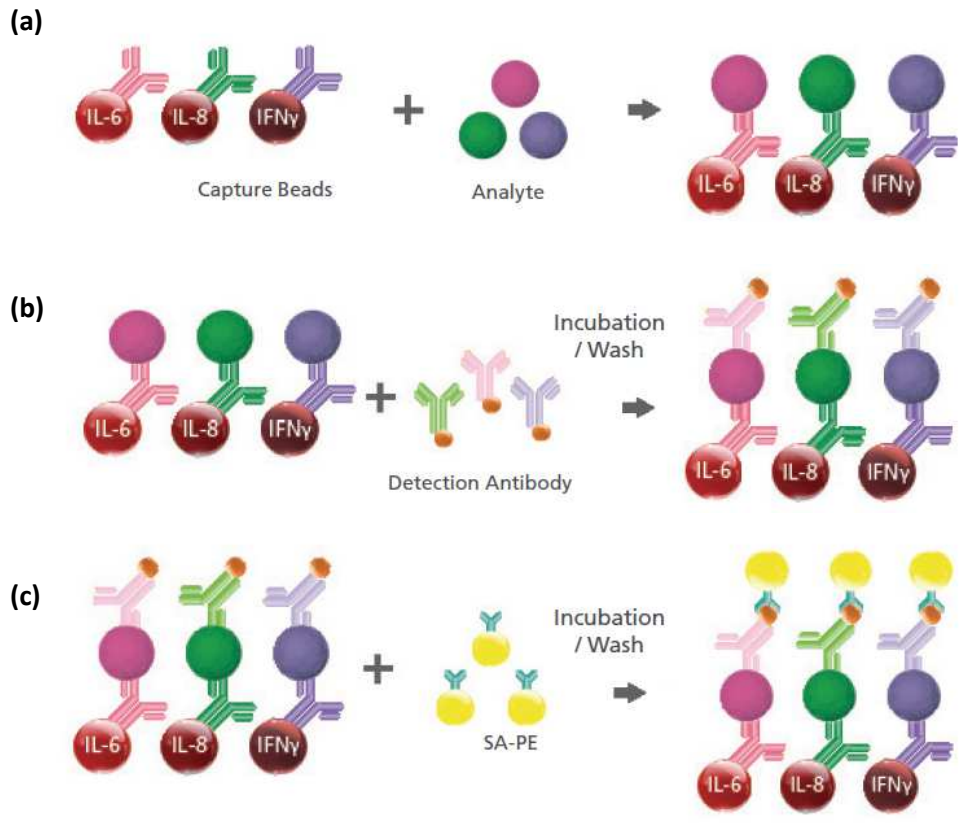

Figure 17. Serum cytokine detection by Luminex technology. (a) Capture beads were incubated with the analyte generating capture beads-analyte complexes. **(b)** This complexes were incubated with the biotinylated detection antibody producing detection complexes. **(c)** These detection complexes were mixed with a fluorescent detection label prior to analyze in a Luminex device. SA: Streptavidin; PE: phycoerythrin.

3.10. Indirect immunofluorescence assay for detection of serum IgG antibodies against B-cell lymphoma

Fourteen days after vaccine treatment, blood samples of treated and untreated mice were collected using capillary collecting tubes. Samples were incubated 30 minutes at room temperature to allow blood to clot, and centrifuged 5 minutes at 10.000x*g*. The levels of specific-IgG antibodies against tumor cells in the mice serum samples were detected using an indirect immunofluorescence assay. To do that, 3µl of each serum sample was incubated with $3x10^5$ 4TOO tumor cells, in 50µl of FACS Buffer during 30 minutes at 4ºC. Following two washes with FACS Buffer during 5 minutes at 1500 rpm, cells were incubated with a PE-conjugated polyclonal anti-mouse IgG antibody (0,125µg/10^6 cells) in 100µl of FACS Buffer, during 30 minutes at 4ºC in the dark. Finally, cells were washed with FACS Buffer during 5 minutes at 1500 rpm and were resuspended in 200µl of FACS Buffer. Data were obtained using the MACSQuant Analyzer 10 and analyzed with the FlowJo software. Negative controls include 4TOO tumor cells with no serum incubation, A20 tumor cells incubated with the different serums and 4TOO/A20 cells incubated with serum from naïve mice. Relative IgG levels were obtained dividing the mean fluorescence intensity (MFI) for treated and untreated serums by the MFI for naïve serum control.

Anti-mouse antibodies	IgG isotype	Clone	Company
F(ab')2 anti-IgG-PE	Goat IgG	Policlonal	eBioscience

Table 9. Specific polyclonal antibody to detect mouse IgG. The detection of mouse IgG in serum samples was done using the polyclonal F(ab')2 anti-mouse IgG antibody conjugated with PE. IgG isotype, cell clone and company are shown. PE: phycoerythrin.

3.11. Statistical analysis

Results are expressed as the mean ± SEM. Kaplan-Meier plots were used to analyze mice survival and the differences between survival curves were assessed by the log-rank test. An unpaired *t* test was done to analyze the differences between experimental groups which have only two values per group. For all other data, Mann-Whitney test was assessed to find statistical differences. All statistical analysis and graphics were performed using GraphPad Prism 5 (Graph Pad Software Inc.). P values <0,05 were considered significant.

4. RESULTS

4.1. The 4TOO and A20 B-cell lymphoma mouse model

Previous to study the antitumor effect of any treatment, it is necessary to generate a tumor mouse model, which allows the evaluation of the efficacy of antitumor treatments by following mice survival and tumor growth. These mice models can be established by the injection of tumor cell lines and the resulting tumor model had to be not only lethal but also slow enough to allow for the best control of mice survival. In our case, two tumor models were established using the 4TOO and the A20 mature B-cell lymphoma cell lines.

In order to determine the optimal dose to generate the B-cell lymphoma mouse model, mice were injected with two different doses of 4TOO tumor cells ($4x10^5$ or $5,5x10^5$ cells/mouse, iv) or A20 tumor cells ($1x10^6$ or $2x10^6$ cells/mouse, sc) (Figure 18).

Mice injected with the two different doses of 4TOO cells exhibited the same survival time (48 days in both doses). In consequence, the selected dose for the survival experiments using this tumor cell line was $4x10^5$ cells per mouse. In the case of A20 B-cell lymphoma model, both doses also exhibited approximately the same survival time (41 days for the lower dose vs. 43 days for the higher dose), but the higher dose was too aggressive and caused an abrupt descent of mice survival. To assure the best monitoring of mice survival in the therapeutic experiments, the lower dose of A20 tumor cells ($1x10^6$) was selected to establish the mice model.

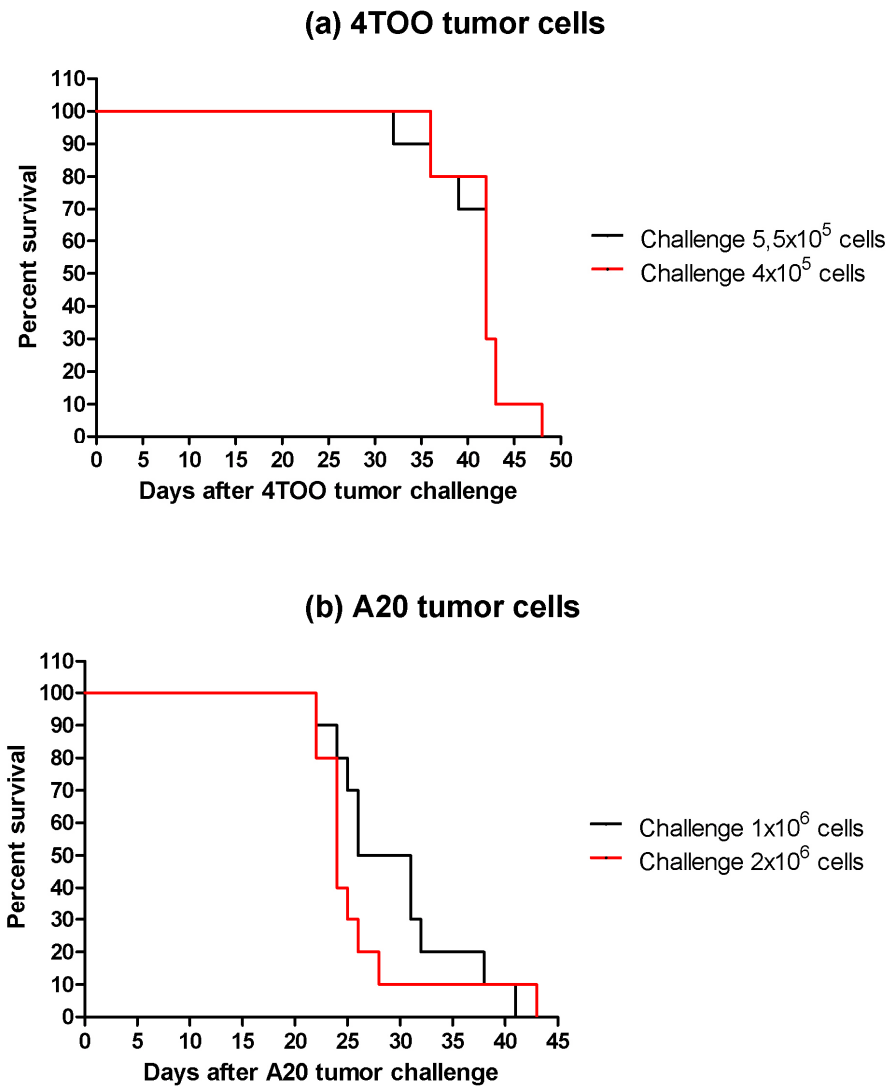

Figure 18. Survival of mice injected with two different doses of 4TOO and A20 tumor cells. **(a)**Two groups of mice (n=10 per group) were injected with different doses of 4TOO tumor cells ($4x10^5$ and $5,5x10^5$ cells/mice, iv). Mice were followed for survival for 50 days. **(b)**Two groups of mice (n=10 per group) were injected with different doses of A20 tumor cells ($1x10^6$ and $2x10^6$ cells/mice, sc). Mice were followed for survival for 45 days.

4.2. Therapeutic treatment against B-cell lymphoma using the Mix+GalCer vaccine

To activate NKT cells, we used the potent NKT-cell activator α-GalCer as a principal component of the therapeutic vaccine. Moreover, it was important to assure that the α-GalCer had an appropriate presentation to activate NKT cells. For this purpose, we assessed the importance of including an APC (DCs or B cells) in the vaccine to enhance the antigen presentation. In addition, we thought to include tumor cells in the vaccine to provide the largest amount of peptide and glycolipid tumor-specific antigens that could be presented to activate T and NKT cells.

4.2.1. Vaccine generation: DCs and tumor cells phenotyping

First of all, we evaluated the APC candidates to include in the vaccine that could guarantee the better α-GalCer presentation. It is known that DCs and B cells have the ability to present different types of antigens, including glycolipid antigens presented by CD1d molecule to NKT cells. Due to the importance of CD1d receptor in α-GalCer presentation and, in consequence, in NKT-cell activation, the expression of this receptor was studied in DCs and also in 4TOO tumor cells, as they are B lymphocytes and they can act as an APC as well.

In order to study the CD1d expression in DCs, they were generated *ex vivo* using bone marrow from Balb/c mice. To identify DCs, the expression of both MHC-II and CD11c molecules were used as the principle markers for this cell type. The analysis of CD1d expression levels in the DC population showed that this molecule was highly expressed on DCs (93,7%), suggesting that they were a good candidate to use in the vaccine as an APC (Figure 19a). The tumor cells 4TOO were also analyzed for the expression levels of CD1d receptor (Figure 19b) and we observed that these tumor cells expressed low levels of CD1d (17,6%). These results demonstrated that DCs were the best option to include in the vaccine as an

α-GalCer presenting cell in comparison to 4TOO tumor cells, which were use in the vaccine only as a tumor-antigen source.

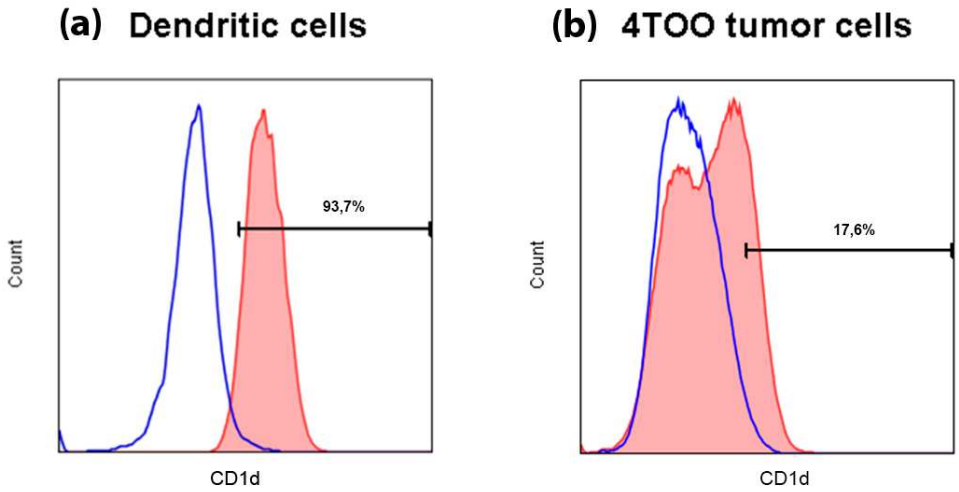

Figure 19. Expression of CD1d receptor in DCs and 4TOO tumor cells. MHC-II$^+$ CD11c$^+$ cells, corresponding to DC population **(a)**, and B220$^+$ cells which are the 4TOO tumor cells **(b)**, were analyzed for CD1d expression by flow cytometry using the CD1d-FITC antibody.

In the process of generate a DC-based vaccine, it is important to know the maturation status of DCs use in the treatment after their culture (Figure 20a), because it determines the antigen presentation and T-cell stimulation capacity. To that purpose, the expression levels of the main costimulatory molecules in DCs, which include CD40, CD80 and CD86, were analyzed. DCs generated from bone marrow progenitors (Figure 20b) presented a moderate expression of CD40 molecule (58% of DCs; Figure 20c) and a high expression of CD80 and CD86 (99,2% and 84,8% of DCs, respectively; Figure 20d-e), corresponding to a semi-mature DC population.

In addition of DC analysis, it was necessary to analyze if other cell types were present at the end of the cell culture. Thereby, the presence of monocytes, T cells and B cells were checked out in our DC culture previous to generate the Mix+GalCer vaccine. After this analysis, we found that there were neither CD4$^+$

and CD8^{+} T cells (0%; Figure 20f), B cells represented a minor population in the culture (4,78% of total cells; Figure 20g) and there was no monocytes (0,97% of total cells; Figure 20h).

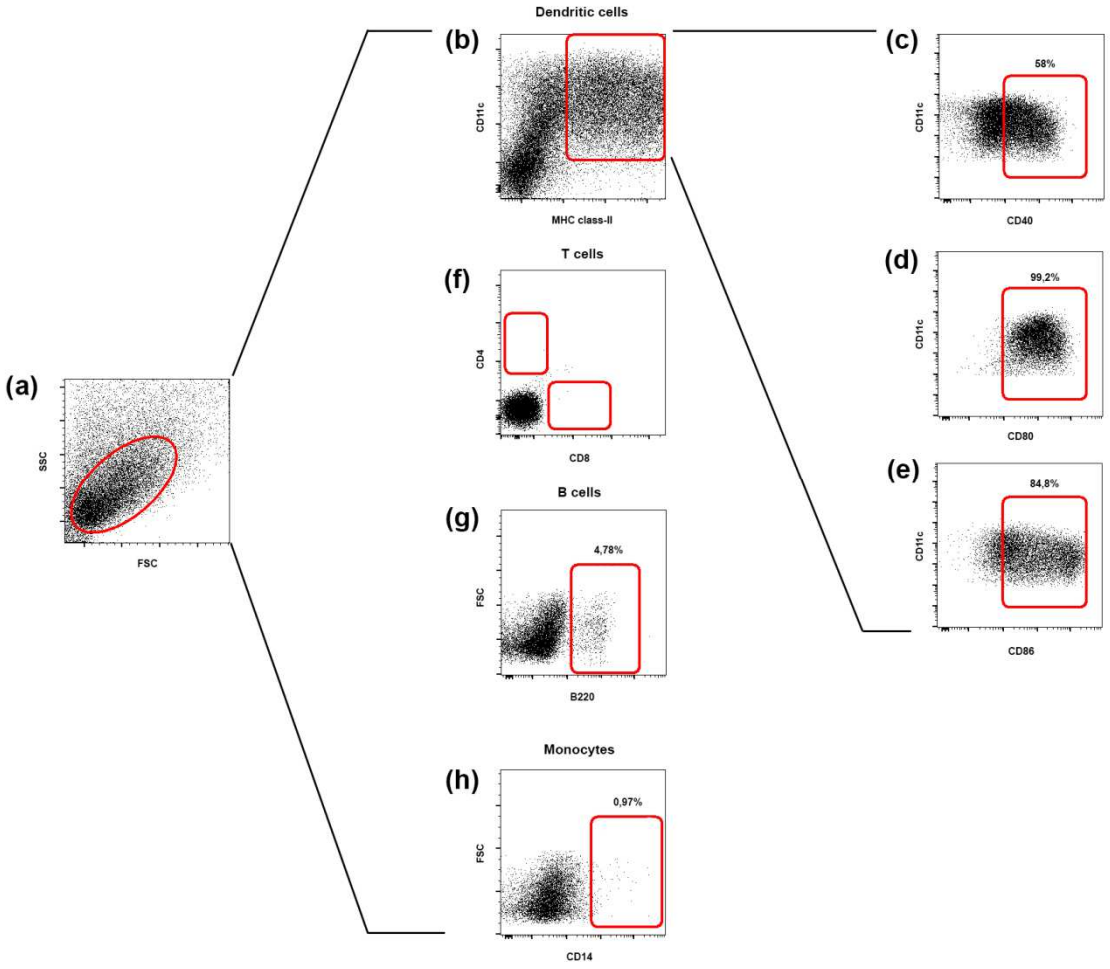

Figure 20. DC phenotyping after bone marrow culture. (a) Bone marrow from Balb/c mice was cultured 7 days with rmGM-CSF (10ng/ml) and the cell composition was analyzed by flow cytometry. **(b)** DCs were gated using MHCII-FITC and CD11c-APC antibodies. The costimulatory molecules on DCs were analyzed using CD40-PE **(c)**, CD80-PE **(d)** and CD86-PE **(e)** antibodies. For the detection of CD4 and CD8 T cells **(f)**, B cells **(g)** and monocytes **(h)**, the CD4-FITC and CD8-VioBlue, B220-APCVio770 and CD14-PE, respectively, were used.

4.2.1.1. Effect of α-GalCer ligation in DC maturation status

The variations in DC maturation status have a direct implication in the capacity of these cells to present antigens and to stimulate T and NKT cell activation. In our case, the semi-mature conditions of generated DCs were the appropriate status to guarantee a good antigen uptake and the best primary T and NKT-cell stimulation.

The glycolipid antigen that we used as NKT-cell activator was α-GalCer and it was the main component in the vaccine to stimulate NKT cells. This glycolipid antigen is presented by CD1d molecules on DCs, so we decided to study the impact of *in vitro* α-GalCer loading in DC maturation. To analyze the possible changes in DC maturation status after α-GalCer ligation, the cells produced after the 7-day culture were incubated with the NKT cell agonist α-GalCer 24 hours. After that, the expression levels of CD40, CD80 and CD86 were checked and we observed that DCs pulsed with the α-GalCer *in vitro* did not present any change in the expression of these costimulatory molecules (Figure 21). Hence, these results showed that the *in vitro* α-GalCer ligation did not generate any alteration in DCs by itself.

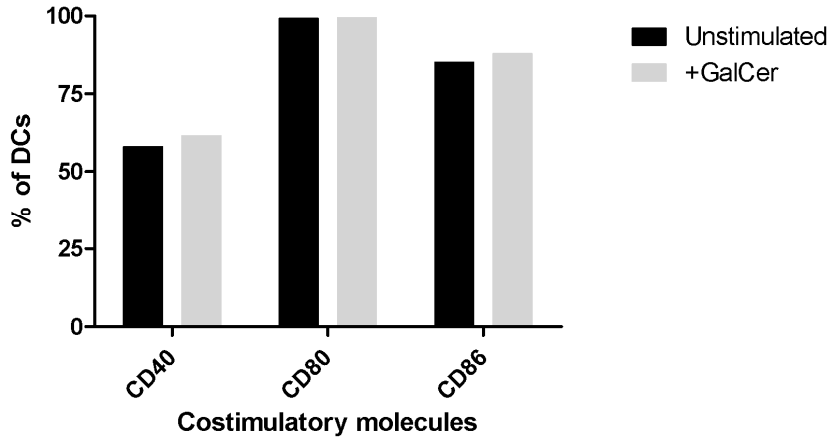

Figure 21. Expression levels of costimulatory molecules in DCs after α-GalCer stimulation *in vitro*. DCs were incubated with α-GalCer (100ng/ml) during the last 24h of the culture. Costimulatory molecules were analyzed by flow cytometry using CD40-PE, CD80-PE and CD86-PE antibodies, both in unstimulated and α-GalCer pulsed DCs, gated as MHC-II$^+$ CD11c$^+$ cells.

4.2.2. *In vivo* antitumor effect of Mix+GalCer vaccine

We generated the therapeutic vaccine by mixing DCs as the better APC, irradiated 4TOO tumor cells, as the better tumor antigen source, and the NKT-cell agonist α-GalCer as a potent NKT-cell activator. The mixture of irradiated tumor cells and DCs was called “Mix” and their combination with α-GalCer was named Mix+GalCer vaccine. All of the components of the vaccine were mixed previous to injection into the 4TOO mice model.

4.2.2.1. Three-vaccination treatment using Mix+GalCer vaccine

It is known that the administration of a therapeutic vaccine followed by, at least, two identical doses few days later, boosts the immune response and can improve the treatment efficacy. Thus, the *in vivo* therapeutic treatment consisted in the administration of Mix+GalCer vaccine on days 2, 6 and 10 after 4TOO tumor challenge (Figure 22). In addition, other vaccine combinations were assessed to observe the contribution of each vaccine component in the antitumor effect. These control combinations included α-GalCer alone, DCs alone, DCs with irradiated tumor cells without α-GalCer, and DCs with α-GalCer, all injected on days 2, 6 and 10 relative to tumor challenge as well. All groups of mice were followed for survival to observe the antitumor efficacy of all treatment. Taking into account that untreated mice died between days 30-45 after tumor challenge, we considered that those mice which survived at least 100 days after tumor injection were tumor-free because they have developed an effective antitumor immune response. As it is shown in figure 6, mice treated with DCs alone, DCs with tumor cells without α-GalCer and α-GalCer alone did not present any antitumor effect (0%, 0%, 10%, respectively). The vaccine consisting on DCs and α-GalCer (DC+GalCer) exhibited 50% antitumor efficacy ($p<0,001$), suggesting that DCs played an important role in α-GalCer presentation to host NKT cells. However, the most effective combination was the vaccine Mix+GalCer, which eradicated the B-cell lymphoma in all treated mice (100% antitumor efficacy, $p<0,001$). This data

demonstrated the antitumor potency of NKT-cell activation against B-cell lymphoma, suggesting a critical implication of the tumor antigens in the vaccine itself, which helps to improve the antitumor immune response.

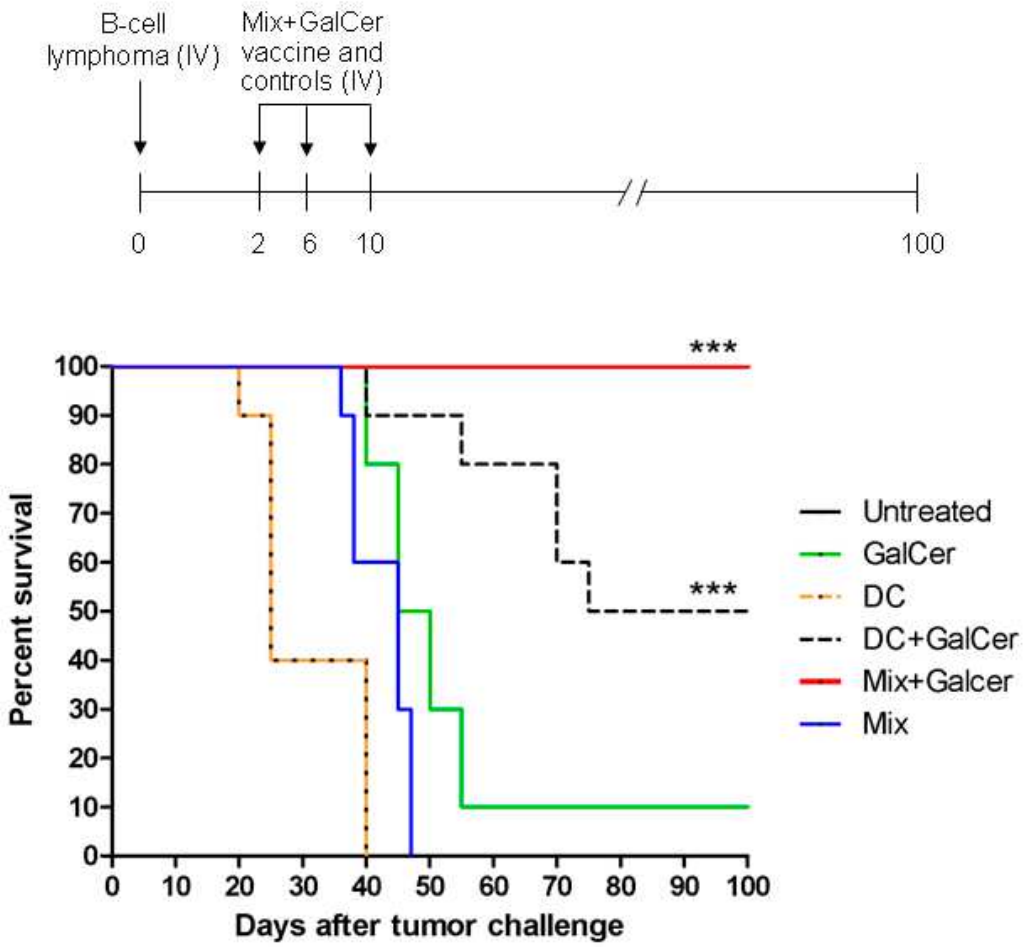

Figure 22. Survival of mice treated with three Mix+GalCer vaccinations. Mice (n=10) were injected with 4TOO B-cell lymphoma ($4x10^5$ cells/mice in 100µl of PBS, iv) and then were treated with the therapeutic vaccine Mix+GalCer (iv) on days 2, 6 and 10 after 4TOO tumor inoculation. Untreated mice received 100 µl of PBS iv. Mice were followed up for survival for 100 days. ***p<0,001.

Furthermore, NKT cells were analyzed in the spleen three days after each Mix+GalCer vaccine injection, which was the most effective treatment, to find

whether Mix+GalCer treatment could induce an increase of NKT cells. The results of this study, shown in figure 23, demonstrated a significant increase of NKT-cell percentage in mice treated with Mix+GalCer vaccine after the first vaccination, in comparison with untreated mice (7,52 ± 0,35% vs. 2,62 ± 0,49%, p=0,0001). Interestingly, the second dose of Mix+GalCer vaccine led to a significant decrease of NKT cells in comparison with the first vaccination (7,52 ± 0,35% vs. 2,61 ± 0,61%, p=0,002), showing the same proportion of NKT cells as the untreated group. The NKT-cell percentage was further reduced after the third Mix+GalCer administration and treated mice exhibited a significant decrease compared to the first dose (0,94 ± 0,06% vs. 7,52 ± 0,35% vs, p=0,0002) and untreated mice (0,94 ± 0,06% vs. 2,62 ± 0,49%, p=0,02).

These results demonstrated a progressive decrease of NKT cells in spleen after the recurrent administration of a vaccine containing α-GalCer. The lack of NKT cell expansion could be a signal of no activation, including the possibility of NKT cell loss.

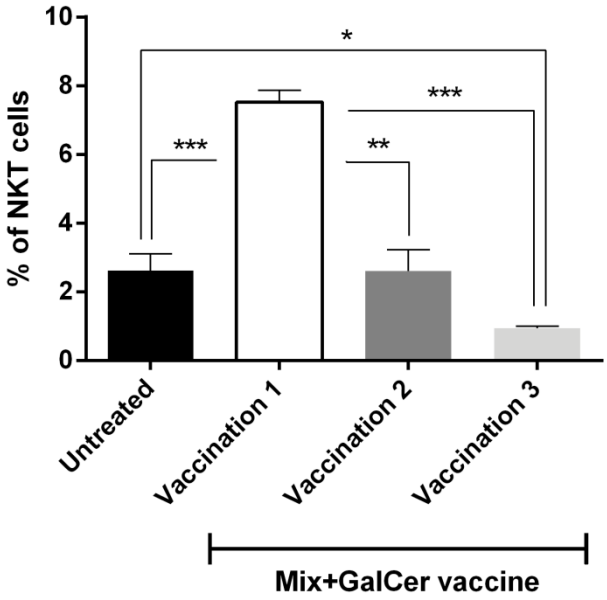

Figure 23. NKT cell expansion after each Mix+GalCer vaccination. Splenocytes of control and treated mice (n=4 per group) were obtained three days after each vaccination. The analysis of the NKT cells was carried out by flow cytometry. *p<0,05, **p<0,005, ***p<0,0005.

4.2.2.2. Treatment with a single-dose of Mix+GalCer vaccine

The decrease of NKT cell expansion observed after the second and third doses of Mix+GalCer treatment suggested the possibility of omitting the boost strategy and test the *in vivo* potency of a single-dose Mix+GalCer treatment. Using this new approach, we wanted to know whether a single dose of Mix+GalCer vaccine had a similar antitumor effect as the three-vaccination model.

With this purpose, mice were treated with the Mix+GalCer vaccine two days after tumor challenge and, additionally, different groups of mice were injected once with the other vaccine controls, including α-GalCer alone, DCs alone, DCs with irradiated tumor cells without α-GalCer and DCs with α-GalCer (Figure 24). Mice treated with a single dose of DCs alone, DCs with tumor cells without the α-GalCer and α-GalCer alone did not show any antitumor effect (0%, 0%, 10%, respectively), whereas the vaccine DC+GalCer exhibited 50% antitumor efficacy ($p<0,001$) Interestingly, the single dose of the Mix+GalCer vaccine was equally efficient in eradicating B-cell lymphoma, showing the 100% of antitumor effect ($p<0,001$).

These results demonstrated the potent antitumor efficacy of our therapeutic vaccine without boost and, importantly, it allowed a simpler and easier procedure.

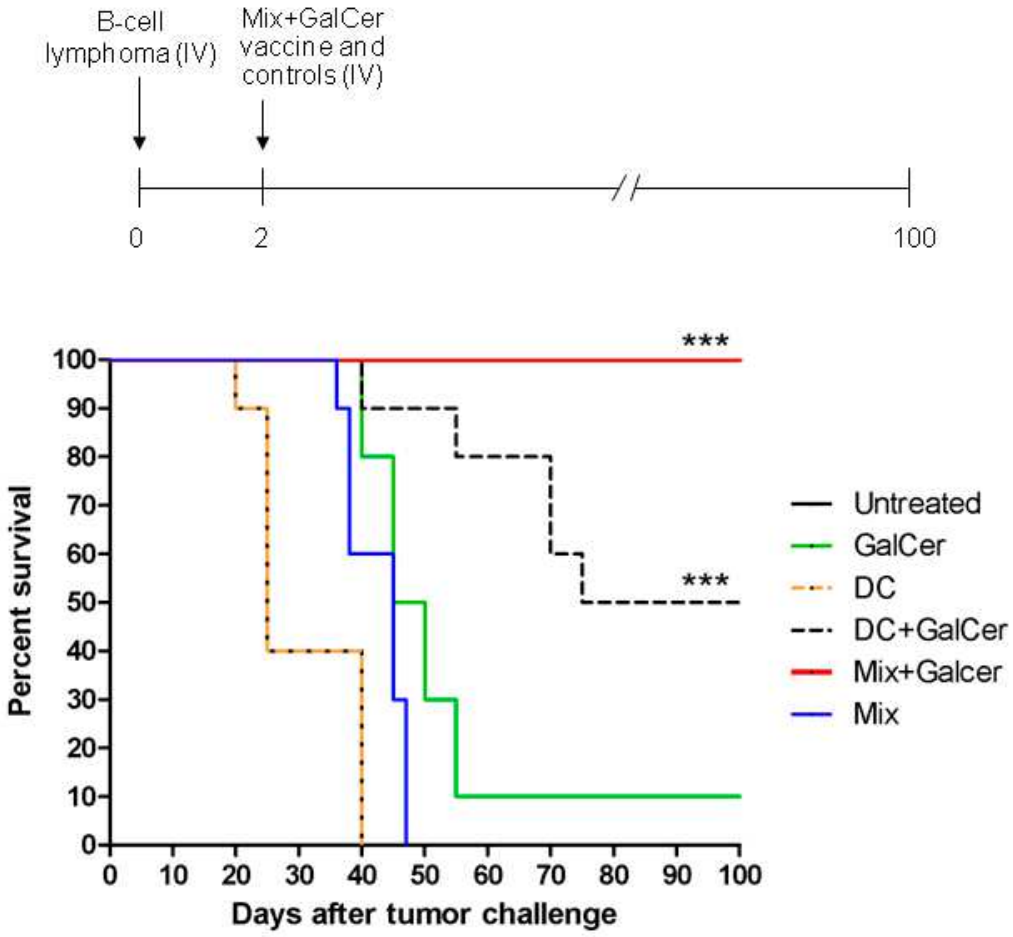

Figure 24. Survival of mice treated with a single dose of Mix+GalCer vaccine. Mice (n=10 per group) were injected with 4TOO tumor cells ($4x10^5$ cells/mice in 100µl of PBS, iv) and were treated 2 days after tumor injection with a single dose of Mix+GalCer vaccine (iv) or control treatments, including α-GalCer alone, DC alone, DC with α-GalCer and DC with irradiated tumor cells without α-GalCer. Untreated mice were injected with 100 µl of PBS iv. Mice were followed up for survival for 100 days. ***p<0,0001.

4.2.2.3. Adaptive memory immunity induced by Mix+GalCer treatment

When we observed the 100% antitumor efficacy of Mix+GalCer vaccine, we wanted to analyze whether Mix+GalCer treated mice could generate a memory immune response. To study that, surviving mice after the Mix+GalCer treatment were challenged again with the 4TOO tumor cell line at the same dose of the first lethal injection ($4x10^5$ tumor cells/mouse, iv). Interestingly, 83% of mice treated

with Mix+GalCer vaccine survived after this second tumor inoculation, suggesting that this treatment was able to induce effective adaptive memory immunity against B-cell lymphoma (Figure 25).

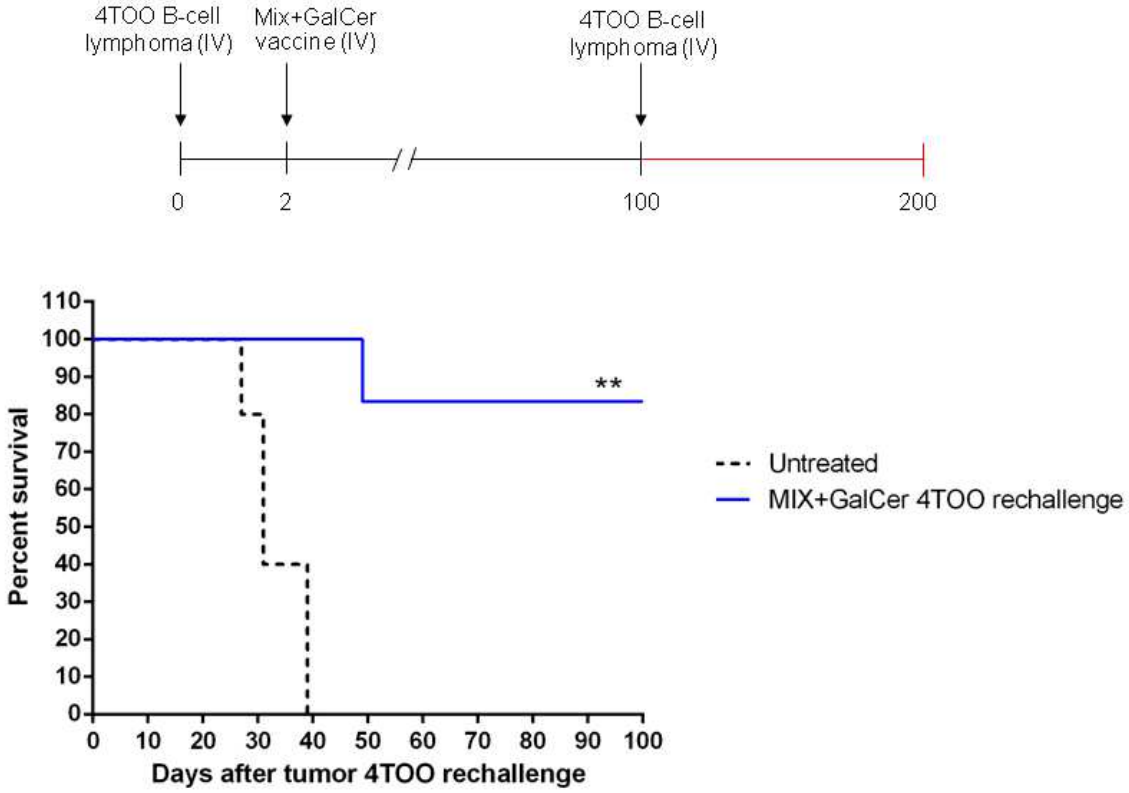

Figure 25. Survival of Mix+GalCer treated mice after 4TOO tumor rechallenge. Mice that survived from the first 4TOO tumor challenge (n=6) were reinjected with 4TOO tumor cells again at the same dose ($4x10^5$ cells/mice in 100µl of PBS, iv) and were followed up for survival for 100 days after tumor rechallenge. Untreated age-matched mice were injected with $4x10^5$ 4TOO tumor cells in 100µl of PBS, iv. **p=0,005.

4.2.2.4. Specificity of the antitumor immune response induced by Mix+GalCer treatment

In addition to memory immunity analysis, the specificity of the antitumor immune response induced by the therapeutic vaccine was also studied. In this case, surviving mice treated with Mix+GalCer vaccine were challenged with a singeneic

tumor cell line, the A20 B-cell lymphoma, 100 days after the 4TOO tumor injection. The results presented in Figure 26 showed that all surviving Mix+GalCer treated mice injected with A20 tumor cells died, suggesting that the immunity induced by Mix+GalCer treatment was 4TOO-specific.

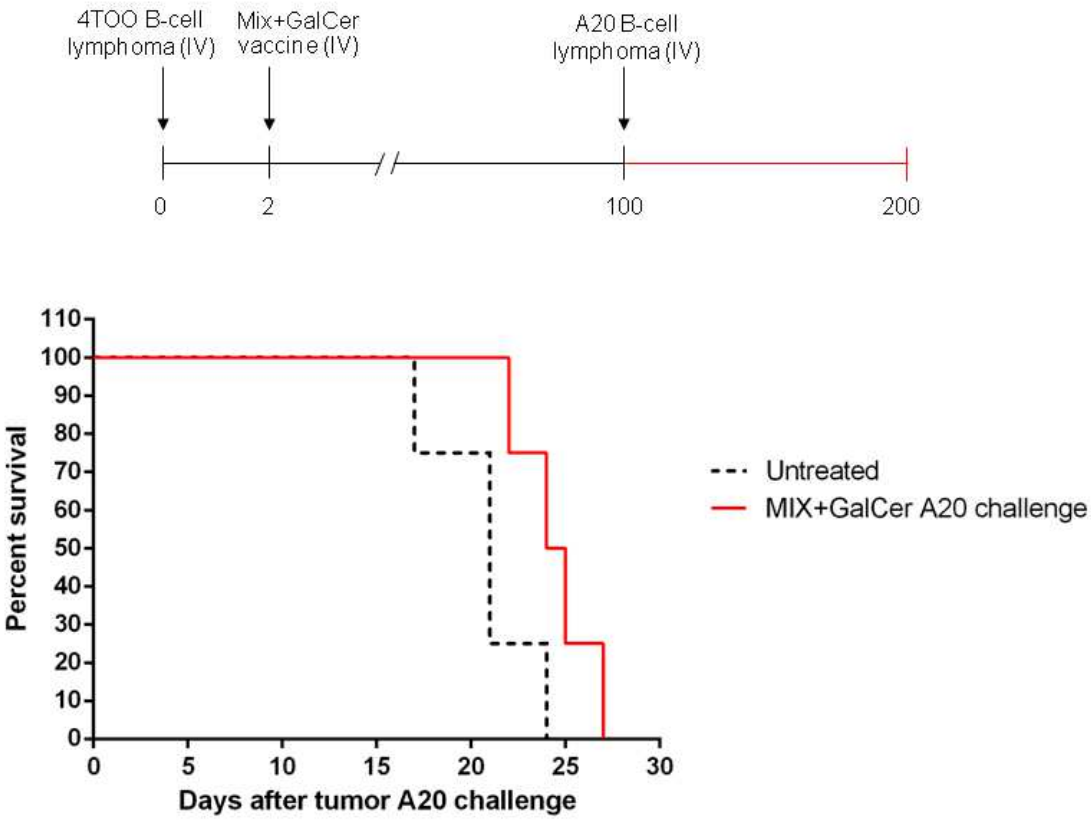

Figure 26. Survival of Mix+GalCer treated mice after the A20 B-cell lymphoma challenge. Mice that survived after the first 4TOO tumor challenge (n=4) were injected with a different tumor cell line, the syngeneic A20 B-cell lymphoma ($1x10^6$ cells/mouse in 100µl of PBS, sc). Untreated age-matched mice were injected with the same dose of A20 tumor cells in 100µl of PBS, iv. Mice were followed up for survival for 30 days from the A20 tumor challenge.

4.2.3. Effector cells and cytokines involved in the antitumor immune response induced by Mix+GalCer treatment

4.2.3.1. Cell expansion after single-dose Mix+GalCer treatment

The NKT cell expansion after a single dose of Mix+GalCer vaccine was studied in spleen of Mix+GalCer treated mice and the other control groups including untreated mice, Mix and α-GalCer treated mice. This analysis showed a significant increase of NKT-cell percentage in α-GalCer treated mice in comparison with the untreated group (2,86 ± 0,06% vs. 1,5 ± 0,05%; p=0,0002) and Mix treated mice (2,86 ± 0,06% vs. 1,27 ± 0,08; p=0,0009), interestingly, the NKT-cell percentage was further increased in the case of Mix+GalCer treated group, in comparison with α-GalCer treated group (6,56 ± 0,46% vs. 2,86 ± 0,06%; p=0,005), Mix treated group (6,56 ± 0,46% vs. 1,27 ± 0,08; p=0,0001) and untreated group (6,56 ± 0,46% vs. 1,5 ± 0,05%; p<0,0001) (Figure 27a-b).

This data suggested that vaccines that include α-GalCer were able to induce an increment of NKT cells in treated mice, being Mix+GalCer the treatment which induced the strongest NKT-cell increment.

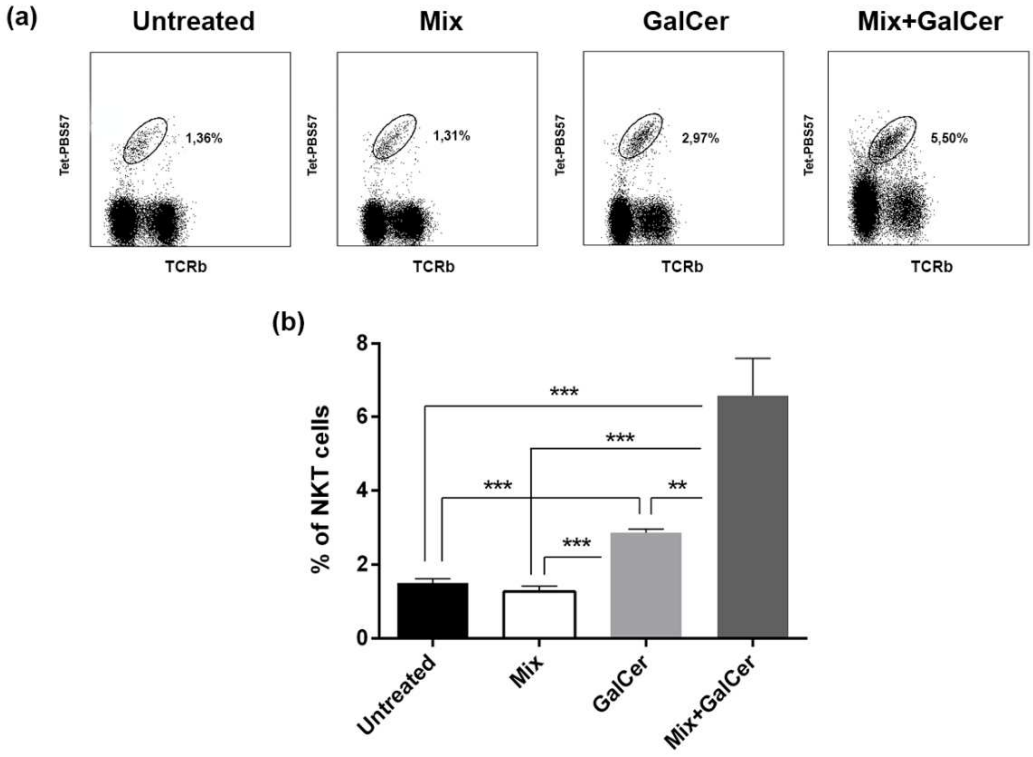

Figure 27. NKT cell percentage after the single-dose Mix+GalCer treatment. NKT cell number was analyzed three days after treatment in spleen of untreated mice and Mix, α-GalCer and Mix+GalCer treated mice, by flow cytometry (n=4 per group). NKT cells were gated as TCRβ^{+} and Tet-PBS57^{+} cells. **(a)** A representative plot of NKT cells and **(b)** the NKT-cell percentage are shown. **p<0,005, ***p<0,0005.

We also studied the number of NK cells in spleen of untreated mice and Mix, α-GalCer and Mix+GalCer treated mice in order to know if the therapeutic vaccine could also induce an increase in the total number of this cell type. As Figure 28a-b shown, Mix+GalCer treated mice exhibited a significant increase in NK-cell percentage compared to untreated group (5,21 ± 0,28% vs. 3,25 ± 0,21%; p=0,01) and Mix treated group (5,21 ± 0,28% vs. 2,68 ± 0,29%; p=0,003). In addition, Mix+GalCer group exhibited a trend to NK-cell increment in comparison to α-GalCer treated mice (5,21 ± 0,28% vs. 3,69 ± 0,71%; p=0,06). These results suggested that Mix+GalCer vaccine could induce an increase of NK cells in treated mice.

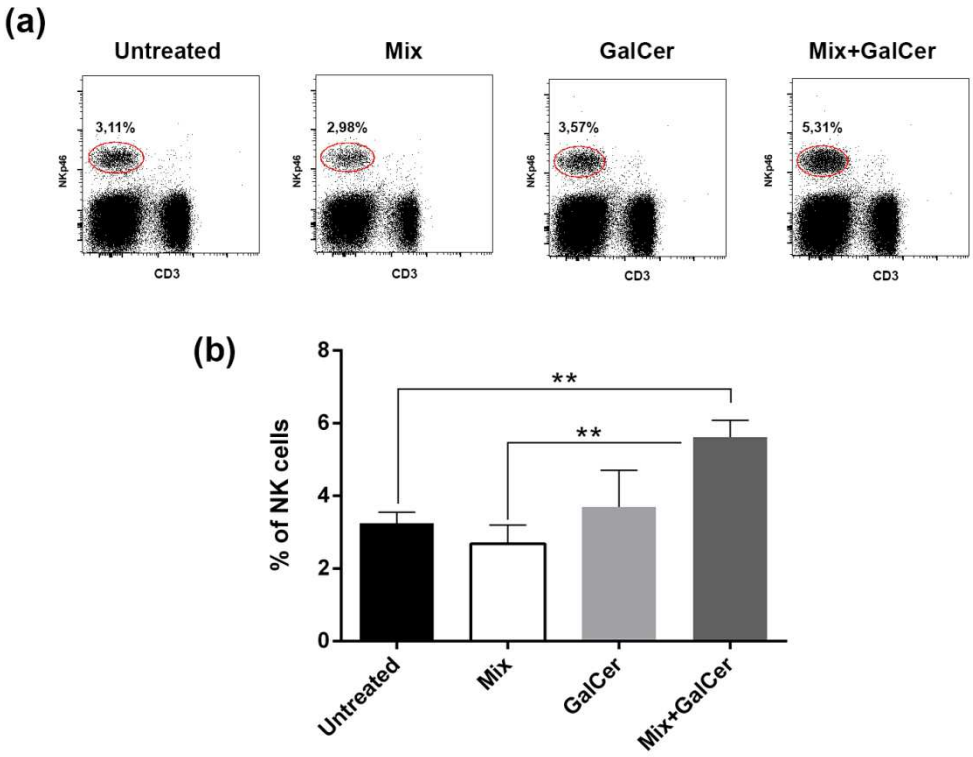

Figure 28. NK cell expansion after the single-dose of Mix+GalCer treatment. NK cell number was analyzed three days after treatment in spleen of untreated mice and Mix, α-GalCer and Mix+GalCer treated mice, by flow cytometry (n=2 per group). NK cells were gated as $CD3^-$ and $NKp46^+$ cells. **(a)** A representative plot of NK cells and **(b)** the NK-cell percentage are shown. **p<0,005.

When $CD4^+$ T cells were studied, a reduction in the percentage of these cells were observed in α-GalCer treated mice in contrast to untreated group (14,55 ± 0,25% vs. 22,75 ± 0,55%; p=0,005) and Mix group (25,15 ± 1,05% vs. 22,75 ± 0,55%; p=0,01), as this reduction was also noticed in Mix+GalCer group compared with untreated mice (13,80 ± 0,49% vs. 22,75 ± 0,55%; p=0,001) and Mix treated mice (13,80 ± 0,49% vs. 25,15 ± 1,05%; p=0,001) (Figure 29a). In the case of $CD8^+$ T cells, we observed a minor decrease of the percentage in α-GalCer and Mix+GalCer groups relative to Mix treated mice (10,95 ± 0,25% vs. 12,80 ± 0,20%; p=0,03, and 11,23 ± 0,26% vs. 12,80 ± 0,20%; p=0,02, respectively), but it was similar to percent levels observed in untreated mice (Figure 29c). These results suggested that treatments containing α-GalCer induced a decrease in the proportion of $CD4^+$ T cells, with no modification of $CD8^+$ T-cell percentage.

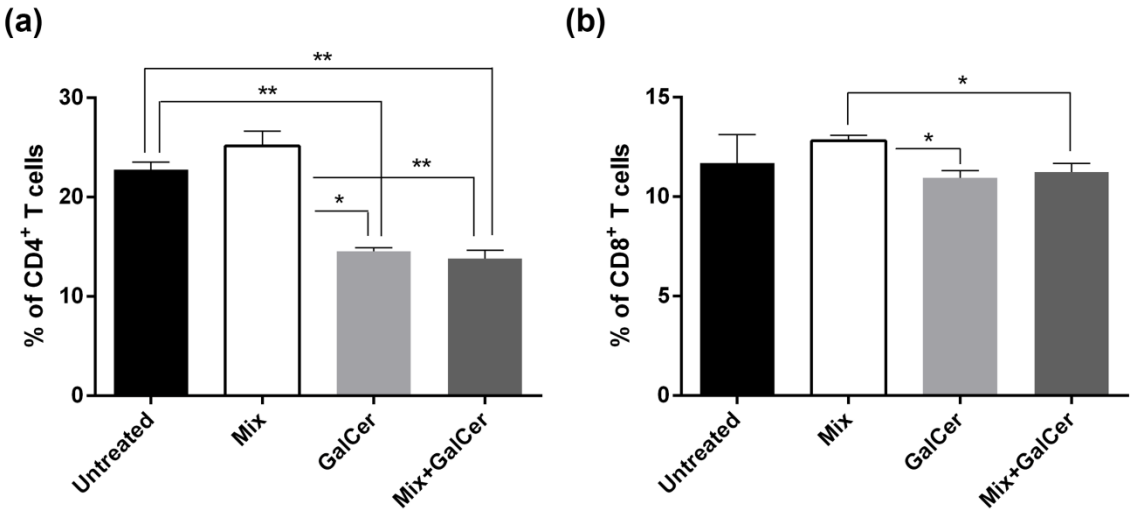

Figure 29. CD4$^+$ and CD8$^+$ T-cell percentage after the single-dose of Mix+GalCer treatment. CD4$^+$ and CD8$^+$ T-cell number was analyzed by flow cytometry three days after treatment in spleen of untreated mice and Mix, α-GalCer and Mix+GalCer treated mice (n=3 per group). The percentage of **(a)** CD4$^+$ and **(b)** CD8$^+$ T cells are shown. *p<0,05, **p<0,005.

4.2.3.2. Cytokine profile induced by Mix+GalCer treatment

After observing the potent antitumor effect of Mix+GalCer vaccine, we wanted to know the cytokines involved in the induction of this specific immune response. We decided to examine the main representative cytokines of a Th1-type response, IFN-γ, IL-12 and TNF-α, the cytokines involved in Th2-type immune response, IL-4, IL-5, IL-6, and also the levels of IL-17, involved in the Th17 polarization.

Serum samples of untreated mice, Mix treated mice and Mix+GalCer treated mice were obtained 12h after vaccination, which was the time that enables detecting enough levels of all cytokines if they were produced. The results of this analysis showed that mice treated with the therapeutic vaccine exhibited a large amount of IFN-γ secretion in comparison with Mix treated mice (58.000 ± 2.000 pg/ml vs 31,50 ± 2,5 pg/ml; p=0,001) and untreated mice (58.000 ± 2.000 ng/ml vs 6,08 ± 3,3 pg/ml, respectively; p=0,001) (Figure 30a). The high serum levels of IFN-γ in mice which received the Mix+GalCer vaccine suggested that this cytokine could play an important role in the antitumor effect observed in the *in vivo* survival

studies. In addition to that, we observed a large increase of IL-12 levels in the serum of Mix+GalCer treated mice in comparison with Mix treated group (31.750 ± 1.750 pg/ml vs 1,3 ± 0,4 pg/ml; p=0,003) and the untreated group (31.750 ± 1.750 pg/ml vs 1100 ± 950 pg/ml; p=0,003) (Figure 30b). This high IL-12 increment in the serum of treated mice is a very interesting data because IL-12 is the main cytokine that induced Th-1 polarization, promoting the secretion of IFN-γ, TNF-α and IL-2 and the resulting antitumor immune response. There was also a significant increase of TNF-α, which is another Th1-type cytokine such as IFN-γ, in the serum of treated mice comparing with Mix treated group (91,52 ± 0,3 pg/ml vs 6,23 ± 0,03 pg/ml; p=0,0001) and the untreated group (91,52 ± 0,3 pg/ml vs 1,1 ± 0,6 pg/ml p=0,0001) (Figure 30c). This cytokine is involved in the induction of inflammation and has inhibitory effects of tumorigenesis, so it is important that the production of this cytokine was also induced by the Mix+GalCer treatment.

The analysis of IL-4 also showed a significant increase of this Th2-type cytokine levels in Mix+GalCer treated mice compared with Mix treated group (8230 ± 490 pg/ml vs 1,03 ± 0,4 pg/ml; p=0,003) and untreated mice (8.230 ± 490 pg/ml vs 0,79 ± 0,7 pg/ml p=0,0002) (Figure 30d). Moreover, an important increase of IL-5 was observed in treated mice comparing to Mix treated mice (106,5 ± 8,5 pg/ml vs 1,1± 0,03 pg/ml; p=0,006) and untreated group (106,5 ± 8,5 pg/ml vs 0,9± 0,1 pg/ml; p=0,006) (Figure 30e). The same was observed in the case of IL-6 analysis, where mice treated with Mix+GalCer vaccine presented high serum levels of this cytokine in comparison with Mix treated mice (592 ± 14,57 pg/ml vs 2,45 ± 2,1 pg/ml; p=0,0006) and the untreated group (592 ± 14,57 pg/ml vs 1,0 ± 0,9 pg/ml; p=0,0006) (Figure 30f). The presence of Th2-type cytokines could be caused by the activated NKT cells, which can secreted both Th1 and Th2 cytokines.

Another interesting result was the significant increase of IL-17 serum levels of Mix+GalCer treated mice compared with the Mix treated group (140,4 ± 5,7 pg/ml vs 6,2 ± 0,03 pg/ml; p=0,001) and untreated mice (140,4 ± 5,7 pg/ml vs 14,8 ± 4,8 pg/ml; p=0,003) (Figure 30g). It is known that IL-17, the main cytokine involved in

Th17 polarization, could be produced by NKT cells and play and important role in inflammatory responses.

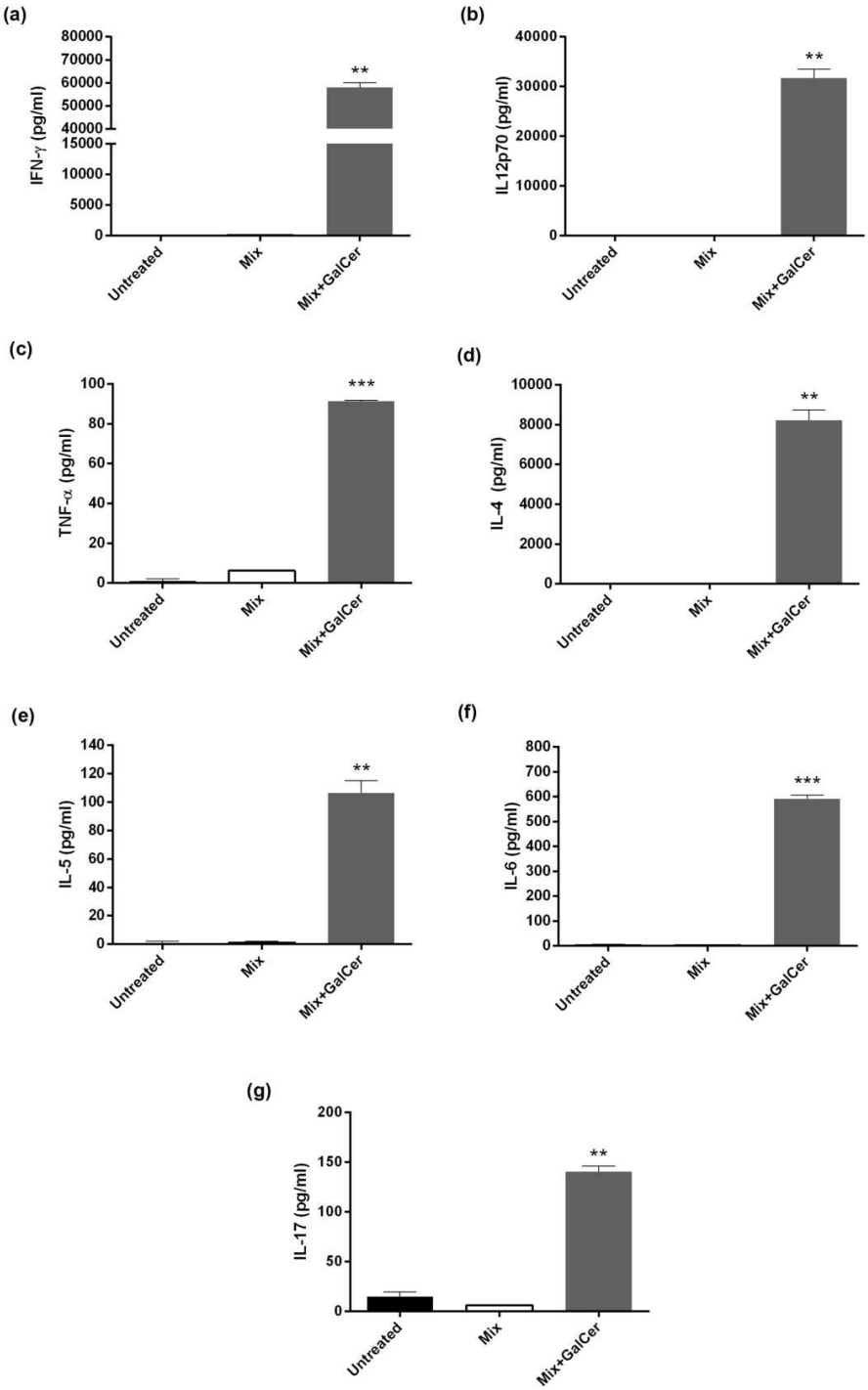

Figure 30. Serum cytokine levels induced by Mix+GalCer therapeutic vaccination. Serum samples of control group, Mix treated mice and Mix+GalCer treated mice (n=2 per group) were analyzed for cytokine levels of **(a)** IFN-γ, **(b)** IL12p70, **(c)** TNF-α , **(d)** IL-4, **(e)** IL-5, **(f)** IL-6 and **(g)** IL-17. Samples were obtained 12h after treatment injection. **p<0,005; ***p<0,005.

4.2.3.3. IFN-γ secreting cells after Mix+GalCer treatment

Mice treated with Mix+GalCer vaccine presented a high level of IFN-γ in serum, which was considered as one of the most relevant cytokine for antitumor immune response. Due to the importance of this cytokine in the antitumor therapeutic setting, we decided to analyze which cells were involved in IFN-γ production. To this purpose, NKT, NK and T cells were analyzed as an IFN-γ source in Mix+GalCer treated mice, as well as in Mix, α-GalCer and untreated groups.

The *ex vivo* analysis of IFN-γ producing NKT cells was done three days after vaccine injection. The study showed a significant increase in the proportion of these cells in spleen of Mix+GalCer treated mice, in comparison with the untreated group (4,59 ± 0,41% vs. 0,92 ± 0,12%; p=0,01) and Mix group (4,59 ± 0,41% vs. 0,38 ± 0,17%; p=0,001), suggesting that NKT cells were implicated in the increment of IFN-γ after Mix+GalCer vaccination. In addition, it was possible to note that Mix+GalCer treatment induced a higher increase of IFN-γ producing NKT-cell number than the group treated with α-GalCer alone (4,59 ± 0,41% vs. 2,35 ± 0,59%; p=0,09), suggesting that this vaccine could induce a higher NKT-cell activation (Figure 31).

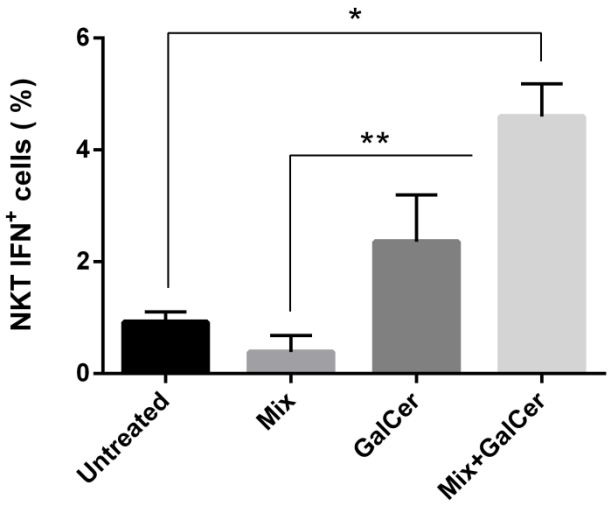

Figure 31. IFN-γ secreting NKT cells after Mix+GalCer vaccine treatment. Untreated, Mix, α-GalCer and Mix+GalCer treated mice (n=2) were sacrificed three days after treatment and splenocytes were assessed for IFN-γ secreting NKT cells by flow cytometry. *p<0,05; **p<0,005.

In addition to that, IFN-γ producing NK cells were also analyzed in all groups. In this case, α-GalCer treated mice exhibited an increase of IFN-γ secreting NK cells in contrast with untreated (19,23 ±1,17% vs. 5,50 ± 0,80%; p=0,01) and Mix treated mice (19,23 ± 1,17% vs. 9,41 ± 0,80%; p=0,006). However, Mix+GalCer vaccine induced a higher increase of IFN-γ producing NK cells compared to untreated mice (23,87 ± 1,64% vs. 5,50 ± 0,80%; p=0,003) and Mix treated mice (23,87 ± 1,64% vs. 9,41 ± 0,80%; p=0,001), although there was no statistical differences between this group and α-GalCer treated group (23,87 ± 1,64% vs. 19,23 ± 1,17%; p=0,1) (Figure 32). These results demonstrated that α-GalCer based treatments stimulated the increase of IFN-γ producing NK cells and Mix+GalCer treatment induced the higher increase of these cells.

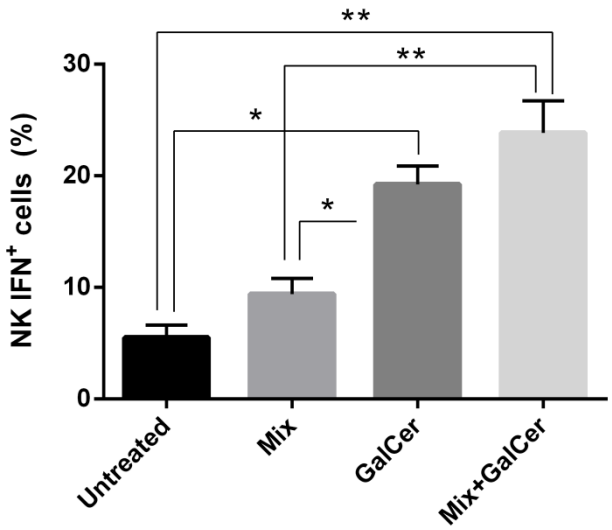

Figure 32. IFN-γ secreting NK cells after Mix+GalCer vaccine treatment. Untreated, Mix, α-GalCer and Mix+GalCer treated mice (n=2) were sacrificed three days after treatment and splenocytes were assessed for IFN-γ secreting NK cells by flow cytometry. *p<0,05; **p<0,005.

Moreover, splenocytes of all mice groups were cocultured together with irradiated tumor cells to study the IFN-γ producing $CD4^+$ and $CD8^+$ T cells. This culture acted as an *ex vivo* tumor stimulus to IFN-γ secreting $CD4^+$ or $CD8^+$ T cells, allowing the assessment of potential tumor-specific T cells. After this analysis, we observed a tendency to increase of IFN-γ secreting $CD4^+$ T cells in mice treated with

Mix+GalCer vaccine in comparison with untreated (1,70 ± 0,63% vs. 0,12 ± 0,02%; p=0,1), Mix (1,7 ± 0,63% vs. 0,46 ± 0,11%; p=0,1) and α-GalCer group (1,7 ± 0,63% vs. 0,17 ± 0,03%; p=0,1) (Figure 33a). When we looked at IFN-γ secreting $CD8^+$ T cells, we also noticed a trend to increase of these cells in Mix+GalCer treated group compared to untreated mice (0,86 ± 0,3% vs. 0,03 ± 0,01%; p=0,1), Mix treated group (0,86 ± 0,3% vs. 0,21 ± 0,03%; p=0,2) and α-GalCer treated group (0,86 ± 0,3% vs. 0,04 ± 0,01%; p=0,1) (Figure 33b). This data suggested that Mix+GalCer treatment could induce the generation of tumor-specific IFN-γ secreting T cells.

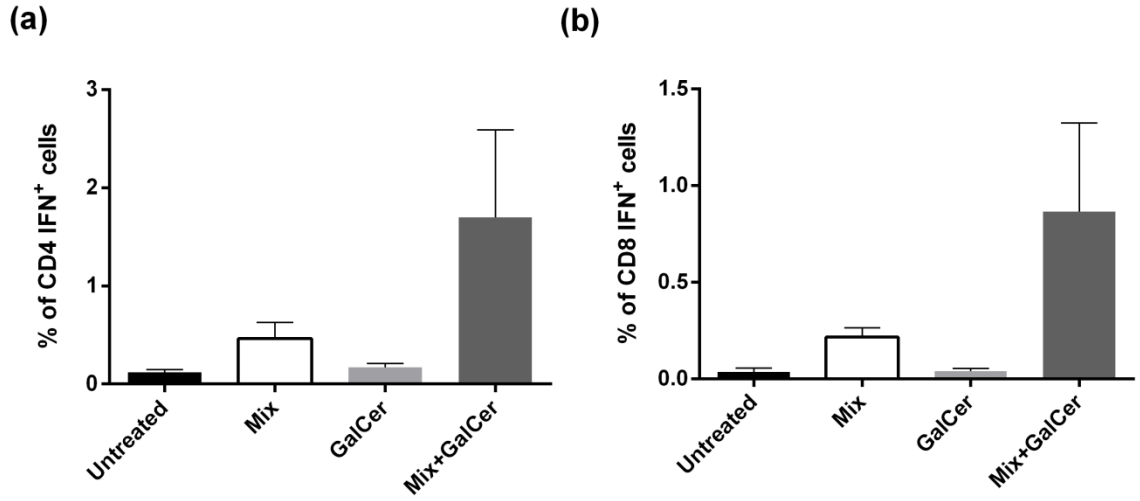

Figure 33. IFN-γ secreting $CD4^+$ and $CD8^+$ T cells after Mix+GalCer vaccine treatment. Untreated, Mix, α-GalCer and Mix+GalCer treated mice (n=2) were sacrificed three days after vaccine injection and splenocytes ($2x10^6$) were cocultured with irradiated tumor cells (2:1 ratio) for 24h. After this time, cells were assessed for IFN-γ secreting **(a)** $CD4^+$ and **(b)** $CD8^+$ T cells by flow cytometry.

4.2.3.4. Involvement of different effector cells in the antitumor effect *in vivo*: depletion assay

The *in vivo* depletion assays were used to elucidate the individual implication of different cell types in the generation of an immune response and are based on the depletion of the specific cell population using specific depleting antibodies.

We decided to analyze the contribution of NK, $CD4^+$ and $CD8^+$ T cells in the antitumor effect of Mix+GalCer treatment to elucidate which cell type played the main role in the establishment of an effective immune response. To analyze the individual implication of the different cell types in the generation of antitumor immune response, an *in vivo* depletion assay was done. These studies are based on the depletion of the specific cell population using specific depleting antibodies that, in our case, were the anti-CD4, anti-CD8 and anti-asialo GM1 to deplete $CD4^+$, $CD8^+$ T cells and NK cells, respectively. It was not possible to study the individual contribution of NKT cells in the antitumor effect of the therapeutic vaccine because of the lack of an available specific NKT-cell antibody.

Previous to *in vivo* study, it was important to determine the depletion efficiency of the monoclonal antibodies used to eliminate NK cells, $CD4^+$ and $CD8^+$ T cells. To do that, mice were injected with depleting antibodies two consecutive days and were sacrificed to analyze the presence of CD4, CD8, NKT and NK cells (Figure 34). Although we did not deplete NKT cells, it was important to know how the other depleting antibodies affected the ratio of NKT cells; especially the anti-CD4 antibody because mouse NKT cells can express the co-receptor CD4. The results shown in figure 15 demonstrated that all monoclonal antibodies depleted their specific population more than 95%, without affecting the other cell types. This step allows us to do the *in vivo* depletion assay with the assurance of a significant elimination of the corresponding population while maintaining the other cell types.

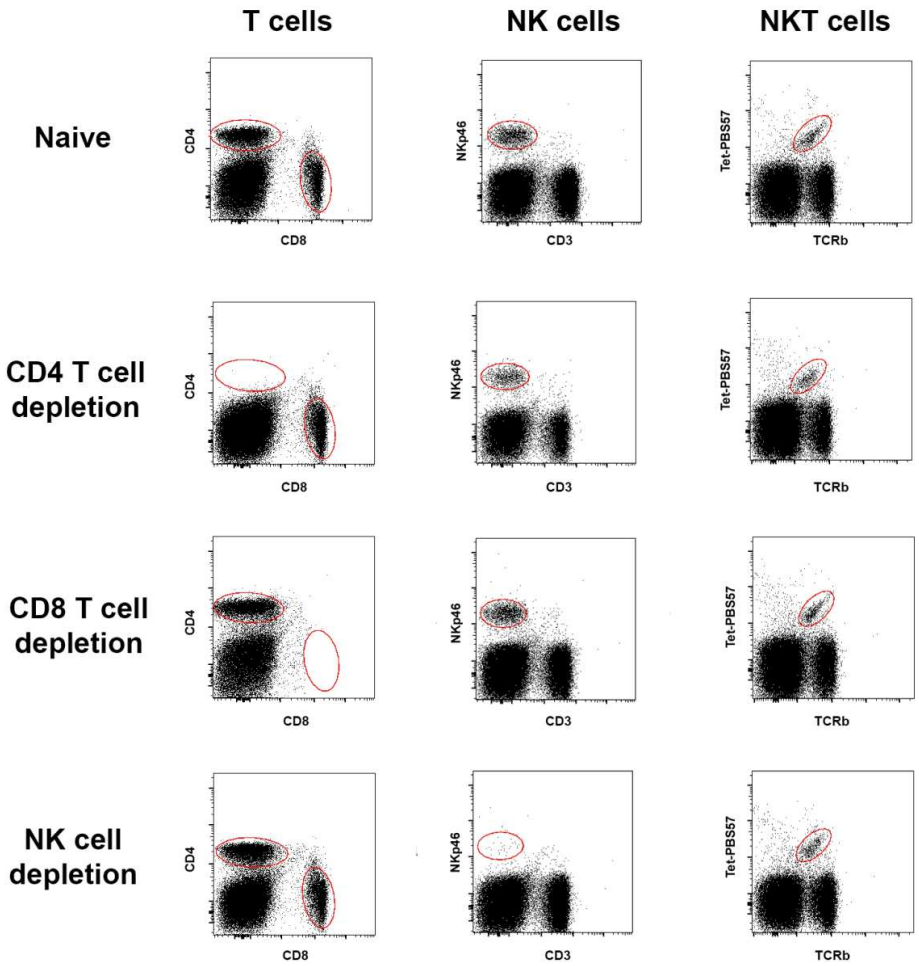

Figure 34. Depletion of T cells and NK cells. Mice (n=2 per group) were injected twice with anti-CD4, anti-CD8 and anti-asialo antibodies to depleted specifically CD4 T cells, CD8 T cells and NK cells, respectively. A naive mice was considering as a control for all cell types. Splenocytes were assessed by flow cytometry for T cells using CD4-FITC and CD8-APC antibodies, for NKT cells using TCRβ-VioBlue and Tet-PBS57-PE antibodies, and for NK cell population using CD3-PE and NKp46-FITC antibodies.

Once checked the efficacy of the depleting antibodies, different groups of mice were treated with the Mix+GalCer vaccine two days after tumor challenge and were depleted for NK, CD4 or CD8 T cells using the specific antibodies on days -2, -1 and 1 relative to tumor injection. After Mix+GalCer vaccination, the depleting antibodies were injected weekly during the rest of the experiment in order to maintain the depletion of the corresponding cell population.

Mix+GalCer treated mice with depletion of CD4 and CD8 T cells exhibited 100% of survival such as Mix+GalCer treated mice without any cell depletion. This observation suggested that, in this model, T cells were not essential for the generation of an effective antitumor immune response (Figure 35). By contrast, all mice treated with Mix+GalCer vaccine which were depleted for NK cells did not reject the tumor, suggesting that NK cells play an important role in the antitumor immune response induced by the Mix+GalCer treatment.

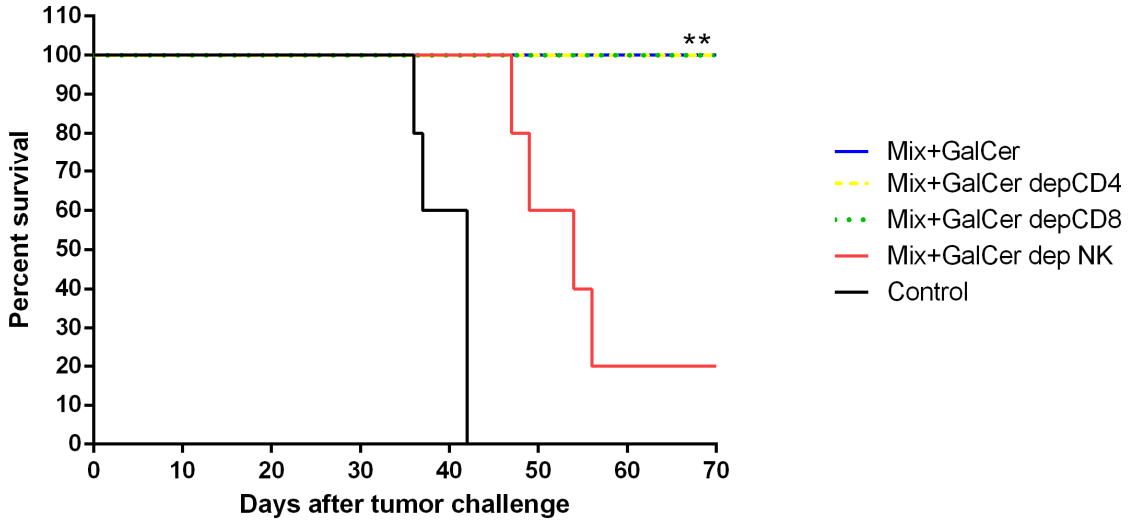

Figure 35. Survival of Mix+GalCer treated mice after CD4, CD8 or NK *in vivo* cell depletion. Mice (n=5) were injected with 4TOO tumor cells ($4x10^5$ cells/mice in 100µl of PBS, iv) and were treated 2 days after tumor injection with a single dose of Mix+GalCer vaccine (iv). Other groups of mice (n=5 per group) received the same regimen of tumor and Mix+GalCer vaccine injection but they were depleted for CD4, CD8 and NK cells using the corresponding depleting antibodies at days -2,-1 and 1 relative to tumor challenge, followed by a weekly antibody injection to maintain the cell depletion. Untreated mice were injected with IgG antibody as a control treatment. Mice were followed for survival for 70 days. **p<0,005

4.2.3.4.1. Analysis of stimulatory and inhibitory NK ligands on tumor cells

After finding the critical role on NK cells in the antitumor immune response after Mix+GalCer vaccination, we wanted to explore the role of NK cells in the direct tumor cell eradication. It is known that NK cells can kill malignant cells by direct recognition of different molecules expressed on these cells through their NKG2A and NKG2D receptors. In this study, we analyzed the expression of the major

inhibitory NK-cell ligand, MHC-I, and the most important activator of NK-cell citotoxicity in mice, Rae-1, in 4TOO tumor cells. The results in Figure 36 showed that 4TOO tumor cells expressed high levels of MHC-I (100%), suggesting that they are unable to trigger NK-cell citotoxicity by the miss-recognition of this inhibitory ligand. By contrast, these cells exhibited a high expression of Rae-1 (99,4%), suggesting that NK cells can be activated by the high exposure of this activating NK ligand. This data suggest that the eradication of 4TOO tumor cells could be, in part, by direct NK-cell killing.

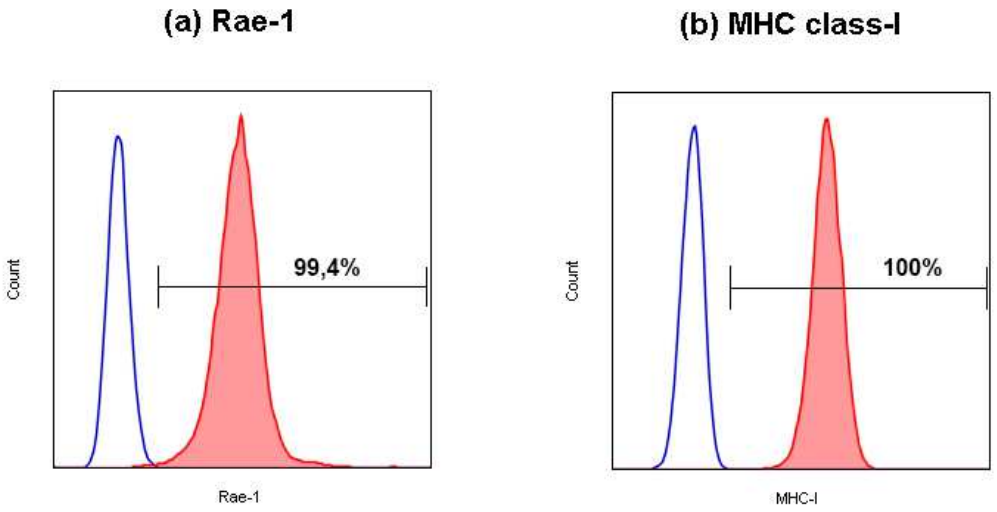

Figure 36. Expression of Rae-1 and MHC class-I in 4TOO tumor cells. 4TOO tumor cells were analyzed for the expression of (a) Rae-1 and (b) MHC class-I by flow cytometry using Rae-1-PE-Vio770 and MHC class-I-eFluor450, respectively.

4.2.3.5. Humoral immunity after Mix+GalCer treatment

To study the role of B cells in the antitumor efficacy of the Mix+GalCer vaccination, serum samples of untreated and treated mice were collected 14 days after treatment in order to analyze the presence of anti-tumor IgG antibodies. As it is shown in Figure 37a-b, there were anti-tumor IgG antibodies in the serum of treated mice since we observed an increase of positive 4TOO tumor cells to IgG-PE labeling, in comparison with the serums from naive (5,4 ± 0,33% vs. 0,53 ±0,03%; p=0,001) and untreated mice (5,4 ± 0,33% vs. 2,13 ± 0,31; p=0,007). If

the MFI was taking in consideration, serum of Mix+GalCer treated mice showed a significant increase in the relative IgG levels which recognize the 4TOO tumor cells compared to serum from untreated mice (1,60 ± 0,12 vs. 1,07 ± 0,06 relative IgG antibody levels, p=0,04) (Figure 37c). The serums from untreated and Mix+GalCer treated mice were also incubated with A20 tumor cells to analyze if IgG antibodies from the serum of treated mice could recognize another type of tumor cells. As Figure 36c shown, both serum samples of control and treated mice did not have IgG antibodies that recognize A20 tumor cells, suggesting that the anti-tumor IgG induced by the Mix+GalCer vaccine were tumor specific. Thus, this data demonstrated that Mix+GalCer vaccine induced the activation of specific B cells that may contribute to the antitumor immune response.

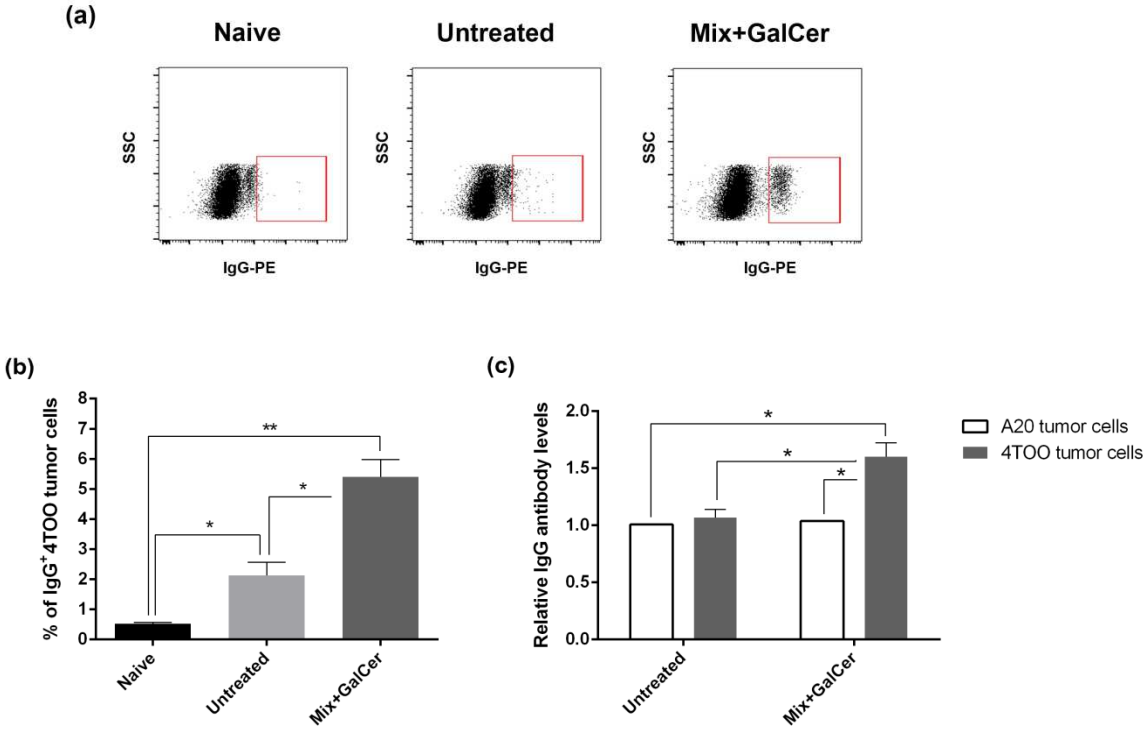

Figure 37. Specific IgG antibodies against 4TOO tumor cells after Mix+GalCer treatment. (a) IgG^+ 4TOO tumor cells were analyzed by flow cytometry using serum samples of naive, untreated and Mix+GalCer treated mice and a secondary anti-mouse IgG-PE antibody. **(b)** Percentage of IgG^+ 4TOO tumor cells after incubation with serum from naive, untreated and Mix+GalCer treated mice. **(c)** 4TOO and A20 tumor cells were incubated with serum samples of naive, untreated and Mix+GalCer treated groups and the relative specific IgG antibody levels were analyze by flow cytometry. *p<0,05, **p<0,005.

4.3. New NKT agonists as a therapeutic treatment against B-cell lymphoma: the NKT14m antibody

In our effort to obtain an efficient treatment to eradicate B-cell lymphoma, it brought our attention a publication related to the generation of a novel NKT agonist: the NKT14m antibody. We had the opportunity of initiating collaboration with NKT Therapeutics to test this new and exclusive NKT-cell agonist antibody to activate NKT cells. This antibody could help us to treat B-cell lymphoma and represent a good alternative to develop an NKT-cell based immunotherapy against B-cell lymphoma with important clinical implications.

4.3.1. Antitumor effect of NKT14m antibody treatment

First of all, we wanted to study the *in vivo* antitumor effect of NKT14m antibody in our B-cell lymphoma mice model. To achieve that, mice were treated with a single dose of the NKT14m antibody two days after tumor injection, following the same schedule of Mix+GalCer treatment. In addition, another group of mice received the NKT14m antibody treatment four days after tumor challenge in order to observe whether a later antibody treatment was as effective as the earlier in presence of more established tumor. In this experiment, control mice received an isotype-matched IgG. After following mice survival for 120 days, mice treated with the antibody on day 4 relative to tumor injection did not survive, suggesting that the NKT14m antibody is not able to cure mice with a large tumor burden. In contrast, treatment with NKT14m 2 days after tumor injection exhibited 37% antitumor efficacy (p=0,04), demonstrating that this antibody had an antitumor effect in an early therapeutic setting (Figure 38).

Previous studies carried out by NKT Therapeutics showed that NKT14m antibody, injected 6 weeks after the first dose, was able to induce NKT-cell activation again, thus reversing the anergy consistently found after NKT-cell activation by α-GalCer. This feature allowed us to retreat mice and study whether the NKT14m

retreatment could improve survival in our B-cell lymphoma mice model. To analyze the *in vivo* antitumor effect of NKT14m retreatment, a group of mice received a first dose of NKT14m antibody on day 2 after tumor challenge and a second dose 42 days after tumor injection. In this case, a 50% tumor efficacy (p=0,002) was observed using the NKT14m retreatment vaccination (Figure 38). We also observed that the mean overall survival, defined as the length of time which half of mice are still alive after tumor injection, were higher on retreated mice than those without retreatment (92,5 vs. 57,5 days, respectively; p=0,0001).

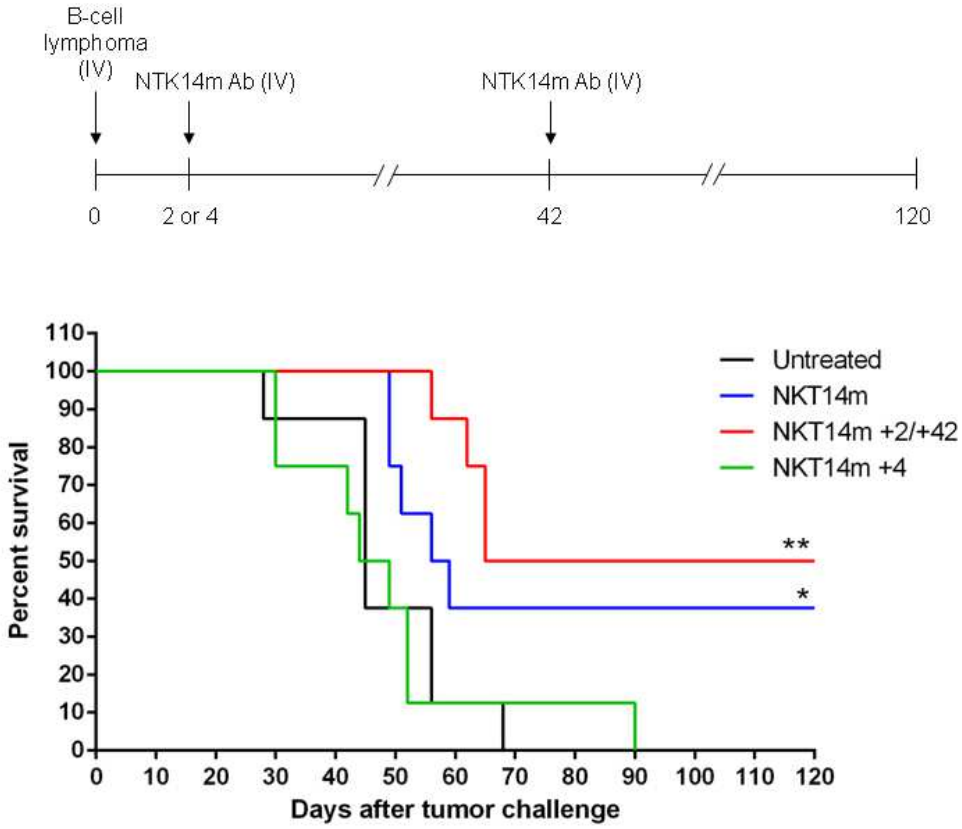

Figure 38. Mice survival after NKT14m therapeutic treatment. Mice (n=8 per group) were injected with 4TOO tumor cells ($4x10^5$ cells/mice in 100µl of PBS, iv) and were treated with a single dose of NKT14m agonist antibody (100ug/mice, iv), 2 days (NKT14m) or 4 days (NKT14m +4) after tumor challenge. One group received a single dose of NKT14m antibody 2 days after tumor challenge and also a second dose of the agonist antibody 42 days after tumor injection (NKT14m +2/+42). Untreated mice were inoculated with an isotype-matched IgG. Mice were followed for survival for 120 days. *p<0,05; **p<0,005.

4.3.2. Therapeutic treatment for B-cell lymphoma using the combination of cyclophosphamide and NKT14m antibody

Cyclophosphamide is a chemotherapeutic agent largely used to treat lymphoma patients. This molecule is able to kill tumor cells, providing tumor antigens to APCs. Moreover, it can stimulate the immune system inducing cytokine release by T cells, as well as B and T cell proliferation. Taking these characteristics in consideration, as well as the importance of cyclophosphamide in established chemotherapeutic protocols in clinical settings, we evaluated the combination of NKT14m treatment with cyclophosphamide in our B-cell lymphoma model.

To test whether the combination of cyclophosphamide and the NKT14m treatment could induce and efficient antitumor response and improve survival, cyclophosphamide was injected into mice 10 days after tumor challenge and the NKT14m antibody was inoculated 24h after cyclophosphamide treatment. The timing and dose of this chemotherapeutic agent was previously assessed to provide a moderate antitumor effect, without eradicating tumors. As a result of this analysis, we observed that cyclophosphamide alone exhibited 35% survival (p=0,02; mean overall survival: 61,5 days), whereas the most effective treatment was the CM+NKT14m combination, which showed 90% survival (p=0,0001) (Figure 39).

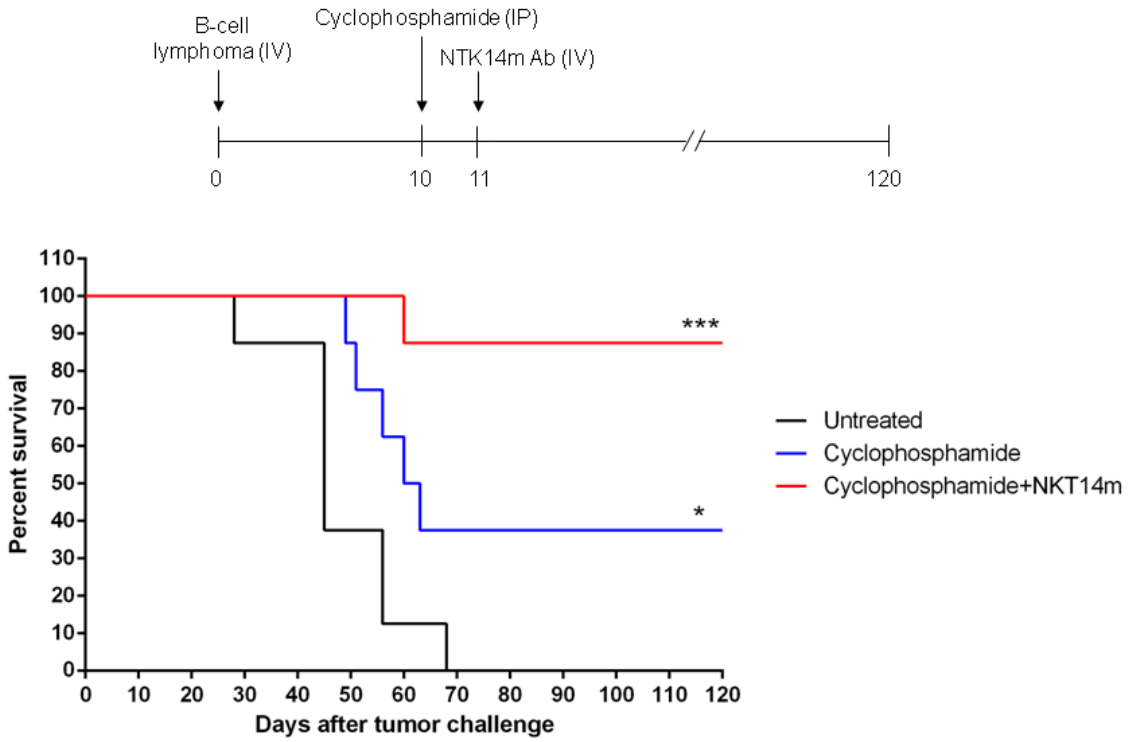

Figure 39. Mice survival after CM+NKT14m therapeutic treatment. Mice (n=8 per group) were injected with 4TOO tumor cells ($4x10^5$ cells/mice in 100µl of PBS, iv) and were treated with cyclophosphamide (70mg/Kg, ip) 10 days after tumor challenge. One group received the cyclophosphamide treatment and 24h later they were treated with a single dose of NKT14m (100ug/mice, iv). Untreated mice were injected with 100µl of PBS iv and this group, together with mice treated with cyclophosphamide alone, received an isotype-matched IgG. Mice were followed for survival for 120 days. *p<0,05; ***p<0,001.

5. DISCUSSION

NKT cells are a small population of lymphocytes that are able to connect the innate and adaptive immune systems since they share characteristics between NK and T cells. This feature allows them to modulate DC function and activation, inducing the expansion of antigen-specific B and T cells (Cerundolo, Silk et al., 2009) and NK-cell activation (Vivier, Ugolini et al., 2012). NKT cells can be activated using the synthetic agonist α-GalCer, a glycolipid that is recognized by the CD1d receptor from DCs. The role of NKT cells in antitumor responses implies the activation of other effector immune cells like T and NK cells and the production of cytokines that promote tumor elimination, such as IL-12 and IFN-γ (Spada, Koezuka et al., 1998; Fujii, Shimizu et al., 2002). Thus, the development of a therapeutic vaccine using NKT cell activation would be an interesting antitumor therapy.

To activate NKT cells it is important to bring them an adequate glycolipid presentation through CD1d receptor (Van Kaer, 2005), which is usually expressed on APC, including dendritic cells, macrophages, granulocytes and B cells (Brossay, Jullien et al., 1997; Roark, Park et al., 1998). To assure the correct α-GalCer presentation, we decided to include an APC in the therapeutic vaccine, being DCs and tumor cells the best candidates. In this study, we observed that DCs expressed high levels of CD1d, supporting the use of this APC in the vaccine to stimulate NKT cells. In fact, DCs are the most potent APC in the immune system and they have been used in the development of different immunotherapy vaccines against cancer (Timmerman, Caspar et al., 2001; Fujii, Shimizu et al., 2002). By contrast, 4TOO tumor cells expressed low levels of CD1d, arguing they would not be the best candidates for activating NKT cells, despite they can act as APC. This observation is not an exception since it is known that some tumors exhibit a low expression of CD1d receptor, impairing the direct NKT cell recognition and activation (Vivier, Ugolini et al., 2012; McEwen-Smith, Salio et al., 2015).

DCs express different types of costimulatory molecules, adhesion receptors and antigen presenting molecules which are essential for an efficient activation of

lymphocytes, including NKT cells. It is important to take into account the maturation status of DCs previous to the development of a DC vaccine, because it is an important feature to direct the correct immune response combining presentation and activation skills (Timmerman and Levy, 1999). To known the maturation grade of DC produced to generate the therapeutic vaccine, the expression of different DC markers was analyzed (CD11c, MHC-II, CD40, CD86 and CD80). These DCs exhibited intermediate levels of MHC-II and expression of CD40, despite they had a high expression of CD80 and CD86. These expression levels suggest that DC population is composed by semi-mature cells (Timmerman and Levy, 1999; Gerlach, Steimle et al., 2012). In addition, we also observed that DCs pulsed with α-GalCer *in vitro* did modify neither the expression levels of costimulatory molecules nor the expression of MHC and CD11c markers. This data suggests that the glycolipid interaction is not enough to induce changes in DC maturation status, and DCs need the cooperation of CD40-CD40L and other cytokine stimuli from host NKT and T cells to complete their maturation (Langenkamp, Messi et al., 2000; Fujii, Liu et al., 2004; Gerlach, Steimle et al., 2012). Observing that *in vitro* α-GalCer did not bring any advantage in DC maturation, we decided to use α-GalCer in vivo directly mixed with the other components (DCs and irradiated tumor cells) previous to mice injection, allowing the host DCs to recognize free α-GalCer and stimulate the host NKT cells as well.

In addition to α-GalCer and DCs, we decided to include tumor antigens in the vaccine to improve the specific antitumor immune response, enhancing the delivery of these specific antigens to DCs. To do that, irradiated whole 4TOO tumor cells were used to assure maximum availability of tumor antigens, although the immunodominant antigen in these tumor cells is still unknown. In fact, previous studies of DC vaccination in lymphoma mice models demonstrated that the use of only one specific and known tumor antigen, such as the B-cell lymphoma idiotype, could not be the immunodominant antigen in the induction of the immune response, so other tumor antigens that still unknown can be act as inducers of antitumor immune responses (Kronenberger, Dieckmann et al., 2002). Thereby, the use of several tumor antigens, whether they are known or not, to stimulate the

immune system through DC presentation would potentially generate a better antitumor effect. Later studies have already used whole tumor cells as an antigen source with good results (Shimizu, Kurosawa et al., 2007; Alvarez, Moga et al., 2010; Hong, Lee et al., 2013). Moreover, the use of whole tumor cells and no requirement of tumor antigen identification make the vaccine development easier and more applicable to the clinic.

Taking all of these reasons in consideration, we decided to develop a therapeutic vaccine against B-cell lymphoma using DCs, as the most important APC to generate an effective immune response, and irradiated tumor cells, as a complete antigen source, with the potent and well-studied NKT cell activator, α-GalCer (Mix+GalCer vaccine). This system allows the tumor-peptide antigen presentation by DCs to stimulate T cells and also the presentation of α-GalCer that is necessary to activate NKT cells.

Antitumor effect of Mix+GalCer treatment against B-cell lymphoma

The antitumor efficacy of Mix+GalCer vaccine and the control vaccines, including α-GalCer alone, Mix alone and DC+GalCer, was studied in a therapeutic setting in order to provide data which may be more relevant to the clinical scenario. In the first part of the study, the treatments were injected 2 days after tumor challenge since this time allows the tumor to disseminate and invade host lymphoid organs. In fact, other studies and our own previous experience applied the same model of vaccination (Gong, Koido et al., 2002; Alvarez, Moga et al., 2010). The prime-boost of all vaccines were injected 6 and 10 days after tumor injection in order to help the immune response to be more efficient, as was done in other studies (Palucka and Banchereau, 2013). When the treatments were evaluated *in vivo*, the administration of α-GalCer alone did not have any antitumor efficacy, in contrast with other studies which showed a considerable antitumor effect of α-GalCer in different tumor models (Kobayashi, Motoki et al., 1995; Fujii, Shimizu et al., 2002). The reason for the lack of antitumor efficacy of α-GalCer alone in our B-cell

lymphoma model, although it is a NKT-cell agonist, could be the lack of CD1d expression on tumor cells. Due to that, the direct recognition of the tumor by NKT cells can be significantly impaired, decreasing the direct and indirect NKT-cell antitumor effect. The reduced antitumor efficacy that we observed in our B-cell lymphoma model using α-GalCer alone mostly resembles the clinic scenario, where patients treated with α-GalCer alone did not showed any antitumor effect although they exhibited a moderate NKT-cell expansion (Giaccone, Punt et al., 2002; Nieda, Okai et al., 2004). Despite of the limited antitumor response induced by α-GalCer, its combination with DCs or Mix greatly improved the efficacy of the treatment. Thus, DC+GalCer vaccine exhibited a 50% antitumor efficacy while Mix+GalCer vaccine showed a 100% of cure, being the best therapeutic approach. Taking this data in consideration, we suggest that, in our B-cell lymphoma model, NKT-cell activation is critically involved in the eradication of B-cell lymphoma, and the simultaneous injection of α-GalCer with DCs and tumor antigens further improves the antitumor immune response, in concordance with previous studies (Liu, Idoyaga et al., 2005; Chung, Qin et al., 2007; Shimizu, Goto et al., 2007). All of these results show the value of having tumor antigens available to DC in order to initiate an antitumor immune response through T cell presentation. Malignant tumor B cells could be also used as α-GalCer presenting cells and this combination showed an effective antitumor response (Chung, Qin et al., 2007; Hong, Lee et al., 2013). However, in contrast to our model, those B cells did express significant levels of CD1d which may account for the different antitumor responses.

Importantly, an increase of NKT cells was observed three days after the first vaccine injection. This large increase of NKT cells is due to NKT cell activation (Parekh, Wilson et al., 2005). By contrast, a reduction of NKT-cell number in spleen of treated mice after the second and third doses was observed, suggesting that recurrent Mix+GalCer vaccination reduced the NKT-cell expansion. As shown in previous studies, the repetitive *in vivo* administration of α-GalCer generates NKT-cell anergy with a drastic reduction of NKT-cell function and IFN-γ secretion (Fujii, Shimizu et al., 2002; Parekh, Wilson et al., 2005). Taking these studies into

account, we believe that the absence of NKT-cell expansion in Mix+GalCer treated mice after the second and third dose was likely due to anergy induced by the presence of α-GalCer in the vaccine.

As an alternative to the three-vaccination strategy, a single-dose of Mix+GalCer vaccine was tested to observe whether it was enough to stimulate an effective antitumor immune response, avoiding the impairment of NKT-cell function. Interestingly, a single-dose of Mix+GalCer vaccine exhibited 100% antitumor efficacy as in the case of the three-dose treatment. These results demonstrated again the strong antitumor effect of NKT cell activation. The ability of a single-dose of Mix+GalCer vaccine to produce a maximum antitumor effect is an important point for clinical applications. Moreover, we observed that the single-dose of Mix+GalCer vaccine induces maximum NKT-cell expansion, as it was induced after the first Mix+GalCer injection in the three-vaccination strategy, and this increment is greater than the one induced by α-GalCer alone. The increment of NKT cells after α-GalCer treatment was observed in experimental studies with the A20 B-cell lymphoma model (Chung, Qin et al., 2007), as well as in patients with advanced myeloma, metastatic hepatocellular cancer, renal cancer, and head and neck cancer after treatment with α-GalCer-loaded DCs (Chang, Osman et al., 2005; Uchida, Horiguchi et al., 2008). In this work, we demonstrated that the combination of Mix+GalCer can induce a greater increase of NKT cells than α-GalCer alone, being probably involved in the better antitumor outcome observed in the survival studies. Moreover, the proportion of NK cells showed the same behavior as NKT cells, showing a moderate increase after α-GalCer treatment and a greater increase in mice treated with Mix+GalCer vaccine. NKT cells can influence NK-cell function after their activation, so it is possible that a higher NKT-cell expansion induced a higher increase of NK cells as well. In fact, previous studies demonstrated that activation of NKT cells could enhanced the NK-cell increment three days after treatment (Smyth et al., 2005), as we also observed. However, α-GalCer and Mix+GalCer treated mice exhibited the same reduction of $CD4^+$ T-cell proportion and no change in $CD8^+$ T-cell percentage. The $CD4^+$ T-cell reduction in spleen might be induced by NKT-cell activation following α-GalCer

administration, which activates $CD4^+$ T cells and could induce their migration to other lymphoid and non-lymphoid tissues.

In addition to these results, a single-dose of Mix+GalCer vaccine was able to protect mice from a second tumor injection, indicating the generation of established adaptive memory immunity against 4TOO tumor cells. Previous studies using DCs or tumor cells loaded with α-GalCer exhibited similar long-lasting immunity in mice models of myeloma and B-cell lymphoma (Chung, Qin et al., 2007; Hong, Lee et al., 2013), as well as in other non-hematological tumor models such as melanoma, colon carcinoma and sarcoma (Shimizu, Kurosawa et al., 2007; Tatsumi, Takehara et al., 2007). In fact, it was described that activated NKT cells are able to improve the generation and proliferation of $CD4^+$ and $CD8^+$ memory T cells (Eberl, Brawand et al., 2000), as well as to enhance B cell function and memory in mice (Galli, Pittoni et al., 2007).

Furthermore, a tumor-specific immune response was observed since Mix+GalCer treated mice did not reject a second challenge of a syngeneic A20 B-cell lymphoma. This data suggests that the specific tumor antigens corresponding to 4TOO tumor cells are involved in the establishment of the memory response. Similar studies also exhibited the generation of tumor-specific immunity using α-GalCer vaccines to activate NKT cells (Shimizu, Kurosawa et al., 2007; Tatsumi, Takehara et al., 2007). However, these studies used α-GalCer treatments against solid tumors and the tumor-specific immunity was studied using a tumor cell line from a different lineage than the original tumor used for challenge. In those cases, tumor cell lines are very different to each other since the tumor antigen variety is also very different, impairing memory immune cells to recognize the second tumor challenge. The analysis of tumor-specific immunity in our lymphoma model was more astringent since we rechallenged mice with A20 B-cell lymphoma, a tumor cell line with similar phenotypic characteristics to 4TOO cells since they also come from a mature B-cell lymphoma.

Antitumor mechanisms of the immune response induced by Mix+GalCer treatment

Once we observed the *in vivo* antitumor effect of Mix+GalCer treatment, we further studied the cytokines involved in the antitumor immune response. This study showed that the cytokine profile induced by Mix+GalCer treatment was a combination of Th1, Th2 and Th17-type responses. This observation is consistent with the different cells that would be activated following the treatment. On one hand, α-GalCer activates NKT cells, which can secrete Th1 and Th2 cytokines, as well as IL-17. On the other hand, tumor antigens presented by DCs, together with the direct contribution of NKT cells, are able to stimulate T-cell activation, which can also produce cytokines related to Th1, Th2 and Th17 responses.

The cytokine that exhibits the major amount levels in Mix+GalCer treated mice was IFN-γ, suggesting a critical involvement in the antitumor effect of Mix+GalCer therapy. As other studies demonstrated, high IFN-γ production is the main common characteristic in α-GalCer based treatments (Shimizu, Kurosawa et al., 2007; Mattarollo, West et al., 2012). In our study, the number of IFN-γ secreting NKT, NK and specific-tumor T cells after Mix+GalCer injection is considerably higher than untreated and Mix treated mice, suggesting that all of these cells are involved in the IFN-γ production observed after Mix+GalCer treatment. Although α-GalCer treated mice exhibited an increase of IFN-γ secreting NK and NKT cells, Mix+GalCer vaccine induced a higher increment of these cells. In addition, Mix+GalCer vaccine strongly activated specific-tumor IFN-γ secreting $CD4^+$ and $CD8^+$ T cells in contrast to α-GalCer treatment. All this data suggested that the combination of α-GalCer together with DCs and tumor cells induces a more potent Th1 response.

We also observed that the expression of IL-4, IL-5 and IL-6 were increased in treated mice compared with Mix and untreated group, suggesting a Th2-type response. In fact, an individual NKT cell can produce both Th1 and Th2 cytokines like IFN-γ and IL-4 (Chen and Paul, 1997). In addition, there was a significant

increase of IL-12 after Mix+GalCer therapy which suggest a pivotal role of DCs in the generation of the antitumor immune response (Shimizu, Kurosawa et al., 2007; Mattarollo, West et al., 2012). IL-12, IFN-γ and IL-4, the most representative cytokines after Mix+GalCer stimulation, may also play an important role in the generation of the potent antitumor immune response observed after the vaccine treatment. The bioactive form IL12p70 acts on T, NKT and NK cells by inducing proliferation, cytotoxicity and IFN-γ production (Trinchieri, 1998). IL-12 is the most important cytokine for induction of Th1 polarization during the initiation of a primary immune response, and plays an important role in resistance to tumors. The major stimuli for IL-12 production by DCs are the CD40-CD40L interaction and the cytokine milieu during their stimulation (Koch, Stanzl et al., 1996). It is shown that IL-4 and IFN-γ together exhibit a strong synergism in increasing IL-12p70 (Hochrein, O'Keeffe et al., 2000; Bocek, Foucras et al., 2004). The increment of IL-12 levels, in turn, leads to IFN-γ secretion by T and NKT cells as well. Thus, we can remark that IL-4 is important to stimulate IFN-γ secretion that, at the same time, enhances IL-12 production. Despite it seems that a Th1 bias is the predominant immune response induced by Mix+GalCer therapy, α-GalCer injection is not an specific Th1 activator and the presence of other Th2 cytokines may be a disadvantage for treating cancer patients with immunotherapy (Venkataswamy and Porcelli, 2010). To solve that and promote a preferential Th1-response of NKT cells, different analogues of α-GalCer were developed, such as a-C-GalCer and HS161. Both of these two molecules have been demonstrated to induce a potent and sustained Th1 cytokine response in mice (Schmieg, Yang et al., 2003; Harrak, Barra et al., 2011; Kerzerho, Yu et al., 2012).

A surprising data was the presence of IL-17 and IL-6 in the serum of Mix+GalCer treated mice. It has been recently demonstrated that NKT cells are able to produce IL-17 and this cytokine production are induced by the presence of TGF-β and IL-6; the latter can be secreted by the NKT cells as well (Monteiro, Almeida et al., 2013). Based on these previous studies, we suggest that the major IL-17 source after Mix+GalCer treatment is NKT cells. In addition, it is also known that IL-17 cytokine can also be secreted by Th17 lymphocytes, which are also activated in

presence of TGF-β and IL-6 (Bettelli, Carrier et al., 2006) (Mattarollo, West et al., 2012) and also have an antitumor activity (Martin-Orozco, Chung et al., 2009), but their presence in Mix+GalCer treated mice was not assessed in our study.

Interestingly, the results from the depletion assay showed a critical role of NK cells in the effective antitumor immune response since Mix+GalCer treated mice depleted for NK cells did not survive after 4TOO tumor injection. In contrast, $CD4^+$ and $CD8^+$ T cell depletion did not have any deleterious effect in antitumor immunity, although they were involved in the establishment of adapted memory immunity. The critical effector-cell that finally mediates tumor eradication depends on the tumor model as well as on the type of treatment. Thus, while other lymphoma models were CD4 and CD8-dependent (Briones, Timmerman et al., 2002; Briones, Timmerman et al., 2003; Chung, Qin et al., 2007; Sagiv-Barfi, Kohrt et al., 2015), our results demonstrated that eradication of tumor cells in our B-cell lymphoma model depends critically on NK-cell activation. In addition, our data are in line with previous studies showing that NK cells are strongly activated by NKT cells, contributing to IFN-γ production, as we also could observed, and probably to tumor cell killing as well (Vivier, Ugolini et al., 2012; Brennan, Brigl et al., 2013). In fact, we observed for the first time that 4TOO tumor cells have a high expression of the activating NK-cell ligand Rae-1, which contributes to trigger the antitumor NK-cell citotoxicity.

Furthermore, the presence of IgG antibodies against 4TOO tumor cells in the serum of Mix+GalCer treated mice suggests that B cells are activated and may also contribute to antitumor immune response. The generation of anti-tumor antibodies was also observed in other preclinical studies (Li, Andreansky et al., 2008; Pinfold, Brown et al., 2014). It is known that NKT cells provide a non-cognate B-cell help because they promote antigen-specific $CD4^+$ T-cell responses that can induce specific B-cell activation (Tonti, Galli et al., 2009). This could explain the presence of specific-tumor antibodies in the serum of Mix+GalCer treated mice since the vaccine activates NKT cells and contains available specific-tumor antigens as well. But more relevant is the fact that NKT cells can provide a

cognate B-cell help by the interaction between their invariant TCR and the CD1d receptor on B cells (Leadbetter, Brigl et al., 2008). Moreover, NKT cells express CD40L, which induces costimulatory signals to B cells, promoting their activation (Kawano, Cui et al., 1997). NKT cells can also secrete IFN-γ, IL-5 and IL-6 cytokines, all of them found in the serum of our Mix+GalCer treated mice, enhancing the B-cell function (Leadbetter, Brigl et al., 2008).

Collectively, the data found by our work and other previous NKT-cell studies clearly shows that NKT-cell activation can orchestrate the entire immune system activation. Specifically, we demonstrate that Mix+GalCer treatment is a strong NKT-cell activator showing an impressive antitumor therapeutic efficacy, involving T-cell and B-cell activation and a critical role of NK cells in tumor eradication.

α-GalCer as a NKT-cell agonist for cellular immunotherapy: general and clinical considerations

The major obstacle that cancer immunotherapy has to deal with is the poor immunogenicity of most tumors and the most important key to overcome this trouble is breaking the immune tolerance. α-GalCer was tested in several clinical trials with cancer patients, demonstrating that it is safe (Giaccone, Punt et al., 2002). Despite of this, only transient NKT cell activation was detected in a minority of patients when α-GalCer was injected alone as a cancer treatment (Giaccone, Punt et al., 2002; Nieda, Okai et al., 2004). Other trials were carried out using DC pulsed α-GalCer and showed improved results, suggesting that is important to enhance α-GalCer presentation using DCs (Chang, Osman et al., 2005; Uchida, Horiguchi et al., 2008). Mix+GalCer therapeutic vaccine offers a promising improvement for DC-based vaccines against lymphoma, since it showed a potent antitumor effect in our B-cell lymphoma model, as well as the capacity of induce a memory and tumor-specific immune response. These last features are very important to avoid relapses and to guarantee a specific immune response against tumor with no damage to healthy tissues. The final goal of this study is to move

Mix+GalCer vaccine into the clinic to treat B-cell lymphoma patients and this can be possible since the vaccine components may be generated for clinical use under good manufacturing practices (GMP) conditions. α-GalCer and some of its analogues are available as a commercial glycolipid in GMP conditions, which is a requirement to initiate a clinical trial with patients. Human DCs can also be produced in GMP conditions and, in fact, this is a well-established procedure that has been used to carry out clinical trials with DC-based vaccines (Nieda, Okai et al., 2004; Chang, Osman et al., 2005; Uchida, Horiguchi et al., 2008). Mix+GalCer treatment has to be considered as a highly personalized medicine, where tumor biopsies from each patient will be taken to introduce the specific antigens into the vaccine. The strategy to generate the Mix+GalCer vaccine using the specific tumor antigens and the importance of them to assure the best antitumor outcome allow us to think whether this vaccine could be tested using other hematological or solid tumors as an antigen source to treat different types of malignancies with a few modifications. This approach opens great possibilities in the cancer immunotherapy-based treatments.

Antitumor effect of a novel NKT14m antibody

NKT14m is the first monoclonal antibody which can specifically activate NKT cells without DC-presentation dependence and it represents a novel tool to assess the therapeutic potential of NKT-cell activation. This monoclonal antibody binds directly to the invariant TCR on NKT cells and induces its activation (Scheuplein, Lamont et al., 2015). To our knowledge, the NKT14m antibody and its human homologue NKTT320 have not been tested in any clinical trial or pre-clinical mice model yet. *In vitro* studies have been done with NKT14m and it was shown that this antibody can trigger IFN-γ production by NKT cells in Balb/c mice. Moreover, human NKTT320 was also tested *in vitro* and it was demonstrated that it can induce NKT cell activation and proliferation in Vα24 transgenic mice (Scheuplein, Lamont et al., 2015).

We studied for the first time the *in vivo* antitumor effect of NKT14m antibody against B-cell lymphoma, using the 4TOO tumor mice model as in the case of Mix+GalCer therapy. Despite the moderate antitumor efficacy observed with a single-dose of NKT14m antibody, the antibody retreatment 6 weeks after tumor injection improved the antitumor efficacy, suggesting the possibility of a recurrent injection of the NKT14m as a promising therapeutic strategy. The possibility of administrating NKT14m antibody without inducing NKT-cell anergy (Scheuplein, Lamont et al., 2015) is a great advantage over α-GalCer since it will allow to perform maintenance treatments that hopefully will contribute to prevent relapses and increase survival of cancer patients. In another effort to improve the NKT14m efficacy, and taking into account the important role of available tumor antigens to build an efficient antitumor response (Shimizu, Kurosawa et al., 2007; Hong, Lee et al., 2013), we tested the combination of cyclophosphamide and NKT14m treatment. The antitumor effect was greatly improved (nearly 90%), suggesting that the availability of tumor antigens after cyclophosphamide treatment is important and effective. This approach has highly relevant clinical consequences since cyclophosphamide is a chemotherapeutic agent broadly use to treat lymphoma patients in clinical settings and this therapeutic strategy offers a promising treatment to move into the clinic. However, more studies are required to better understand the mechanisms that undergo the antitumor effect of NKT14m antibody and its therapeutic combination with cyclophosphamide.

Final remarks

Our studies using new therapeutic approaches for B-cell lymphoma treatment elucidate the large efficacy of NKT cell activation to induce an effective antitumor response. If the Mix+GalCer and NKT14m treatments are considered, we noted that α-GalCer therapy with DCs and tumor antigens induced a potent IFN-γ response, as well as a better survival than the NKT14m antibody treatment. In fact, a single-dose of Mix+GalCer vaccine causes the major antitumor effect that is possible in a therapeutic setting, suggesting that is a good therapy against B-cell

lymphoma in mice. Although NKT14m treatments seemed to be less efficient than Mix+GalCer vaccine, it is a useful and promising therapy that could brings clinical advantages. Thus, in contrast to the NKT-cell anergy induced by α-GalCer, the NKT14m antibody therapy allows the retreatment 6 weeks after the first injection, which is a very interesting feature in the clinical setting. Moreover, the antibody allows retreating mice using the same antibody or α-GalCer (Scheuplein, Lamont et al., 2015), making feasible the combination of NKT14m and Mix+GalCer treatments to improve their antitumor effect. In addition, the humanized agonistic NKT14 antibody is ready for clinical use in patients, which can facilitate the translation of this approach to treating patients with cancer.

6. CONCLUSIONS

1. A vaccine consisting of DCs, tumor cells and the NKT-cell agonist α-GalCer (Mix+GalCer) induces a potent, long-lasting and tumor-specific immune response that eradicates B-cell lymphoma *in vivo*.

2. The Mix+GalCer vaccine induces an *in vivo* NK and NKT-cell expansion in spleen.

3. The cytokine profile induced by Mix+GalCer treatment is composed by the combination of Th1-type cytokines (mainly IFN-y and IL-12), Th2-type cytokines (IL-4, IL-5 and IL-6) and IL-17 which is according to a strong activation of NKT cells.

4. The Mix+GalCer vaccine induces an increase of IFN-γ secreting NKT, $CD4^+$ and $CD8^+$ T cells.

5. In our B-cell lymphoma model, NK cells play a critical role in the antitumor effect induced by Mix+GalCer treatment, whereas $CD4^+$ and $CD8^+$ T cells are not essential to eradicate the tumor.

6. In addition to a cellular response, the Mix+GalCer treatment stimulates a humoral response that specifically recognizes the tumor cells.

7. The novel agonistic NKT cell antibody NKT14m shows an effective antitumor efficacy that is improved by its combination with chemotherapy (i.e., cyclophosphamide) treatment.

8. This work supports the use of NKT cell agonists as immunotherapy for the treatment of patients with B-cell lymphoma.

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