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# HARNESSING iNKT AND CD8 T CELLS FOR IMMUNOTHERAPY OF CANCER

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**Département d'Oncologie Fondamentale**

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## Monsieur Benjamin Tschumi

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**Harnessing iNKT and CD8 cells for Immunotherapy of Cancer**

Lausanne, le 13 mars 2018

pour le Doyen
de la Faculté de biologie et de médecine

Valentin Rousson

Prof. Valentin Rousson

# A Résumé (français)

Mon projet de thèse s'inscrit dans le cadre de l'immunothérapie du cancer qui consiste en différents traitements visant à optimiser la réponse immunitaire du patient contre ses cellules cancéreuses.

Mon travail de thèse s'articule autour de trois projets liés à l'immunothérapie anti-cancer. Dans le premier projet, mon intérêt s'est porté sur la manipulation contre les tumeurs des lymphocytes invariant Natural Killer T cells (iNKT). En l'occurrence, j'ai étudié les mécanismes responsables de l'induction de l'anergie des cellules iNKT suite à une première stimulation par le ligand $\alpha$-galactosylceramide, qui limite grandement l'efficacité de ces cellules. Notre étude comparative d'analogues de l'αGalCer a démontré que les glycolipides de structure chimique plus soluble connus pour induire une réponse de type Th2, provoquaient beaucoup moins d'anergie que les ligands de type Th1 plus hydrophobes. En outre, nous avons montré que n'importe quelle cellule présentatrice d'antigènes exprimant le CD1d est capable d'induire l'anergie des cellules iNKTs lorsque chargée avec un ligand « Th1 », et que la présentation des glycolipides dans les domaines « lipid rafts » particulièrement présents dans les cellules dendritiques (DCs) ne semble pas être un prérequis pour l'induction de l'anergie des lymphocytes iNKTs. Notre hypothèse est que l'induction de l'anergie par différents analogues de l'αGalCer résulte de l'interaction du ligand avec le récepteur de cellules T (TCR) iNKT. Dans le second projet, j'ai investigué comment améliorer l'effet adjuvant des cellules iNKT sur la réponse antitumorale des lymphocytes T CD8. Dans ce cadre, j'ai choisi un analogue αGalCer de type Th1, qui confère aux cellules iNKT de meilleures propriétés transactivatrices sur la maturation des DCs. J'ai pu confirmer l'efficacité des DCs chargées avec l'αGalCer en tant que vaccin anticancer. De plus j'ai démontré de manière innovante une importante amélioration de l'effet antitumoral en ajoutant à la vaccination αGalCer/DCs, un traitement anti-Programmed cell death protein 1 (PD1), connu pour restaurer la fonctionnalité des lymphocytes T dans les tumeurs. Mes résultats montrent aussi que l'importance de l'effet additif entre ces deux traitements dépend de l'immunogénicité du modèle tumoral.

Finalement, mon troisième projet investigue la fonctionnalité *in vivo* du TCR endogène des lymphocytes T CD8 lorsque transduits avec un récepteur d'antigène chimérique (CAR). Nous avons constaté que lorsque ces lymphocytes T co-expriment un CAR et que leur TCR est activé par une infection bactérienne (Listeria-OVA) ou virale (LCMV), ces cellules meurent par apoptose 7 jours après l'infection. Nous avons découvert que ces lymphocytes, mais pas les cellules contrôles, expriment de hauts niveaux de Fas et FasL et meurent généralement entre les jours 6 et 7 post-infection. L'apoptose massive des cellules CAR lorsque activées par leur TCR ne peut pas être évitée par le blocage de Fas, suggérant qu'il existe des mécanismes parallèles à Fas induisant leur apoptose. L'ensemble de mes résultats a amélioré la compréhension des lymphocytes iNKT et de leur potentiel en immunotherapie du cancer, et a révélé un mécanisme très important pour l'optimisation de l'immunothérapie utilisant des CAR.

# B Summary (english)

My PhD project has been conducted in the context of cancer immunotherapy which aims to manipulate the immune system of the cancer patient against his tumor.

More specifically, my thesis work has focused on three projects related to cancer immunotherapy. In the first project, I have studied the mechanisms underlying the onset of anergy in invariant Natural Killer T lymphocytes (iNKT) after a single injection of the CD1d ligand α-galactosylceramide (αGalCer). Our comparative study of various analogs of αGalCer has shown that rather polar ligands known to generate a Th2 type of iNKT cell response were also inducing less anergy than the apolar Th1 type αGalCer ligands. We also observed that all antigen presenting cells (APCs) induced similar levels of iNKT cell anergy when loaded with Th1 glycosphingolipids (GSLs). Finally, we observed that enriched GSL presentation within lipid raft domains of APCs, which is a hallmark of Th1 GSLs, is likely not required to induce iNKT cell anergy. Our hypothesis is that the induction of iNKT cell anergy might rather depend on the iNKT T cell receptor (TCR) recognition of the glycolipid.

My second project aimed at improving the adjuvant activity of the iNKT cell agonist glycolipid αGalCer, when formulated as a dendritic cell (DC) vaccine against cancer. In this concept, I chose a Th1-type αGalCer analog, as we demonstrated their superior capacity to induce the transactivation of DCs by iNKT cells. I could confirm the efficacy of αGalCer-loaded DCs to generate an antitumor CD8 T cell response. Importantly, I revealed the superior antitumor activity of the combined iNKT/DC vaccine with Programmed cell death protein 1 (PD1) blockade, known to restore the functionality of intratumoral T cells, as compared to the treatments alone. My results also showed that the combined antitumor activity between DC/αGalCer vaccine and anti-PD1 depended on the immunogenicity of the tumor model.

Finally, my last project investigated the functionality of the resident TCR in CD8 T cells transduced with a chimeric antigen receptor (CAR). We observed that CAR CD8 T cells undergo massive apoptosis *in vivo* when activated via their TCR in the context of a bacterial or viral infection. Furthermore, CAR T cells, but not mock-transduced T cells, express high levels of Fas and FasL from day 6 post infection. Finally, the *in vivo* CAR T cell deletion upon TCR stimulation could not be rescued by the blockade of Fas signalling, suggesting that parallel signalling pathways contribute to the apoptosis of TCR-engaged CAR T cells. Altogether, my results have improved the comprehension of iNKT cells and in particular their potential for therapeutic cancer vaccines. Moreover, the results of the third project have revealed an important mechanism in CAR T cell therapy, which may allow new improvements for its application to solid tumors.

# C Résumé Large Public (français)

De nombreuses études ont démontré l'énorme potentiel thérapeutique des approches d'immunothérapie du cancer. Celles-ci se fondent sur des méthodes visant à améliorer la réponse immunitaire du patient contre sa tumeur. Toutefois, des progrès dans la compréhension du système immunitaire et de son interaction avec les tumeurs sont nécessaires afin d'utiliser au mieux ces nouvelles thérapies. Ce projet de thèse traite de trois problématiques relatives à l'immunothérapie. La première étudie les lymphocytes invariant Natural Killer T (iNKT) qui représente une intéressante cible immunothérapeutique car, une fois activées par le glycolipide αGalactosylCeramide (αGalCer), ces cellules activent la maturation des cellules dendritiques, qui sont très importantes pour l'initiation d'une réponse immunitaire adaptative. Malheureusement, après une première activation, les lymphocytes iNKT deviennent anergiques, ce qui signifie qu'ils ne peuvent plus répondre à des stimulations ultérieures. Nous avons découvert que l'anergie induite par le glycolipide αGalCer ne dépend pas de la nature de la cellule qui le présente aux lymphocytes iNKT, ni de la durée de cette interaction, ni du domaine membranaire dans lequel il est présenté. Notre hypothèse basée sur nos résultats est qu'elle dépend de facteurs intrinsèques aux glycolipides utilisés. Dans le second projet, nous démontrons que la vaccination de souris avec des cellules dendritiques chargées avec le glycolipide αGalCer combiné à un antigène tumoral est très efficace dans l'induction d'une réponse immunitaire dirigée contre la tumeur médiée par les lymphocytes T CD8. De plus, nous montrons que ce vaccin peut être amélioré par l'ajout d'un traitement anti-Programmed cell death protein 1 (PD1) connu pour restaurer la fonctionnalité des lymphocytes T à la tumeur. Finalement, dans le troisième projet, nous étudions la fonctionnalité du récepteur endogène des lymphocytes T transduits pour exprimer un CAR, qui est un récepteur antitumoral artificiel de haute affinité. Cette approche a montré des effets cliniques spectaculaires dans le traitement de leucémies. Dans ce projet, nous démontrons que l'activation des lymphocytes T CD8 via leur CAR ne pose aucun problème *in vivo*, mais que leur activation via leur récepteur TCR endogène induit le suicide de ces cellules, qui reçoivent un signal de mort. La voie de signalisation d'apoptose Fas/FasL semble impliquée mais pas suffisante, car son blocage ne permet que partiellement la survie des cellules. Finalement, ces résultats expliquent pourquoi les essais d'activation combinées TCR et CAR ont échoué et sont donc de première importance pour les développements futurs de thérapies utilisant les CARs pour le traitement de tumeurs solides.

# D Acknowledgements

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# E Abbreviations

| | |
|---|---|
| ACT | Adptive cell transfer |
| ADCC | Antibody-dependent cell-mediated cytotoxicity |
| AICD | Activation-induced cell death |
| αGalCer | -GalactosylCeramide |
| β2m | β2-microglobulin |
| CAR | Chimeric antigen receptor |
| CTLA-4 | Cytotoxic T lymphocyte-associated molecule-4 |
| DAG | Diacyl glycerol |
| DAMPs | Damage-associated molecular patterns |
| DC | Dendritic cell |
| EDTA | Ethylenediaminetetraacetic acid |
| FCS | Fetal calf serum |
| GSL | Glycosphingolipid |
| HER2 | Human Epidermal Growth Factor Receptor-2 |
| Hexb | β-hexosaminidase |
| iGb3 | Isoglobotrihexosylceramide |
| IL-2/7/15 | Interleukin-2/7/15 |
| IL7R α | IL7 receptor subunit α |
| IDO | Indoleamine 2,3-dioxygenase |
| $IP_3$ | Inositol 1,4,5-trisphosphate |
| ITAM | Immunoreceptor tyrosine-based activation motif |
| KLRG1 | Killer cell lectin-like receptor subfamily G member 1 |
| MDSC | Myeloid-derived suppressor cell |
| MPL | Monophosphoryl lipid A |
| mTORC1 | Mechanistic target of rapamycin complex 1 |
| NK | Natural Killer |
| NSCLC | Non-small-cell lung cancer |
| $PIP_2$ | phosphatidylinositol 4,5-bisphosphate |
| PD1 | Programmed cell death 1 |
| PKC θ | Protein Kinase C θ |
| PTEN | Phosphatase and tensin homolog |
| RasGRP | RAS guanyl nucleotide- releasing protein |
| ROR γ | RAR-related orphan receptor gamma |
| SHP-2 | SH2-domain containing tyrosine phosphatase 2 |
| TAA | Tumor-associated antigen |
| TAM | Tumor-associated macrophage |
| TCR | T-cell receptor |
| TILs | Tumor-associated lymphocytes |
| TLR | Toll-like receptor |

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# G Introduction

## G.1 Invariant Natural Killer T cells

Invariant Natural Killer T cells or iNKT cells belong to the family of innate-like T cells, such as γδ T cells and MAIT. iNKT cells recognize lipid antigens presented on CD1d and express a semi-invariant T cell receptor: the invariant Vα14Jα18 chain in mouse or Vα24Jα18 in human combined with Vβ2/7/8.2 in mouse and Vβ11 in human[1]. They usually co-express NK-specific receptor such as NK1.1 or DX5 in mice and NK1.1 or CD56 in humans[2,3], and were identified by their recognition of the marine sponge-derived glycosphingolipid (GSL) α-GalactosylCeramide (αGalCer) in the context of CD1d[3,4].

| | **CD4 T cells** | **CD8 T cells** | **iNKT cells** |
|---|---|---|---|
| Antigen recognition | peptides on MHC II | peptides on MHC I | lipids on CD1d |
| Co-receptor expression | CD4 | CD8 | DN or CD4 in mice<br>DN, CD4 or CD8 in humans |
| Localization | Lymphoid organs | Lymphoid organs | Lymphoid organs and liver |
| Frequency | 35% in spleen | 40% in spleen | 2.5% of T cells in the spleen and 30% in the liver[3] |
| Subsets | Th1, Th2, Th17 or Treg | Cytotoxic T lymphocytes | NKT1, NKT2, NKT17, NKT10[5–8] |

Table 1 - Comparative description of iNKT cells and conventional T lymphocytes.

### G.1.1 Antigen recognition

The iNKT TCR recognize lipid antigens presented on CD1d, which is structurally very similar to MHC I[9]. iNKT cells were identified because of their reactivity to the prototypic agonist αGalCer, which has the optimal binding capacity to CD1d[10] and was originally discovered because of its antitumor properties[11]. It consists of an acyl and a sphingosine chain of 26 and 15 carbons respectively, bound to a galactose sugar ring by an α glycosidic bond, which is not present in mammals.

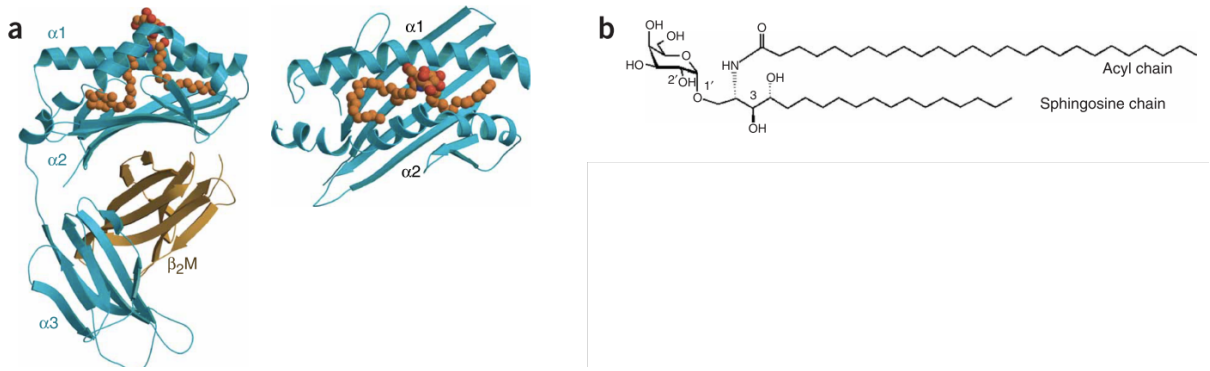


Figure 1 - a: the crystal structure of human CD1d bound to αGalCer from the side and from the top. b: the chemical structure of αGalCer. Adapted from [10].

Like for MHC I molecules, the α1 and α2 domains of CD1d form the antigen binding site, while the α3 domain is responsible for the interaction with β2-microglobulin (β2m)[12,13] (Figure 1). However, instead of the peptide binding groove present in MHC I and II molecules, the CD1d molecule is characterized by a two deep pockets named A' and F' in mouse and A' and C' in human, which are very hydrophobic[12] and interact with the lipid chains of the GSL[10,13]. For instance, a study of the crystal structure of human CD1d loaded with αGalCer showed that the acyl chain of the glycosphingolipid is buried in the A' pocket and the sphingosine chain in the C' cavity, while the galactose ring protrudes outside of the binding pocket and is exposed to the TCR[10]. Indeed, the combined crystal structure of the human iNKT TCR bound to CD1d/αGalCer pointed out that the TCR invariant α chain plays the major role in the interaction with CD1d/αGalCer with the CDR1 domain interacting only with αGalCer, and the CDR3 loop recognizing both CD1d and αGalCer[14], while Indeed, the CDR2 and 3 domains of the β chain interact exclusively with CD1d.

Co-incubation experiments of DCs, iNKT cells and heat-killed bacteria showed that some bacteria species such as *Ehrlichia muris* and *Sphingomonas capsulata* could induce iNKT cell activation even when DCs were deficient for the Toll-like receptor (TLR) signalling components MyD88 and Trif, while others such as *Salmonella typhimurium* required functional MyD88 and Trif signalling in DCs to properly activate iNKT cells. However, in all cases, CD1d expression on DCs was always required for iNKT cell activation[15], and DCs deficient in the β-hexosaminidase (hexb) enzyme required for the synthesis of the self-derived glycolipid iGb3[16], were not able to stimulate iNKT cells when co-incubated with *Salmonella* bacteria[15]. Along the same line, a study combining *in vitro* and *in vivo* data showed that activation of iNKT cells by DCs upon *Salmonella typhimurium* infection depended on both the presentation of endogenous

glycolipids and IL-12 secretion by DCs[17]. Furthermore, blockade of iGb3 recognition with a lectin precluded the activation of iNKT cells by DCs when the cells were cocultured with *Salmonella*, but not *Ehrlichia* or *Sphingomonas* species[15,16]. Collectively, these observations demonstrate that iNKT cell activation by bacterial components can occur either via the direct recognition of bacterial glycolipids presented by CD1d-expressing DCs, or indirectly via IL-12 released by TLR- activated DCs combined with the CD1d presentation of the self-GSL iGb3. The two models of iNKT activation are illustrated on Figure 2.

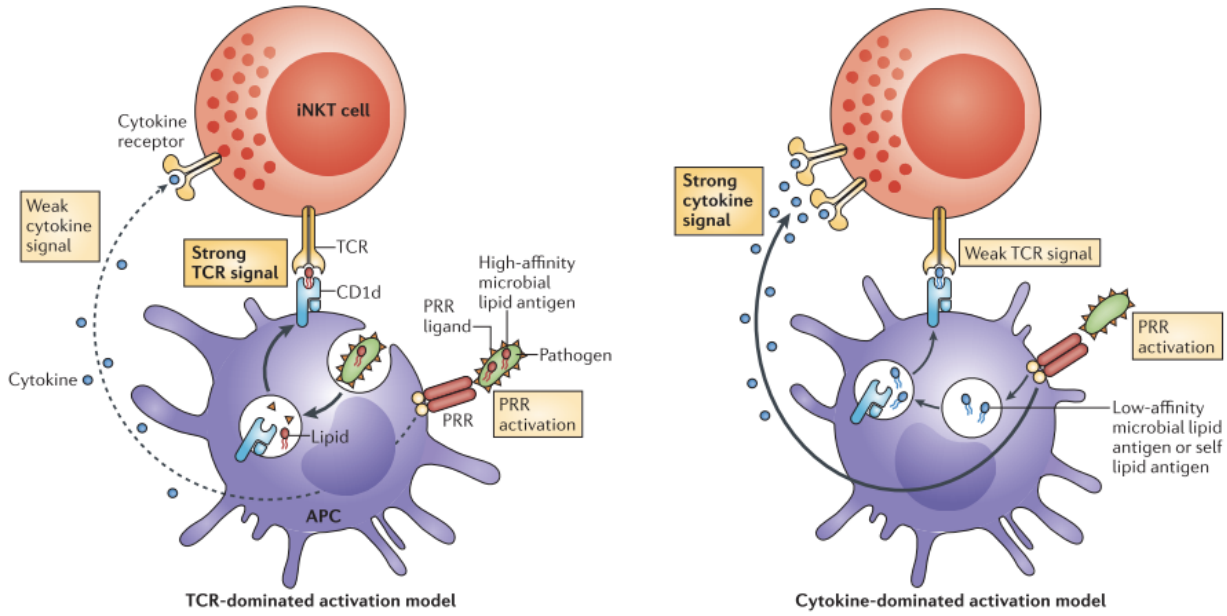


Figure 2 - The two models of iNKT cell activation. iNKT cells can be activated by the direct presentation of bacteria-derived lipids or by self-lipids and cytokines. Adapted from [18].

### G.1.2 Ligands presented to iNKT cells: self, microbial or artificial

Most glycolipids presented on CD1d to iNKT cells are glycosphingolipids (GSLs), which have an α anomeric bond between the sugar head and the sphingolipid tail, such as αGalCer and α-galacturonosyl ceramide, which is found in *Sphingomonas* bacteria[19]. Another class of CD1d ligands are glycerolipids, such as diacylglycerolipids found in the pathogenic bacteria *Borrelia burgdorferi*[20,21].

Mammalians are not able to synthesize glycolipids with an α anomeric bond[22], but self-derived glycolipids with a β-anomeric bond have been shown to bind to CD1d and activate iNKT cells. A well characterized example is isoglobotrihexosylceramide (iGb3). It is present in the mouse thymus[23] and was proposed to play a role in the positive selection of iNKT cells[24]. The reason why molecules with different structures, such as pathogen-derived α-linked glycolipids and self-derived β-linked lipids, can be recognized by the iNKT TCR came from the resolution of

the crystal structure of CD1d/iGb3/TCR. Indeed, it revealed that the interaction with the TCR modifies the orientation of the β-linked sugar moiety towards an orientation that resembles that of α-linked lipids[25], explaining how a single TCR can recognize structurally different antigens.

### G.1.3 The crosstalk of iNKT cells with innate and adaptive immunity

iNKT cells play pleiotropic roles in the immune system by interacting with multiple immune cells, of both myeloid and lymphoid lineages.

First, the crosstalk of iNKT cells with DCs has been extensively described. Indeed, DC presentation of αGalCer/CD1d to iNKT cells combined with CD40-CD40L interaction induces the production of IL-12 by DCs and the upregulation of the IL-12 receptor, with a concomitant up-regulation of CD40L and increased IFNγ secretion by iNKT cells[26]. Hence blockade of IL-12 or CD40 results in a strong decrease of iNKT cell activation[27].

Importantly, the crosstalk between iNKT cells and DCs, results in a fast and potent transactivation of NK cells via both the IL-12 secreted by DCs and the massive production of IFNγ by activated iNKT cells[28].

iNKT cells also interact with B cells. A study has shown that coculture of iNKT cells and B cells enhances the maturation of the B cells and also immunoglobulin production[29]. Another study showed that vaccination with αGalCer increases the antibody response, and this effect is mediated by CD40L presentation on iNKT cells to B cells. Furthermore iNKT cells support the homeostatic maintenance of memory B cells[29].

Finally, iNKT cells also influence the function of macrophage. In a model of *Borrelia burgdorferi* infection in mice, the IFNγ secretion by activated iNKT cell upon recognition of bacteria-derived lipid antigens[30], directly enhanced the phagocytic activity of macrophages and also increased their CD1d expression, suggesting a positive feedback loop between macrophages and iNKT cells. In contrast, resident iNKT cells in adipose tissue rather exhibit an anti-inflammatory M2 macrophage polarization and prevent macrophage-induced inflammation through IL-10 production[31,32]. Overall, the outcome of iNKT cell activation is context-dependent, whether in pathogenic or homeostatic conditions.

### G.1.4 The development of iNKT cells

iNKT cells differentiate from T cell progenitors and their specific fate is determined at the double-positive (DP) stage, when immature thymocytes express both CD4 and CD8 and rearrange their T cell-receptor[33]. The key signal in acquiring the iNKT cell identity is the

presentation of self-glycolipids by other DP thymocytes, which will select for the mature semi-invariant TCR[34]. Immature T cells expressing the iNKT TCR will undergo a specific differentiation program, starting at the stage 0, characterized with the expression of the surface markers CD4, CD69 and CD24. Then, stage 1 is defined by the downregulation of CD24. Afterwards, stage 2 cells upregulate CD44, a marker of effector lymphocytes. Finally, stage 3 is associated with the acquisition of NK-associated markers. Differentiation to stage 3 occurs both in the thymus and in the periphery[33] (Figure 3). A key transcription factor involved in the constitutive effector functions of iNKT cells is PLZF and its ablation impairs iNKT cell thymic expansion[35]. PLZF is highly expressed in stages 1 and 2 of iNKT cell maturation and its expression decreases at stage 3. Interestingly, PLZF-deficient iNKT cells show a phenotype comparable to naïve T cells, characterized by a deficient secretion of effector cytokines and by the expression of CD62L which relocate them to lymph nodes[35]. The induction of PLZF expression is directly induced by Egr2, which results from the strong TCR signalling in early iNKT cell precursors[33]. The high TCR signalling in iNKT cell precursors does not induce their cell death them by negative selection, as it does during thymic development of CD4 and CD8 T cells. Their survival likely result by their selection mediated by DP thymocytes, which do not express CD80 or CD86 in contrast to thymic stromal cells, responsible for the thymic selection of CD4 and CD8 T cells[36,37]. Instead iNKT precursors receive a signal through Slamf1 (CD150) and Slamf6 (Ly108), which is necessary for their development[38]. In addition to Slam molecules, several pathways are selectively required for iNKT cell development, but not for conventional T cells, such as c-Myc[39] or mTORC1[40].

Finally, RORγt plays also a central role in iNKT cell development. Indeed, this transcription factor increases the lifespan of thymocytes undergoing TCR rearrangement. Its ablation prevents thymocytes from rearranging the Vα14 and the Jα18 gene fragments together[41].

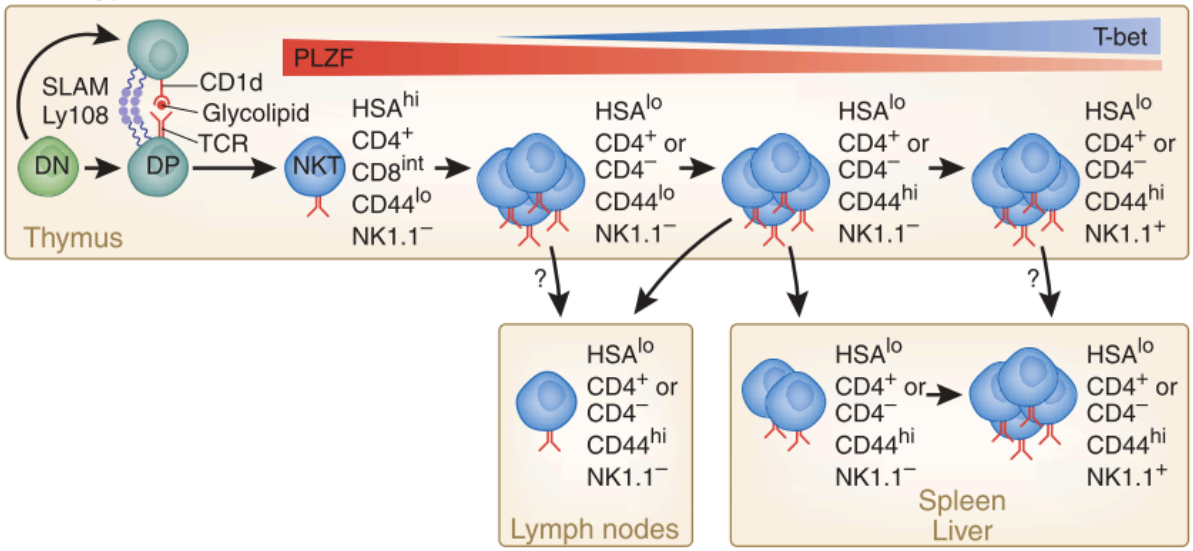


Figure 3 - iNKT cell development and maturation occurs through distinct stages, which can be identified with the expression of specific surface markers. Adapted from [42].

### G.1.5 iNKT cell subsets

Like CD4 T cells, iNKT cells are subdivided into different functional subsets, characterized by defined cytokine profile and expression of transcription factors. In mice, NKT1 cells express the transcription factor T-bet, often express NK1.1 and CD122 and secrete IFNγ. They can be $CD4^+$ or $CD4^-$. iNKT2 cells express CD4 and IL-17RB and produce typical Type-2 cytokines such as IL-4 and IL-13[18]. iNKT17 cells do not express CD4 or NK1.1 and secrete IL-17A[5]. Finally, a regulatory subset of iNKT cells has been identified and named NKT10. These NKT10 express surface molecules similar to regulatory CD4 T cells, but do not express their master transcription factor FoxP3. They exert a regulatory function through IL-10 secretion[6].

Unlike mouse iNKT cells, their human homologs can express CD8. $CD8^+$ and $CD4^-CD8^-$ have the highest expression of NK-related receptors such as CD56 and NK1.1. They also secrete more Type-1 cytokines than their $CD4^+$ counterparts. Alternatively, $CD4^+$ iNKT cells are more potent to secrete Type-2 cytokines than the other subsets[43].

### G.1.6 αGalCer analogs differentially polarize iNKT cells

Several derivatives of αGalCer have been synthesized and characterized by their ability to induce preferentially a Th0, Th1 or Th2 response in murine iNKT cells, which has been defined by the ratio between the serum levels of IFNγ at 18h and IL-4 at 2h post injection, and also by their ability to transactivate NK cells. The mechanisms underlying the differential capability of such antigens to bias the polarization of iNKT cell are still debated. The group of Steven Porcelli has shown that Th2-biasing αGalCer analogs are more polar than their Th1 counterparts and

can load on CD1d directly at the cell surface of APCs, while the more hydrophobic Th1 GSLs are internalized and require endosomal lipid transfer factors mainly of the saposin family to be loaded on CD1d (Figure 4). As a result, Th2 GSLs result in a fast and transient iNKT cell activation with a peak of IFNγ at 6h, while Th1 GSLs lead to a delayed iNKT cell response with the peak of IFNγ at 18h post injection. In addition, CD1d loaded with Th1-biasing GSLs were shown to be preferentially localized within lipid rafts[44], while Th2-biasing GSLs were excluded from these cholesterol-rich membrane micro-domains, and were quickly detached from CD1d upon endosomal trafficking[45]. However, upon neutralization of endosomal acidification, increased localization of Th2 GSLs in lipid rafts was obtained which resulted from their stabilized CD1d presentation and thus allowed them to acquire Th1 properties[46]. These results clearly suggest that the site of CD1d loading and presentation likely explains the properties of various αGalCer analogs.

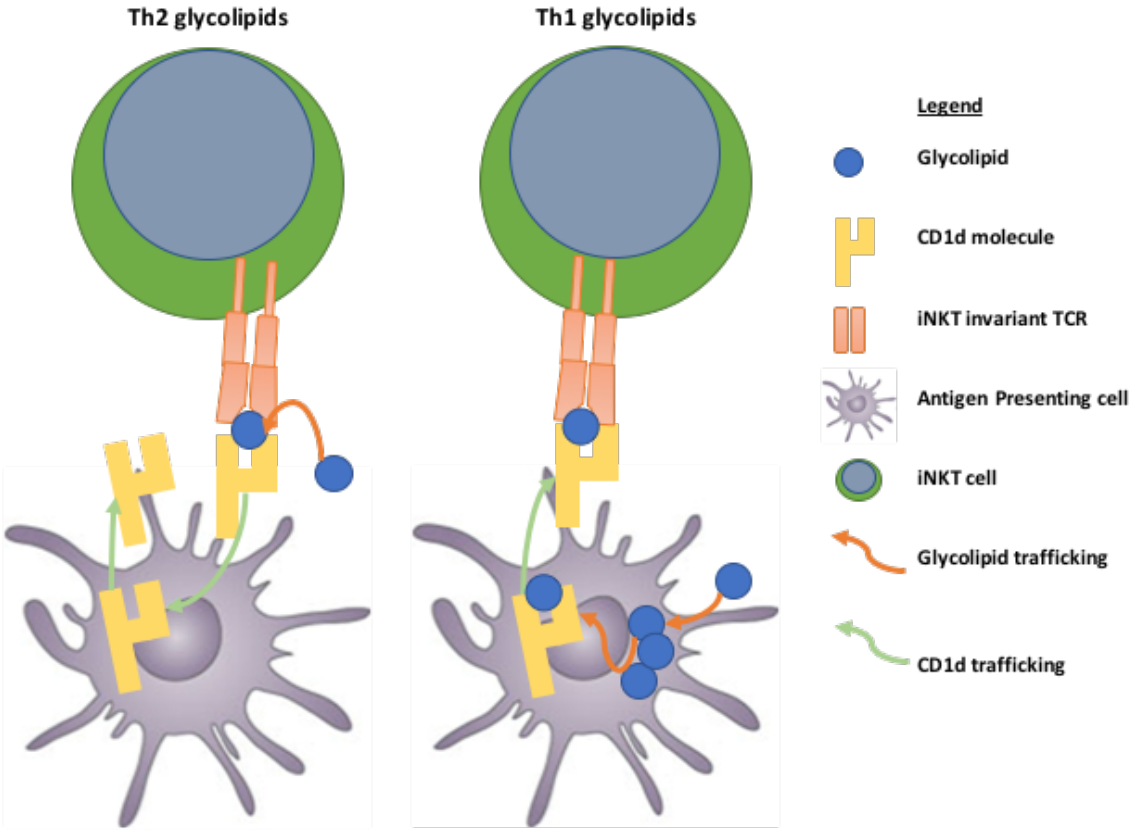


Figure 4 - Th1 glycolipids require endocytosis and are loaded on CD1d in acidic endosomes, while their Th2 counterparts are surface-loaded but rapidly unloaded upon CD1d recycling through endosomes.

Th1 or Th2 αGalCer analogs also exhibit different affinities for APCs, as Th1 GSLs load preferentially on DCs, which are rich in lipid rafts. Another study by Bendelac *et al* postulated that Th1 GSLs were mainly presented by DCs *in vivo* and Th2 GSLs by non-professional APCs,

such as B cells, explaining the different ability of these molecules to bias the cytokine response of iNKT cells[47]. This hypothesis was however challenged by a study from Porcelli *et al.* demonstrating that CD8α DCs are the main APCs responsible for both Th1 and Th2 GSLs presentation *in vivo*[48], which could also result from their highest CD1d expression, as compared to B cells or macrophages.

### G.1.7 iNKT cell anergy

The injection of αGalCer in mice strongly activates iNKT cells resulting in cytokine secretion and cell proliferation. However, iNKT cells rapidly downregulate their TCR or die by apoptosis and within one week, their frequency is back to steady state. Interestingly, after this initial antigen stimulation, iNKT cells become unresponsive to a second challenge with αGalCer, as they fail to proliferate, secrete cytokines and transactivate other cells such as NK cells[49]. This phenomenon is referred as iNKT cell anergy or hyporesponsiveness. The mechanisms underlying iNKT cell anergy are still poorly understood. However, some molecular players have been identified. Indeed, blockade of PD1 during the priming with αGalCer partially prevents the induction of iNKT hyporesponsiveness. However, iNKT cell anergy cannot be reverted by PD1 blockade once it has been induced[50]. Later, it was hypothesized that TCR signalling with CD28 costimulation is more potent at rendering iNKT cells anergic than TCR signalling alone and thus DCs loaded with αGalCer are better iNKT cell anergy inducers than B cells[51]. Another study showed that Cbl-b-deficient iNKT cells are resistant to anergy since cbl-b was shown to promote the degradation of CARMA-1, an key molecule of the NF- κB pathway[52]. Finally, the deficiency of TSC1, a negative regulator of mTORC1 renders iNKT cells resistant to anergy induction. Indeed these cells failed to upregulated PD-1, Egr2 and Grail, which are negative regulators of T cell activation[53].

Interestingly, one recent study has shown that Th2-biasing αGalCer derivatives induce less iNKT cell anergy than their Th1-biasing counterparts[54], suggesting that the kinetic of iNKT cell activation might directly influence the responsiveness of iNKT cell to re-challenge. In support of this hypothesis, studies in our group have shown that CD1d-antitumor fusion proteins lead to potent but transient iNKT cell activation, which maintained them reactive to multiple antigen restimulation, similarly to Th2 αGalCer analogs[55,56]. Finally, one recent study revealed that instead of becoming anergic, iNKT cells may rather change their cytokine profile upon antigen stimulation, and differentiate into a regulatory phenotype resembling that of regulatory T cells[57].

### G.1.8 Lipid antigen presentation by CD1d

CD1d expression is restricted to thymocytes and APCs, such as DCs, B cells and macrophages[3]. Molecules of the CD1 family are folded and assembled with β2m in the endoplasmic reticulum, helped by the chaperones calnexin and calreticulin[58,59]. After folding, CD1d molecules traffic to both the cell membrane and late endosomes[60]. However, most CD1d molecules go first to the cell membrane and are then rapidly re-internalized and targeted to endosomes before going back to the membrane[61]. The endosomal loading of lipid antigens on CD1d depends mainly on the lipid transfer saposin B[62]. On the cell surface, association with the adaptor proteins AP2 and AP3 is required for the targeting of CD1d from the membrane to late endosomes[63,64].

### G.1.9 The adjuvant effect of iNKT cells on adaptive immunity

The interaction between iNKT cells and DCs results in the increased maturation of DCs. Thus, presentation of αGalCer to iNKT cells and T cell peptide antigens by the same DC drastically increases the T cell response[65,66], as DC maturation is a key step for the initiation of T cell immunity.

This occurs through a well-described mechanism in which the recognition of glycolipids loaded on CD1d on DCs by iNKT cells triggers their secretion of IFNγ, which favours the production of IL-12 by DCs[67]. The presentation of CD40L by iNKT cells to DCs enhance the maturation of the latter. This process occurs through a positive feedback loop as enhanced IL-12 secretion and costimulatory molecules presentation on DCs induces more CD40L expression on iNKT cells. This process finally results in the generation of mature APCs that can efficiently prime T cells[68,69].

As mentioned previously, the presentation of iNKT cell ligands by B cells to iNKT cells enhances the maturation of B cells and their antibody production[70,71]. Thus, on top on their adjuvant role on T cell immunity, iNKT cells are as well potent boosters of the humoral immunity provided by B cells.

## G.2 CD8 T cell-mediated immunity

CD8 T cells are cytotoxic lymphocytes playing a central role in the control of viral infections, intracellular pathogens and neoplasms by the adaptive arm of the immune system. Unlike iNKT cells, they express a very diverse range of TCRs enabling them to recognize a large panel of peptides on MHC-I molecules. Furthermore, in contrast to iNKT cells, CD8 T cells reach the periphery with a naïve phenotype and require antigen priming by professional APCs to acquire their effector functions[72].

### G.2.1 The development of CD8 T cells

Unlike B lymphocytes, which mature in the bone marrow, a portion of lymphoid progenitors migrate to the thymus where they acquire the T lymphocyte identity[73]. As these T lineage-committed cells do not express yet either the CD4 or CD8 coreceptor, they are named DN for double-negative. DN T cells differentiate through 4 steps, identified by the expression of CD25 and CD44: DN1 are CD44+ CD25-, DN2 are CD44+ CD25+, DN3 are CD44- and CD25+ and DN4 are negative for both molecules[74]. DN3 thymocytes express a rearranged β TCR chain, which is expressed at the cell surface with a surrogate α chain[75]. A functional signalling by this pre-TCR complex allows the thymocytes to undergo somatic rearrangement of the TCR α chain[76,77]. Late DN4 thymocytes express a T-cell receptor with a rearranged α and β chains which differentiate into the DP $CD4^+CD8^+$ stage[78].

DP thymocytes undergo several selection steps to ensure that their TCR is functional, reactive to self-MHC, but not reactive to self-derived peptide antigens. In the first selection process called positive selection, thymocytes bearing TCRs with weak reactivity for self MHC/peptides commit apoptosis because of insufficient TCR signalling. On the other hand, excessive TCR signalling also leads DP thymocytes to apoptosis during the negative selection step, allowing the removal of potentially harmful self-reactive T cells[77]. Finally, DP thymocytes undergo the lineage choice by losing the expression of either CD4 or CD8 provided they were selected by respectively MHC I or MHC II molecules, resulting in mature $CD4^+$ or $CD8^+$ SP thymocytes. Evidences have shown that the correct co-receptor choice to pair with the TCR is guided by differences in signalling through CD4 and CD8[79,80]. Mature CD4 or CD8 SP T cells finally leave the thymus to the periphery. An overview of the steps of T cell development in the thymus is illustrated on Figure 5.

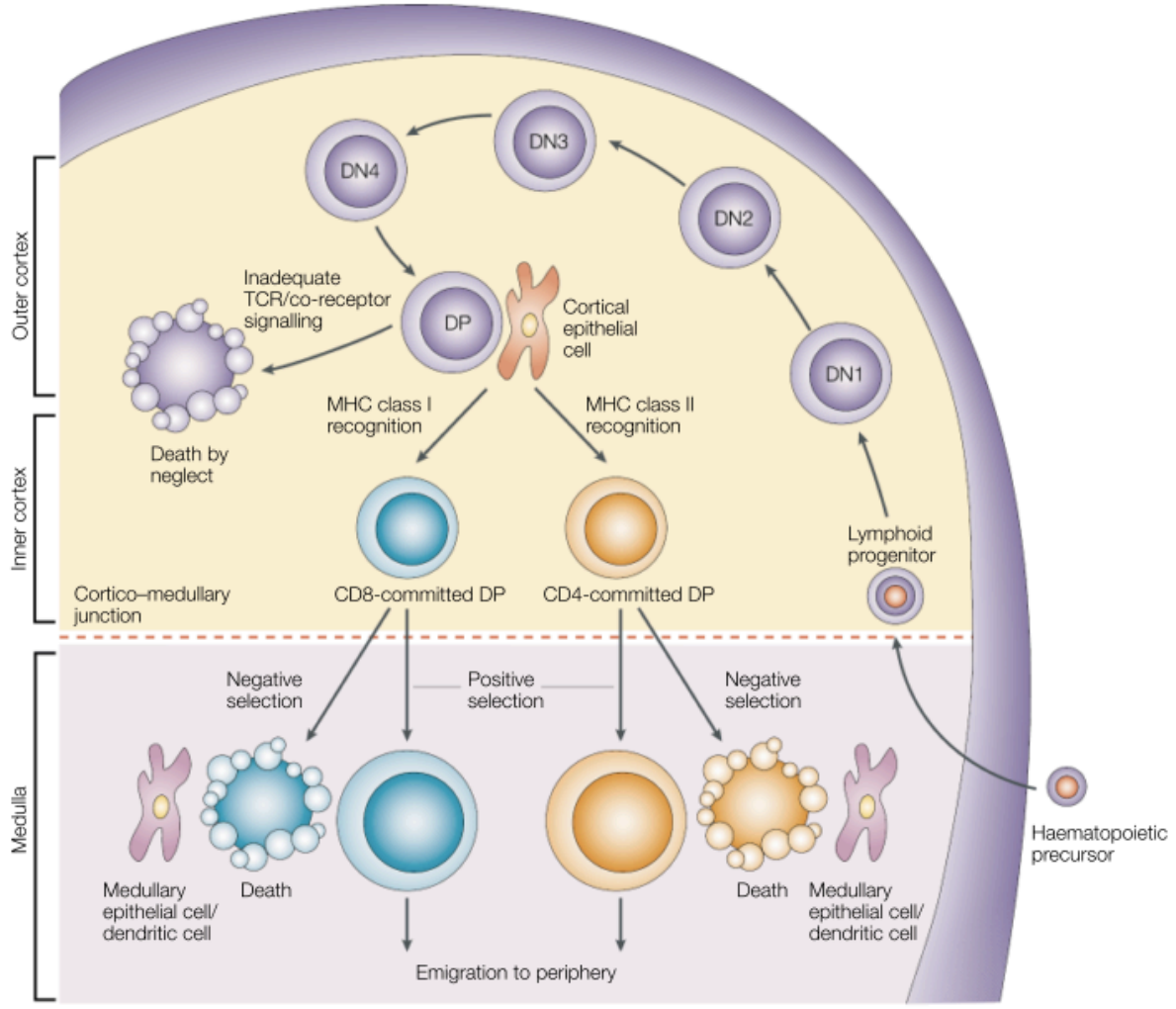


Figure 5 - Schematic representation of T cell development in the thymus. Adapted from [77].

### G.2.2 CD8 T cell priming and differentiation

Naïve CD8 T cells continuously circulate between secondary lymphoid organs via the blood and lymph vessels where they patrol for DCs presenting antigens they are specific for[81]. DCs capture antigens in the periphery which when combined with the sensing of danger signals via TLR receptors, lead to their maturation in pro-inflammatory DCs trafficking to lymph nodes[82]. Hence the presentation of peptide antigens by mature DC to naïve T cells in secondary lymphoid organs is the initiating event of a T-cell response. The priming of T cells requires at least two signals. Signal 1 is the recognition of the MHC/peptide complex on the APC by the TCR and signal 2 is a costimulatory signal provided only by mature APCs, which is generally the ligation of CD28 by CD80 or CD86[83,84]. Upon priming, antigen-specific T cells clonally expand by dividing up to 20x. During this stage, CD8 T cells also undergo a differentiation process in which they acquire the expression of effector molecules, such as

Granzyme B, Perforin, Interferon γ and TNF α. They finally leave the secondary lymphoid organs to the periphery in order to kill infected cells at the sites of infection[85]. This phenomenon is partly mediated by the downregulation of the selectin CD62L and the chemokine receptor CCR7, which are key homing molecules targeting T cells to secondary lymphoid organs. Instead, they express homing molecules targeting inflamed endothelium, such as CD44, which binds P- and E-Selectin and chemokines receptors, such as CXCR3, which facilitates the migration of effector cells towards inflamed tissues producing CXCL9/10[86]. But upon antigen clearance, most of effector CD8 T cells will die by apoptosis in a process called contraction and only a subset of effector CD8 T cells will survive and form a long-lasting memory T cell pool, able to quickly divide and form effectors upon antigen re-challenge by the same pathogen[87] (Figure 6). In some infection models such as *Listeria monocytogenes*, it was demonstrated that at the peak of the effector response (*i.e.* when there is the highest frequency of effector CD8 T cells in the periphery), a population of effectors express high level of killer cell lectin-like receptor G1 (KLRG1) and are very susceptible to die during the contraction phase. They are called short-lived effector cells (SLECs). On the other hand, some cells express high levels of IL-7 receptor subunit-α (IL-7Rα or CD127) and are more likely to survive the contraction phase and become memory T cells. These cells are called memory precursor effector cells (MPECs)[88,89]. The remaining memory T cells do not require signalling via their antigen receptor and interleukin-2 (IL-2) to survive as it is the case for effector cells. In contrast, memory Cd8 T cells require IL-7 and IL-15, similarly to naïve T cells, which depend on IL-7 for their survival[90].

Different subsets of memory T cells exist. Indeed, central memory T cells reside solely in secondary lymphoid organs and have a great potency to proliferate and form a novel wave of effectors upon antigen re-encounter. Effector memory T cells are also found in secondary lymphoid organs, but also in the periphery. Upon antigen re-challenge, they have a reduced proliferative capacity as compared to central memory cells, but they keep the expression of effector molecules[87]. Finally, a third subset of memory T cells has been identified, called resident memory T cells. These cells reside in the previously infected tissues and do not recirculate in secondary lymphoid organs or in other peripheral sites. They respond immediately to reinfection in those exposed tissues[91].

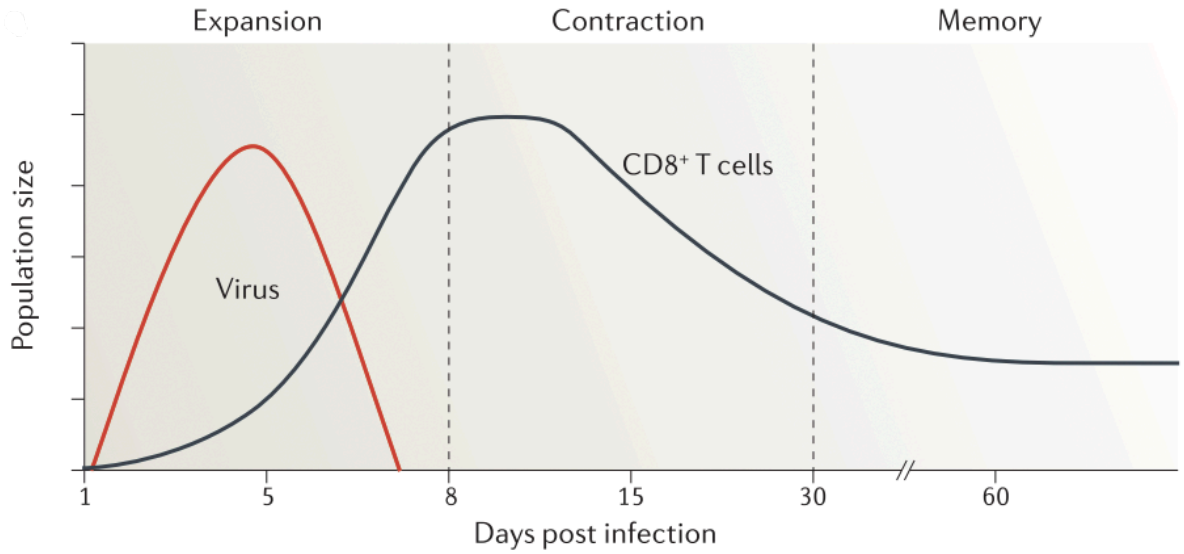


Figure 6 - CD8 T cells clonally expand upon viral infection. Most of the effector T cells then die upon pathogen clearance. A fraction of these cells will survive to form memory cells. Adapted from [87].

### G.2.3 TCR Signalling

The engagement of a T-cell receptor triggers a cascade of signalling events, which will determine the fate of the T cell, which will vary according to several factors such as the differentiation status of the cell or the engagement of co-stimulatory receptors.

The TCR does not contain signalling modules by itself, but is associated with the CD3 complex on the cell surface, which consists of membrane proteins containing immunoreceptor tyrosine-based activation motifs (ITAMs). The triggering TCR signalling occurs when these ITAMs are phosphorylated by tyrosine kinases such as Lck and Fyn. Lck associates with the intracellular part of the CD4 or CD8 co-receptor and is brought close to the TCR upon contact of the TCR and co-receptor with the peptide-loaded MHC molecule on the APC[92,93]. Phosphorylated ITAMs by Lck and Fyn enable the docking of ZAP70, which is also phosphorylated by Lck and Fyn[94]. An important negative regulator of the initiation of these early events in TCR signalling is the phosphatase CD45, which dephosphorylates the proximal TCR signalling components and has to be excluded from the active TCR signalling complex[95]. The activated ZAP70 phosphorylates the scaffold protein LAT and the adapter protein SLP-76[96], which initiates the branching of the TCR signalling cascade towards three distinct pathways: NFAT, NF-κB and AP-1. For instance, the recruitment of phospholipase C γ (PLCγ) by LAT and SLP-76 will catalyse the conversion of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate ($IP_3$). The messenger molecule $IP_3$ will trigger the release of calcium from the endoplasmic reticulum, leading to the activation

of NFAT, while DAG recruits and activates both Protein Kinase C θ (PKCθ) and RAS guanyl nucleotide- releasing protein (RasRGP), which respectively activate the NF-κB and AP-1 signalling pathways. Finally, NFAT, NF-κB and AP-1 are the main transcription factors orchestrating the T cell response upon TCR triggering[93,97]. The main signalling events downstream of the TCR activation are depicted in Figure 7.

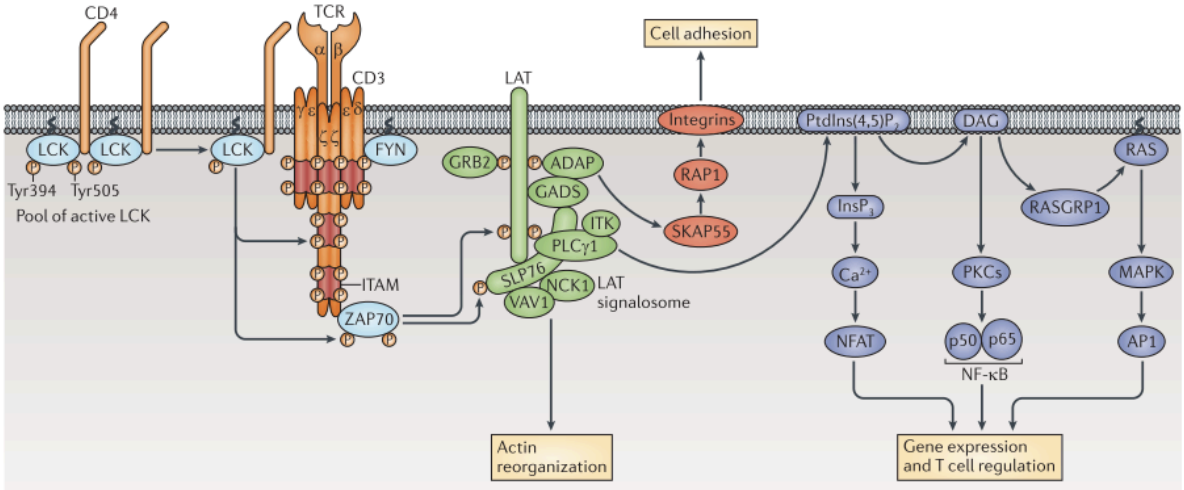


Figure 7 - An overview of the early events in TCR signaling. TCR triggering recruits the kinases Lck and Fyn, which phosphorylate the CD3 complex, leading to the recruitment and the phosphorylation of ZAP70, which in turn phosphorylates LAT and SLP76. The next key step is the activation of PLC γ, which coordinates initiation of the signaling through 3 critical pathways: NFAT, NF-κB and AP-1. Adapted from [93].

### G.2.4 T cell dysfunction: anergy, exhaustion and activation-induced cell death

T cell anergy is an important mechanism of peripheral tolerance. It occurs when naïve T cells are primed without proper co-stimulatory signalling, such as CD28[98] when interacting for example with tolerant DCs lacking CD80/86 expression. As a consequence, anergic T cells fail to proliferate, secrete IL-2 and exert effector functions[99,100]. Interestingly, anergy can be reverted by *in vivo* IL-2 treatment[101]. Molecularly, the TCR activation in absence of CD28 signalling leads mainly to the NFAT signalling pathway, with a defect in AP-1 and NF-κB pathways. The regulation of transcription by NFAT is altered when AP-1 pathway is aborted, which prevents the expression of genes critical for T cell priming and effector differentiation, such as IL-2[102]. Indeed, CD28 mainly acts through the activation of the Phosphoinositide 3-kinase (PI3K) pathway, which increases the activation of PLC γ, leading to optimal NF-κB and AP-1 signaling[103–105].

T cell exhaustion is, like T cell anergy, a dysfunctional state of T cells, which does not occur at the priming phase but in chronically stimulated effector T cells. It was first described by the

group of R. Ahmed in a mouse model of chronic viral infection, in which they showed that chronic exposure to antigens led to a gradual loss of effector function and proliferation capacity in CD8 T cells[106].

The inhibitory receptor Programmed cell death 1 (PD1) was later shown to be an important player in the induction of T cell exhaustion, as the blockade of the interaction with its ligand PD-L1 could restore the function of T cells in a model of chronic infection[107]. Other factors also positively influence the onset of exhaustion in T cells, like the lack of CD4 T cell help[108]. Furthermore, it was later found that tumour-infiltrated lymphocytes (TILs) were often exhausted both in mice and human tumours and that in addition to PD-1, they co-expressed a broad set of inhibitory receptors, such as CTLA4, LAG3, TIM3 and BTLA. Indeed, the induction of T cell exhaustion in the tumour micro-environment (TME) is a major mechanism used by tumours to escape rejection by the immune system[109,110].

Alternatively, activation-induced cell death (AICD) is a crucial mechanism regulating the homeostasis of peripheral T cells. Upon activation, T cells upregulate both FasL and its receptor, Fas. Upon activation, T cells upregulate the apoptosis-mediating receptor Fas. Depending on their activation status (like costimulatory support), they can be more or less susceptible to Fas-mediated apoptosis. The triggering of AICD can occur via the expression of FasL on T-cells themselves (in the contraction process) or in immunopriviledged organs. Finally, other molecules such as TNF-α can induce AICD in certain circumstances[111].

### G.2.5 PD1 signalling

As previously explained, PD1 is highly expressed by exhausted T cells, but its expression is also increased upon TCR triggering[112]. The inhibitory action of PD1 mainly acts through its downstream effector SH2-domain containing tyrosine phosphatase 2 (SHP-2), which will prevent the phosphorylation of the early TCR signalling modules CD3, ZAP70 and PKCθ, but also PI3K, which is downstream of CD28[113]. The effect of PD1 signalling is seen as a reduction of effector function and survival[114].

PD-L1 is the main ligand of PD1 and its expression pattern is seen on both hematopoietic and non-hematopoietic cells. A variety of cells, including tumors, upregulate PD-L1 expression in response to IFNγ exposure[114,115]. But oncogenic signalling in tumors like increased PI3K activity through the loss of Phosphatase and tensin homolog (PTEN) does also enhance PD-L1 expression[116].

Interestingly, PD1 ligands also induce signalling in APCs. For example, PD-L1 and PD-L2 ligation on DCs reduces their maturation, resulting in an impaired T cell priming[117].

## G.3 Cancer

Cancer is the second cause of mortality worldwide and results from the abnormal proliferation of transformed cells, which then eventually invade other parts of the body, ultimately disrupting the organization and the function of the invaded structures[118].

### G.3.1 Epidemiology of cancer

Cancers are caused by genetic mutations, which can be inherited or generated in somatic cells. Certain viruses can also promote tumorigenesis by expressing viral oncogenes upon cell infection.

Somatic mutations are caused by errors during DNA replication and by environmental cues. Indeed, the World Health Organization reported that "high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, and alcohol use" are the cause of one third of cancer deaths. Furthermore, "cancer causing infections, such as hepatitis and human papilloma virus (HPV), are responsible for up to 25% of cancer cases in low- and middle-income countries"[119]. These statistics emphasize the important role of environmental factors such as lifestyle and pathogens in the onset of cancer. However, random mutations occurring in somatic cells are estimated to be the main cause of cancer-causing mutations[120].

### G.3.2 The development of cancer

The development of cancer is a stepwise process in which transforming cells acquire key features allowing them to form local neoplasms (newly formed masses) and distant metastasis. In 2000, Hanahan and Weinberg have proposed a list of capabilities that a transforming cell need to acquire to form a tumor, referred as "hallmarks of cancer"[121] (Figure 8). In 2011, they adapted this list, taking into account the most recent knowledge in the understanding of cancer biology[122].

Some of these hallmarks are not intrinsically acquired by the tumor cells, but also by the stroma, which defines non-transformed cells recruited by the tumor and actively participating in the tumorigenesis[122]. The stroma is also referred as "tumor microenvironment" and is composed of a diversity of normal cells, such as macrophages, T cells, B cells and fibroblasts[123].

The first hallmark of cancer is the ability of tumor cells to sustain proliferative signalling. Indeed, cell proliferation is a tightly controlled mechanism that includes several checkpoints

to prevent abnormal cell proliferation. This can be overcome by the onset of mutations driving the constitutive activation of oncogenes (genes positively regulating the cell cycle). The next hallmark proposed by Hanahan and Weinberg is the evasion of growth suppressors: tumor suppressors are genes that negatively regulate the progression through the cell cycle, like p53, which ensures the control of cell division. Furthermore, the resistance to cell death is a hallmark which defines mechanisms involved in the resistance to apoptosis in cancer cells, via defective signalling pathways which generally lead to cell death. Another associated hallmark is the replicative immortality, by which tumor cells bypass the limited replicative capacity of normal cells, generally by expressing telomerase, an enzyme responsible for the maintenance of telomere length which controls cell longevity. The next hallmark is the ability of tumors to form new blood vessels, referred as the neovasculature, to allow the supply of oxygen and nutrients to the growing tumor mass. This capability is often provided by macrophages in the stroma[124]. Finally, a crucial hallmark of major interest in my project is the ability of tumors to escape elimination by the immune system, which will be described later in the introduction[122]. To summarize this part, transforming cells must acquire several characteristics to form tumors. Some of these characteristics are acquired by mutations in the tumor cells themselves and some others are provided by the recruited normal somatic cells, which form the tumor stroma.

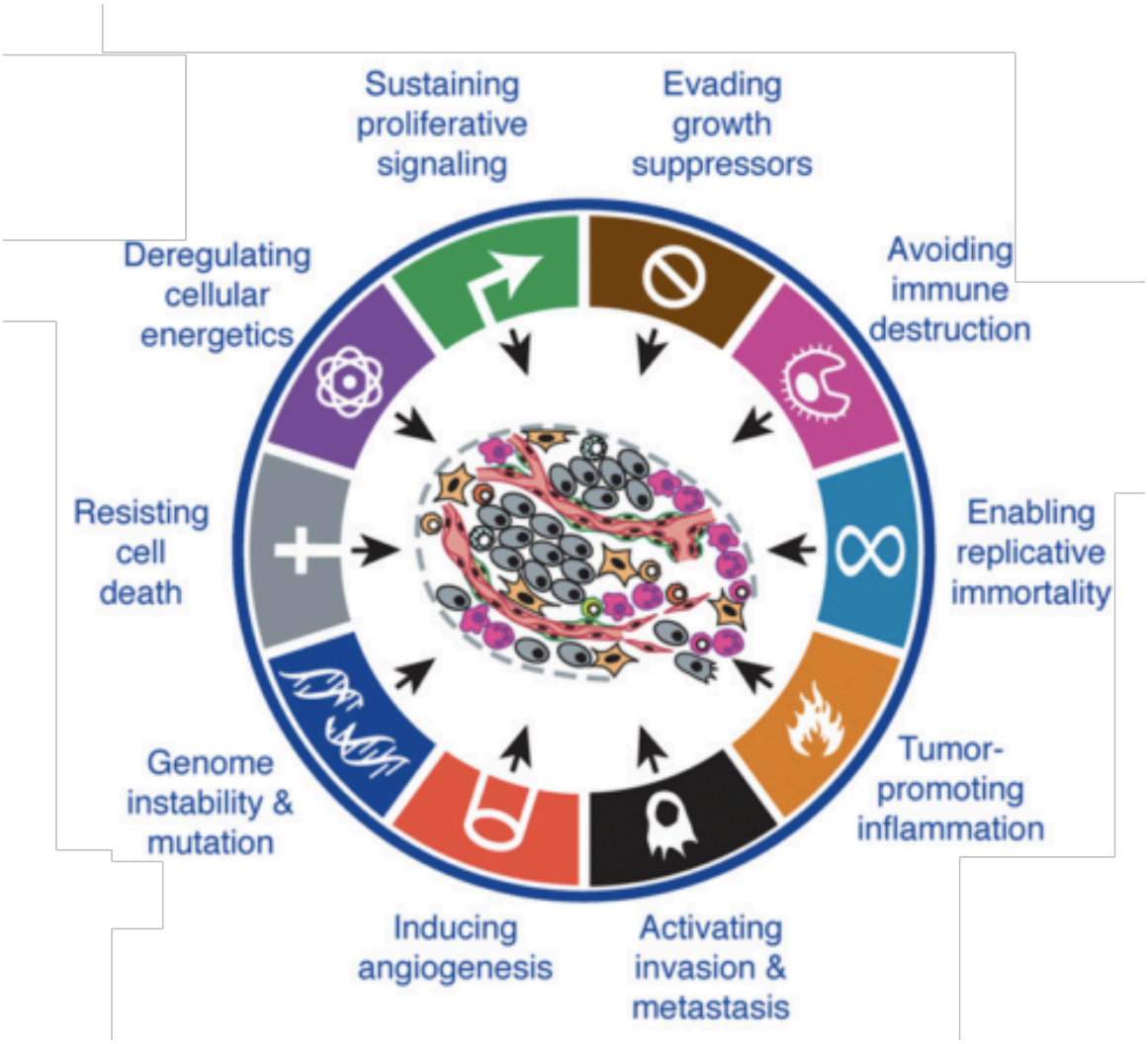


Figure 8 - The hallmarks of cancer: these characteristics have been described by Hanahan and Weinberg as key features required for tumor initiation. Adapted from [122].

### G.3.3 Conventional cancer therapies

Cancer patients are first treated with "conventional therapies", either as single or combined treatments, depending on the tumor type, the tumor stage and the status of the patient. Conventional cancer therapies include surgery, radiotherapy and chemotherapy[125]. Recently, other treatments have emerged, such as targeted therapies, which target specifically oncogenic signalling in tumors rather than broad cytotoxic drugs such as chemotherapeutic agents[126]. When possible, these tumor targeting therapies have resulted in a reduced toxicity towards normal cells and thus lower off-target toxicity.

## G.4 Cancer immunity

The immune system was primarily shaped to discriminate self versus non-self in order to protect and tolerate its host while attacking invading pathogens such as bacteria or fungi. Despite the fact that tumors are self-derived, the immune system has developed mechanisms to recognize and attack them[127]. However, the antitumoral immunity is often insufficient to reject established neoplastic lesions, for reasons which will be explained in the next sections. The first pieces of evidence that the immune system plays a key role in the surveillance of tumors came in 1994 when a study showed that mice deficient for IFN γ receptor[128] or for perforin, a cytotoxic effector molecule in T and NK cells, had a higher tumor incidence when treated with a carcinogen[129]. Few years later, the key role of lymphocytes in tumor immunosurveillance was demonstrated[130].

### G.4.1 Cancer recognition by the innate immunity

NK cells are very important for the early control of viral infections, as unlike T lymphocytes, they can kill target cells without prior sensitization[131]. However, their role in the surveillance of malignant transformation has been demonstrated by several studies on carcinogen-induced or transplanted tumors[132,133].

To recognize malignant or virally-infected cells, NK cells express a set of germline-encoded receptors. These NK receptors transmit either inhibitory or activatory signals to the NK cell, which altogether will decide whether the target will be killed or not[131] (Figure 9). In mouse, the main inhibitory signalling molecules inducing NK cell tolerance are the receptors of the Ly49 family, which recognize MHC I molecules. Therefore, the loss of MHC I expression often occurring on tumor cells renders them more susceptible to NK cell-mediated lysis[134,135]. The activation of NK cells also depends on a wide set of receptors, which recognize stress signals on target cells that are virally infected or undergoing malignant transformation[136]. For instance, NKG2D binds to stress-related ligands upregulated upon DNA damage response in cancer cells or upon TLR stimulation in infected cells[137]. NK cells are also important mediators of Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) through the recognition of the Fc domain of IgG1 antibodies by the CD16 receptor[136].

In addition to NK cells, myeloid cells such as granulocytes and macrophages are highly present in the tumor stroma. These myeloid cell subsets are highly plastic, and their effector functions depend on their environment and the signals they receive. For instance, upon microbial infection, macrophage activation occurs via exposure to IFNγ, which increases their

antimicrobial properties and their secretion of pro-inflammatory cytokines[138]. Alternatively, macrophages exposure to Type-2 cytokines such as IL-4 and IL-13, was shown to also result in a high phagocytic activity but without secretion of pro-inflammatory cytokines[139,140]. The classical and alternative activation of macrophages were named M1 and M2, mirroring the Th1 and Th2 type of immune responses. More recently, it was shown that like IL-4 and IL-13, the immunosuppressive cytokines such as IL-10 and TGFβ also induced the M2 phenotype[141,142]. The consensus today is that macrophage differentiation is rather a continuum between M1 and M2[143]. In tumors, M1 macrophages are pro-inflammatory, inhibit angiogenesis and support the adaptive immune response, while their M2 counterparts are immunosuppressive and support angiogenesis[144,145].

DCs also play an important role in tumor immunity by stimulating the T cell-mediated response. As for macrophages, the immunosuppressive stimuli in the tumor microenvironment induce a suppressive type of DCs characterized by the lack of T cell co-stimulatory signals and the expression of the inhibitory molecules PD-L1, Arginase and IDO[146]. In order to acquire a pro-inflammatory phenotype and induce protective T cell responses, DCs need to sense Pathogen-Associated Molecules Patterns (PAMPs), which are generally lacking in the tumor environment. However, in certain conditions, tumor cell death can trigger DC maturation in a process called immunologic cell death (ICD). Indeed, although cell death does generally not induce an immune response, it can become immunogenic in certain circumstances. Indeed, the release of damage-associated molecular patterns (DAMPs) stimulates DCs to generate an immune response. DAMPs are certain cellular components, which are released upon apoptosis. Nucleic acids, ATP or heat-shock proteins are example of DAMPs[147]. For instance, the treatment of tumor cells with the chemotherapeutic agent anthracyclin induces an immunogenic cell death signal able to trigger an immune response. This signal critically depends on the DAMP Calreticulin (CRT), released by the dying tumor cells[148].

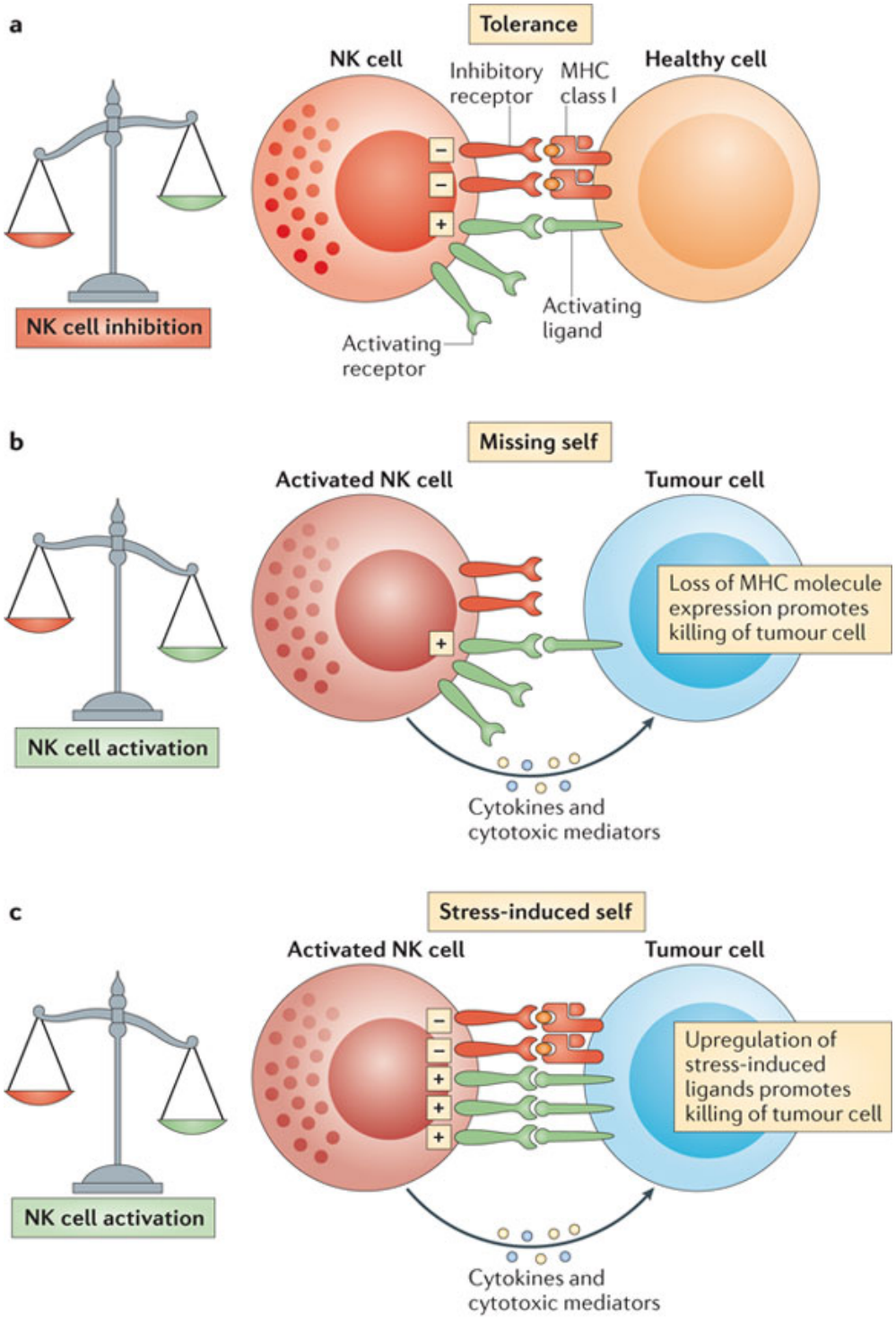


Figure 9 - NK cells play a major role in tumor immunosurveillance. Lack of MHC I expression (missing self) decreases the inhibition of NK cells, leading to their activation. Alternatively, the upregulation of stress-related ligands on transformed cells activates NK cells cytotoxicity. Adapted from [149].

### G.4.2 Cancer recognition by the adaptive immunity

The adaptive immune system encompasses B and T lymphocytes, which play a key role in the control of tumors.

The main adaptive players in adaptive immunity-mediated tumor rejection are CD8 T cells. In a majority of tumors, like in colorectal cancer[150], high T cell infiltration correlates with a better overall survival (OS) or progression-free survival (PFS). It is now clear that cancer patients mount T cell responses against tumor antigens, such as cancer-testis antigens (i.e. NY-ESO I), differentiation antigens (i.e. Melan A), and neoantigens (i.e. mutated epitopes)[151,152]. In order to mount an effective antitumoral T cell response, several steps need to be efficiently accomplished. They are described by Chen and Mellmann as the Cancer-Immunity cycle (Figure 10). The first step of the cycle is the released of tumor antigens, upon tumor cell death, and their processing by DCs. As said above, this step requires immunogenic signals to induce DC maturation. The next step is the DC antigen-presentation to T cells, resulting in the generation of effector CD 8 T cells. Primed T cells will traffic to the tumor, where they will exert cytotoxicity, release inflammatory signals and tumor antigens to feed the cycle. However, the Cancer-Immunity cycle is not optimally running in cancer patients, as their endogenous T cell response are not able to cure progressive disease [151]. The aim of cancer immunotherapy is to optimize this cycle in order to improve the rejection of established tumors by the immune system.

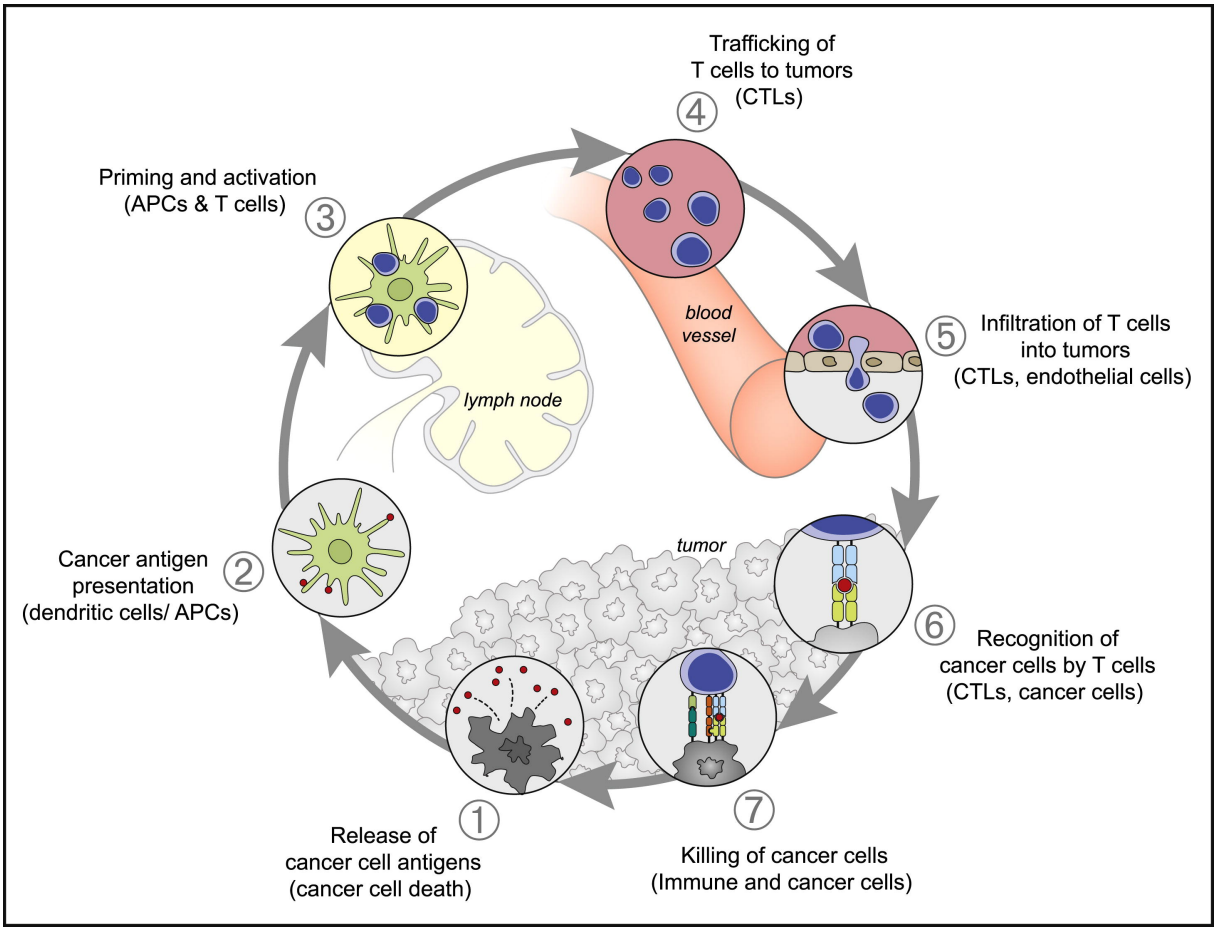


Figure 10 - The cancer immunity cycle. This cycle was proposed by Chen and Mellmann to illustrate the single steps required to induce an effective antitumoral T cell response. Adapted from [151].

### G.4.3 The cancer immunoediting hypothesis

The concept that the immune system is a sentinel of tumor formation is old[153] and now widely accepted. However, despite the mechanisms of immune surveillance of tumors, cancer still arise in immunocompetent hosts. This can be explained by the cancer immunoediting hypothesis, proposed by Schreiber *et al*. Briefly, the immune response shapes the tumor as shown by several studies showing that tumors induced in an immunodeficient host grow slower when transplanted in an immunocompetent host, in contrast to tumors induced in an immunocompetent host, which are more aggressive[130,154]. As viruses, tumors have the ability to change and adapt because of their genetic instability. The pressure of the immune system drives the selection of variants that can withstand immune attack, for example by reducing their immunogenicity. Thus, the immune system does not only protect the organism against neoplasms, but it also shapes these neoplasms by selecting immune-resistant variants. In that view, Schreiber *et al*. propose three steps: the three Es of cancer immunoediting (Figure 11). The first E, Elimination, occurs when the immune system successfully rejects a tumor.

However, when the lesion persists and grows, the immune system may actively attack the tumor, as described by the second E: Equilibrium. The tumor is sufficiently big to generate a diversity of tumor cells, among which immune escape variants will be selected due to the pressure of the immune system. As a result, the third E: Escape describes the step in which only the tumor cells that highly resist to the immune system are selected and allow the progression of the disease[155].

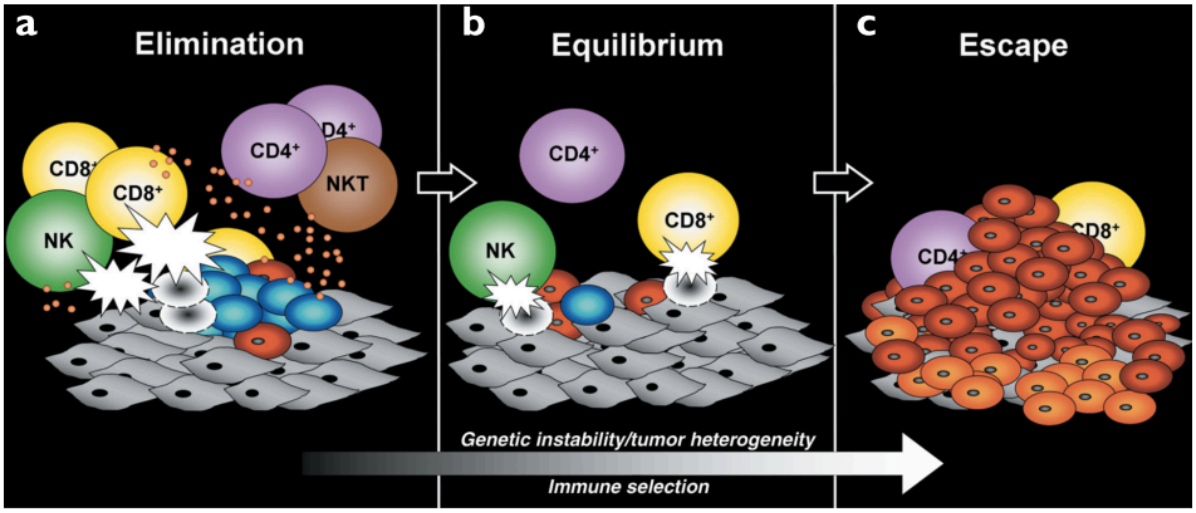


Figure 11 - The three Es of cancer immunoediting describe the key steps of the selection of immune-resistant tumors by the immune system attack. Adapted from [155].

### G.4.4 Immune escape mechanisms

A big variety of mechanisms are used by tumors cells or by their supporting stroma to sabotage the cancer immunity cycle and counteract T cell-mediated rejection. For instance, in absence of immunogenic signals, tumor-derived DCs remain immature and tolerant and induce T cell anergy rather that effector differentiation[156]. It was even observed that peripheral DCs from cancer patients (out of the tumor bed) had defects in maturation as their DCs could not efficiently prime autologous T cells against tetanus toxoid or influenza peptides *ex vivo*, when compared to healthy donors.[157].

Myeloid-derived suppressor cells (MDSC) are important mediators of tumor immunosuppression, as immature myeloid precursor cells are rapidly recruited to the tumor. They exert their immunosuppressive functions through the secretion of the immunosuppressive cytokines TGF-β and IL-10 and the expression of the Arginase 1, which depletes L-Arginine from the stroma. They also produce nitric oxide through the expression of the iNOS enzyme[158].

Defined subsets of lymphoid cells are also involved in tumor immunosuppression, among which the $CD4^+$ regulatory T cells (Treg) are the best characterized. They are naturally occurring cells, unlike MDSC, and are considered as an important brake of the immune system to prevent excessive inflammation and autoimmunity. They are however hijacked by tumors to induce a tolerogenic environment. Tregs exert their suppressive activity through the secretion of Adenosine and the immunosuppressive cytokines IL-10 and TGF-β, cytolysis of effector CD8 T cells via Granzyme B and Perforin, local IL-2 depletion and the induction of tolerogenic DCs[159].

Another key immunosuppressive mechanism in tumors is the expression of PD-L1 by both tumor cells and stromal cells. Although PD-L1 expression is usually constitutive, it is largely induced by IFN-γ as a response to T cell attack[160] (Figure 12). Its ligation on its receptor PD-1 negatively regulates the activation of CD8 T cells, as described before.

Another key effector of tumor immunosuppression is the enzyme Indoleamine 2,3-dioxygenase (IDO), which is mainly expressed by myeloid cells and by the tumor cells themselves. IDO acts by recruiting MDSC and by depleting L-tryptophan, which is essential for effector T cell survival[160].

Finally, another example of immunosuppressive mechanism in tumors is the expression of FasL in the tumor endothelium, which kills T cells migrating to the tumor site. FasL expression does not occur in normal endothelium but is induced in the tumor neo-vessels as a consequence of the presence of immunosuppressive factors[161].

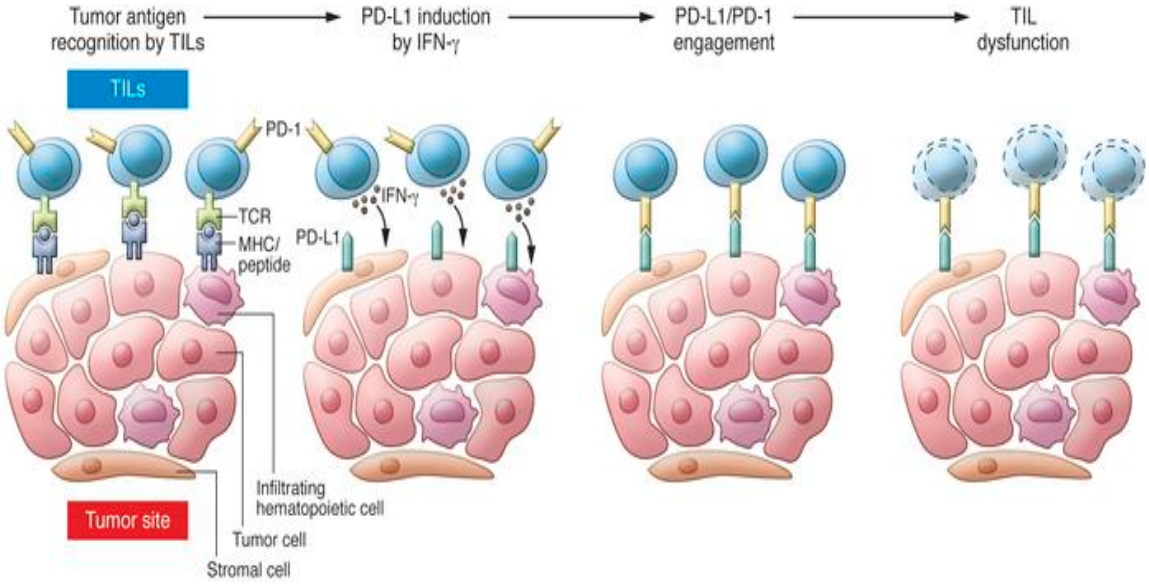


Figure 12 - PD-L1 is induced by IFNγ and is therefore upregulated by tumor cells upon T cell attack. It is therefore a key mediator of the immune adaptive resistance, as it serves as a molecular shield to protect tumor cells from T cells. Adapted from [162].

## G.5 Cancer immunotherapy

The principle of cancer immunotherapy relies on the use of the host immune system to attack tumors, and can be subdivided into two categories: active and passive immunotherapy. Active immunotherapy takes advantage of the immune system of the host to mount an antitumoral response, giving the possibility to generate an immunological memory as well, like cancer vaccines. Passive immunotherapy does not rely on the generation of an immune response and instead provides the molecules or the effector cells, such as respectively therapeutic antibody treatments and the adoptive transfer of effector T cells. However, the frequent combination of several therapies makes this distinction obsolete.

Immunotherapy was discovered more than 100 years ago by William Coley, when he obtained complete remission of several cancer patients by injecting in the tumors the live bacteria *Streptococcus pyogenes*. However, this protocol was rapidly abandoned because of its toxicity and its unknown mechanism of action, and surgery and radiotherapy became standard of care[163].

In 1976, the idea to use bacteria to treat cancer was again exploited for the treatment of bladder cancer via the intra-vesical instillation of the Bacillus Calmette-Guérin (BCG) tuberculosis vaccine. This protocol is still indicated for the treatment of non-muscle-invasive bladder cancer, and the major local inflammation prevents tumor recurrence in 70% of patients[164,165]. A key event in the history of immunotherapy was the cloning in 1999[166] of the melanoma-derived antigen MZ2-E (MAGE-A1), which was associated with antigen-specific T cell responses in several melanoma patients, showing that tumor-derived antigens can be targeted by T cells.Another milestone was the approval of T cell-stimulating cytokine IL-2 by the FDA also in 1999 for the treatment of metastatic melanoma[167,168]. Recent strategies include adoptive T cell therapies and checkpoint blockade treatments, which will be described below.

### G.5.1 Therapeutic Cancer vaccines

Therapeutic cancer vaccines aim to generate or amplify an antitumoral T cell response. A vaccine requires one or multiple antigens and an adjuvant, which makes the antigens immunogenic by activating the innate immune system.

Different vaccine strategies have been tested in clinical trials, but often with moderate efficacy. Several reasons can explain this limited success. First, most tumor-associated antigens (TAAs) are self-antigens expressed by somatic cells and overexpressed by cancer

cells. Thus, central tolerance mechanisms preclude the generation of high affinity T cells towards these antigens. Second, strong adjuvants need to break the peripheral tolerance towards tumor-derived antigens in tumors. Third, cancer immunoediting mechanisms select tumor cells that are poorly immunogenic[169], and the immunosuppressive tumor environment generate the so-called exhausted T cell phenotype seen in tumor-infiltrating T cells.

So far, a large number of clinical trials have and are testing therapeutic cancer vaccines. However, very few have proven to efficiently improve the survival of cancer patients and have been moved to clinical application.

For instance, the Sipuleucel-T therapeutic was FDA-approved in 2010 for the treatment of metastatic castration-resistant prostate cancer[170]. Briefly, APCs and peripheral blood mononuclear cells (PBMC) are pulsed with a fusion protein made of the prostate acid phosphatase antigen and the granulocyte-macrophage colony-stimulating factor (GM-CSF). The cell suspension is then injected into the patients. Another strategy tested in a phase II clinical trial, made use of viral vectors encoding the prostatic antigen PSA, the co-stimulatory molecule CD80 and the adhesion molecules LFA-1 and ICAM-1. The viral system allowed the *in vivo* delivery of genes and acted as a strong adjuvant[171].

Several clinical trials involving therapeutic vaccines were also conducted in breast cancer patients. in particular a phase II trial involving a HER2-derived peptide formulated with GM-CSF[172].

Concerning lung malignancies, several vaccination strategies have been tested, with however poor clinical outcome. For instance, Tecemotide, which contains a peptide derived from the TAA antigen MUC1 and the adjuvant monophosphoryl lipid A (MPL), failed to significantly improve the survival of patients with unresectable non-small-cell lung cancer (NSCLC)[173].

In view of the immunogenicity of melanoma tumors, therapeutic vaccines have also been clinically tested, such as a phase II clinical trial combining systemic IL-2 administration and vaccination with the TAA antigen gp100 formulated with the adjuvant Montanide™ ISA 51, which led to significant improved the survival of melanoma patients[174]. Another strategy implied the loading of peptides derived from the gp100 and tyrosinase melanoma-associated TAAs on autologous DCs before reinfusion into the patients, showing also clinical beneficial clinical outcome[175]. Finally, a phase II trial on melanoma patients showed that the delivery of the cancer-testis (CT) antigen NY-ESO-1, expressed in various tumors, by viral vectors

significantly improved the survival of the patients, yielding 14% of objective response rate and 52% of disease stabilisation[176].

### G.5.2 Neoantigens

As described before in the introduction, the high mutational load in the tumor cells generates a panel of mutated proteins called neoantigens, of which peptides can be detected by the CD8 T cells of the host and generate a specific antitumor response. In mouse tumor models, it was demonstrated by the group of Schreiber that checkpoint blockade therapies promote tumor regression mainly by enhancing the activity of neoantigen-specific T cells[177]. The same is true in cancer patients as it was reported that high mutational load is associated with better response to checkpoint blockade therapy in melanoma, colon cancer and NSCLC[178–180]. Progresses in sequencing technology and in predictive tools able to select mutations with high probability of being presented on the MHC molecules on tumor cells will allow to generate personalized vaccines targeting mutations specifically displayed by tumors in individual patients. A very recent trial has tested the feasibility of this strategy and showed very encouraging clinical results in a cohort of melanoma patients[181].

### G.5.3 Tumor lysates

Another approach to elicit an antitumoral T cell response is to inject *ex vivo*-matured DCs loaded with tumor lysates. The rationale is that the DCs will present mutated antigens specific to the tumor of the patient which will elicit a T cell response against the specific antigens presented by the tumor. This strategy has been tested by the group of Prof G. Coukos in a phase I clinical trial with patients bearing recurrent ovarian cancers and showed modest but encouraging efficacy[182]. This strategy is very promising as it will allow to vaccinate patients specifically against their own tumor antigens without the need to sequence and analyse the mutanome of their tumors, which would be much faster and cost-effective.

### G.5.4 iNKT cells in cancer

It is now evident in both mouse and humans that iNKT cells play a role in tumor immunosurveillance. For instance, mice lacking iNKT cells have a higher onset of spontaneous tumors induced by a deficiency of p53[149]. The role of iNKT cells in tumor immunosurveillance has been outlined as well in different models[183,184] and cancer patients often display reduced iNKT cell number and defective activation[185,186].

On top of tumor immunosurveillance, iNKT cells can be harnessed against cancer by exploiting their transactivating properties of the innate and adaptive arms of the immune system, as described in G.1.9.

It was first demonstrated that the injection of αGalCer in mice bearing EL4 or B16 tumors prolonged their survival. As well, a group demonstrated that in a mouse myeloma model, the injection of irradiated tumor cells loaded with αGalCer in tumor-bearing mice promoted the regression of the implanted tumors by inducing both a humoral and a cytotoxic antitumoral response[187].

The groups of V. Cerundolo and others demonstrated the potent adjuvant role of αGalCer as an adjuvant for therapeutic cancer vaccines. For instance, they showed that the combined presentation of the OVA antigen and αGalCer by DCs effectively raised an *in vivo* OVA-specific CD8 T cell response able to control established OVA-expressing tumors[65].

Alongs the same line, our group has demonstrated that iNKT cells can be targeted to the tumors via fusion proteins made of a tumor-targeting antibody single chain such as HER2 and of CD1d, loaded with αGalCer. These fusion proteins were able to induce the recruitment of iNKT cells to the tumor site and induce tumor regression. Furthermore, the combination of an anti-HER2/CD1d fusion protein with OVA/CpG vaccination in a tumor model expressing both HER2 and OVA synergistically expanded OVA-specific T cells, NK cells, increased the serum levels of IL-12 and promoted a strong control of the tumor[55,56,188].

The group of François Trottein has also shown that targeted iNKT cell activation with the co-delivery of peptide tumor antigens and αGalCer to CD8α DCs induces a very strong T cell immunity and tumor rejection[189].

iNKT cells can also have a direct effect on the tumor stroma. Indeed, the group of Metelitsa has highlighted in a xenograft model of neuroblastoma[190], that iNKT cells can kill tumor-associated macrophages (TAMs), when loaded with tumor lysate.

Since iNKT cells have proven to be potent immunotherapeutic agents in mouse models, several clinical trials have been conducted to harness iNKT cells in human cancer patients. A clinical trial has evaluated the effect of the injection of αGalCer-loaded mature DCs in cancer patients. This treatment significantly increased the IL-12 concentration in serum and promoted the proliferation of iNKT cells. There was also evidence of T cell transactivation as seen by the expansion of CMV-specific CD8 T cells in some patients[191]. Another study combined the administration of *ex vivo*-expanded iNKT cells and αGalCer-loaded DCs in cancer

patients. Although, the cohort of treated patients was rather small, this treatment resulted in the regression of the tumors in half of them[192].

### G.5.5 Checkpoints blockade

In 2013, the world's leading Science magazine nominated immunotherapy as the breakthrough of the year, following the discovery of checkpoint blockades as immunotherapeutic agents (Figure 13).


Figure 13 - Science nominated cancer immunotherapy as the « breakthrough of the year » in 2013, following the discovery of checkpoint blockades as immunotherapeutic agents. "Antibodies (pink) zoom toward a T cell (gray, with CTLA-4 receptor proteins shown in light blue), giving the T cell a push to attack tumor cells. Legend and picture are adapted from [193].

Checkpoint blockades refer to antibodies that specifically target molecular brakes on T cells to prevent their activation by ligand binding. They are effective for CTLA-4 and PD1 receptors blockade.

CTLA-4 is a receptor which is almost exclusively expressed on T lymphocytes upon T cell activation[194]. CTLA-4 binds to the same ligands CD80 and CD86 expressed on APCs, as CD28,

although with a higher affinity[195]. Because of its structural similarity to CD28, CTLA-4 was first believed to be a co-stimulatory molecule[196]. It was however later discovered that CTLA-4 inhibits T cell activation first by competing with CD28 for the costimulatory ligands CD80 and CD86 presented on APCs, depriving T cells of costimulation. Second, CTLA-4 recruits the SHP-2 phosphatase, which decreases the phosphorylation of the proximal TCR signaling complex, whereby it dampens T cell activation and also reduces IL-2 production and cell division[196,197].

Ipilimumab is a monoclonal antibody blocking CTLA-4. In 2010, a phase III clinical trial with stage III and IV melanoma patients demonstrated that Ipilimumab could significantly improve the survival of patients vaccinated with the TAA antigen gp100, as compared to vaccination alone[198]. In the same time, another clinical trial showed that Ipilimumab plus the chemotherapeutic agent dacarbazine significantly improved the overall survival of melanoma patients compared to dacarbazine alone[199]. Finally, Ipilimumab (Yervoy) was FDA-approved in 2011 for the treatment of unresectable or metastatic melanoma.

PD1 is another checkpoint in T cells and its mechanism of action has been described above (Chapter G.2.5). PD-1 is up-regulated on T cells during chronic inflammation such as occurring in tumors, and its binding to PD-L1 and PD-L2 on tumor cells, render T cells exhausted and dysfunctional [200].

Two anti-PD1 monoclonal antibody have been approved by the FDA to treat cancer: the first one, Nivolumab, from Brystol-Myers Squibb and the second one, Pembrolizumab from Merck. Nivolumab was the first anti-PD1 treatment to show efficacy for multiple cancers. It was able to induce up to 40% of objective response rate (ORR) in patients with metastatic melanoma[201,202]. It was also tested in patients with squamous non-small-cell lung carcinoma (NSCLC) and its efficacy was demonstrated to be superior than standard chemotherapy in this disease[203]. Its competitor, Pembrolizumab, was also extensively tested and shown to be efficient for melanoma[204], NSCLC[205], head and neck cancers[206] and triple-negative breast cancers[207].

Finally, the combination of CTLA-4 and PD-1 blockade has also been investigated in metastatic melanoma patients and was shown to be even more efficient than the single drugs alone, with however more important immune-related toxicities[208–210]. In addition, recent data showed that anti-PD1 treatments for metastatic melanoma are more efficient and cause less adverse effects than anti-CTLA-4[204].

### G.5.6 TILs therapies

Adoptive T cell therapies involving the transfer of tumor-infiltrating lymphocytes were first tested in mice thirty years ago by the group of S. Rosenberg. They showed that lymphocytes extracted from various types of tumors could be expanded *ex vivo* using IL-2 before being re-infused into the tumor-bearing hosts to mediate tumor rejection[211]. They later demonstrated that transferred TILs were more than 50x more efficient at rejecting established tumors than IL-2-activated peripheral blood lymphocytes[212]. In 1988, the first clinical trial involving TILs therapy in cancer patients was published and displayed encouraging results, as a subset of the patients experienced tumor regression with minimal side effects[213]. Later, TILs therapy was improved thanks to a better understanding of the biology of T lymphocytes. Indeed, prior-lymphodepletion of the host and recombinant IL-2 infusions allowed a better engraftment, survival and antitumoral activity of the transferred T cells[214]. Several additional clinical trials have been conducted in patients with metastatic melanoma. The refinement of the protocols for the culture and selection of tumor-reactive TILs, for the patient pre-conditioning and for the transfer TILs together with IL-2 administration allowed to strongly increase the efficacy of the therapy. Indeed, in 2008, in a trial on metastatic melanoma, 70% of overall response rate (ORR) was achieved within the group of patients that received the most radical lymphodepletion, with some patients experiencing complete remission[215]. An example of the response of a melanoma patient to TIL therapy is presented on Figure 14.

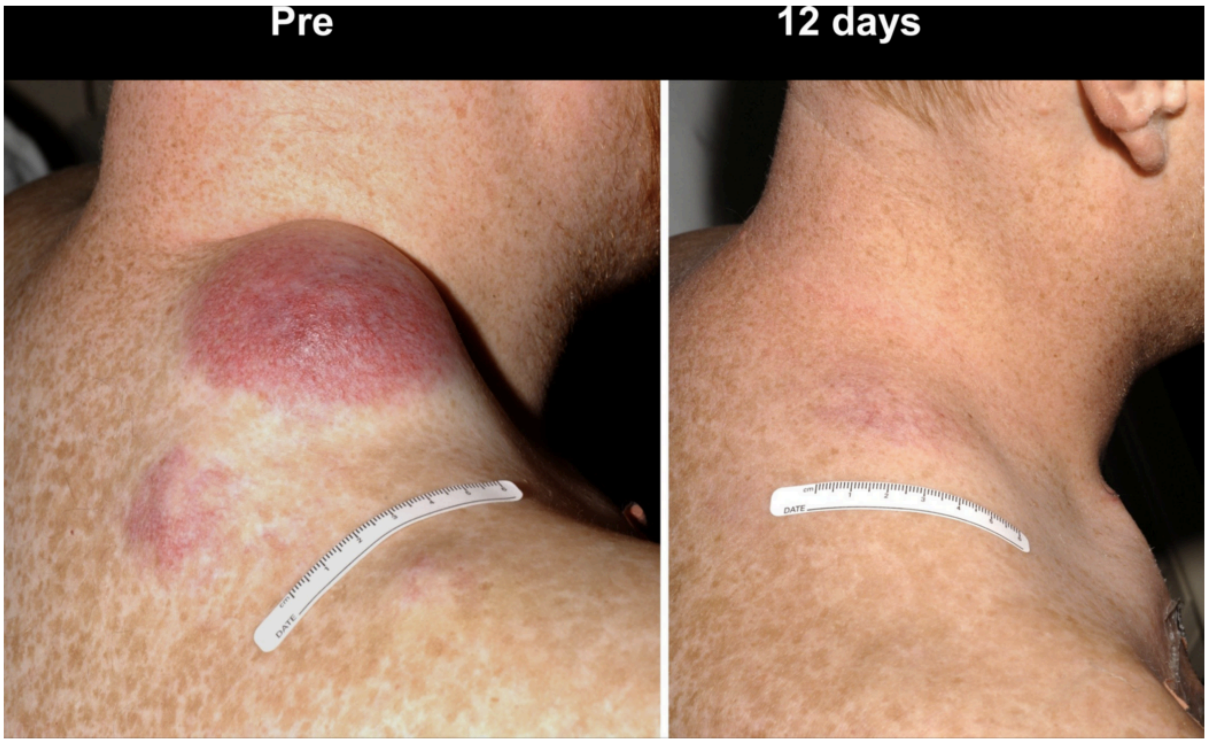


Figure 14 - Example of a spectacular response in a patient suffering from metastatic melanoma, treated with autologous TIL within a clinical trial conducted by the group of Rosenberg. Adapted from [216].

TIL therapies have shown also encouraging results in other malignancies like in ovarian [217] and cervical[214] cancers.

### G.5.7 CAR T cells

Chimeric antigen receptors (CARs) are artificial proteins constructed from an antigen recognition domain, made of an antibody single chain (scFv), a hinge region and a transmembrane domain, generally derived from CD8α or CD28. The intracellular part of a CAR comprises T cell-signalling domains. First generation CARs only include CD3ζ, while second generation CARs add a co-stimulatory domain on top of CD3ζ, generally 4-1BB or CD28. Third generation CARs use two co-stimulatory domains (Figure 15). CARs are transduced into peripheral blood-derived autologous CD8 T cells with replication-incompetent γ-retroviruses or lentiviruses[218]. T cells are then expanded *ex vivo* and infused back to the patient in combination with a lymphodepleting chemotherapy[219] (Figure 16).

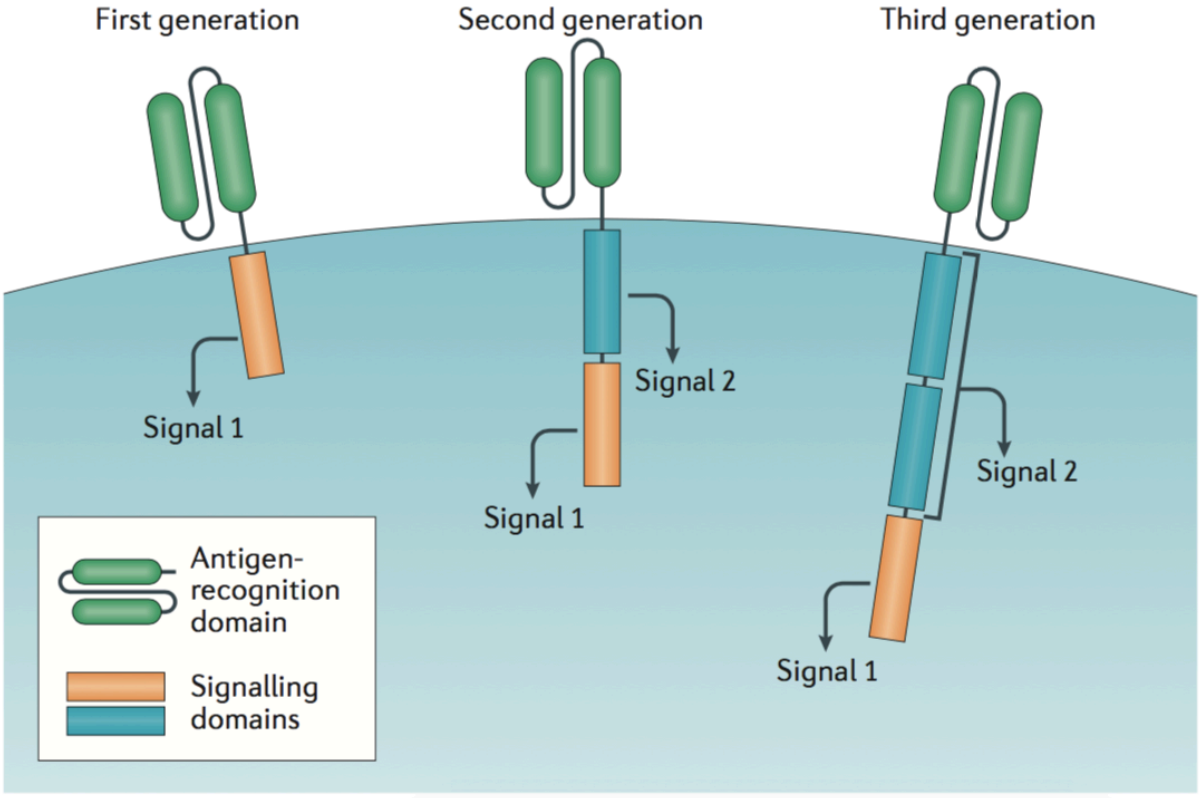


Figure 15 - Structure of a chimeric antigen receptor. Adapted from [220].

CAR T cell-mediated immunotherapies show very impressive results for the treatment of B cell malignancies. The first report of a patient successfully treated with CAR T cells was published in 2010. This patient after multiple-relapses of follicular lymphoma received a lymphodepleting chemotherapy followed by the infusion of autologous CTLs transduced with a second generation anti-CD19 CAR, which target the CD19 surface molecule on B cells and most B cell-derived malignancies[221]. In another study by the same group, it was demonstrated that CD19 CAR CTLs could efficiently eliminate lymphoma masses as well as tumor cells in the bone marrow, resulting in long term remission. This impressive clinical efficacy is however often associated with significant systemic toxicities caused by an important rise in the level of serum pro-inflammatory cytokines such as TNF-α, leading to the so-called cytokine-release syndrome (CRS)[218].

It is important to note that first generation anti-CD19 CAR T cells containing only the CD3ζ activatory domain failed to eradicate tumors in patients with follicular lymphomas because these cells could not expand and persist well *in vivo*[222,223]. Mostly, CD28 and 4-1BB are used as costimulatory domains in second generation CARs. 4-1BB was however shown to improve

more T cell persistence than CD28[224–226]. Later, multiple clinical trials involving patients with B cell malignancies have been conducted with second generation CD19 CAR CTLs, showing sometimes up to 60% of complete responses[218]. Recently, Novartis received the FDA approval to treat B cell lymphomas with a CD19 second generation CAR originally developed at the University of Pennsylvania[227].

Despite the very impressive results of CAR T cell therapy against haematological malignancies, clinical trials using CAR T cells to treat solid tumors have so far failed to demonstrate efficacy. Indeed, unlike CD19 in B cell malignancies, it is less obvious to find surface antigens expressed homogeneously by most tumor cells in a solid tumor and not by normal somatic cells. Furthermore, migration of CAR T cells into solid tumors is often inefficient because they lack the appropriate chemokine receptors required to enter the lesion. Finally, solid tumors are highly immunosuppressive (as describe above) and preclude the normal lytic function and survival of CAR T cells[228].

Yet, multiple clinical trials are testing the optimization of CAR therapy for solid malignancy. For instance, EGFRvIII is a mutated form of the epithelial growth factor receptor (EGFR), expressed solely on tumor cells in certain glioblastomas and is tested in a phase I clinical trial[229]. Another example is the Human Epidermal Growth Factor Receptor-2 (HER2), which is expressed at low level on somatic cells, but overexpressed on certain malignancies such as breast cancers[230]. Unfortunately, the low level expression of HER2 on lung epithelial cells led to fatal respiratory distress in a case report of a patient with metastatic colon cancer treated with anti-HER2 CAR T cells[231]. However, another study showed no toxicity but only moderate efficacy of anti-HER2 CAR therapy in patients with sarcoma[232].

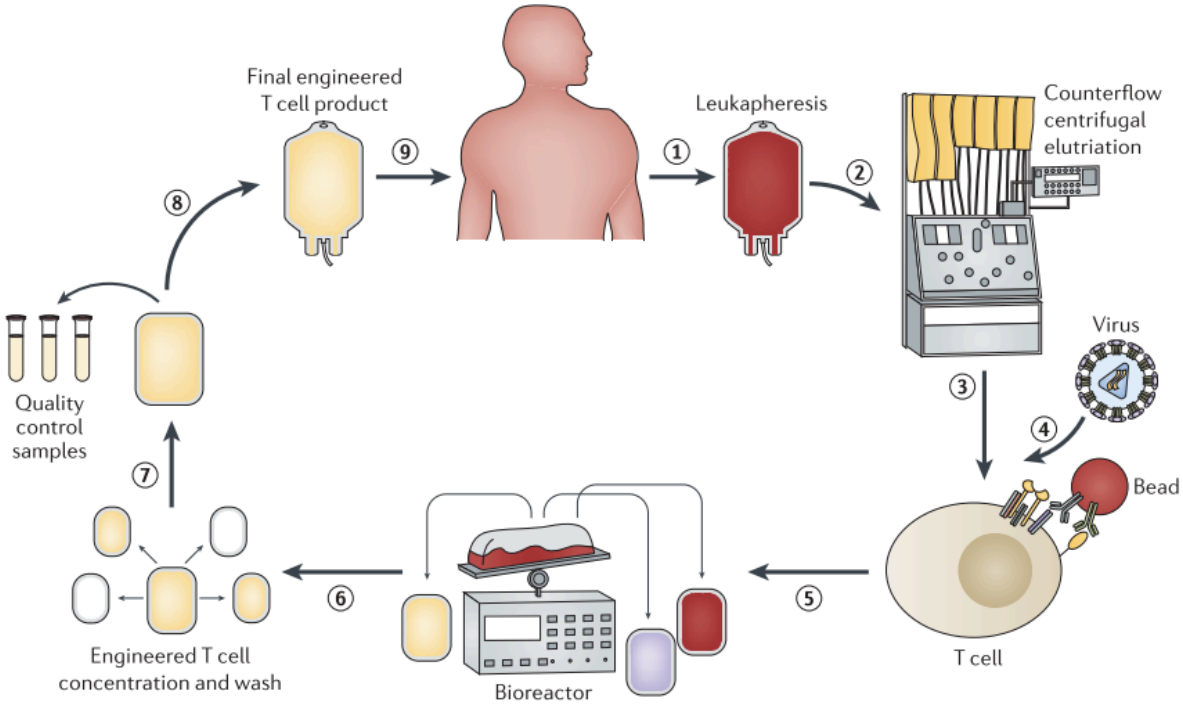


Figure 16 - The complex preparation of CAR T cells: T cells are collected from peripheral blood and purified. They are next activated with beads coated with anti-CD3 and CD28 antibodies and transduced with lentiviruses encoding for the CAR. Then, T cells are expanded, tested and reinfused in the patient. Adapted from [219].

# H My PhD projects

Cancer immunotherapy comprises several strategies to generate an antitumoral immune response, such as cancer vaccines, checkpoints blockade or T cell engineering. These immunotherapy modalities target different aspects of the T cell-mediated antitumoral response.

My PhD thesis involved three main projects, which encompassed these three strategies of cancer immunotherapy.

The first two projects were devoted to the study of iNKT cells. Indeed, our laboratory has a strong expertise in exploiting iNKT cells against cancer, and in particular their adjuvant properties on the adaptive immune response. However, their rapid anergy induction after an initial strong activation by the CD1d/iNKT agonist αGalCer has so far limited their clinical use. Therefore, my first project has focused on the understanding of iNKT cell anergy, which may allow improving the manipulation of these non-conventional lymphocytes in therapeutic cancer vaccines.

My second project was also related to iNKT cells with the development of a DC vaccine. The adoptive transfer of ex vivo generated DCs pulsed with an antigen and/or cytokines have now entered clinical application in cancer immunotherapy and have provided promising results when involving the transactivation of iNKT cells. In this context, I tested an innovative combined treatment involving a DC/αGalCer vaccine and PD-1 checkpoint blockade.

Finally, my third project was related to CAR T cell engineering. While CAR T cell therapy have entered the clinics for the treatment of B cell malignancies, it still needs optimization for the treatment of solid malignancies. Several groups including ours aimed at combining the activation of a CAR and the endogenous TCR on the same T cell to improve the activity of CAR T cells. In this project, I investigated how the T cell receptor copes with the presence of the CAR and if the presence of the CAR disturbs the activation of the T cell when engaged via its TCR.

Altogether, the general purpose of my PhD thesis was to optimize T cell-mediated cancer immunotherapy either via cancer vaccine or T cell engineering.

# I Regulation of iNKT cell anergy

## I.1 Aim

iNKT cell anergy, which is described in the introduction, is a hurdle for efficient iNKT cell-mediated immunotherapy. Indeed, a single dose of αGalCer in mice renders iNKT cells hyporesponsive to subsequent treatments[49]. Furthermore, a proportion of anergized iNKT cells acquire a regulatory phenotype, which promotes tumor tolerance[49]. As discussed in 6.1.7, several molecular players have been demonstrated to play a role in the onset of anergy in iNKT cells, such as PD-1 up-regulation, the lack or excess of co-stimulation to iNKT cells by APCs[50–53]. However, no clearly-defined mechanism has been proposed so far to explain the molecular mechanisms behind iNKT cell hyporesponsiveness. Interestingly, our previous results demonstrated that CD1d-antitumor fusion proteins induce a fast and sustained iNKT cell activation, which allowed repeated treatments[55,56]. Similarly, we and others showed that Th2 GSLs which quickly load on the APC cell surface, resulted in diminished iNKT cell anergy (unpublished, and [54]).

In view of these results, we investigated the mechanisms of iNKT cell anergy induction by comparing analogs of αGalCer inducing either a Th1 or a Th2 response.

## I.2 Material and methods

### I.2.1 B cell isolation

To isolate B cells, C57BL/6 spleens were smashed on a 40µm cell strainer. Red blood cells were lysed in RBC lysis buffer (Qiagen 158904) for 5min and then washed in full medium. After that, Fc receptors were blocked for 20min using the supernatant of the rat anti-mouse CD16/32 2.4G2[233] hybridoma. Cells were then stained with 5µg/ml CD19-FITC (Biolegend 115506) for 30min and then washed 3 times in FACS buffer (PBS, 5% FCS and 5mM EDTA). Finally, B cells were sorted with anti-FITC microbeads according to the manufacturer protocol (Miltenyi 130-048-701).

### I.2.2 DC isolation from spleen

DCs were isolated from the spleens of Flt3L transgenic mice[234]. Spleens were harvested and digested by injecting 0.32mg/ml Liberase and 0.12 mg/ml DNAse in plain RPMI medium and incubated for 20min at 37°C. The organs were then smashed on a 100µm cell strainer. Red blood cells were lyzed and Fc receptors blocked as described above. Finally, DCs were sorted with CD11c microbeads (Miltenyi 130-108-338) according to the manufacturer protocol.

### I.2.3 DC generation from bone marrow

To generate DCs from bone marrow, the bones from the back legs of C57BL/6 mice were collected, isolated from muscles and washed in full media. The bone marrow was flushed out with a 1ml insulin syringe (Beckton Dickinson 324912) and filtered through a 100µm cell strainer. Red blood cells were lyzed as described previously. $2x10^6$ cells were plated in each well of a 6 well plate in 5ml of IMDM medium supplemented with 10% FCS, 50U/ml Penicillin-Streptomycin (Gibco 15070-063), 0.075% Sodium Bicarbonate (Thermo Fisher Scientific 25080094), 10mM HEPES (Gibco 15630080), 1x MEM NEAA (Gibco 11140050), 50µM β-mercapto-ethanol (Gibco 31350010), 10 ng/ml recombinant mouse GM-CSF (Peprotech) and 20 ng/ml recombinant mouse IL-4 (Peprotech). The medium was changed on day 3 and 5. Cells were harvested on day 6. PBS-5mM EDTA was used to detach the DCs that were generated.

### I.2.4 GSL loading on APCs

To pulse APCs with αGalCer analogs, GSLs were first heated for 5min at 65°C to reduce micelles before dilution. Cells were incubated with 200ng/ml GSLs (or less, depending on the experiment) for 20h in full medium. The proper loading of GSLs was verified by flow cytometry: the cells were washed, incubated with the FcR blocking supernatant as described

previously and incubated for 20min on ice with 2µg/ml of L363-PE antibody (Biolegend 140505). Cells were washed twice with FACS buffer prior to cytofluorimetric acquisition.

Depending on the experiment, the cells were fixed before or after the loading of GSLs: cells were washed twice in cold PBS and incubated for 10min on ice in PBS 2% paraformaldehyde. The cells were then washed twice in cold complete medium.

### I.2.5 Restimulation *in vivo* and flow cytometry analysis of iNKT cells

To assess the anergy status of iNKT cells, mice were re-challenged *i.v.* with 0.8 to 1µg of DB03-4 or KRN7000 depending on the experiment. The animals were sacrificed 3h later and spleens were harvested and smashed through a 70µm filter. Red blood cells were lysed as described previously. Single cell suspensions were put in culture at 37°C in full medium for 4.5h with Golgi Stop (BD Biosciences 554724) and Golgi Plug (BD Biosciences 555029), both at a 1:1000 dilution. Afterwards, cells were stained for flow cytometry analysis: dead cells were excluded with Fixable Viability Dye eFluor™ 506 (eBioscience 65-0866-14). iNKT cells were gated using CD3e-Alexa Fluor 700 (Biolegend 100215) and CD1d/αGalCer multimer PE (home-made), and B cells were excluded with the B220-APC antibody (Biolegend 103211). To stain for intracellular cytokines, cells were fixed using a fixation buffer for intracellular staining (Biolegend 420801) according to the manufacturer protocol, before being stained with the antibodies IFNγ-PerCP/Cy5.5 at 0.5µg/ml (Biolegend 505821), TNFα-Pacific Blue at 1µg/ml (Biolegend 506318) and IL-10-PE/Cy7 at 1µg/ml (Biolegend 505025) diluted in permeabilization buffer (Biolegend 421002) for 30min on ice. Cells were finally washed twice in permabilization buffer and resuspended in FACS buffer prior to acquisition.

### I.2.6 Serum cytokines measurement

To measure the serum levels of IFN-γ, blood was collected from the tail veins and centrifuged in microvette tubes to separate the serum from the cells (Sarstedt 20.1344). IFN-γ was assessed with the ELISA Ready-SET-Go kit (eBioScience 50-173-19). TNF-α and IL-6 were measured by flow cytometry using the Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences 560485).

### I.2.7 CD1d and lipid raft flow cytometry analysis on APCs

CD1d levels were analyzed using the anti-CD1d-PerCP/Cy5.5 (BioLegend 123513) at the concentration of 1µg/ml for 20min on ice. Lipid rafts were stained with Cholera Toxin B

labelled with Alexa Fluor 647 (Thermo Fisher Scientific C-34778) at the concentration of 2µg/ml for 20min on ice.

### I.2.8 DC vaccination and OT-1 T cell transfer

To vaccinate mice, DCs were generated from bone marrow as described previously. They were then loaded for 20h with the OVA SIINFEKL peptide at the concentration of 10µM together with 200ng/ml of either DB03-4 or KRN7000. The cells were then washed 3 times and resuspended in plain RPMI medium for *i.v.* injection.

OT-1 CD8 T cells were extracted from the spleen of OT-1 transgenic mice[235]. CD8 T cells were purified using the EasySep™ Mouse CD8+ T Cell Isolation Kit (StemCell 19853) according to the manufacturer protocol.

## I.3 Results

### I.3.1 Th1 and Th2-biasing αGalcer analogs with different kinetic of iNKT cell activation

To investigate the onset of anergy induction in iNKT cells by Th1 and Th2-biasing αGalCer analogs, we focused on two GSLs, DB03-4 and KRN7000. While KRN7000, originally isolated from a marine sponge, induces a mixed Th1/Th2 response in iNKT cells, DB03-4 skews the iNKT cell response towards Th2[236]. In addition to the cytokine profile, B cells incubated with 200ng/ml of DB03-4 or KRN7000 showed very different kinetic of presentation, as monitored with the antibody L363, specifically recognizing CD1d when loaded with αGalCer[237]. Indeed, DB03-4 is presented at the cell surface after already 2h, while the presentation of KRN7000 is delayed, as it appears at 20h (Figure 17 A). The same kinetic of iNKT cell activation occurs *in vivo*. Mice received either 1µg of DB03-4 or KRN7000 *i.v.* and serum was collected at 2h and 21h. IFNγ was measured by ELISA as a surrogate of iNKT cell activation. At 2h, DB03-4 induced more IFNγ than KRN7000, while the opposite pattern was observed at 21h (Figure 17 B). These results support the hypothesis that Th2-biasing glycolipids are presented faster than their Th1 counterparts as they can load directly on CD1d at the cell surface without requiring internalization[44].

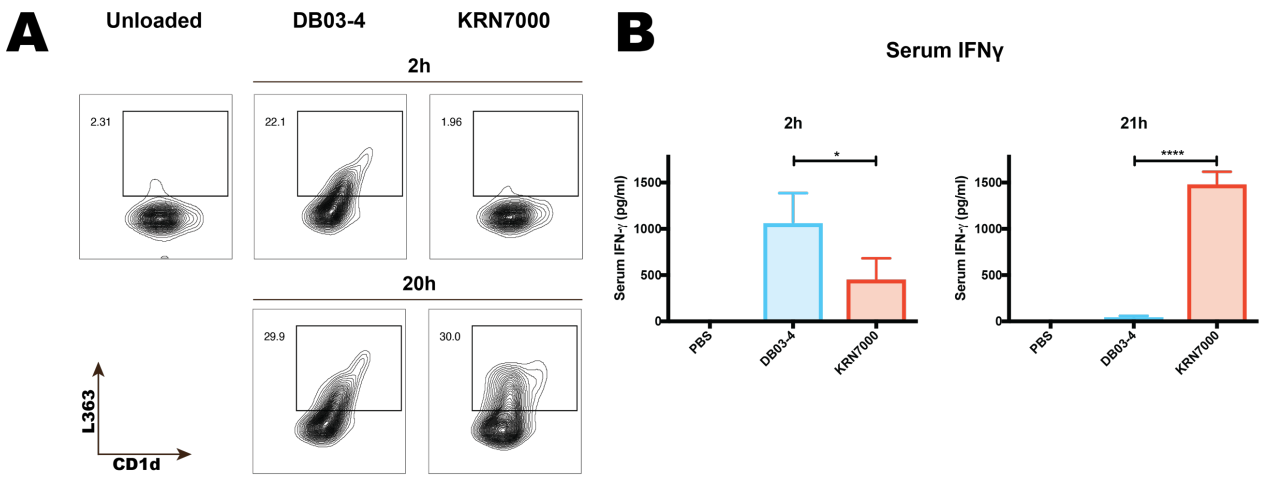


Figure 17 – Different kinetics of the two αGalCer analogs DB03-4 and KRN7000. A) B cells were incubated at 37°C with 200ng/ml of either DB03-4 or KRN7000 for 2h and 20h. The dot plots indicate the loading or not of αGalCer on CD1d by L363 mAb staining. B) C57BL/6 mice received 1µg of either DB03-4 or KRN7000 *i.v.* and were bled at 2h and 21h. Serum IFNγ was measured by ELISA.

To investigate the whether DB03-4 or KRN7000 differentially induce iNKT cell anergy, we challenged mice 3 times with each of the GSLs. We performed a cytokine measurement in the serum of mice 6h after the first injection of DB03-4 or KRN7000, which shows that the two

GSLs induce IFNγ and TNF-α, while only KRN7000 induces IL-6 at this time point (Figure 18). However, 3h after the 3rd GSL injection, only mice that received DB03-4 display a secretion of these cytokines in the serum. These results show that repeated injections of DB034 induce less iNKT cell anergy than KRN7000.

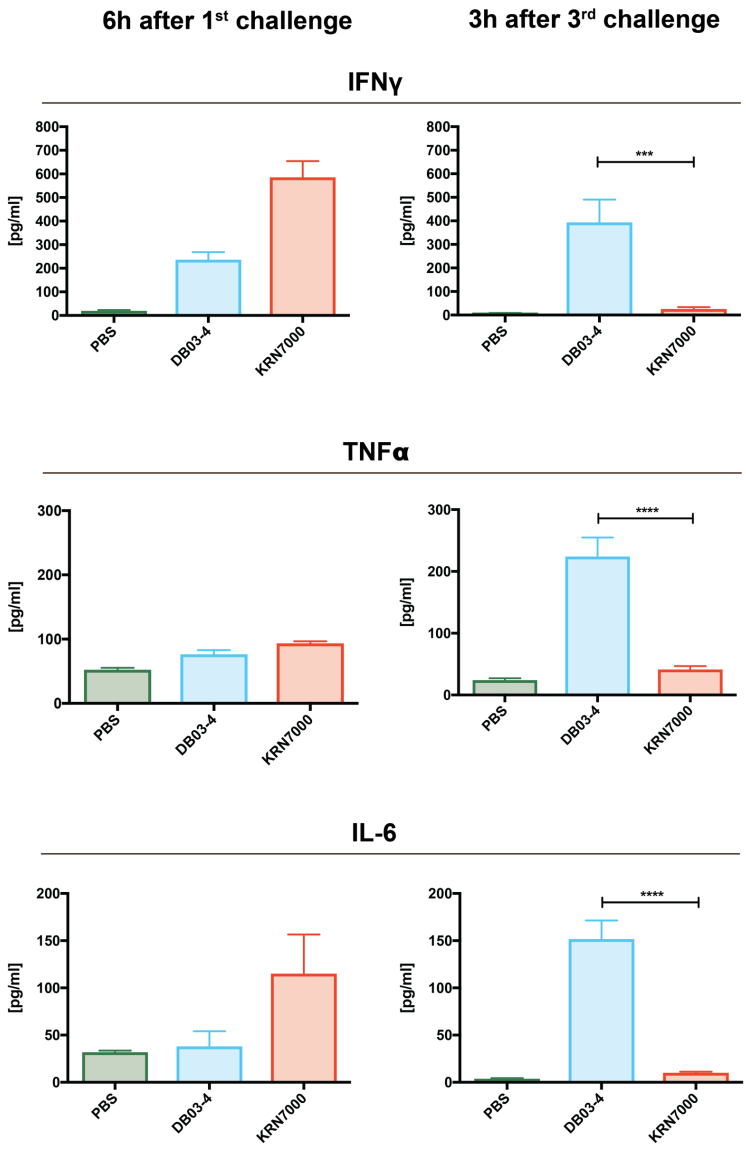


Figure 18 - DB03-4 keeps iNKT cells responsive to multiple challenges. Serum cytokines measured in mice 6h after the first and 3h after the third injection of either DB03-4 or KRN7000.

### I.3.2 Th2/polar αGalCer analogs induce less iNKT cell anergy than their Th1/apolar counterparts

To extend the notion that Th1 and Th2 αGalCer analogs induce differential iNKT cell anergy, we studied a panel of αGalCer analogs, which differ in their solubility as a result of slight structural differences in their lipid tail (Figure 19). c-GC, KRN7000 and SKC08-27 are apolar analogs and induce preferentially a Th1 response, while DB03-4, DB05-14, DB03-5 and BF1308-84 are Th2-skewed. Finally, 7DW8-5 has a mixed phenotype.

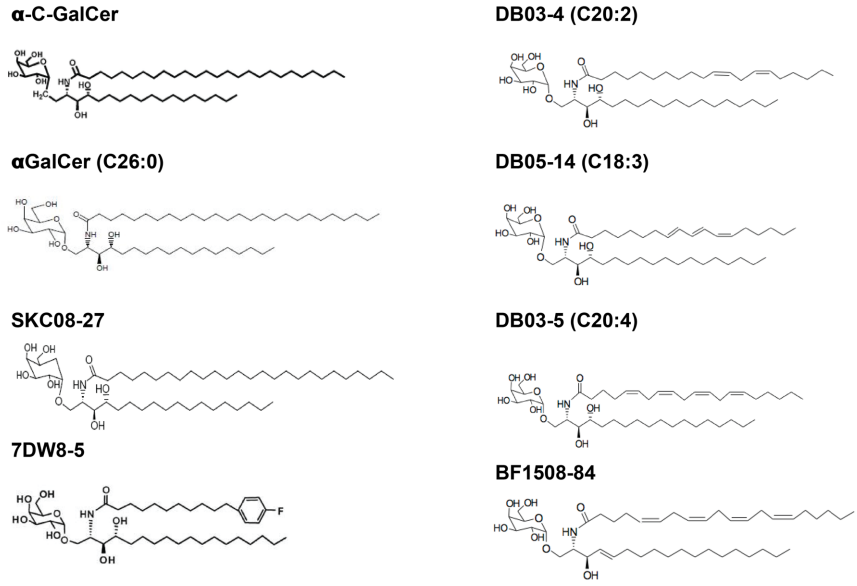


Figure 19 - Chemical structure of the αGalCer analogs tested in this project.

C57BL/6 mice were injected twice with 0.4µg of glycolipids *i.v.* on day 0 and 5. They were then rechallenged with a dose of 0.8µg of each of the GSLs (Figure 20 A). Three hours after the 3$^{rd}$ challenge, iNKT cells failed to upregulate the activation marker CD69 after repeated injections of Th1 analogs, while its expression remained high with Th2-biasing GSLs (Figure 20 B). The same pattern was also observed with intracellular INFγ. These results show that Th2 glycolipids induce significantly less iNKT cell anergy than Th0/1 analogs.

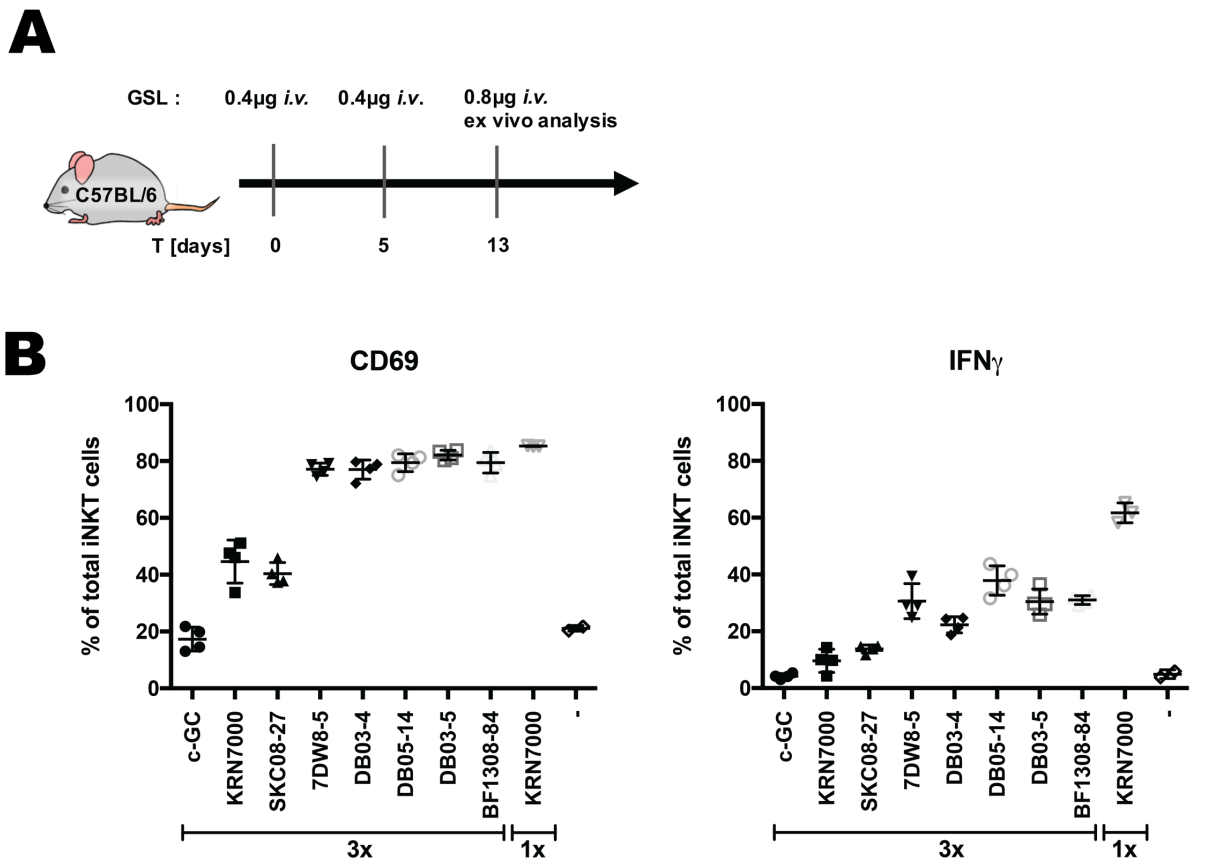


Figure 20 – The hydrophobicity of αGalCer analogs controls the induction of iNKT cell anergy. A) Schematic representation of the experiment: mice received two doses of 0.4µg of each of the GSLs at day 0 and 5. They were rechallenged on day 13 with 0.8µg of each GSLs and cytokine accumulation in spleen iNKT cells was monitored by flow cytometry. B) Surface staining of CD69 and intracellular cytokine staining of splenic iNKT cells after 3 injections of GSLs.

### I.3.3 Professional and non-professional APCs similarly induce iNKT cell anergy when loaded with KRN7000

The group of Kronenberg demonstrated that professional APCs such as DCs induce more iNKT cell anergy than other APCs such as B cells[51]. Later, the group of Kronenberg showed that neither B cells or DCs were required for the induction of iNKT cells *in vivo* and that it could be induced by an unidentified hematopoietic population[54]. However, we do not confirm these findings.

To evaluate the affinity of different APCs for polar and apolar GSLs, splenocytes were loaded with DB03-4 or KRN7000 for 20h to evaluate the GSL presentation in several APC populations. Interestingly, the L363 staining showed that B cells and $CD8\alpha^-$ DCs presented more efficiently DB03-4 than $CD8\alpha^+$ DCs. However, $CD8\alpha^+$ DCs were the most efficient APCs at presenting KRN7000 (Figure 21).

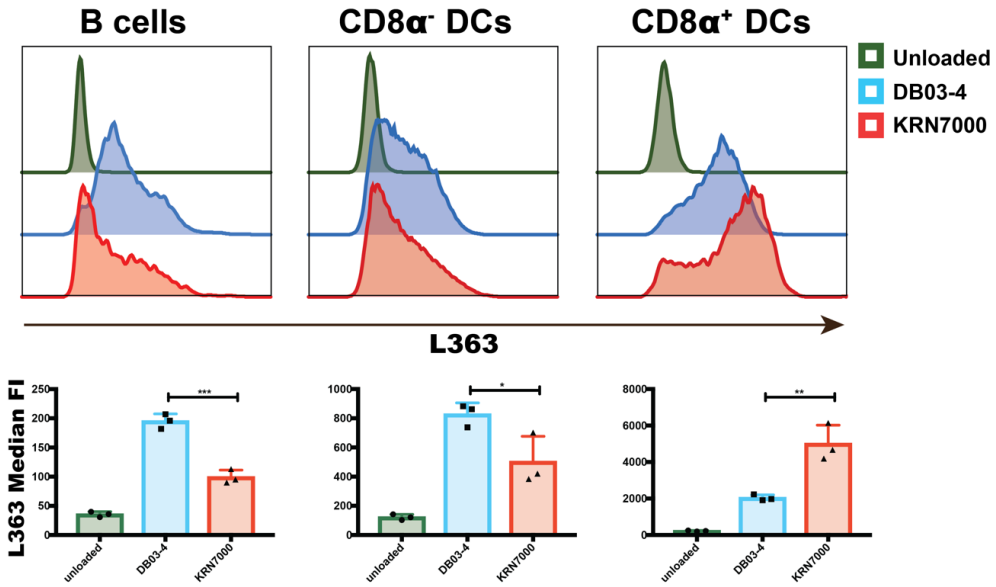


Figure 21 - APCs have different affinities for the polar and apolar GSLs DB03-4 and KRN7000. L363 staining of splenic B cells (CD19+), CD8α⁻ or CD8α⁺ CD11c⁺ DCs after 20h of coincubation with 200ng/ml DB03-4 or KRN7000.

This difference might be explained by the increased CD1d expression on CD8α⁺ DCs (Figure 22 A), as well as their higher lipid raft content, as revealed by the staining with Cholera Toxin B (Figure 22 B), as apolar GSLs are reported to be preferentially presented on lipid rafts[44].

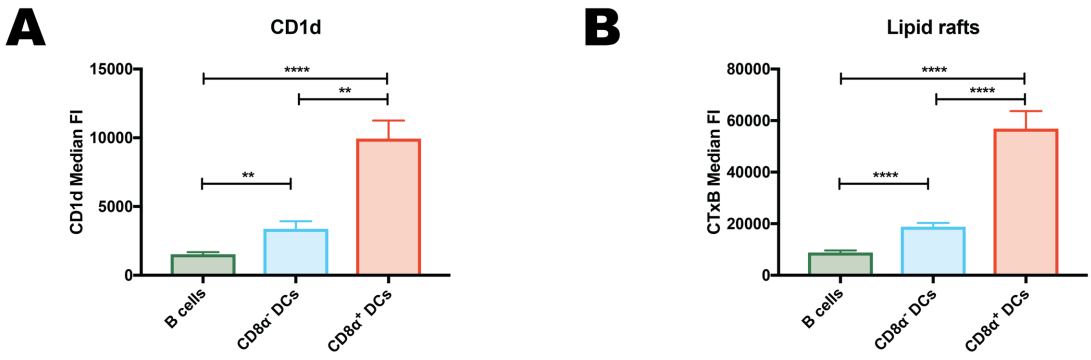


Figure 22 - A) CD1d and B) Cholera Toxin B staining in APC populations in spleen.

To assess whether a differential affinity of APCs for either DB03-4 or KRN7000 could explain the preferential iNKT cell anergy induction by KRN7000, we injected several types of APCs pulsed with either DB03-4 or KRN7000 in mice (Figure 23 A).

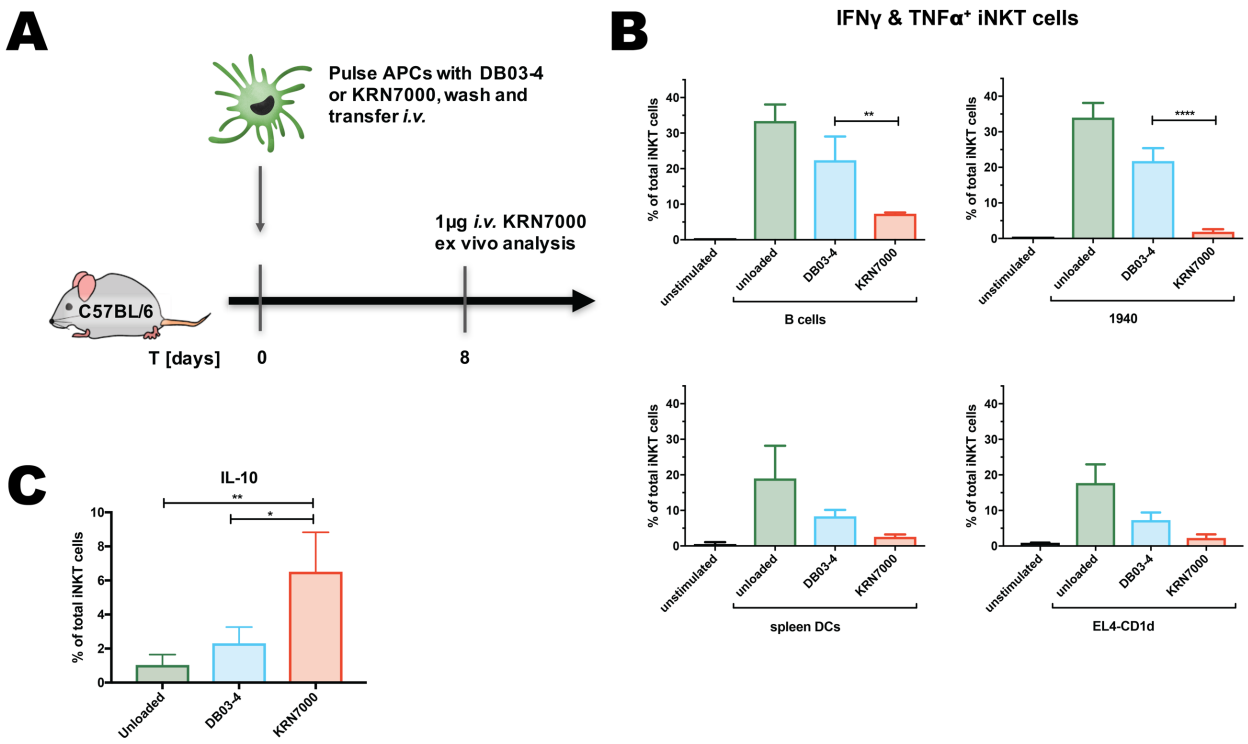


Figure 23 - Different APCs induce similar levels of iNKT cell anergy. A) Schematic representation of the experiment: APCs were loaded for 24h with 200ng/ml of either DB03-4 and KRN7000, washed 3 times and injected *i.v.* in mice. Animals were rechallenged 8 days later. B) and C) Intracellular cytokine staining of *ex vivo* splenic iNKT cells restimulated *in vivo* with 1µg of KRN7000 for 3h and *ex vivo*-cultured for 4h with Brefeldin A and Monensin.

Mice were rechallenged one week later to monitor the anergy in iNKT cells. Surprisingly, B cells and splenic DCs, as well as the CD8α$^+$ DC cell line 1940[238], or the T cell lymphoma EL4-CD1d, transduced to express high levels of CD1d (Figure 24), similarly induced iNKT cell anergy when loaded with KRN7000, but not with DB03-4 (Figure 23 B). Furthermore, IL-10 production was increased in iNKT cells when anergized with KRN7000-pulsed DCs (Figure 23 C). These results show that the phenomenon of anergy induction is not related to the nature of the APC and that any APC has the ability to induce it.

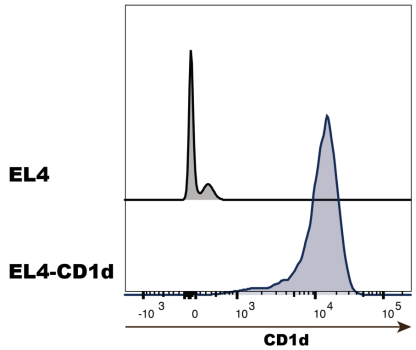


Figure 24 - CD1d expression level in EL4 and EL4 transduced with mouse CD1d.

### I.3.4 Fixation of DCs pulsed with DB03-4 sustains its presentation

A study by the group of Porcelli reported that surface-loaded Th2-biasing glycolipids, which are more polar, are not stably presented on the surface of APCs because they are quickly unloaded in acidic endosomes during the recycling of CD1d[45]. To assess that in our system we blocked CD1d recycling via PFA fixation of DB03-4-loaded DCs (Figure 25 A). Indeed, we showed that, while live DCs quickly lost the presentation of DB03-4 upon withdrawal of the GSL from the media, PFA fixation allowed the retention of DB03-4-loaded CD1d molecules at the cell surface (Figure 25 B).

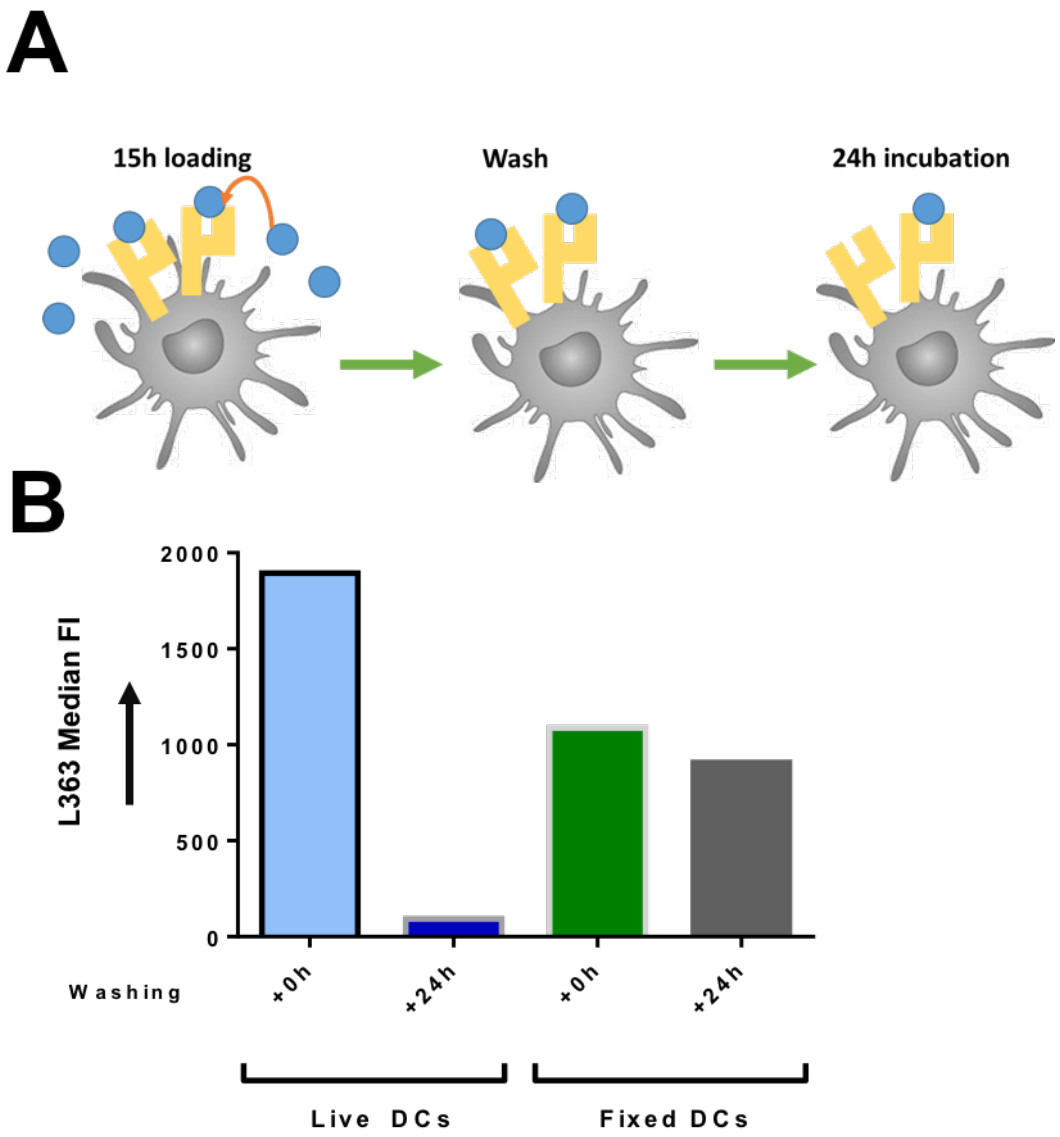


Figure 25 – Fixation allows the retention of DB03-4 at the surface of DCs. A) Schematic representation of the experiment: live or fixed DCs were pulsed for 20h with 200ng/ml of DB03-4 and then washed. 24h later, the presentation of DB03-4 was assessed by flow cytometry using the L363 antibody. B) L363 staining of live or PFA-fixed splenic DCs loaded with 200ng/ml DB03-4 for 15h and washed.

### I.3.5 Sustained presentation of DB03-4 is not sufficient to induce iNKT cell anergy.

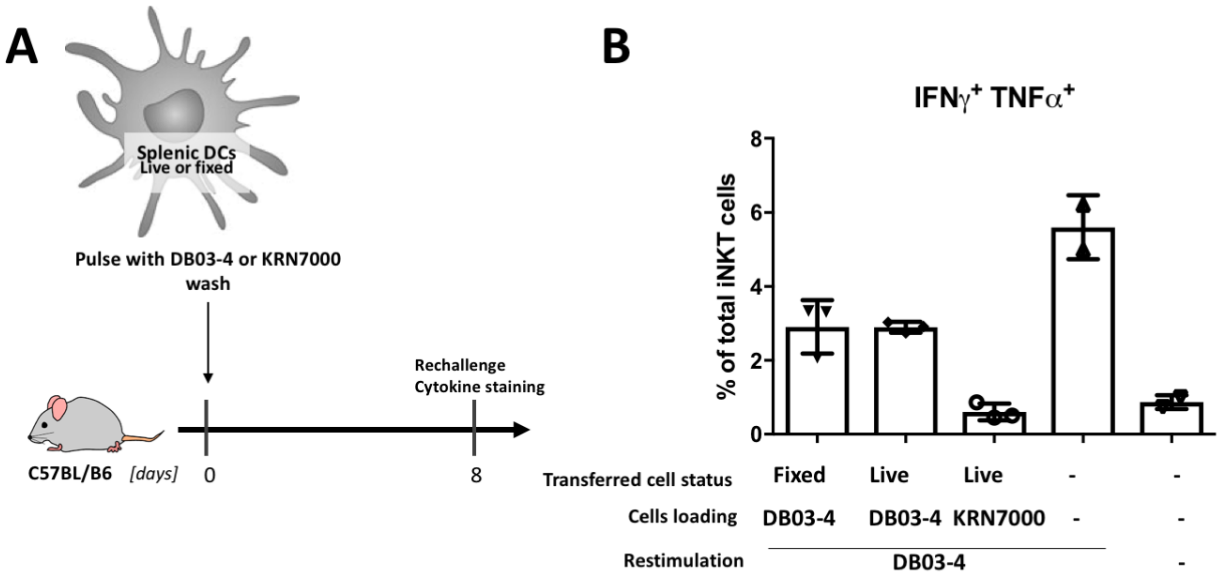


Figure 26 – Retention of DB03-4-loaded CD1d at the surface of DCs with PFA fixation is not sufficient to induce iNKT cell anergy. A) Schematic representation of the experiment: live or PFA-fixed splenic DCs were loaded with DB03-4 or KRN7000, washed and transferred into mice hosts *i.v.* Mice were rechallenged 8 days later with 1µg DB03-4 *i.v.* to assess for iNKT cell anergy. B) Intracellular cytokine staining of *ex vivo* splenic iNKT cells restimulated *in vivo* with 1µg of DB03-4 for 3h and *ex vivo*-cultured for 4h with Brefeldin A and Monensin.

To assess whether the short presentation of DB03-4 was the reason why this GSL induces less iNKT cell anergy than KRN7000, we pulsed live or PFA-fixed splenic DCs with DB03-4 or KRN7000 and injected these cells into C57BL/6 hosts (Figure 26 A). Anergy was assessed one week later via a restimulation with DB03-4, as described earlier. Interestingly, mice that received fixed APCs loaded with DB03-4 did not display more iNKT cell anergy than the animals that received live DCs loaded with DB03-4 (Figure 26 B). We also confirmed that fixed APCs were able to induce iNKT cell anergy by injecting in mice fixed KRN7000-loaded DCs, which induced iNKT cell hyporesponsiveness similarly to live DCs loaded with KRN7000 (data not shown).

These results show that prolonged presentation of the polar GSL DB03-4 via fixation does not induce more anergy in iNKT cells.

### I.3.6 High presentation of DB03-4 is not sufficient to induce iNKT cell anergy

To fully exclude that saturating and/or sustained GSL presentation to iNKT cells mediates iNKT cell anergy, we compared two cell lines as APCs: EL4-CD1d pulsed with DB03-4 presented 200x more DB03-4 than 1940 DCs, due to their much higher CD1d expression (Figure 21). This presentation was still very high when EL4-CD1d were fixed, which allowed a stable presentation over time, as shown before. Similarly, EL4-CD1d had a higher surface presentation of KRN7000 than 1940 DCs (Figure 27 B). Mice were then challenged twice with APCs fixed or alive and loaded with DB03-4 or KRN7000 (Figure 27 A). Interestingly, EL4-CD1d which presented very high amounts of DB03-4 at their surface, did not induce iNKT cell anergy, whether live or stabilized with fixation. Furthermore, 1940 DCs which presented much lower amount of KRN7000 presentation, significantly induced iNKT cells anergy. These results demonstrate that the induction of iNKT cell anergy does not depend on the amount or the duration of the antigen presentation, but may rely on the processing of the glycolipid which depends on its solubility.

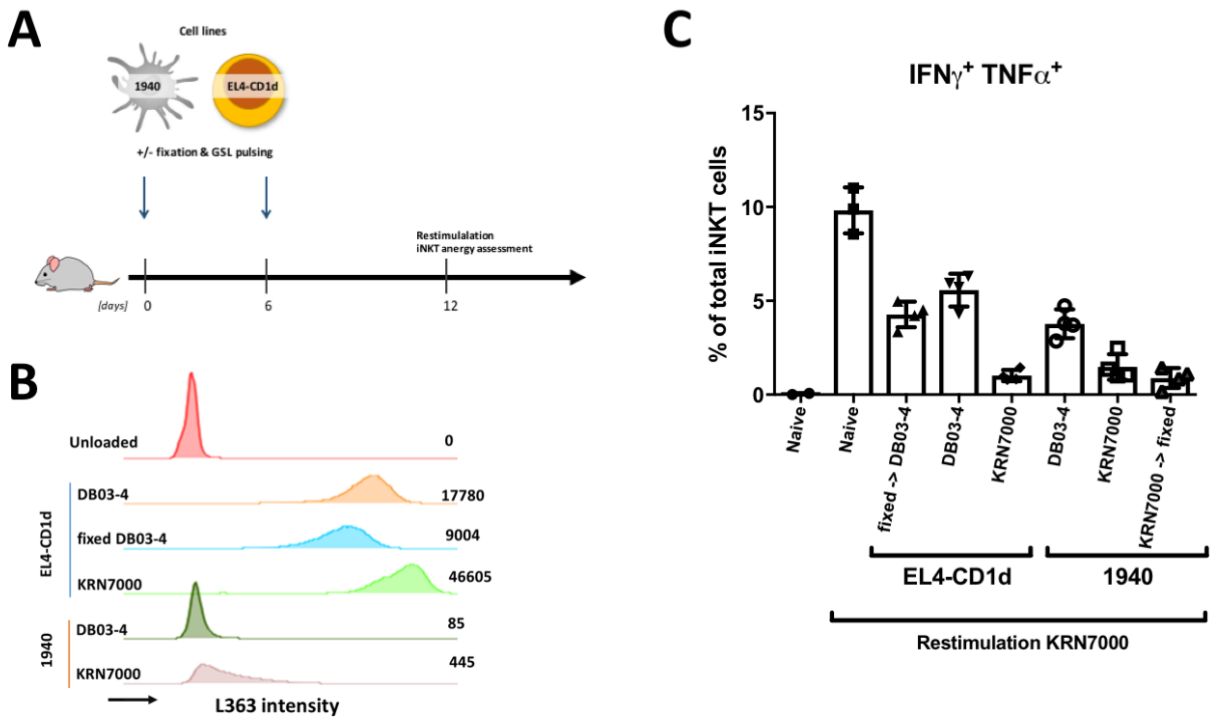


Figure 27 – High and sustained DB03-4 presentation is not sufficient to induce iNKT cell anergy. A) Schematic representation of the experiment: 1940 and EL4-CD1d cell lines were fixed and pulsed with either DB03-4 or KRN7000. These cells were injected on day 0 and 6. Mice were restimulated on day 12 with KRN7000 to assess for iNKT cell anergy. B) L363 staining of the different GSL-loaded APCs before transfer in mice. C) Intracellular cytokine staining of *ex vivo* splenic iNKT cells restimulated *in vivo* with 1µg of KRN7000 for 3h and *ex vivo*-cultured for 4h with Brefeldin A and Monensin.

### I.3.7 iNKT cell anergy induction by KRN7000 does not rely on a mechanism induced by the processing of the glycolipid in APCs.

Apolar glycolipids, which polarize the iNKT cell response towards Th1[44] and induce iNKT cell anergy (Figure 20), require intracellular processing in APCs to be loaded on CD1d[44–46]. We hypothesized that the intracellular antigen processing might participate to the anergy induction of iNKT cells. To test this, we loaded EL4-CD1d with DB03-4 or KRN7000 for a very short period of time (1.5h) before fixation with PFA. We hypothesized that the short incubation time would not allow antigen processing but only surface loading, which would be frozen by subsequent PFA fixation. Different doses of GSLs were used to allow a similar surface loading of DB03-4 and KRN7000, in view of their different solubility (Figure 28 A). As a control, live cells were also loaded with the same high amount of DB03-4 or KRN7000 for different durations. Pulsed EL4-CD1d cells were fixed or not and injected in mice. Anergy was assessed 8 days later by rechallenging mice with 1µg KRN7000 *i.v* (Figure 28 B). The results showed that EL4-CD1d fixed after being loaded with KRN7000 for a short period of time induced similar levels of iNKT cell anergy than EL4-CD1d loaded for 8h with KRN7000 and kept alive (Figure 28 C). These results suggest that the intracellular processing of apolar GSLs is unlikely to be responsible for iNKT cell anergy.

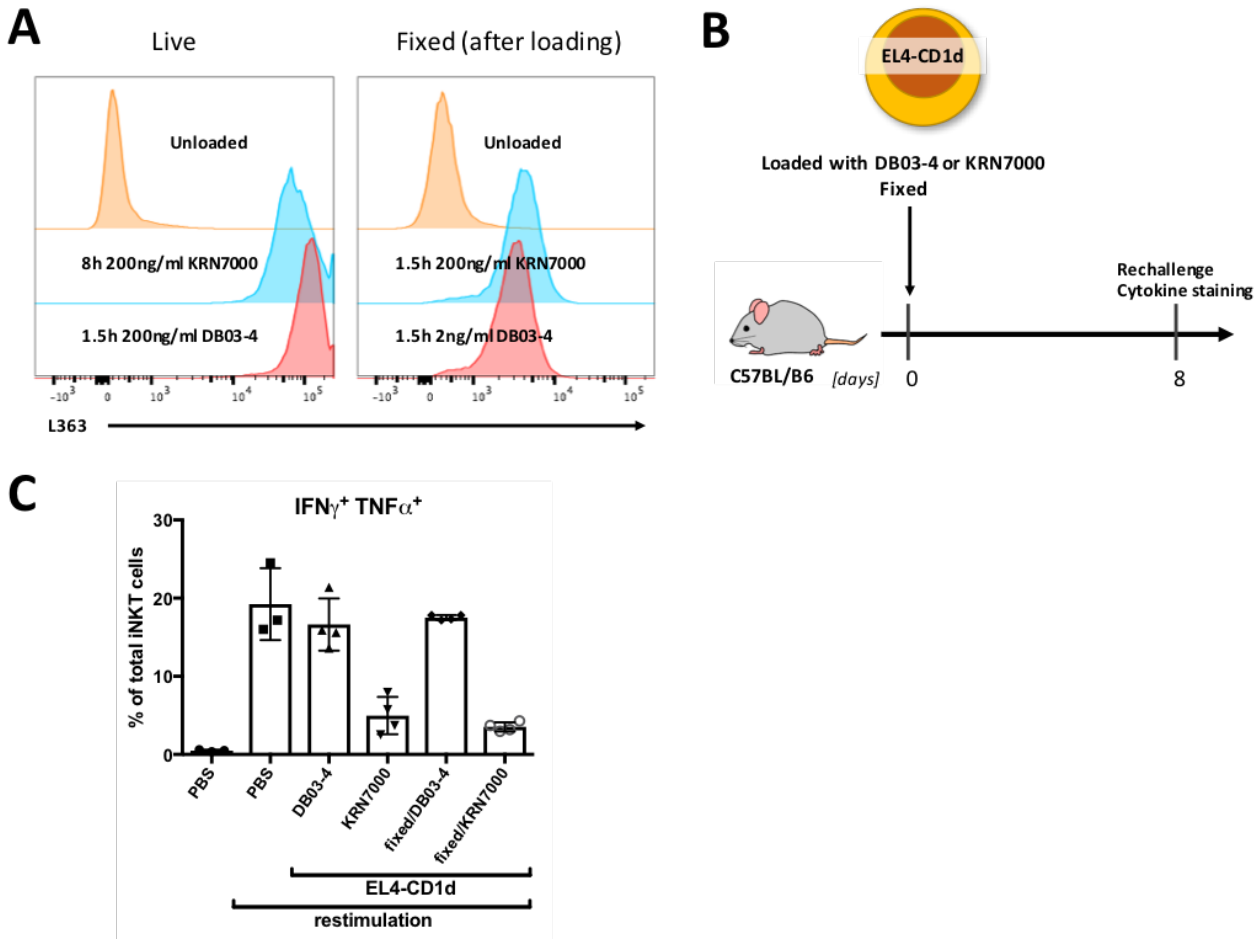


Figure 28 - iNKT cell anergy induction by KRN7000 does not seem to rely on a mechanism induced by the processing of the glycolipid in APCs. A) GSL presentation of EL4-CD1d loaded with KRN7000 or DB03-4 with or without fixation post antigen-pulsing. B) Scheme of the *in vivo* experiment: Live or fixed GSL-pulsed EL4-CD1d were transferred *i.v.* into recipient C57BL/6 hosts. C) Mice were re-challenged 8 days later with KRN7000 to assess for iNKT anergy. iNKT cell anergy is reported as intracellular accumulation of IFN-γ and TNF-α in iNKT cells.

### I.3.8 KRN7000 is a more potent inducer of DC maturation than DB03-4 *in vivo*

We finally asked whether DB03-4 was as potent as KRN7000 analog for the maturation of pro-inflammatory mature DCs and the priming of T cells. To do so, we injected 1µg of either DB03-4 or KRN7000 in Flt3L transgenic mice, which have higher numbers of DCs in the spleen[234]. Spleens were collected 20h later and DCs maturation was analysed by flow cytometry (Figure 29 A). DCs from mice treated with KRN7000 up-regulated significantly more CD80 and CD86 than with DB03-4 (Figure 29 B), indicating their better transactivation as compared to DB03-4 injection.

To assess if the superior DC transactivation by KRN7000 over DB03-4 also resulted in a better T cell priming, we transferred OT-1 T cells in C57BL/6 mice and vaccinated them 5 hours later with the transfer of bone marrow-derived DCs pulsed with the OVA SIINFEKL peptide and

DB03-4 or KRN7000. OT-1 expansion was analysed 7 day later in the spleens (Figure 29 C). As for DC maturation, KRN7000 induced significantly more OT-1 expansion than DB03-4 (Figure 29 D). These results show that KRN7000 is more potent than DB03-4 at priming T cell immunity, despite the fact that it induces more iNKT cell anergy.

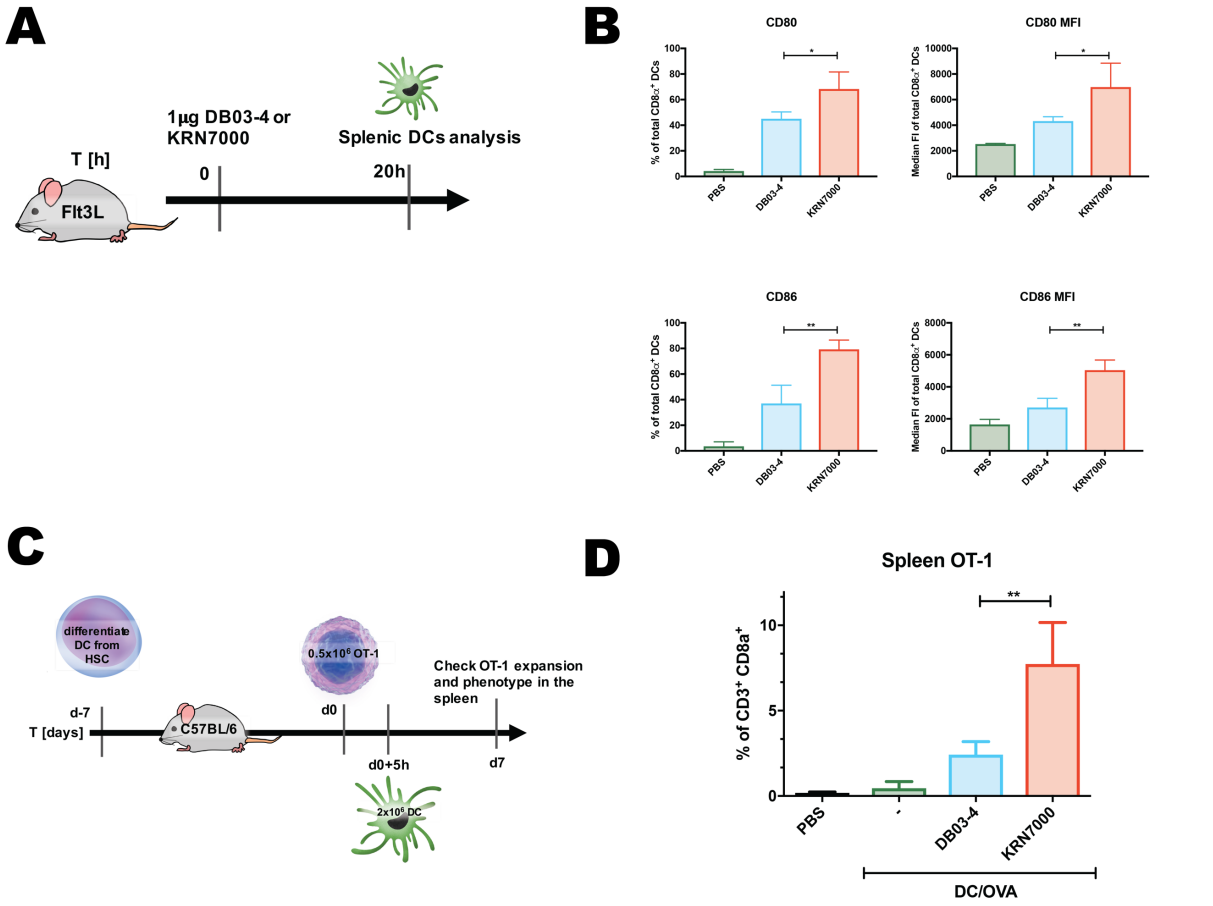


Figure 29 - DB03-4 is weaker than KRN7000 at maturing DCs and promoting T cell immunity. A) Scheme of the experiment: Flt3L mice received 1 µg of DB03-4 or KRN7000 *i.v.* Splenic DCs were collected and analysed 20h later. B) CD80 and CD86 expression in spleen DCs harvested 20h after GSL injection. C) Scheme of the experiment: DCs were differentiated from HSC for 6 days with GM-CSF and IL-4. They were then loaded with the SIINFEKL OVA peptide (10 µg/ml) and DB03-4 or KRN7000 (200ng/ml) for 20h. $0.5x10^5$ CD45.1 OT-1 T cells were injected in recipient CD45.2 C57BL/6 mice, followed by the DCs. OT-1 expansion was assessed 7 days later in the spleen. D) Percentage of spleen OT-1 T cells 7 days after vaccination.

## I.4 Discussion

In this project, we aimed at comparing the capacity of αGalCer analogs at inducing iNKT cell anergy with regard to their hydrophobicity and capacity to induce a Th1/Th2 bias. Based on their respective characteristics, we tried to identify a possible cellular mechanism on the APC, which may control the induction of anergy in iNKT cells.

We mainly focused on KRN7000 and DB03-4, as representative Th1 and Th2 analogs. We first confirmed that KRN7000 mainly required internalization by APCs for its efficient CD1d presentation via the endosomal pathway, which translated in a slow kinetic. In contrast, the more polar Th2 analog DB03-4 quickly loaded on the cell surface of the APCs[44]. The slow versus fast kinetic of CD1d presentation of Th1 versus Th2 analogs also correlated with the delayed versus fast iNKT cell activation, and more strikingly with the differential capacity of these GSLs to induce iNKT cell anergy. Indeed, testing a larger panel of Th1 and Th2-biasing αGalCer analogs, we demonstrated that Th1 αGalCer analogs are more potent to induce iNKT cell anergy, which was simultaneously shown by the group of Kronenberg[54].

In view of these observations, we investigated whether there was a link between anergy and antigen processing by the APC. Several hypotheses have been proposed to explain iNKT cell anergy, such as the type of APCs, PD-1 up-regulation or excess of co-stimulation[50–54]. Surprisingly, we found that any APC, even a lymphoma cell line transduced with CD1d, could induce iNKT cell anergy to the same extent provided they were loaded with a Th1-biasing αGalCer analog.

The nature of the APC was also proposed to be a mechanism explaining how different αGalCer analogs induce either a Th1 or Th2 iNKT response. We indeed found that the Th1-biasing GSL KRN7000 loaded preferentially on CD8α DCs, while DB03-4 loaded more on B cells and CD8α$^-$ DCs. This might be explained by the high density of lipid rafts in CD8α DCs, which quickly recruit glycolipid micelles[44,48]. CD8α DCs were proposed as the main APCs for iNKT cells, which also probably results from their higher expression of CD1d, as compared to other DCs and B cells. However, we show that independently of the presence of rafts, or level of CD1d expression, any APC is able to induce anergy in iNKT cell, when loaded with an apolar GSL. Similarly, Wingender *et al.* recently showed that neither B cells or DCs are required for the anergy induction of iNKT cells[54]. Collectively, data from the literature and our results demonstrate that the selective loading of GSLs on different APCs is not the main mechanism involved in the Th1/Th2 bias or the onset of anergy in iNKT cells.

We then asked whether the sustained presentation of Th1 GSLs was required for the onset of iNKT cell anergy. Indeed, we showed that the Th1-biasing αGalCer analog is stably presented on CD1d in contrast to the Th2-biasing GSL DB03-4. The mechanism accounting for this differential presentation has been elucidated by Porcelli *et al*[44,45], by showing that polar Th2-biasing GSLs mainly load on the cell surface and quickly unload from CD1d in acidic endosomes during the dynamic CD1d turnover. We observed that the fixation of APCs presenting DB03-4 allowed the stabilisation over time of CD1d/DB03-4 complexes, mimicking the stable presentation of Th1 GSLs. However, fixed DB03-4-pulsed DCs did not induce more iNKT cell anergy than their live counterparts. Even when using EL4 T cell lymphoma transduced to express very high level of CD1d, both live and fixed EL4-CD1d loaded with DB03-4 failed to induce iNKT cell anergy. These data strongly suggest that the strength and duration of GSL presentation is not a prerequisite for the induction of iNKT cell anergy.

Porcelli *et al*. showed that neutralization of endosome acidification with chloroquine or $NH_4Cl$ increased the localization of CD1d/Th2 GSLs to lipid rafts, as CD1d molecules trafficking from endosomes towards membrane lipid rafts were able to keep Th2 GSLs loaded. Furthermore, chloroquine-treated APCs loaded with Th2 GSLs induced some features of Th1 GSLs *in vivo*[46]. Unfortunately, we were not able to confirm those results of DB03-4 presentation on raft domains in APCs when treated with chloroquine (data not shown). Instead, we showed that loading for a short period of time of 90 min high amounts of KRN7000 mainly allowed its surface loading without preferential enrichment on raft domains. Interestingly, we found out that surface-loaded KRN7000 still induces iNKT cell anergy, which suggests that preferential localization of CD1d/GSL complexes in raft domains is not a prerequisite for anergy induction in iNKT cells. Furthermore, the short pulsing of KRN7000 immediately followed by cell fixation likely exclude that phenotypical changes in APCs are not required for iNKT cell anergy induction. Such a mechanism was proposed to play a role in the capacity of DCs to bias the iNKT cell response towards Th1 or Th2 when loaded with apolar or polar GSLs, respectively. Apolar GSLs were described to induce CD86 and Rae-1 on DCs, while their polar analogs induced PD-L1 and PD-L2[48]. We were however not able to confirm phenotypic changes of DCs when loaded with either DB03-4 or KRN7000.

To conclude, the mechanism underlying the induction of iNKT cell anergy by apolar Th1-biasing GSLs remains elusive as it does not involve a specific APC population, nor a long duration or strength of presentation, nor enriched presentation in rafts or phenotypical

differences in APCs. The difference between apolar and polar GSLs may reside in their nature, as apolar GSLs have a longer hydrophobic tail. Indeed, crystallographic analysis showed that the acyl chain of αGalCer fully fills the A' pocket of human CD1d, which induces a conformational change in CD1d, when compared with the unloaded molecule[10]. Polar GSLs have generally smaller acyl tails or insaturations, which decrease their affinity for CD1d and might induce weaker or different conformational changes on CD1d, which would then be seen differently by the semi-invariant TCR and induce a different outcome in iNKT cells.

Importantly, we showed that, while Th1 αGalCer analog KRN7000 induces iNKT cell anergy, it is also a much better adjuvant for DC maturation and T cell priming than Th2-biasing DB03-4, which induces less anergy. Therefore, for vaccine development, the Th1-biasing αGalCer analogs are much preferred and actively tested in pre-clinical[239–241] and clinical studies[191,192].

# J PD1 blockade combines with the iNKT cell activation to enhance the CD8 T cell-mediated anti-tumoral response

## J.1 Aim

The aim of this project was to find a way to improve the adjuvant effect of αGalCer on adaptive immunity shown by us and others[55,56,65–69,188]. DC vaccines involving the ex vivo maturation and antigen pulsing of pro-inflammatory DCs have reached clinical usage[170,242–244], and also showed promising cell responses in conjunction with iNKT/CD1d ligands[187,192,245]. In this project, we combined a DC/αGalCer vaccine with PD1 blockade and hypothesized that the two treatments would synergistically enhance T cell-mediated tumor rejection. Indeed, on one hand the crosstalk between iNKT cells and DCs would promote a potent T cell priming, while PD-1 blockade would restore the effector functions of the primed T cells within the tumor micro-environment.

## J.2 Material and methods

### J.2.1 Tumor engraftment

B16F10 or MC-38 tumors were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco 10566-016) with 10% FCS, 50U/ml Penicillin-Streptomycin, 50µM β-mercapto-ethanol and 100mM HEPES. Prior to engraftment, cells were detached with PBS 5mM EDTA, washed 3 times with ice-cold PBS. $10^5$ B16F10 or $7x10^5$ MC-38 were then resuspended in 200µl cold PBS and engrafted *s.c.* on the flank of C57BL/6 mice with a 25G syringe needle (Braun, 041-921019).

### J.2.2 Subcutaneous peptide vaccination

The vaccination was prepared with 10µg $Trp2_{180-188}$ (SVYDFFVWL) and 50µg poly(I:C) HMW (Invivogen tlrl-pic-5) or 1µg αGalCer (Enzo Life Sciences BML-SL232-1000) diluted in 200µl of PBS. Mice were subcutaneously vaccinated at the base of the tail with a 25G syringe needle.

### J.2.3 *Ex vivo* DC maturation and loading with Trp2 peptide for vaccination

DCs were isolated from Flt3L transgenic spleens as described in I.2.2. Splenocytes were cultured in IMDM medium supplemented with 10% FCS, 50U/ml Penicillin-Streptomycin (Gibco 15070-063), 0.075% Sodium Bicarbonate (Thermo Fisher Scientific 25080094), 10mM HEPES (Gibco 15630080), 1x MEM NEAA (Gibco 11140050), 50µM β-mercapto-ethanol (Gibco 31350010) at the concentration of $2x10^6$ cells/ml in poly-lysine-coated 6 well plates. The

medium was supplemented with 5µg/ml poly(I:C) and 10µg/ml of the indicated peptide. After 20h of culture, cells were detached with cold PBS 5mM EDTA, washed 3 times with PBS.

Correct maturation and viability were checked by flow cytometry with the CD80-PE (BioLegend 104707) and CD86-FITC (BioLegend 105005) antibodies and Fixable Viability Dye eFluor 506 (eBioscience 65-0866-14). $2x10^6$ cells were resuspended in 200µl of plain RPMI medium and injected *i.v.* into mice.

$2x10^6$ cells were resuspended in 200µl of plain RPMI medium and injected *i.v.* into mice.

### J.2.4 DC loading with αGalCer and Trp2 peptide for vaccination

DCs were isolated and cultured as in J.2.3. The medium was supplemented with 10µg of the indicated peptide and 200ng/ml αGalCer. After 20h of culture, cells were washed 3 times with cold PBS. Viability and αGalCer loading were checked by flow cytometry with Fixable Viability Dye eFluor 506 and the L363-PE antibody. $2x10^6$ cells were resuspended in 200µl of plain RPMI medium and injected *i.v.* into mice.

### J.2.5 Lymphodepletion and Pmel transfer

One day prior to adoptively transfer *h*gp100-specific CD8 T cells, mice were irradiated with 5Gy in a RS2000 X-Ray irradiator (Rad Source). On the next day, *h*gp100-specific CD8 T cells were purified from spleen of Pmel transgenic mice using the EasySep™ Mouse $CD8^+$ T Cell Isolation Kit (Stem Cell 19853) according to the manufacturer protocol. The concentration of *h*gp100-specific T cells in the bulk CD8 T cells was determined by flow cytometry and *h*gp100 multimer staining. $10^5$ *h*gp100-specific CD8 T cells were transferred *i.v.* in plain RPMI medium.

### J.2.6 anti-PD1 therapy

200µg of anti-PD1 clone RMP1-14 (Bio X Cell BE0146) were diluted in 200µl of PBS and injected *i.p.* in mice using a 26G syringe needle (Terumo 041-050101).

### J.2.7 Tumor dissociation

Tumors were collected and separated from skin. Single cell suspensions were obtained with the Mouse Tumor Dissociation Kit (Miltenyi 130-096-730) according to the manufacturer protocol. Single cells were then resuspended in 40% Percoll (36% Percoll [GE Life Sciences 17-0891-01], 4% 10x PBS, 60% full DMEM medium). 70% Percoll (63% Percoll, 7% 10x PBS, 30% full DMEM medium) was carefully poured on top, avoiding the mix of the two phases. Afterwards, the tubes were centrifuged at the speed of 950rcf. Finally, the lymphocytes accumulating at the interphase were collected and washed in full medium.

### J.2.8 Ex vivo restimulation of spleen and tumor T cells

To stain for cytokines, around 0.5 to 1x$10^6$ spleen and tumor single cells were plated in flat-bottom 96well plates with 10µM of the indicated peptides. 30min later, the Monensin and Brefeldin A-containing reagents Golgi Stop and Golgi Plug (Beckton Dickinson 554724 and 555029) were added at the final concentration of 1:1000. Cells were further incubated for 4h prior to staining for flow cytometry.

### J.2.9 Flow cytometry staining of spleen and tumor T cells

Cells were washed with FACS buffer (PBS, 2% FCS and 2.5mM EDTA) and incubated for 15min with 2.4G2 supernatant to block the Fc receptors CD16 and CD32[233]. Next, cells were stained with Fixable Viability Dye eFluor 506 according to the manufacturer protocol.

Consecutively, the cells were stained with the indicated PE-labelled MHC-I/peptide multimers. The cells were then surface-labelled with the antibodies CD3ε-Alexa Fluor 700 at 10µg/ml (BioLegend 100215), CD8α-Brilliant Violet 650 at 0.5µg/ml (BioLegend 100741), PD1-APC at 1µg/ml (BioLegend 135209), CD44-APC/eFluor 780 at 2µg/ml (eBioScience 47-0441-82) and CD62L-PE/Cy7 at 0.5µg/ml (BioLegend 104417).

To stain for intracellular cytokines, cells were fixed using a fixation buffer for intracellular stainings (Biolegend 420801) according to the manufacturer protocol, before being stained with the antibodies IFNγ-PerCP/Cy5.5 at 0.5µg/ml (Biolegend 505821), TNFα-Pacific Blue at 1µg/ml (Biolegend 506318) and Granzyme B-PE/Texas Red at 1:200 (according to the indications of the manufacturer, Thermo Fischer Scientific GRB17) diluted in permeabilization buffer (Biolegend 421002) for 30min on ice. Cells were finally washed twice in permabilization buffer and resuspended in FACS buffer prior to acquisition.

CD4 T cells were stained with the CD4-PerCP/Cy5.5 antibody (BioLegend 100433) at 0.5 µg/ml. The cells were permeabilised for nuclear staining using the Foxp3 / Transcription Factor Staining Buffer Set (eBioscience 00-5523-00) according to the manufacturer protocol. Afterwards, cells were stained with FoxP3-Alexa Fluor 488 at 2.5µg/ml (BioLegend 126405) and T-bet-APC at 1µg/ml (BioLegend 644813) in FoxP3 permeabilization buffer (perm buffer) (eBioscience 00-5523-00). Cells were finally washed twice in FoxP3 perm buffer and finally resuspended in facs buffer prior to analysis on a Beckton Dickinson LSRFortessa flow cytometer.

## J.3 Results

### J.3.1.1 αGalCer-pulsed DCs are superior to poly(I:C) for CD8 T cell priming

In order to illustrate the advantage of using αGalCer as an adjuvant for cancer vaccines, we compared it with the TLR3 agonist poly(I:C). To do so, we engrafted B16F10 tumors in mice and vaccinated them with the $Trp2_{180-188}$ peptide, which is a tumor-associated antigen in B16 melanoma[246]. We tested different vaccination strategies: *s.c.* immunization with Trp2 and poly(I:C), as previously described by our group[247], or with Trp2 and αGalCer. In addition to *s.c.* peptide immunization, we also compared DC vaccine, either loaded with the Trp2 peptide and αGalCer, or DCs matured *ex vivo* with poly(I:C) and loaded with Trp2 (Figure 30 A). DCs viability and loading with αGalCer was verified by flow cytometry before transfer. The maturation status of *ex vivo* DCs matured with poly(I:C) was confirmed by the upregulation of CD80 and CD86 (Figure 30 B). The efficacy of the vaccination strategies was tested 7 days later by comparing the percentage of H2-$K^b$/$Trp2_{180-188}$-specific CD8 T cells in the blood (Figure 30 C). Both groups of mice vaccinated *s.c.* with the peptide and αGalCer or poly(I:C) had very low levels of peripheral Trp2-specific CD8 T cells, in view of the low frequency of Trp2-specific T cell precursors. The expansion of Trp2-specific T cells remained quite low for poly(I:C)-matured DCs loaded with Trp2 peptide. In contrast, *ex vivo* DCs loaded with both the Trp2 peptide and αGalCer induced significantly more Trp2-specific T cells than all the other groups. These mice also displayed the best tumor control, although differences were not statistically significant (Figure 30 C).

These data show that DCs loaded with both αGalCer and a peptide antigen are very potent at inducing an endogenous antigen-specific CD8 T cell response.

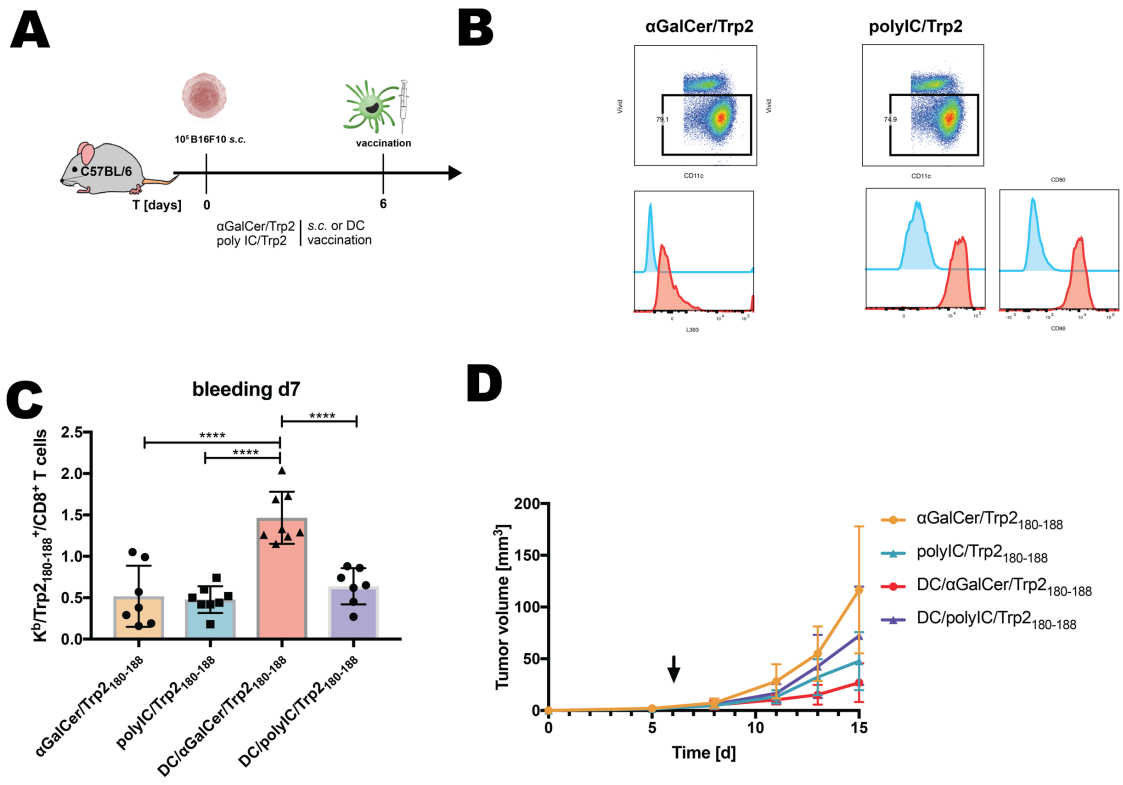


Figure 30 - Better CD8 T cell priming with transferred DCs loaded with αGalCer than matured with poly(I:C). A) Scheme of the experiment: $10^5$ B16F10 tumors were engrafted *s.c.* in the flank of C57BL/6 mice. One week later, mice were vaccinated with either DCs matured with poly(I:C) and loaded with the $Trp2_{180\text{-}188}$ peptide, or DCs loaded with αGalCer and Trp2 peptide or with *s.c.* injection of $Trp2_{180\text{-}188}$ peptide and poly(I:C) or αGalCer. B) Viability and L363 staining of the DCs loaded with αGalCer prior to injection (left) and viability and CD80 and CD86 staining of DCs matured with poly(I:C) (right). C) H2-$K^b$/ $Trp2_{180\text{-}188}$ multimer staining of blood CD8 T cells 7 days after vaccination. D) Kinetic of tumor growth.

### J.3.2 Adoptive T cell transfer and DC/αGalCer vaccine strongly rejects established tumors

In the Trp2 model, vaccination expanded the endogenous pre-existing Trp2-sepcific T cells. We then investigated how DCs loaded with αGalCer with or without peptide would expand a pool of transferred tumor-specific CD8 T cells. To do so, we engrafted B16F10 in C57BL/6 mice. To favour the engraftment of adoptively-transferred T cells[248], we lymphodepleted the hosts by X-ray irradiation of 5Gy on day 8. On day 9, we transferred $10^5$ H2-Kb restricted Pmel T cells specific for the human gp100 melanoma TAA, which also crossreact the murine gp100 epitope expressed in B16F10 tumors[249]. On the next day, mice were vaccinated with $2x10^6$ DCs loaded with αGalCer with or without the human $gp100_{25\text{-}33}$ peptide, as the murine ortholog is not immunogenic in mice[249] (Figure 31 A). Interestingly, the vaccination with αGalCer-pulsed DCs in the absence of gp100 peptide already induced a very strong tumor control, significantly better than the non-vaccinated group, which also received tumor-specific Pmel T cells. These

results suggest that the αGalCer/DCs were able to prime Pmel T cells against the B16 tumors. Most importantly, the vaccination with DCs loaded with both αGalCer and *h*gp100$_{25\text{-}33}$ was even more potent, as it induced almost complete tumor rejection (Figure 31 B). In line with this tumor inhibition, we observed that Pmel T cells were significantly enriched in the spleen and tumors of mice vaccinated with both αGalCer and peptide-pulsed DCs. Additionally, a higher proportion of Pmel had an effector phenotype in the spleen of mice treated with the combined vaccination (Figure 31 C). Finally, mice in this group presented a higher Pmel/Treg ratio in both the spleen and tumors, which is generally associated with successful tumor control[247]. Collectively, these data show that DC/αGalCer enhance the tumor control by priming and expanding adoptively transferred tumor-specific T cells. However, this effect can be greatly potentiated by adding a tumor peptide antigen to the vaccine.

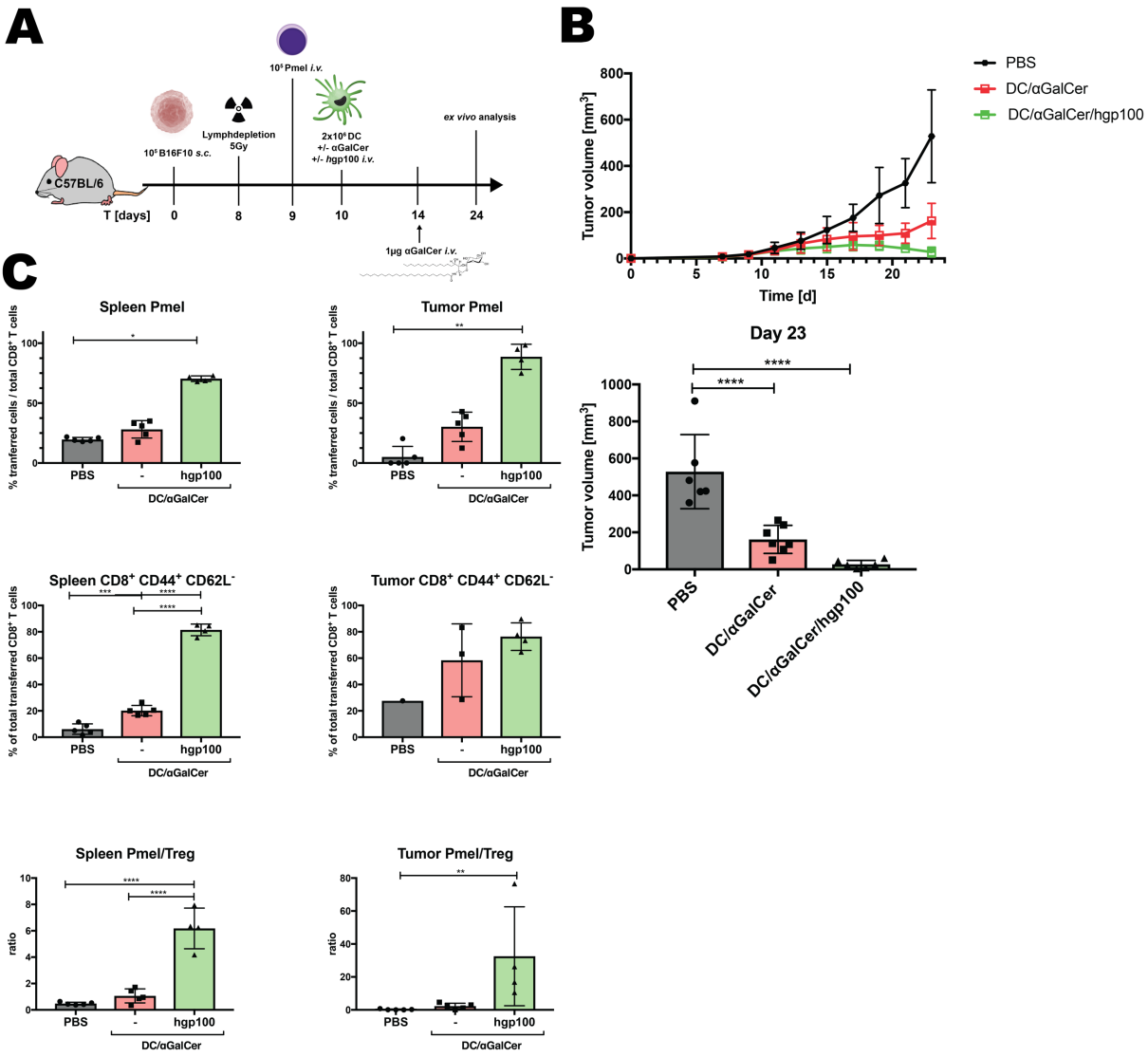


Figure 31 - Adoptive T cell transfer and DC/αGalCer vaccination induces a strong antitumor T cell immunity. A) Scheme of the experiment: $10^5$ B16F10 tumors were engrafted. On day 8, mice were lymphodepleted with a single dose of 5Gy X-ray. $10^5$ Pmel CD8 T cells were injected *i.v.* on day 9. The mice were then vaccinated on day 10 and rechallenged with 1µg of αGalCer on day 14. B) Tumor growth kinetics. C) Spleens and tumors were collected on day 24 and cells were analysed by flow cytometry.

### J.3.3 Antitumor additive effect between DC/aGC/Trp2 vaccine and PD-1

To investigate whether the immunotherapeutic effect of DCs loaded with αGalCer could be further improved, we tried to combine it with the checkpoint blockade anti-PD1. Indeed, as we observed that our DC/αGalCer vaccine was able to prime efficiently CD8 T cells, we hypothesized that anti-PD1 therapy would further support those T cells in the tumor microenvironment. Therefore, we checked whether the potent antitumor effect generated by the aGC/Trp2/DC vaccine could be further enhanced by PD-1 blockade. Mice were engrafted with B16F10 tumors and vaccinated one week later with DCs loaded with αGalCer $^{+}$/$_{-}$ $Trp2_{180-}$

$_{188}$ peptide. Mice were treated twice with anti-PD1 one week later, at 3 days interval (Figure 32 A). In the absence of the Trp2 peptide, DC/αGalCer treatment reduced the tumor burden compared to the control group, although not significant. Moreover, the addition of anti-PD1 to DC/αGalCer did not improve the control of the tumors, suggesting that T cell priming by the B16 tumor was too weak. However, when the Trp2 peptide was added to the DC/αGalCer vaccine, the control of the B16 melanoma tumors was significantly improved. Most importantly, the addition of anti-PD1 further improved the tumor control in the DC/αGalCer/Trp2 group (Figure 32 B), although not statistically significant. In line with these therapeutic results, we saw a higher frequency of H2-$K^b$/$Trp2_{180-188}$-specific CD8 T cells in the spleen of DC/αGalCer/Trp2 + anti-PD1 treated mice (Figure 32 C), which was however not confirmed in the tumors (Figure 32 D). Nevertheless, after restimulation with the Trp2 peptide, we saw that tumor isolated Trp2-specific T cells from the DC/αGalCer/Trp2 + anti-PD1 group significantly expressed more Granzyme B and secreted more IFNγ and TNFα than the other groups.

Collectively, these data show that DC/αGalCer/peptide vaccination is enhanced by the anti-PD1 treatment in the B16 model by promoting on one hand a better priming and on the other hand improved effector functions at the tumor site.

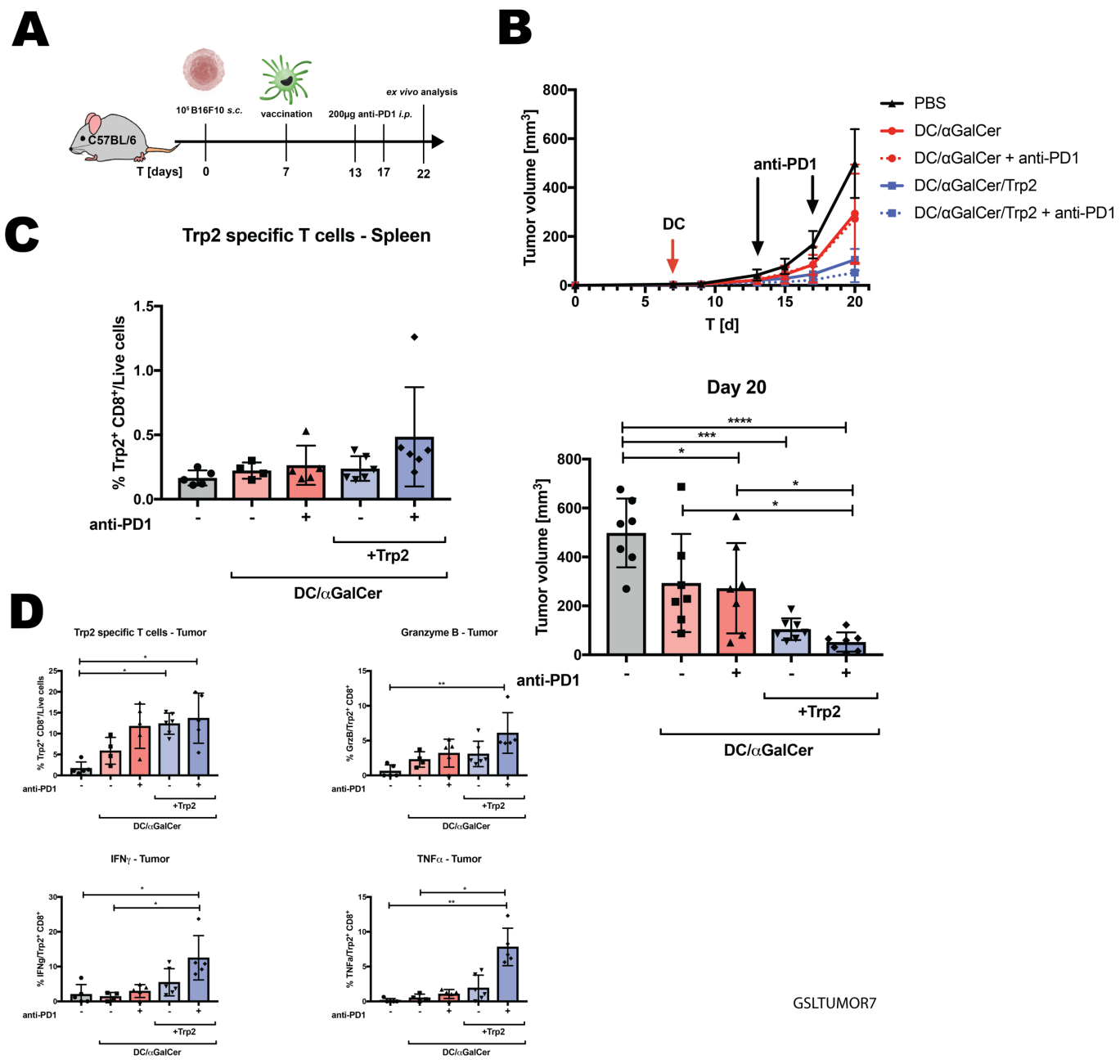


Figure 32 - DC/αGalCer/Trp2 vaccination is enhanced by anti-PD1 treatment against B16 melanoma tumors. A) Scheme of the experiment: $10^5$ B16F10 tumors were engrafted in C57BL/6 mice. The animals were vaccinated one week later with DCs loaded with αGalCer $^{+}/_{-}$ $Trp2_{180\text{-}188}$ peptide. Mice were treated with 200µg of anti-PD1 *i.p.* on days 13 and 17. Animals were sacrificed on day 22. B) Tumor growth kinetic and tumor volumes on day 20. C) *Ex vivo* staining of H2-$K^b$/$Trp2_{180\text{-}188}$-specific CD8 T cells in the spleen and D) in the tumor. D) Intracellular cytokine staining of tumor Trp2-specific T cells restimulated with the $Trp2_{180\text{-}188}$ peptide.

### J.3.4 The control of established MC-38 tumors by DC/αGalCer vaccination is improved when combined with anti-PD1

We then checked whether our DC/αGalCer + anti-PD1 treatment without peptide was sufficient to induce an antitumor response in a tumor model that is more immunogenic than

B16. Therefore, we engrafted mice with the MC-38 colon adenocarcinoma cell line and vaccinated the mice one week later. On the next day, mice were treated with anti-PD1 3x at 3 days of interval (Figure 33 A). Mice treated with DC/αGalCer or unloaded DCs with anti-PD1 similarly controlled the growth of MC-38 tumors. However, DC/αGalCer and anti-PD1 induced a better control of MC-38 tumors, when combined (Figure 33 B).

These data show that anti-PD1 significantly enhances the antitumor immune response driven by the vaccination with DCs loaded with αGalCer. However, we were not able to track the tumor-specific CD8 T cell response in these mice.

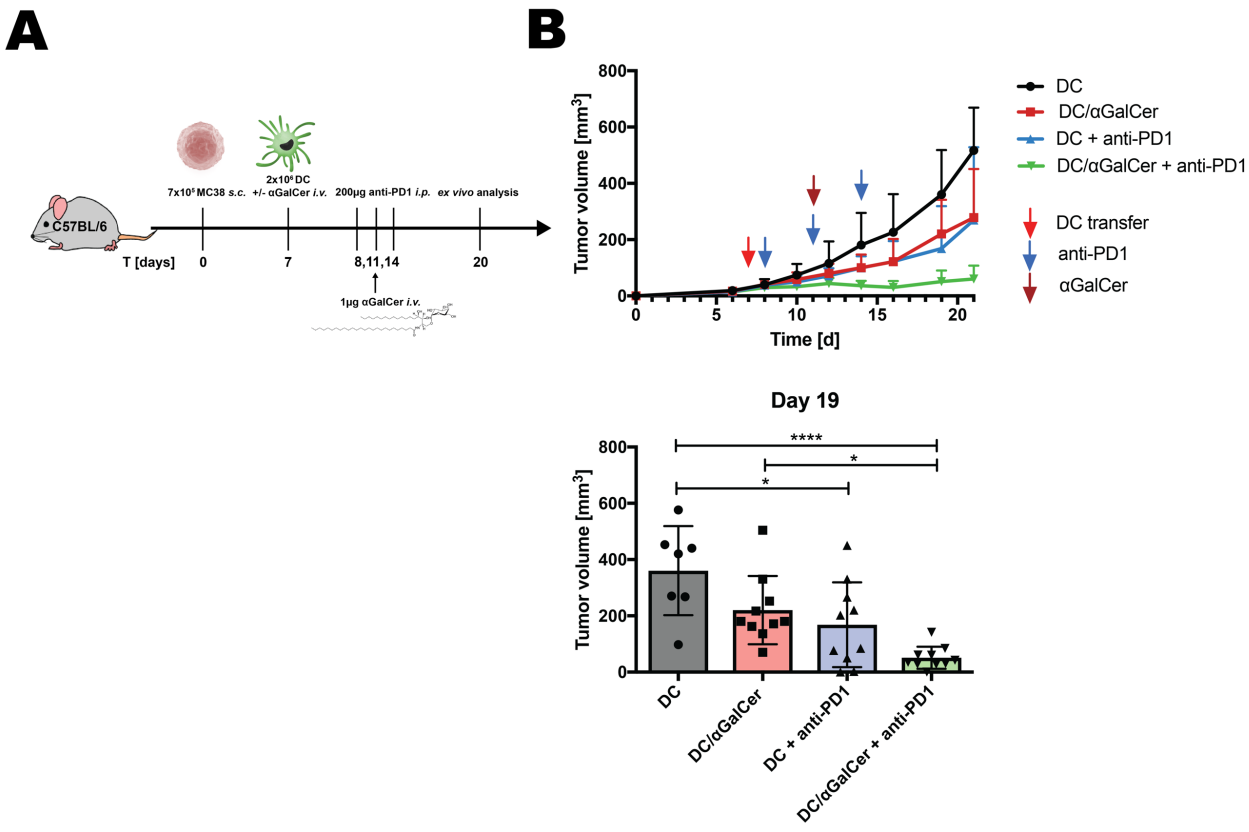


Figure 33 - DC/αGalCer vaccination controls better established MC-38 tumors when combined with anti-PD1. A) Scheme of the experiment: $7x10^5$ MC-38 tumors were engrafted *s.c.* Mice were vaccinated on day 7 and received 3 anti-PD1 doses of 200µg *i.p.* on days 8, 11 and 14. The mice were rechallenged with 1µg of free αGalCer *i.v.* on day 14. B) Tumor growth.

### J.3.5 DC/αGalCer vaccination increases the functionality of neoantigens-specific CD8 T cells in the MC38 tumor model

With the aim to identify the tumor antigens which raised the potent CD8 T cell response in the MC38 model, we tested some neo-antigens that were recently described for this tumor. The first one is a single amino acid substitution in the Adpgk gene, the ADP-specific glucokinase, which generates a neoantigen[250].

| Gene | Peptide | MHC allele | $IC_{50}$ (mut) | $IC_{50}$ (WT) | Mutation position | Immunogenicity prediction |
|---|---|---|---|---|---|---|
| *Dpagt1* | SIIVFNL[V/**L**] | *H-2K*$^b$ | 8 | 34 | Anchor (P8) | – |
| *Reps1* | AQL[P/**A**]NDVVL | *H-2D*$^b$ | 9 | 100 | Solvent (P4) | + |
| *Adpgk* | ASMTN[R/**M**]ELM | *H-2D*$^b$ | 2 | 3 | Solvent (P6) | + |
| *Cpne1* | SSP[D/**Y**]SLHYL | *H-2D*$^b$ | 211 | 685 | Solvent (P4) | – |
| *Irgq* | AALLNSA[G/**V**]L | *H-2D*$^b$ | 3 | 52 | Solvent (P8) | – |
| *Aatf* | MAPIDHT[A/**T**]M | *H-2D*$^b$ | 30 | 102 | Solvent (P8) | – |
| *Med12* | DPSSSVLFE[D/**Y**] | *H-2K*$^b$ | 38,300 | 39,411 | No structure | – |

Figure 34 –Neoantigens predicted in the MC-38 model. Adapted from [250].

The second one is a peptide derived by the gp70 retroviral gene expressed in several mouse tumor cell lines including MC-38 [251,252]. Mice bearing MC-38 tumors were vaccinated with DC/αGalCer $^{+}/_{-}$ Adpgk neoantigen peptide. The animals were treated subsequently with anti-PD1 (Figure 35 A). As in previous experiments, we saw that DC/αGalCer and anti-PD1 significantly work together to control the tumor burden. However, the antitumor effect was not improved by the addition of the Adpgk neoantigen peptide on DCs (Figure 35 B). Surprisingly, although DC/αGalCer/Adpgk + anti-PD1 did not enhance the tumor control, it clearly induced H2-D$^b$/Adpgk-specific CD8 T cells in seen in the spleen (Figure 35 C). However, Adpgk-specific CD8 T cells were not detected in the absence of the adpgk peptide in the DC vaccine although it induced the same effective tumor control in the group DC/αGalCer + anti-PD1, suggesting that other tumor antigens may be seen. In the tumor, neither of the two groups displayed a consistent enhanced frequency of Adpgk-specific CD8 T cells. However, upon *ex vivo* restimulation of TILs with the Adpgk peptide, these two groups displayed high Granzyme B expression and IFNγ secretion in CD8 T cells, suggesting that the adpgk neoantigen may participate to the CD8 T cell mediated tumor control (Figure 35 C).

DC/αGalCer + anti-PD1-treated mice, which displayed the best tumor control did not accumulate H2-K$^b$/gp70-specific CD8 T cells in spleen or tumor (Figure 35 D). However, similarly to Adpgk, the restimulation with the gp70 peptide revealed an increased Granzyme B expression and IFNγ secretion in tumor CD8 T cells, suggesting that this viral antigen may also participate to the tumor control.

Collectively, these results suggest that multiple tumor antigens likely participate to the immune response against the MC38 tumors, which raises the possibility to pulse DC/αGalCer with a pool of peptides in order to increased efficacy of DC/αGalCer vaccines.

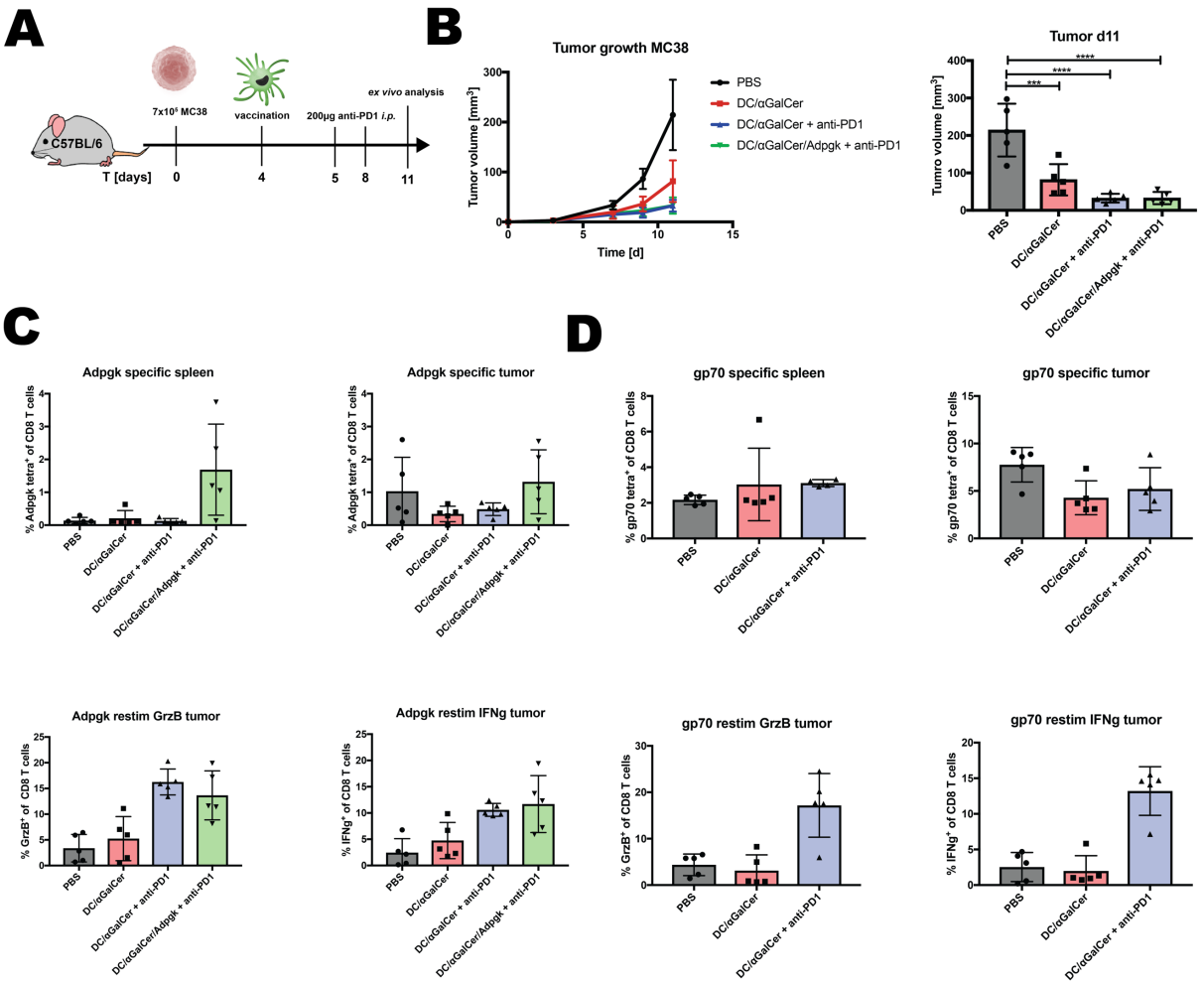


Figure 35 - DC/αGalCer vaccination increases the functionality of neoantigens-specific CD8 T cells in the MC38 tumor model. A) Scheme of the experimental plan: 7x10$^5$ MC38 tumors were engrafted in C57BL/6 mice. Animals were vaccinated 4 days later with DC/αGalCer $^{+}$/$_{-}$ Adpgk peptide. Animals were treated twice with anti-PD1 on days 5 and 8 and were sacrificed on day 11. B) Tumor growth and volume at day 11. C) and D) Frequency in spleen and tumors and *ex vivo* restimulation of TILs seen by intracellular cytokine stainings with regard to Adpgk and gp70-specific CD8 T cells, respectively.

## J.4 Discussion

As shown by other studies[253,254], we confirmed the potency of DC vaccines, as compared to conventional formulation of peptide/adjuvant[247]. Furthermore, we demonstrated that pulsing immature DCs with αGalCer instead of inducing their *in vitro* maturation with poly(I:C) resulted in much better CD8 T cell priming. The major reason is that TLR-mediated activation of DCs make them prone to apoptosis rapidly after their maturation[255], resulting in their poor survival after *in vivo* transfer. In contrast, immature DCs pulsed with αGalCer will proceed to their maturation only upon their encounter with iNKT cells[66–69] after their *in vivo* transfer. As a consequence, αGalCer-loaded immature DCs will likely survive better and thus have a prolonged interaction with T cells, which would result in a better T cell priming.

αGalCer and its Th1-biasing derivatives are potent adjuvants[11,239,241,256], which allows the transactivation of DCs and efficient T cell priming before iNKT cells become unresponsive. Therefore, in the context of therapeutic cancer vaccine, consecutive iNKT cell anergy is not a major issue, since their helper T cell function is exploited. In order to further optimize the functionality of CD8 T cells primed by αGalCer/DC vaccination, we included PD-1 checkpoint blockade, with the hypothesis that these two modalities would synergize as they function at different steps of the cancer-immunity cycle, proposed by Chen and Mellman to mount a successful antitumor T cell-mediated immunity[151].

To this aim, we tested αGalCer/DC vaccination involving two different tumor antigens in the poorly immunogenic and aggressive B16 melanoma model, including the ACT of T cell precursors. Briefly, DC loaded with αGalCer and with and without *h*gp100 peptide epitope was performed with prior ACT of *h*gp100-specific Pmel CD8 T cells. The transfer of Pmel T cell precursors was optimised by prior X-ray irradiation, which played an important role in their efficient antigen priming and resulted in the almost complete rejection of established B16 melanomas, especially when the *h*gp100 peptide was added to the DC/αGalCer vaccine.

Several clinical trials have taken advantage of TILs as tumor-specific T cells to treat cancer patients when adoptively transferred after an *ex vivo* expansion process[211–215]. Our data indicate that DCs loaded with αGalCer could potentially be beneficial in combination with adoptive TILs transfer to further improve their therapeutic effect. Alternatively, DC/αGalCer could be loaded with oxidized tumor lysates[182,257], which could boost an existing antitumor response in cancer patients. As TILs are generally exhausted[258], they might benefit from the strong pro-inflammatory phenotype of DC/αGalCer after their *in vivo* encounter with iNKT

cells. In this model, we were however unable to show synergy between DC/αGalCer vaccine and checkpoint blockade (data not shown), which might have resulted from the already maximized efficacy.

Since adoptive T cell transfer in cancer patients requires heavy and costly infrastructures and handling, we tested the melanoma-specific model antigen Trp2 which is able to raise an endogenous T cell response resulting in significant tumor inhibition[247]. The detection of Trp2-specific T cells in spleen and tumors, as well as the resulting tumor inhibition, were greatly increased by the addition of the Trp2 peptide in the αGalCer/DC vaccine formulation. However, the addition of PD-1 blockade only slightly improved the therapeutic effect. At the cellular level, PD-1 blockade did not improve the frequency of Trp2-specific T cells at the tumor and only slightly improved their functionality. This lack of synergy with PD-1 blockade likely result from the low MHC I expression and low immunogenicity of the B16 melanoma model[259] as well as to the poor delivery of tumor antigens to the lymph nodes in this model, which is required for antitumor immunity[260–262]. Similar observations have been made in clinical settings showing high tumoral MHC-I expression, a pre-existing T cell response and a high mutational load correlate with improved response rate to PD1 checkpoint blockade[263–269].

To address this aspect of tumor immunogenicity, we tested the tumor model of the colon carcinoma-derived MC38 cell line, which expresses about ten times higher MHC I levels. Strikingly, there was a strongly improved tumor control when combining DC/αGalCer and anti-PD1 treatment even in the absence of any added tumor antigen, suggesting a strong T cell priming by antigens released by the tumor itself.

With the aim to identify the antigen specificity of the T cell response against MC38 tumors, we tested the neoantigen epitope Adpgk, described to be immunogenic in a screening study combining *in silico* prediction and peptide elution from MHC to discover new epitopes in the MC38 tumor model[250]. Despite the detection of increased Adpgk-tetramer specific CD8 T cells in the spleen of mice vaccinated with DC/αGalCer/Adpgk and treated with anti-PD1, the tumor control was not better than in mice treated with DC/αGalCer + anti-PD1. These results suggest that additional MC38-derived antigens participate to the T cell response induced by DC/αGalCer vaccination. However, recent identification of personalized neoantigens-derived peptides will offer a new effective vaccine approach in cancer patients, especially when combined with anti-PD1[181].

Altogether, these results show that depending on the tumor model, the addition of peptide to the DC/αGalCer vaccination and combined with anti-PD1 treatment, may increase or not the efficacy of the treatment. The reasons behind this difference are still elusive.

Collectively, we show for the first time that DC/αGalCer vaccination may be strongly benefit from anti-PD1 immunotherapy, especially against immunogenic tumors.

Furthermore, we aim at challenging our immunotherapeutic combo with a melanoma model that recapitulates the biology of human melanoma such as the YUMM1.7 line[270] and unlike B16. The carcinogenesis of this cell line is driven by Braf activation, Pten and Cdkn2a inactivation. As B16, these tumors are poorly immunogenic. Therefore, we expect to observe a cooperative effect of DC/αGalCer + anti-PD1 therapy only when coupled to peptide vaccination, as in B16.

We aim also at testing whether our combo immunotherapy induces antigen spreading. Indeed, antigen spreading was shown to happen both in mouse models and clinical studies and was generally associated with good prognosis[271–275]. To test this hypothesis, we plan to engraft B16F10 sufficient and deficient for Trp2 on both flanks of the same mice. We would then monitor whether vaccination with DC/αGalCer/Trp2 + anti-PD1 induces the control as well of the Trp2-deficient tumors.

# K CAR expression interferes with T cell receptor signalling *in vivo* in CD8 T cells

## K.1 Aim

The original aim of this project was to combine CAR and TCR activation within the same T cell. We initially created a CAR reactive to CD1d/αGalCer based on the L363 antibody[237], mimicking the semi-invariant iNKT TCR. We hypothesized that the combination of CAR and TCR would enhance the *in vivo* recall to a vaccine containing both antigens which would increase the cytotoxicity against tumors expressing CAR and TCR-specific antigens. However, OT-1 CAR T cells failed to expand *in vivo* upon OVA/CpG vaccination. These preliminary results suggested that the CAR domains interfered with the TCR signalling upon TCR activation even without CAR triggering. Therefore, in this project, we aimed at understanding the mechanisms hindered the expansion of CAR CD8 T cells upon TCR stimulation. We found out that *in vivo* TCR stimulation of CAR T cells results in Fas-mediated AICD, which was so far not described and which may have crucial implications for further developments of CAR-mediated immunotherapy.

## K.2 Material and methods

### K.2.1 Retroviral constructs

BFP fluorescent protein, HER2 and CEA-specific CARs were cloned in the MSGV retroviral transfer vector[276] under the control of the 5' LTR promoter. The plasmid pIG6-4D5, containing the scFv fragment derived from the mouse anti-HER2 antibody 4D5, was used as template[277] (kind gift from A. Pluckthun, University of Zurich, Switzerland) for cloning the HER2 binding domain of the CAR. For the CEA-CAR, the scFv MFE23[278] (kindly provided by R.H. Begent) was used. The single chain antibody domains of the CARs were fused to CD8α hinge and transmembrane domains followed by intracellular TCR signalling endodomains.

The miRNAs against Fas were co-expressed with ZsGreen in pLMN expression vectors (Transomic, shERWOOD-UltramiR).

### K.2.2 Retrovirus preparation

For each retroviral preparation, $8x10^6$ Phoenix ECO cells (ATCC, CRL-3214) were plated in a T150 tissue culture flask in RPMI medium supplemented with 10% FCS, 10mM HEPES and 50U/ml Penicillin-Streptomycin. On the next day, cells were transfected with 21µg of the retroviral construct and 14µg pCL-Eco with Turbofect transfection reagent (Thermo Fischer

Scientific, R0532), according to the manufacturer protocol. pCL-Eco[279] was a gift from Inder Verma (Addgene, plasmid #12371). The medium was changed daily and collected at 48h and 72h post transfection. 48h and 72h virus supernatants were pooled and sedimented at 22000rcf for 2h at 4°C. Finally, retrovirus pellets were resuspended in 2ml of full RPMI medium and divided in 8 aliquots of 250µl each, which were snap-frozen on dry ice and stored at -80°C.

### K.2.3 Mouse CD8 T cells transduction

Spleens from CD45.1xOT1 or CD45.1xP14 transgenic mice were smashed on a 40µm cell strainer. CD8 T cells were then purified using the EasySep™ Mouse $CD8^+$ T Cell Isolation Kit (StemCell, 19853) according to the manufacturer protocol. $0.5x10^6$ CD8 T cells were plated in 48well plates in 0.5ml of RPMI 1640 medium containing GlutaMAX (Thermo Fischer Scientific, 61870-010) and supplemented with 10% FCS, 50U/ml Penicillin-Streptomycin (Gibco, 15070-063), 10mM HEPES (Gibco, 15630080), 1x MEM NEAA (Gibco, 11140050), 50µM β-mercapto-ethanol (Gibco, 31350010), 2mM L-Glutamine (Thermo Fischer Scientific, 25030081), 1mM Sodium Pyruvate (Gibco, 11360070), and 50 IU/ml of recombinant human IL-2. Finally, Mouse T-Activator CD3/CD28 Dynabeads for T-Cell Expansion and Activation (Gibco, 11452D) were added at a ratio of 2 beads per cell. Retroviral infection was conducted at 37°C for 24h.

Non-tissue culture-treated 48well plates were coated for 24h with 20µg/ml of recombinant human fibronectin (Takara Clontech, T100A) at 4°C, followed by PBS 2% BSA for 30min at RT and finally washed with PBS. One aliquot of 250µl of concentrated retroviruses were plated in each of the fibronectin-coated 48well plates and centrifuged for 90min at 2000rcf and 32°C. Then, $0.5x10^6$ of 24h-activated CD8 T cells were added on top of the viruses and spun for 10min at 400rcf and 32°C.

The medium was renewed daily, and cell density was kept below $2x10^6$ cells/ml. On day 3, the medium was supplemented with 10 IU/ml recombinant human IL-2, 10ng/ml recombinant human IL-7 and 10ng/ml recombinant human IL-15. From day 5 post activation, the cells were fed with only IL-7 and IL-15.

### K.2.4 Listeria-OVA and LCMV infection

*Listeria*-OVA N4 and T4 were generated as described in [280]. 2000*cfu* were injected *i.v.* in 200µl of PBS with a 1ml insulin syringe (Beckton Dickinson, 041-BD-324827). Lymphocytic choriomeningitis virus (LCMV), strain Armstrong, was prepared as described in [281]. $2x10^5$*pfu* were injected *i.p.* in 200µl of PBS with 26G syringe needles (Terumo, 041-050101).

### K.2.5 Adoptive T cell transfer

CD8 T cells were harvested and CD3/CD28 Dynabeads were removed with an EasySep™ Magnet (Stem Cell, 18000). Cells were counted and washed 3 times in PBS. $0.2x10^6$ cells were resuspended in 200µl of plain RPMI medium and injected in the mouse tail vein using 1ml insulin syringes.

### K.2.6 Fas-Fc treatment

Human Fas linked to a human IgG1 Fc-tag (Adipogen, AG-40B-0082) or the human IgG1 Fc control (Adipogen, AG-35B-0007) were resuspended in PBS 1% BSA at the concentration of 1mg/ml. 100µg were injected in mice *i.v.* with 1ml insulin syringes.

### K.2.7 Sample preparation, flow cytometry staining and acquisition

Spleens were collected at indicated days post *Listeria*-OVA or LCMV infection and smashed on 40µm cell strainers to obtain single cell suspensions. Red blood cells were lysed using a RBC Lysis Solution (Qiagen, 158902). Then, the cells were labelled with Zombie Yellow™ Fixable Viability Kit (Biolegend, 423103) to discriminate dead cells. HER2-specific CARs were labelled with HER2-Fc (Sino Biological, 10004-H02H-50) at 5µg/ml on ice for 20min and then washed 3 times with facs buffer (PBS 2% FCS and 2.5mM EDTA). Next, cells were labelled with Brilliant Violet 421 anti-human IgG Fc Antibody (BioLegend, 409317) at 2µg/ml for 20min on ice and washed again 3 times. The CEA-specific CAR was instead stained with Fluorescein AffiniPure Goat Anti-Mouse IgG, $F(ab')_2$ (Jackson ImmunoResearch, 115-095-072) at 1µg/ml on ice for 20min and then washed 3 times.

After CAR staining, transduced cells were labelled with CD3e-Alexa Fluor 700 at 10µg/ml (BioLegend, 100215), CD8α-PE/TexasRed at 1/500 dilution (Thermo Fischer Scientific, MCD0817), CD45.1-APC/eFluor 780 at 2µg/ml (eBioScience, 47-0453-82), CD45.2-PerCP/Cy5.5 at 2µg/ml (BioLegend, 109827), Fas-APC at 2µg/ml (BioLegend, 152603) and FasL-Biotin at 5µg/ml (BioLegend, 106603). Cells were then washed once and labelled with Streptavidin-PE/Cy7 at 0.4µg/ml (BioLegend, 405206). Finally, cells were washed again and stained with Annexin V-PE (BioLegend, 640907) according to the manufacturer protocol, and acquired on a BD LSR2 flow cytometer.

### K.2.8 In vitro cytokine release assay for CAR T cells

$0.2x10^6$ OT-1 CAR T cells were plated in flat-bottom 96well plates with either $0.1x10^6$ B16, B16-OVA or B16-HER2 tumor cells. 30min later, the Monensin and Brefeldin A-containing

reagents Golgi Stop and Golgi Plug (Beckton Dicksinon, 554724 and 555029) were added at the final concentration of 1:1000. Cells were further incubated for 4h prior to staining for flow cytometry.

The cells were then labelled for viability and with HER2-Fc as described previously. They were also surface-labelled with the antibodies anti-human IgG Fc-FITC (BioLegend, 409309), CD3ε-Alexa Fluor 700 at 10µg/ml (BioLegend, 100215) and CD8α-Brilliant Violet 650 at 0.5µg/ml (BioLegend, 100741).

To stain for intracellular cytokines, cells were fixed and permeabilized (Biolegend, 420801) according to the manufacturer protocol, before being stained with the antibodies IFNγ-PerCP/Cy5.5 at 0.5µg/ml (Biolegend, 505821) and TNFα-Pacific Blue at 1µg/ml (Biolegend, 506318) diluted in permeabilization buffer (Biolegend, 421002) for 30min on ice. Cells were finally washed twice in permabilization buffer and resuspended in FACS buffer prior to acquisition.

## K.3 Results

### K.3.1 The CD3ζ endodomain mediates the CAR effector functions

To address the effect of the presence of a CAR in CD8 T cells when engaged via their TCR, we cloned a CAR reactive to human HER2 using the 4D5 scFv[277] antibody fragment fused to different TCR-associated signalling domains, respectively 4-1BB and CD3ζ (BBz), CD28 only (28), 4-1BB only (BB) and CD3ζ only (z). The CAR functionality requires the CD3ζ endodomain[282], but we also tested CAR configuration with single domains other than CD3ζ to evaluate their influence on TCR signalling. In line with what is published, only the BBz and z HER2 CAR T cells secrete IFNγ and TNF-α when cocultured with B16 expressing HER2, whereas 28 and BB CAR T cells were not activated (Figure 36).

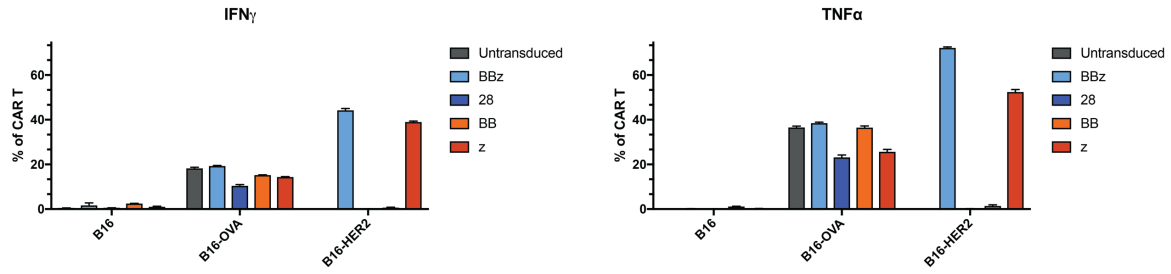


Figure 36 - Chimeric antigen receptors encoding the CD3z endodomain mediate effector function in CD8 T cells. Untransduced or BBz, 28, BB or z CAR OT-1 T cells were cocultured with B16, B16-OVA or B16-HER2 for 4h and stained for (a) IFNγ and (b) TNF-α intracellularly.

### K.3.2 Several CAR configurations cause T cell deletion *in vivo* upon TCR engagement

To address the effect of the presence of a CAR in T cells upon TCR engagement, we transduced OT-1 CD45.1 CD8 T cells with the BFP fluorescent protein as a control or with the HER2-specific CAR containing the signaling domains mentioned in the previous paragraph. These cells were transferred in CD45.2 recipient hosts, which were subsequently infected with *Listeria*-OVA to trigger transferred cells via their TCR but not via their CAR (Figure 37 A and B). As suspected from our preliminary results in vaccine setting, we observed that, while BFP-transduced OT-1 T cells persisted well in mice 7 days post *Listeria*-OVA infection, CAR T cells with all CAR configurations has disappeared, as illustrated by the drop of CAR positive cells in the transferred OT-1 population (Figure 37 C). Next, we addressed the kinetic of the *in vivo* CAR T cell deletion upon TCR engagement and saw that initially CAR OT-1 T cells expanded well until day 5, while from day 6, they started to disappear and were totally gone at day 7 post infection (Figure 37 D). We investigated the cause underlying the death of CAR T cells upon TCR engagement *in vivo* and first checked the Fas/FasL pathway, which is a major mediator of T cell activation-induced cell death (AICD) [111]. Interestingly, we found that CAR T cells upregulated Fas from day 6 and FasL at day 7 post infection, which was not the case for BFP-transduced T cells (Figure 37 E). We also observed that these cells upregulated the early apoptosis marker phosphatidylserine, detected by Annexin V and about 20% of CAR-T cells were already apoptotic on day 6, as seen with the Vivid staining. In contrast, there was very low Annexin and/or Vivid staining in BFP-transduced cells (Figure 37 E), demonstrating that AICD is intrinsic to CAR-T cells.

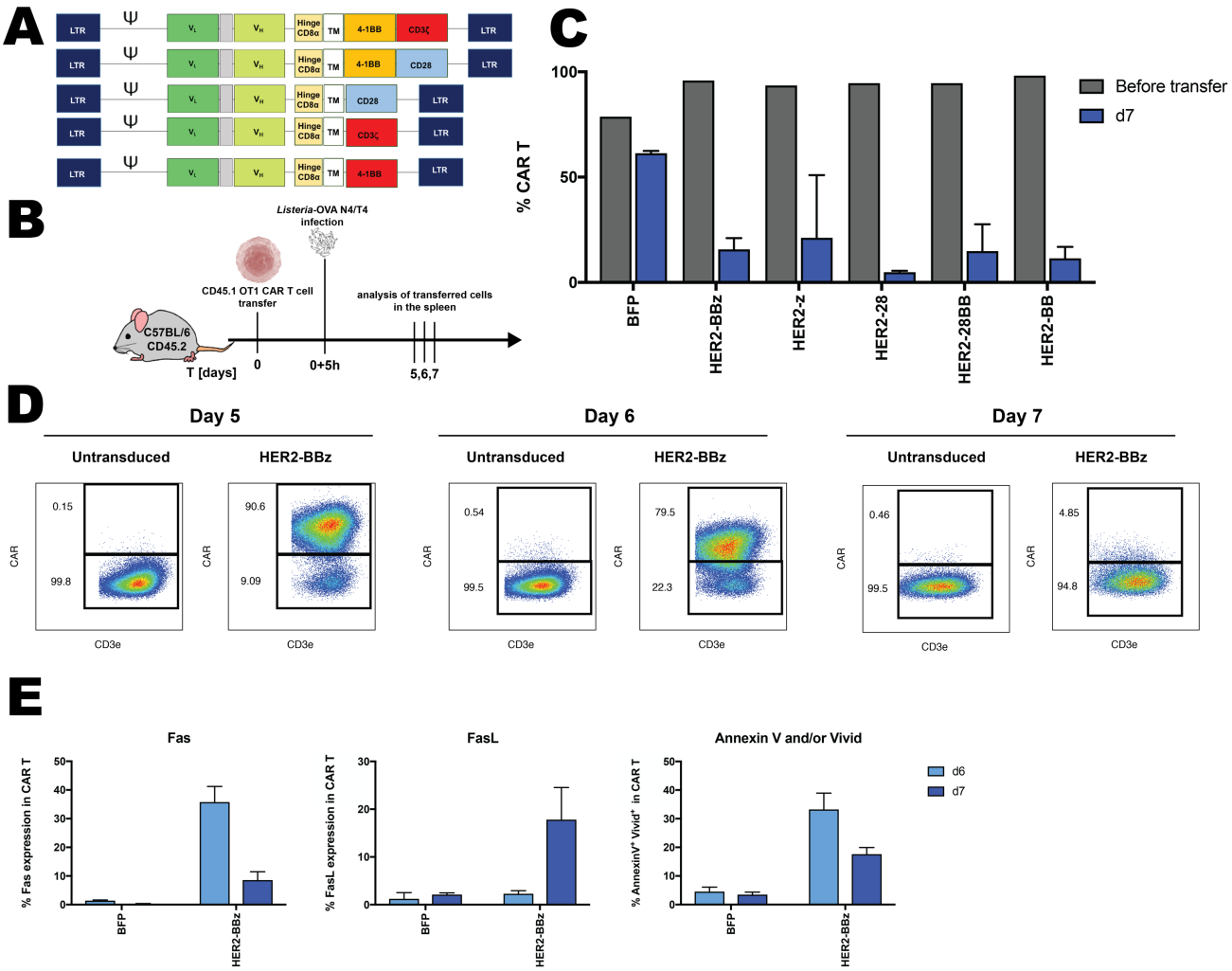


Figure 37 - HER2-specific CAR T cells undergo apoptosis upon *in vivo* TCR activation (a) Schemes of HER2-CAR configurations. (b) CD45.1 OT-1 T cells transduced with BFP or HER2-CAR were transferred in CD45.2 C57BL/6 hosts, subsequently infected with *Listeria*-OVA (c) Frequencies of BFP or CAR T cells in transferred CD45.1 OT-1 population at day 7 post infection. (d) dot plots of frequencies of CAR–T cells at day 5,6 and 7 in the transferred OT-1 population. (e) Fas, FasL and Annexin V expression and Vivid viability staining in BFP or CAR-positive OT-1 T cells at day 6 and 7 post infection

Since OT-1 T cells have a very high TCR avidity for the OVA epitope SIINFEKL expressed by *Listeria*-OVA, we tested whether CAR T cells would also be deleted when triggered via a decreased TCR signal, such as a self-derived tumor antigen. We therefore repeated the same experiment, using the *Listeria* -OVA expressing the mutant epitope SIITFEKL T4, for which OT-1 T cells have a 70x decreased avidity[280]. We confirmed the reduced avidity of OT-1 T cells for *Listeria*-T4 compared to the N4 strain, as seen by a significantly weaker OT-1 expansion *in vivo* (Figure 38 A). However, CAR T cells were similarly deleted *in vivo* whether the OT-1 TCR was triggered via the wild-type N4 and the low affinity T4 epitope (Figure 38 B).

Collectively, these results show that CAR T cells are deleted *in vivo* upon TCR engagement whatever the CAR configuration and even upon low avidity TCR triggering.

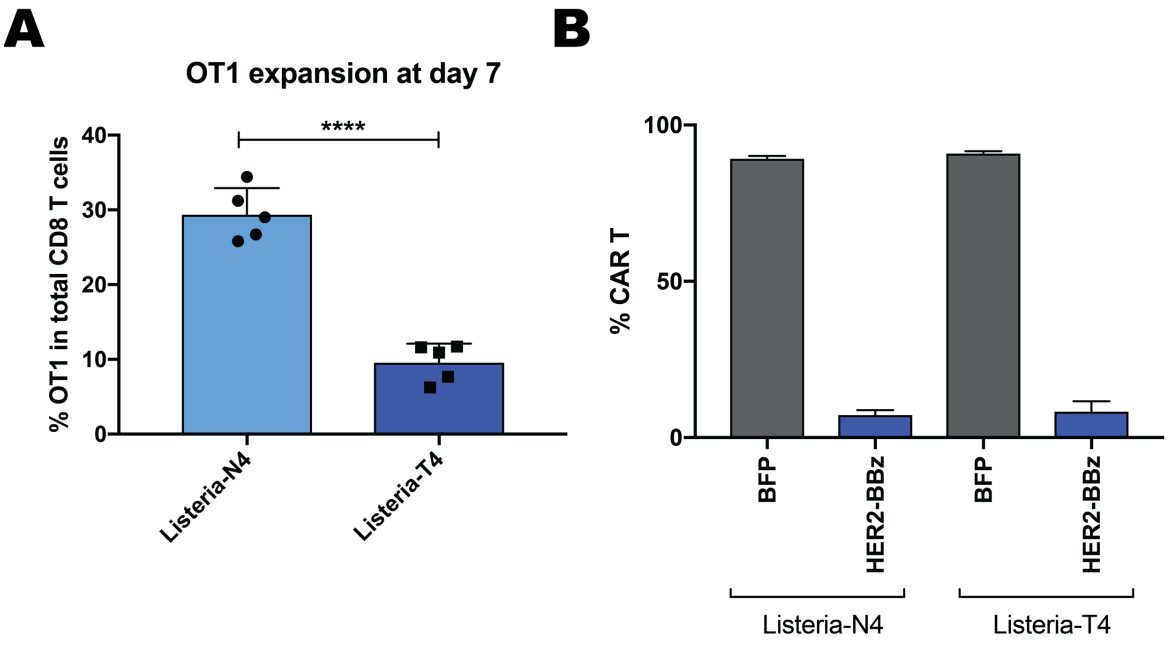


Figure 38 – Low avidity TCR engagement renders a similar CAR T cell deletion *in vivo* than high avidity TCR. (a) *Listeria*-T4 expresses a low affinity OVA antigen and induces less proliferation of OT-1 T cells *in vivo* than *Listeria*-N4. OT-1 T cells were transferred in mice that were infected with either *Listeria*-N4 or T4. The graph shows the percentage of OT-1 CD45.1 T cells in the total CD8 spleen population. (b) Percentage of BFP or HER2-BBz-positive OT-1 CD45.1 T cells at day 7 post infection with either *Listeria*-OVA N4 or T4.

### K.3.3 The deletion of CAR-T cells upon TCR triggering is independent of the TCR and CAR specificity

To address whether the CAR T cell deletion that we observed was a general phenomenon, we transduced the HER2-BBz CAR in P14 CD8 cells specific for the LCMV gp33 epitope[281]. HER2-CAR P14 cells were transferred in mice, which were then infected with LCMV Armstrong (Figure 39 A). In line with our previous results in OT-1 T cells, we observed that CAR P14 T cells were almost completely deleted at day 8 post LCMV infection (Figure 39 B and C). In contrast, control BFP-transduced P14 cells survived well the expansion phase. Additionally, we also observed Fas, FasL and Annexin V and Vivid upregulation in CAR T cells but not in BFP controls at day 7 post infection (Figure 39 D).

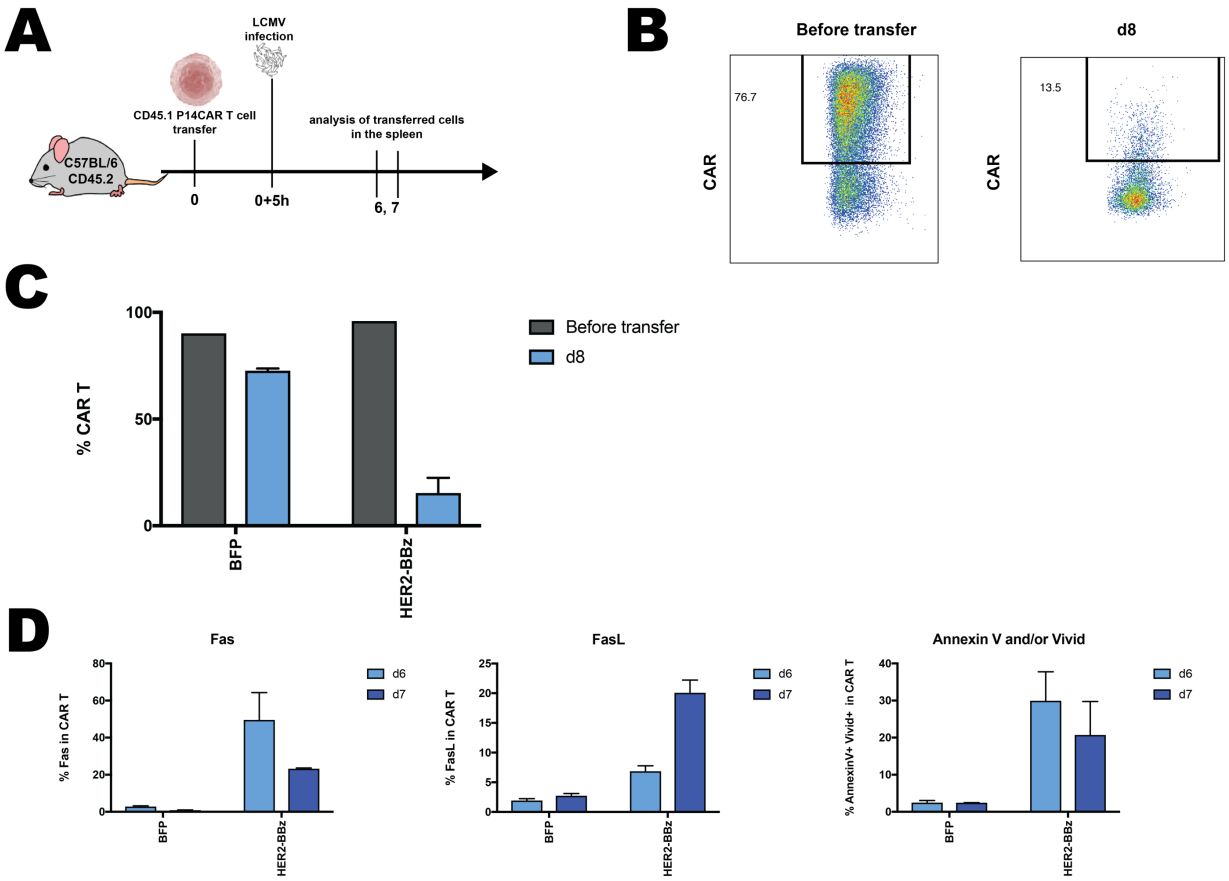


Figure 39 - CAR-P14 CD8 T cells also die upon *in vivo* TCR triggering (a) CD45.1 P14 CD8 T cells transduced with BFP or with HER2-BBz CAR were transferred in CD45.2 C57BL/6 hosts, subsequently infected with LCMV Armstrong. (b) Dot plots showing CAR-positive P14 T cells before transfer and at day 8 post infection. (c) BFP or CAR expression before transfer and in the spleen at day 8 post infection as % CD45.1 T cells. (d) Fas, FasL and Annexin V and/or viability staining in either BFP or CAR-positive transferred P14 T cells at day 6 and 7 post infection.

Next, we tested whether CAR T cell apoptosis upon TCR engagement was independent of the CAR specificity. Therefore, we cloned a human CEA-specific CAR with the BBz endodomains, based on the MFE23 scFv[278]. OT-1 T cells were transduced and transferred in mice, which were infected with *Listeria*-OVA (Figure 40 A). Similarly to HER2-CAR, CEA-specific CAR T cells were also deleted *in vivo* upon TCR engagement (Figure 40 B and C). Accordingly, they also upregulated Fas, FasL and Annexin V and Vivid at day 6 and 7 post infection (Figure 40 D). Collectively, these results show that the inability of CAR T cells to persist *in vivo* upon TCR engagement is independently of the CAR and the TCR specificity.

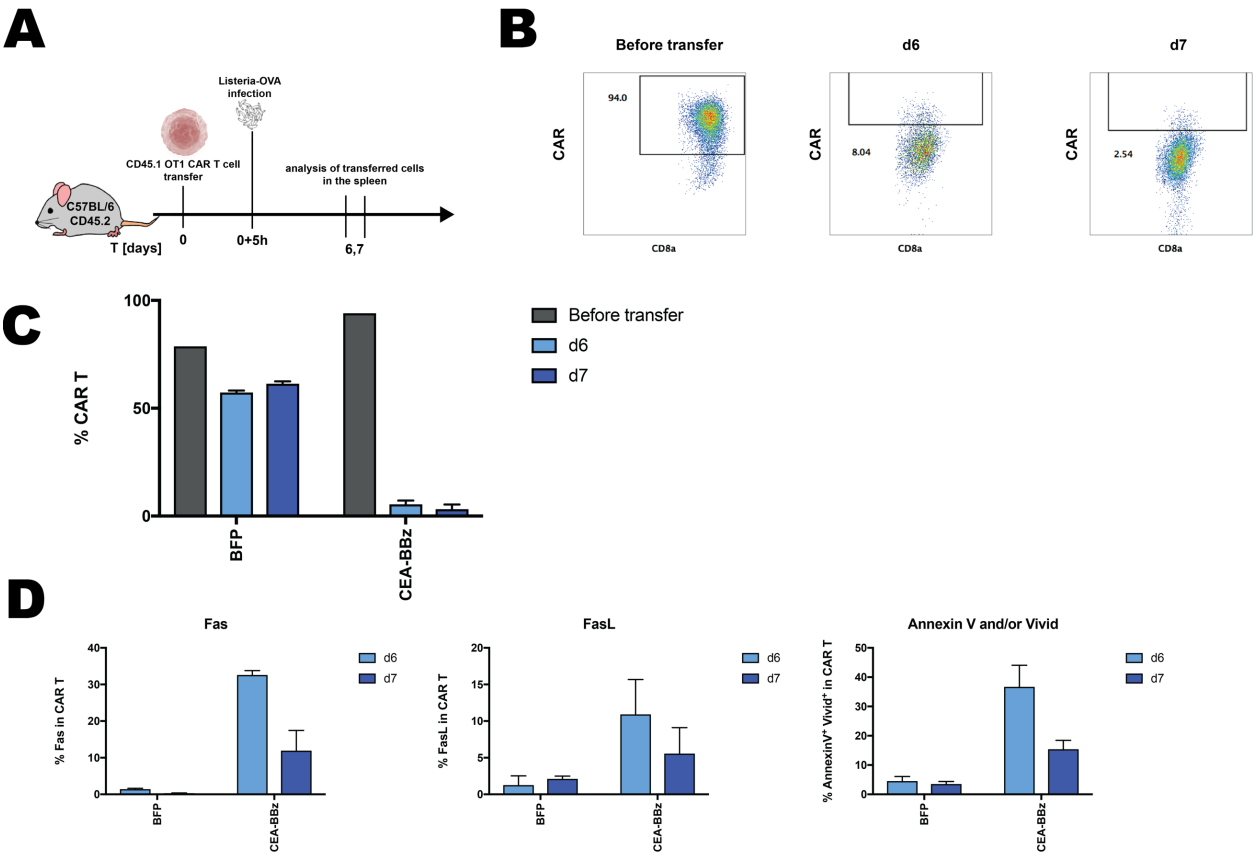


Figure 40 - CEA-BBz OT-1 CD8 T cells also die when triggered *in vivo* via their TCR. (a) CD45.1 OT-1 CD8 T cells transduced with BFP or CEA-BBz CAR were transferred in CD45.2 C57BL/6 hosts, subsequently infected with *Listeria*-OVA. (b) Dot plots showing CAR expression before transfer and at day 6 and 7 post infection in the transferred OT-1 population. (c) Frequencies of BFP or CAR OT1 T cells before transfer and in the transferred CD45.1 OT-1 population at day 6 and 7 post infection. (d) Fas, FasL and Annexin V and/or viability staining in either BFP or CAR-positive transferred OT-1 T cells at day 6 and 7 post infection.

### K.3.4 Fas blockade partially rescues CAR T cells from TCR-mediated AICD

As we observed that CAR T cells upregulated Fas and FasL *in vivo*, which are key mediators of T cell contraction and AICD[283], we asked whether Fas blockade would prevent the apoptosis of CAR OT-1 T cell upon challenge with *Listeria*-OVA. To this aim, we co-transduced OT-1 T cells with two retroviral particles, one expressing the BFP or the HER2-BBz CAR, and the second expressing ZsGreen fused to a miR-based shRNA either scrambled or specific for silencing Fas expression. *In vitro* validation of these constructions showed that all three miRs against Fas decreased the expression of Fas in CAR T cells, with however different efficiencies (Figure 41).

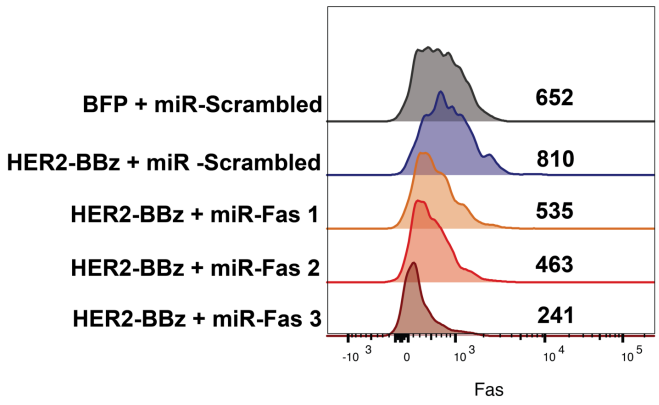


Figure 41 - Efficient Fas silencing of miR-based shRNA vectors against Fas. OT-1 T cells were co-transduced with BFP or HER2-BBz CAR together with vectors encoding ZsGreen and miR-based shRNAs against Fas or a scrambled miR. The expression of Fas was assessed at day 5 post-transduction in cells transduced with the two vectors.

Before *in vivo* transfer, CAR-ZsGreen co-transduced OT-1 T cells had to be sorted since only a small proportion of cells co-expressed both the CAR and the ZsGreen-miR-based shRNA which allowed transferring only 1500 cells. After cell transfer, mice were challenged with *Listeria*-OVA (Figure 42 A). At day 7 post infection, we observed that OT-1 cells transduced with BFP and the scrambled miR retained higher levels of BFP expression among the transferred cell population, while as expected, most CAR T cells expressing the scrambled miR, had disappeared. Most importantly, about 40% of CAR T cells co-transduced with two independent miR-based shRNA against Fas were still detected (Figure 42 B and C). In a subsequent experiment, the efficiency of co-transduction of miRs and CARs has been improved, which allowed to transfer more CAR-miR T cells (30000) in host mice to test their *in vivo* rescue of CAR T cells via Fas blockade. Unfortunately, this new experiment did not allow confirming the rescue of TCR-activated CAR T cells, indicating that Fas might not be the only player.

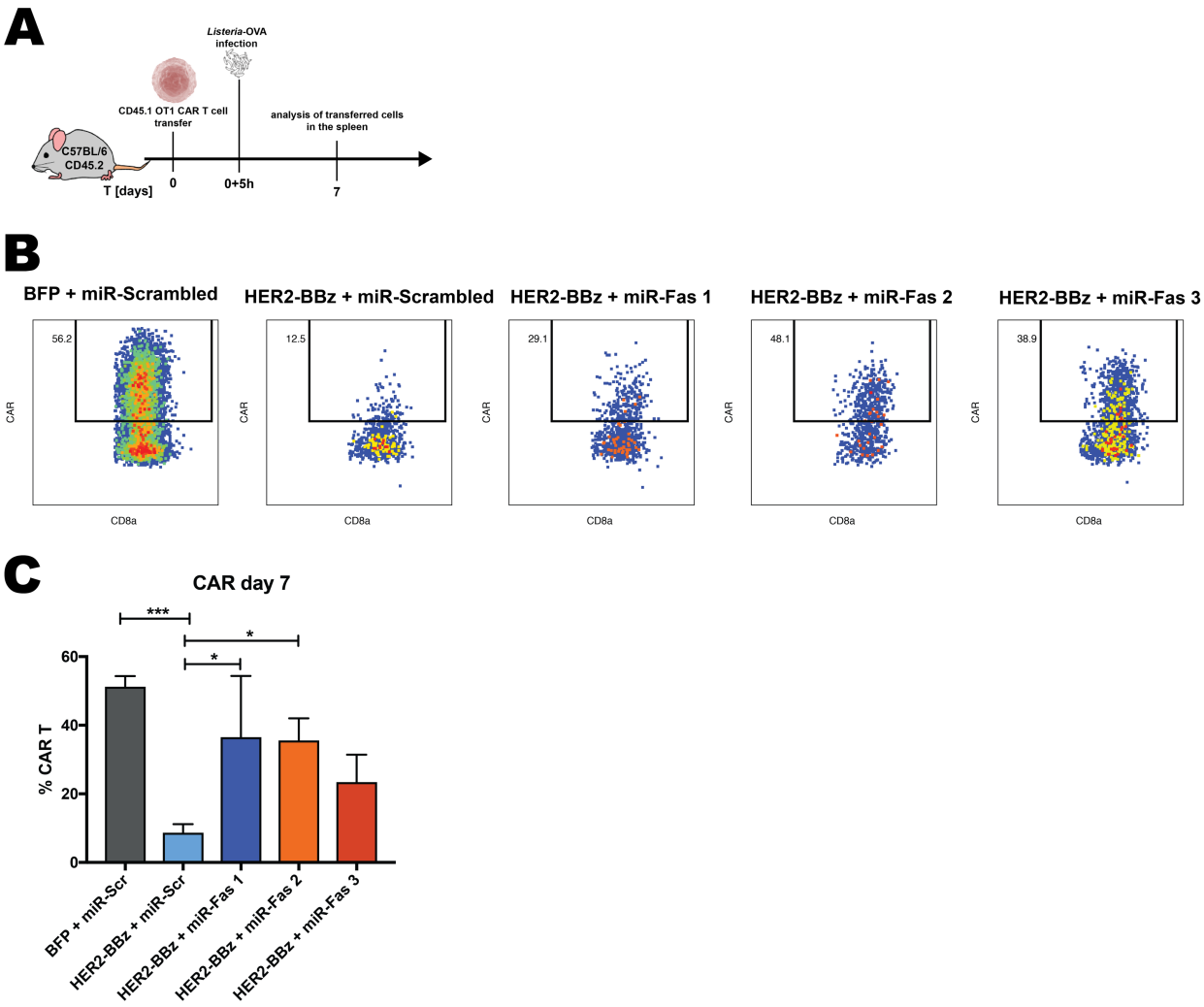


Figure 42 - shRNA-mediated Fas blockade partially rescues TCR-activated CAR T cells. (a) CD45.1 OT-1 CD8 T cells were co-transduced with BFP or HER2-BBz CAR together with vectors encoding ZsGreen and miR-based shRNAs against Fas or scramble. The cells were sorted for the expression of both the CAR and the miR and transferred in CD45.2 C57BL/6 hosts, followed by *Listeria*-OVA infection. (b) Dot plots showing the BFP or CAR expression at day 7 post *Listeria*-OVA infection in spleens of mice. (c) Frequencies of BFP or CAR T cells in the transferred CD45.1 OT-1 population at day 7 post infection.

As a second attempt to block Fas-mediated AICD of CAR T cells, we treated mice with Fas-Fc post CAR-T cell transfer and *Listeria*-OVA infection. Briefly, OT-1 T cells transduced with BFP or HER2-BBz CAR were transferred in C57BL/6 hosts and infected with *Listeria*-OVA. At days 4 and 5 post infection, the mice received 100µg of Fas-Fc or a Fc tag alone as a control (Figure 43 A). As expected, CAR T cells were deleted *in vivo* at day 7 post infection in the control group, while we saw a partial rescue of CAR T cells in mice treated with Fas-Fc, which however

was not statistically significant (Figure 43 B). Unfortunately, the rescue of TCR-engaged CAR T cells upon Fas-Fc treatment could not be confirmed in repeat experiments.

Collectively, these results show that the blockade of the Fas/FasL pathway is not consistently able to rescue the AICD of CAR T cells upon *in vivo* TCR engagement, which suggests that additional pathways may be involved in the deletion of TCR-triggered CAR T cells.

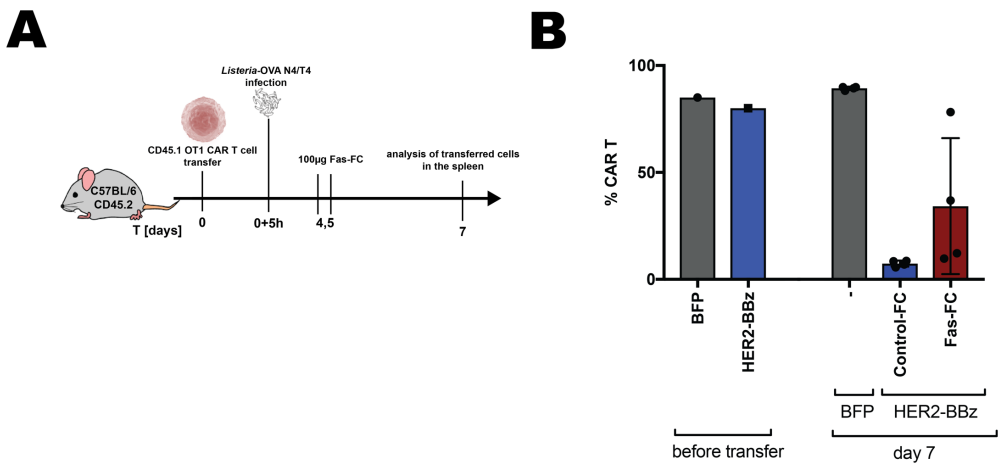


Figure 43 - Fas-Fc-mediated Fas/FasL blockade partially rescues CAR T cells *in vivo* upon TCR engagement. (a) CD45.1 OT-1 CD8 T cells were transduced with BFP or with HER2-BBz CAR and transferred in CD45.2 C57BL/6 hosts, followed by Listeria-OVA infection. Mice were treated with 100µg of control-Fc or Fas-Fc at day 4 and 5. (b) Percentage of BFP or CAR expression before transfer and at day 7 post infection in the transferred population.

## K.4 Discussion

Upon *in vivo* TCR engagement, we observed that CAR T cells initially expand normally like control cells. However, from day 6 post TCR stimulation, they undergo massive apoptosis in a Fas-dependent mechanism that resembles AICD. We showed that this phenomenon is independent of the TCR and CAR specificity, and also occurs upon low affinity TCR stimulation. These observations made in infectious models suggest that AICD of CAR T cell may also occur in tumor settings in which tumor antigens are generally recognized by low affinity TCRs. So far only indirect evidences from several studies have suggested that concomitant TCR and CAR engagement on the same T cell is not effective in terms of additive or synergistic antitumor effects. For instance, in the B16 melanoma tumor model, the group of Rosenberg could not show better antitumor effects of gp100-specific Pmel T cells when transduced with a VEGFR2 CAR. Synergistic antitumor effects were only obtained when Pmel T cells were co-transferred

with polyclonal T cells transduced with the VEGFR2-CAR[284]. The authors concluded that the different localization of the two antigens hindered a synergistic activation of CART-Pmel T cells. However, our observations suggest that TCR activation of Pmel-VEGFR2 CAR T cells by gp100-expressing tumor cells led to their AICD, independently of the CAR activation. Furthermore, a study tested the feasibility of transferring virus-specific (Epstein-Barr virus, cytomegalovirus, and adenoviruses) CAR CD8 T cells, hypothesizing that viral reactivation would enhance the expansion and persistence of CAR T cells. However, they found that viral activation induced only a modest expansion and antitumor activity of the infused CAR T cells[285]. Another indirect evidence of TCR-driven AICD of CAR T cells came from observations made in allogeneic hematopoietic stem-cell transplantation (alloHSCT) in leukemic paediatric patients[286]. Indeed, the severity of GvHD was much diminished when allogeneic HSCs were transduced with a CD19-CAR, as compared to untouched alloHSCs, suggesting that CAR T cells bearing a host-reactive TCR were deleted in the patients. Short time after, the same group has recapitulated their observations in mouse models, showing that CD28-containing CAR T cells expressing a host-reactive TCR progressively lost their effector functions and underwent clonal deletion[287]. However, they did not fully demonstrate the TCR-mediated apoptosis of CAR-T cells.

Strikingly, we found that a single CD3z, CD28 or 4-1BB CAR domain was sufficient to mediate AICD of CAR T cells upon TCR engagement. This observation was particularly surprising in the case of the 4-1BB-containing CAR since 4-1BB signalling is known to enhance the survival and delays exhaustion of effector T cells[288]. Along this line, the introduction of 4-1BB domain in CARs has conferred long persistence of CAR T cells in lymphoma patients[289]. However, in certain circumstances, 4-1BB can induce a Fas-mediated apoptosis[290], which was also shown to happen in CAR T cells[291]. These authors reported that when a CAR is expressed under the control of the retroviral 5' LTR promoter, it promotes a positive feedback loop in which the CAR triggering activates the NF-κB pathway, which promotes more CAR expression and overactivates the signalling through NF-κB. Finally, excessive NF-κB signalling leads to the upregulation of Fas and apoptosis, suggesting that CAR-related toxicity is dose-dependent. However, these mechanisms were not seen in clinical CAR therapy, because the CD19-CAR containing the 4-1BB domain[292] was expressed in a sin lentiviral vector in which the LTR promoter is inactivated after genome insertion.

Until very recently, our observations of TCR-mediated CAR T cell AICD were not published. Unfortunately, while writing our manuscript, a publication from the group of Terry Fry reported that CD19-CAR CD8 T cells originating from female mice failed to reject murine lymphoma tumors when transferred in males in which the HY-specific TCR was activated resulting in CAR T cell death[293]. These results are very similar to what we obtained using infectious models. However, they did not show the involvement of Fas/FasL mediated apoptosis and instead showed caspase 3/7 activation. Interestingly, they reported that unlike CD8 CAR T cells, CD4 CAR T cells survived TCR activation and retained their antitumoral activity via CAR activation by the tumor. Our system using bacterial or viral infection resulted in a large initial expansion of CAR T cells upon TCR stimulation which allowed us to better visualize CAR-T cell deletion and dissect the Fas/FasL-dependent mechanism. In particular, we showed that blocking Fas/FasL pathway could partially rescue CAR T cells engaged via their TCR. However, the exclusive involvement of Fas/FasL could not be consistently confirmed, which suggests that in addition to Fas/FasL, other apoptotic pathways might be involved in the deletion of TCR-engaged CAR T cells. As a consequence, the need to block several apoptosis pathways would require heavy and costly interventions, which would not be applicable in a clinical setting. An alternative would be to use CD4 T cells as a CAR platform for combined TCR and CAR activation[293]. However, if this study showed that CD4 CAR T cells could exert an antitumor effect when triggered through their TCR, they ultimately did not survive, which would only allow a transient therapeutic effect. As an alternative to CD4 and CD8 T cells, iNKT cells have been shown to be a safe platform for CAR immunotherapy in a pre-clinical study[294], and it would be interesting to evaluate whether CAR-iNKT cells would undergo AICD upon iNKT TCR activation. Finally, as published in the case of a 4-1BB containing CAR[291], the dose of CAR expressed on the T cell surface was instrumental in mediating CAR T cell apoptosis upon TCR stimulation. Therefore, controlling the dose of CAR expressed in CD8 T cells may prevent their TCR-induced apoptosis. Indeed, our preliminary results (data not shown) indicate that a decreased CAR expression greatly improve the survival of TCR-triggered CAR T cells without significantly impeding the functionality of the CAR. Therefore, a tight control of the level of CAR expression may allow targeting solid tumors via both a tumor-specific TCR and a CAR.

Finally, although it was shown that CAR signalling can also use the endogenous CD3 complex[295], there are no report of excessive activation of the CAR leading to AICD. The best demonstration that this does not occur is the success of CAR-mediated immunotherapy, which

showed that CAR T cells may persist quite long in mice and cancer patients. Therefore, it is likely that the activation threshold required for AICD is not achieved upon CAR activation. Our hypothesis is that high numbers of CAR molecules might be recruited at the TCR immunological synapse formed upon TCR activation. The accumulation of CAR co-stimulatory domains may greatly amplify the signal initiated by the TCR, thus accelerating exhaustion, and Fas/FasL pathway because of excessive signalling. To evaluate the recruitment of the CAR at the TCR synapse, we have developed a CAR, which is fused to the ZsGreen fluorescent protein. By confocal microscopy, we will investigate whether TCR engagement indeed recruits the CAR at the TCR synapse, hence increasing TCR signalling. More in depth biochemical studies will allow dissecting proximal and distal TCR signalling events in CAR T cells engaged via their TCR and evaluate whether the presence of the CAR co-stimulatory domains modifies the signalling events downstream of the TCR.

# L General conclusion

My first two projects aimed at improving therapeutic cancer vaccines by exploiting the adjuvant properties of iNKT cells. In the first project, we aimed at understanding how Th1 and Th2 αGalCer analogs induce or not iNKT cell anergy, in order to design new analogs which would allow vaccinating cancer patients repeatedly. Unfortunately, we found that Th2 analogs which only weakly induce iNKT cell anergy, also confer iNKT cells with a poor adjuvant activity on DC maturation. In this context, several groups have tried to optimize the structure of αGalCer to improve its adjuvant properties. For instance, the analog 7DW8-5 induced a superior priming of CD8 T cells than αGalCer[239], and its addition to an Adenovirus-based vaccine against malaria strongly enhanced the CD8 T cell response in primates[296]. Interestingly, our results on this glycolipid also revealed that it induces less iNKT cell anergy than αGalCer, although its structure is rather apolar. These results indicate that it is possible for an αGalCer analog to combine a strong Th1 phenotype with a reduced anergy induction. In contrast, c-GalCer, which is a potent Th1 agonist[241], strongly induces iNKT cell anergy. Altogether, the chemical and cellular parameters which control the adjuvant activity versus anergy induction of iNKT cells are not fully understood and it is not clear yet to which extent these processes can be dissociated.

In the second project, we confirmed the superiority of therapeutic cancer vaccines based on the intravenous transfer of *ex vivo* DCs loaded with αGalCer and peptide, as compared to subcutaneous peptide and poly(I:C) formulation. For instance, DCs loaded with αGalCer and the TAA NY-ESO-1 were recently challenged in a phase I clinical trial, which demonstrated their potency to induce a CD4 and CD8 T cell response against the NY-ESO-1 antigen in melanoma cancer patients. These encouraging results led to the setup of a phase II trial, which is now recruiting patients. Importantly, we showed that the antitumor response mediated by the adjuvant activity of iNKT cells is strongly improved by the addition of PD1 checkpoint blockade, which to our knowledge was not yet shown. The combined vaccination and anti-PD1 treatment improved both the priming of T cells and their effector functions inside the tumor These results are promising since DC vaccines are yet suboptimal[297,298], and only one modality, Sipuleucel-T[299], has reached the market so far. As seen already in the clinic, we found that the efficacy of PD1 checkpoint blockade largely depends on the immunogenicity of the tumor and its degree of T cell infiltration. However, the simultaneous DC loading with αGalCer and

peptide antigen could enhance therapeutic response of poorly immunogenic tumors. A promising alternative will be to pulse DCs with oxidized tumor lysates as tested clinical trials in ovarian cancer patients[182,257]. The combined loading of αGalCer with tumor lysates might further enhance T cell response against poorly immunogenic tumors.

The third project has demonstrated that TCR engagement in CAR CD8 T cells leads to Fas-mediated AICD, which precludes the combined TCR and CAR activation, as an improved strategy of CAR therapy against solid tumors. However, several alternatives may allow the rescue of CAR T cells upon TCR triggering. Interestingly, we showed that Fas/FasL blockade could not consistently rescue CAR T cells when activated through their TCR, which suggests that several pathways in addition to Fas/FasL are involved in the deletion of TCR-engaged CAR T cells. Therefore, blocking more than one pathway in CAR T cells would require heavy interventions that may not be applicable in a clinical condition. Alternatively, it would be interesting to combine CAR and TCR activation in CD4 T cells, which were shown by Fry *et al*.[293] to be less prone to TCR-mediated apoptosis. Moreover, the number of CAR molecules expressed in a given T cell might greatly influence the level of activation of its TCR, and a tight control of the dose of CAR expression may limit the TCR-induced AICD of CAR T cells as shown previously with regard to 4-1BB-containing CARs[291]. Indeed, our preliminary data show that a low CAR expression prevents the apoptosis of TCR-triggered CAR T cells without impeding the function of the CAR. Thus, a controlled CAR expression in tumor-specific T cells may allow TCR/CAR combined activation against tumors expressing both antigens. Additionally, iNKT cells were shown by others as a safe CAR platform[294], and it would be interesting to test whether iNKT TCR engagement in the presence of a CAR would or would not lead to apoptosis. Finally, a better understanding as how the TCR machinery copes with the presence of CAR co-stimulatory domains may certainly help finding ways to avoid CAR T cell AICD upon TCR engagement.

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