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Centre Ludwig pour la recherche sur le cancer

HARNESSING iNKT CELLS WITH RECOMBINANT CD1d FUSION PROTEINS TO REDIRECT INNATE AND ADAPTIVE IMMUNITY TO THE TUMOR

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Stéphanie CORGNAC

Diplomée en Master de Biologie (MSc), Université de Montpellier, France

Jury

Prof. Romano Regazzi, Président
Prof. Pedro Romero, Directeur de thèse
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Lausanne, le 31 mai 2013

pour Le Doyen
de la Faculté de Biologie et de Médecine

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Résumé large public

Exploiter les cellules iNKT avec une protéine CD1d recombinante pour l'immunothérapie du cancer

Les thérapies du cancer, comme la radiothérapie et la chimiothérapie, sont couramment utilisées mais ont de nombreux effets secondaires. Ces thérapies invasives pour le patient nécessitent d'être améliorées et de nombreuses avancées ont été faites afin d'adapter et de personnaliser le traitement du cancer. L'immunothérapie a pour but de renforcer le système immunitaire du patient et de le rediriger de manière spécifique contre la tumeur. Dans notre projet, nous activons les lymphocytes Invariant Natural Killer T (iNKT) afin de mettre en place une immunothérapie innovatrice contre le cancer. Les cellules iNKT sont une unique sous-population de lymphocytes T qui ont la particularité de réunir les propriétés de l'immunité innée ainsi qu'adaptative. En effet, les cellules iNKT expriment à leur surface des molécules présentes aussi sur les cellules tueuses NK, caractéristique de l'immunité innée, ainsi qu'un récepteur de cellules T (TCR) qui représente l'immunité adaptative. Les cellules iNKT reconnaissent avec leur TCR des antigènes présentés par la molécule CD1d. Les antigènes sont des protéines, des polysaccharides ou des lipides reconnus par les cellules du système immunitaire ou les anticorps pour engendrer une réponse immunitaire. Dans le cas des cellules iNKT, l'alpha-galactosylceramide (αGC) est un antigène lipidique fréquemment utilisé dans les études cliniques comme puissant activateur. Après l'activation des cellules iNKT avec l'αGC, celles-ci produisent abondamment et rapidement des cytokines. Ces cytokines sont des molécules agissant comme des signaux activateurs d'autres cellules du système immunitaire telles que les cellules NK et les lymphocytes T. Cependant, les cellules iNKT deviennent anergiques après un seul traitement avec l'αGC c'est à dire qu'elles ne peuvent plus être réactivées, ce qui limite leur utilisation dans l'immunothérapie du cancer. Dans notre groupe, Stirnemann et al ont publié une molécule recombinante innovante, composée de la molécule CD1d soluble et chargée avec le ligand αGC (αGC/sCD1d). Cette protéine est capable d'activer les cellules iNKT tout en évitant l'anergie.
Dans le système immunitaire, les anticorps sont indispensables pour combattre une infection bactérienne ou virale. En effet, les anticorps ont la capacité de reconnaître et lier spécifiquement un antigène et permettent l'élimination de la cellule qui exprime cet antigène. Dans le domaine de l'immunothérapie, les anticorps sont utilisés afin de cibler des antigènes présentés seulement par la tumeur. Ce procédé permet de réduire efficacement les effets secondaires lors du traitement du cancer. Nous avons donc fusionné la protéine recombinante αGC/CD1d à un fragment d'anticorps qui reconnaît un antigène spécifique des cellules tumorales. Dans une étude préclinique, nous avons démontré que la protéine αGC/sCD1d avec un fragment d'anticorps dirigé contre la tumeur engendre une meilleure activation des cellules iNKT et entraîne un effet anti-tumeur prolongé. Cet effet anti-tumeur est augmenté comparé à une protéine αGC/CD1d qui ne cible pas la tumeur. Nous avons aussi montré que l'activation des cellules iNKT avec la protéine αGC/sCD1d-anti-tumeur améliore l'effet anti-tumoral d'un vaccin pour le cancer. Lors d'expériences *in vitro*, la protéine αGC/sCD1d-anti-tumeur permet aussi d'activer les cellules humaines iNKT et ainsi tuer spécifiquement les cellules tumorales humaines.
La protéine αGC/sCD1d-anti-tumeur représente une alternative thérapeutique prometteuse dans l'immunothérapie du cancer.

Résumé

Les cellules Invariant Natural Killer T (iNKT), dont les effets anti-tumoraux ont été démontrés, sont de puissants activateurs des cellules Natural Killer (NK), des cellules dendritiques (DC) et des lymphocytes T. Cependant, une seule injection du ligand de haute affinité alpha-galactosylceramide (αGC) n'induit une forte activation des cellules iNKT que durant une courte période. Celle-ci est alors suivie d'une longue phase d'anergie, limitant ainsi leur utilisation pour la thérapie. Comme alternative prometteuse, nous avons montré que des injections répétées d'αGC chargé sur une protéine recombinante de CD1d soluble (αGC/sCD1d) chez la souris entraînent une activation prolongée des cellules iNKT, associée à une production continue de cytokine. De plus, le maintien de la réactivité des cellules iNKT permet de prolonger l'activité anti-tumorale lorsque la protéine αGC/sCD1d est fusionnée à un fragment d'anticorps (scFv) dirigé contre la tumeur. L'inhibition de la croissance tumorale n'est optimale que lorsque les souris sont traitées avec la protéine αGC/sCD1d-scFv ciblant la tumeur, la protéine αGC/sCD1d-scFv non-appropriée étant moins efficace.

Dans le système humain, les protéines recombinantes αGC/sCD1d-anti-HER2 et anti-CEA sont capables d'activer et de faire proliférer des cellules iNKT à partir de PBMCs issues de donneurs sains. De plus, la protéine αGC/sCD1d-scFv a la capacité d'activer directement des clones iNKT humains en l'absence de cellules présentatrices d'antigènes (CPA), contrairement au ligand αGC libre. Mais surtout, la lyse des cellules tumorales par les iNKT humaines n'est obtenue que lorsqu'elles sont incubées avec la protéine αGC/sCD1d-scFv anti-tumeur. En outre, la redirection de la cytotoxicité des cellules iNKT vers la tumeur est supérieure à celle obtenue avec une stimulation par des CPA chargées avec l'αGC.

Afin d'augmenter les effets anti-tumoraux, nous avons exploité la capacité des cellules iNKT à activer l'immunité adaptive. Pour ce faire, nous avons combiné l'immunothérapie NKT/CD1d avec un vaccin anti-tumoral composé d'un peptide OVA. Des effets synergiques ont été obtenus lorsque les traitements avec la protéine αGC/sCD1d-anti-HER2 étaient associés avec le CpG ODN comme adjuvant pour la vaccination avec le peptide OVA. Ces effets ont été observés à travers l'activation de nombreux lymphocytes T CD8+ spécifique de la tumeur, ainsi que par la forte expansion des cellules NK. Les réponses, innée et adaptive, élevées après le traitement avec la protéine αGC/sCD1d-anti-HER2 combinée au vaccin OVA/CpG ODN étaient associées à un fort ralentissement de la croissance des tumeurs B16-OVA-HER2. Cet effet anti-tumoral corrèle avec l'enrichissement des lymphocytes T CD8+ spécifiques observé à la tumeur.

Afin d'étendre l'application des protéines αGC/sCD1d et d'améliorer leur efficacité, nous avons développé des fusions CD1d alternatives. Premièrement, une protéine αGC/sCD1d dimérique, qui permet d'augmenter l'avidité de la molécule CD1d pour les cellules iNKT. Dans un deuxième temps, nous avons fusionné la protéine αGC/sCD1d avec un scFv dirigé contre le récepteur 3 du facteur de croissance pour l'endothélium vasculaire (VEGFR-3), afin de cibler l'environnement de la tumeur.

Dans l'ensemble, ces résultats démontrent que la thérapie médiée par la protéine recombinante αGC/sCD1d-scFv est une approche prometteuse pour rediriger l'immunité innée et adaptive vers le site tumoral.

Summary

Invariant Natural Killer T cells (iNKT) are potent activators of Natural Killer (NK), dendritic cells (DC) and T lymphocytes, and their anti-tumor activities have been well demonstrated. However, a single injection of the high affinity CD1d ligand alpha-galactosylceramide (αGC) leads to a strong but short-lived iNKT cell activation followed by a phase of long-term anergy, limiting the therapeutic use of this ligand. As a promising alternative, we have demonstrated that when αGC is loaded on recombinant soluble CD1d molecules (αGC/sCD1d), repeated injections in mice led to the sustained iNKT cell activation associated with continued cytokine secretion. Importantly, the retained reactivity of iNKT cell led to prolonged antitumor activity when the αGC/sCD1d was fused to an anti-tumor scFv fragments. Optimal inhibition of tumor growth was obtained only when mice were treated with the tumor-targeted αGC/CD1d-scFv fusion, whereas the irrelevant αGC/CD1d-scFv fusion was less efficient.

When tested in a human system, the recombinant αGC/sCD1d-anti-HER2 and –anti-CEA fusion proteins were able to expand iNKT cells from PBMCs of healthy donors. Furthermore, the αGC/sCD1d-scFv fusion had the capacity to directly activate human iNKT cells clones without the presence of antigen-presenting cells (APCs), in contrast to the free αGC ligand. Most importantly, tumor cell killing by human iNKT cells was obtained only when co-incubated with the tumor targeted sCD1d-antitumor scFv, and their direct tumor cytotoxicity was superior to the bystander killing obtained with αGC-loaded APCs stimulation.

To further enhance the anti-tumor effects, we exploited the ability of iNKT cells to transactivate the adaptive immunity, by combining the NKT/CD1d immunotherapy with a peptide cancer vaccine. Interestingly, synergistic effects were obtained when the αGC/sCD1d-anti-HER2 fusion treatment was combined with CpG ODN as adjuvant for the OVA peptide vaccine, as seen by higher numbers of activated antigen-specific CD8 T cells and NK cells, as compared to each regimen alone. The increased innate and adaptive immune responses upon combined tumor targeted sCD1d-scFv treatment and OVA/CpG vaccine were associated with a strong delay in B16-OVA-HER2 melanoma tumor growth, which correlated with an enrichment of antigen-specific CD8 cells at the tumor site.

In order to extend the application of the CD1d fusion, we designed alternative CD1d fusion proteins. First, a dimeric αGC/sCD1d-Fc fusion, which permits to augment the avidity of the CD1d for iNKT cells and second, an αGC/sCD1d fused to an anti vascular endothelial growth factor receptor-3 (VEGFR-3) scFv, in order to target tumor stroma environment.

Altogether, these results demonstrate that the iNKT-mediated immunotherapy via recombinant αGC/sCD1d-scFv fusion is a promising approach to redirect the innate and adaptive antitumor immune response to the tumor site.

Abbreviations

Ab	Antibody
ACT	Adoptive cell transfer
ADCC	Antibody-dependent cellular cytotoxicity
Ag	Antigen
αGC	α-galactosylceramide
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
ArgI	Arginase-I
β_2m	Beta 2-microglobuline
BiTE	Bispecific T cell Engager
BTLA	B and T lymphocyte attenuator
CAR	Chimeric antigen receptor
CBA	Cytometric Bead Array
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CEA	Carcino-embryonic antigen
CLL	Chronic lymphocytic leukemia
CMV	Cytomegalovirus
CNS	Central nervous system
CpG ODN	Oligodeoxynucleotide-containing CpG motif
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DN	Double negative
DP	Double positive
dsDNA	Double-strand DNA
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr Virus
EGFR	Epidermal growth factor receptor
Fab	Fragment antigen binding
FACS	Fluorescence activated cell sorter
FAP	Fibroblast activation protein
Fc	Crystallizable fragment
FcR	Fc receptor
FSC	Fetal calf serum
FDA	US Food and drug administration
FOXP3	Forkhead box P3
FR	Folate receptor
GITR	Glycocorticoid-induced TNFR-related protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEK	Human embryonic kidney cells
HER2	Human epidermal growth factor receptor-2
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HVEM	Herpes virus entry mediator
IBD	Inflammatory bowel disease
IFN	Interferon

Ig	Immunoglobulin
iGb3	Isoglobotrihexosylceramide
IL	Interleukin
i.m.	Intramuscularly
iNOS	Inducible nitric oxide synthase
IP-10	IFNg-inducible protein
i.v.	Intravenously
KO	Knock-out
LAG3	Lymphocyte activation gene-3
LCMV	Lymphocytic choriomeningitis virus
LN	Lymph node
LPS	Lypopolysaccharide
Mab	Monoclonal antibody
MAGE	Melanoma-associated gene
MAIT	Mucosal-associated invariant T
MCA	Methylcholanthrene
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MIP-1b	Macrophage inflammatory protein
MOG	Myelin oligodendrocyte glycoprotein
MR-1	MHC-class I-related molecule
MUC-1	Mucin-1
NHL	Non-Hodgkin lymphoma
NK	Natural Killer
NKG2D	NK group 2D
NKT	Natural Killer T
NO	Nitric oxide
NSCLC	Non-small cell lung carcinoma
OVA	Ovalbumin
PAMP	Pathogen-associated molecular patterns
PBL	Peripheral blood leukocytes
PBMC	Peripheral blood mononuclear cell
PC	Phosphatidylcholine
PD-1	Programmed cell death-1
PD-L	Programmed cell death ligand
PE	Phycoerythrin
scFv	Single chain fragment variable
TAA	Tumor associated antigen
TAM	Tumor-associated macrophage
TCR	T cell receptor
Teff	Effector T cell
TGF	Transforming growth factor
TIL	Tumor-infiltrating lymphocyte
TIM-3	T cell immunoglobulin mucin domain containing molecule-3
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAMP	Transgenic adenocarcinoma of the mouse prostate
Treg	Regulatory T cell
TRP-2	Tyrosine-related protein

TSA	Tumor specific antigen
PR	Partial response
RAE-1	Retinoic acid early transcript-1
Rag2	Recombination-activating gene-2
RNA	Ribonucleic acid
ROS	Reactive oxygen species
s.c.	Subcutaneously
S.E.M	Standard error of the mean
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
WT	Wild-type

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GENERAL INTRODUCTION

Part I: Cancer Immunity

1. Cancer immunosurveillance

In the 1880s Campbell De Morgan argued that cancer has a focal origin and can be dispersed in the body through lymphatic vessels. De Morgan also reported the fact that cancer occasionally spontaneously regresses (1). These observations provide evidence that host's defense can react against cancer. In 1909, Paul Ehrlich was the first scientist to declare that the immune system can recognize and prevent tumor formation. The idea of Ehrlich had to wait until 1950's when Medawar demonstrated the role of cellular components of the immune system in mediating allograft rejection (as reviewed by (2)). The development of syngeneic mouse strains and their immunization against tumor transplant gave rise to the discovery of tumor-specific antigens, distinct from alloantigens (3). Altogether, these discoveries have built the hypothesis of "cancer immunosurveillance" proposed by Burnet and Thomas. They postulated that lymphocytes have the role of detecting and eliminating constant arising newly transformed cells (4).

2. Tumor antigens

Coming with the concept of immunosurveillance, the discovery of tumor antigens was a significant step in the knowledge of cancer biology and brought an important input in the large field of cancer immunotherapy. Tumor antigens can be divided in two groups based on their pattern of expression: tumor-specific antigens (TSA) and tumor-associated antigens (TAA). TSAs consist of antigens expressed exclusively on tumor cells, arising from mutations that result in novel and abnormal protein production. TAAs are normal proteins, which are

also found in healthy cells, but they are ectopically expressed or overexpressed in tumor cells. These antigens are mostly tissue-specific antigens, lineage differentiation antigens or developmental antigens that are no longer expressed by normal cells in the adult stage or only weakly and in restricted sites.

The epidermal growth factor receptor (EGFR) family is expressed in some normal tissues and has a function in proliferation and differentiation of cells. The EGFR member 2, or HER2, which is expressed during fetal development, was found to be overexpressed in many human cancers, especially in 30% of breast cancer and ovarian cancer, and is a marker of bad prognosis. Herceptin is a humanized monoclonal antibody (mAb), targeting the antigen HER2, and is currently used to treat breast cancer patients. Herceptin treatment has demonstrated significant clinical benefits, mostly in combination with chemotherapies (5). HER2 forms heterodimer with EGFR-1 receptor, which is also a prognostic factor in epithelial cancers (6, 7). Another TAA is the carcino-embryonic antigen (CEA) involved in cell adhesion, which is also normally expressed during fetal development. CEA is a glycoprotein overexpressed in several human tumors and its detection in the serum serves as tumor marker in colorectal carcinoma (8).

In 1991, the first human tumor antigen recognized by lymphocytes T of patient was identified on human melanoma tumor cells and called melanoma-associated antigen-1 (MAGE-1). MAGE-1 was recognized by several CTL derived from tumor patients, and restricted by human leukocyte antigen (HLA)-A1 (9). Subsequently, several other antigens of the MAGE family as well as the NY-ESO-1 were identified by the reactivity of lymphocytes from patients. Interestingly, these antigens were expressed only by tumor cells and by testicular cells thus there were given the name of cancer testis antigens.

Today, numerous tumor antigens have been discovered and are now classified in five different categories. Some of them are being tested as target for cancer immunotherapy (10, 11) (table 1).

Table 1: Examples of human cancer antigens classified into 5 groups

Category	**Examples of tumor antigens**
Mutated antigens	p53, ras, BCR-ABL
Overexpressed antigens	EGFR, HER2
Cancer-testis (CT) antigens	MAGE, NY-ESO-1
Differentiation antigens	Tyrosinase, Melan-A, gp100, CEA
Viral antigens	HPV E6-E7, EBV proteins

3. Cancer immunoediting

The immune system has the capacity to eliminate nascent tumor cells based on the appearance of tumor antigens or molecules induced by stress. This process is defined as tumor immune surveillance. Unfortunately, cancers can arise from these transformed cells even with the presence of a competent immune surveillance. These observations led to the concept of immunoediting. This concept is divided into three phases, named the 3Es of cancer immunoediting: elimination, equilibrium and escape (figure 1).

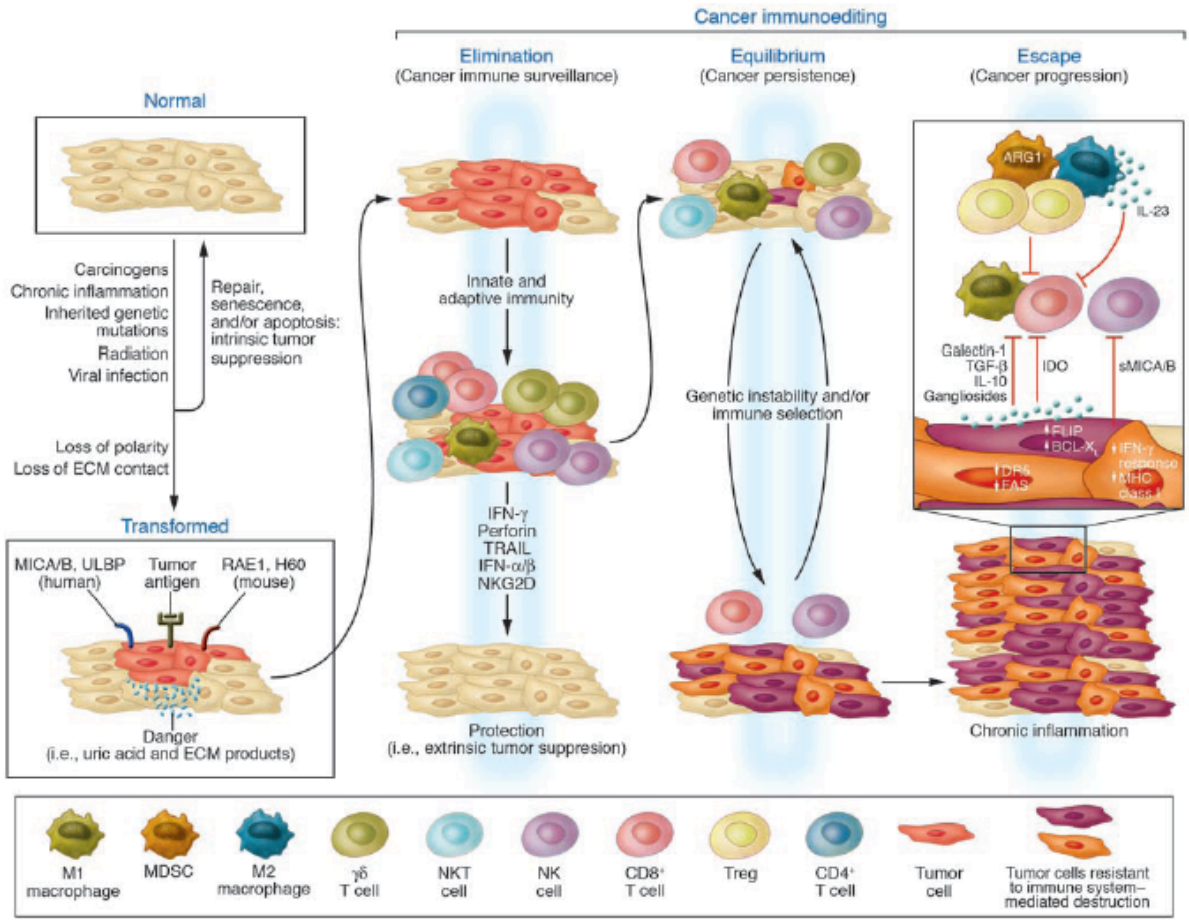

Figure 1: The three phases of the cancer immunoediting: Elimination, Equilibrium and Escape. Tumor cells arise from normal cells after an oncogenic transformation caused by different processes (carcinogen, virus…). These transformed cells express TAA or danger signals that initiate the cancer immunoediting process. First, during the elimination phase, innate and adaptive immune cells recognize and eliminate tumor cells leading to the protection of the host. However, if the elimination process is not complete, tumor cells enter the equilibrium phase where the immune pressure leads to the selection of resistant tumor variants. Finally, these newly variants develop escape mechanisms to evade the immune system and become prolific. Adapted from (12).

3.1. Elimination

The elimination process is described as the cancer immunosurveillance. Immunodeficient mice have been useful to determine the role of immune cells in destroying tumors. For instance, lymphocyte-deficient mice, which have a defect in the recombination-activating gene 2 (Rag2), were shown to develop spontaneous sarcomas more rapidly and more

frequently compared with WT mice when treated with the chemical carcinogen methylcholanthrene (MCA) (13). These observations underline the essential role of adaptive immune cells for the protection against cancer development. The innate part of the immune system is also effective against tumor apparition as shown by the enhanced cancer susceptibility of RAE1 transgenic mice, which lack functional natural killer (NK) cells, and of $J\alpha18^{-/-}$ mice, that are deficient in natural killer T (NKT) cells (14). At the molecular level, NK cells play a role in cutaneous tumor immunosurveillance through their activating receptor NKG2D (15). There is also evidence demonstrating that immunological components of lymphocytes, as interferon (IFN)-γ and perforin, are involved in the prevention of tumor and metastasis development (16) (14). Various immune gene-targeted KO mouse models were used to assess the role of other lymphocytes and immune effector pathways including tumor necrosis factor (TNF)- related apoptosis-inducing ligand (TRAIL), interleukin (IL)-12 and type I IFN in the control of tumor emergence (12). Altogether, these results highlight the complexity and the synergy between all components of the immune system in eliminating tumors.

3.2. Equilibrium

With such evidence of tumor immunosurveillance, the question concerning the eventual apparearance of tumors is asked. The equilibrium phase is an immune latent period following incomplete tumor destruction, which precedes tumor escape. During this phase, an inhibitory activity of the immune cells on tumor cells has been demonstrated, leading to the selection of resistant tumor variants. Through escape mechanisms, tumors become less immunogenic and are better adapted to survive in immunocompetent hosts. In parallel, lymphocytes are overwhelmed and cannot prevent appearance of new tumorigenic cells. The paradox of immunoselection of tumor variants has been well described. To reveal the influence of the

adaptive immune system in the immunogenicity of tumors, MCA-induced tumors originated from $Rag2^{-/-}$ or WT mice were transplanted in either $Rag2^{-/-}$ or in WT hosts. When grafted into $Rag2^{-/-}$, both $Rag2^{-/-}$ and WT-derived sarcomas developed with the same progression. In contrast when transplanted into immunocompetent hosts, 40% of $Rag2^{-/-}$ tumors were rejected around 8-9 days after graft, while all WT-derived tumors grew (13). This observation clearly indicated that WT derived sarcomas went through an immunoediting process. The innate system is also implicated in the immune pressure exerted on tumors during the equilibrium phase. A recent study has demonstrated that NK cells producing IFNγ promoted the immunogenicity of MCA-induced tumors through the attraction of tumor-associated macrophages (TAM). Even in $Rag2^{-/-}$ mice, tumors were edited by Th1-phenotype TAM also called M1 macrophages, which expressed high level of major histocompatibility complex (MHC)-class II and produced TNFα. (17). Overall, these observations show that tumors derived from immunocompetent mice are poorly immunogenic indicating that the adaptive immune system shapes the resistant phenotype of tumors, with an additional contribution of innate immunity.

3.3. Cancer escape

At some point of the equilibrium stage, tumor variants evade the immune pressure and become more aggressive. Several mechanisms were described to explain how tumors evade or counteract the immune system. First, immunosuppressive tumor environment is organized by the appearance of regulatory immune cells together with suppressive factors released by tumors themselves. Second tumor cells have the capacity to down-regulate MHC-class I molecules or to present an altered phenotype of MHC-class I and by consequence failing to correctly present antigens (reviewed in (18)). T cell-based therapies exert a pressure leading to the selection of immune-resistant tumor variants, as does the natural immune response.

Indeed, patients who relapsed after undergoing cancer immunotherapy, provided evidence that tumors can lose the expression of the targeted antigens. In a clinical study of a cancer vaccine against melanoma, one patient who was described positive for gp100 and TRP-2 antigens, displayed tumor variants with a total loss of HLA-class I or the disappearance of melanoma antigens at the end of the therapy process (19). The presence of immunosuppressive cytokines, the impact of immune-regulatory cells, such as regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC), and the inhibitory receptors expressed by T cells play together to dampen the immune system.

Regulatory T cells

Treg cells are well known to have a role in the maintenance of peripheral tolerance. Their regulatory effects are essential to prevent autoimmune disorders and moderate inflammation (20). Treg cells are described as T lymphocytes expressing CD4 and CD25 as well as the transcription factor forkhead box P3 (FOXP3) that is needed for their development and for the expression of multiple genes involved in the regulatory functions. Natural Treg develop in the thymus and express a diverse TCR repertoire specific for self-antigens, but some suppressive T cells, also called Treg, are induced or converted in the periphery, from antigen-specific effector T cells, depending on the microenvironment (21). Suppression mechanisms used by Treg to regulate the immune system are classified in three distinct groups: the production of inhibitory cytokines, metabolic disruption and the targeting of dendritic cells (DC) (22). Treg cells have been studied in different situations of chronic inflammation such as inflammatory bowel disease (IBD) or experimental autoimmune encephalomyelitis (EAE). In those pathological situations, their protective activity was shown to be mediated by the production of IL-10 and transforming growth factor (TGF)-β (23). Normally assigned to NK and CTL, cytolitic activities are also found in Treg. Several studies have shown that Treg can suppress

effector T cells by killing, through granzyme B and perforin pathways. As Treg express a high level of IL-2 receptor α chain CD25, it has been demonstrated that they can induce IL-2 deprivation by consuming this cytokine that in turn leads to a poor survival of effector T cells (24). An indirect suppressive function on T cells is also attributed to Treg. Indeed, Treg also express the co-inhibitory molecule cytotoxic T-lymphocyte antigen 4 (CTLA-4), which interacts with DC through CD80 and CD86, to modulate their maturation and their functions. Treg also contribute to render DC tolerogenic or to attract regulatory macrophages, which in turn damped the activities of effector T cells (reviewed by (22)) (figure 2).

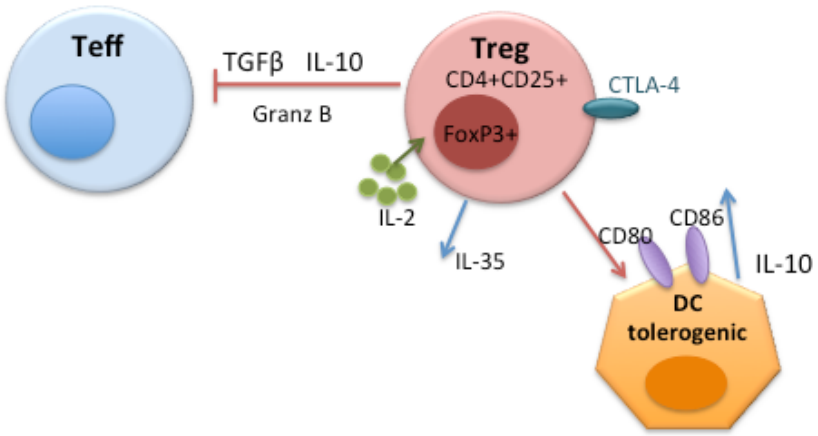

Figure 2: Mechanisms of suppression by regulatory T cells.

Among their primary role in the maintenance of peripheral tolerance, Treg were also found to suppress antitumor activities and to be a major obstacle in the success of cancer immunotherapy. As Treg are supposed to be specific for self-antigens, it is expected to find TAA specific Treg in the tumor environment. For instance, a study identified Treg specific for Melan-A antigen in the peripheral blood and in tumor-infiltrated lymph nodes of melanoma patients (25). Interestingly, the frequency of TAA-specific Treg was largely reduced after immunotherapy based on Melan-A peptide vaccination (25). Altogether the potential sources of Treg in the tumor result from the trafficking of thymus-derived Treg, the expansion of Treg

in the tumor and the differentiation or the conversion of effector T cells, induced by different factors in the tumor like IL-10, TGFβ or vascular endothelial growth factor (VEGF).

To prevent the immunosuppressive activity of Treg within the tumor, immunotherapies targeting Treg were developed. Briefly, monoclonal antibodies targeting CD25, the glucocorticoid-induced TNFR-related protein (GITR) and CTLA-4, defined as markers of Treg, showed an efficient reduction of tumor burden in mouse and human, and represent promising adjuvant cancer therapies. Low-dose of the alkylating agent cyclophosphamide, currently used as a chemotherapy agent, was reported to induce a depletion of Treg cells, but these observations remain to be confirmed and understood. The combination of immunotherapy in parallel with the depletion of Treg is under investigation and is an attractive choice in the treatment of cancer patients (reviewed by (26)).

Myeloid-derived suppressor cells

MDSC are a suppressive subset of myeloid cells also involved in tumor escape. A large variety of myeloid precursors give rise to MDSCs, including myeloid progenitor cells, immature macrophages, immature granulocytes and immature DC. In mice, MDSCs are characterized by CD11b and Gr1 markers, while in humans their phenotypes are LIN-HLA-DR-CD33+ or CD11b+CD14-CD33+. MDSC have been shown to strongly expand in tumor-bearing mice both in peripheral organs and tumor tissue, and increased MDSCs frequencies were shown in the blood of cancer patients (27). Among the CD11b+Gr1+ MDSCs, two major subsets have been described in several mouse tumor models: the granulocytic $Ly6G^{+}Ly6C^{low}$ MDSC and the monocytic $Ly6G^{-}Ly6C^{high}$ MDSC, which might use different suppressive mechanisms to impair effector T cell responses (28). MDSCs produce high quantities of the two enzymes; inducible nitric oxide synthase (iNOS) and arginase I (ArgI), which both degrade L-arginine and generate nitric oxide (NO) and urea respectively, leading

to the direct suppression of T cell responses (figure 3). Reactive oxygen species (ROS) and the oxidant product peroxynitrite are also important mediators used by MDSCs for their suppressive activities (figure 3)(29).

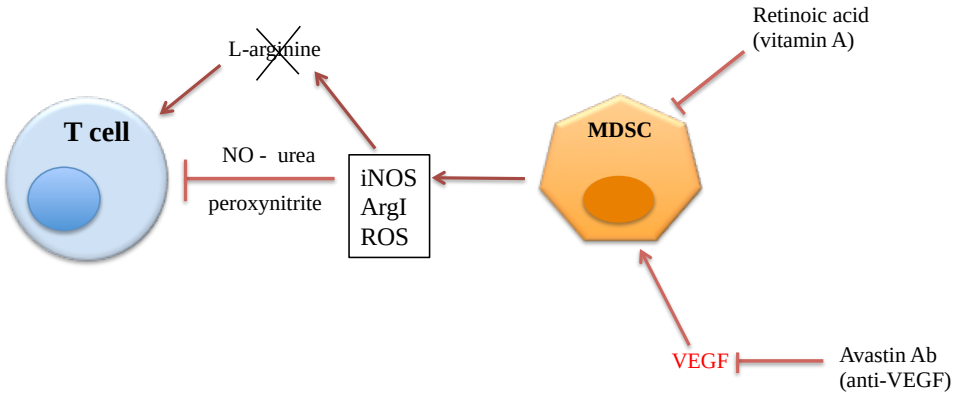

Figure 3: Mechanisms of MDSC-induced suppression of T cell functions and inhibition of MDSC functions.

In addition to enzymatic activities directly impairing T cell functions, MDSCs also promote the expansion of other immunosuppressive cell populations such as Treg (reviewed by (30)). To target MDSC and promote a better tumor immune response, several approaches were tested. The first one is to differentiate MDSC into mature myeloid cells that do not display suppressive functions. The best characterized compound for this purpose is retinoic acid, derived from vitamin A, which has been shown to decrease the number of MDSC in tumor-bearing mice and in patients with cancer (31, 32). Retinoic acid increased significantly the production of glutathione, a potent antioxidant, within the cells and thus reduced the level of ROS, which in turn led to the differentiation of MDSC (33). The blocking of VEGF, a major factor in the promotion of MDSCs, by using the anti-VEGF antibody (avastin), also led to a decrease of MDSC in the peripheral blood of cancer patients (34). Another approach is the inhibition of the function of MDSC. Inhibitors of ArgI, iNOS and ROS are being developed

to reduce the immunosuppressive effects of MDSC and ameliorate the antitumor immune response. Finally, a direct depletion of MDSC has been observed in mice after the administration of the chemotherapy gemcitabine (reviewed by (30)). Altogether, these results suggest an important potential of targeting MDSC to enhance immunotherapeutic effects.

Inhibitory receptors

During the immune response, a balance between co-stimulatory and co-inhibitory receptors expressed on T cells regulates the quality of the response. A broad diversity of co-inhibitory receptors was described, which negatively regulate T cell responses to protect from autoimmune diseases. Among the CD28/B7 family, belonging to the immunoglobulin (Ig) superfamily, programmed cell death-1 (PD-1) and CTLA-4 receptors are the two negative immune regulators commonly studied and used in the cancer immunotherapy setting (figure 4). Additional co-inhibitory receptors are also known to negatively regulate T cells. T cell immunoglobulin mucin domain containing molecule-3 (TIM3) receptor binds to galectin 9, expressed on many types of cancer, to inhibit T cells functions (35). The B and T lymphocyte attenuator (BTLA) also induces negative signaling in T cells by interacting with the herpes virus entry mediator (HVEM) (36).

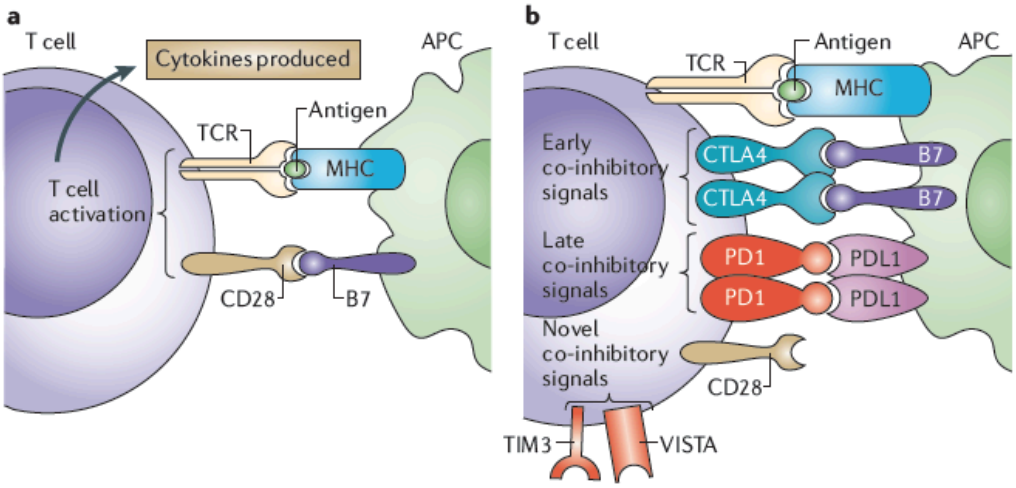

Figure 4: T cell stimulation and inhibition. a) T cell activation needs a "signal 1" induced by the TCR/Ag-MHC-I interaction and the costimulatory "signal 2" formed by the binding of CD28 on B7 ligands. b) Co-inhibitory molecules PD-1 and CTL-4 are upregulated after T cell activation and bind to their respective ligands PD-L1 and B7 to negatively modulate T cell activation (adapted from (37)).

In cancer, the ligands for inhibitory receptors, which dampen T cell functions, are often overexpressed in tumor tissue and then used as an escape mechanism (38). This control used by tumor leads to the appearance of unresponsive T cells, which can no longer mount an antitumor response. The non-responsive T cells are characterized by a prolonged upregulation of co-inhibitory receptors as well as defect in proliferation and in the production of cytokines, but these features can be reversed by using blocking antibodies directed against these co-inhibitory receptors.

PD-1 receptor limits T cell effector function by binding to PD-1 ligand 1 (PD-L1) and PD-L2 during an inflammatory response. The expression of PD-1 is upregulated when T cells become activated and acts mainly through the phosphatase SHP2 (39). In the tumor microenvironment, the increased expression of PD-1 in tumor-infiltrating lymphocytes (TIL) was shown to correlate with impaired cytokine production that reflected an anergic state of T cells compared to T cells in normal tissues (40). Similarly, the expression of PD-L1 by tumor cells could be used as a prognostic factor for melanoma (41).

CTLA-4 is expressed by activated T cells and negatively regulates the amplitude of T cell activation. CTLA-4 binds the same ligands as the co-stimulatory receptor CD28: CD80 and CD86 that are up-regulated on mature antigen presenting cells (APC). CTLA-4 exhibits a higher affinity for its ligands than CD28 and acts as a competitor of CD28, during the contraction phase of the T cell response. The binding of CTLA-4 to its ligands activates the SHP-2 and PP2A pathways that will stop the TCR activation. Moreover, CTLA-4 is highly expressed on Tregs and plays an important role in their immunosuppressive activity (reviewed by (39)).

The targeting of co-inhibitory receptors is a promising approach for cancer immunotherapy that is being studied further by targeting several different co-inhibitory receptors at once or by using it in combination with other immunotherapies.

Part II: Cancer immunotherapies

1. Objectives of immunotherapy

Over the past decade, immunotherapy is still in progress for the treatment of cancer and revealed promising successes. Compared to conventional cancer treatments like chemotherapy, radiation or surgery that are invasive for the patient, immunotherapy aims to be specific for the cancer and to limit the side effects. The principle of the immunotherapy is to activate or modulate the immune system in order to redirect it to the tumor site. Different strategies have been developed and belong to the large field of cancer immunotherapy: monoclonal antibodies, immune adjuvants, cancer vaccines or adoptive transfer of immune cells. The discovery of new tumor antigens has permitted a large variety of cancers to be targeted and extended the therapeutic approaches.

2. Tumor vaccines

The discovery of TAA has lead to the development of specific tumor vaccines that can prime the immune system against cancer. Prophylactic cancer vaccines are well established experimentally and they are efficient in protecting from tumor challenge in MCA-treated animals or from tumor onset in genetically modified animals, which otherwise spontaneously develop tumors (42). In the human, vaccination of young girls against virus-induced tumors, such as human papillomavirus (HPV), is currently in clinical use and efficiently protects women from the development of cervical cancers (43). Preliminary therapeutic benefits were also reported with a HPV vaccine (44). However, the development of therapeutic vaccines against non-virally induced cancers remains more challenging, as the immune system has to

overcome the tolerance to self-antigens and the immunosuppression induced by the tumor. Nevertheless, the use of tumor-specific antigens as vaccines, in the form of peptide or recombinant proteins mixed with a potent adjuvant, is efficient to elicit anti-tumor T cell responses (45). To enhance anti-tumor effects it is necessary to find the right formulation. This implies the combination of the appropriate tumor antigen with a potent adjuvant and to determine the best route of vaccination for each vaccine. In particular, vaccination of non-small cell lung carcinoma (NSCLC) patients with a recombinant MAGE-A3 protein mixed in a saponin-based adjuvant improved the disease-free survival (46). Detailed analysis in these patients revealed a high-titer of anti-MAGE-A3 antibodies as well as a potent CD4+ and CD8+ MAGE-A3-specific response. These vaccinated patients have been boosted with MAGE-A3 plus adjuvant and the authors concluded that this vaccine was efficient to mount long-term B and T cell memory responses. Melanoma, ovarian and breast cancer patients receiving a vaccination with recombinant protein NY-ESO-1, combined with CpG ODN and montanide, developed a rapid CD4+ T cell response and later, a fraction of these patients showed a specific CD8+ T cell response (47). Active immunization of patients with HER2 overexpressing tumors using HER2/neu peptides elicited a robust CD4+ and CD8+ T cell response (48). This acquired immunity specific for HER2 persisted during a long period in patients, most likely due to the activation of helper specific CD4+ T cells. Taken together, these vaccine strategies underlined the efficacy to promote and enrich the T cell response against cancers. Continuous efforts are ongoing to test TAA-derived peptide based vaccines in cancer patients. In the context of melanoma, these studies use peptides derived from melanocyte differentiation antigens such as gp100, Melan-A or different members of the MAGE family as well as NY-ESO-1, in combination with standard adjuvants and immunostimulants (45) (49). Peptides from HER2, CEA or mucin-1 (MUC1) are being tested

for ovarian and breast cancers. In general, there are no toxicities observed after the administration of the vaccines (50).

Another way to elicit antitumor immune responses is the administration of DC as a vaccine. This strategy aims to induce antigen-specific T cell responses by transplanting autologous DC previously pulsed *in vitro* with the peptide or the protein of interest (51). Because DCs are important to stimulate adaptive responses, their administration in patients permits to extend and amplify CD8+ T cell responses. The activation of DC before autologous transfer is an important step to improve antigen presentation, migratory capacity and the up-regulation of co-stimulatory molecules that help to confer potent adaptive immunity. Numerous clinical trials with DC vaccines have been directed in cancer patients, especially in melanoma, but few clinical responses were observed, largely attributed to the advanced stage of the cancer (51, 52). However, in 2010, a DC-based vaccine, leading to a prolonged median survival in castration-resistant prostate cancer patients, was approved by the FDA. The Sipuleucel-T immunotherapy (Provenge®) consists of an autologous PBMCs transfer, previously activated *ex vivo* with a recombinant prostate antigen fused to GM-CSF (53).

Alternative strategies consist in the use of genetically modified autologous tumor cells that secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) as a vaccine. One example is the study showing that irradiated autologous melanoma cells secreting GM-SCF enhanced anti-tumor immunity in melanoma patients (54). Named GVAX, this whole-tumor vaccine is also applied in clinical trials for the treatment of prostate cancer (55). This approach gives the advantage of presenting multiple tumor antigens specific for each patient together with a DC stimulation, but like DC vaccine, it is a personalized approach difficult to adapt to standardized cancer treatment.

The results obtained with cancer vaccines are encouraging even if poor clinical benefits are yet observed in cancer patients. The combination of this strategy with adoptive transfer of

immune cells or with blockade of immunosuppressive pathways might result in a more effective adaptive anti-tumor response.

3. Adoptive cell transfer

The *in vitro* expansion of T cells led to the development of adoptive cell transfer (ACT). Briefly, autologous T cells are expanded *ex vivo*, activated or genetically modified *in vitro* and re-infused in lympho-depleted patients. The ACT immunotherapy was improved by the re-infusion of expanded tumor-infiltrating lymphocytes (TILs), with increased specificity against the tumor. The aim is to generate TIL cultures from excised tumor biopsies and obtain a large number of highly tumor reactive T cells (56). A study reported that treatment with autologous TILs plus IL-2 led to durable complete responses in 22% of patients with metastatic melanoma. Moreover, 56% of these patients had an objective clinical response (57). Unfortunately, the accumulation of TILs is peculiar to melanoma tumors that is much less observed in other solid tumors, with high variability among individuals. One limitation is that TILs often display an exhausted phenotype acquired in the immunosuppressive tumor microenvironment, characterized by the up-regulation of co-inhibitory receptors and the deficient production of IFNγ (58). This lack of naturally occurring TIL populations and their weak antitumor reactivity prompted the development of genetically engineered T cells to express tumor-specific TCRs or chimeric antigen receptors (CAR). Briefly, autologous T cells are transduced with a selected high affinity TCR using a retrovirus, expanded *in vitro* and re-infused in cancer patients (figure 5).

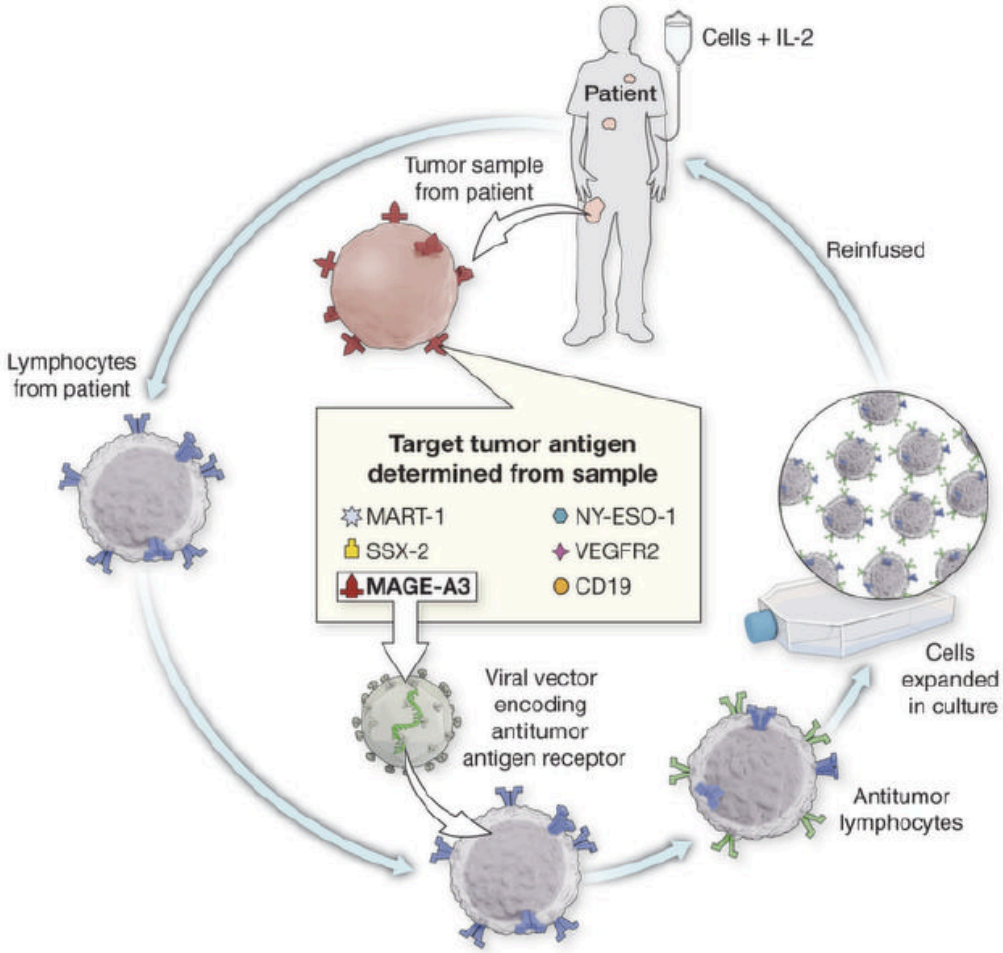

Figure 5: Schematic steps of adoptive cell therapy from (59).

The TCR candidates can be selected either from the natural repertoire or after mutagenesis of their antigen recognition domain in order to increase the affinity of T cells (60). A clinical trial was performed in patients with metastatic melanoma or metastatic synovial cell sarcoma. Patients were treated with the transfer of autologous T cells engineered to express NY-ESO-1-specific TCR (61). Results showed objective clinical responses in 50% of patients and two complete regressions among melanoma patients. One month after cell transfer in patients, NY-ESO-1 specific and tumor-specific IFNγ positive responses were still observed in peripheral blood mononuclear cells (PBMCs). Another similar study demonstrated the potent anti-tumor effects of ACT in patients with metastatic colorectal cancer (62). In this case, high

affinity TCR directed against human CEA were generated in HLA transgenic mice and the selected TCR were transduced in peripheral blood leukocytes (PBLs) from patients. In the three patients treated, tumor regressions were observed but transient inflammatory colitis was induced in all patients, which limit the use of anti-CEA T cell transfer.

As an alternative, chimeric antigen receptors (CAR), containing the antigen recognition domain of an antibody can be transduced by retrovirus into T cells from patients. CARs are composed of a single-chain variable fragment (scFv) of an antibody, fused to an intracellular domain that activates T cells (figure 6). CAR engineered T cells can be directed to the tumor via the antigen specificity of the scFv and activated by the intracellular motif of the CD3ζ chain domain. Since this approach is not MHC restricted, a single CAR can be used in all patients.

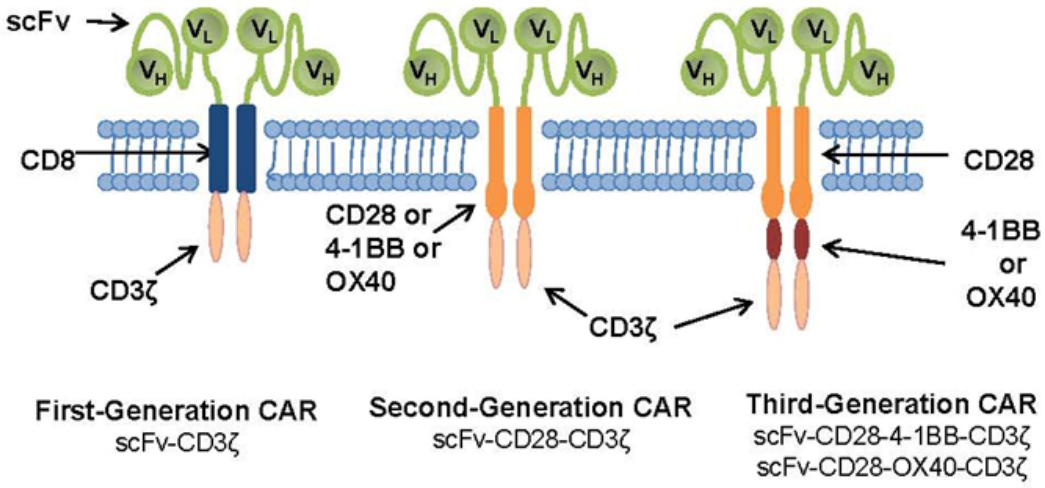

Figure 6: Structure of chimeric antigen receptors (CARs). First-generation CARs are typically composed of an scFv fragment specific to a TAA, fused to an inert transmembrane domain of the CD8 linked to a cytoplasmic signaling domain of the CD3 ζ chain. Second-generation CARs include a co-stimulatory signaling domain such as CD28, 4-1BB or OX40, while third-generation CARs contain tandem cytoplasmic signaling domains from 2 co-stimulatory receptors **(**from (63)).

A clinical study in a patient who had chronic lymphocytic leukemia (CLL) showed a complete remission after an anti-CD19 CAR-transduced T cell transfer (64). The CAR was constructed to specifically target CD19 on B cells and to drive the CD3ζ chain activating signaling into T cells. The 4-1BB signaling domain was also included in the construction to enhance the anti-tumor activities of re-engineered T cells. CAR19 T cells were able to expand *in vivo* and remained detectable six months after the transfer. The use of CAR-modified T cells can be extended to other tumor targets such as the folate receptor alpha (FRα) in ovarian cancer patients (65). Experimental results show that a CAR anti-VEGFR-2 targeting the vasculature could inhibit the growth of vascularized syngeneic tumor in mice (66). Recently, the same group reported an enhanced efficacy of the CAR system by co-transducing T cells with an anti-VEGFR-2 CAR and the IL-12 gene with strong anti-tumor effects in different mouse tumor models (67).

ACT represents a highly personalized and promising therapy to treat cancers. This immunotherapy can be adapted to each patient and each tumor depending of the choice of tumor antigens. Moreover, the extension of CAR targeting tumor stroma antigens might be a good alternative to treat several types of cancer (67). Altogether, the recent data have shown the feasibility and the efficacy of the transfer of anti-tumor T cells as a new form of cancer immunotherapy but this technology is still limited by the cost.

4. Antibody-based therapy:

4.1. Monoclonal antibodies targeting tumor

Today, a large collection of monoclonal antibodies (mAbs) has become standard pharmaceuticals for treating a wide variety of cancers. Thanks to new technologies, it is now possible to obtain chimeric, humanized or fully human mAbs (68). These technologies have

permitted enhanced efficacy of antibody-mediated immunotherapy due to reduced immunogenicity, increased effector functions and prolonged half-life. Based on the tolerance of the human immune system to antibodies and their targeting properties, nine pharmaceutical mAbs targeting five TAA and a growth factor (VEGF) have been approved by the US Food and Drug Administration (FDA) in anti-cancer therapy (table 2).

A representative example is the Trastuzumab antibody (Herceptin) targeting the HER2 antigen, which is overexpressed in several tumors including breast and ovarian cancers. It was approved by the FDA in 1998 for the treatment of invasive breast cancers which are positive for HER2 expression. Used in combination with chemotherapy in the treatment of breast cancer, trastuzumab induced a complete and partial response rate of 50% compared to 32% with the chemotherapy alone (5). Moreover, patients who received the combination had a median survival increased by five months compared to the chemotherapy alone.

Suggested mechanisms of Ab induced antitumor effects are the interaction between the Ab and its antigen, that interfaces with intracellular signaling of the targeted protein, the activation of complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). The last two mechanisms are induced by the crystallizable fragment (Fc) part of the Ab. In ADCC the Fc fragment reacts with Fc receptors (FcR) leading to the activation of effectors cells such as NK cells, monocytes and probably neutrophils. This property plays an important role in mAb antitumor activity. Moreover, different FcR polymorphisms, in patients treated with traztuzumab, strongly influence the clinical response by enhancing the ADCC activity (69). Naked mAbs are those without any material attached to them, whereas conjugated mAbs are those joined to a chemotherapy drug, radioactive isotope or toxin (table 2). The targeting of CEA antigen using Iodine^{131}-labeled antibodies was also extensively investigated for colon cancer therapy and for specific tumor localization (70). The binding between the Ab and tumor-associated antigen targets the drug or toxin to tumor cells.

Table 2: Monoclonal therapeutic antibodies approved by FDA in oncology classed according to the year of approval.

Generic name (Trade name)	Type	Target	Indication	Year of approval
Panitumumab (Vectibix)	Human IgG2	EGFR	Colorectal cancer	2006
Bevacizumab (Avastin)	Humanized IgG1	VEGF	Colorectal cancer	2004
Cetuximab (Erbitux)	Chimeric IgG1	EGFR	Colorectal cancer	2004
Alemtuzumab (Campath1H)	Humanized IgG1	CD52	CLL	2001
Trastuzumab (Herceptin)	Humanized IgG1	HER2	Breast cancer	1998
Rituximab (Rituxan)	Chimeric IgG1	CD20	NHL	1997
Tositumomab-I^{131} (Bexxar)	Murine IgG2a	CD20	NHL	2003
Ibritumomab tiuxetan (Zevalin)	Murine IgG1	CD20	NHL	2002
Gemtuzumab ozogamicin (Mylotarg)	Humanized IgG4	CD33	AML	2000

Ab-mediated immunotherapies are restricted to the availability of surface antigens mainly expressed on tumor cells and poorly by healthy tissue. New strategies have been developed to enhance the CDC or ADCC activities or to lengthen their half-life and thus ameliorate antitumor effects (71). The combination of mAbs with radiotherapy, chemotherapy or other immunotherapy reveals promising results and are rarely given as single therapy.

Different antibody (Ab) formats result from enzymatic digestion or from molecular biology modifications, retaining or not effector functions (figure 7). The main structure used in fusion proteins is the scFv, for instance in the development of CARs. The scFv is composed of the

variable domains of the heavy and the light chains of mAb linked by a peptide spacer, and represents the smallest structure (25 kDa) with antigen recognition. If the Ab serves in diagnostics, a protein with a low molecular weight, such as the scFv or the diabody, will be favorable due to its rapid pharmacokinetics. In contrast, the intact antibody gives advantage in the therapeutic setting with the presence of Fc-mediated effector functions.

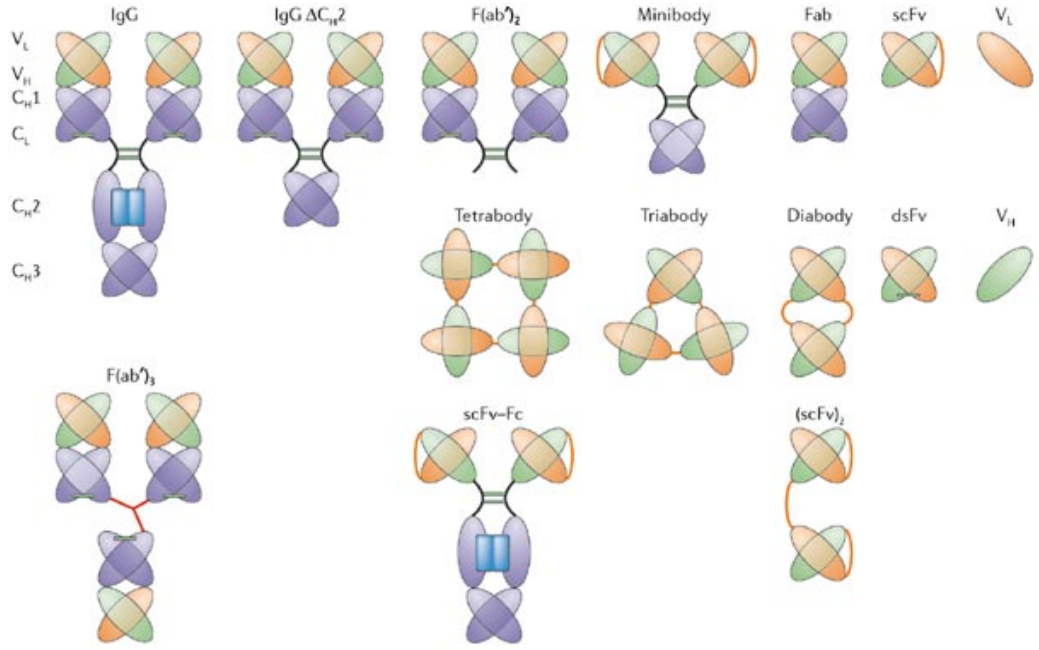

Figure 7: Representative examples of antibody structures. The constant or the variable domains of the Ig are combined or used alone to create a vast range of antibody constructs. Thus, this strategy leads to antibody fragments revealing different molecular weights from 12 to 150 kDa and valencies, which confer specific biological properties (adapted from (71)).

4.2. Immune-modulating antibodies

Recently, a novel class of mAbs that modulates the immune response is under development. The majority of these mAbs aim to block the negative function induced by co-inhibitory receptors (see chapter 3.3) and thus should restore the anti-tumor activity of immune cells (figure 8).

In 2010, the first immune-modulating Ab was approved by the FDA for the treatment of metastatic melanoma. It is named ipilimumab and is directed against the co-inhibitory receptor CTLA-4. Allison and colleagues were the first to demonstrate experimentally and then clinically the potent anti-tumor effects induced by the blockade of CTLA-4. In mice grafted subcutaneously with tumor, treatment with anti-CTLA-4 resulted in tumor rejection and protection against a re-challenge (72). Later, this group and others demonstrated that the blockade of CTLA-4 influences Treg cell and effector T cell outcomes leading to impressive and synergistic anti-tumor effects (73). Nevertheless, anti-CTLA-4 treatment alone had a low impact in the poorly immunogenic B16 melanoma model. This weak effect was enhanced when anti-CTLA-4 was combined with a GM-CSF-expressing tumor vaccine. This therapeutic combination allowed the eradication of established B16 tumors and correlated with a better tumor-specific CD8 T cell response. In this study, a depigmentation of mice was observed, associated with auto-reactive T cells. This phenomenon indicated an autoimmune disease, vitiligo, often observed in melanoma patients who positively respond to cancer therapy (74). The encouraging pre-clinical studies gave rise to the development of ipilimumab. In a clinical trial, fourteen melanoma patients were administered with anti-CTLA-4 and gp100 peptides but only two complete responses were observed. Importantly, nine of these patients showed toxicity associated with autoimmune disorders (75). However, a phase III trial in advanced melanoma patients treated with ipilimumab, combined or not with gp100 peptide vaccine, demonstrated a significant improvement of the survival (3.5 months survival benefit) compared to vaccine alone (76). The potential of anti-CTLA-4 antibody in cancer treatment is now well demonstrated and its use in combination with conventional therapy or with cancer vaccines is promising. The autoimmune toxicities observed after anti-CTLA-4 treatment highlight the important role of this co-inhibitor receptor to maintain

peripheral tolerance. The adverse events can be controlled and reversed by specific drugs, however careful monitoring is required.

The second promising mAb designed to manipulate the immune response is directed against the co-inhibitory receptor PD-1. PD-1 is highly expressed on TILs, reflecting an anergic or exhausted state, and the PD-1 ligand, PD-L1 is up-regulated also on cells from different human tumors. These observations provided a robust rationale for blocking PD-1 in the context of cancer therapy. Similar to ipilimumab, the anti-PD-1 mAb exerts a blocking effect on the receptor and prevents the inhibition of T cell functions. The anti-PD-1 in combination with the GM-CSF-secreting tumor cell vaccine improved the survival of B16 melanoma and CT26 colon carcinoma-bearing mice. The anti-tumor immune response induced by the tumor cell-based vaccine was enhanced by the blocking of PD-1 (77).

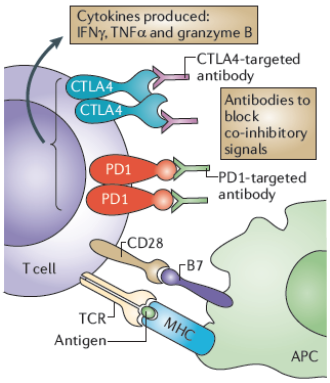

Figure 8: Blocking antibodies against PD-1 or CTLA-4 negative pathways demonstrate potent effects to restore T cell functions (37).

Another example of anti-PD-1 blockade combined with a cancer vaccine demonstrated a synergic effect of the two types of immunotherapy (78). In this study, recombinant lentivectors served as a support to efficiently present Trp2 antigen and prime a tumor-specific CD8+ T cell response. When associated with anti-PD-1 and anti-PD-L1 Ab therapy, the tumor growth in mice was slowed (78). Clinical trials are ongoing to confirm the efficacy of blocking PD-1 in cancer therapy. So far, anti-PD-1 treatment has led to anti-tumor benefits in patients with NSCLC, melanoma and renal-cell cancer, and minor immune-related toxicities

were observed (79). Interestingly, a correlation between the PD-L1 expression on tumor and the objective response was demonstrated; indeed in patients with PD-L1 negative tumors, no clinical response was seen (79).

Altogether, the strategies aiming to modulate CTLA-4 and PD-1 immune checkpoints promoted the targeting of other inhibitory molecules known to dampen the anti-tumor effects. Thus, the blocking of BTLA, TIM3, CTLA-4 or LAG3 is being studied and also showed an enhancement of T cell functions in mice (figure 9).

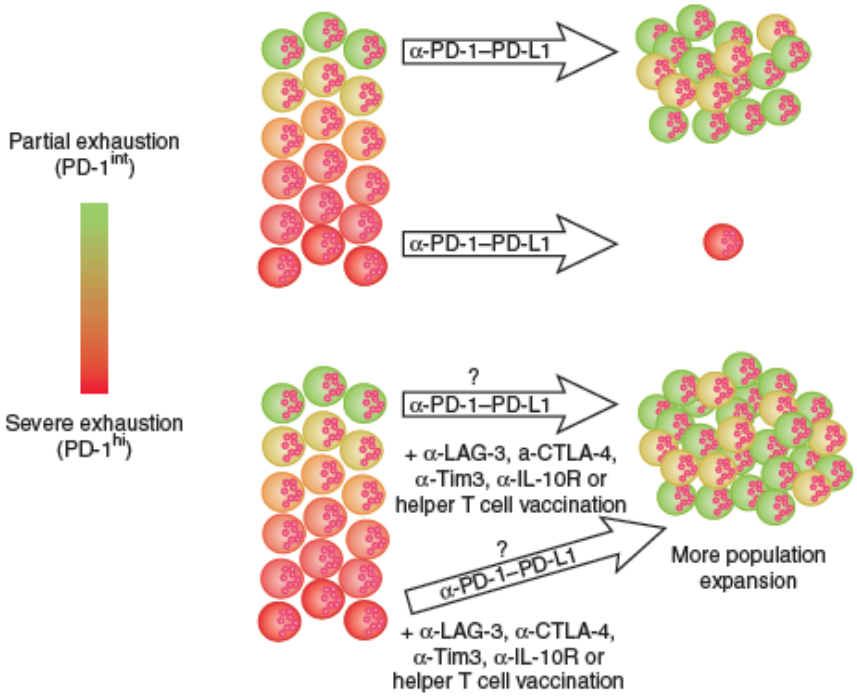

Figure 9: Antibody blockade of the PD-1 pathway. Anti-PD-1 and anti-PD-L1 antibodies reverse exhaustion and seem to selectively expand a subset of PD-1^{int} exhausted T cells (green and yellow cells), whereas PD-1^{hi} exhausted T cells (red cells) respond poorly (top). Many strategies have combined blockade of the PD-1 pathway with antibody blockade (α-) of other inhibitory receptors or of negative regulatory cytokines (such as IL-10) or therapeutic vaccination. Such strategies might augment the population expansion and/or survival of PD-1^{int} exhausted $CD8^+$ T cells already recovered by blockade of the PD-1 pathway or could lead to additional recovery of cells in the PD-1^{hi} subset of exhausted $CD8^+$ T cells (bottom). Tim3, inhibitory molecule; IL-10R, receptor for IL-10 (80).

Based on the demonstration of synergistic effects of two co-inhibitory receptors in the suppression of T cell responses, several studies are focused on the combined treatment with a pair of immune modulating Abs. The combined targeting of PD-1 and TIM-3 with Abs showed synergistic anti-tumor effects on CT26 tumor model in mice (81). Synergistic blockade of PD-1, CTLA-4 and PD-L1 co-inhibitory molecules coupled with B16-Flt3 (Fvax) vaccination improved the survival of B16 melanoma-tumor bearing mice (82). The simultaneous blockade also resulted in favorable Teff/Treg and Teff/MDSC ratios within the tumor (82).

Finally agonist mAb targeting stimulatory receptors have also been shown to reverse the tumor immune suppression. As an example, an Ab targeting the tumor necrosis factor (TNF) receptor family member CD40 induced potent anti-tumor effects both in mice and humans with pancreatic adenocarcinoma (83).

4.3. Bispecific antibodies

In addition to native Abs, which target one antigen, bi-specific antibodies have recently been engineered to target concomitantly a tumor antigen and an effector function (84).

TriomAbs antibodies, which display a trifunctionnal design, represent an association of two subclasses of immunoglobulin: a mouse IgG2a and a rat IgG2b. TriomAbs have a double specificity, one for a TAA and the second for the CD3 complex on T cells (figure 10). The particular design of these complexes permits to specifically target tumor and attract both innate and adaptive immune cells to the tumor. Indeed, the Fc part will mediate the attraction of NK cells and macrophages while the anti-CD3 Ab will recruit and activate T cells.

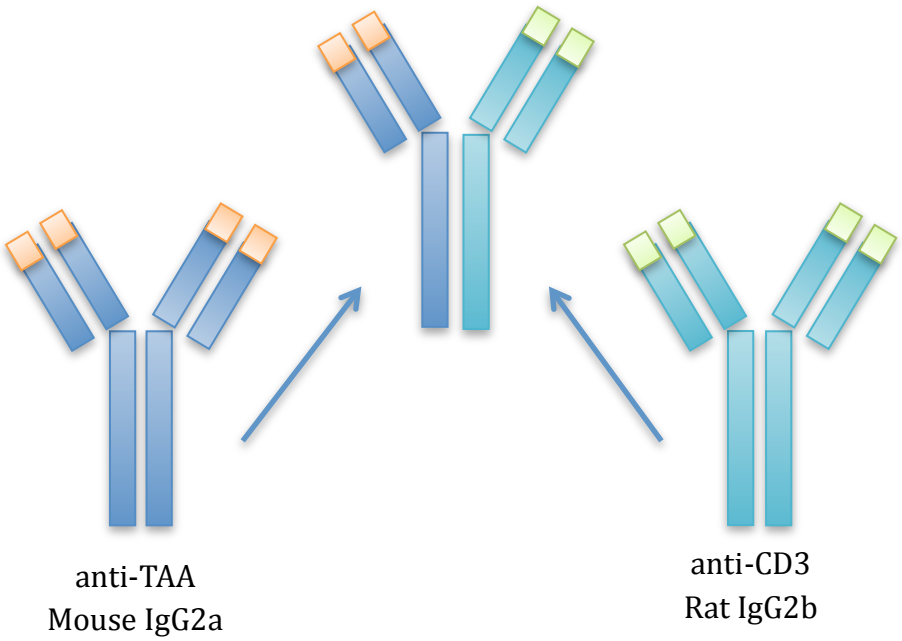

Figure 10: TriomAb bi-specific complex. TriomAbs are composed by IgG2a and a IgG2b parts that confer them three specificities. First, their Fc fragment is able to activate macrophage and NK cells by binding to the Fc receptor and promote ADCC and CDC. Second, triomAbs attract and activate T cells by targeting the CD3 and finally this complex target the tumor via the anti-TAA Ab part.

For example, Ertumaxomab (Rexomum) is a triomAb able to target the HER2 tumor antigen and the CD3 complex (85). Intraperitoneal administration of ertumaxomab in patients with malignant ascites resulted in complete elimination of tumor cells, without any severe adverse event. In addition, an *in vitro* study showed the best efficiency of triomAb to kill tumor cells compared to the anti-HER2 Ab (trastuzumab), particularly against tumors with a low HER2 expression (86).

Ab designers provided smaller complexes, made by the genetic fusion of two scFv, which encountered more success in clinical trials. BiTEs, bispecific T cell Engagers, are composed of an anti-CD3 scFv fused to an anti-TAA scFv with a short peptide linker. The first BiTE developed was blinatumomab, an anti-human CD3 x anti human CD19, which is being applied as a treatment in lymphoma and leukemia. A report in NHL patients demonstrated the successful anti-tumor effects induced by blinatumomab treatment in all patients (87). Partial

and complete tumor regressions were reached at a very low dose of blinatumomab (from 0.0005 to 0.06mg/m^2 per day), underlying the strong potential of BiTEs in cancer therapy. The second BiTE construct, approved for clinical trials, is Catumaxomab, which target EpCAM expressed on the majority of epithelial cells (88).

In addition to conventional antibodies, bispecific Abs have the advantage, via the anti-CD3 part, to activate cytotoxic T cells at the tumor site. Combination of several factors exploiting multiple anti-tumor effects reveals a promising use of bispecific Abs in numerous cancers.

At a preclinical level, the group of Jean-Pierre Mach at UNIL has focused on the fusion of anti-TAA Ab fragment to MHC or MHC-like molecules recognized by adaptive or innate effector cells. Promising results obtained with an anti-TAA Fab-MHC/gp33 peptide conjugates that were able to redirect antiviral T cell response to tumor cells by the Fab targeting. In mice infected with lymphocytic choriomeningitis virus (LCMV), the murine colon carcinoma (MC)-38-CEA^+ growth was inhibited by systemic injection of the anti-CEA Fab x H-$2D^b$/gp33 conjugates. The manipulation of the antiviral response was also extended to the influenza virus response (89) and could be applied in the clinic by redirecting against the tumor the cytotoxic T lymphocyte (CTL) response against endemic virus, such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV), often present in cancer patients. The potent activation of CTL by these anti-TAA-MHC/peptide conjugates is mediated predominantly by the oligomerization of MHC/peptide complexes on the surface of tumor cells (90).

To redirect NK effector cells against tumor, a bifunctional conjugate was constructed with an anti-HER2 or CEA scFv and the murine NKG2D ligand H60, linked together with an Fc part.

The fusion protein specifically bound to tumor cells and induced their lysis via effector NK cells activated by the ligand H60 (91).

More recently, a fully recombinant bifunctionnal fusion protein, composed of an scFv anti-HER2 and the MHC-like CD1d molecule loaded with alpha-galactosylceramide (αGC) was synthetized. This bifunctional protein had the property to attract invariant natural killer T (iNKT) to the tumor and showed potent anti-tumor effects *in vivo* (92). The iNKT cells, which are at the junction between innate and adaptive immunity will be described in the part III.

In this PhD study, our strategy of cancer immunotherapy was inspired from these promising results obtained with bifunctional recombinant proteins that combine different and important functions to eradicate cancers.

Part III: CD1d-restricted invariant NKT cells

1. Non-conventional T cells

In the 1980s, several groups studied and defined a new subset of αβ-TCR positive cells in mice, expressing the NK-cell marker NK1.1 and lacking the expression of CD8 co-stimulatory molecule. In 1995, Taniguchi was one of the first scientists to report the expression of an invariant Vα14+ TCR in the NK1.1+ T cell population, which is refer today under the name of natural killer T (NKT) cells (figure 11) (93). TCR of NKT is restricted by the MHC-related molecule CD1d, which is presenting various glycolipids instead of peptide antigens. Other T lymphocytes sharing NKT cell characteristics have also been described. Mucosal-associated invariant T cells (MAIT) express the Vα19-Jα33 TCR α-chain and are found in gut mucosa. These NKT-like cells are restricted to the MHC class I-related-l molecule (MR1) and possess a mature phenotype (reviewed by (94)). Recently, microbial derivatives of vitamin B have been identified as MAIT antigens, presented by the MR1 (95).

Among non-conventional T cells, a population of T cells expressing γδ TCRs also display innate-like features. γδ T cells recognize non-peptide antigens, such as microbial phosphoantigens, presented by MHC molecules or MHC-related molecules. Their innate phenotype also permits their activation by stress-induced NK ligands and/or pathogen-associated molecular patterns (PAMPs). Another important characteristic of γδ T cells is their specific location in epithelial tissues, such as skin and mucosae (96).

To summarize, a broad variety of non-conventional T cells exists in all species, bringing the capacity to respond differentially against several kinds of disease.

2. CD1d-restricted T cells

Different subsets of CD1d-restricted T cells have been identified, expressing invariant or variable TCR and the NK1.1 molecule. However all subsets are referenced under the name of NKT even though they probably display different functions (97, 98).

2.1. Invariant NKT or type I NKT cells

Among T lymphocytes, type I NKT cells or invariant NKT (iNKT) cells are a unique subpopulation of cells that express a semi-invariant αβ TCR and NK cell markers (figure 11). In mice, the TCR is composed by the Vα14-Jα18 chain associated with a semi-invariant Vβ chain restricted to Vβ7, 8 or 11. In human, the αβ chain is determined by the homologue Vα24-Jα18 and Vβ11. iNKT cells are reactive to glycolipid antigens presented by the MHC class-I-like molecule CD1d, especially the synthetic ligand α-galactosylceramide (αGC). iNKT cells are well characterized and defined by their immunoregulatory properties (figure 14), indeed, they rapidly produce a broad range of cytokines such as IFNγ, IL-4 and TNFα. They have a phenotype of conventional effector memory T cells with expression of surface markers such as CD44 and CD69 and a low expression of CD62L (reviewed by (99)). In contrast to T cells, iNKT cells do not need pre-activation by an antigen to reach an effector state. Preformed cytokine and lytic granule-encoding mRNAs are stocked in the cytoplasm of iNKT cells leading to rapid effector responses after TCR engagement (100). Moreover, activation of iNKT cells does not require co-stimulatory signals (101).

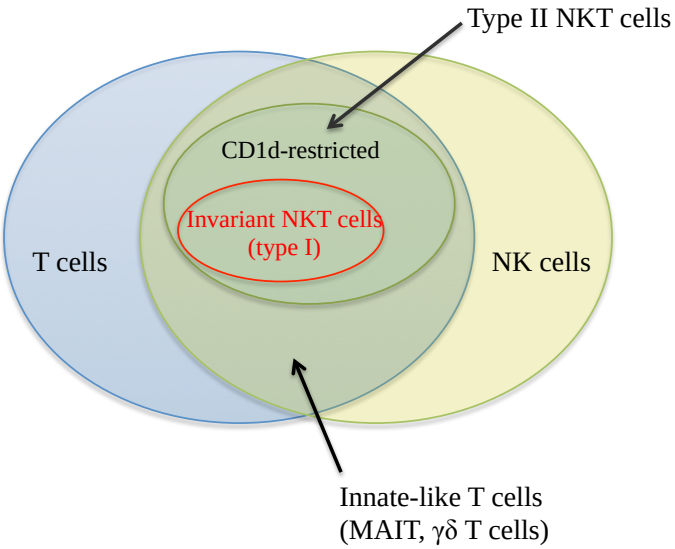

Figure 11: Invariant NKT cells definition among non-conventional T cells. iNKT cell is a T lymphocyte defined with a semi-invariant αβ TCR, restricted to lipid/CD1d, and NK cell.

2.2. Subsets of iNKT cells

iNKT cells differ in their expression of CD4 and CD8 molecules in different species. In mice, iNKT cells express CD4 or are double negative (DN) (97). At this time, no CD8 expression has been found in mouse CD1d-restricted iNKT cells, probably resulting from an intrathymic selection (102). These two subgroups exist in different proportions depending of the tissue. The functional difference between CD4+ and DN iNKT remains unclear. However, it appears that CD4+ iNKT cells have a tendency to induce Th2 response while DN iNKT cells have more Th1-like functions. A study has shown that the CD4+ fraction seems to produce more IL-4 after CD3-antibody stimulation in vitro (103), although this characteristic was not observed *in vivo* after αGC stimulation (104).

In contrast, human iNKT cells can express CD8 and are divided among CD4+, CD8+ and DN subsets. Different functions have also been observed in human Vα24+ iNKT cells according to the expression of CD4 (105). In PBMCs, CD4+ iNKT subset produced higher levels of IL-2, IL4 and IL-13, while DN and CD4+ were able to produce IFNγ and TNFα. iNKT cells

could also be separated depending on their pattern of chemokine receptors, NK cell receptors, or by their killing capacity. In the invariant NKT cell fraction, a small population was observed both in the thymus and in the periphery of mice that does not express the NK1.1 marker. *In vitro* activation of these NK1.1 negative iNKT cells led to higher production of IL-4 and lower levels of IFNγ than the NK1.1+ subset (106). In the thymus and in the periphery, NK1.1 negative Vα14-Jα18+ cells are characterized as precursors that give rise to the well-known populations of iNKT (107). A recent study, conducted by the Immunological Genome Project, has demonstrated that iNKT cells shared a similar transcriptional program with NK cells, as well as memory CD8 T cells and innate immune γδ T cells. In addition, at the final maturation stage of iNKT cells in the thymus, a large proportion of activating and inhibitory NK-cell receptors were found to be upregulated, showing the innate side of iNKT. However, a unique set of genes was found in iNKT cells underling their particular functions in the immune system (108).

2.3. Type II NKT

In addition to the conventional iNKT cells, named type I iNKT, non Vα14-Jα18 but CD1d-restricted NKT cells have been identified in mice. A diverse TCR repertoire can be expressed by this population of cells and importantly these TCRs are not reactive to αGC and they cannot be identified by αGC-CD1d tetramers (97). Also described as type II NKT cells, they display distinct properties from their type I counterparts (figure 11). In general, type II NKT cells are specific for antigens that are not agonists for the type I NKT (109). A study in mice showed, that they had immunosuppressive functions and protected mice from autoimmune diseases. Non Vα14 CD1d-restricted NKT cells were enriched in the central nervous system (CNS) of mice during EAE and treatment with sulfatide, a sulphated glycolipid, prevented the disease by inhibition of Th1 and Th2 cytokines produced by myelin oligodendrocyte

glycoprotein (MOG)-reactive T cells (110). At this moment, sulphatide is the most well-known lipid antigen that activates type II NKT cells. It is found in neuronal tissue and has been described in the EAE mouse model. To study the two major types of NKT cells specifically, mouse models were developed possessing different characteristics. The CD1d-deficient mouse is depleted of the two type, I and II, NKT cell subsets. In contrast, Jα18$^{-/-}$ mice lack only the type I iNKT cell part (111, 112). Type II NKT cells have been reviewed by Berzofsky and Terabe, demonstrating their important role in suppressing cancer immunosurveillance. They can also regulate the protective function of type I iNKT cells. The regulatory effect of the type II NKT cells is mediated in part by the production of IL-13, which induces TGFβ expression by CD11b+Gr1+ myeloid cells (113).

2.4. Other CD1d-restricted NKT cells

Recently, a new subset of CD1d-restricted cells was discovered. Uldrich and colleagues described αGC-reactive NKT cells that express a canonical Vα10-Jα50 TCR α-chain. This subset was found in Jα18-/- mice and displayed similar characteristics to the invariant Vα14-NKT cells, like the production of IFNγ and IL-4 under stimulation with αGC. However, the Vα10 NKT cells showed a preference for glucose- and glucuronic acid-containing glycolipids such as α-GlcCer and produced more IL-4 and IL-13 in response to αGlcA-DAG ligand compare to type I iNKT cells (114).

3. The lipid-presenting molecule CD1d

CD1d molecule belongs to the large CD1 family encoded by five non-polymorphic genes located on chromosome 1: CD1a, CD1b, CD1c, CD1d and CD1e. In mice and rats, only the CD1d genes are expressed (115). The CD1 genes display a similar sequence structure to

MHC class I genes and encode for proteins with significant homology to MHC-I and MHC-II proteins (116, 117). The three-dimensional structure of the mouse CD1d, established by X-ray crystallography, revealed that CD1d proteins are composed by α1 and α2 domains supported by an Ig-like α3 domain. The three α domains are associated with the β2-microglobulin to confer an efficient folding very similar to the MHC-I molecule (118). However, the CD1d ligand binding groove displays a deeper cavity, in contrast to MHC-I, mainly formed by hydrophobic amino acids leading to the presentation of hydrophobic ligands such as lipids (reviewed by (119)). CD1d proteins are expressed on hematopoietic cells both in humans and mice and at high levels on thymocytes (120). The majority of APC including DC, macrophages and B cells also constitutively express the CD1d molecules (121). While MHC molecules present peptidic antigens to conventional T cells, CD1d molecules present lipidic antigens to NKT cells.

4. CD1d ligands

Due to the specific hydrophobic binding pocket of CD1d, it is now well established that CD1d presents lipids or glycolipids to NKT cells. The field of NKT ligands largely remains to be explored, however, chemical approaches led to the isolation of several ligands from different origins (self ligands or bacterial ligands). The diverse structure of these lipids influences the binding to the CD1d and modulates the activation of NKT cells (98, 122).

4.1. Self-ligands

As NKT cells exert a low level of autoreactivity, it becomes evident that NKT cells could recognize self-antigen presented in the context of CD1d. They can be activated *in vitro* by CD1d-presenting cells without additional exogenous lipid (123, 124). Natural lipids are also

required for the CD1d-dependent positive selection of NKT cells in the thymus (125). The discovery of natural self-lipids presented by CD1d has to be extended but potential candidates include phospholipid antigens or glycosphingolipid antigens. Belonging to phospholipid antigens, phosphatidylcholine (PC) and sphingomyelin were identified by mass spectrometry to be major CD1d-associated ligands extracted from cells (126). Lysosomal glycosphingolipids also appeared as natural ligands for mouse and human NKT cells. The best studied is the isoglobotrihexosylceramide (iGb3), this glycolipid weakly activates CD1d-restricted cells *in vitro*. Furthermore, mice lacking the enzyme to process iGb3 have a defect in NKT cell development, suggesting that iGb3 lipid is an important biological ligand required to select NKT cells in the thymus (127). However, these conclusions are controversial since another study demonstrated that mice deficient for the iGb3 synthase showed a normal development of NKT cells (128).

4.2. Microbial glycolipids

Several findings support the idea that NKT cells could be activated by a broad range of antigens specific to microbes. Microbe-associated glycolipids include both ceramide-based lipids and diacyglycerol antigens. The discovery of glycolipids present in *Sphingomonas* bacteria that activate NKT cells provides evidence of a specific role of NKT cells in antimicrobial immunity (129). A diacylglycerol antigen derived from the pathogenic bacteria *Borrelia burgdorferi*, which causes Lyme disease, activates mouse and human iNKT cells. Moreover, iNKT cells were able to bind CD1d tetramers loaded with *B. burgdorferi* glycolipid-2 (BbGL-II). The iNKT cell activation by the bacteria was TLR-independent, proven by the activation in Myd88-/- mice, and resulted from the triggering of iNKT TCR by specific lipids (130). iNKT cells are also involved in the recognition of glycolipids from the highly pathogenic gram-positive bacteria; *S.pneumoniae* and group B *Streptococcus.* iNKT

cells were activated *in vivo* after administration of the bacteria and were needed for the pathogen clearance, which was dependent on CD1d (131). Altogether, iNKT cells play an important role in the protection against diverse pathogens.

4.3. α-galactosylceramide (αGC)

αGC was first identified as a potent immune activator in extracts of the marine sponge *Agelas mauritianus*. KRN7000 is the synthetic form of αGC, slightly modified, and it is a powerful tool to identify and activate the type I population of mouse and human iNKT cells (132). Structurally, αGC is composed of a galactose combined with a ceramide base in an α-configuration (an 18-carbon phytosphingosine and a 26-carbon acyl chain) (figure 12).

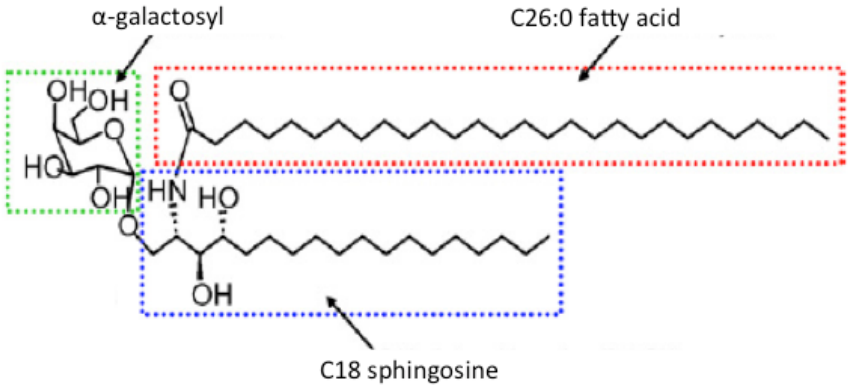

Figure 12: Structure of the synthetic αGC ligand (KRN7000) (adapted from (122))

Until now, αGC is the best ligand known to induce a strong activation of iNKT cells. Several studies have exploited the capacity of iNKT cells to rapidly produce Th1 and Th2 cytokines after αGC stimulation (133, 134). Particularly, mice bearing lung nodules after i.v. B16 melanoma inoculation showed a better survival after *in vivo* activation of iNKT cells by αGC (133). Different analogs of αGC are currently being tested to polarize the iNKT cell activation and elicit either Th1 or the Th2 responses. Modifications of the basic lipid chain of αGC results in a profound change in the type of cytokine produced (135). For example, the C20:2

αGC derived from αGC and containing a di-unsaturated C20 fatty acid was shown to induce a Th2 cytokine biased response by human and mouse iNKT cells (136).

5. Control of iNKT cell responses by ligands

iNKT cells are able to generate different responses and direct the outcome of an immune response, from protective immunity to immune tolerance. Depending on the structure of the glycolipid antigen, iNKT cells preferentially produce Th1 or Th2 cytokines. Several studies addressed the mechanisms of the iNKT cell control and three points have been highlighted. The first one involves the TCR affinity as a critical factor. Indeed, the binding affinity of the invariant TCR to the CD1d molecule and thus the iNKT cell activation is strongly influenced by the headgroup and length of the lipid chain. The increase in the length of the phytosphingosine improved the binding affinity of the iNKT TCR (137). The second factor influencing the outcome of iNKT cell response is the presentation of antigens by different types of APCs. It has been shown that αGC needs to be taken-up by APC and requires intracellular loading onto CD1d before it can be presented at the surface (138). However, Th2-biasing analogs are reportedly loaded rapidly and directly onto CD1d molecules expressed on the cell surface, without intracellular processing (135). Lipid raft localization is also demonstrated to be essential in shaping iNKT cell responses. CD1d molecules loaded with αGC were found in the lipid raft fraction of the plasma membrane. In contrast to the Th2-type antigens such as αGC C20:0 or αGC C10:0, which are mostly located outside of the lipid raft domains (135) (figure 13).

The previous observation raises the possibility that Th2-type antigens can be presented by non-professional APCs such as B cells. A study has shown that presentation of αGC by B cells induced more IL-4 cytokine production and no IFNγ production. This B cell presentation

also dampened DC-mediated activation of iNKT cells (139). Recently, cd1d1$^{fl/fl}$ mice have been exploited to give more information about the cytokine bias between Th1 and Th2 ligands. Specific ablation of CD1d from DC and macrophages completely abrogated the Th1 cytokine production after αGC injection. In contrast, processing of Th2 ligands, such as αGC acC8, are independent of DC and macrophages and explaining why they failed to induce IL-12 production by DCs which in turn enhances the Th2 response, leaning towards the IL-4 balance (140).

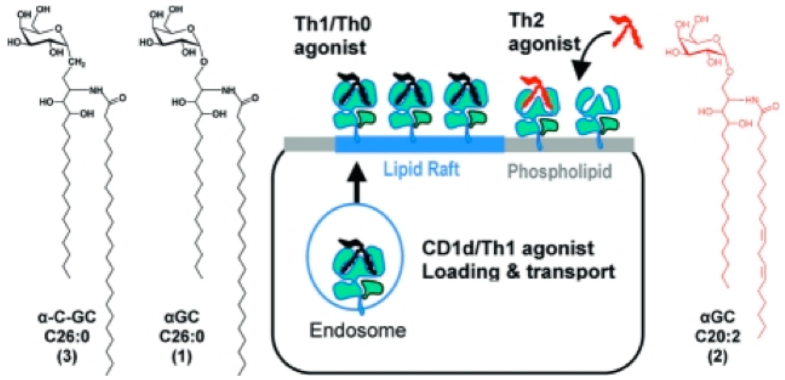

Figure 13: Mechanism of loading of Th1 or Th2 variants of αGC. Th1 ligand is intracellularly loaded onto CD1d and processed at the surface by endosome. In contrast, Th2 ligands are directly loaded onto surface CD1d molecules (141).

6. iNKT cell development

Similar to CD8+ and CD4+ T cells, iNKT cells develop in the thymus. Nude mice, which lack a thymus and thymectomized mice do not possess any iNKT cells. NKT cells derive from conventional T cells precursors, the double positive (DP) thymocytes, and thus diverge at the time of TCR expression (142, 143). The canonical TCRα expression on iNKT cells is the result of the random rearrangement of the Vα14 and the Jα18 gene segments. This recombination event might be favored by the close position of the two segments in the TCRα locus. The Vα14-Jα18 recombination occurs only within a 24-48 hour period before birth and

explains the delayed appearance of iNKT cells in the thymus (144). Moreover, the low frequency of iNKT cells in the thymus also supports the idea of a random expression of the invariant TCR Vα14-Jα18 (145).

CD1d is expressed by both epithelial cells and DP thymocytes in the thymus, but studies have shown that iNKT cells are positively selected at the DP stage by CD1d-expressing DP cells themselves (146). As mentioned above, self-lipids, such as lysosomal glycosphingolipids, might be candidate self-antigens for selecting iNKT cells in the thymus, as reviewed in (143). During DP-DP interactions, the SLAM-associated proteins are involved in the expansion and the differentiation of iNKT cells. This signal also contributes to the innate-phenotype acquired by iNKT cells in the thymus (147, 148). Once selected, iNKT cell precursors follow different stages of maturation leading to the upregulation of CD24, CD44 and DX5. The last maturation step is the acquisition of the NK1.1 marker that begins in the thymus but largely occurs in the periphery (143).

7. Functional role of NKT cells

Within the immune system, iNKT cells connect innate and adaptive immune responses. iNKT cells have mostly immunoregulatory functions, through their rapid production of a broad range of cytokines and chemokines, and they also exert a potent direct killing activity trough perforin, granzyme B and FasL pathways (figure 14). These characteristics allow iNKT cells to influence many other cells of the immune system such as NK cells, DC and B cells as well as conventional CD4 and CD8 T cells (figure 14) (149, 150). It is well established that cytotoxicity associated with the activation of iNKT cells is predominantly mediated by the activation of NK cells and their high cytotoxic effects (151). In addition, through DC maturation, activated iNKT cells promote CD8+ CTL cells and can enhance antigen-specific

CD8+ T cells against malaria (152) (153). Furthermore, iNKT cells interact with B cells and have a role of adjuvant in antibody production. Used as an adjuvant, αGC ligand protected mice from influenza by inducing a higher antibody response and enhancing memory B cell frequencies (154). Briefly, iNKT cells also have the capacity to counteract the immunosuppressive effects of IL-10-producing neutrophils and tumor-associated macrophages by direct or indirect interactions with these immune cells, leading to better anti-tumor responses (155) (156). Altogether these findings demonstrate the high potential of CD1d-restricted iNKT cells to balance the immune response and fight efficiently against a broad variety of infectious and malignant diseases.

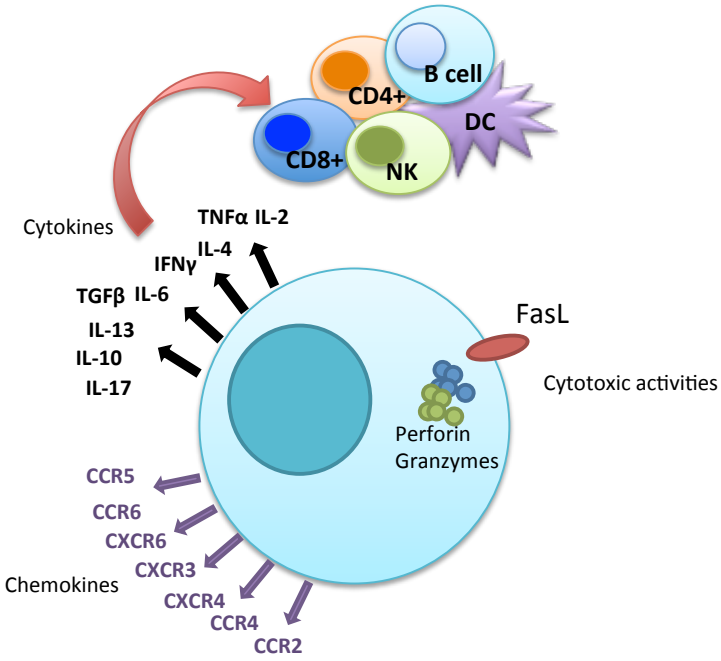

Figure 14: Immune functions of iNKT cells. NKT cells are able to produce a large variety of cytokines that in turn transactivate other immune cells including NK cells, conventional T cells, B cells and DCs. They also express a broad diversity of chemokine receptors, most of which are Th1-associated chemokine receptors.

8. NKT cells in pathological conditions

8.1. iNKT as effectors of tumor immunity

Numerous reports have demonstrated that iNKT cells contribute to anti-tumor immunity and represent promising therapeutic targets. In various tumor models iNKT cells have been recognized to play a role in tumor immunosurveillance as their absence predisposes to cancer onset. Decreased survival in an iNKT-deficient background was shown in MCA-induced fibrosarcomas and transgene induced adenocarcinomas such as the mouse prostate (TRAMP) cancer model. Here iNKT cell deficiency is associated with more frequent and bigger tumors than the WT background (14, 157). In MCA-induced tumors, protection by iNKT cells against tumor development is in part mediated by their IFNγ production and by the activation of NK cells and CD8 T cells (158). Recently, the role of iNKT cells in preventing MCA-induced tumors has been questionned because there is no clear evidence about the activation of iNKT cells in this model (159). Nevertheless, iNKT cells are able to directly recognize CD1d-expressing tumor cells and render them sensitive to lysis without addition of αGC (160). Expression of CD1d by tumors increases the number of circulating iNKT cells and iNKT tumor infiltration are associated with a better prognosis in humans (161, 162). In addition, evidence that iNKT cells activated by a strong agonist can lead to anti-tumor effects are numerous (163, 164). As mentioned previously, this effect results from the presentation of the strong ligand by DC and it is mainly mediated by NK cells rather than direct cytotoxic effects of iNKT cells. To summarize, antitumor activities of iNKT cells result either from a direct activation of the innate cells by CD1d+ tumor cells or by APCs. Activation is followed by a direct killing by cytotoxic iNKT cells and/or by an indirect killing mediated by NK cells or by controlling the suppressive activities of TAMs (reviewed by (165)) (figure 15).

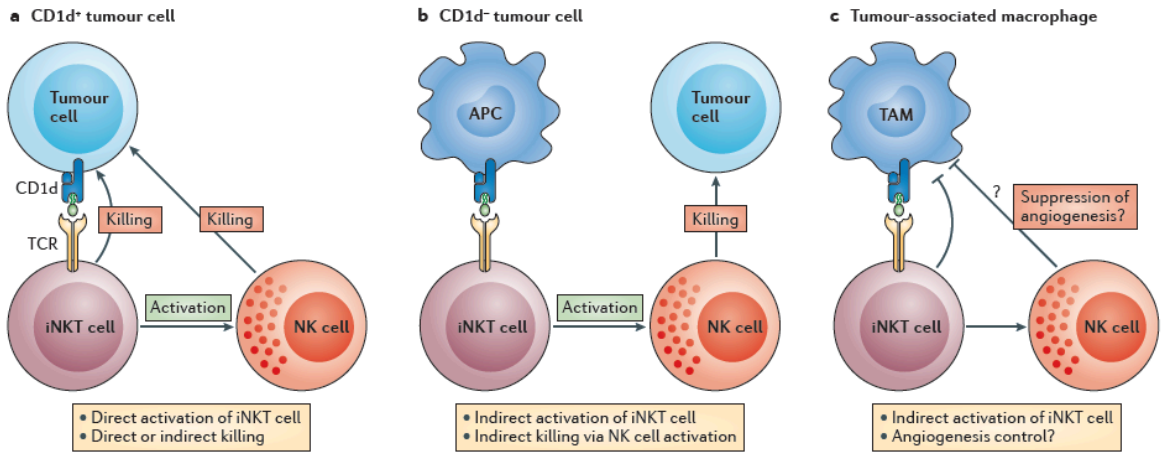

Figure 15: Antitumor activities of iNKT cells. a) Tumor cells expressing CD1d can present agonist glycolipid to iNKT cells and be killed by direct lysis or indirectly by NK cells, which have been activated by iNKT. b) APCs can activate iNKT cells via glycolipid/CD1d presentation, resulting in activation of cytotoxic NK cells, which in turn will kill the tumor. c) Tumor-associated macrophages can be directly or indirectly killed by iNKT cells, which limits the angiogenesis process (adapted from (165)).

8.2. Regulatory functions of NKT cells

In addition to antitumor activity through NK cells or direct cytotoxicity, some reports have described the opposite effects. These studies demonstrated that NKT cells might have a suppressor role in antitumor immunity (figure 16). In patients with hepatocellular carcinoma or bearing metastases from melanoma and colon carcinoma, CD4+ NKT cells were found to be enriched within the tumor compared to the DN or CD8+ subsets. They produced high levels of Th2 cytokines and demonstrated a weak lysis capacity against CD1d-expressing cells (166). In a murine T-cell lymphoma model, CD1d-deficient mice or Jα18-/- mice exhibited a better survival than the WT mice (167). In general, the immunosuppressive effects of NKT cells are attributed to type II NKT cells but without specific tools to identify them, the exact mechanisms are unknown.

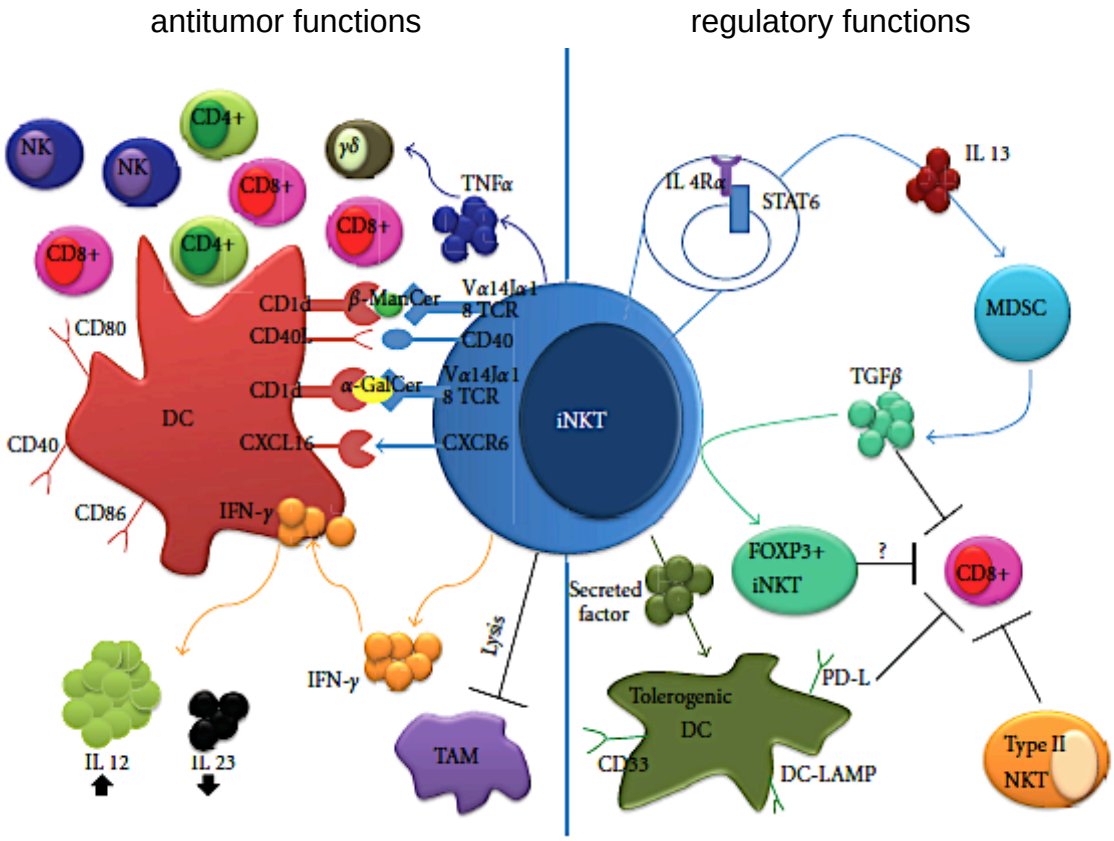

Figure 16: iNKT cell functions against cancer. iNKT cells exert two different roles in cancer: they can have anti-tumor effects (left) or immunosuppressive effects (right). Pro-inflammatory functions of iNKT cells result from their activation by agonist ligands, such as αGC loaded on CD1d-expressing DC. Through the release of cytokines, they can transactivate other immune effector cells like NK, CD8+ and CD4+ T cells or directly kill tumor cells by their own lytic properties. On the other hand, NKT cells (mostly type II) can inhibit CTL activity by producing IL-13. They also trigger the acquisition of a tolerogenic phenotype by DCs (adapted from (168)).

9. iNKT cells in cancer treatment

Based on pre-clinical studies, several phase I/II clinical trials of cancer immunotherapy were initiated using iNKT cells as effector cells. Three strategies have been tested: the injection of the ligand αGC as a free ligand, the infusion of autologous αGC-loaded DC and the infusion of autologous iNKT cells expanded *in vitro* with glycolipids (reviewed by (168)). All these treatments were well tolerated with no severe toxicities but the clinical outcome was poor, even though an activation of iNKT and NK cells was often observed (table 3). The most

important limitation of using iNKT cells is their low number in the majority of cancer patients and, in some cases, their failure to proliferate after ligand stimulation (169). To overcome this problem, the option of adoptive transfer of *ex vivo* expanded iNKT cells into patients is promising and opens new ways to iNKT cell-based immunotherapies.

Table 3: Summary of phase I-II studies of iNKT cell-based immunotherapy. Adapted from (168)

Tumor	Treatment	Summary of results	Ref.
Solid tumors	αGC i.v (50-4800μg/m^2)	- no dose limiting toxicities - increased serum levels of GM-CSF and TNFα - biological effects depend on NKT cell numbers - no clinical responses	(170)
Non-small cell lung cancer	i.v. infusion (2x) of *in vitro* expanded autologous iNKT with αGC and IL-2 ($1x10^7$ or $5x10^7$ NKT/m^2)	- no major toxicities - expansion of iNKT cells in blood - increase in IFNγ-producing cells - no clinical responses	(171)
Non-small cell lung cancer	i.v. infusion of *in vitro* expanded autologous DC loaded with αGC dose escalation: $5x10^7$, $2.5x10^8$ or $1x10^9$ APCs/m^2)	- no major toxicities - expansion of iNKT cells in blood - increase in IFNγ-producing iNKT cells - no clinical responses	(172)
Head and neck cancer	infusion into the nasal submucosal of *in vitro* expanded autologous APCs loaded with αGC (2x $1x10^8$ APCs)	- no major toxicities - expansion of iNKT cells in blood - increase in IFNγ-producing cells - only one PR	(173)
Non-small cell lung cancer	i.v. infusion of αGC-pulsed autologous PBMCs cultured in IL2/GM-CSF (4 injections, $1x10^9$ PBMCs/m^2)	- no major toxicities - expansion of iNKT cells in blood - better survival in patient with IFNγ-producing cells after αGC stimulation - no clinical responses	(174)
Metastatic cancer	Autologous αGC-pulsed monocyte-derived DCs ($1x10^6$ DC)	- no severe toxicities - activation of iNKT cells, increase in NK and T cell activation and cytotoxicity - increased serum levels of IFNγ and IL-12	(175)

Myeloma, anal cancer and renal cell cancer	Autologous αGC-pulsed mature DC	- no severe toxicities - sustained expansion of iNKT cells in blood and tumor - increased serum levels of IL-12, IP-10 and MIP-1b - increased in memory CMV-specific CD8 T cells	(176)
Advanced lung cancer	i.v. infusion of autologous αGC-pulsed APC ($1x10^9$ APCs/m^2)	- no severe toxicities - expansion and activation of iNKT cells in blood - strong infiltration and activation of NKT cells into the tumor after treatment	(177)
Head and neck cancer	Intra arterial infusion of expanded autologous NKT cells ($5x10^7$) + infusion in intranasal submucosa of αGC-pulsed APCs ($1x10^9$)	- one grade 3 adverse event - NKT and NK cells responses in blood (NKT expansion and IFNγ production) - 3 PR but relapses, 4 SD and 1 PD	(178)
Myeloma	i.v. infusion of autologous αGC-pulsed DC ($1x10^7$ DC) + lenalidomide orally for 21 days (10mg/day)	- one grade 3 adverse event - activation of iNKT and NK cells - increase in monocytes and eosinophiles - reduction in tumor-associated monoclonal Ig	(179)

10. Future in iNKT cell immunotherapy

Other strategies are currently being tested to improve immunotherapy using iNKT cells. A recent clinical study investigated the combination of αGC-loaded DC with the conventional chemotherapy lenalidomide and they showed evidence that these treatments synergized to enhance immune effects (179). Taking into consideration the numerous functions of iNKT cells, the use of a modified glycolipid, which polarizes iNKT cells toward a Th1 cytokine profile, is a promising approach. Glycolipids as vaccine adjuvants also showed powerful properties in enhancing specific CD8 T cells and the overall protective immune response (150). In two mouse models of ovarian cancer, treatment with αGC combined with a tumor-

cell based vaccine significantly enhanced the protective and the therapeutic antitumor effects (180). These effects were dependent on T cells and NK cells as demonstrated by the absence of anti-tumor response when CD4, CD8 or NK1.1+ cells were depleted, and by the augmentation of tumor-specific CD8+ T cells producing IFNγ in response to the αGC vaccine (180). Interestingly, the use of CD1d-expressing tumor cells as a vector to present αGC demonstrated encouraging results (181). These authors have shown that B16 melanoma and EL-4 thymoma cells transfected with CD1d were able to activate NKT and NK cells *in vitro* and *in vivo*, independently of the CD40, CD80 and CD86 co-stimulatory molecules. Moreover, i.v. vaccination with live αGC-loaded B16-CD1d tumor cells protected mice from the development of B16 lung metastasis. Similarly, in a glioma model, the vaccination with irradiated tumor cells pulsed with αGC also resulted in potent therapeutic effects through iNKT activation and a CD4 T cell-mediated response (182).

Therefore, exploiting the antitumor functions of iNKT cells in cancer immunotherapy displays great potential. However, the regulatory activities of NKT cells still need to be understood.

11. iNKT cell anergy

The phenomenon of iNKT cell anergy has greatly limited the therapeutic use of these lymphocytes. Upon *in vivo* stimulation with the superagonist αGC, iNKT cells produce large amounts of cytokines within only a few hours and reach a maximum expansion after 2-3 days. Following this period, iNKT cells enter a state of unresponsiveness that precludes re-stimulation (183). Indeed, after a second challenge of mice with αGC, iNKT cells failed to produce cytokines and were inefficient to protect mice from B16 lung metastasis (183). The mechanism of this long-term anergy remains unclear despite several studies. To understand

the mechanism of iNKT cell anergy, studies focused on markers characteristic of the exhaustion of conventional T cells. As a first candidate, PD-1 has been suspected to be involved in the negative regulation of CD8+ T cells, and it was also proposed that PD-1 and PD-L1 pathways play a role in the anergy of iNKT cells (184). The authors observed an upregulation of PD-1 and PD-L1 upon αGC stimulation that correlated with the anergy of iNKT cells. Moreover, *in vitro* and *in vivo* blocking of PD-1 and PD-L1 with antibodies restored the IFNγ and IL-4 production, and the proliferation of iNKT cells as well as their protective effects against B16 lung metastasis. A second group confirmed the implication of the PD-1/PD-L1 co-stimulatory pathway by showing that iNKT cells in PD-1 KO mice did not become unresponsiveness when treated with αGC (185). In these studies, combined NKT therapy and PD-L1 blockade led to better antitumor effects in a melanoma model. Furthermore in a more recent study, tumor-bearing mice treated with PD-L1$^{-/-}$DC loaded with αGC showed a better tumor inhibition as compared with mice injected with WT DC loaded with αGC (186). However, the inhibitory effect of PD-1 seems to be reversed by CD28 co-stimulatory receptor engagement on iNKT cells, which probably changes the balance between positive and negative signals (187). The role of PD-1 in the anergy of iNKT cells was further contradicted by other results, which argued that a strong TCR signal plus co-stimulation is responsible for the induction of the unresponsiveness (188). They confirmed the PD-1 up-regulation, but not the reversal effect of the PD-1 blockade on the iNKT cell anergy. In addition, injection of a high dose of αGC in PD-1-deficient mice resulted in the anergic state of iNKT cells, demonstrating that PD-1 up-regulation was not sufficient. This last report was in contradiction with the results published by Parekh et al. (185). To explain the difference, the authors argued that the dose of ligand might influence the duration of the unresponsiveness. They showed the importance of co-stimulatory signals for inducing anergy.

For instance, the stimulation of iNKT cells with anti-CD3 did not render them unresponsive but concomittant anti-CD28 stimulation induced anergy of iNKT cells (188).

Intracellular mediators such as the E3 ubiquitin-protein ligase Cbl-b has also been shown to play a role in the induction of iNKT cell anergy (189). Accumulation of Cbl-b was observed in αGC-treated iNKT cells correlating with their reduced function. Interestingly, IFNγ production, but not IL-4, was restored in Cbl-b-deficient iNKT cells stimulated with αGC, and this was associated with the ability of αGC-pretreated iNKT cells to prevent B16 lung nodules (189). Cbl-b plays an important role in the NF-kB pathway; the authors demonstrated that CARMA1, a critical regulator in NF-kB activation, is a target of Cbl-b. CARMA1 was degraded during the anergic state of iNKT cells, preventing further stimulation and IFNγ production.

As mentioned before, different αGC analogs have been exploited for their ability to differentially regulate the activation of iNKT cells. The Th2-biasing derivative αGC C20:2 stimulated iNKT cells more rapidly and resulted in a profound defect in producing cytokines already 48h after *in vivo* injection of the ligand, whereas αGC induced anergy after 72h. However, further kinetic analyses demonstrated that iNKT cells recovered more rapidly from anergy when stimulated by the analog C20:2 and interestingly, they expressed PD-1 and PD-L1 during a shorter period compared to the long-lasting expression on αGC-treated iNKT cells (190).

In summary, iNKT cells display important properties that could be helpful for cancer immunotherapy. In addition, Stirneman et al. in our group have developed a recombinant CD1d protein loaded with the αGC and fused to an anti-TAA scFv, which demonstrated potent anti-tumor effects. Moreover, this approach avoided the anergic state of iNKT induced

when αGC is injected as free ligand. The manipulation of iNKT cells in the treatment of cancer patients is promising (92).

OBJECTIVES OF THE THESIS

The general aim of the project is to develop a cancer immunotherapy based on the sustained activation of iNKT cells with the αGC ligand loaded on a recombinant CD1d protein. Moreover, the CD1d protein is fused to an scFv anti-TAA in order to redirect the adaptive response and the NKT-mediated anti-tumor effects to the tumor site.

Rational

Why use iNKT as effector cells?

Stirnemann et al and others have highlighted the potent effects of iNKT cells on the immune system and against cancer.

- iNKT cells rapidly produce cytokines as IFNγ, TNFα, IL-4, and IL-2 upon TCR triggering by high affinity ligand αGC presented on the CD1d molecule
- iNKT cell activation leads to the maturation of DC, which upregulates MHC-I and II molecules, as well as the co-stimulatory molecules CD80, CD86, CD40.
- iNKT cells activate the highly cytotoxic NK cells.
- iNKT cells promote the adaptive immune response

iNKT cells display cytotoxic effects by the upregulation of FasL and the production of granzyme B and perforin.

Stirnemann et al have developed a recombinant CD1d-anti-HER2 fusion protein loaded with the αGC ligand (αGC/CD1d-anti-HER2), which has the properties to bind tumor cells expressing HER2 antigen and to activate iNKT cells by triggering the invariant TCR. They have shown potent anti-tumor effects in a B16 lung metastasis model and on subcutaneous tumors in mice. Importantly, recombinant αGC/CD1d-anti-HER2 fusion promote the sustained activation of iNKT cells after several injections, as compared to the anergic state

induced after one injection of free αGC. The re-activation of iNKT cells with αGC/CD1d-anti-HER2 and the specific targeting to the tumor permitted the design of a promising cancer immunotherapy (92).

Aims of the thesis

Chapter 1- The tumor targeting with an αGC/CD1d-anti-tumor scFv fusion redirects NKT-mediated cytotoxicity.

Based on the promising results obtained by Stirnemman et al, we developed αGC/CD1d fusion directed against the CEA antigen to extend tumor targeting to other types of tumors. We investigated the potency of the αGC/sCD1d-anti-CEA fusion;

- Its therapeutic anti-tumor effects in pre-clinical models.
- Its comparison with the irrelevant αGC/sCD1d-anti-HER2 fusion to further assess the advantages to target tumors via a specific scFv.
- The ability of both αGC/CD1d-anti-TAA fusions to activate human iNKT cells.
- The need to target iNKT cells to the tumor via the specific αGC/CD1d-anti-TAA fusion to promote the killing of human tumor cells.

Chapter 2- Combination of the αGC/sCD1d-scFv fusion with cancer vaccines.

iNKT cells are potent activators of adaptive immunity and evidence of an adjuvant property of iNKT cells in cancer vaccine have been demonstrated.

In this part of the study we investigated in a pre-clinical model:

- The ability of the αGC/sCD1d-anti-HER2 fusion to act as an adjuvant in the priming of OVA-specific CD8 T cells.

- The combination of the αGC/sCD1d-anti-HER2 fusion with CpG ODN as adjuvant for the cancer vaccine.
- The antitumor effects promoted with the combination of αGC/sCD1d-anti-HER2 fusion treatments with a cancer vaccine.
- The specific redirection of the innate and the adaptive responses to the tumor site within tumor-targeted αGC/sCD1d-anti-HER2 fusion treatments after OVA/CpG vaccination.

Chapter 3- Alternatives to recombinant CD1d fusion.

In order to extend the tumor targeting by the recombinant CD1d, we have designed two alternative CD1d fusion proteins.

- First, we fused the CD1d to the Fc domain of an IgG1 to obtain a dimeric protein with enhanced cytotoxic effects and better avidity for iNKT cells.
- Secondly, we took advantage of an scFv directed against the protein VEGFR-3 present in the tumor stroma to enlarge the iNKT targeting to several tumors.

RESULTS

CHAPTER 1

CD1d-antibody fusion proteins target iNKT cells to the tumor and trigger long-term therapeutic responses.

In a pre-clinical model, we assessed the need to activate iNKT cells at the tumor site to obtain the better anti-tumor response by comparing treatments with the tumor-targeted αGC/sCD1d-anti-CEA or with the irrelevant αGC/sCD1d-anti-HER2 fusion proteins. The tumor specificity of the αGC/sCD1d-scFv was required to obtain an optimal reactivity of iNKT cells, regarding the cytokine release and more importantly the tumor inhibition. In human settings, the αGC/sCD1d-scFv fusion was able to expand iNKT cells from healthy donor PBMCs and activate iNKT cell clones without the presence of APCs, in contrast to free αGC. Moreover, *in vitro* experiments also gave evidences that the human tumor cell killing was dependent of the tumor targeting of iNKT cells via the anti-TAA αGC/sCD1d-scFv.

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ORIGINAL ARTICLE

CD1d-antibody fusion proteins target iNKT cells to the tumor and trigger long-term therapeutic responses

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Abstract Despite the well-established antitumor activity of CD1d-restricted invariant natural killer T lymphocytes (iNKT), their use for cancer therapy has remained challenging. This appears to be due to their strong but short-lived activation followed by long-term anergy after a single administration of the CD1d agonist ligand alpha-galactosylceramide (αGC). As a promising alternative, we obtained sustained mouse iNKT cell responses associated with prolonged antitumor effects through repeated administrations of tumor-targeted recombinant sCD1d-antitumor scFv fusion proteins loaded with αGC. Here, we demonstrate that CD1d fusion proteins bound to tumor cells via the antibody fragment specific for a tumor-associated antigen, efficiently activate human iNKT cell lines leading to potent tumor cell lysis. The importance of CD1d tumor targeting was confirmed in tumor-bearing mice in which only the specific tumor-targeted CD1d fusion protein resulted in tumor inhibition of well-established aggressive tumor grafts. The therapeutic efficacy correlated with the repeated activation of iNKT and natural killer cells marked by their release of TH1 cytokines, despite the up-regulation of the co-inhibitory receptor PD-1. Our results demonstrate the superiority of providing the superagonist αGC loaded on recombinant CD1d proteins and support the use of αGC/sCD1d-antitumor fusion proteins to secure a sustained human and mouse iNKT cell activation, while targeting their cytotoxic activity and cytokine release to the tumor site.

Keywords Cancer immunotherapy · iNKT cells · CD1d · Tumor targeting · Fusion protein

Introduction

Human Vα24-invariant natural killer T lymphocytes (iNKT), and their murine counterparts Vα14-iNKT cells, represent a particular sublineage of T lymphocytes activated by self- and microbial-derived glycolipids in the context of the monomorphic MHC-related molecule CD1d. Their importance in the transactivation of innate and adaptive immune responses has been extensively described [1, 2], as well as their protective or pathological role in various conditions [3]. In particular, their antitumor activity has been well documented in a number of mouse tumor models [4–6], and several clinical observations also indicate their protective role against cancer progression. Furthermore, low numbers and impaired proliferative capacity of iNKT cells were reported in cancer patients compared to normal donors [7, 8], which in some studies were correlated with poor clinical outcome [9, 10]. These preclinical and clinical observations have prompted

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testing of iNKT cell-directed therapies, mainly through their strong activation by the synthetic glycosphingolipid CD1d ligand, alpha-galactosylceramide (αGC) [11–13]. Phase I clinical trials involving the autologous transfer of αGC-pulsed monocyte-derived dendritic cells (moDC) were conducted in patients with different types of cancer [4, 11, 13–16]. No severe adverse effects were seen and the transient expansion and activation of iNKT cells, obtained in non-small-cell lung cancer (NSCLC) and head and neck squamous cell cancer (HNSCC) patients, correlated with some clinical benefit. Unfortunately, however, iNKT cell-mediated tumor immunotherapy has been limited by the short-lived cytokine response of iNKT cells to αGC stimulation, followed by a long-term anergy [4, 17, 18]. Recently, we have instead showed that sustained mouse iNKT cell responses could be induced by repeated stimulations with recombinant αGC-loaded sCD1d fusion proteins [19]. This prolonged responsiveness of iNKT cells resulted in potent antitumor activity when CD1d was targeted to the tumor site by its fusion to an anti-HER2 antitumor antibody fragment [19]. In the present study, recombinant CD1d proteins are shown to expand and activate human iNKT cells without the need of antigen-presenting cells (APCs). Importantly, we show that human iNKT cells exhibit a potent direct cytotoxicity only against cancer cells coated with the specific sCD1d-antitumor scFv fusion protein. The importance of CD1d tumor targeting to promote sustained activation of iNKT cells and prolonged tumor inhibition is further characterized in mice in therapeutic settings.

Materials and methods

Mice and human samples

Female mice C57BL/6J (B6) 6–8 weeks old (Harlan, Zeist, Holland) were maintained in specific pathogen-free conditions. All animal experiments were conducted according to institutional guidelines and under an authorization delivered by the Swiss veterinary department. Fresh human PBMC were obtained from healthy donor blood, isolated by density centrifugation using Lymphoprep (Axis-Shield PoC AS, Norway).

Tumor cell lines and human iNKT cell lines and clones

The murine colon carcinoma MC-38 cell line transfected with human CEA (MC38-CEA) was a kind gift from J. Primus [20]. The human cell lines KATO III (gastric carcinoma) and SK-BR-3 (breast carcinoma) were obtained from the ATCC. The human B lymphoma cell line C1R stably transfected with human CD1d was used as APC. Alternatively, moDCs were generated as described by Shao et al. [21]. Human iNKT cell lines were established starting with fresh PBMC from healthy donors cultured with αGC (100 ng/ml) or αGC/sCD1d proteins (40 μg/ml) in RPMI medium with 8 % human serum, recombinant IL-2 (20 U/ml) and IL-7 (10 ng/ml). Human iNKT cell clones had been previously generated by limiting dilution after sorting from peripheral blood lymphocytes (PBLs) of healthy donors by anti-CD3, anti-Vα24, and anti-Vβ11 mAbs staining [22].

Reagents and antibodies

The αGC analog KRN7000 (Alexis Biochemicals Corp) was dissolved in PBS-0.5 % Tween-20. Cytokine levels were measured either individually by ELISA (ELISA ready-set-go, eBiosciences), or as multiple cytokine measurements using BD Cytometric Bead Array kit TH1/TH2/TH17 (CBA, BD Biosciences). All fluorochrome-labeled antibodies were purchased from Becton–Dickinson (BD Biosciences) or eBiosciences. The humanized mAbs anti-HER2 Herceptin (Trastuzumab) was from Roche Ltd and anti-CEA (X4) from Ciba-Geigy [23]. Cells were analyzed with a FACSCalibur, FACSCanto or LSRII (BD Biosciences) and the acquired data were processed using FlowJo software (Tree Star Inc.).

Recombinant CD1d fusion proteins

Genetic fusion of mouse β2 microglobulin (β2 m) with the soluble part of mouse CD1d (sCD1d) has been described previously [19]. In the original pEAK8-β2m-sCD1d-anti-HER2-6xHIS construct, the anti-HER2 scFv located between the Gly-Ser spacer and the 6xHIS was replaced by the anti-CEA scFv MFE23 (kindly provided by R.H. Begent [24]). Recombinant CD1d fusion proteins produced by transient transfection of the human cell line HEK293-EBNA (Cellular Biotechnology Laboratory, EPFL, Switzerland) were purified and loaded with αGC as previously reported [19] (Fig. S1). The CD1d tetramer was developed by engineering a BirA consensus sequence at the C-terminus of the soluble mouse CD1d protein. The CD1d monomer was biotinylated by the BirA enzyme (Avidity, Denver, CO), and after loading with αGC, it was tetramerized on streptavidin-PE (Invitrogen) using a molar ratio of 4:1.

In vitro proliferation

Human iNKT cells were labeled with 1 μM CFSE for 6 min at 37 °C and washed three times. Labeled iNKT cells were incubated in a 12-well plate at 1×10^6 cells/ml in RPMI with 8 % HS, 30 U/ml IL-2, and 10 ng/ml IL-7 at 37 °C. iNKT cells were stimulated with either 8×10^5 irradiated αGC-loaded C1R-CD1d, plastic-coated sCD1d fusion proteins (40 μg), or 200 ng/ml αGC. The dilution of CFSE was analyzed by flow cytometry.

iNKT cell cytotoxicity

For chromium release experiments, iNKT clones, $CD4^+$ or double negative (DN), were thawed the day before and kept overnight in RPMI 8 %HS supplemented with recombinant IL-2 (150 U/ml) and IL-7 (10 ng/ml). On the day of the experiment, target cells were labeled with ^{51}Cr for 1 h at 37 °C and washed three times in medium before incubation (10^3 cells per well) in 96 V-bottom well plates either with effector cells at an E:T ratio of 10:1 and different concentration of CD1d-recombinant molecules, or in the presence of different E:T ratios and a fixed concentration of CD1d-recombinant molecules (10 μg/ml). Supernatants were collected after 4 h of incubation at 37 °C, and released radioactivity was measured in a γ-counter. For Annexin V analysis, 2×10^5 NKT cells were incubated with 1×10^4 tumor cells in the presence of either αGC/sCD1d-antitumor scFv at 10 μg/ml in RPMI 8 % human serum or 5×10^4 αGC/C1R-CD1d cells or 5×10^4 αGC/moDCs for 4 h at 37 °C. After incubation, tumor cells and APCs were analyzed using Annexin V kit (BD Pharmingen) and CD1d-tetramer-positive iNKT cells were stained for CD107a and intracellular content of IFNγ and TNFα.

Antitumor therapy

Mice were grafted s.c. in the right flank with 7×10^5 MC38-CEA cells. When all tumors were palpable, mice were treated i.v. with 200 μl of either PBS alone, equimolar amounts of αGC (0.4 μg), αGC/sCD1d (25 μg), or αGC/CD1d-antitumor scFv fusion proteins (40 μg). Systemic treatment was repeated at 3- to 4-day intervals. Mean tumor volume measured every 2 days was calculated using the following formula: (length × width × thickness)/2.

Statistical analysis

Results are expressed as mean ± SEM. Statistical significance between the groups was determined with student's *t* test or one-way -ANOVA test with Bonferroni correction (GraphPad Prism, GraphPad software). Tumor progression statistics were calculated with two-way ANOVA test with Bonferroni correction (GraphPad Prism, GraphPad software).

Results

Human iNKT cells efficiently proliferate in the presence of αGC-loaded CD1d protein

To validate the usefulness of soluble recombinant CD1d proteins for clinical immunotherapy, we investigated the reactivity of human iNKT cells to mouse αGC/sCD1d or αGC/sCD1d-antitumor scFv proteins. Irrespectively of whether fused or not to an antitumor scFv fragment, all sCD1d fusion proteins in solution were able to expand iNKT cell lines from freshly isolated human PBMC. The kinetics of expansion was similar to that observed following exposure to free αGC (Fig. 1a), with approximately 40 % iNKT cells on day 7 and 60 % on day 14 of culture. All iNKT cell lines, whether expanded with free αGC or αGC-loaded sCD1d fusion proteins, retained the same subset composition, with a majority of DN and a minority of $CD8^+$ iNKT cells (Fig. 1b). Importantly, recombinant αGC/sCD1d proteins could directly expand pure iNKT cell populations, as seen by CFSE dilution (Fig. 1c) and increased numbers of iNKT cells over 5 days of culture (data not shown), whereas the addition of irradiated APCs was required for free αGC to induce iNKT cell proliferation. These data indicate that αGC-loaded recombinant CD1d proteins directly trigger the semi-invariant TCR of human iNKT cells, and thus represent a promising tool for rapid and potent expansion of human iNKT cells from patients for subsequent adoptive cell transfer.

Soluble CD1d proteins directly activate human iNKT cell clones without requirement for APCs

As suggested by the expansion of human iNKT cells, αGC-loaded sCD1d proteins did not require the presence of APCs and were sufficient to activate human iNKT cell clones to release IFNγ after 18-h incubation (Fig. 2a). In contrast, αGC as a free drug was unable to activate iNKT cell clones in the absence of APCs (Fig. 2a) and required the presence of CD1d-expressing cells such as the human lymphoma C1R transfected with CD1d (Fig. 2b). These data fully established that the activation of human iNKT cells by soluble CD1d proteins did not result from the transfer of αGC to endogenously expressed CD1d, but rather from the direct TCR triggering by the soluble fusion proteins. As shown for iNKT cell proliferation, plastic-coated sCD1d proteins were even more efficient than soluble proteins in inducing iNKT cell clones to release a panel of cytokines such as IFNγ, TNFα, IL-2, and IL-4 (Fig. 2b). Still, when compared to αGC loaded on C1R-CD1d APCs, sCD1d proteins remained about threefold weaker in activating iNKT cells, likely resulting from the lack of adhesion mechanisms and molecular aggregation provided by cell–cell interaction.

Human iNKT cells efficiently kill tumor cells only when coated with the sCD1d-antitumor fusion protein

In view of the rare expression of CD1d on tumor cells, the direct cytotoxicity of iNKT cells against tumors has been disregarded, and instead, the immediate antitumor activity

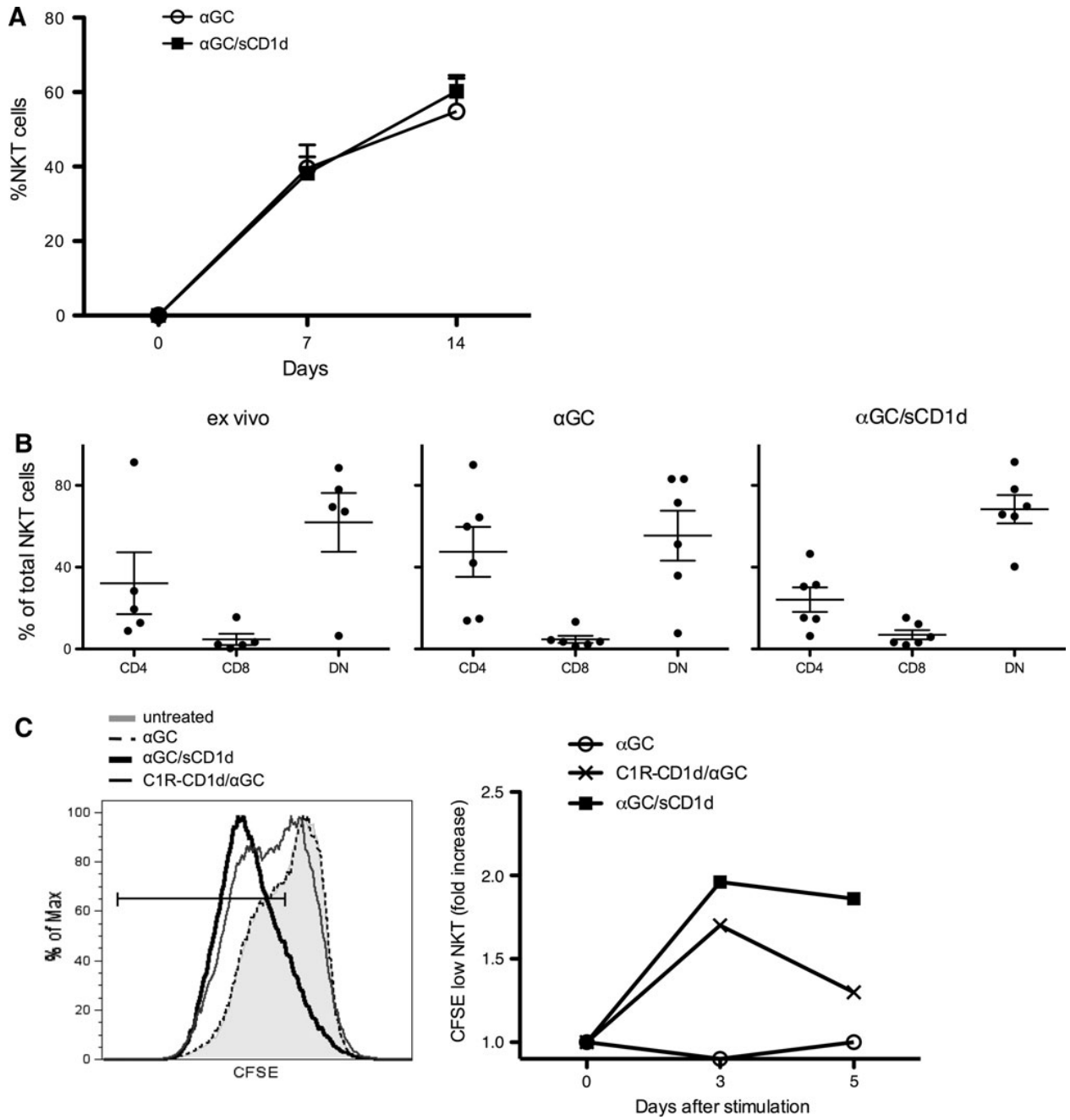

Fig. 1 Expansion of human iNKT cell lines by αGC/sCD1d proteins. **a** PBMCs from healthy donors were stimulated with medium alone, αGC (100 ng/ml) or αGC/sCD1d protein (10 μg/ml). Frequency of iNKT cells in total PBMC was assessed as CD1d-tetramer^{+}CD3^{+} at day 0, 7, and 14 of culture. Results are shown as mean ± SEM of four donors. **b** Distribution of human iNKT cell subsets ex vivo and after 14 days of culture as described in **a**. *Dots* represent percentages of CD4, CD8, and DN iNKT cells in total CD1d-tetramer^{+}CD3^{+} from normal individuals or expanded cell lines, and *bars* show mean ± SEM. **c** CFSE-labeled iNKT cells were incubated for 5 days with the different stimuli, and CFSE dilution was analyzed by FACS on gated CD1d-tetramer^{+}CD3^{+} cells. *Left panel* illustrates CFSE fluorescence in iNKT cells on day 5 of culture. *Right panel* shows the kinetic of CFSE dilution as the fold increase of CFSE^{low} iNKT cells. Results shown are representative of three independent experiments

of iNKT cells was shown to be largely mediated by the transactivation of natural killer cells [5, 19, 25]. However, direct cytotoxicity of human iNKT cells has been well demonstrated, especially against CD1d-expressing leukemia in vitro and in vivo [26, 27]. Here, we show that the killing capacity of human iNKT cells can be extended

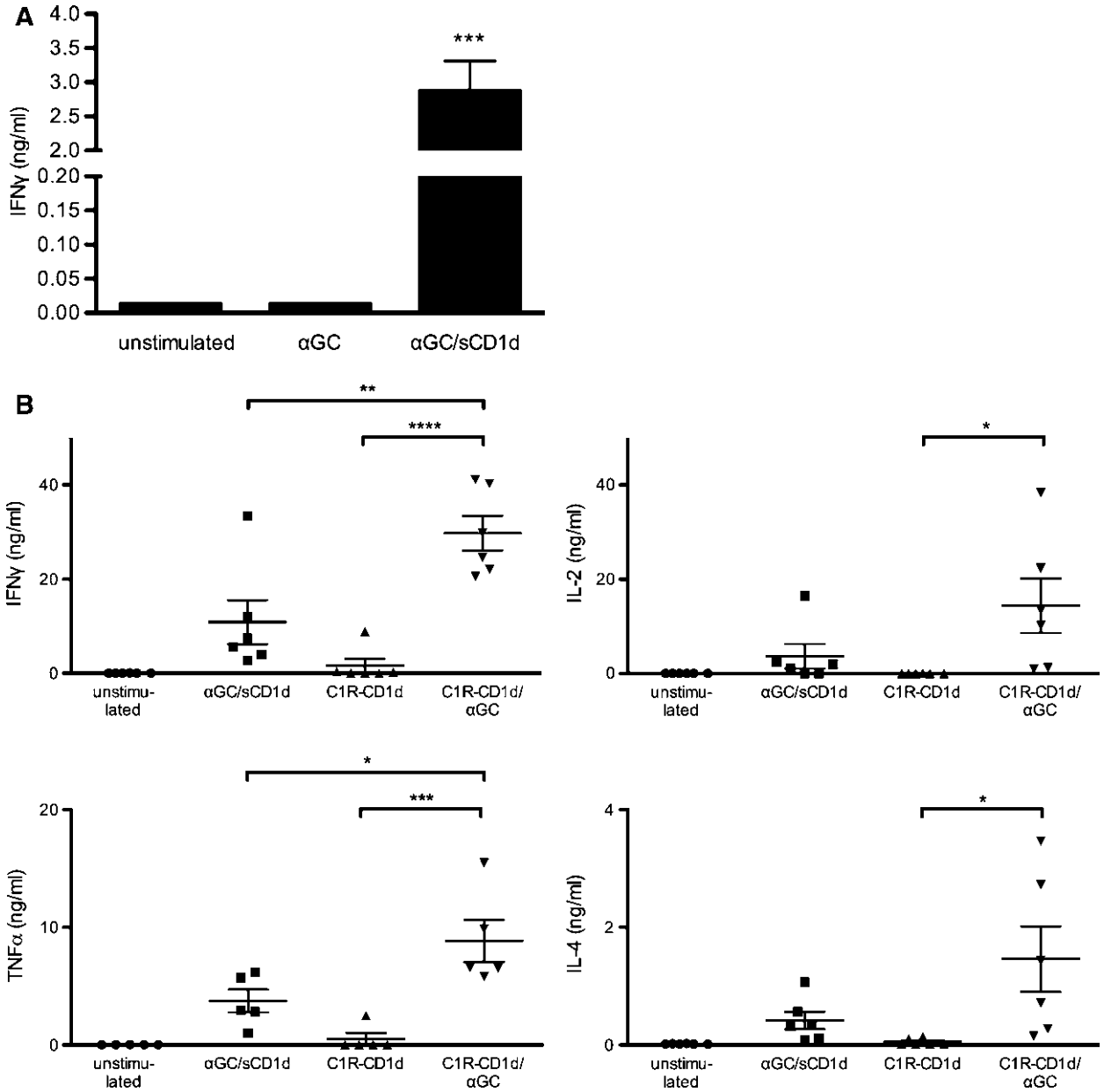

Fig. 2 Human iNKT cells are directly activated by recombinant αGC/sCD1d proteins. **a** iNKT cell clones (10^5) were incubated for 18 h with αGC (100 ng/ml) or αGC/sCD1d proteins in solution (10 μg/ml). *Graph* shows the level of IFNγ in the supernatant as the mean ± SEM of three different human iNKT cell clones. ***$P < 0.001$. **b** Activation of iNKT cells by αGC/sCD1d proteins or αGC-pulsed APCs. Human iNKT cell clones (10^5) were incubated with no stimulus, with plastic-coated αGC/sCD1d proteins (10 μg) or with the B-cell lymphoma cell line C1R-CD1d (5×10^4) pulsed or not with αGC. After 24 h, cytokines were measured in supernatants by CBA. *$P < 0.01$**$P < 0.005$, ***$P < 0.001$, ****$P < 0.0001$

against CD1d-negative tumor cells by their coating with αGC/CD1d-antitumor scFv fusion proteins. Two human tumor cell lines were selected based on their expression of HER2 and/or CEA (Fig. 3a). The pancreatic tumor cell line KATO III expresses both HER2 and CEA, as shown by the binding of the specific antibodies, as well as of the corresponding sCD1d-anti-HER2 and the newly developed sCD1d-anti-CEA fusion proteins (Fig. S1). In contrast, the breast cancer cell line SKBR-3 highly expresses HER2 but is negative for CEA, which provided the possibility of evaluating targeted versus untargeted iNKT cell-mediated cytotoxicity. After 4-h incubation with iNKT cells, tumor cells were killed only when coated with the relevant sCD1d-scFv fusion proteins. Indeed, KATO III tumor cells

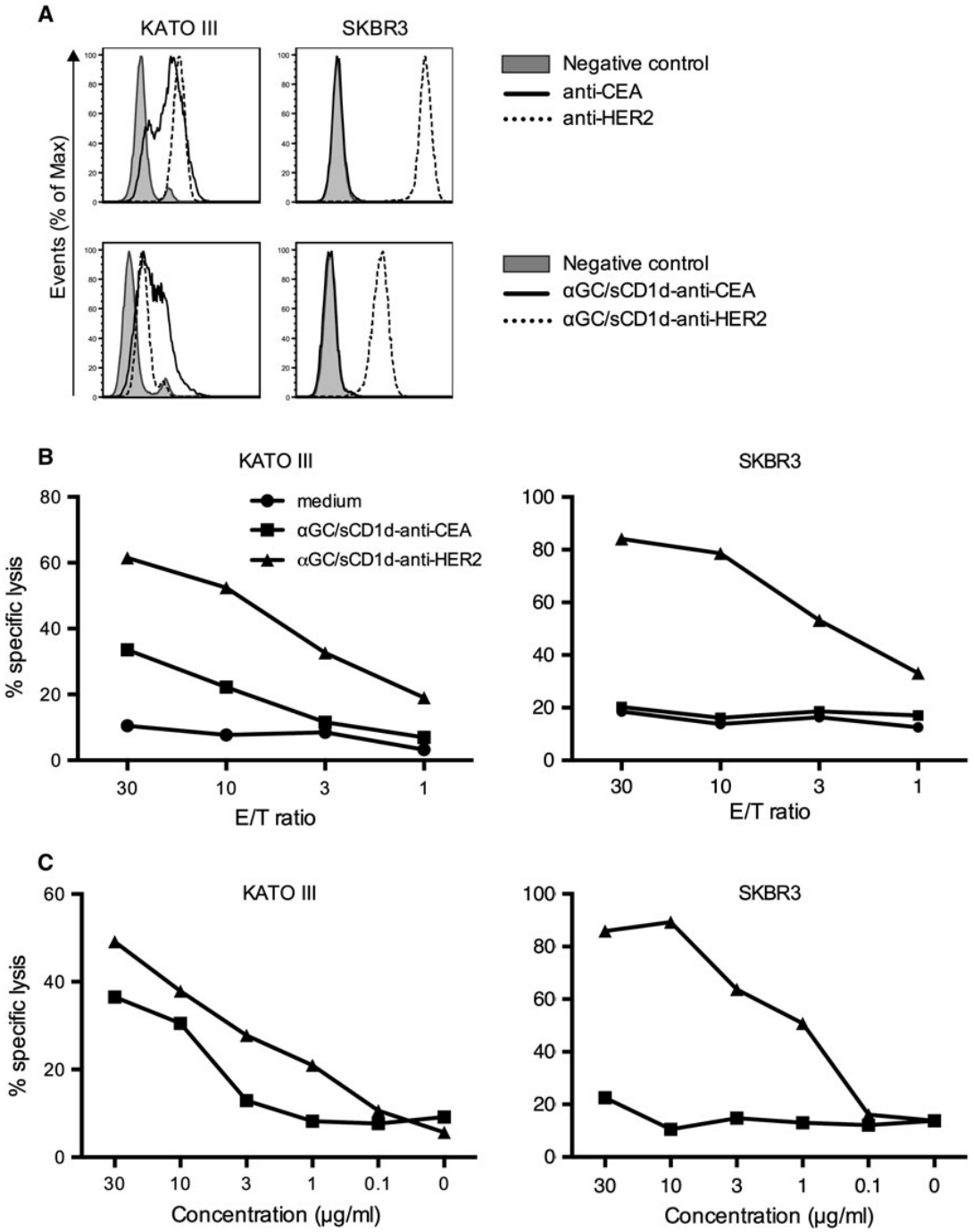

Fig. 3 Human iNKT cells efficiently kill tumor cells only when coated with the αGC/sCD1d-antitumor fusion protein. **a** Expression of HER2 and CEA by KATO III and SKBR3 human tumor cells revealed either by the anti-HER2 (Herceptin) and anti-CEA (X4) mAbs (*upper panels*), or by the binding of sCD1d-anti-CEA and sCD1d–anti-HER2 fusion proteins, revealed by FITC-labeled anti-CD1d (*lower panels*). **b** ^{51}Cr release assay after 4-h incubation of iNKT cell clones with the tumor cell lines described in **a**. *Graph curves* show percent killing of KATO III (*left panel*) and SKBR-3 cells (*right panel*) with decreasing effector-to-target ratio (*E/T*) with 2×10^3 target tumor cells and 10 μg/ml of sCD1d fusion proteins. **c** *Panels* show percent killing of KATO III (*left*) and SKBR-3 cells (*right*) with decreasing concentrations of sCD1d fusion proteins at an E/T ratio of 10/1. Results shown are representative of three independent experiments

co-expressing HER2 and CEA were killed in the presence of either of the two αGC/sCD1d-antitumor proteins (Fig. 3b, left panel), with 40 and 60 % of tumor cells killed at an E/T ratio of 30/1 and 10 μg/ml of αGC/sCD1d-anti-HER2 and αGC/sCD1d-anti-CEA fusion proteins, respectively. In contrast, the SKBR-3 tumor cells expressing only HER2 were exclusively killed when incubated with the αGC/sCD1d-anti-HER2 protein (Fig. 3b, right panel), with 80 % of cells eliminated, while co-incubation with the αGC/sCD1d-anti-CEA protein resulted in only background ^{51}Cr release similar to medium alone. At an E/T ratio of 10/1 (Fig. 3c), 20 % targeted killing of KATO III (left panel) and 50 % killing of SKBR-3 (right panel) tumor cells was still obtained with 1 μg/ml (13 nM) of HER2 and/or CEA-targeted CD1d fusion proteins, demonstrating the sensitivity of this approach. All iNKT cell clones tested in cytotoxic assays were $CD4^+$ or DN and showed similar capacity of tumor-targeted cell killing.

Activated iNKT cells exhibit poor bystander cytotoxicity and selectively kill CD1d-positive target cells

In addition to ^{51}Cr release experiments, the killing of SKBR3 tumor cells was evidenced by their Annexin V^+ 7-AAD^- profile, while the state of iNKT cell activation was evaluated by CD107a expression, and secretion of TNFα and IFNγ (Fig. 4). After 4-h incubation with human iNKT cells, an average of 60 % of SKBR3 tumor cells was apoptotic when incubated with the tumor-targeted αGC/sCD1d-anti-HER2 fusion protein, while there was a similar background of tumor cell death with the untargeted αGC/sCD1d-anti-CEA or with unstimulated iNKT cells (Fig. 4a). Interestingly, when instead of recombinant CD1d proteins, iNKT cells and SKBR-3 tumor cells were co-incubated with αGC-pulsed C1R-CD1d cells or moDCs, less than 20 % of SKBR3 cells became Annexin V positive (Fig. 4a), although the percentages of CD107a, IFNγ and TNFα-positive iNKT cells (Fig. 4b, c) were similar after incubation with the tumor-targeted αGC/sCD1d-anti-HER2 protein or with αGC/C1R-CD1d cells. The iNKT cell activation was slightly weaker in the presence of αGC/moDCs (Fig. 4b, c), likely resulting from their hundred times lower CD1d expression than the C1R-CD1d transfectant. In addition, the state of activation of iNKT cells incubated with the αGC/sCD1d-anti-CEA did not differ from unstimulated iNKT cells (Fig. S2). In conclusion, the similar percentages of activated iNKT cells stimulated by αGC-pulsed APCs or by tumor-targeted αGC/sCD1d-anti-HER2 protein did not correlate with a similar killing of SKBR3 tumor cells, but rather with the concomitant elimination of the αGC/APCs, as shown by, respectively, 26 and 36 % of Annexin V^+ αGC/C1R-CD1d and αGC/moDCs (Fig. 4a). Altogether, these data further confirmed the requirement of CD1d on the surface of the target cell for efficient killing, whether naturally expressed on the surface or bound via its fusion to an antitumor scFv fragment. The activation of iNKT cells by tumor-targeted CD1d molecules was also evidenced by their cytokine content (Fig. 4c). Indeed, in the presence of SKBR3 tumor cells coated with αGC/sCD1d-anti-HER2, about half of iNKT cells was positive for TNFα and IFNγ, while no intracellular cytokines were detected in the presence of the irrelevant αGC/sCD1d-anti-CEA fusion protein. Altogether, these results support the relevance of sCD1d-antitumor fusion proteins for cancer therapy, as seen by the strong activation of human iNKT cells, revealed by direct tumor cytotoxicity and cytokine release.

In vivo targeting of αGC-loaded CD1d proteins to the tumor site is required for prolonged iNKT cell-mediated tumor inhibition

The importance of targeting αGC/sCD1d fusion proteins to the tumor was further investigated in C57BL/6 mice grafted with the MC38 colon carcinoma cell line stably transfected with human CEA (MC38-CEA). Mice with established tumors (>100 mm^3) were treated either with αGC/sCD1d-anti-CEA fusion protein (Fig. S1), αGC alone, untargeted αGC/sCD1d or αGC/sCD1d-anti-HER2 fusion proteins. After a total of six injections given over 3 weeks, all mice treated with αGC/sCD1d-anti-CEA protein retained small tumors barely exceeding 200 mm^3, and hence 60 % smaller than in untreated animals (700 mm^3) (Fig. 5a). In marked contrast, αGC alone and untargeted sCD1d were unable to inhibit tumor growth. The requirement of tumor-targeted CD1d treatment to achieve a therapeutic effect was best demonstrated in the mice treated with the irrelevant αGC/sCD1d-anti-HER2 fusion protein, as all animals had fast tumor growth. The prolonged reactivity of iNKT cells was tested in the spleen by the detection of ex vivo IFNγ production 1 h after the sixth injection. After repeated injections of αGC, only few iNKT cells still produced IFNγ, confirming the induction of anergy upon repetitive stimulations (Fig. 5b) [18, 19]. Similarly, no significant IFNγ was detected after untargeted αGC/sCD1d and irrelevant αGC/sCD1d-anti-HER2 treatments, though in this case likely resulting from a weak activation of iNKT cells rather than anergy. In contrast, 15 % of spleen iNKT cells isolated from mice treated with tumor-targeted αGC/sCD1d-anti-CEA were positive for IFNγ, which correlated with the fact that antitumor activity was exclusively obtained in this group. Although tumor-infiltrating iNKT cells were too few to be functionally analyzed, the prolonged reactivity of iNKT cells only in the spleens of mice treated with tumor-targeted fusion protein

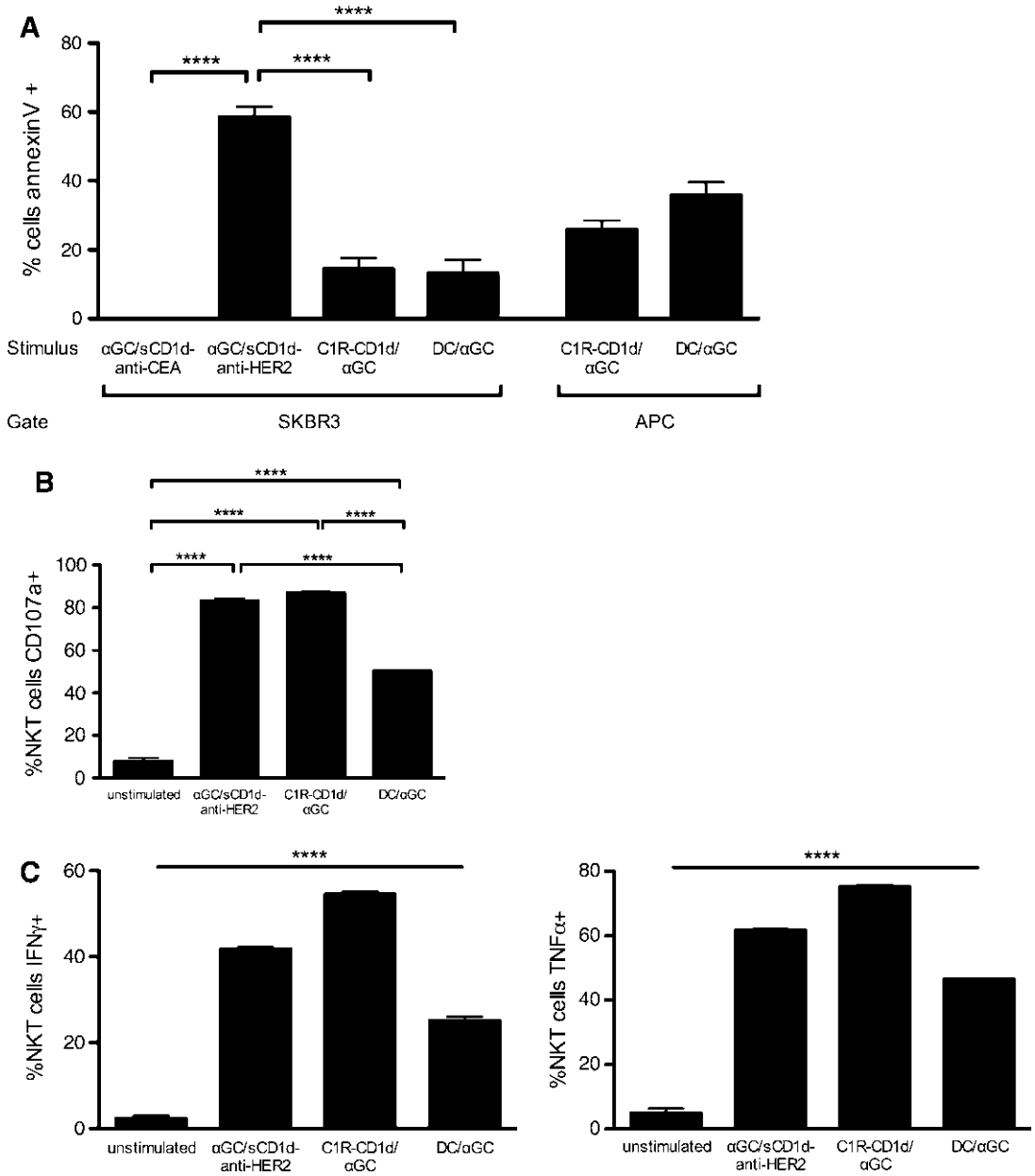

Fig. 4 Activated iNKT cells kill αGC/sCD1d-loaded tumor cells as well as CD1d-positive APCs. **a** Apoptosis of SKBR3 tumor cells or APCs after 4-h incubation with iNKT cell clones, in the presence of αGC/sCD1d-CEA, αGC/sCD1d-HER2, αGC-pulsed C1R-CD1d cells, or αGC-pulsed moDCs. Results are shown as percentages of Annexin V^+ $7AAD^-$ cells gated on $HER2^+$ SKBR-3 cells, $CD20^+$ C1R-CD1d, or $CD11c^+$ moDCs, after subtracting the respective backgrounds obtained with unstimulated iNKT cells. $^{**}P < 0.005$. **b** Cytotoxic activity of iNKT cells in the same experiment described in **a** was determined by assessing CD107a expression on the $CD3^+CD1d$-tetramer$^+$ effector cells. $^{***}P < 0.001$. **c** The activation state of iNKT cells was also evaluated by ICS for IFNγ and TNFα. The unstimulated group consists of the pooled data of iNKT cells incubated with unpulsed C1R-CD1d cells or moDCs. $^{****}P < 0.0001$ between all groups

suggested that these cells had been repeatedly activated at the tumor site. In view of previous studies that attributed αGC-induced iNKT cell anergy to the up-regulation of the co-inhibitory receptor programmed death-1 (PD-1) [28, 29], we tested its expression level on spleen iNKT cells (Fig. 5c). Strikingly, PD-1 expression was up-regulated not only on the vast majority of iNKT cells after six injections of αGC alone, but also after repeated injections of recombinant αGC/sCD1d proteins, including iNKT cells activated by the tumor-targeted αGC/sCD1d-anti-CEA

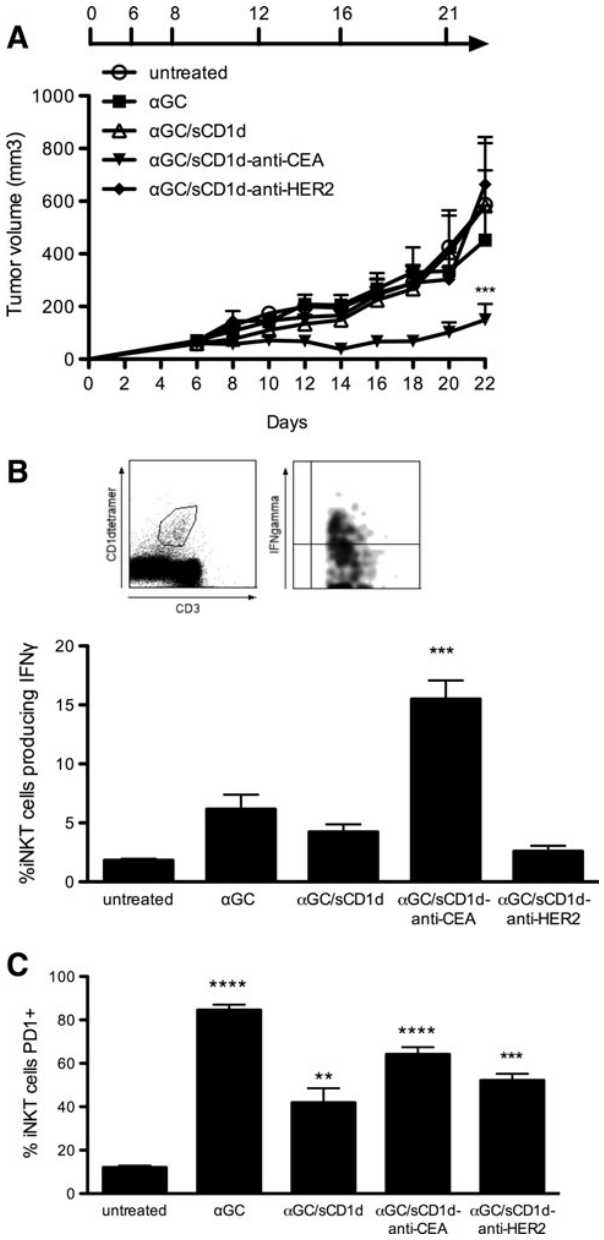

Fig. 5 In vivo antitumor activity of αGC/sCD1d-anti-CEA fusion protein. **a** Mice were grafted s.c. with 7×10^5 MC38-CEA tumor cells. I.v. injections of PBS (untreated), or equimolar amounts of αGC, αGC-loaded sCD1d, αGC/sCD1d-anti-HER2, and αGC/sCD1d-anti-CEA were started 6 days later when all tumors were well established and were repeated for a total of 5 injections as specified. Tumors were measured every 2 days and the graph represents the kinetic of tumor growth (mm^3) as the mean of 7 mice per group. $***P < 0.001$ versus untreated group. **b** Ex vivo IFNγ production by spleen iNKT cells at the end of the antitumor experiment (day 22). Splenocytes were isolated 1 h after the sixth injection of each treatment and iNKT cells were stained with CD1d-tetramer-PE and anti-CD3-FITC, and intracellularly with anti-IFNγ-APC. Results are expressed as the mean percentage of IFNγ-producing iNKT of three mice per group. $***P < 0.001$ versus all groups. **c** The same samples as in **b** were analyzed for the expression of the co-inhibitory receptor PD-1 on spleen iNKT cells. The *graph* represents the percentage of PD-1 positive iNKT cells from three mice per group. $**P < 0.005$, $***P < 0.001$, $****P < 0.0001$ versus untreated group

protein (Fig. 5b). Therefore, increased PD-1 expression did not correlate with the state of unresponsiveness of iNKT cells. At this point, it is interesting to mention that the majority of human iNKT cells were found positive for PD-1 expression when analyzed ex vivo in normal donor PBMCs (Fig. S3), likely resulting from previous in vivo stimulations. These observations suggest that PD-1 up-regulation on mouse and human iNKT cells is rather a marker of activation and is not in itself sufficient to mediate iNKT cell anergy.

The prolonged reactivity of iNKT and NK cells to repeated stimulations is optimized by tumor bound CD1d proteins

The requirement for CD1d tumor targeting to provide sustained reactivity of iNKT despite PD-1 up-regulation was further characterized. As already reported [18, 19], a single injection of αGC induced a fast and potent activation of iNKT cells, as revealed by the presence of 40 % splenic iNKT cells producing IFNγ (Fig. 6a). However, after three injections of αGC and despite similar numbers of iNKT cells (data not shown), only 12 % of iNKT cells still produced IFNγ (Fig. 6a), confirming the induction of anergy upon repetitive stimulations [18, 19]. In contrast, 25 % of iNKT cells from MC-38-CEA tumor-bearing mice treated with αGC/sCD1d-anti-CEA fusion protein still produced IFNγ (Fig. 6a), while only 9 % iNKT cells were $IFN\gamma^+$ in mice treated with the irrelevant αGC/sCD1d-anti-HER2 fusion protein. Importantly, after three treatments, percentages of IFNγ-producing cells were similar whether gated on total or on $PD\text{-}1^+$ iNKT cells (Fig. 6a), confirming that PD-1 up-regulation was not sufficient to block the restimulation of iNKT cells. In view of their fast transactivation by iNKT cells, NK cells were tested in the same groups of mice (Fig. 6b). After three injections of αGC, spleen NK cells also failed to produce IFNγ, as a consequence of iNKT cell anergy. In contrast, in mice treated with either αGC/sCD1d-anti-CEA or αGC/sCD1d-anti-HER2 fusion proteins, NK cells remained reactive, as seen by increased IFNγ production. The repeated activation of NK cells upon treatment with the irrelevant fusion protein αGC/sCD1d-anti-HER2 indicated that sustained systemic activation of iNKT cells had occurred, although to a weaker extent than with the tumor-targeted treatment. Serum cytokines measured 1 h after the third injection also reflected the prolonged reactivity of iNKT cells to tumor-targeted recombinant CD1d proteins (Fig. 6c), as shown by significant serum levels of IFNγ and IL-4 in mice treated

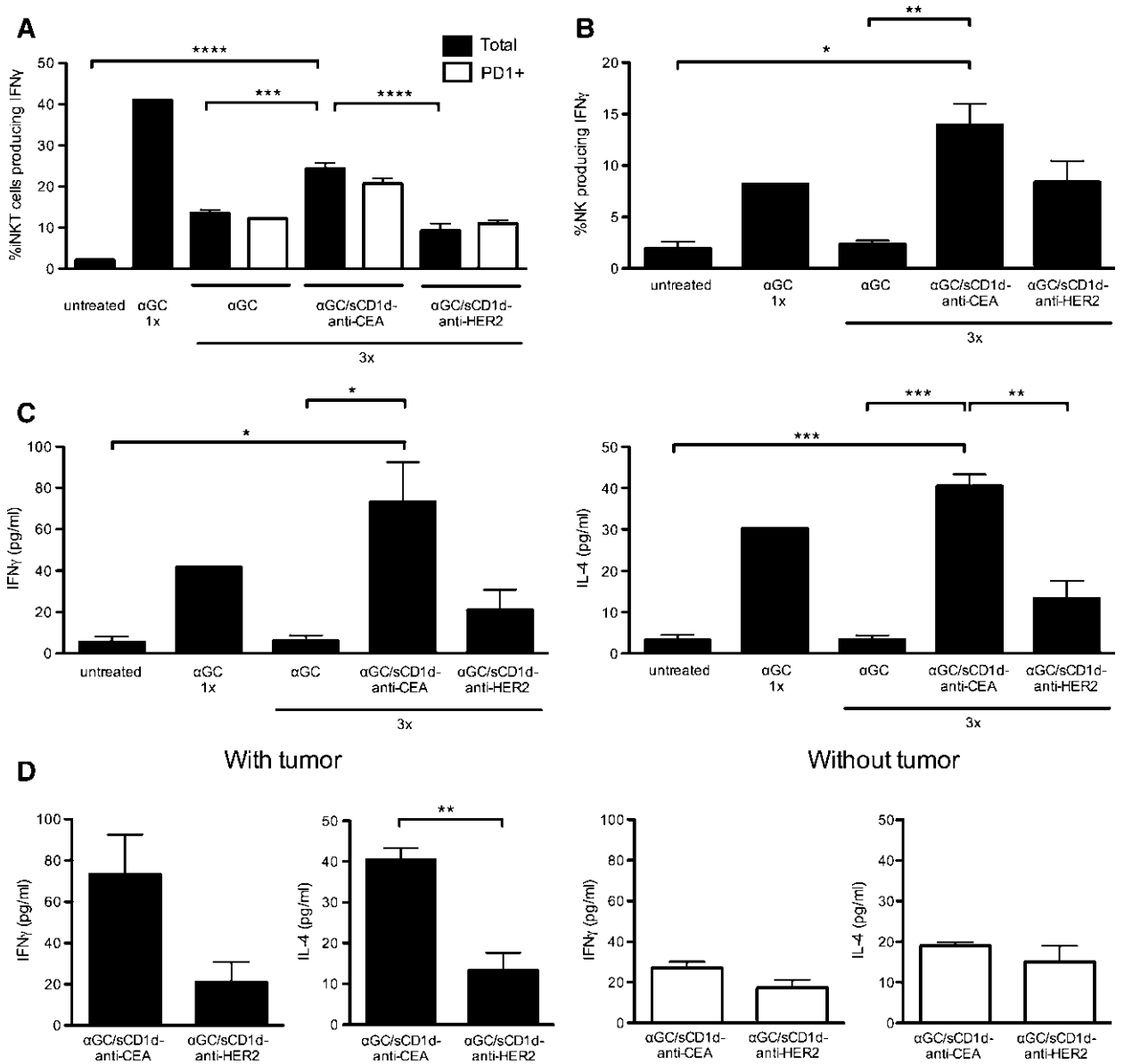

Fig. 6 Sustained activation of iNKT and NK cells upon repeated injections of tumor-targeted αGC/sCD1d-anti-CEA. Mice bearing MC38-CEA tumors were treated three times, following the same protocol as in the tumor therapy experiments (Fig. 5), and splenocytes were analyzed 1 h after the third injection. As a positive control for IFNγ production, a naïve mouse was injected only once with αGC. **a** Percentage of IFNγ-producing cells gated on total (*black bars*) or PD-1$^+$ (*empty bars*) iNKT cells. **b** Percentage of IFNγ-producing NK cells as gated on CD3$^-$NK1.1$^+$. **c** Cytokines were measured by CBA in the sera of mice taken 1 h after the third treatments, as described in **a**. *Graphs* show the concentration of IFNγ (*left panel*) and IL-4 (*right panel*). **d** Serum levels of IFNγ and IL-4 were compared between mice-bearing or not MC38-CEA tumors, and treated with targeted or non-targeted αGC/sCD1d fusion proteins administered as in **a**. All data are shown as the mean ± SEM of 3 mice per group, $*P < 0.01$, $**P < 0.005$, $***P < 0.001$, $****P < 0.0001$

with αGC/sCD1d-anti-CEA protein, while barely any cytokines could be measured after three injections of αGC/sCD1d-anti-HER2 protein. Regarding αGC as a free drug, a single injection induced a fast release of IFNγ and IL-4, but almost no cytokine production was detected after three injections, confirming the induction of iNKT cell anergy. Most importantly, in the absence of MC38-CEA tumor grafts, repeated treatments with αGC/sCD1d-anti-CEA fusion protein did not lead to significant release of cytokines, which did not differ from mice treated with the irrelevant αGC/sCD1d-anti-HER2 protein (Fig. 6d). Altogether, these results demonstrate the importance of targeting CD1d to the tumor site to favor a strong and prolonged reactivity of iNKT cells.

Discussion

The present study demonstrates the therapeutic efficacy of recombinant sCD1d-antitumor scFv fusion proteins via the sustained activation of murine and human cytolytic iNKT cells. The attractiveness of this strategy resides in two main beneficial characteristics. First, recombinant CD1d proteins have the capacity to keep iNKT cells reactive through multiple stimulations, in contrast to their unresponsiveness after repeated challenge by αGC-loaded APCs. Second, the targeting of CD1d molecules to cancer cells by their fusion to an antitumor scFv fragment efficiently redirects activated iNKT cells to the tumor site, promoting a local innate immune response, including direct lysis of targeted tumors by iNKT cells, release of large amounts of cytokines and transactivation of NK cells, altogether leading to prolonged antitumor effects.

So far, the therapeutic use of iNKT cells has been limited by their short-lived cytokine response to αGC stimulation, followed by a long-term anergy [4, 17, 18]. Several mechanisms have been proposed for the induction of iNKT cell unresponsiveness, and the controversial results suggest that multiple factors are likely involved. At first, the fast and long-term up-regulation of PD-1 upon activation of iNKT cells was proposed as the main mechanism since their anergic state could be prevented or reverted by PD-1/PD-L1 blockade and did not occur in PD-1 KO mice [[28], [29]]. However, the exclusive role of PD-1 in the control of iNKT cell anergy was not confirmed in all systems [30], and the involvement of co-stimulation through CD28 or co-inhibitory receptors such as BTLA was suggested [30–32]. In this respect, our present results also demonstrated the strong and long-term up-regulation of PD-1 upon activation of murine iNKT cells, which however did not correlate with the state of anergy. Indeed, iNKT cells remained reactive to multiple injections of recombinant CD1d fusion proteins despite similar up-regulation of PD-1 as was found on anergic iNKT cells after treatment with αGC as a free drug. Clinical trials in cancer patients have preferred the autologous transfer of αGC-pulsed DC, which showed prolonged iNKT cell activation, as compared to the glycolipid alone [4, 11, 13, 14, 33]. However, a maximum of two subsequent rounds of iNKT cell stimulations were seen despite three to four autologous transfers of αGC-pulsed DC in some studies, suggesting that iNKT cells became progressively unresponsive to further challenge. Unfortunately, in vitro experiments with human iNKT cells do not allow monitoring the induction of anergy by αGC, likely due to the presence of IL-2 required for the maintenance of human and mouse iNKT cells in vitro. Indeed, IL-2 has been shown to prevent or revert the state of anergy of murine iNKT cells both in vivo and in vitro [18, 34]. However, it is interesting to note that around 70 % of human iNKT cells were already expressing PD-1 when tested ex vivo on total PBMC from healthy donors, probably resulting from previous in vivo antigen stimulations. Since it is unlikely that the large majority of iNKT cells present in normal donors are anergic, the expression of PD-1 on activated human and mouse iNKT cells is probably not sufficient to mediate iNKT cell unresponsiveness. So far, the exact mechanism that mediates the αGC-induced anergy of iNKT cells or, conversely, their prolonged reactivity to recombinant αGC/CD1d fusion proteins remains unclear.

Importantly, our data demonstrated a direct activation of human iNKT cells by recombinant soluble CD1d proteins in the absence of any APC, which excluded the possibility of loss of αGC from the recombinant CD1d proteins and its loading onto endogenous CD1d expressed by APCs. In this regard, the capacity of a monomeric CD1d molecule to activate iNKT cells seems to be unique to this antigen-presenting molecule, as soluble MHC Class I and Class II monomers were shown to be unable to activate antigen-specific T cells, in contrast to dimeric and multimeric forms [35, 36]. The question remains whether the iNKT cell activation by monomeric αGC/sCD1d proteins is peculiar to the strong agonist αGC and whether it would not occur with a more physiological CD1d ligand. Nevertheless, activation of human iNKT cells by soluble αGC/CD1d proteins remained significantly less efficient than plastic-coated or tumor bound CD1d fusion proteins and αGC-pulsed APCs, which likely resulted from the lack of molecular CD1d aggregation in the absence of plastic or cell surface, as well as from the absence of co-stimulation. In support of this, systemic treatments of mice with untargeted αGC/sCD1d or irrelevant αGC/sCD1d-scFv fusion proteins led to a two to threefold weaker release of cytokines, as compared to treatment with the tumor-targeted αGC/sCD1d-scFv protein. Rather than a drawback, the lower potency of soluble monomeric CD1d molecules will be rather favorable in limiting a potentially detrimental systemic activation of iNKT cells, while promoting their sustained activation when targeted at the tumor site.

The second attractive characteristic of our immunotherapy strategy is the targeting of recombinant CD1d fusion proteins to tumors by fusion of the extracellular part of CD1d to an antibody scFv fragment specific for a tumor antigen. Indeed, the present data show both in vitro with human iNKT cell clones and in vivo in a therapeutic mouse tumor model that the strongest tumor inhibition is obtained with tumor-targeted recombinant sCD1d-antitumor scFv fusion protein as compared to an irrelevant sCD1d fusion protein. This finding fits with the prerequisite of CD1d expression by tumors to promote efficient iNKT cell-mediated killing, which has been previously reported in humans in the context of CD1d-expressing lymphomas

[26, 37, 38], and in mice with tumor models transfected with CD1d [39, 40]. In this context, our strategy using CD1d-antitumor scFv fusion proteins opens the possibility to target CD1d-negative tumors and render them susceptible to iNKT cell attack. The effectiveness of this approach was best demonstrated by the direct cytotoxicity of human iNKT cell clones against tumor cells when coated with the αGC/sCD1d-scFv fusion protein specific for the tumor antigen expressed on their surface, while the untargeted αGC/sCD1d-scFv was unable to induce antitumor cytotoxic activity. More importantly, although iNKT cells were strongly activated by αGC-pulsed APCs, such as C1R-CD1d or moDCs, as seen by CD107a expression and cytokine release, they rather killed the APCs and to a lesser extent the tumor cells, indicating that bystander killing by iNKT cells was inferior to the CD1d-mediated cytotoxicity. These observations suggest that the strategy of iNKT cell activation by autologous transfer of αGC-pulsed DCs, as tested so far in clinical trials, may direct the intrinsic cytotoxic activity of iNKT cells preferentially against the transferred DCs and not against tumors. Because of the relatively low numbers of peripheral iNKT cells in humans, this aspect was probably not a major issue, and these protocols of DC transfer have instead successfully induced the immunomodulatory functions of iNKT cells, such as the transactivation of NK and T cells. However, our results in mice demonstrated that the stronger iNKT cell activation by tumor-targeted sCD1d treatment also led to higher NK cell activation, which are known to greatly participate in tumor inhibition [19]. Therefore, the targeting of CD1d molecules to the tumor site not only triggers iNKT-mediated tumor lysis, but also favors local innate and adaptive antitumor responses, as suggested by the accumulation of iNKT, NK, and T cells at the tumor site [19]. The importance of tumor targeting and local activation of iNKT cells was underlined by recent clinical trials in which αGC-pulsed DC and/or ex vivo expanded iNKT cells were delivered in the vicinity of the tumor [15, 33]. The best clinical results were obtained in HNSCC patients in whom αGC-pulsed DCs were administered via the nasal submucosa and iNKT cells via the tumor-feeding arteries [15, 41]. In addition to the local delivery of iNKT cells, this encouraging clinical benefit also demonstrated the need to increase the numbers of iNKT cells by autologous transfer of in vitro expanded iNKT cell lines, since the frequency of iNKT cells is often very low or even undetectable in advanced cancer patients [8, 42]. To this aim, recombinant sCD1d-antitumor fusion proteins appear as promising tools: first, in vitro to expand large numbers of human iNKT cells for adoptive transfer, and second, in vivo to redirect these transferred iNKT cells to the tumor site.

Finally, the efficient tumor targeting of CD1d molecules requires the over-expression of a tumor antigen for which a high-affinity antibody scFv has been developed. So far, we have used two of the highest affinity existing scFv fragments specific for the HER2 and CEA, which are often over-expressed, respectively, in breast cancers and gastric cancers. As alternatives to tumor-associated markers, antigens over-expressed in the tumor stroma and/or neovessels would be additional good candidates for the targeting of CD1d molecules to the tumor site.

In conclusion, the present results propose and support monomeric CD1d-scFv antitumor fusion proteins as a potent tool to effectively harness iNKT cells against cancer.

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Conflict of interest All co-authors concur with the submission with no conflicting financial interest.

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CHAPTER 2

Advantages of NKT activation mediated by αGC/CD1d-anti-HER2 fusion to improve cancer vaccine and redirect the adaptive immune response to the tumor.

Introduction

The identification of TAA epitopes recognized by T cells led to the development of specific therapeutic vaccine (191). The aim of such strategy is to elicit a potent adaptive immune response that can mediate the killing of tumor. The attractive feature of this strategy is the specificity driven by the utilization of antigen restricted to the tumor, which limits undesirable side effects. Protein and peptide-based vaccine represents an efficient system to induce specific T cells. They can be easily processed by APCs and loaded on MHC-class-I or II to prime CD8 and CD4 T cells. However, peptides and recombinant proteins as such are poorly immunogenic and therefore need to be administered in combination with an adjuvant to activate the immune system machinery. Adjuvants play two roles in the vaccination strategy. First, they favor the duration of the antigen exposition to the immune system that in turn strengthens the priming of CD8 and CD4 T cells. Second, adjuvants can trigger the maturation of DC that ameliorates the migration to lymph nodes to initiate a robust T cell response. Today, a limited number of adjuvants is approved for human vaccine, including aluminum salts (Alum) and Incomplete Freund's adjuvant (IFA) that create a depot that prolongs the antigen presentation. However, another class of adjuvant is developed which acts as immunostimulants that ameliorate vaccine activities. So far, mostly Monophosphoryl lipid-

A (MPL), constituted by an active part of bacterial cell wall constituent, is used as adjuvant in human cancer vaccine and elicits effective immune responses (192). But there are many other immunostimulants including pathogen-associated molecular patterns (PAMPs) that are sensed by Toll-like receptors (TLRs), and several components belonging to the PAMP family are currently tested in clinical trials with vaccine (193). Toll-like receptors have an important role in the innate immune system and are expressed by macrophages, DC, and monocytes. They are able to recognize conserved pathogen features (figure 1), which are not found in the host, such as DNA motifs of CpG oligodeoxynucleotides (CpG ODN), double-strand DNA (dsDNA) or LPS (194).

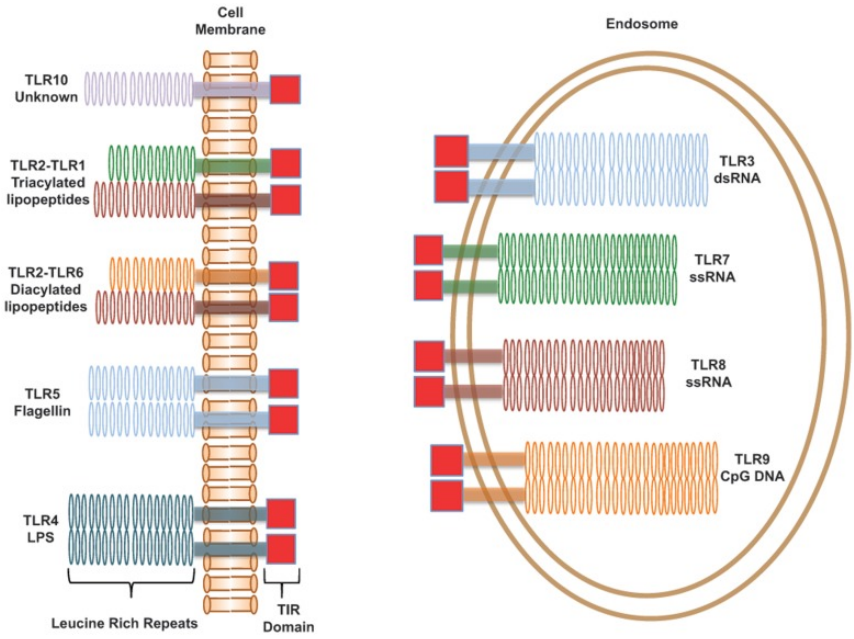


Figure 1: Schematic representation of Toll-like receptors. TLRs are divided in two classes, cell-surface receptors that recognize bacterial cell wall components, and endosomal receptors that recognize bacterial and viral nucleic acids (adapted from (195))

For example, the receptor TLR9 is expressed by B cells and DC and is localized in the endosomal-compartment of the cells where it interacts with pathogen-derived products gained after phagocytosis. TLR9 detects the unmethylated CpG motif specific of bacterial DNA and its activation results in the maturation of DC (196). The innate immune response following the differentiation and the activation of DCs is characterized by a strong production of Th1-

like cytokines, including IL-6, TNFα, IFNγ and IL-12, and the activation of NK cells and T cells (197). CpG ODN is a potent stimulus for pDC subset, which can produce high amounts of the pro-inflammatory cytokine IL-12 and type I IFN (198). Taken together, TLR9 activation boosts vaccine effects and supports immune responses against cancers (199).

An alternative strategy to the activation of DC by pathogens-derived molecules is the activation of iNKT cells, which are potent modulators of the adaptive response through the maturation of DCs. This approach renders these cells attractive as vaccine adjuvant. Different cancer vaccines using the iNKT ligand αGC showed potent effects to enhance CD8 T cell priming and therefore anti-tumor immunity (200) (150). Indeed, a potent cross talk between iNKT cells and DCs happens after aGC stimulation, and mediates the full maturation of DCs. This link requires principally the CD40L-CD40 interaction, which triggers IL-12 production that in turn enhances the NK and NKT cell responses (figure 2)(201) (202).

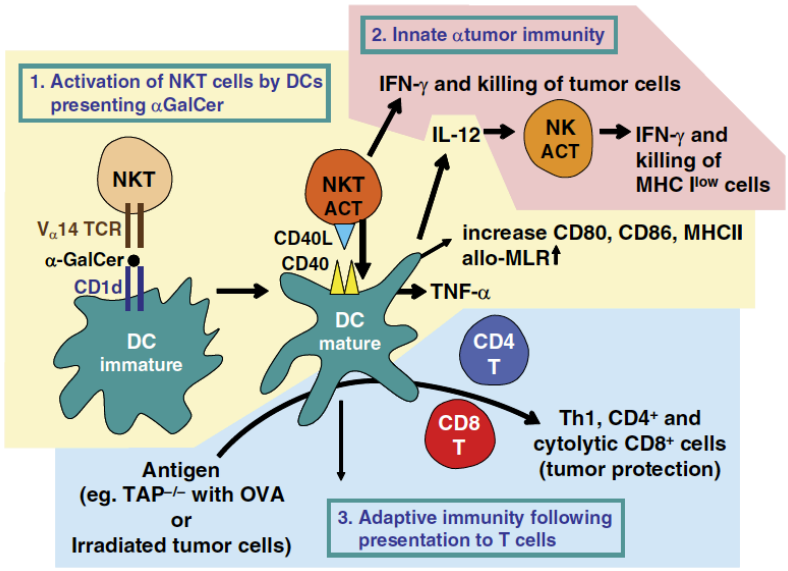


Figure 2: Activation of iNKT cells with αGC links the innate and the adaptive immunity via DC maturation (203).

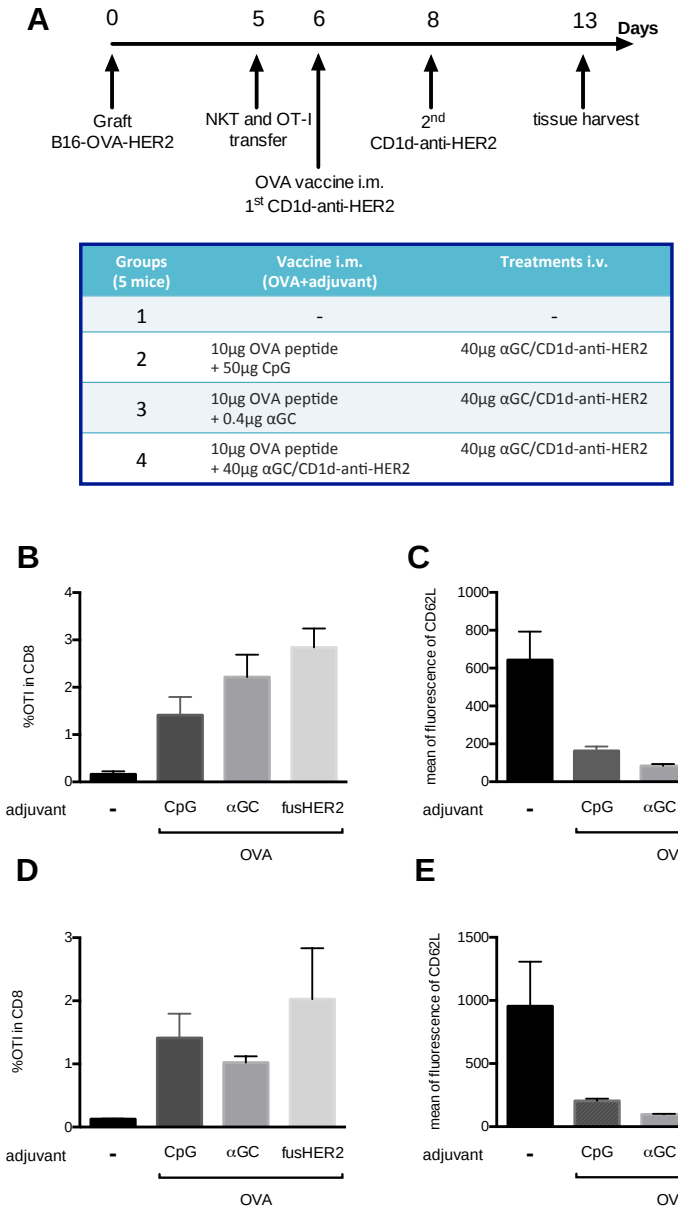
There is also evidence that the combination of TLR9 ligand together with the activation of iNKT cells results in an enhanced maturation of DC as well as their immunostimulatory functions (204). In this study, we investigated the property of the recombinant αGC/CD1d-

anti-TAA fusion to activate iNKT cells and to act as an adjuvant for cancer vaccine. As a model, we used the peptide-based vaccine of the chicken ovalbumin (OVA) antigen peptide SIINFEKL. The transgenic mouse model OT-I served as a source of OVA-specific CD8 T cells to be adoptively transferred in WT mice together with iNKT cells purified from the Vα14Jα18 TCR transgenic mice (205). In this study, the peptide vaccine formulated with CpG-ODN adjuvant, combined with the recombinant αGC/CD1d-anti-HER2 therapy, enhanced the priming of antigen-specific CD8 T cells. The tumor targeted iNKT cells directed by the αGC/CD1d-anti-HER2 fusion also resulted in an improved enrichment of antigen-specific CD8 T cells and NK cells into the tumor, which led to better tumor inhibition.

Results

CD8 T cells priming is effective with the aGC/CD1d-anti-HER2 fusion as adjuvant

The microbial stimuli CpG-ODN is well characterized as a potent adjuvant in cancer vaccine by increasing the CD8 T cell responses (206). The CpG-ODN also promoted antibody production as well as the activation of Th-1 helper CD4 T cells that therefore led to a better CD8 T cell responses (47). First, we examined the ability of recombinant αGC-loaded CD1d-anti-HER2 fusion (αGC/CD1d-anti-HER2) to promote the priming of specific CD8 T cells when it is co-administrated with the OVA peptide and compare it with CpG ODN and αGC as adjuvant. For this purpose, iNKT cells from Vα14Jα18 TCR transgenic mice and OVA-specific CD8 T cells were transferred into mice bearing B16-OVA-HER2 tumor. Six days after the tumor graft, mice were vaccinated i.m. with PBS or the OVA peptide in combination of either CpG-ODN (OVA-CpG), the free ligand αGC (OVA-αGC) or the αGC/CD1d-anti-HER2 fusion (OVA-αGC/CD1d-anti-HER2) **(figure 1A)**. Seven days after the vaccine, at the peak of the response, we analyzed in the periphery the priming of OVA-specific CD8 T cells measured by using MHC class-I/SIINKFEL tetramers. The data showed that the αGC/CD1d-anti-HER2 fusion was as efficient as the CpG-ODN and the ligand αGC alone to induce the expansion of OVA-specific CD8 T cells in the blood **(figure 1B)**. The profound downregulation of the lymphoid-homing molecule CD62L, occurring during effector T cell differentiation, indicated that OVA-specific CD8 T cells were activated by all different vaccinations **(figure 1C)**. Likewise, the adjuvant effect of the αGC/CD1d-anti-HER2 fusion was observed in the spleen of vaccinated mice, resulting in 2% of effector MHC-I/OVA tetramer positive cells among CD8 T cells, that was similar to the percentage obtained with CpG-ODN or free αGC as adjuvant **(figure 1D, E)**.



Groups (5 mice)	Vaccine i.m. (OVA+adjuvant)	Treatments i.v.
1	-	-
2	10µg OVA peptide + 50µg CpG	40µg αGC/CD1d-anti-HER2
3	10µg OVA peptide + 0.4µg αGC	40µg αGC/CD1d-anti-HER2
4	10µg OVA peptide + 40µg αGC/CD1d-anti-HER2	40µg αGC/CD1d-anti-HER2

Figure 1: The αGC/sCD1d-anti-HER2 fusion is a potent adjuvant to prime OVA-specific CD8 T cells. Recipient CD45.1 mice were grafted s.c. with 500 000 B16-OVA-HER2 tumor cells. Five days later, splenocytes from T414 and OT-I transgenic mice containing respectively 500 000 NKT and 100 000 OVA-specific CD8 T cells were co-transferred. One day later, mice were immunised i.m. with OVA peptide alone or in combination with different stimulation and treated systemically every 2-4 days with the αGC/CD1d-anti-HER2 fusion (fusHER2). Mice were sacrificed at day 13 and lymphocytes from blood and spleen were analyzed by flow cytometry. **A.** Experimental protocol (upper panel) and description of the different treatment combinations (lower panel). **B.** Frequency of H-2K^b/OVA-specific CD8 T cells among total CD8 lymphocytes in the blood on day 13. **C**. Mean of fluorescence of CD62L on the H-2K^b/OVA-specific CD8 T cell population in the blood. **D**. Frequency of H-2K^b/OVA-specific CD8 T cells among total CD8 cells in the spleen. **E.** Mean of fluorescence of CD62L on the H-2K^b/OVA-specific CD8 T cell population in the spleen. Bar graphs show frequencies or mean of fluorescence, as mean +/- SEM of groups of 3 mice.

The combination of αGC/CD1d-anti-HER2 fusion with CpG-ODN rapidly activates the innate and the adaptive immune responses.

In view of the ability to CpG-ODN and the αGC/CD1d-anti-HER2 fusion proteins to induce potent priming of antigen-specific CD8 T cells, we investigated their combined effects on lymphocytes activation and DC maturation. Total splenocytes from Vα14Jα18 transgenic mouse were cultured for 6h in presence of either αGC/CD1d-anti-HER2 fusion proteins, CpG-ODN or the combination of the two stimuli and the expression of the early activation antigen CD69 was analyzed on the surface of iNKT, CD3 and NK cells **(figure 2)**. While CpG ODN stimulation was unable to directly activate iNKT cells, αGC/CD1d-anti-HER2 fusion proteins induced rapid up-regulation of CD69 on their surface **(figure 2A)**. Furthermore, a strong synergistic effect was observed upon the combination of CpG-ODN and αGC/CD1d-anti-HER2 fusion resulting in an enhanced expression of CD69 on iNKT cells **(figure 2A)**. While iNKT cells were not stimulated by CpG-ODN, T lymphocytes and NK cells were activated by TLR9 ligand-mediated activation of DC and macrophages **(figure 2B, C)**. We noticed that the αGC/CD1d-anti-HER2 fusion-mediated activation of iNKT cells was more efficient to promote CD8 T lymphocytes and NK cells activation compared to CpG-ODN alone, as showed by the higher expression of CD69 **(figure 2B, C)**. As observed on iNKT cells, the effects of each stimulation acted together to induce the better activation of T lymphocytes **(figure 2B)** and NK cells **(figure 2C)**.

For each stimulation, we observed an up-regulation of CD86, a marker of DC maturation, on CD11c+ DCs compared to non-stimulated condition **(figure 2D)**, however, no synergistic effect was induced with the combination of CpG-ODN and αGC/CD1d-anti-HER2 fusion. But interestingly, the production of the pro-inflammatory cytokine IL-12 was two-fold enhanced with the combined stimulus CpG-ODN and αGC/CD1d-anti-HER2 fusion compared to the CpG-ODN alone **(figure 2E)**. These results demonstrated that iNKT cell

activation, together with the TLR9 ligand, represents a promising approach to reach a maximal activation of immune cells.

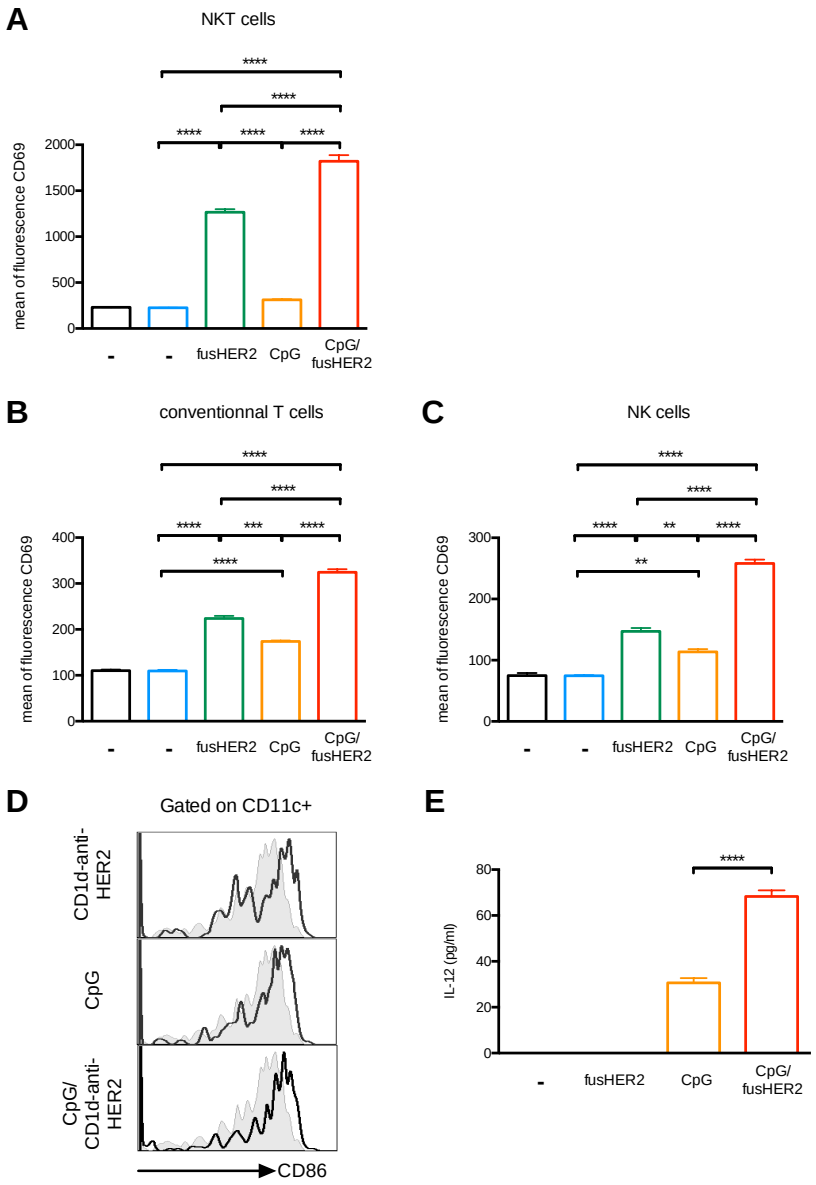


Figure 2: The combination of αGC/CD1d-anti-HER2 fusion with CpG ODN rapidly activates immune cells. 200 000 splenocytes from T414 mouse (Vα14Jα18 transgenic mouse) were cultured in complete DMEM for 6 or 24 hours in presence either of plate-coated αGC/CD1d-anti-HER2 fusion (40µg/ml), CpG-ODN (5µg/ml) or the combination of the two stimuli. Cells were recovered at 6h and analyzed by flow cytometry. **A.** Mean of fluorescence of CD69 on CD1d tetramer+ NKT cell population. **B**. Mean of fluorescence of CD69 on CD3+ T cell population. **C.** Mean of fluorescence of CD69 on NK1.1+ NK cell population. **D**. Dot plot representative of the surface expression of CD86 on CD11c+ cells. **E**. Supernatants were recovered at 24h and IL-12 level (pg/ml) was analyzed using CBA assay. Bar graphs show mean of fluorescence or cytokine concentration, as mean of triplicates +/- SEM.

Sustained activation of iNKT cells combined with a vaccine enhances the priming of antigen-specific CD8 T cells.

Recipient mice bearing B16-OVA-HER2 tumor were injected i.v. with iNKT and OT-I cells and then vaccinated i.m. with the OVA peptide in presence of either PBS, CpG-ODN alone or CpG-ODN combined with αGC/CD1d-anti-HER2 fusion proteins (OVA-CpG+αGC/CD1d-anti-HER2), before i.v. treatments with the αGC/CD1d-anti-HER2 fusion **(figure 3A)**. We hypothesized that the innate signal through TLR9 and iNKT cell-mediated cytokines would act synergistically in the modulation of DC maturation and promotion of the adaptive T cell immune responses. At two time points, day 3 and day 7 after the vaccination, we assessed the proliferation of transferred antigen-specific CD8 T cells. As expected, the peptide OVA without adjuvant failed to prime OVA-specific CD8 T cells and the αGC/CD1d-anti-HER2 fusion treatment without the OVA peptide did not stimulate OVA-specific CD8 T cells (figure 3B). In contrast, we observed a potent priming of OVA-specific CD8 T cells 3 days after vaccination with OVA-CpG or with OVA-CpG+αGC/CD1d-anti-HER2 fusion **(figure 3B, C)**, which at this early time point, were not yet fully activated as seen by high CD62L expression **(figure 3D)**. Moreover, 7 days after the vaccination, OVA-specific CD8 T cells still proliferated **(figure 3E)** and exhibited a fully effector phenotype demonstrated by the downregulation of the CD62L **(figure 3F)**.

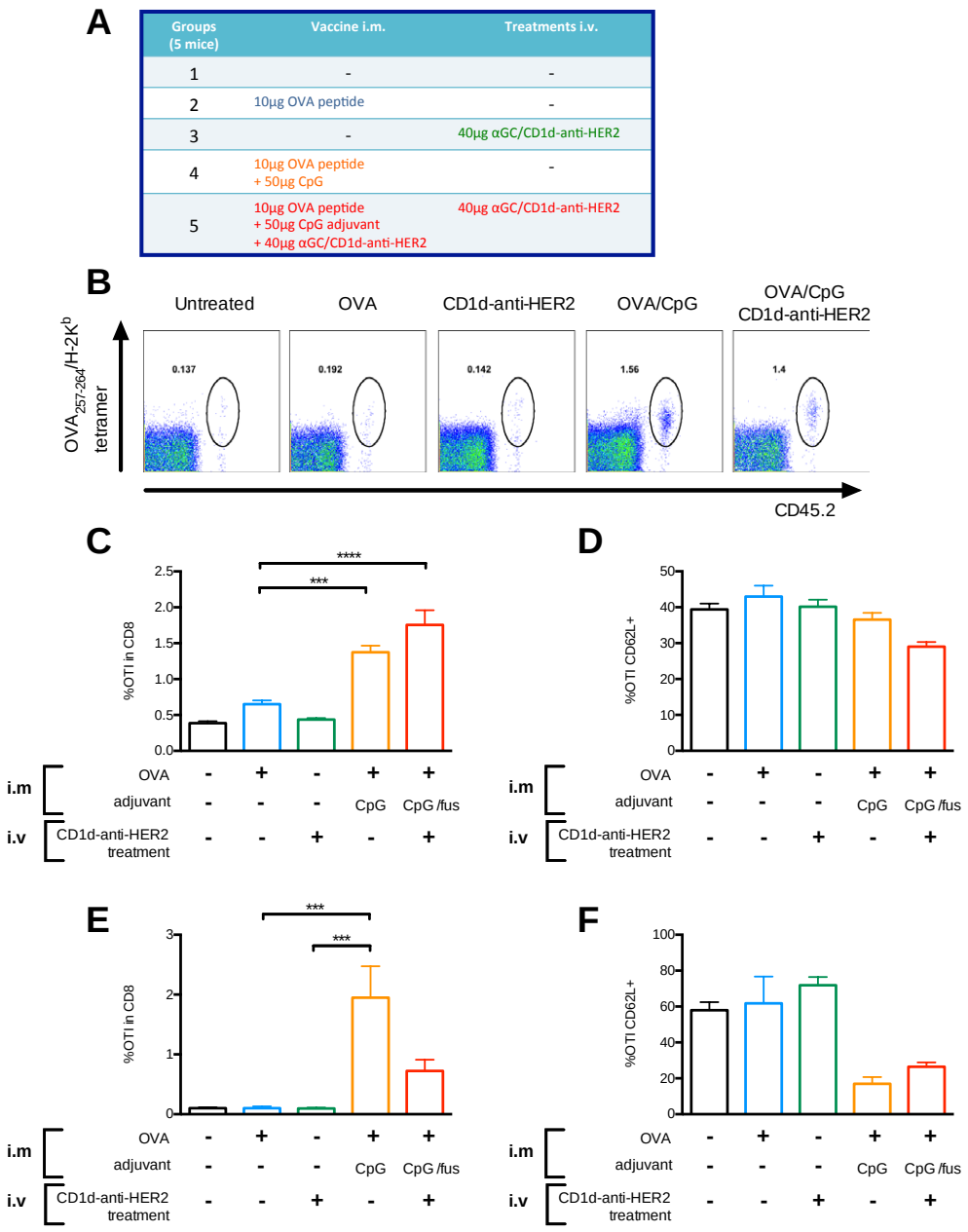


Figure 3: Vaccination of mice with OVA/CpG combined or not with the αGC/CD1d-anti-HER2 fusions leads to the priming of H-2K^b/OVA-specific CD8 T cells. Schedule of the experimental was the same as in figure 1. Mice were bled at day 10 and day 14 after the tumor graft, and lymphocytes were analyzed by flow cytometry. **A**. Description of the different treatment combinations **B.** Dot plot representative of one mouse per group showing the H-2K^b/OVA tetramer positive CD45.2+ CD8 T cells in the blood at day 10. **C.** Frequency of H-2K^b/OVA-specific CD8 T cells among total CD8 cells in the same time point. **D.** Mean of fluorescence of CD62L on the H-2K^b/OVA-specific CD8 T cell population **E.** Frequency of H-2K^b/OVA-specific CD8 T cells among total CD8 cells in the blood at day 14. **F.** Mean of fluorescence of CD62L on the H-2K^b/OVA-specific CD8 T cell population in the blood at day 14. Bar graphs show frequencies or mean of fluorescence, as mean +/- SEM of groups of 5 mice.

Sustained activation of iNKT cells combined with a vaccine leads to the expansion of NK cells.

In parallel, we tested the frequency of iNKT and NK cells in the blood following the different vaccination protocols. The percentage of iNKT observed at day 3 and day 7 after treatment did not change, except a weak decrease with the vaccination alone at day 3 and an increased percentage of iNKT cells after CpG-ODN and αGC/CD1d-anti-HER2 fusion treatment at day 7 **(figure 4A, B)**. Moreover and as expected, the activation of iNKT cells with the αGC/CD1d-anti-HER2 fusion led to the transactivation of NK cells, which were required for anti-tumor effects (92). Indeed, the percentage of NK cells in the blood increased significantly, exclusively in the two groups of mice treated with the αGC/CD1d-anti-HER2 fusion alone or with CpG-ODN **(figure 4C)**, and rapidly reached 10% of total lymphocytes, 3 days after the vaccine. Interestingly, the expansion of NK cells remained stable at day 7 only in the group with the combination of the OVA-CpG vaccine and the αGC/CD1d-anti-HER2 fusion treatment **(figure 4D)**. These data indicated that the CpG-ODN adjuvant effects synergized with the activation of iNKT cells leading to a better activation of the NK cells.

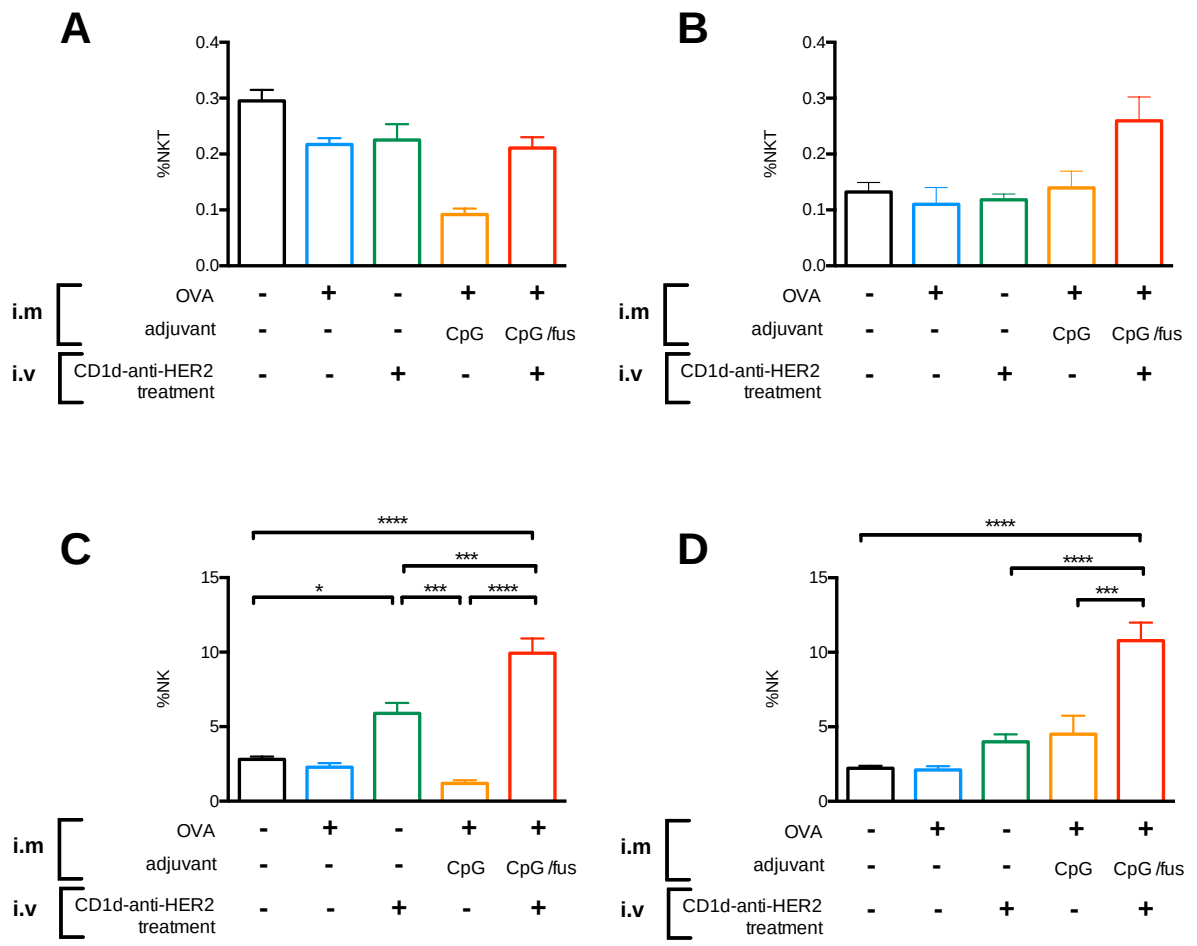


Figure 4: Administration of OVA-CpG vaccine combined with the αGC/CD1d-anti-HER2 fusion induces the proliferation of NK cells. NKT and NK cells from the mice described in figure 3 were analyzed by flow cytometry. **A.** Frequency of NKT cells (CD1dtetramer+CD3+) in the blood at day 10. **B.** Same as in A at day 14. **C.** Frequency of NK cells (NK1.1+CD3-) in the blood at day 10. **D.** Same as in C at day 14. Bar graphs show frequencies as mean +/- SEM of groups of 5 mice.

The synergistic effects obtained with the combination OVA-CpG+αGC/CD1d-anti-HER2 fusion was also revealed by the large amount of Th1 cytokines released in the blood as seen 20h after vaccination **(figure 5)**. As expected, triggering iNKT cells with the αGC/CD1d-anti-HER2 fusion resulted in the strong production of IFNγ **(figure 5A)**, while TLR-9 signal induced by CpG-ODN only led to a marginal production of IFNγ in the serum **(figure 5A)**. In contrast, IL-6 and TNFα were produced upon treatment either with the αGC/CD1d-anti-HER2 fusion or the OVA-CpG vaccine, which led to a synergistic effect **(figure 5B, C)**.

Furthermore, in correlation with the strong proliferation of NK cells **(figure 4)**, IL-6 and TNFα productions were two fold increased after the combination treatment compared to each separated treatment **(figure 5B, C)**.

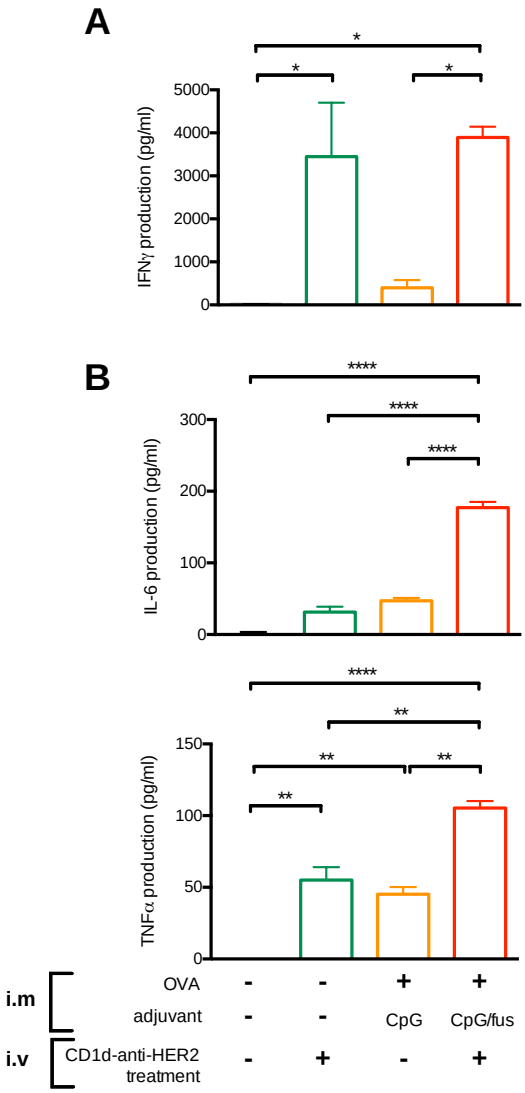


Figure 5: αGC/CD1d-anti-HER2 fusion combined with CpG-ODN induces strong Th1 cytokine production early after immunisation. Sera from mice described in figure 3 were collected 20 hours after the first immunisation and cytokines were measured using a Th1/Th2/Th17 CBA assay. **A.** Serum IFNγ level **B.** Serum IL-6 level **C.** Serum TNFα level. Bar graphs show cytokine level in pg/ml as mean +/- SEM for 3 samples per group.

Synergistic effects of OVA-CpG vaccine and αGC/CD1d-anti-HER2 fusion treatment is confirmed in the spleen.

At day 7 after vaccination, we observed a proliferation of OVA-specific CD8 T cells in the spleens of mice vaccinated with OVA-CpG, and their frequency was further enhanced when

enhanced when the vaccination was combined to the activation of iNKT cells **(figure 6 B)**. Indeed, the frequency as well as the absolute cell number of OVA-specific CD8 T cells were two fold increased in the combination group compared to the vaccine alone **(figure 6B, C)**. In all vaccinated groups, CD8 T cells displayed an effector phenotype as seen by the down-modulation of CD62L **(figure 6D)**.

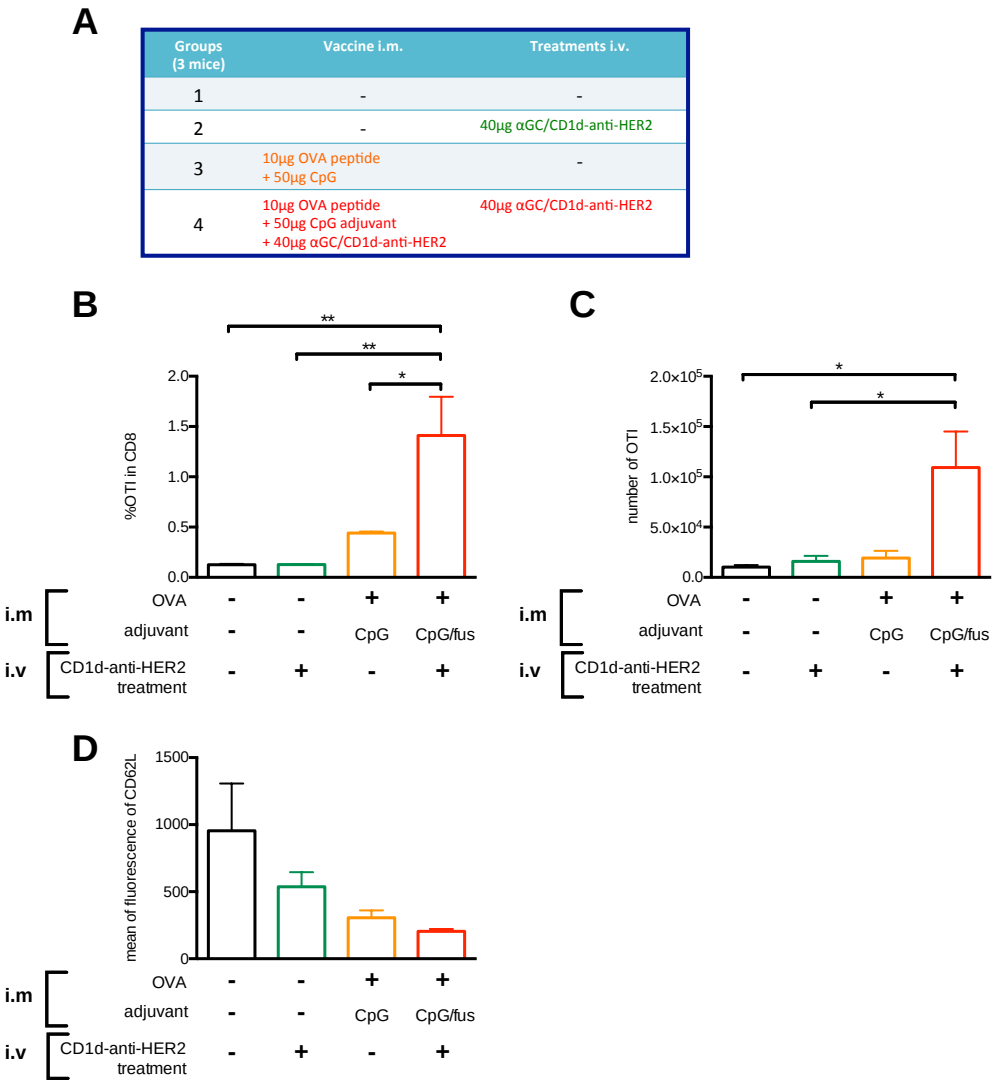


Figure 6: Vaccination of mice with OVA/CpG combined with the αGC/CD1d-anti-HER2 fusion efficiently primes H-2K^b/OVA-specific CD8 T cells in the spleen. Schedule of the experimental was the same as in figure 1 **A.** Table describing the different treatment combinations. **B.** Frequency of H-2K^b/OVA-specific CD8 T cells among total CD8 cells in the spleen on day 13. **C.** Absolute cell number of H-2K^b/OVA-specific CD8 T cells in the spleen. **D**. Mean of fluorescence of CD62L into the H-2K^b/OVA-specific CD8 T cell population. Bar graphs show results as mean +/- SEM of groups of 3 mice.

In all groups receiving i.v. treatments with the αGC/CD1d-anti-HER2 fusion, expansion of iNKT cells was enhanced as shown by their increased percentage and absolute cell number **(figure 7A, B)**. Moreover, as observed in the blood, the population of NK cells was highly augmented in mice treated with the combined treatment, as seen in both the frequency and absolute cell number **(figure 7C, D)**. Altogether, these results confirmed the synergistic effects on innate and adaptive immune cells between OVA-CpG vaccination and the activation of iNKT cells with the αGC/CD1d-anti-HER2 fusion.

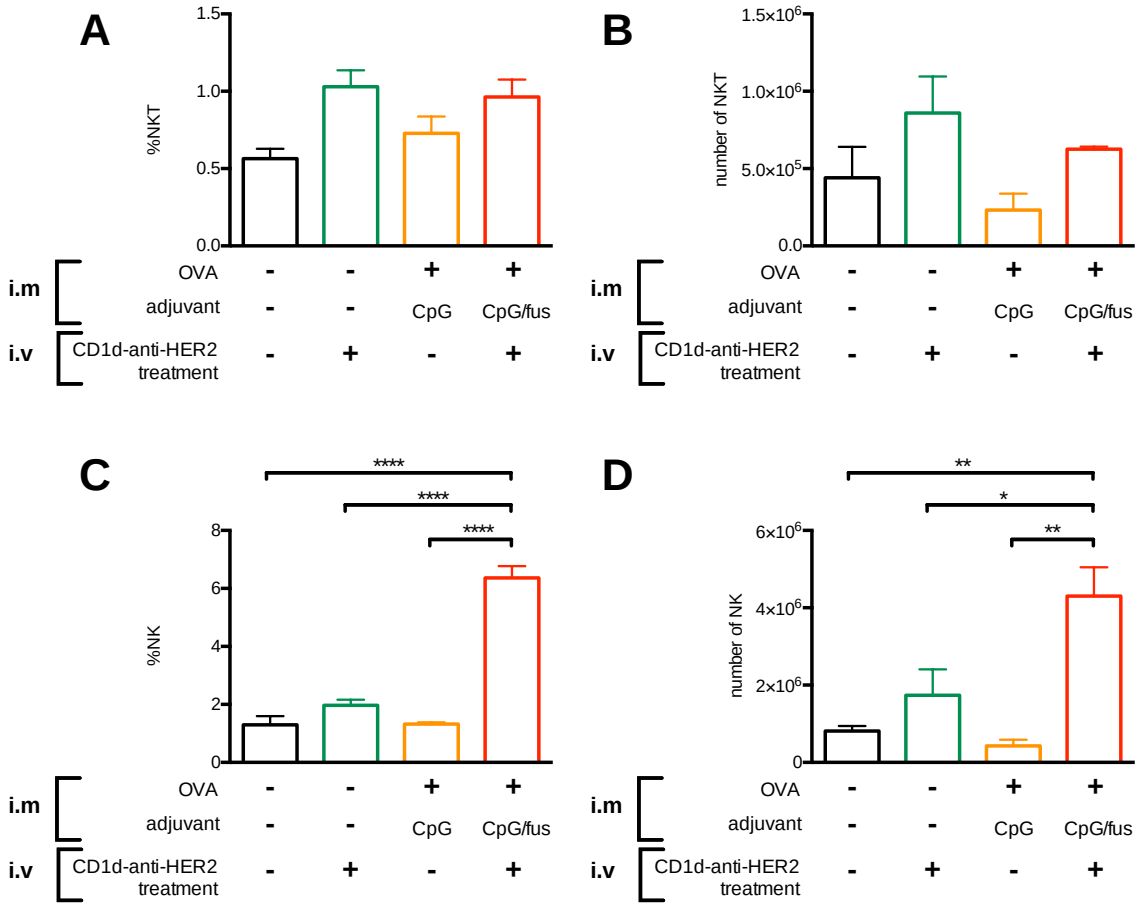


Figure 7: The combination of OVA-CpG vaccine with activation of iNKT cells enhances the proliferation of innate NK cells. NKT and NK cells were analyzed on day 13 in the spleen of the same mice as in figure 6. **A.** Frequency of NKT cells (CD1dtetramer+CD3+). **B.** Absolute cell number of NKT cells. **C.** Frequency of NK cells (NK1.1+CD3-). **D.** Absolute cell number of NK cells. Bar graphs show result as mean +/- SEM of groups of 3 mice.

OVA-specific CD8 effector T cells are specifically redirected to the tumor following injection of tumor-targeted αGC/CD1d-anti-HER2 fusion.

At day 7 after the vaccination, during the peak of the adaptive immune response (data not shown), we isolated tumor-infiltrating lymphocytes (TILs) and analyzed by flow cytometry the proportion of OVA-specific CD8 T cells at the tumor site **(figure 8 A)**. The highest percentage of OVA-specific CD8 T cells (45% of CD8 T cells) was achieved in response to the combined therapy **(figure 8A, B)** as compared to vaccine alone. Spontaneous tumor infiltration also occurred in the vaccine OVA-CpG group certainly resulting from the activated phenotype of specific CD8 T cells attracted by OVA antigen presentation by the tumor. The mean number of antigen-specific CD8 T cells per milligram (mg) of tumor in the combined group was increased 2-fold as compare to vaccine alone **(figure 8C)**.

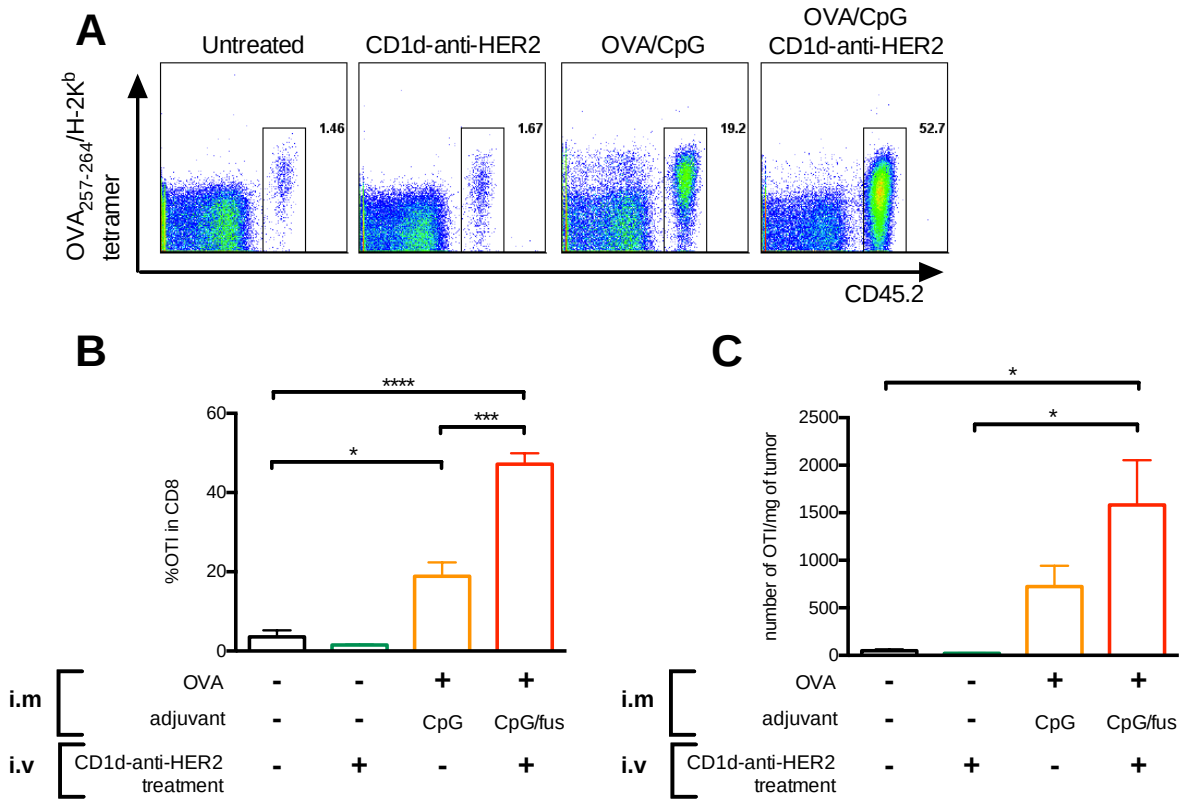


Figure 8: Treatment of mice with the tumor-specific αGC/CD1d-anti-HER2 fusion redirects primed OVA-specific CD8 T cells at the tumor site. Mice treated as described in figure 6 were sacrificed at day 13, and the percentage of H-2K^b/OVA-specific CD8 T cells was determined in the tumor tissue by flow cytometry. **A.** Dot plot representative of one mouse per group showing the accumulation of H-2K^b/OVA-specific CD8 (tetramer H-2K^b/OVA+ CD45.2+). **B**. Frequency of H-2K^b/OVA-specific CD8 among total CD8 T cells in tumors. **C.** Absolute cell number of H-2K^b/OVA-specific CD8 T cells per milligram of tumor. Bar graphs show results as mean +/- SEM of groups of 3 mice.

We also observed a discrete tendency of NKT cells to be enriched into the tumor after the αGC/CD1d-anti-HER2 treatment with or without the OVA-CpG vaccine **(figure 9A, B)**. However, when we analyzed NK cells, we found that the specific activation of iNKT cells at the tumor site by the αGC/CD1d-anti-HER2 treatment markedly increased the percentage and the absolute cell number of NK per mg of tumor, independently of the combination with the OVA-CpG vaccine **(figure 9C, D)**. In contrast the vaccine OVA-CpG alone did not lead to the activation and proliferation of NK cells and their attraction to the tumor site **(figure 9C)**.

The overall better tumor infiltration lymphocytes indicated the specific role of the αGC/CD1d-anti-HER2 fusion to redirect the iNKT cell response to the tumor site to induce the tumor targeting of CD8 and NK effector cells.

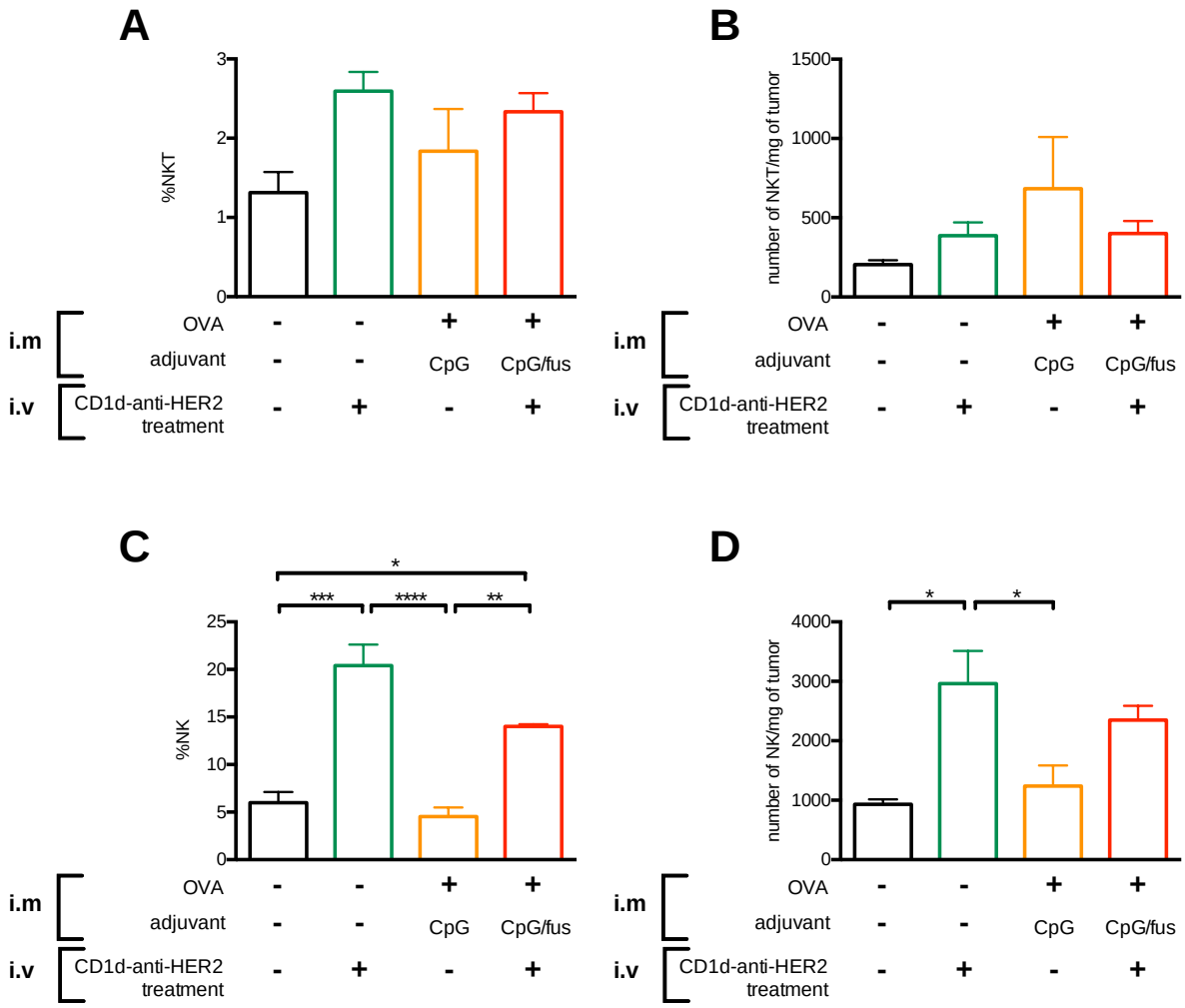


Figure 9: Activation of iNKT cells by the tumor-specific αGC/CD1d-anti-HER2 fusion attracts innate immune cells at the tumor site. Mice treated as described in figure 6 were sacrificed at day 13 and the percentage of NKT and NK cells was determined in the tumor tissue by flow cytometry. **A.** Frequency of NKT cells (CD1dtetramer+CD3+). **B.** Absolute cell number of NKT cells per milligram of tumor. **C.** Frequency of NK cells (NK1.1+CD3-). **D.** Absolute cell number of NK cells per milligram of tumor. Bar graphs show result as mean +/- SEM of groups of 3 mice.

The tumor-targeted αGC/CD1d-anti-HER2 fusion treatment synergizes with the tumor vaccine to reduce tumor growth.

The cross talk between activated iNKT cell and DC results in a large production of the IL-12 pro-inflammatory cytokine by the latter cells (203). This IL-12 production is further enhanced in the presence of bacterial activation and triggers a stronger production of IFNγ by iNKT cells (207). We expected that the increased activation of iNKT cells and their capacity to release Th1 cytokines could lead to potent anti-tumor effects upon OVA-CpG+αGC/CD1d-anti-HER2 treatment. To verify this hypothesis, recipient mice were grafted s.c. with B16-OVA-HER2 tumor cells and treatments were initiated when all tumors were palpable. Treatment regimens included either with PBS, the OVA peptide alone, the peptide plus OVA-CpG, the αGC/CD1d-anti-HER2 fusion alone or the combination OVA-CpG and αGC/CD1d-anti-HER2 fusion **(figure 10A)**. Untreated mice or those vaccinated with the OVA peptide without adjuvant, all had fast tumor growth **(figure 10B)**, while the addition of the immunostimulant CpG-ODN to the OVA peptide resulted in a delayed tumor progression **(figure 10B)**, which correlated with a weak infiltration of OVA-specific CD8 T cells into the tumor. The αGC/CD1d-anti-HER2 fusion alone had a weak anti-tumor effect on this aggressive melanoma model **(figure 10B)**. However, the tumor growth was best inhibited in mice, which received the combined treatment **(figure 10B)**. The long-term protection was increased when compared to the vaccination alone and fusion alone. These strongest effects against the B16 melanoma model likely resulted from the synergistic action of innate and adaptive immune responses redirected to the tumor site. Indeed, the tumor-targeted iNKT cells activation promoted the antitumor cytotoxicity both by NK cells and by the enhancement of the adaptive immune response induced by the OVA tumor vaccine.

We have previously reported that the tumor targeting of the recombinant αGC/CD1d-antitumor fusion protein was essential to obtain an optimized activation of iNKT cells and resulting in better anti-tumor effects (208). Now, to confirm the importance of the targeting, we performed an anti-tumor experiment, where mice-bearing B16-OVA-HER2 were vaccinated with OVA-CpG and treated i.v. either with the tumor-specific αGC/CD1d-anti-HER2 fusion or the irrelevant αGC/CD1d-anti-CEA fusion protein. Synergistic antitumor effects between the CTL induction and the systemic iNKT cells activation were observed for the two CD1d-scFv fusion proteins **(figure 10C)**. However, the inhibition of tumor growth was more efficient in mice treated with the tumor-targeted αGC/CD1d-anti-HER2 fusion as compared to the irrelevant αGC/CD1d-anti-CEA fusion protein **(figure 10C)**. Altogether, these results demonstrated the advantage of the activation of iNKT cells by a tumor-targeted αGC/CD1d-anti-TAA fusion in combination with vaccination based on TLR9 ligand for an optimal tumor immunotherapy.

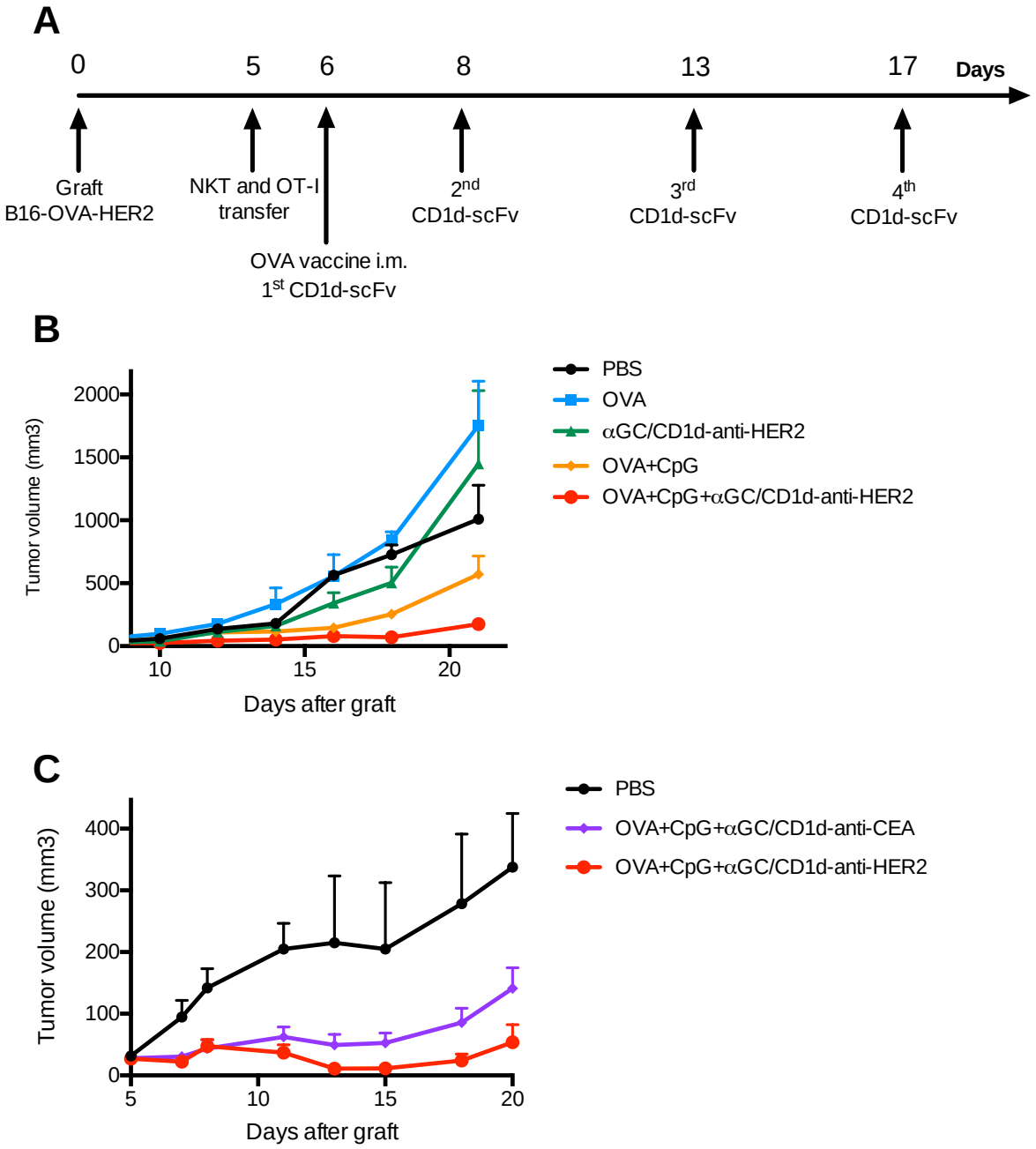


Figure 10: The combination of OVA-CpG vaccine with the tumor-targeted αGC/CD1d-anti-HER2 fusion lead to enhanced anti-tumor effects. Mice were treated following the protocol experiment described in figure 1 **A.** Experimental schedule. **B.** Kinetic of tumor growth shown as mean tumor sizes +/- SEM. **C.** Kinetic of tumor growth comparing the OVA-CpG vaccine combined with i.v. injections of the tumor-targeted αGC/sCD1d-anti-HER2 or the irrelevant αGC/sCD1d-anti-CEA fusion proteins. Graph represents tumor growth kinetic as the mean tumor sizes +/- SEM. For all graphs, tumors were measured every two days and the values represent the mean of 5 mice per group.

Discussion

The characterization of increasing numbers of tumor-associated antigen opens the way for their use as antigenic component in preventive and therapeutic cancer vaccines. Protein and peptide-based vaccines have demonstrated clinically relevant effects, and the improvement of their immunogenicity is under extensive investigation through the use of adequate adjuvants. In the present study, we reported that the activation of iNKT cells via the recombinant αGC-loaded CD1d protein improved antigen-specific CD8 T cell responses when co-administrated with the OVA peptide. The capacity of iNKT cells to act as a potent adjuvant was already described by using the iNKT-cell ligand αGC as a free drug or loaded on DCs (200, 209). The activation of iNKT cells led to the enhancement of specific CD8 and CD4 T cell responses when αGC ligand was co-injected with the antigen (209). The increased vaccine efficacy is likely resulting from the cross talk between iNKT cells and DC including the CD40L-CD40 interaction, as well as IFNγ and IL-12 secreted respectively by activated iNKT cells and DCs (202). On the other hand, TLR ligands are known to activate the innate immune system and to greatly promote the activation and maturation of DCs (210) (211), which has made them as adjuvants of choice in many vaccine settings (193). Using the same model antigen, we have demonstrated that iNKT cells activation was as efficient as the TLR9 ligand CpG-ODN to prime specific CD8 T cell responses when co-administrated with the OVA peptide. Importantly, the combination of αGC/CD1d-anti-HER2 fusion with the CpG-ODN led to synergistic activation of iNKT cells, T lymphocytes and NK cells. This combination induced a better *in vivo* priming of specific-CD8 T cells than the vaccine alone. These results correlated with a more efficient DC maturation, observed with the upregulation of costimulatory molecules and the enhanced IL-12 production after stimulation with αGC/CD1d-anti-HER2 fusion and CpG-ODN. The efficacy of the combined iNKT cell

activation and cancer vaccine was seen by enhanced production of Th1 cytokines in the serum of mice, coming rapidly after the co-injection of the OVA-CpG vaccine and the αGC/CD1d-anti-HER2 fusion. The high production of IFNγ, IL-6 and TNFα upon the combined treatment resulted in effective innate immune response and DC maturation that lead to a strong priming of CTLs. In all, these results provided evidence that the TLR9-mediated priming of CD8 T cells response was potentiated by iNKT cells activation.

In addition to the choice of adjuvant, the route of vaccination is also a major parameter in the design of a vaccine. Previous report has shown that oral injection of antigen together with αGC is efficient to elicit CD8 T cell responses (150). However, this activation required an important dose of protein as well as a significant quantity of the glycolipid αGC (150). In addition, oral vaccine may result in a mechanism of mucosal tolerance and, in the rapid degradation of soluble forms of antigenic proteins (212). In contrast, the subcutaneous vaccination promotes an optimal uptake of the antigen by skin resident DCs, which migrate to the lymph node and engage strong CD8 T cell responses. However, iNKT cells poorly traffic to the skin and lymph nodes and i.v. injection of αGC-loaded-DCs was preferred to the subcutaneous route (213). Importantly, when OVA-CpG vaccine was administrated s.c. and αGC/CD1d-anti-HER2 fusion by i.v. injection, no synergic effects were observed regarding either the CD8 T cell priming in the blood or the anti-tumor effects (data not shown). These results likely suggested that synergistic effects required interaction between activated iNKT cells and the DC at the vaccination site. Therefore, we decided to choose the muscle as site of injection. Most of human vaccine demonstrated good efficacy when injected i.m. and a recent study in mouse has characterized conventional and monocyte-derived DCs in the muscle with a strong migratory capacity into the draining lymph node (LN) (214). In contrast to DCs, little is known about the activation of iNKT by this route. However, when OVA peptide and αGC/CD1d-anti-HER2 fusion were co-injected i.m., a good expansion and activation of

OVA-specific CD8 T cells and NK cells were observed, indicating that their iNKT-mediated transactivation had occurred. Most importantly the i.m. co-administration of OVA-CpG vaccine and αGC/CD1d-anti-HER2 fusion led to the enhanced priming of OVA-specific CD8 T cells and expansion of NK cells. We hypothesized that the strong vascularization of the muscle allows an efficient systemic activation of iNKT cells and their cross talk with both muscle and splenic DCs.

The well-described iNKT-mediated transactivation of NK cells was confirmed in the chapter 1, as well as in the present study following repeated treatments of the αGC/CD1d-anti-HER2 fusion. In the other hand, activation of DCs via TLR signaling induces their production of IL-12, which also largely activate NK cells (210) (215). Indeed, the present data demonstrated that OVA-CpG also activated NK cells in addition to the priming of CD8 T cells. Moreover, a strong synergistic effect on the expansion of NK cells occurred between iNKT cell activation by αGC/CD1d-anti-HER2 fusion and CpG-ODN signaling. The enhanced expansion of NK cells likely resulted by the cross talk between NKT cells and DCs. The expansion of iNKT cells after *in vivo* stimulation with the αGC/CD1d-anti-HER2 fusion remained marginal and their adjuvant activity rather resides in their potent immunostimulatory functions rather than in their own proliferation.

Overstimulation of iNKT cells with one injection of free αGC ligand is known to induce an anergic state that impedes their restimulation and their use in cancer therapeutic (183). However, DCs or whole cancer cells loaded with the αGC injected *in vivo* were shown as good alternatives to activate iNKT cells and therefore transactivate NK cells in a more sustained manner (175). As an example, whole cancer cells loaded with αGC were an efficient system to vaccine against glioma (182). Tumor cells presented antigens and the CD8 priming was boosted by the activation of iNKT cells (182). However, this procedure involves an expansive personalized medicine and αGC-loaded DCs as cancer therapeutic mainly

promoted clinical responses when administrated in the vicinity of the tumor (174, 216) (217). Unlike free αGC, the recombinant αGC/CD1d-scFv fusion circumvents the anergy of iNKT cells observed after one αGC injection, and their sustained activation by repeated injections of the αGC/CD1d antitumor fusion leads to significant anti-tumor effects (92). Therefore, αGC/CD1d-scFv fusion represents a better alternative to αGC-loaded DCs to promote activation of iNKT cells in vaccine settings. Indeed, we observed that activation of iNKT cells with the αGC/CD1d-anti-HER2 fusion redirected efficiently the innate and adaptive immune responses to the tumor site. In particular, when the OVA-CpG vaccine was combined with the specific αGC/CD1d-anti-HER2 fusion, the percentage and numbers of OVA-specific CD8 T cells infiltrated into the tumor was two fold increased compared to the vaccine alone. As expected, treatments of tumor-bearing mice with the tumor-targeted αGC/CD1d-anti-HER2 fusion also attracted NK cells to the tumor, which was not observed with the vaccine OVA-CpG alone. Like in periphery, increased numbers of iNKT cells was hardly seen at the tumor site, likely resulting from their weak proliferation and also from their progressive TCR downregulation making them difficult to track.

To evaluate the therapeutic effects of the OVA-CpG vaccine combined with the sustained iNKT cells activation, we proceeded to a tumor inhibition experiment. In order to test the combined immunisation in therapeutic settings, mice were grafted with the melanoma model B16F10, transfected with the OVA protein and the HER2 antigen. The melanoma tumor B16 is an aggressive tumor model, which is poorly immunogenic (218) and hence difficult to eradicate. At the peak of the OVA-specific CD8 T cells response, a delay in tumor growth was observed in mice vaccinated with OVA-CpG likely resulting from the OVA antigen presentation by the tumor. Overall, the anti-tumor effects correlated with the increased infiltration of OVA-specific CD8 T cells at the tumor site compared to untreated group.

Consistent with the higher frequencies of NK and OVA-specific CD8 T cells infiltrated at the tumor, the combined treatment group exhibited the best anti-tumor effects.

We have shown in the Chapter 1 the need to activate iNKT cells at the tumor site via the recombinant αGC/CD1d protein fused to the specific anti-TAA scFv in order to obtain the best anti-tumor effects. In addition, the reactivity of iNKT cells, as seen by their cytokine production, was optimal when activated with the tumor specific αGC/CD1d-scFv fusion in tumor-bearing mice. In the present study, the anti-tumor effects of the OVA-CpG vaccine against B16-OVA-HER2 tumors were best ameliorated when combined to the tumor-specific αGC/CD1d-anti-HER2 fusion as opposed to the combination with the irrelevant αGC/CD1d-anti-CEA fusion. These results underline the need to re-direct the overall immune response to the tumor site in order to develop an efficient cancer therapy.

Altogether, our study demonstrates that αGC/CD1d-anti-TAA fusion protein may improve therapeutic cancer vaccine strategies as adjuvant during the T cell priming and also as therapeutic agent to redirect immune responses to the tumor. Moreover, the synergic effects of CpG-ODN and iNKT cell activation may be useful to reinforce the innate immune response against the tumor.

Materials and Methods

Mice

All mice were maintained in specific pathogen-free conditions. C57BL/6J (B6), CD45.1 congenic B6-SJ ptp-rca mice were obtained from Harlan laboratories (The Netherlands) and maintained in our animal house. TCR-transgenic OT-I mice were maintained in our animal house (219). The Vα14Jα18 (T414) transgenic mice were kindly provided by A.Bendelac (University of Chicago, Chicago, USA) (205) and maintained in our animal house. All animal experiments were conducted according to institutional guidelines and under an authorization delivered by the Swiss veterinary department.

Cell line

The melanoma cell line B16-OVA was obtained from B.Huard (University medical center, Geneva, Switzerland) (220) and stably transfected using infection with a retroviral pLPCX containing the human HER2 antigen (kind gift from Y.Yarden, The Weizman Institute of Science, Israel). B16-OVA-HER2 cells were maintained in complete DMEM medium supplemented with 1mg/ml G418 and 1μg/ml puromycin. Expression of HER2 was monitored by FACS using the humanized mAb anti-HER2 Herceptin (Trastuzumab, Roche Ltd).

Reagents

The αGC analog KRN7000 (Alexis Biochemicals Corp) was dissolved in PBS-0.5% Tween-20. Peptide of OVA ($OVA_{257-264}$, H-2Kb restricted; amino acid sequence SIINFEKL) was synthetized in house at the Protein and Peptide Chemistry Facility (PPCF) of the UNIL (Lausanne Switzerland), resuspended in 10%DMSO at 1mg/ml and

stored at -80°C. The TLR9 ligand CpG-ODN 1826 was purchased from Coley Pharmaceuticals (Wellesley, MA). Cytokines levels were measured by fluorescence-based multiplex assay using BD Cytometric Bead Array kit TH1/TH2/TH17 (CBA, BD Biosciences).

Flow cytometry

The CD1d tetramer was developed with engineering a BirA consensus sequence at the C-terminus of the soluble mouse CD1d protein. The CD1d monomer was biotinylated by the BirA enzyme (Avidity, Denver, CO), and after loading with αGC, it was tetramerized on streptavidin-PE (Invitrogen) using a molar ratio of 4:1. The MHC-class-I/SIINFEKL tetramer was purchased from TCmetrix company (Lausanne, Switzerland).

Cells were stained first with the MHC-class-I/SIINFEKL tetramer at RT for 30 min, then CD1d tetramer was added for additional 30 min on ice. Surface staining antibodies were added without wash and incubated for 20 min on ice. Cells were washed once with PBS 2%FCS and resuspend in 200µl of PBS 2%FCS for acquisition.

All fluorochrome-labeled antibodies were purchased from Becton Dickinson (BD Biosciences) or eBiosciences. Flow cytometric analyses were performed with a FACSCalibur, FACSCanto or LSRII cytometer (BD Biosciences) and the acquired data were processed using FlowJo software (Tree Star Inc.).

Recombinant CD1d fusion proteins

Genetic fusion of mouse β2-microglobulin (β2m) with the soluble part of mouse CD1d (sCD1d) and the anti-HER2 or the anti-CEA scFv has been described previously (92) (208). Recombinant CD1d fusion proteins produced by transient transfection of the human cell line HEK293-EBNA (Cellular Biotechnology Laboratory, EPFL, Switzerland) were

affinity purified on the Sartobind His-Tag membrane adsorbers for exchange chromatography (Sartorius AG, Germany) and loaded with αGC as previously reported (92).

In vitro experiments

Cell suspensions from spleens of the indicated mouse strains were incubated in cDMEM for 6h or 24h with the following stimulations: cDMEM alone, 1μg/ml $OVA_{323-339}$ peptide alone, OVA in combination either with 5μg/ml CpG-ODN, 40μg/ml of coated αGC/CD1d-anti-HER2 fusion proteins or OVA+CpG-ODN+coated αGC/CD1d-anti-HER2 fusion proteins Supernatants were recovered at indicated time-points and frozen to perform cytokine measurements using CBA assay and cells were analyzed by flow cytometry.

Adoptive cell transfers

OVA-specific CD8+ and NKT cells (CD45.2) were isolated from spleens of OT-I and Va14-Ja18 mice, respectively. Single cell suspensions were obtained by disrupting the lymphoid tissue and the frequency of transgenic T cells was determined by flow cytometry. Cells were labeled with Vα2 and Vβ5.1/5.2 antibodies for OT-I cells and CD1d tetramers and CD3 antibody for NKT cells. Naive B6-SJ ptp-rca (CD45.1) recipient mice received an i.v. transfer of $1x10^5$ OT-I cells and $5x10^5$ NKT in 200 μl DMEM, as indicated.

In vivo therapy

CD45.1 congenic mice were grafted s.c. in the right flank with $5x10^5$ B16-OVA-HER2 cells. Five days after the graft, a mix of $5x10^5$ NKT cells and $1x10^5$ OT-I CD8 T cells were transferred into the mice. One day after the transfer, mice were vaccinated intra-muscularly (i.m.) with 50μl of either PBS, OVA peptide (10μg), OVA peptide (10μg) and

CpG-ODN (50μg) alone or in combination with αGC/CD1d-scFv fusion protein (40 μg). Vaccination was immediately followed by i.v. treatments with 200μl of either PBS alone or αGC/CD1d-antitumor scFv fusion proteins (40μg). Systemic treatment with the αGC/CD1d-antitumor scFv fusion proteins (40μg) was repeated at 2 to 4-day intervals. Mean tumor volume, monitored every two days, was calculated using the following formula (length x width x thickness)/2.

Preparation of PBMC

Mice were bled from the lateral tail vein. Erythrocytes were removed from PBMCs by 10 min incubation at 37°C with red blood cell lysis solution (Qiagen) and PBMCs were washed in PBS 2%FCS buffer. Leukocytes from blood were directly stained with antibodies for the flow cytometry assay.

Preparation of splenocyte suspension

Spleens were disrupted through a 70μm filter using a 1ml syringe plunger and cells were washed twice with PBS 2%FCS buffer. Splenocytes were incubated for 5 min at RT in red blood cell lysis solution (Qiagen), washed with PBS 2%FCS buffer and stained with antibodies for the flow cytometry assay.

TIL extraction

At day 7 after the vaccination, tumors were harvested and digested for 40 min at 37°C, according to the Tumor dissociation kit protocol (MACS Miltenyi Biotec, Germany). Tumors were crushed on 100μm cell strainers and washed twice with PBS 2%FCS. Single cell suspensions were enriched for CD45+ cells using the autoMACS system (Miltenyi Biotec, Germany). Briefly, cells from maximum 500mg of tumor tissue were labeled with anti-CD45

microbeads (Miltenyi Biotec), as per the manufacturer's protocol, and purified using the POSSEL program on the AutoMACS. The positive fraction was recovered for TILs analysis by flow cytometry.

Statistical analysis

Results are expressed as mean ± SEM. Statistical significance between the groups was determined with the one-way-ANOVA test with Bonferroni correction (GraphPad Prism, GraphPad software). Tumor progression statistics were calculated with the two-way-ANOVA test with Bonferroni correction (GraphPad Prism, GraphPad software). Significant differences are indicated by * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$), **** ($P<0.0001$).

CHAPTER 3

Alternative recombinant αGC/sCD1d fusion proteins

In order to extend the application of αGC/sCD1d-scFv fusion protein, alternative CD1d fusions were constructed in our laboratory. The following chapter is divided into two parts and aims to present two different approaches of αGC/sCD1d fusion proteins. First, we investigated the targeting of the tumor stroma environment via the targeting of vascular endothelial growth factor receptor-3 (VEGFR-3) and second, we developed a dimeric αGC/sCD1d fusion to augment the avidity for iNKT cells.

1-Tumor neo-vasculature targeting by αGC/sCD1d-anti-VEGFR-3 fusion

One alternative, to extend the targeting strategy to several different tumors, is to fuse the sCD1d protein to an scFv directed against tumor stromal antigens. Indeed, the tumor is not only constituted of tumor cells but also of stroma cells, which are composed of fibroblasts, endothelial cells or tumor-associated macrophages (TAMs). These cells are assisting tumor development by secreting growth factors, which promote proliferation of neoplastic cells, angiogenesis and lymphangiogenesis. The tumor stroma is a source of induced or over-expressed proteins that could be potential targets for immunotherapy (221). Instead of TAAs, which characterize specific tumors, a large number of stromal antigens are expressed in a wide variety of tumors. As an example, the fibroblast activation protein (FAP) appears as a target structure expressed by activated fibroblasts in a large number of epithelial cancers (222). Transferred CD8 T cells transduced with the CAR F19, which is composed of the anti-

FAP scFv fragment F19, is under investigation in a phase I trial for patients with malignant pleural mesothelioma (223). This study is based on the potent capacity of the sibrotuzumab, the humanized version of F19 antibody, to accumulate specifically in FAP-positive hepatic metastasis of colorectal cancer patients (224) (225).

The targeting of such antigens would allow to treat multiple cancers and extend the number of patients that could benefit from the treatment. We hypothesized that the use of αGC/sCD1d-fusion targeting stromal antigens could redirect immune effector functions to the tumor stromal microenvironment, resulting in local cytotoxicity and consequently in the deprivation of tumor nutriment.

To this aim, we obtained an anti-VEGFR-3 scFv that has the capacity to cross-react on human and mouse VEGFR-3 (kind gift from professor K. Ballmer-Hofer, Paul Scherrer Institute, Villigen-PSI, Switzerland) (226). VEGFR-3 is an important receptor expressed on endothelial cells of lymphatic vessels that surround tumors and is activated by the ligand VEGF-C (227). Inactivation of the VEGFR-3 by administration of blocking antibodies in mice resulted in an inhibition of metastasis and a reduction of lymphatic vasculature in the primary tumors (228). The inhibition or normalization of tumor vasculature already showed clinical efficiency with the humanized antibody directed against the VEGF ligand (Bevacizumab, Avastin; Genentech). It was approved for use in colorectal, breast and lung cancer patients (229). Zehnder-Fjallman and colleagues demonstrated that the scFv anti-VEGFR-3 accumulated into VEGFR-3-transfected tumors (226). Altogether, these studies argued that VEGFR-3 is a potent target for cancer immunotherapy.

The αGC/sCD1d-anti-VEGFR-3 fusion was constructed by replacing the scFv anti-CEA of the previous fusion construct with the scFv anti-VEGFR-3 in the plasmid pEAK8 containing the β2 microglobulin and the sCD1d protein **(figure 1)**. The production in HEK293 EBNA

cells and the purification of the αGC/sCD1d-anti-VEGFR-3 fusion were done as described in Stirnemann et al (92).

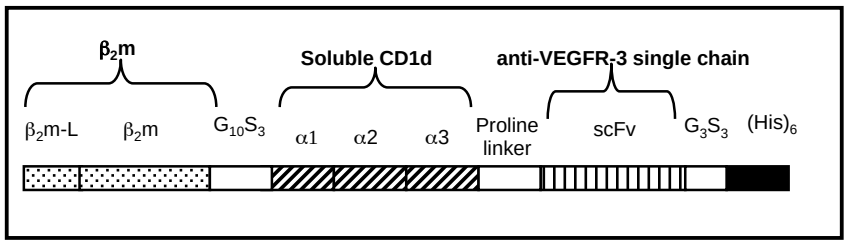


Figure 1: Design of the genetic fusion of mouse β2 microglobulin with the sCD1d and the scFv anti-VEGFR-3.

We investigated the VEGFR-3 expression on the tumor environment of B16 melanoma **(figure 2A)** and Tyr:Nras melanoma **(figure 2B)** by immunofluorescence staining. Tumor sections were stained with the αGC/sCD1d-anti-VEGFR-3 fusion and the CD31, as a marker of endothelial cells to assess their co-localization.

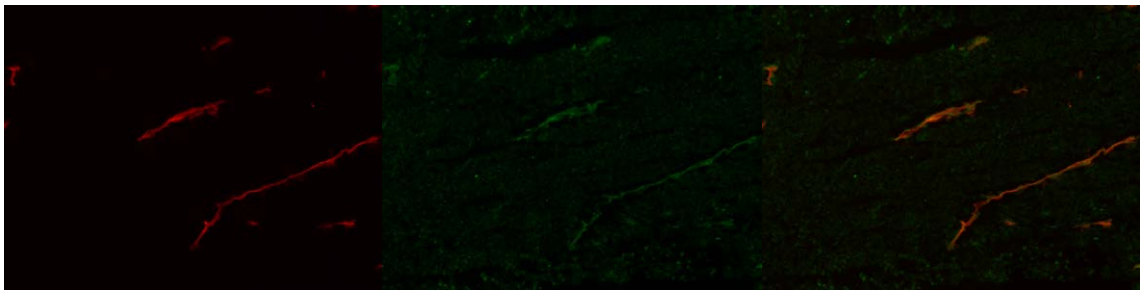


Figure 2A: Staining of B16 grafted tumor by anti-CD31 antibody visualized in red, sCD1d-anti-VEGFR-3 fusion protein visualized in green and the overlap in orange, 20x.

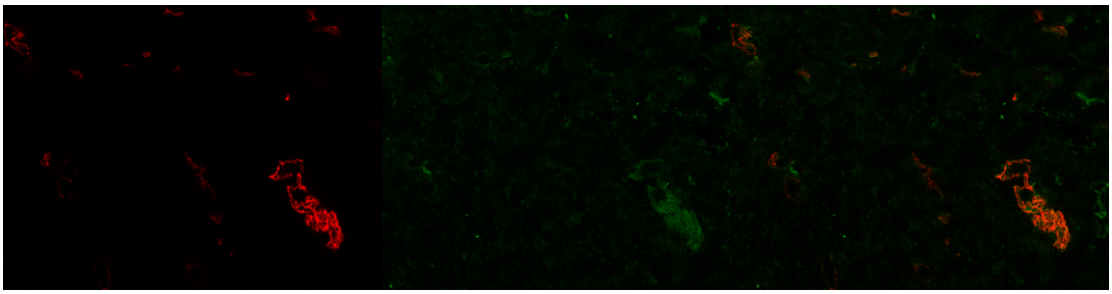


Figure 2B: Staining of Tyr:Nras transgenic tumor by anti-CD31 antibody visualized in red, sCD1d-anti-VEGFR-3 fusion protein visualized in green and the overlap in orange, 20x

The binding to VEGFR-3 on tumor sections by the αGC/sCD1d-anti-VEGFR-3 fusion was only reached after incubation with 70 to 150μg/ml of the fusion, representing a large quantity of protein. The poor VEGFR-3 visualization by immuno-staining suggested that the scFv anti-VEGFR-3 has a weak avidity. Moreover, the expression of VEGFR-3 in our tumor section was weak. Indeed, Zehnder-Fjallman and colleagues have demonstrated a specific tumor accumulation on VEGFR-3 transfected variants of tumor, which certainly do not reflect the natural expression of the receptor in WT tumors (226). With regard to VEGFR3, the two requirement of high avidity scFv and high expression of VEGFR-3 were not fulfilled, likely explaining the weak antitumor effects obtained.

As it is important for the design of an antibody in cancer immunotherapy, the high affinity of the scFv and the high expression of the targeted protein favor anti-tumor effects (230). For example, the high affinity antibody Herceptin, directed against the HER2 antigen, showed clinical benefits only in patients who displayed an overexpression of HER2 (5).

Another option would be to target components of the extracellular matrix domain such as fibronectin and tenascin-C splice variants, which are reported to be expressed specifically in the neo-vasculatures of aggressive tumors, such as melanoma, with a high level of expression (231). A fusion protein, composed of an anti-ED-B domain of fibronectin scFv, used to the delivery of a coagulation-inducing protein tissue factor (TF), mediated eradication of solid tumors in mice (232). Antibody-mediated targeting of tumor vasculature showed several advantages; such as a better diffusion of the antibody within tumors and consequently enhanced tumor toxicity. Thus far, the targeting of tumor microenvironment represents a promising alternative strategy to the targeting of TAAs (225, 231).

2-The dimeric αGC/CD1d-Fc fusion

The goal was to increase the activation of iNKT cells by creating a dimeric αGC/sCD1d complex and hence augment the half-life and the avidity of the protein for the NKT invariant TCR. We know that untargeted αGC/sCD1d protein is able to promote a systemic activation of iNKT cells after several injections, but fails to have antitumor effects against aggressive tumors, like in B16 melanoma model (92). Based on the hypothesis that this lack of antitumor effect might be due to a shorter half-life of αGC/sCD1d in addition to the absence of tumor targeting, we developed a dimeric molecule composed of the αGC/sCD1d fused to the Fc fragment from mouse IgG1 (figure 3).

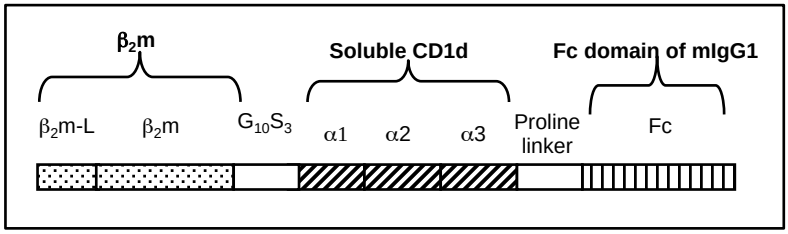


Figure 3: Design of the genetic fusion of mouse β2 microglobulin with the sCD1d and the mouse Fc domain of IgG1.

As expected, the monomeric molecule of sCD1d-Fc readily dimerizes on the Fc part in solution. Because the Fc domain has the property to prolong the half-life of the recombinant protein *in vivo*, this strategy was thus proposed to lead to a better activation of iNKT cells and a stronger tumor protection.

A preliminary experiment was done on s.c. grafted B16-F10 melanoma model. As soon as tumors were palpable, mice were treated i.v. with either 40μg of αGC/sCD1d-Fc or 25μg of αGC/sCD1d or no treatment. As usually, i.v. injections were repeated every 3 or 4 days. Whereas monomeric sCD1d had poor antitumor effect, treatment with αGC/sCD1d-Fc

protein led to a more potent retardation of tumor growth comparable to that obtained with αGC/sCD1d-antitumor scFv fusion (figure 4).

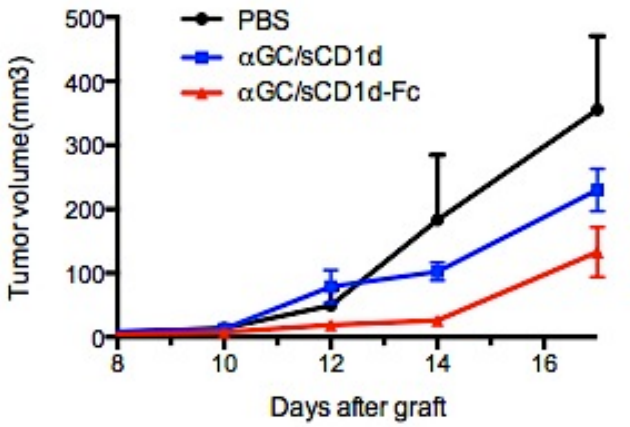


Figure 4: Kinetic of B16-F10 tumor growth.
Mice were treated at day 7 after tumor graft with αGC/sCD1d-Fc (red line) or αGC/sCD1d (blue line) or PBS (dark line). Graph represents tumor kinetic as the mean of tumor volume of 5 mice per group +/- SEM.

This result suggested that due to the increased avidity and/or stability of αGC/sCD1d-Fc dimer, systemic activation of iNKT cells could result in antitumor effect, despite the absence of tumor targeting. This approach may allow the treatment of tumors for which no tumor antigen is known or no scFv available.

The Fc domain has the advantage the property of dimerization, however, since the recombinant αGC/sCD1d fusion binds to the TCR of iNKT cells, we cannot exclude the possibility that effector cells such as NK, activated by the Fc domain, may exert their cytotoxicity directly against iNKT cells and dampen the effects of the CD1d fusion. In the same time, Fc-mediated effector effects could provoke toxicity against normal Fc receptor bearing cells. Indeed, after treatments with the αGC/CD1d-Fc fusion, we have often observed a rapid death of mice. To prevent these toxic effects, we have mutated the Fc residue Asn^{174} (Ala) that is critical for the FcγRIII/CD16 binding, which permits to suppress the ADCC (233). Further experiments are needed to evaluate the toxicity of the αGC/sCD1d-Fc dimer, and compare it to the αGC/sCD1d-mutated-Fc dimer.

Other possibilities were developed to extend the half-life of therapeutic proteins, especially those with a small molecular mass, rapidly cleared from circulation. It includes the conjugation of polyethylene glycol chains, the addition of N-glycosylation sites and the fusion to the albumin protein or albumin-binding domain, which generate more neonatal FcR recycling process (234). These extended derivatives led to a better distribution of bispecific single-chain diabody in the tumor and therefore its cytotoxicity (234). Such alternatives could be employed to prolong the half-life of our recombinant αGC/sCD1d and it should result in the enhanced activity of iNKT cells and thus to better anti-tumor effects.

In conclusion, the increase of affinity of the targeting scFv, the choice of the targeting of stromal cells from tumors and the addition of Fc dimerization, as well as the increase of circulating half-life of the recombinant CD1d protein are promising aspects, which might improve the antitumor benefits induced by the αGC/sCD1d-scFv fusion.

GENERAL DISCUSSION AND PERSPECTIVES

The major goal of my PhD project was to develop a cancer immunotherapy based on the activation of iNKT cells by recombinant CD1d fusion protein loaded with glycolipid ligand αGC. First, we assessed the antitumor efficacy of CD1d tumor targeting by its fusion to an antitumor scFv antibody fragment. Second, we evaluated the combination of this therapy to a cancer vaccine, and finally, we investigated alternatives of CD1d fusion proteins.

In Chapter 1, we demonstrated the potential of targeting recombinant CD1d-antitumor scFv fusion proteins in a tumor mouse model, as well as in human settings. Our strategy offers two main potent features, which are attractive in harnessing iNKT cells against cancer (92). First, the recombinant αGC/sCD1d-scFv fusion has the capacity to activate iNKT cells without inducing anergy (92). Indeed, when the αGC is injected as a free ligand, iNKT cells rapidly encounter an anergic state that prevents them to be restimulated later (183). Several studies reported that the anergy of iNKT cells was due to an overstimulation and resulted from up-regulation of the co-inhibitor receptor PD-1 (185). In our experiments, iNKT cells activated by the αGC/CD1d-scFv fusion showed similar up-regulation of the PD-1 receptor, but they still produced IFNγ upon repeated injections, suggesting that PD-1 is not sufficient to mediate the iNKT cell anergy. Similarly, we showed that human iNKT cells were fully activated by αGC/CD1d-scFv fusion, despite high expression of PD-1. Indeed, before *in vitro* activation, human iNKT cells from PBMCs of healthy donors already expressed PD-1 receptor, which was likely due to previous *in vivo* activations. Recently, the involvement of PD-1 in T cell anergy and exhaustion has been tempered and is now rather described as an activation marker, which is expressed on effector memory CD8 T cells of healthy donors, and does not correlate with the exhausted phenotype (235). One reason for the sustained reactivity of iNKT cells to our αGC/CD1d-scFv fusion could result from the direct triggering of the iNKT invariant TCR

without the need of APC, which may avoid the interaction between co-inhibitory receptors such as PD-1 and their respective ligands expressed on the majority of APCs. We also have observed that recombinant αGC/CD1d-scFv fusion induced much faster high IFNγ and TNFα cytokine production after treatment than free αGC ligand. This last observation implies a mechanistic difference in the activation of iNKT cells depending on whether it occurs via endogenous CD1d presented by APC or via the recombinant αGC/CD1d-scFv fusion. For instance, it was shown that free αGC requires intracellular processing to be loaded on CD1d before being exported to the surface of APC (135), which certainly takes more time than the direct triggering of the αGC/CD1d-scFv fusion to activate iNKT cells. We thus propose that the direct triggering of the invariant TCR by the αGC/CD1d-scFv fusion in the absence of co-stimulatory molecule provided by APCs, does not impact on the amplitude of the activation or duration of the invariant TCR signaling, but likely prevents the negative feedback exerted by APCs.

The second advantage of the αGC/CD1d-scFv fusion resides in the tumor targeting via its anti-TAA scFv, which allows redirecting the immune response to the tumor. We have shown that the specific tumor targeting of the CD1d fusion, as compared to the non-targeted CD1d fusion, resulted in a higher reactivity of iNKT cells upon several stimulations, as shown by the increased cytokine production associated with better anti-tumor effects in mice (208). This observation was also confirmed by *in vitro* experiments with human iNKT cells in which only the tumor-targeted CD1d fusion redirected the killing to tumor cells. For instance, the KATO III tumor cell line that expressed both HER2 and CEA antigens, was efficiently killed by iNKT cells upon incubation with both αGC/CD1d-anti-HER2 and -CEA fusions. In this context, human iNKT cells were highly activated and cytotoxic, likely due to the aggregation

of the recombinant αGC/CD1d-anti-tumor scFv fusion on the surface of tumor cells, which potentiated the iNKT cell stimulation.

So far, the clinical benefits of several strategies based on iNKT cell-mediated immunotherapy have remained modest (170, 177). The first clinical trial relied on the direct injection of the αGC as a free ligand, which showed a correlation between the production of Th1 cytokines and the iNKT cell number, without however clinical response (170). The discovery of the anergy-induced by free αGC injection promoted the strategy of autologous transfer of αGC-loaded DCs. Administration of αGC-loaded DCs in cancer patients resulted in prolonged activation of iNKT cells, and thus potent transactivation of NK cells, which produced IFNγ. However, these outcomes were made only after two successive iNKT stimulations, which suggested a gradual induction of the anergic state (172, 174). Our approach consisting in the repeated administration of recombinant αGC/CD1d-scFv treatments would permit to bypass the anergic state of iNKT cells and avoid individualized heavy and onerous cell-mediated therapy. Altogether, the sustained iNKT cell activation associated with the potent transactivation of other immune cells at the tumor site, should lead to an enhanced clinical response.

An alternative to CD1d tumor targeting is to enhance iNKT cell activation by the construction of a dimeric recombinant CD1d, which would display a better avidity for the invariant TCR. However, the association of two CD1d molecules by its fusion to an Fc domain may also lead to systemic toxicity resulting by Fc-mediated effector functions. Mutating the amino acid residue required for FcR binding should prevent the Fc-mediated ADCC while retaining the avidity of the dimeric CD1d and the improved circulating half-life provided by the Fc domain. The dimeric CD1d-Fc protein does not have the property to target the tumor but

could be tested against various tumors for which there are no surface antigen and/or high avidity scFv available.

Another attractive option is to target the CD1d molecule via its fusion to an scFv with high affinity for antigens over-expressed in the tumor stroma or neo-vessels. However, our attempt to target CD1d to VEGFR-3 as a marker of tumor neo-vessels and neo-lymphatics was unsuccessful, likely because of the too low affinity of the anti-VEGFR-3 scFv used and by the weak VEGFR-3 expression. Over-expressed stromal proteins belonging to the extracellular matrix such as tenascin C or fibronectin spliced variants, would be good candidates (231).

Additional aspects are envisaged to improve the efficacy and stability of the recombinant αGC/CD1d-scFv fusion proteins, in particular the use of different αGC analogs and the cross-linking of the glycolipid ligand to the recombinant CD1d-scFv protein.

The development of glycolipid analogs with discrete modifications of the glycosyl head group and/or the lipid tail were shown to polarize iNKT cells towards a Th1 or a Th2 cytokine profile (122, 135, 136). In this respect, we want to assess if Th1 or Th2-biased αGC analogs loaded on recombinant CD1d-scFv fusion could improve the anti-tumor effects obtained after repeated treatments with αGC/CD1d-scFv fusion proteins, Preliminary results with the DB03-4 αGC analog, provided by Professor S. Porcelli (Albert Einstein College of Medicine, New York), showed a better Th-1 cytokine profile when loaded on the recombinant CD1d-scFv fusion as compare to the conventional αGC used so far. Several studies investigated the mechanisms regarding the iNKT cell response to different ligands (141, 236). One important finding was that high affinity glycolipids for CD1d promoted a prolonged interaction with the invariant TCR and therefore skewed the cytokine pattern towards TH1 profile (237). In contrast to MHC molecules, the CD1d displays some degree of flexibility and can fit the pocket to galactose-modified glycolipids that results in superior Th-1 and sustained iNKT cell

responses (237). We hypothesize that the αGC analog could also result in a profound change of the binding stability when loaded on the recombinant CD1d protein, and therefore modify the interaction with the invariant TCR resulting in the sustained activation of iNKT cells.

A second important aspect is the stability of our recombinant CD1d-scFv fusions that depends on its correct loading with the glycolipid ligand especially when injected *in vivo*. Indeed, a loss of αGC from the fusion protein would on one hand induce its unfolding and degradation, and on the other hand, the free ligand would get presented by CD1d-expressing cells resulting in iNKT cell anergy. In this context, Professor S. Porcelli and his colleagues at the Albert Einstein College in New York, have developed an αGC analog containing a chemical modification that permits the ultra-violet cross linking of the ligand inside the CD1d pocket. This strategy allows stabilizing the interaction between the recombinant CD1d fusion and the ligand, which should optimize the sustained activation of iNKT cells. Indeed, our preliminary results are showing an enhanced Th-1 cytokine release and a better sustained activation in mice treated with the cross-linked αGC/CD1d-scFv compared to the loaded αGC/CD1d-scFv fusion protein.

The second chapter of this PhD thesis focused on the combination of the αGC/CD1d-anti-TAA fusion therapy with therapeutic cancer vaccine in order to possibly gain synergic anti-tumor protection.

Many factors contribute to the escape and the growth of tumor (12), which support the rationale for combined immunotherapies in order to simultaneously attack the tumor from different sides. As an example, the use of immune-modulating doses of chemotherapy, like cyclophosphamide or doxorubicin, which is commonly used as single treatment in several cancers, have demonstrated a potent amelioration of vaccine responses and is now often used

in combination with antibody-mediated therapies (238) (239). Recently, it was shown that the chemotherapeutic drug lenalidomide (LEN) synergized with αGC-loaded DC infusion to enhance clinical tumor regressions (240). In addition, numerous studies investigated the combination of NKT-based therapy with other treatments such as radiation and CTLA-4 blockade, immune-modulating antibodies or Treg depletion (241-243). All of these combination strategies considerably enhanced therapeutic cancer treatment. But so far, it remains unclear whether we could obtain synergic anti-tumor effects by using a NKT-based therapy together with a cancer vaccine. Here, we have demonstrated the advantage of combining our NKT-therapy, based on the sustained iNKT cell activation, and a simultaneous cancer vaccine, as shown by increased antitumor effects against B16 aggressive tumor grafts. In concordance with results showing the potent capacity of activated iNKT cells to promote the adaptive immunity (203), we have shown that the recombinant αGC/CD1d-scFv fusion was efficient to act as a vaccine adjuvant. Importantly, the major advantage of our fusion to activate iNKT cells is driven through the specific TAA targeting, which permits to redirect both innate and adaptive immune responses at the tumor site. Indeed, after the efficient priming of antigen-specific CD8 T cells induced by the OVA peptide vaccine formulated with the TLR ligand CpG-ODN and the recombinant αGC/CD1d-scFv fusion, TILs were enriched in antigen-specific CD8 T cells. Previous studies reported an enhanced antitumor effects by using αGC-loaded DC or αGC-loaded tumor cells as vaccine adjuvants, but did not show enrichment of antigen-specific CD8 T cells in the tumor (150, 182). In contrast, in our settings, almost 50% of tumor infiltrated CD8 T cells were antigen specific, already 7 days after the vaccination. These findings correlated well with the strongest anti-tumor effects obtained with the combined therapy.

Our combination strategy also included a TLR agonist, which was shown to be a great modulator of the DC maturation (193). Previous report showed that co-injection of αGC

together with TLR ligands, such as Poly I:C, CpG-ODN or flagellin, resulted in enhanced maturation of splenic DC and therefore increased their immunomodulatory effects by inducing a highly Th-1-biased immune response (204). We also found an increased production of IFNγ, IL-6 and TNFα in the serum of mice after combination of αGC/CD1d-scFv fusion and OVA-CpG vaccine. Moreover, we observed a strong proliferation of NK cells that was likely mediated by pro-inflammatory cytokines released after NKT activation and matured DC.

Altogether, our present strategy underlies the importance to promote innate and adaptive responses to obtain a synergic and efficient anti-tumor response. In addition, the tumor targeting allows the immunotherapy to be more efficient with no adverse toxicity effects. Further experiments are required to evaluate the respective role of either NK cells or antigen-specific CD8 T cells on anti-tumor effects.

Tumor cells have evolved to evade the immune surveillance by multiple mechanisms. In this context, it will be interesting to evaluate whether the sustained activation of iNKT cell by αGC/CD1d-anti-TAA fusion and the subsequent highly inflammatory environment helps to overcome the immunosuppressive environment at the tumor site. For instance, several studies have demonstrated that the depletion of immunosuppressive Treg cells, elicited a better tumor protection when combined with a NKT-based therapy (182, 243).

Promising data were also generated by treating cancer patients with an immune checkpoint blockade antibody against PD-1 (244). Moreover, anti-PD-1/PD-L1 antibody therapy was shown to be efficient to enhance iNKT cell activity and consequently anti-tumor effects resulting from iNKT cell stimulation (185). After αGC/CD1d-anti-TAA fusion treatment, we observed an up-regulation of PD-1 on iNKT cells, likely resulted from activation. Treatments with anti-PD-1 and anti-PD-L1 antibodies could act simultaneously with αGC/CD1d-anti-

TAA fusion to enhance the sustained activation and also prevent a possible exhaustion of CD8 T cells infiltrated into the tumor. In addition, the strong increase of PD-L1 on tumor cells, upon CD1d therapy, would support the use of combined NKT activation with an anti-PD-L1 antibody treatment.

Encouraging studies were made by adoptively transferring autologous CD8 T cells, which display antitumor properties and particularly the transfer of expanded autologous TILs (245). On the other hand, best clinical responses were obtained in recent clinical trials involving the autologous transfer of *in vitro* expanded iNKT cells from cancer patients (171). Administration of activated iNKT cells resulted in enhanced IFNγ production and one recent study showed good clinical responses in HNSCC patients (217).

To conclude, we have demonstrated that αGC/CD1d-anti-TAA fusion is a promising approach for cancer treatment. The tumor targeting of CD1d molecules offers the advantage of redirecting the innate and the adaptive immune responses to the tumor site, which should increase efficacy and clinical benefits. Furthermore, this NKT therapy could be associated with other immunotherapeutic approaches to obtain synergic anti-tumor effects.

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