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Natural Killer cells and monoclonal antibodies: two partners for successful antibody dependent cytotoxicity against tumor cells.

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Abstract

Monoclonal antibodies targeting tumors are one of the most important discoveries in the field of cancer. Although several effective antibodies have been developed, a relapse may occur. One of their mechanisms of action is Antibody Dependent Cell Cytotoxicity (ADCC), by engaging the Fc γ receptor CD16 expressing Natural Killer cells, innate lymphoid cells involved in cancer immunosurveillance and able to kill tumor cells. A lack of NK cells observed in many cancers may therefore be a cause of the low efficacy of antibodies observed in some clinical situations. Here we review clear evidences of the essential partnership between NK cells and antibodies showed *in vitro*, *in vivo*, and in clinical trials in different indications, describe the hurdles and ways to enhance ADCC and the evolution of monoclonal antibody therapy. NK cell adoptive immunotherapy combined with monoclonal antibodies may overcome the resistance to the treatment and enhance their efficacy.

1. Introduction

Tumor targeted antibodies are one of the most important development in the field of cancer therapy in the last 20 years[1]. Their first clinical success was in the treatment of B cell malignancies. Since then, many new antibodies have enter the clinical practice for colorectal, breast, lung, hematopoietic malignancies and many others indications, with a list increasing each year. Although several effective antibodies have been developed, long term durable response remain elusive and relapse still occurs, representing a major limitation of this therapy. One of the anti-tumor mechanisms of IgG1 monoclonal antibodies is antibody dependent cell cytotoxicity (ADCC) through the binding of Fc γ receptors. ADCC is mainly performed by Natural Killer (NK) cells and cytotoxic T lymphocytes like $\gamma\delta$ T cells representing 1 to 6% of the circulating leukocytes that express Fc γ RIIIa/CD16A as predominant Fc γ Receptor. So the anti-tumoral efficacy of monoclonal antibody therapy relies of the presence and functionality these cells.

In this review, we focused on the partnership between NK cells and therapeutic antibodies to exert anti-tumoral effects. This review is divided in the following five main sections: (1) a brief summary of NK cells main features, with a focus of the ADCC mechanism mediated by these cells, (2) the main indications of therapeutic antibodies and their interaction with NK cells, *in vitro*, *in vivo* the results of published clinical trials and eventually the design of ongoing ones described on *clinical trials.gov* based on a combination therapy between NK cells and antibodies, (3) the hurdles to the ADCC mechanisms, (4) the ways to enhance ADCC and (5) the perspective of the treatment with antibodies.

1.1 Main features of NK cells

CD56+ CD3- Natural Killer cells are known to play a role in cancer immune surveillance and their trafficking around the body is a major component of their anti-metastatic function. Their role in the specific context of cancer was discovered in NK deficient or depleted animal models[2] where lack of NK cells results in a lack of tumor cell rejection. Moreover, it was shown that various NK cell abnormalities correlate with an increased risk of cancer and aberrant functions of NK cells have been found in cancer patients[3]. Dysfunctional NK cells have been detected in some solid tumors and hematological malignancies. For some of these cancers, it has been suggested that endogenous NK cell function can be restore after treatment with cytoreductive chemotherapy or targeted drugs or corrected with the help of an adoptive cell transfer. Thus, the important role of NK cells in tumor recognition, metastasis control, cancer cell destruction and new discoveries about their functional enhancement, has made them to be considered as a treatment option in the field of immunotherapies although some questions still remain about their use (Fig. 1).

NK cells are ideal candidates for immunotherapy as they are not HLA restricted on the opposite to T cells, recognize and lyse tumor targets immediately and are known to induce graft versus tumor effect without graft versus host effect. As innate lymphoid cells, they don't need a first contact with an antigen, and can distinguish tumor or virus infected cells from healthy cells through activating and inhibitory receptors that bind to self-ligands expressed on the cell surface[4]. Activating receptors include natural cytotoxicity receptors (NCR; i. e NKp30, NKp44, NKp46), NKG2D, CD16, activating KIR (Killer Immunoglobulin like Receptor), CD226 (DNAM-1) and CD244, belonging to SLAM family receptors. Inhibitory receptors involve inhibitory KIR, CD94/NKG2A, T cell immunoglobulin and mucin domain containing molecule-3 (Tim-3), Lymphocyte

Activation Gene -3 (LAG-3), T cell Immunoreceptor with IgG and ITIM domain (TIGIT), CD96, CTLA-4 and Programmed Death-1 (PD-1), the latter are considered as immune checkpoints. Two main mechanisms have been described for NK cell recognition of tumor cells. One is the “missing self-mechanism”, whereby NK cells detect the loss or the down regulation of MHC class I molecule. The other is the “induced self” where NK cells recognize activating ligands present or overexpressed by tumor cells with the activating receptors. Next, a balance between inhibitory and activating signals determines NK cell engagement and cytotoxicity based on the release of granzymes and perforin leading to the final apoptosis of the tumor target. Furthermore, some activating receptors seemed to be involved in the recognition of potentially metastatic cells. During epithelial to mesenchymal transition, these cells lose inhibitory receptor ligand expression such as cadherin1 but also increase multiple NKG2D ligands such as ULBP2 or MHC Class I polypeptide related sequence A (MIC-A), rendering them more detectable by NK cells[5]. NK cells can also be cytotoxic directly through the FASL/FAS or the TRAIL/TRAILR pathway, or through indirect activation of immune cells by the release of cytokines like IFN- γ .

Among the activating receptors, Fc receptors and particularly CD16, represents one of the most powerful one. This receptor is encoded by FCGR3 gene and acquired during maturation. It is called Fc γ RIII because of its ability to bind the constant fragment of immunoglobulins (Ig) G1 and G3. The FcR γ IIIa isoform of the receptor is mainly expressed by CD56 dim NK cells, a subpopulation of cytotoxic NK cells mainly found in the peripheral blood[6]. CD16a on NK cells is composed with 2 Ig like extracellular domains, a transmembrane portion and a cytoplasmic tail associated with a disulfide linked homodimers containing an ITAM (Immunoreceptor Tyrosine based Activation Motif) [7]. Upon IgG cross linking, the receptors become close to lipid rafts where they co-localize with Src kinases. Src kinases phosphorylate tyrosines within the ITAM, providing docking site for Syk. Multiple molecules can next be phosphorylated by Syk like Phosphatidylinositol 3 kinase, Vav1, Phospholipase c γ and SLP76. These molecules activate signaling pathways leading to cellular response like cytokine secretion or ADCC mechanism. Since most therapeutic monoclonal antibodies are IgG1 molecules, they can engage CD16a expressed by NK cells and trigger ADCC by this way.

2 Main indications of therapeutic antibodies and their interaction with NK cells

2.1 NK cells and myeloma

Multiple Myeloma (MM) is usually treated with a combination of therapeutic approaches like chemotherapy, steroids, and radiations. In the last few years, novel drugs including proteasome inhibitors (PI) such as bortezomib and carfilzomib, and immunomodulatory agents such as thalidomide, lenalidomide, and pomalidomide have entered the clinical routine. Recently, the introduction of monoclonal antibodies in MM therapy, represented a promising step forward in improving treatment outcomes.

2.1.1 *elotuzumab*

Elotuzumab, a monoclonal antibody targeting the glycoprotein CS1 can be combined with the drugs cited above. CS1 also known as SLAMF7 (CD319) belongs to the SLAM family receptors comprising 9 members[8]. Given

its high and very common expression on myeloma cells, SLAMF7 was considered as a target for myeloma therapy. Of note, it is also expressed on other lymphocyte subsets, including T cells and NK cells and can be also shed into the circulation. Despite SLAMF7 expression on NK cells, it seems that there is no significant fratricide between NK cells themselves in presence of elotuzumab. Actually, elotuzumab mediates either antibody dependent cell mediated cytotoxicity (ADCC) of CS1 expressing myeloma cells by NK cells or activates them by binding to their own CS1. In the first situation, the interaction of elotuzumab with NK cells is mediated by the binding of the Fc part of this IgG1 antibody to the Fc γ RIIIa / CD16 isoform [9]. *In vitro* studies have shown that elotuzumab promotes NK cell degranulation that correlates with SLAMF7 expression and that it requires the Fc fraction of the antibody. Furthermore, better results were observed with non-fucosylated elotuzumab, which has a higher affinity binding to CD16[10]. In the second situation, elotuzumab can cause myeloma cell death via NK cell direct activation[11].

Regarding animal models, few studies evaluated the effect of a combination therapy based on NK cells and elotuzumab as the antibody is not able to bind mouse SLAMF7. Bezman et al[12] developed a mouse tumor cell line expressing hSLAMF7. They observed that Fc γ receptor-expressing cells (NK cells and CD8⁺ T cells) are necessary to induce an anti-tumoral effect in combination with a murine immunoglobulin G2a version of elotuzumab. Beneficial effects were also observed in combination with an anti-PD-1 antibody such as an increase of NK cells found inside the tumor. Therefore, future investigation of anti-PD-1/ elotuzumab bitherapy in MM patients could be of specific interest. Future studies using knock-in mice expressing hSLAMF7, or elotuzumab similar antibodies binding mouse SLAMF7, will help to determine the role of SLAMF7 activation to elotuzumab-mediated antitumor effect.

Two clinical trials were designed for the use of NK cells in combination with elotuzumab. One is a phase 2 trial from the Anderson Cancer Center with an estimated enrollment of 60 participants (NCT01729091). Patients with high risk myeloma will be treated with elotuzumab, then chemotherapy and infusion of umbilical cord blood (UCB) derived NK cells followed by an autologous stem cell transplant. The primary objective is to determine the maximal tolerate dose of UCB-derived NK cells and the secondary objective to quantify NK cells persistence into patients. The second study is set up by the University of Arkansas and starts the recruitment of patients. This phase II trial has an estimated enrollment of 10 patients (NCT03003728). Elotuzumab infusions will be performed before and after an autologous stem cell transplant (ASCT), and autologous expanded NK cells will be infused 2 days after ASCT. In addition, patients will receive 4 subcutaneous infusions of an IL-15 superagonist (ALT803) at one week interval. The designers of the trial aim to see a myeloma response within 100 days post-NK cell infusion and aim also to collect the frequency and severity of adverse events during the patient follow up.

2.1.2 Daratumumab

Daratumumab and its use in combination with other agents is becoming a new standard of care for treatment of MM.

Daratumumab is a human IgG1 κ antibody directed against CD38, a 46-kDa type II transmembrane glycoprotein. CD38 structure relies on a long extracellular domain, a transmembrane region and a short cytoplasmic tail [13]. CD38 has been showed to have different roles. Among them, CD38 was shown to be a receptor of CD31, also

known as PECAM-1, expressed on endothelial cells and other tissues. The CD38–CD31 interaction is involved the migration of leukocytes through the endothelial barrier and triggers their activation and proliferation. CD38 plays also a role of an ecto-enzyme, hydrolyzing nicotinic acid adenine dinucleotide phosphate[14]. CD38 is expressed on lymphoid and myeloid cells and on non-hematopoietic tissues. The protein can be found on hematopoietic progenitor cells, not on mature cells although it was observed on activated lymphocytes. Eighty percent of resting NK cells expressed low level of CD38. CD38 is also found at high levels on B differentiated plasma cells, interesting in the context of myeloma, a plasma cell disease.

At this moment, several anti-CD38 antibodies are evaluated in clinical trials, with daratumumab already approved alone or in combination with conventional treatments of MM. This drug was originally developed by Genmab and received the status of Orphan Drug by the US FDA and the EMA in this indication. It is indicated for patients with MM after more than 3 lines of treatment, including a PI and an immunomodulatory drug (IMiD) or who are refractory to both [15]. Daratumumab has lots of mechanisms of action described in the review of Sanchez et al [16]. Briefly, daratumumab is able to induce complement (a component of serum) dependent cytotoxicity and antibody dependent phagocytosis mediated by macrophages. This last mechanism seems to be particularly important in the bone marrow where these cells have an Fc-dependent anti-tumor function[17]. The antibody can also initiate apoptosis of MM cells through Fc mediated cross-linking [18].

This anti-CD38 antibody mediates also ADCC, where NK cells play a critical role, assessed by many studies showing a clear benefit of the NK cell-daratumumab combination for MM treatment. Other parameters like tumor microenvironment or the co-stimulation of NK cells via other activation pathways have also been examined as they can influence NK response towards MM. Mahaweni et al [16] focused on the influence of the tumor microenvironment (TME) on NK cell ADCC. The tumor microenvironment is known to be immunosuppressive and a potential hurdle of many anti-cancer treatments [19]. They hypothesized that it may be a synergy between the ADCC mechanism and killer-immunoglobulin like receptor (KIR)-ligand mismatched NK cells to potentiate the NK cell anti-tumor effect against MM and overcome the immunosuppressive effect of the TME. They selected 3 factors known to play a role in the TME, lactate, hypoxia and PGE2 and analyzed their influence on daratumumab mediated ADCC. They observed that TME Factors reduced NK cell cytotoxicity but that the addition of daratumumab enhanced their cytotoxicity with a limited benefit when using KIR ligand mismatched NK cells. This study suggested that choosing KIR ligand mismatched NK cells in combination with therapeutic antibody could improve the anti-tumor response especially since the tumor could down regulate the antigen targeted by the antibody.

As described for other antibodies, NK cells acquired the activation marker CD137 upon contact with daratumumab coated surface or daratumumab bearing MM cells. Ochoa et al[20] explored this phenomenon and showed that the anti-CD137 antibody urelumab synergized with the anti-CD38 and enhanced CD25 expression and IFN production. Although they did not observe any increase in ADCC *in vitro*, they developed an original mouse model based on the intravenous administration of human NK cells, the anti-CD38 followed by the administration of the anti-CD137 antibody after injection of luciferase transfected myeloma cells. They observed a benefit in adding the anti-CD137 close to daratumumab treatment with a reduction of tumor growth and an enhanced survival of the animal model suggesting an advantage of the co-stimulation of NK cell activation pathway to enhance anti-CD38 therapeutic efficacy.

However, regarding the recent published data, NK cells exposure to daratumumab seems to have either positive or negative effects on their functions [21]. Actually, CD38, the target of daratumumab, is expressed by NK Cells and there is some evidence that NK cell fratricide can be observed during daratumumab therapy. The data collected during 2 single agent daratumumab studies Gen501 and SIRIUS [22] showed a relationship between the dose of daratumumab and the decrease in patient peripheral blood NK cell counts. The observed decrease was reversible after the end of the treatment. This was also described by Wang et al[23], as they detected a significant reduction of NK cells percentage in the peripheral blood of daratumumab treated patients and that the remaining NK cells were mainly CD38-/low. The data suggested that CD38 high NK cells were susceptible to daratumumab mediated depletion but not CD38-/low cells and that CD16 expression of NK cells is essential to daratumumab mediated apoptosis as no effect was seen in presence of an Fc digested fragment of the antibody. However, the CD38-/low NK cells have very interesting properties as they have a high expansion capacity and are able to kill MM cells using ADCC in presence of daratumumab, *in vitro* and *in vivo*. Therefore, the authors concluded that the injection of expanded autologous CD38-/low NK cells may overcome the NK cell fratricide induced during daratumumab treatment.

Taken together these studies suggested that daratumumab helped to overcome the immunosuppressive environment of the tumor and that it synergized with urelumab to potentialize NK cell response. Furthermore, adoptive immunotherapy with NK cells helps to overcome the decrease in these cells observed during daratumumab treatment. Nevertheless, until now, no clinical trials were designed to study the impact of an NK cell immunotherapy combined with daratumumab, on the MM response to treatment.

2.2 NK cells and B cell malignancies

Anti-CD20 monoclonal antibody based immunotherapies have represented a breakthrough in the treatment of several B cell malignancies expressing this antigen. Rituximab is considered as the reference molecule for the comparison with new generation of antibodies, designed to overcome rituximab refractoriness, like Obinutuzumab which has now reached clinical use for untreated LLC or refractory lymphoma[24]. The anti-lymphoma effect of this antibody mediated therapy is based on an increase degranulation of NK cells. Regarding the interaction of NK cells with anti-CD20 antibodies, some studies have been published trying to decipher the different modalities of this interaction[25] .

Binding of anti-CD20 antibodies to CD16 expressing cells like NK cells seemed to be conditioned by different parameters. Obinutuzumab is a humanized IgG1K monoclonal antibody with a core fucose removed in the Fc portion of the antibody to enhance the binding affinity to CD16 thus improving ADCC. Compared to rituximab, it was shown to bind more efficiently CD16 especially when serum complement was present. Obinutuzumab was shown to be able to bind and activate NK cells and to induce ADCC in presence of serum whereas rituximab was not[26]. Another parameter that can influence obinutuzumab or other anti-CD20 antibodies binding to NK cells is the other receptors expressed by NK cells. For example, NK cells express a wide array of inhibitory or activating receptors and licensing status of NK cells affects their capacity of activation through Fc γ RIII as well as inhibitory signals derived from KIR/ HLA interaction may influence ADCC. Compared to rituximab, obinutuzumab mediated ADCC is less influenced by licencing status and target cell depletion does not seem to be influenced by KIR/ HLA interaction[27].

Some studies have been published aiming to address the therapeutic potential of a co-administration of NK cells and anti-CD20 antibodies and some clinical trials are currently ongoing trying to address this issue (Table 1). Bachanova et al[28] reported 2 studies based on patients treated with haploidentical NK cells, rituximab and IL2. One was a phase I/II clinical trial (NCT 00625729) with 6 enrolled patients with non-Hodgkin lymphoma or chronic lymphocytic leukemia designed to determine if allogeneic NK cells infused following chemoimmunotherapy can be safely expanded *in vivo* with IL-2 (Aldesleukin). Unfortunately, this study did not reach the primary outcome as no NK cells were detected *in vivo* after infusion. The second one was a phase II clinical trial (NCT01181258) on 16 patients. The treatment was well tolerated and produced a remission in 1 out of 4 patients with refractory non Hodgkin lymphoma after 2 months. Transient highly functional CD16 expressing NK cells were detected in most of the patients whereas autologous NK cells exhibit poor functions. Some factors supposed to influence the response to therapy were also highlighted like the level of T Reg or myeloid derived stem cells correlating with worse outcome or cytokine level like IL15, correlating with a clinical response. Some questions that remain to be asked in clinical studies are the need of a lymphodepleting therapy before NK cells infusion, the use of cytokine like IL2 or IL15 to sustain NK cell proliferation *in vivo* and the best source of NK cells.

2.3 NK cells and metastatic colorectal carcinoma

Colorectal cancer (CRC) is the third most frequent cancer, with more than 1 million cases per year in the world according to the World Health Organization. Despite the development of new therapeutic strategies, colorectal cancer remains a disease far from being controllable. One of the challenges is to reduce the risk of recurrence or to prolong the survival of patients with liver metastases. The improvement of therapeutic strategies could come from a better understanding of the immune cell behavior in colorectal cancer and by the development of new immunotherapies. For example, the tumor and metastasis infiltration by T cells and NK cells was found to be associated with a better prognosis [29], as well as a high amount of circulating NK cells was inversely correlated with the presence of metastasis at clinical presentation[30]. NK cells were also shown to be able to target cancer initiating cells, involved in tumor recurrence, because of their high expression of ligands for the natural cytotoxic receptors NKp30 and NKp44 and their low expression of MHC Class I molecules[31]. On the opposite, an under expression of CD16, NKG2D, CD161, DNAM-1, Nkp46 and Nkp30 [32] and a decrease in NK cell cytotoxicity was associated with tumor relapse in CRC patients[33]. These observations were then followed by the discovery that chemotherapy commonly used to treat this disease like 5-fluorouracil, sensitized cancer cells to NK cell mediated lysis[34]. This was confirmed in a clinical trial based on patients treated with a combination of chemotherapy and adoptive immunotherapy based on autologous NK cells. An improvement in the progression free survival (PFS) and overall survival (OS) was seen for patients receiving more than 3 courses of NK cells[35].

Regarding monoclonal antibodies that have been developed for the treatment of CRC, cetuximab is a good candidate to be combined with NK cells for ADCC against CRC. Cetuximab is a chimeric IgG1 monoclonal antibody binding the extracellular domain of the epidermal growth factor receptor(EGFR). This receptor is expressed on normal cells as well as on tumor cells with a different intensity of expression. When EGFR binds its ligand EGF, this leads to the activation of signaling pathways like the RAS-RAF- mitogen activated protein kinase (MAPK) and the PI3K-PTEN-AKT-pathways both involved in cell survival and proliferation[36]. Thus,

any dysregulation in the EGFR cascade can result in rapid cell division supporting tumor growth. EGFR is overexpressed in 85% of CRC patients and its expression is correlated with a poor prognosis, making it a good target for monoclonal antibody therapy. Cetuximab has been approved as a first line treatment of EGFR positive metastatic colorectal cancer[37]. However, anti-EGFR therapy have no effect in metastatic patients presenting a tumor with mutation in the KRAS gene[38]. KRAS gene activating mutation constitutively activates the RAS signaling pathway and can predict resistance to anti-EGFR antibodies. These KRAS mutations are found in approximately 35 to 40% of the patients with CRC[39]. Therefore, development of new strategies of treatment for KRAS mutated tumors are highly desirable.

Cetuximab mechanism of action not only relies on ADCC but also on its interaction with immune cells[40]. The treatment by cetuximab modifies the immune cell repartition in CRC patients and enhances tumor infiltrating CD56+ cells when used as first line. After treatment, CRC patients showed an increase in CD3, CD8 T cells and NK cells. Furthermore, CD137 and CD107a expression of NK cells were increased after 4 weeks of cetuximab treatment suggesting that cetuximab treatment could promote the cytotoxic activity of PBMC and especially NK cells[41]. Other studies have confirmed the benefic interaction between cetuximab and NK cells. *In vitro*, resting NK cells isolated from CRC patients were shown to be unable to display cytotoxicity against CRC, but overnight or 5 days incubation with IL-2 or IL-15, was able to restore their cytotoxicity against the tumor cells, further enhanced in presence of cetuximab[38]. Other *in vitro* experiments showed that activated peripheral blood NK cells combined with anti-EGFR antibodies (cetuximab or panitumumab) were cytotoxic against a wide range of tumor cell lines, regardless of the KRAS or BRAF status. As expected, cetuximab enhanced NK cell mediated lysis of EGFR positive cell lines, either on RASwt, RASmut or BRAFmut, in a CD16 dependent manner[42]. This effect is mainly due to the fact that NK cells recognize the surface bound antibodies and are able to kill tumor cells independently of the EFGR pathway activation. In addition, exposure of colon cancer cell lines to cytotoxic drugs like irinotecan, 5-fluorouracil or gemcitabine upregulated their EGFR expression, and rendered them more susceptible to cetuximab mediated ADCC, independent from the KRAS status[43]. Together, these data build a rationale to strengthen NK immunotherapy with cetuximab for RAS or BRAF mutant CRC patients and have paved the way to clinical trials. Two clinical trials have been published but no clinical trials are ongoing on the NK cells and cetuximab combination to treat CRC patients.

Upon the trials already published, a dose-escalating phase I trial on 9 patients was designed to evaluate the safety, toxicity and immunological response of the administration of autologous NK cells in combination with trastuzumab or cetuximab in patients with advanced colorectal or gastric cancer[44] (table 2). NK cells were expanded with IL-2, frozen before administration, and infused into patients 3 times at triweekly intervals 3 days after administration of the monoclonal antibody and a chemotherapy based on capecitabine. The most common adverse events observed were thrombocytopenia, fatigue and peripheral neuropathy which occurred at a similar rate to those previously described with that type of chemotherapy. A clinical benefit was observed for 4 patients. Regarding patient immunomonitoring, no follow up of NK cells was performed after administration but a decrease in peripheral T regs was observed after combination therapy and an increase of the IFN γ /IL-4 and IFN γ /IL-10 ratio was seen. Another phase I trial studied the effect of the intrahepatic infusion of allogeneic NK cells isolated after CD3+cell depletion and IL2 overnight activated to treat liver metastasis of gastrointestinal origin[45]. Prior to NK cell enriched infusion, 9 patients received a lymphodepleting chemotherapy based on

cyclophosphamide and fludarabine. Cetuximab was administered once weekly for 7 weeks and well tolerated. No dose limiting toxicity was observed. One patient showed a partial response of liver metastasis, one a dissociated response, one a stable and 5 a progressive disease. The immunomonitoring showed an expansion of Tregs and PD1+ T cells probably due to high dose of IL-2. Altogether, these trials showed that the combination of NK cells with cetuximab is safe and well tolerated but their limitation is clearly the small number of patients included. Additional studies on a higher number of patients are necessary to assess the clinical benefit of this association with an immunomonitoring of the patients to study the impact of the treatment on the immune cells, cytokine release and NK cell follow up *in vivo*.

2.4 NK cells and NSCLC

Non small cell lung cancer (NSCLC) is the major subtype of lung cancer, representing the leading cancer diagnosis and related death in the world[46]. In the recent years, biological therapies or immunotherapies have been evaluated in the treatment of NSCLC since outcomes are poor, especially for patients with EGFR overexpressing NSCLC. Regarding monoclonal antibodies, attention has been focused on anti-EGFR or VEGF antibodies, especially cetuximab and bevacimumab respectively but only bevacimumab is used as first line of treatment[47]. Regarding immunotherapy, this approach is mainly considered for patients not eligible for surgery. Two recent papers have been published aiming to evaluate the benefit of a combined approach of these two treatments. The first one is an *in vitro* study which uses a NK92 CD16 transduced cell line with cetuximab to increase the cytotoxic effect. Most of NSCLC cell lines used in the study were efficiently killed in presence of cetuximab through an ADCC effect. The second one is a clinical trial on 54 patients in 2 groups : group A with 2 courses of 3 injections of allogeneic KIR ligand mismatch NK cells combined with cetuximab, group B with cetuximab alone[48]. The treatment was well tolerated with nausea, fever or hypersensitivity reactions reported but no significant differences observed between the 2 groups. The results showed a significant difference between Group A and B regarding PFS and OS. Also, the combination therapy significantly reduced circulating tumor cell (CTC), carcinoma embryonic antigen and neuron specific enolase level, suggesting that NK cells in the blood stream are able to target CTC. These encouraging results suggested to evaluate the clinical benefit of the combination therapy on a larger cohort with a long term follow up. Currently, 4 clinical studies are recruiting, 3 in China and 1 in Korea, evaluating the safety and efficacy on NK cell injections to treat NSCLC patients and among then, 1 is planning to use anti-PD1 antibody with NK cells but none with anti-EGFR antibodies (NCT03958097).

2.5 NK cells and breast cancer

Every year, more than 1 million women developed a breast cancer around the world, despite an increasing rate of early detection. Among breast cancer, 2 types can be eligible for monoclonal antibody treatment: the triple negative breast cancer (BC) expressing high level of EGFR which can be targeted by the use of cetuximab and the Epidermal growth factor receptor 2 / HER2+ breast cancer for which trastuzumab or pertuzumab is used.

Patients with triple negative BC defined as estrogen, progesterone and HER negative cannot benefit from anti-HER2 or anti-estrogen therapy and this phenotype is associated with a more aggressive clinical course and a

worst outcome. Triple negative BC often expresses the EFGR (HER1) and cetuximab is able to bind this receptor. Despite strong preclinical data, its clinical efficacy remained questioned. Two phase II clinical trials showed a response in 20% of the patients when cetuximab was used in combination with chemotherapy[49], [50].

One way to improve the clinical results of cetuximab therapy is to enhance the cetuximab mediated immune mechanisms with Fc receptor bearing effector cells like NK cells. Indeed, NK cells are able to infiltrate the breast tumor and this infiltration often correlates to the treatment response and this population could be responsible for preventing metastatic spread[51]. However, the exploration of the cetuximab dependent cytotoxicity mechanism *in vitro* using cancer patient NK cells showed that ADCC is impaired probably because of an increased expression of the inhibitory receptor CD85j. But the inhibitory effect of CD85j can be reverted using anti-CD85J antibody and the cytotoxicity can be increased after IL-2 or IL-15 pretreatment of NK cells, by enhancing the expression of activating receptor such as CD16[52]. The benefit of a pre-conditioning by IL-2 or IL-15 was confirmed by a second study published by the same team and suggested that the combination of cetuximab with one or 2 cytokines may potentialize the clinical efficacy of cetuximab in triple negative BC[53]. Regarding HER2+ BC, this type of cancer overexpresses HER2, a member of the ErbB family, involved in oncogenic transformation and tumor growth and found in 20 to 30% of the patients. This overexpression is often associated with metastases and a reduced OS[54]. The humanized IgG1 anti-HER2 antibody trastuzumab (Herceptin) has been approved for the treatment of HER2+ breast cancer. The addition of HER2 antibodies like trastuzumab or pertuzumab to conventional treatments was shown to improve the relapse free and OS. Trastuzumab binds to HER2 transmembrane domain inhibiting its dimerization whereas pertuzumab binds to another site involved in the dimerization with other members of HER receptors[55]. Trastuzumab enhances HER2 degradation, and the inhibition of the (MAP) kinase pathway observed after its binding to HER expressing cells leads to an inhibition of the cell division [56]. The recruitment of NK cells and the induction of ADCC can contribute to its efficacy as observed *in vitro* studies and confirmed by the observation of an increased number of NK cells into the tumor after trastuzumab treatment [57]. The importance of the ADCC effect for the anti-tumor activity was also demonstrated in animal models as Knock-out mice for Fc γ RIII receptor showed a dramatic decrease of trastuzumab efficacy[58].

Based on these preclinical data, a clinical trial (NCT02844335; table 2) evaluated the benefit of a combination between cryoablation, allogeneic KIR mismatch NK cells and trastuzumab for the treatment of 48 patients assigned in 3 groups (cryoablation, cryoablation and NK cells alone or with the anti-HER2 antibody).

The evaluation of the safety showed that the treatment was well tolerated and no significant difference regarding the occurrence of adverse events was observed between the 3 groups. All the reported adverse events were below grade 3. Compared to cryoablation alone, the combination therapy improves the anti-tumor effects, enhances the immune function of patients significantly[59] and results in significant prolongation of PFS.

Nethertheless, it is important to note that only around 20% of the patients respond to the treatment, that some patients become resistant or less sensitive to anti-HER2 therapy and experienced relapse or disease progression. In addition, it has been shown that although encouraging ADCC results obtained *in vitro* in presence of IL2 or

IL12 and trastuzumab, no correlation was observed with clinical responses[60]. This raised the question on how to improve clinical responses to anti-HER2 treatment. Some interesting results showed that 2 anti-HER2 antibodies can synergize together as their binding on distinct epitopes of the HER extracellular domain provides more efficient response on tumor growth than one antibody alone. Furthermore, the combination of trastuzumab, pertuzumab and NK cells was shown to increase ADCC against HER2+ cells lines compared to each antibody alone and was able to target cancer stem cells[61] that were resistant to trastuzumab mediated cell cytotoxicity.

Among the clinical trials ongoing on breast cancer declared on *clinical trials.gov*, 2 have been achieved with results and relied on NK cells infusion combined with chemotherapy, 2 have an unknown status, one is recruiting but relying on NK cells alone expanded from cord blood. Taken together these preclinical results, a clinical trial based on a tri-therapy combining the two anti-HER2 antibodies and NK cells on a large cohort of patients would be of specific interest.

2.6 NK cells and brain or neuroectodermal origin tumors

Brain tumors are intracranial neoplasm located in the brain or surrounding meninges accounting for at least 1.4% of all cancers. Despite advances in current standard treatments, patients' outcome remains poor, due to recurrence of the disease and malignant progression. One of the challenges in brain tumor treatment is the presence of the blood brain barrier responsible for the limited access to systemically administered drugs, and for the protection against immune system surveillance. As such, the central nervous system has been considered as an immune privileged site because intracerebral injection of antigen failed to generate a classical immune response. But recently, this concept has been revised as immune cells can infiltrate brain tumors [62], [63]. According to different papers, NK cells are able to infiltrate brain tumors but the intensity of the infiltration clearly depends on the type of the tumor and their functionality main be affected by the tumor microenvironment. As an example, TGF β secreted by tumor cells and / or immunosuppressive cells has been shown to induce a downregulation of NKG2D expressed by NK cells[64] in gliomas.

2.6.1 Glioblastoma

Glioblastoma multiforme is a frequent brain tumor found mainly in adults with a median survival rate of 14 to 16 months following diagnosis. Aberrant activation of the EGFR pathway plays an important role in glioblastoma leading to tumor progression, metastatic spread and poor survival. Sixty percent of glioblastoma overexpress the common form of EGFR whereas 40% express the EGFR variant III mutant. But despite the expression of this therapeutic target, glioblastoma often shows a resistance to EGFR inhibitors, including tyrosine kinase inhibitors as well as antibodies like cetuximab, probably because of a limited access of the brain to hydrophilic molecules and to the redundancy of activating pathways into the tumor cells[65]. So monoclonal antibody therapy based on this growth factor receptor seems to be disappointing in this indication.

Regarding cellular immunotherapy of glioblastoma, different types of immune cells have been evaluated such as lymphokine activated killer (LAK)[66], $\gamma\delta$ T cells or NK cells. However, despite the increasing evidence that NK cells can infiltrate the tumors, no study has been published regarding the use of NK cells combined with

monoclonal antibodies able to bind Fc receptors for the treatment of glioblastoma. Another approach seems to gain a new interest in this indication: the development of Chimeric Antigen Receptor NK cells targeting EGFR.

2.6.2 Neuroblastoma

Neuroblastoma, a neuroectodermal malignancy is accounting for 8% of all childhood cancers with a half of the patients presenting a metastatic disease at diagnosis. Despite offensive multimodal therapies, including multi-agent dose intensive induction chemotherapy and surgical resection to obtain remission, progression free survival (PFS) is under 50%. Furthermore, treatment induced sequelae limit their use. Immunotherapy, as monoclonal antibodies can be an attractive treatment option. Some of them have been developed to target neoantigens expressed by neuroblastoma and neuroblastoma is the first pediatric tumor for which monoclonal antibody therapy has been proven effective[67]. GD2, belonging to the ganglioside family remains the most targeted antigen. In normal tissues, GD2 expression is restricted to peripheral sensory nerve fibers, neurons and melanocytes. GD2 is also highly expressed on neuroblastoma cells and on some adult tumors like retinoblastoma, glioma and melanoma. Regarding its functionality, GD2 is thought to have a role in tumorigenesis through an enhancement of cell motility and invasion particularly observed in osteosarcoma[20]. GD2 also influences the tumor infiltration by myeloid derived suppressor cells known to have immunosuppressive properties shown on effector cells [68]. This tumor antigen was also ranked as 12 out of 75 potential targets for cancer therapy based on immunogenicity, oncogenicity, expression level and percentage of antigen-positive cells by the National Cancer Institute pilot program [69]. Despite its high relevance for immunotherapy, there are some challenges to develop anti-GD2 antibodies. First, antibodies should be generated with sufficient affinity to mediate Fc receptor dependent cytotoxicity against tumor cells. Second, GD2 expression on normal tissues is responsible for a high anti-GD2 antibody related toxicity. Pain, fever, and urticaria have been reported after monoclonal antibody therapy. Despite these hurdles, anti-GD2 antibodies have been developed such as 3F8, hu3F8, ch14.18 and hu14.18. 3F8 and 14.18 are murine IgG3 antibodies and were first described in the 80's. The antibody 3F8 was the first antibody tested into patients and demonstrated some anti-tumor potential in phase I and II trials but was accompanied by substantial neuropathic pain and by a high immunogenicity with the development in the majority of patients of human anti-murine antibodies [70]. The antibody Ch14.18 (dinutuximab) was next developed in order to circumvent the immunogenicity associated with murine antibodies. With a comparable affinity to GD2, it shows a superior ability to mediate ADCC, at least *in vitro* [71]. The difference between ch14.18 and Hu14.18 is that hu14.18 is a humanized antibody with the hypervariable regions of the murine antibody placed into a human IgG1 framework. Ch14.18 also has a mutation of a single aminoacid substitution in the Fc part abrogating complement activation, leading to less neuropathic pain into patients [72].

Some clinical trials are ongoing where anti-GD2 are tested alone or in combination with IL2, GM-CSF or isotretinoin. The cytokines, IL2 [73] and GM-CSF were added to the immunotherapeutic regimen to expand effector cells involved in tumor cell killing. Among the effector cells, some clues indicated an important role of NK cells in the therapeutic effects of anti-GD2 therapies.

The first evidence was a discovery in a phase II trial of the Children Oncology Group. They observed that an autologous killer immunoglobulin like receptor (KIR) / KIR ligand mismatch was associated to an improved

outcome after an autologous stem cell transplant for neuroblastoma whereas no improvement was seen in patients who were matched. These data were consistent with a role of NK cells in this clinical response [74], further confirmed by a study [75] showing that in presence of an anti-GD2 antibody, unlicensed NK cells (NK cells lacking KIR for self HLA molecules) were able to induce a strong ADCC and play a key anti-tumor role in patients undergoing anti GD2 therapy, explaining the benefit of the missing KIR ligand found in these patients. Then other relationships between neuroblastoma and NK cells have been described such as the expression of NK cell receptor ligands by this tumor, for example B7H6, ligand of NKp30, or the polyomavirus receptor PVR (CD155), ligand of DNAM-1 [76]. The demonstration of NK cell cytotoxicity against neuroblastoma was also confirmed *in vitro* using neuroblastoma cell lines as targets [77] and *in vivo* into animal models. Liu et al showed an improved survival of NOD/SCID mice treated with autologous NK cells expanded from neuroblastoma patients in combination with ch14.18 antibody [78]. Next, a benefit of cytokine activated NK cells combined with dinutuximab was demonstrated in a mouse model after surgical resection of the primary tumor with an improved survival in the treated animals [79] suggesting an interest of NK cell immunotherapy for the treatment of any residual disease.

Regarding clinical studies with NK cells and anti-GD2 antibodies, 2 were recently published. One is a pilot trial (NCT01576692) aiming to treat refractory neuroblastoma with hu14.18 combined with chemotherapy, IL-2 and haploidentical NK cells. Thirteen patients were enrolled in this study, eleven received 29 infusions of allogeneic NK cells. The infusions were well tolerated and NK cell persistence was observed until day 14 demonstrating the feasibility, tolerability and safety of administering NK cells with anti GD2, chemotherapy and IL-2. The adverse events observed were expected and related to chemotherapy or to hu14.18 but this trial was limited by the small number of patients enrolled. The other is a phase I study (NCT00877110), based on haploidentical NK cells and murine monoclonal 3F8 antibody administered to 35 patients with high risk neuroblastoma. The objective of the study was to determine the highest NK cell dose tolerated. Patients received a lymphodepleting chemotherapy based on cyclophosphamide prior to NK cell infusion. Although the study was not designed to evaluate the anti-tumor effects of NK cells since the regimen included chemotherapy and anti-GD2 antibody, a NK cell dose response seemed to be present with an improved PFS in patients receiving $>10 \times 10^6$ NK cells/kg suggesting that higher NK cell number may be more beneficial although circulating NK cells could not be detected after day 14. Based on the lessons learned from this study, regarding NK cell isolation and the regimen toxicity, a follow up study is ongoing, with cyclophosphamide conditioning, haploidentical NK cells, hu3F8 and IL-2 ((NCT02650648).

3 Hurdles to ADCC mechanism

Different reasons can be responsible to a loss of ADCC efficacy. Among them, a diminished expression of the target, hindrance of the antibody binding, competing circulating immunoglobulins for Fc receptors, undesired binding of inhibitory Fc receptors instead of activating ones, cleavage of CD16 from the surface of NK cells by matrix metalloproteinases or diminished affinity to antibodies due to CD16 polymorphism can be cited. In this review, we focused on the latter.

3.1 CD16 shedding

As seen in the part above, the ADCC is clinically important as one of the mechanisms of action of therapeutic antibodies. For example, for rituximab, the anti-CD20 antibody, the engagement of Fc receptors has been shown to be vital for its activity *in vivo*[58]. The Fc receptor on which relies the ADCC mechanism mediated by NK cells is the CD16a, the only Fc G receptor they express[80]. CD16 undergo a very rapid and efficient proteolytic cleavage upon NK cell activation by various stimuli such as mitogen stimulation or co-culture with malignant targets[81, p. 16]. This process may be important for rapid modulation of the cell surface density of the receptor, and in turn the activation status and effector functions of NK cells. Downmodulation of CD16 is caused by a proteolytic cleavage of its extracellular domain by different enzymes such as A Disintegrin And Metalloproteinase ADAM17 or membrane type 6 metalloproteinase MMP25. ADAM17 has been shown to be expressed by NK cells, especially CD56 dim NK cells, and is thought to be a regulatory checkpoint of NK cell function through ADCC. Given the fact that CD16 expression by effector cells is essential to mediate ADCC, the loss of CD16 could be considered as a hurdle to the efficacy of therapeutic monoclonal antibodies. Therefore, there have been several attempts to use ADAM17 inhibitors in the clinic but none has been proven successful so far. Recently, new function blocking antibodies of ADAM17 have been generated, more specific and with a longer half-life than ADAM17 small molecule inhibitors first designed to avoid the release of HER receptor ligand[82]. The results published in some studies showed that NK cells treated with ADAM17 blocking antibodies produced higher level of IFN γ in presence of antibody bound tumor cells, indicating that blocking ADAM17 in NK cell could result in an increased production of IFN γ in the tumor micro-environment.

However some recent results showed that the shedding of CD16 increases the NK cell motility and facilitates detachment from target cells, allowing more target to be engaged and killed in a given time[83].

3.2 Fc γ RIIIa/ CD16a polymorphism

A significant correlation between functional polymorphism of Fc γ RIIIa/ CD16a activating receptor and the outcome of anti-tumor mAb therapy has been reported in many studies[84], [85]. Fc γ RIIIA polymorphism is closely associated with the affinity to IgG-Fc. This polymorphism is located on the extracellular domain of Fc γ RIIIa and aminoacid 158 is involved in the interaction with CH2 domain of human IgG1. So the presence of a valine (V) or a phenylalanine (F) influences qualitatively the binding of IgG1 to the Fc receptor by modifying its affinity. Fc γ RIIIA 158V/V allele or V/F allele conjugates easily with IgG-Fc to induce ADCC efficiently, whereas Fc γ RIIIA 158F/F conjugates weakly to IgG-Fc, resulting in weak ADCC[86]. It was also shown that Fc γ RIIIA polymorphism induces quantitative differences such as CD16 cell surface expression, binding of the antibody or ADCC activity[87] with better results observed for individuals expression at least one valine at Fc γ RIIIA 158. Thus, the polymorphism of Fc γ RIIIA should be taken into account or at least explored, as it may influence the response to mAb therapy and the clinical outcome observed in the clinical trials.

4 Potentialization of ADCC

Different ways have been explored to potentialize the efficacy of therapeutic antibodies through an optimization of the ADCC mechanism (Figure 2). Thus, combination with immunoadjuvants like cytokines or simultaneous engagement of activating pathways like the CD137L/CD137 pathway have been investigated to overcome resistance to therapeutic antibodies or to improve ADCC efficacy.

4.1 Combination with cytokines

Cytokines are small peptides or glycoproteins involved in survival, growth, differentiation signals to immune cells. Cytokines can act through autocrine or paracrine effects. They bound to high affinity receptors expressed by target cells and then triggered an intracellular signal, resulting in activation of gene transcription able to modify cellular functions. Among the cytokines able to potentiate immunotherapies, IL-2 and IL-15 are the 2 main cytokines used to enhance NK cells proliferation[88] and cytotoxicity. IL-2 has received FDA approval and is currently used to treat melanoma or renal carcinoma[89] but its use is restricted by grade 3 and 4 adverse events, including chills and fever. IL2 is known to stimulate T lymphocytes effector functions but has also a dual role, with an effect on immunosuppressive and tolerogenic regulatory T cells (Tregs) especially when low or intermediate dose are used. Indeed, an increase of the number of regulatory T cells was seen in many clinical trials using IL-2 to maintain NK cell expansion after infusion[45] [90]. IL-15 is one of the 6 common gamma (γ) chain cytokines activating T cells and NK cells. The IL-15 receptor shares 2 chains with the IL-2 receptor, the β and γ chains leading to similar effects on the proliferation of T cells, on the induction of cytotoxic T cells, and on the expansion of NK cells[91] but has a cytokine specific α chain. Functional differences are mainly on expansion and maintenance of T regs by IL-2 but not by IL-15, activation induced cell death by IL-2 but not IL-15 and a control of the lymphocyte pool size even after lymphodepleting therapy by IL-15 not by IL-2[92]. IL-15 was shown to be essential for NK cell differentiation and survival, as no NK cells are found in IL15 KO or IL-2/IL15 R γ c or β mouse model. Regarding the clinical development, IL-15 has been tested as intravenous(IV) bolus or SC infusions in adult cancer patients with a maximal tolerated dose lower for IV than for SC. The main adverse events reported are leukopenia, lymphopenia, neutropenia (IV), hypophosphatemia or hypertension (SC).

The anti-tumoral effect of these cytokines can be direct, by targeting the proliferation or survival of cancer cells or indirect through the potentialization of ADCC by stimulating, expanding *in vivo* populations of NK cells, macrophages and monocytes. When used in combination with antibodies, IL2 or IL-15 induced ADCC at lower effector to target ratio, and are able to induce the up-regulation of NK cell activating receptor CD16 or NKG2D. This effect was seen *in vitro*, in a study using KRAS mutated breast cancer cell line in presence of cetuximab and IL-2 or IL-15 activated PBMC[93]. The synergy between therapeutic antibodies and cytokines was also recently confirmed in another study aiming to compare the immunoadjuvant effect of IL-2 and a complexed form of IL-15 on NK cell mediated lysis on neuroblastoma cell in culture[94]. This was explored because IL-2 and anti-GD2 increases the survival of children with high risk neuroblastoma but therapy failure and IL-2 related toxicity needs to consider other treatment option. To this end, IL-15 was integrated in the current therapy regimen for neuroblastoma. The cytokine exhibits a higher anti-tumor activity than IL-2 in an *in vivo* xenografted mouse model. This effect probably occurred because IL-15 promoted the maturation of tumor infiltrating NK cells. Taken together, these studies and the known advantages of IL-15 compared to IL-2, suggested that this cytokine in its simple form or complexed with IL-15R α could be a feasible IL-2 substitute in immunotherapy regimen including therapeutic antibodies to enhance NK cell ADCC.

4.2 CD137 agonists

CD137(4-1BB; tnsfr9) is a co-stimulatory receptor belonging to the TNF family receptor. CD137 recognizes its ligand CD137L (4-1BBL, tnsf9) mostly expressed by macrophages, dendritic cells and B cells[95]. Ligation of 4.1BB induces an activating signal in NK cells and CD8+ T cells resulting in an increase in cytokine release, cytolytic functions and ADCC. Thus targeting 4-1BB with CD137 agonists results in “pressing the accelerator” of immune cells, analogously to immune check points “removing the brakes” on immune cells and its expression can be stimulated by different agents notably IL-2 or FcγRIII ligation on NK cells[96]. As an enhancement of CD137 expression has been observed in patients after treatment with monoclonal antibodies, it has been suggested that a synergy may occur between anti-CD137 antibodies and anti-tumor monoclonal antibodies, resulting in an increased ADCC. This hypothesis has been tested *in vitro* and *in vivo* to enhance ADCC mediated by trastuzumab. *In vitro*, this was tested against a HER2 low expressing pancreatic carcinoma cell line [97]. Upon exposure to trastuzumab, an increase expression of CD137 was observed and the combination between anti-CD137 antibody and trastuzumab enhances ADCC against pancreatic cell line. However, the analysis of individual results showed that the up-regulation of CD137 did not already occur and in that case, no increase of ADCC was observed, suggesting interindividual variation of the results. *In vivo*, this was tested in a Balb/c transgenic ErbB2 animal model. In this study, a synergic effect on tumor size was observed between trastuzumab and anti-PD1 or anti-CD137 antibodies. This suggests that anti-CD137 mAb could be used to capitalize on the immune mediated effects of trastuzumab but need to be further evaluated in clinical trials. A phase I dose escalating study (NCT 03650348) is actually recruiting, testing the safety of a bispecific fusion protein targeting CD137 and HER2 developed by Pieris Pharmaceutical (PRS-343) in combination with atezolizumab, an anti-PD-L1 antibody in 70 patients presenting HER2 positive advanced or metastatic solid tumors.

5 Future evolutions of monoclonal antibody therapy

5.1 Bispecific antibodies, Bispecific Killer Engager (BiKE) or trispecific Killer Engager (TriKE)

Bispecific antibodies have been developed to selectively trigger a distinct activating Fcγ Receptor and to allow the recruitment of immune cells bearing this receptor. As immune engagers that have reached the clinical routine, catumaxomab (CD3, EpCAM trifunctional antibody) and blinatumomab can be cited. However, they are linked to an anti-CD3, leading to T cell activation with consecutive cytokine toxicity like cytokine release syndrome and nervous system events including seizure and encephalopathy, adverse events very similar to CAR T cell Therapy and of note, catumaxomab marketing authorization was withdrawn in the EU. So it could be of specific interest to develop a drug engaging NK cells instead of T cells. Based on a structure including a CD16 component, an (HER2)2xCD16 tribody has been developed[98] to target HER2 expressing cancer cells. Compared to trastuzumab, the tribody enhanced the cytotoxicity of NK cells and γδ T cells and present the advantage to target innate cells as well as cells linking innate and adaptative response.

Bi and tri specific killer engagers (BiKE and TriKE) are different from full length antibodies and are considered as the next generation of monoclonal antibody therapy. These small molecules contain a variable portion of an antibody linked to one (Bi) or two variable portions (Tri) of another antibody. BiKE and TriKE have some advantages compared to full length antibodies including increased biodistribution due to their smaller size, with a higher tissue penetration allowing these molecules to cross the extracellular matrix to reach the tumor. They are also non- immunogenic and rapidly engineered. Due to a good knowledge of the ADCC mechanism, BiKE

and TriKE are designed with a CD16 component to engage NK cells whereas Bispecific T cell Engager (biTE) are designed with a CD3 component. The first BiKE that has been designed is a BiKE signaling through CD16 and targeting CD33 expressed by acute myelogenous leukemia targets. CD16*CD33 BiKE was specific for NK cell activation and specifically engaged CD33+ target cells *in vitro*. Furthermore, an increase of NK cell cytotoxicity and cytokine release was observed when an ADAM17 inhibitor was added to prevent CD16 shedding [99]. The CD16*CD33 BiKE was next compared to a CD16*CD33*15 TriKE. In this TriKE, IL-15 was chosen as a linker between CD16 and CD33 variable fraction. CD16*CD33*15 TriKE was shown to induce higher cytotoxicity, degranulation and cytokine production against CD33+ targets as well as an increase survival of NK cells *in vivo* in a xenografted model[100]. This new approach was next followed by a recent publication of the same team[101], where a CD16*CD19*15 triKE was tested against chronic lymphoid leukemia targets. Similar to the previously developed TriKE, a specific expansion and activation of NK cells was observed and compared to rituximab, a better tumor cells killing was observed *in vitro*. These encouraging results suggested that a CD16*CD19*15 triKE could be considered as a future alternative to CAR T cell therapy.

As such, TriKE can be considered as a potential evolution of monoclonal antibody therapy, by providing a cost effective and easy to use product for enhancing the NK cell therapy selectivity in myeloid malignancies like AML but can find also an application in solid tumors, although their therapeutic efficacy has to be proven in clinical trials.

6 The place of NK cells and antibodies regarding in tumor progressive or metastatic disease

Tumor progression and metastasis remains a therapeutic challenge despite the emergence of new treatments. These events are mainly due to immunosubversion and immune escape. The immunosubversion relies on the different mechanisms developed by tumor cells and their microenvironment that directly or indirectly suppress NK cell immunosurveillance. The release of soluble ligands and immunosuppressive factors (i.e TGF β , adenosine, IL-10...) were shown to be involved in tumor progression. The immune escape relies on strategies to avoid immune system recognition such as the loss, downregulation, or mutation of an antigen formerly recognized by immune cells. So the main question is how to reinforce NK cells anti-tumor activity in this context? In this review, we put in light some clues. First, regarding patient's own NK cells, a preincubation with cytokines is able to restore NK cells cytotoxicity *in vitro*, although there are some limitations in restoring autologous NK cell full functionality due to an impregnation by the immunosuppressive microenvironment of the tumor. Furthermore, cytokine administration was shown to cause some dose limiting toxicities into patients. This phenomenon can be circumvented by the infusion of allogeneic NK cells, curing the main cause of poor innate immunity in cancer patients by bolstering NK activity against cancer through the restoration of a higher number of functional NK cells. Choosing this option as a therapeutic treatment leads to other indirect immune mechanisms that follow allogeneic cell infusion such as a recruitment of dendritic cells to tumor site through chemokine secretion leading to an interaction between dendritic cells and NK cells that can promote a better uptake of tumor antigens after lysis, and at the end a better presentation by T cells facilitating a second step in the anti-tumor response. However, the use of allogeneic NK cells is not as simple as it seems, as due to their foreign origin, they must be infused after a lymphodepleting therapy to avoid rejection which is not easy to perform in patients after many lines of treatment. Second, a synergy of monoclonal antibodies and NK cells was observed in different clinical contexts, that we described in this review. The main roles of monoclonal antibodies

are twice: they allow a better infiltration of the tumor by NK cells, as it was observed in CRC and a better targeting of cancer stem cells, involved in tumor progression. Also, NK cells still allow the use of monoclonal antibodies even when a resistance to the treatment appears due to a mutation in the signaling pathway targeted by the antibody. NK cells combined with antibodies, by exerting ADCC, provide a second chance to avoid or limit tumor escape. However, there are also some hurdles of monoclonal antibody therapy such as ligand reduction expression due to internalization or shedding, and reduced or limited ligand specificity. This last point is taken into account by the developers of new antibodies. Indeed, many antibodies directed against the same target are now available on the market. However, they cannot be considered as equal as they often are not directed against the same site of the target, like for example trastuzumab and pertuzumab, suggesting that it may be a synergy between them and that when the efficacy of one is lost, the other can still be used. These critical parameters should be addressed in future clinical trials and taken into account in the future development of new antibodies. Indeed, an improvement of antibody efficacy with glycol optimization, or the evolution of existing antibodies (cetuximab, trastuzumab,,) in bispecific BiKE for example, will reinforce their interaction with immune cells and allow a better targeting of the primary tumors and even distant metastases.

In this review, we showed that NK cells and monoclonal antibodies are two partners for successful antibody dependent cytotoxicity but other aspects of the immune response clearly influence the quality of the partnership. Therefore, testing other combination therapies with immune checkpoint antibody (i.e nivolumab), Toll Like Receptor 5 or 7/8 agonists, or anti-CD137 (urelumab) can further enhance the intensity of the anti-tumor response and would be certainly evaluated in the next years.

7. Conclusion

Today NK cells are part of the therapeutic arsenal based on cellular immunotherapies. As the most common of cellular immunotherapies, obstacles limit their effectiveness, such as the tumor microenvironment, the difficulty in infiltrating a tumor mainly in hypoxia, as well as all the immunosubversion mechanisms implemented by cancer cells. However, numerous studies show a renewed effectiveness when these immunotherapies are combined with other approaches such as immune checkpoints, cytokines or monoclonal antibodies as we have been able to describe in this review. The combination of NK cells with monoclonal antibodies clearly shows a benefit in terms of efficiency by overcoming the numerical or functional deficit of autologous NK cells as well as the resistance mechanisms appearing during treatment. In addition, the possibility of arming NK cells with antibodies *in vitro* using CD16 which they express makes them a potential alternative to CAR T cells, but the effectiveness of this concept has yet to be demonstrated in future clinical trials.

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Table 1: Clinical trials evaluating the co-administration of rituximab and NK cells.

Clinical trial	phase	status	NK cell origin	Lymphodepleting therapy	Cytokine	indication
NCT00383994	I	completed	allogeneic	no	GM-CSF	CD20+ LLC or non Hodgkin lymphoma post non myeloablative HSCT
NCT03019640	II	recruiting	Cord blood derived	Yes (autologous stem cell transplant)	GM-CSF	B lymphoma candidate to autologous stem cell transplant
NCT01181258	II	completed Published	haploidentical	Yes (fludarabine/ cyclophosphamide)	IL2	Refractory non Hodgkin lymphoma leukemia
NCT00625729	I	completed	Haploidentical	Yes (fludarabine/ cyclophosphamide)	IL2	non Hodgkin lymphoma chronic lymphocytic leukemia
NCT02843061	I/II	completed	autologous	no	no	B cell lymphoma
NCT02280525	I	Active, recruiting	not Cord blood derived	Yes (fludarabine/ cyclophosphamide)	no	B cell lymphoma

Table 2 published trials on EGFR family antibodies and NK cell treatment.

Clinical trial	disease	phase	Type of EGFR family antibody	Other treatment	NK cell origin	dose	NK status/ cytokine used	Phenotype at injection (median values)	Clinical response
UMIN000013378. Ishikawa 2018	Metastatic gastric and CRC	I (9 patients)	Cetuximab and tratuzumab	Capecitabine and cisplatin Capecitabine and oxaliplatin	Autologous	0.5 to 2 10^9 /injection , 3 times , triweekly	Expanded until 24 days (IL2); No cytokine post-NK cell infusion	CD3-CD56+ 93% Lag3+(35%); TIGIT+ (81%); CXCR3+(73.4%)	6 patients evaluable with 4 with stable disease (SD) (target lesions decreased in 3 patients) and 2 with progressive disease.
NCT 02845999 Adotevi 2018	CRC liver metastases and pancreatic adenocarcinoma	I (9 patients)	cetuximab	Cyclophosphamide (60 mg/kg) + fludarabine (25 mg/m ² /day during 5 days)	Allogeneic	3, 8 and 12 10^6 /kg (0.3 to 1.5 10^9 total cells)	Overnight activated (IL-2); IL2 post infusion (6 injections)	CD3-CD56+(20%)	One partial response, one dissociated response one stable and 5 progressive disease.
NCT02845856 Liang 2018	Lung cancer	II (54 patients) 2 groups: NK cells with cetuximab (A) And cetuximab alone (B)	cetuximab	none	Allogeneic (KIR /HLA mismatch)	3 injections day 13 to day 15 and a second course from day 28 to 30	Expansion 12 days in presence of lethally radiated K562-mb15-41BBL stimulatory cells	CD3-CD56+ >85% Cell viability >80%	None achieved CR. RECIST evaluation showed a clinical efficacy of group A superior to group B. Group A: 4 with PR, 17

NCT02844335 Liang 2017	HER2 overexpressing Breast cancer	II (48 patients) 3 groups: (1)Cryoablation, (2)cryoablation with NK cells, (3) cryoablation with NK cells and herceptin	trastuzumab	none	Allogeneic (KIR /HLA mismatch)	10 ⁹ in 3 injections day 13 to day 15	Expansion 12 days in presence of lethally radiated K562- mb15- 41BBL stimulatory cells	CD3-CD56+>85% Cell viability >90%	with SD, 6 with PD. Group 3: 43.7% achieved partial regression, 31.25% SD. According to RECIST, the clinical efficacy in group 3 was superior to that in group I.
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Abbreviations: MM: mismatch, CR : complete remission, CRC : colorectal carcinoma, RECIST: response evaluation criteria in solid tumor. SD : stable disease, PD progressive disease, PR partial response.

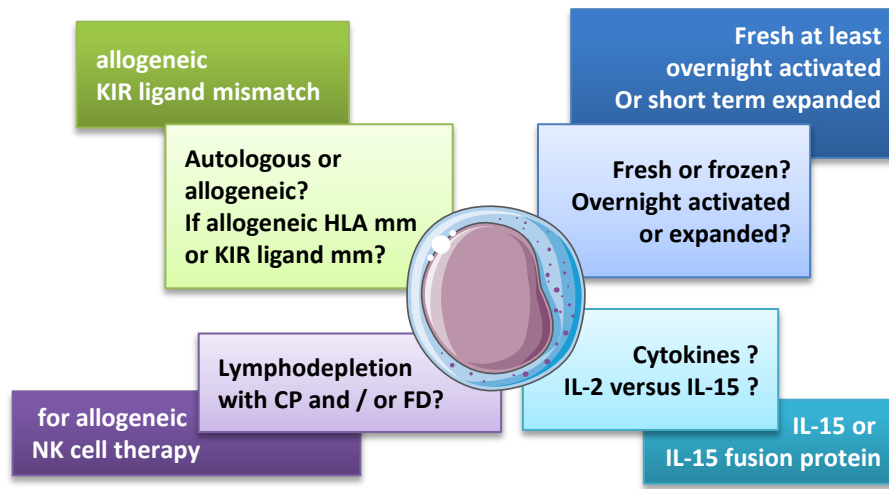


Fig. 1: Remaining questions on Natural Killer cell infusion and proposed answers. HLA: Human Leucocyte Antigen; KIR: Killer Immunoglobulin like Receptors; IL: Interleukin. Mm: mismatch.

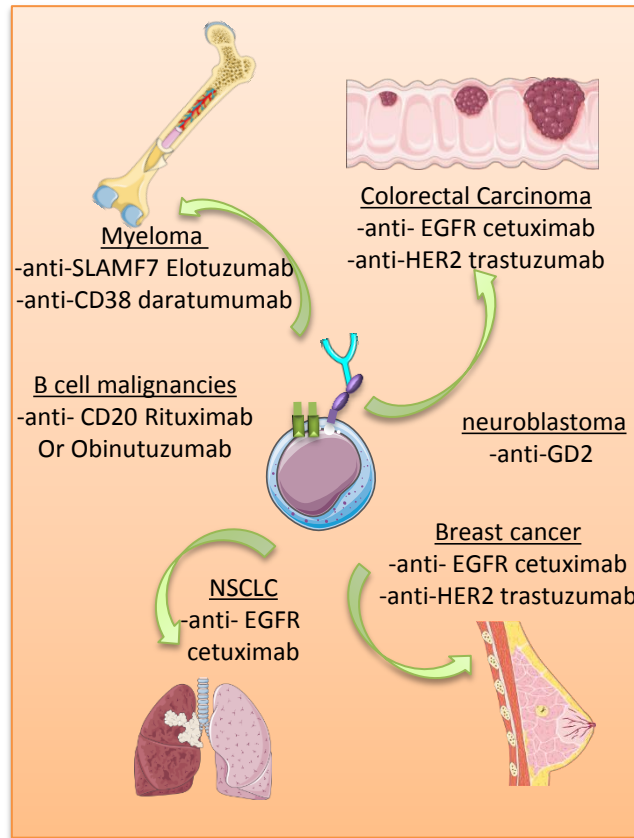


Fig. 2. Indications of monoclonal antibody therapy and corresponding targets. SLAMF7: SLAM Family member 7; NSCLC: Non Small Cell Lung Cancer; EGFR : Epidermal Growth factor Receptor.

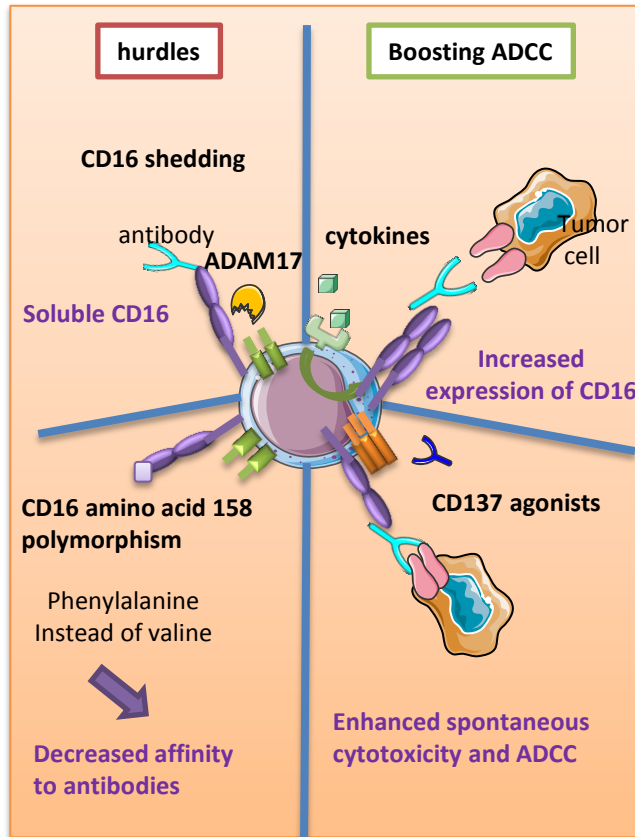
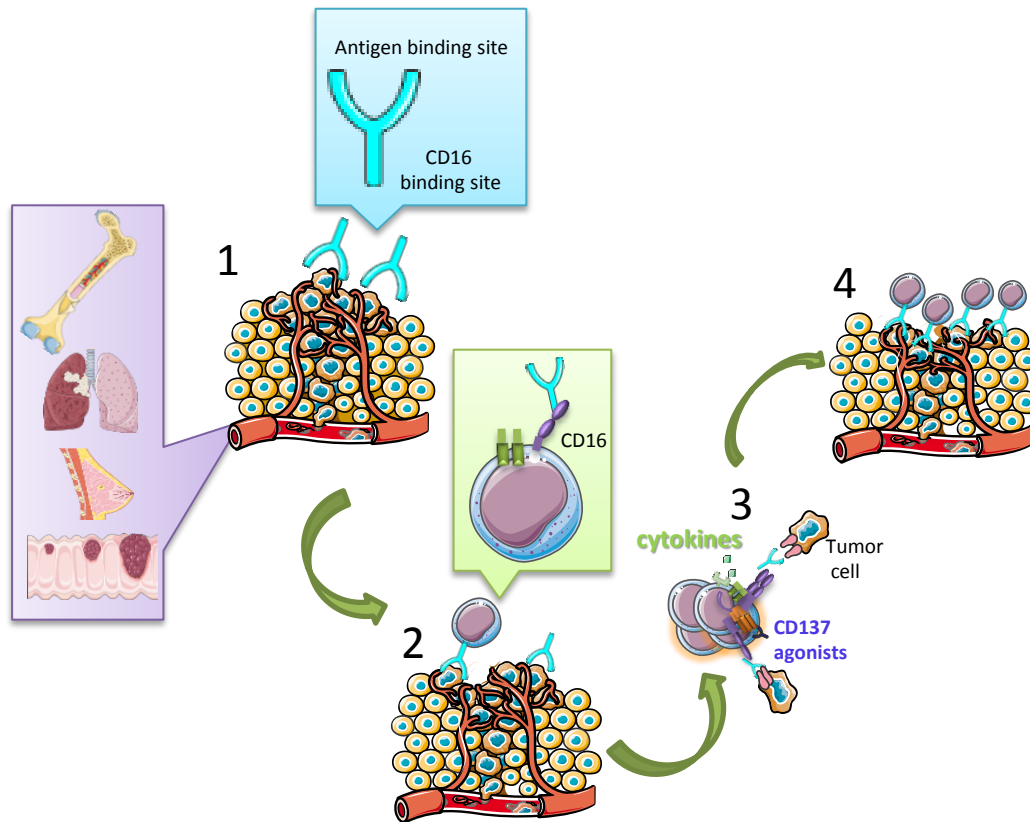


Fig. 3 Summary of hurdles and ways to boost antibody dependent cytotoxicity (ADCC). The main hurdles of ADCC are the shedding of CD16 by A disintegrin and Metalloproteinase 17 (ADAM17), inducing the release of soluble CD16 and the polymorphism of the amino acid 158 leading to a decrease of CD16 affinity to antibodies when it is a phenylalanine instead of a valine. The ways to boost ADCC include: NK cell stimulation with cytokines to increase CD16 expression and the use of urelumab (CD137 agonist) to enhance ADCC.



Graphical abstract:

1, Monoclonal antibodies targeting tumors are one of the most important discoveries in the field of cancer therapy in the last decade.

2, A lack of Natural Killer cells observed in many cancers may therefore be a cause of the low efficacy of antibodies observed in some clinical situations.

3, The ways to enhance the ADCC mechanism and the perspective of the treatment with therapeutic antibodies : enhancement of CD16 expression by cytokines or anti-CD137 antibody

4, A combination of NK cell adoptive immunotherapy and monoclonal antibodies may overcome the resistance to the treatment and enhance their efficacy.

Conflict of interest

The authors declare they have no conflict of interest.

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Sample CRediT author statement

M. Gauthier : Writing- Original draft **C. Laroye**: Editing **D. Bensoussan**: reviewing **C. Boura**: Reviewing **V. Decot**: conceptualization, writing and editing.

Highlights

- Therapeutic antibodies are used to treat solid tumors and hematologic malignancies.
- A lack of Natural killer cells diminishes the antibody efficacy against the tumor.
- NK cells combined with antibodies can overcome a resistance to the antibody.
- Cytokine use or preventing CD16 shedding may potentialize the NK cell efficacy.