University of Padova

Department of *Surgery, Oncology and Gastroenterology*

PhD SCHOOL IN
ONCOLOGY AND SURGICAL ONCOLOGY
XXVIII CICLO

A DONOR-DEPENDENT SUBSET OF CYTOKINE-INDUCED KILLER (CIK) CELLS EXPRESS CD16 AND CAN BE RETARGETED TO EXERT A POTENT ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY (ADCC)

Director of the School: Ch.mo Prof. Paola Zanovello

Supervisor: Ch.mo Prof. Antonio Rosato

Co-Supervisor: Roberta Sommaggio

PhD Candidate: Elisa Cappuzzello

INDEX

SUMMARY

Cancer adoptive cell therapy (ACT) relies on the infusion of immune cell populations mediating direct antitumor effects, such as cytotoxic $CD8^+$ T lymphocytes (CTL), natural killer (NK) cells and Cytokine-Induced Killer (CIK) cells. In this study, we aimed at improving CIK cell potential for adoptive immunotherapy strategies. CIK cells are a heterogeneous population of *ex vivo* expanded lymphocytes, which share phenotypic and functional features with both NK and T cells. They exert a potent MHC-independent antitumor activity against both hematological and solid malignancies, but not normal tissues and hematopoietic precursors. Several clinical trials have demonstrated the feasibility and the therapeutic efficacy together with low toxicity of CIK cells infusion, supporting CIK cells as a very promising cell population for adoptive immunotherapy. In this work, CIK cells were obtained from PBMCs of healthy donors by the timed addition of IFN-γ, anti-CD3 antibody and IL-2. Analyzing their phenotype, we demonstrated for the first time a relevant expression of CD16 in a donor-dependent manner and, based on this observation, we proved the ability of CIK cells to kill tumors by an Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) mechanism. Indeed, the concurrent administration of clinically therapeutic mAbs, such as trastuzumab or cetuximab, led to a significant improvement of their antitumor activity *in vitro* against both ovarian and breast cancer cell lines. To formally prove that the CD16 receptor is functional and directly involved in the ADCC, an anti-CD16 blocking antibody was added to the assays. NK cell depletion from bulk cultures confirmed that the ADCC activity is accountable to the CIK $CD16^+$ subpopulation. This novel function of CIK cells, never exploited before, was assessed for therapeutic efficacy in mouse models of human ovarian carcinoma xenografted in NOD/SCID common γ chain knockout (NSG) mice. Co-administration of CIK cells and mAbs significantly increased the survival of tumor-bearing mice, as compared to animals receiving CIK cells alone. CIK cell antitumor activity *in vitro* was also enhanced by the combination with bispecific antibodies and immunoligands, which are able to target both a tumor-associated antigen and activating receptors expressed by effector cells. Taken together, these data envisage new perspectives for adoptive immunotherapy where antigen-specific retargeting of T cells can be achieved by a combination therapy with clinical-grade monoclonal antibodies already widely used in cancer therapy, and CIK cell populations that are easily expandable in very large numbers, inexpensive, safe and do not require genetic manipulations. In conclusion, this new therapeutic strategy for the ACT treatment of different types of tumors could find wide implementation and application, and be expanded to the use of additional therapeutic antibodies.

RIASSUNTO

La terapia cellulare adottiva (Adoptive Cell Therapy, ACT) si basa sulla somministrazione di popolazioni di cellule immunitarie in grado di mediare un effetto antitumorale in modo diretto, ad esempio linfociti T $CD8^+$ citotossici (CTL), cellule natural killer (NK) e cellule killer indotte da citochine (Cytokine-Induced Killer cells, CIK). Lo scopo di questo lavoro è stato quello di incrementare il potenziale delle cellule CIK nelle strategie di immunoterapia adottiva. Le cellule CIK sono una popolazione eterogenea di linfociti espansi *ex vivo* che condividono caratteristiche fenotipiche e funzionali sia con le cellule NK sia con le cellule T. Queste cellule esercitano una potente citotossicità MHC-indipendente nei confronti di tumori sia ematologici sia solidi, ma non di tessuti normali e precursori ematopoietici. Diversi trial clinici hanno dimostrato l'attuabilità, l'efficacia terapeutica e la bassa tossicità delle infusioni di cellule CIK, supportandole come popolazione cellulare molto promettente per l'immunoterapia adottiva. In questo lavoro, le cellule CIK sono state ottenute da cellule mononucleate del sangue periferico (Pheripheral Blood Mononuclear Cells, PBMCs) di donatori sani mediante l'aggiunta di interferone gamma (Interferon-γ, IFN-γ), anticorpi anti-CD3 e interleuchina 2 (Interleukin-2, IL-2). Analizzando il fenotipo, abbiamo dimostrato per la prima volta una rilevante espressione donatore-dipendente del recettore CD16 e, basandoci su questa osservazione, abbiamo analizzato la capacità delle cellule CIK di uccidere cellule tumorali mediante citotossicità cellulo-mediata anticorpo-dipendente (Antibody-Dependent Cell-mediated Cytotoxicity, ADCC). Infatti, abbiamo osservato che la simultanea somministrazione di anticorpi monoclonali terapeutici, come il trastuzumab e il cetuximab, portano ad un significativo incremento dell'attività antitumorale *in vitro* delle CIK nei confronti di linee cellulari di tumore ovarico e mammario. Per dimostrare che il CD16 è funzionale ed è direttamente coinvolto nell'ADCC, è stato aggiunto al saggio un anticorpo bloccante anti-CD16. La deplezione delle cellule NK ha confermato che l'ADCC è attribuibile alla sottopopolazione $CD16^+$ delle cellule CIK. Questa nuova funzione delle cellule CIK, descritta qui per la prima volta, è stata valutata per la sua efficacia terapeutica in un modello murino di carcinoma ovarico umano trapiantato in topi NOD/SCID knockout per la catena comune γ (topi NSG). La co-somministrazione di cellule CIK e anticorpi monoclonali ha aumentato significativamente la sopravvivenza dei topi con tumore, in confronto ai topi trattati soltanto con le CIK. Inoltre, l'attività antitumorale *in vitro* delle cellule CIK è stata incrementata mediante la

combinazione con anticorpi bispecifici e immunoligandi, in grado di legare contemporaneamente un antigene associato al tumore e un recettore attivatore espresso dalle cellule effettrici.

Complessivamente, questi dati prospettano nuove possibilità per l'immunoterapia adottiva, in cui il reindirizzamento antigene-specifico dei linfociti T può essere ottenuto mediante la combinazione di anticorpi monoclonali di utilizzo clinico, già ampiamente utilizzati per la terapia antitumorale, con popolazioni di cellule CIK, che sono facilmente espandibili, economiche, sicure e non richiedono manipolazioni genetiche. In conclusione, questa nuova strategia terapeutica per trattamento di diversi tipi di tumori mediante terapia cellulare adottiva potrà trovare ampie possibilità di implementazione e applicazione, e potrà essere estesa all'utilizzo di ulteriori anticorpi terapeutici.

INTRODUCTION

Cancer immunotherapy

Cancer is a major cause of mortality worldwide, and current standard cancer therapies, such as surgery, radiation and chemotherapy, are effective in a consistent fraction of patients by efficiently removing primary tumors. However, complete removal of malignant tissues is not always possible, conventional therapies becoming ineffective and often leading to a high toxicity, severe side effects and, eventually, to a relapse of the tumor. In this context, alternative approaches are indispensable; among these, cancer immunotherapy has shown promising results against different tumors, and the last few years have witnessed a renewed interest in targeting and eliminating cancer using immunologic strategies[1].

Cancer immunotherapy can be divided into active and passive approaches. Active immunotherapy aims at eliciting a specific host immune response by employing cancer vaccines or cytokines. The systemic delivery of cytokines, such as interleukin-2 (IL-2)[2] and interferon-α (IFN-α)[3], boosts immune responses through the recruitment and the induction of immune effector cells. Cancer vaccines stimulates patient's own immune system to recognize and eliminate malignant cells, and can lead to immunological memory and long-lasting antitumor response[4]. Examples of antitumor vaccines are the prophylactic vaccines against hepatitis B virus (HBV), which prevents HBV-induced hepatocellular carcinoma[5], and Gardasil® and Cervarix®, which protect against human papillomavirus (HPV)-induced cervical carcinoma[6]. Most recent vaccine strategies are dealing with therapeutic, rather than prophylactic, applications, to treat patients with already apparent tumors. Several strategies for cancer vaccination and vaccine delivery have been developed, such as recombinant proteins and peptides, viral vectors, pulsed dendritic cells (DC), DNA or RNA-based vaccines[7,8].

Passive immunotherapy involves the administration of therapeutic agents, such as monoclonal antibodies and effector cells, which can directly affect the tumor. Monoclonal antibodies can target tumor-expressed antigens, vascular growth factors, and tumor-associated stroma, inhibiting tumor cell growth[9]. However, recently, antibodies targeting immune checkpoints, such as PD-1 (Programmed Death protein-1) and CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4), have been developed. These antibodies are able to elicit a specific host immune response and, therefore, can not be considered as a passive, but rather active, immunotherapy approach.

Adoptive cell transfer (ACT) therapies are based on the administration of *in vitro* expanded, activated and, if necessary, genetically modified effector cells, such as TCR- or Chimeric Antigen Receptor (CAR)-transduced T lymphocytes[10], Tumor-Infiltrating Lymphocytes (TIL)[11], Natural Killer (NK) cells[12], γδ T cells[13], or Cytokine-Induced Killer (CIK) cells[14,15]. Depending on the cell type, the effectors can be obtained from donors (allogeneic cells) or from the patients themselves (autologous cells), and are armed to specifically eliminate tumor cells.

Cell-based immunotherapy strategies have been proven to be highly effective against both hematological and solid malignancies in many clinical trials[16]. Three critical issues need to be addressed by all ACT strategies in order to be effective in clinical applications: first, sufficient numbers of immune effector cells must be obtained; second, they must reach the tumor site and recognize the targets and third, kill the tumor cells[17]. The laborious and expensive production of effector cells, mostly restricted to autologous T-cell products, is an important disadvantage for T-cell therapies and an impediment to their commercialization. These obstacles could be overcome by using CIK cells; they are a population of effector cells obtained from peripheral blood mononuclear cells (PBMCs) after *ex vivo* stimulation, which show unique and interesting features supporting their role as a promising tool for ACT approaches.

Cytokine-Induced Killer (CIK) cells

CIK cells are a heterogeneous population of *ex vivo* expanded lymphocytes capable of MHC-unrestricted antitumor activity, which share phenotypic and functional features with both NK and T cells. These cells were first obtained by Schmidt-Wolf *et al.* in 1991 from PBMCs by the timed addition of IFN-γ, monoclonal antibodies directed against CD3 (OKT3) and IL-2[18]. During the first 24 hours, PBMCs are stimulated with interferon-γ (IFN-γ) that induces the expression of IL-2 receptors and increases cytotoxicity. On the next day, cells are exposed to a mitogenic signal by engagement of CD3 with OKT3 antibody, and further activated and maintained with IL-2 during the entire expansion phase. After 2-3 weeks of expansion, the bulk population is composed mainly by $CD3^+CD56^+$ CIK cells and $CD3^+CD56^-$ T cells, and only a small fraction (<10%) of $CD3^-CD56^+$ NK cells (Figure 1)[19].

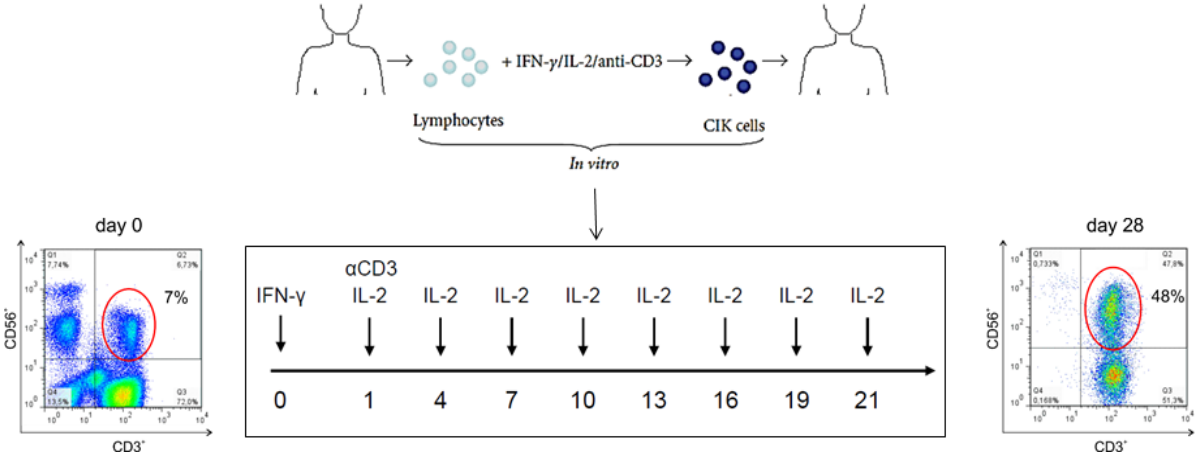

Figure 1: CIK cell *in vitro* generation and expansion. CIK cells are obtained from healthy donor PBMCs and expanded *ex vivo*. Cells are stimulated by the timed addition of IFN-γ, anti-CD3 antibodies and IL-2 to the culture medium. Starting from a small subset (*left dot plot graph*), $CD3^+CD56^+$ cells progressively expand to comprise a large percentage of the cell population present at the end of culture period (*right dot plot graph*).

To identify the subpopulation giving origin to CIK cells, the $CD3^+CD56^-$ T cell and $CD3^-CD56^+$ NK cell subsets present in culture at the beginning of expansion were purified from PBMCs, and stimulated with IFN-γ, OKT3 and IL-2. Under these conditions, T cells acquired the expression of CD56, while NK cells did not show phenotypic changes[20]. Similar experiments were performed sorting $CD3^+CD56^+$ cells, demonstrating that this subset is able to maintain its phenotype but does not further expand in respect to the total cell number. Thus, $CD3^+CD56^+$ CIK cells derive from T cells present in PBMCs, and not from NK cells or from the few $CD3^+CD56^+$ cells present in the starting culture[21].

In vitro-generated $CD3^+CD56^+$ CIK cells are mostly $CD8^+$, and express the αβ and γδ TCR in proportion similar to that found in the peripheral blood T cells. CIK cells are also characterized by high levels of Natural-killer group 2 member D (NKG2D) and CD11[21]. The antitumor activity of CIK cells is associated with the $CD3^+CD56^+$ subset[20], which exert a potent cytotoxicity only against both hematological and solid malignancies without prior exposure or priming, but not against normal tissues and hematopoietic precursors[21,22]. Functional assays using blocking antibodies against CD3, CD8, CD56, TCR α/β, and MHC class I and II molecules failed to inhibit the cytotoxic activity, demonstrating that CIK cells recognize target cells by TCR- and MHC-independent mechanisms; on the other hand, a significant inhibition was obtained blocking CD11 and ICAM-1, suggesting that cell-to-cell contact is strongly required for cytolysis[23,24].

The molecule that plays the most important role in tumor recognition is likely NKG2D, a member of the c-type lectin-activating receptor family that is expressed on all NK cells. It recognizes at least 6 counter ligands, which include the MHC-class I-like molecules, MICA and MICB, and members of the UL-16 protein binding family (ULBP1-4), named for the ability of some members to bind the UL-16 protein of cytomegalovirus[25]. Interestingly, the ligands for NKG2D appear to have an expression pattern relatively restricted to malignant tissues[26,27]. NKG2D has a significant role in the triggering of IL-2-activated NK cells inducing calcium flux, cytokine release, and cytotoxicity[28]. NKG2D expression is upregulated in CIK cells by the presence of IFN-γ, high-dose IL-2, and TCR-crosslinking with OKT3 Ab. Studies with anti-NKG2D blocking antibodies, small interfering RNA, or redirected cytolysis, indicated that most CIK cell cytotoxicity is exerted through the NKG2D rather than TCR engagement[25,29]. While NKG2D mediates the interaction between CIK cells and tumor targets, the final cytotoxic mechanism is perforin- and granzyme-mediated. In fact, CIK cells generated from perforin-knockout mice are completely lacking their tumor killing ability in *in vitro* experiments. Similarly, the adoptive infusion of CIK cells generated from FASL-deficient or wild-type mice, protected the animals from a lethal dose of lymphoma cells, while no tumor protection was observed following the infusion of perforin-deficient CIK cells[30].

When tested as a bulk population, CIK cells have been reported to maintain an alloreactive proliferation similar to that observed with fresh lymphocytes. However, when the $CD3^+CD56^-$ T cell and $CD3^+CD56^+$ CIK cell subsets were tested separately, it became evident that most of the observed proliferation was due to T cells, while CIK cells showed only minimal alloreactivity capacity[22]. One possible explanation for the low graft-versus-host disease (GVHD) incidence is the abundant production of IFN-γ, spontaneously occurring in expanded CIK cells, and known to be protective against GVHD[31,32]. CIK cells generated from IFN-γ-knockout mice, rapidly induced lethal GVHD when infused across MHC-barriers, in contrast to the wild-type counterpart that confirmed a minimal GVHD potential[32,33].

With the availability of bioluminescence imaging (BLI) technologies, *in vivo* functional activities of CIK cells have been visualized in a real-time fashion in mice inoculated with bioluminescent gene-transfected tumor cells and treated with CIK cells for cellular immunotherapy. Using the same strategy, CIK cells were transfected with the *luciferase* genes (luc^+) to visualize their *in vivo* trafficking by BLI. Following intravenous injection in tumor-bearing mice, luc^+ CIK cells first reached the lungs within 30 minutes, to thereafter generally distribute to other sites of the body within the next 16 hours. By 72 hours, a population of the labeled CIK cell infiltrated the tumor and remained

detectable by BLI at this site for an additional period of 9 days with resultant tumor regression[34]. CIK cells retain NKG2D-mediaded antitumor activity *in vivo*, as their killing activity was inhibited by the *in vivo* administration of NKG2D blocking antibodies[33].

Several clinical trials have assessed the feasibility of CIK cell application in immunotherapy protocols (Table 1). CIK cells can be successfully generated from healthy donors, as well as from patients treated with chemotherapy for various malignancies[35]. Feasibility for large-scale expansion was also reported for cord blood, and even from washout of leftover mononuclear cells from cord blood unit bags[19]. Different clinical trials have been carried out in patients affected by different malignancies[36], in both the allogeneic and autologous settings, in several cases getting significant therapeutic success with complete responses, stabilization of disease and prolonged survival. Noteworthy, the most common side effects, such as fever, headache and fatigue, were not severe and did not last beyond 24 h, being easily controllable with symptomatic treatments. Mild GVHD occurred in some patients but was responsive to corticosteroids[36].

Table 1 (see next page): Therapeutic effects of CIK cell immunotherapy in phase I/II clinical trials sorted by tumor entity. Therapeutic success was defined as "High" if it was observed (i) complete remission (CR) in more than 10 % and/or (ii) significantly prolonged overall survival in more than 20 % of patients who underwent CIK cell treatment as compared with control group. The therapeutic success was defined as "Very high" in tumor entities in which we obtained data about (i) CR in more than 20 % of patients and/or (ii) significantly prolonged overall survival in more than 40 % of patients as compared with control group. All other clinical responses as partial remission, minor remission, stable disease and progressive disease were not defined as therapeutic success. CR: complete remissions in patients treated with CIK cells; PR: partial remissions in patients treated with CIK cells; OS: significantly prolonged overall survival in patients treated with CIK cells as compared with control group (from [36]).

Study reference	Cancer disease	Patients (*n*) total	Patients (*n*) treated with CIK cells	Clinical outcome			Therapeutic success
				CR (*n*)	PR (*n*)	OS (*n*)	
Jiang et al. (2005)	Acute leukemias (not specified)	41	19	–	–	–	–
Linn et al. (2012a, b), Laport et al. (2011), Introna et al. (2007, 2010)	Acute lymphocytic leukemia	7	7	1	1	–	High
Linn et al. (2012a, b), Laport et al. (2011), Introna et al. (2007, 2010), Yang et al. (2012, 2014b), Wang et al. (2013)	Acute myelogenous leukemia	49	40	3	1	–	–
Laport et al. (2011), Yang et al. (2012), Cai et al. (2012)	Chronic lymphocytic leukemia	10	10	5	1	–	Very high
Linn et al. (2012a, b), Introna et al. (2007)	Chronic myelogenous leukemia	13	13	1		–	–
Schmidt-Wolf et al. (1999), Linn et al. (2012a, b), Laport et al. (2011), Yang et al. (2010, 2012), Leemhuis et al. (2005), Huang et al. (2006), Lu et al. (2012), Zhang et al. (2012), Olioso et al. (2009)	Non-Hodgkin lymphoma	60	60	28	4	–	Very high
Linn et al. (2012a, b), Laport et al. (2011), Introna et al. (2007), Yang et al. (2012), Leemhuis et al. (2005), Huang et al. (2006), Olioso et al. (2009)	Hodgkin lymphoma	20	20	3	2	–	High
Laport et al. (2011), Yang et al. (2012, 2014a, b), Zhong et al. (2012), Zhou et al. (2013)	Myeloma	68	38	3	2	30	Very high
Laport et al. (2011), Introna et al. (2007), Yang et al. (2012), Liu et al. (2011)	Myelodysplastic syndrome	15	15	3	2	–	High
Zhang et al. (2012), Olioso et al. (2009), Hao et al. (2010), Niu et al. (2011), Shi et al. (2004), Zhou et al. (2006), Hui et al. (2009), Qiu et al. (2011), Pan et al. (2013), Yu et al. (2014)	Hepaotocellular carcinoma	936	497	1	16	352	Very high
Zhang et al. (2012), Niu et al. (2011)	Gallbladder carcinoma	3	2	–	–	1	Very high
Olioso et al. (2009)	Pancreatic cancer	3	3	–	–	–	–
Zhang et al. (2012), Niu et al. (2011), Gao et al. (2014), Shi et al. (2012a), Jiang et al. (2006), Zhao et al. (2013), Liu et al. (2013)	Gastric cancer	511	233	–	–	153	Very high
Schmidt-Wolf et al. (1999), Niu et al. (2011), Gao et al. (2014), Zhu et al. (2013)	Colorectal carcinoma	58	43	–	–	15	High
Schmidt-Wolf et al. (1999), Zhang et al. (2012), Olioso et al. (2009), Zhan et al. (2012), Liu et al. (2012), Su et al. (2010), Wang et al. (2014), Zhu et al. (2013)	Renal cell carcinoma	328	209	21	38	120	Very high
Zhang et al. (2012)	Ureter carcinoma	1	1	–	–	–	–
Zhang et al. (2012), Niu et al. (2011), Shi et al. (2012b), Zhong et al. (2011), Li et al. (2012), Yang et al. (2013)	Non-small cell lung cancer	403	203	–	–	156	Very high
Zhang et al. (2012), Niu et al. (2011), Pan et al. (2014)	Breast cancer	93	47	–	–	46	Very high
Niu et al. (2011), Liu et al. (2014)	Ovarian cancer	94	47	–	–	–	–
Niu et al. (2011)	Cervical cancer	2	1	–	–	–	–
Huang et al. (2006)	Rhabdomyosarcoma	2	2	–	–	–	–
Zhang et al. (2012)	Sarcoma	2	2	–	–	–	–
Zhang et al. (2012), Niu et al. (2011)	Melanoma	10	8	–	–	1	–
	Total	2,729	1,520	69	67	874	

Therapeutic monoclonal antibodies and Antibody-Dependent Cell-mediated Cytotoxicity (ADCC)

In the last twenty years, the use of monoclonal antibodies (mAbs) to treat patients with hematological and solid tumors has achieved considerable success. To date, more than 20 antibodies have received FDA approval for the treatment of various types of tumors, and even more molecules are under evaluation in different stages of clinical trials[37]. The therapeutic mAbs target and attack tumor cells through various mechanisms of action, including the inhibition of cell proliferation, the blocking of immune cell inhibitory signals, and the recruitment of immune cells to kill targets through Antibody-Dependent Cell-mediated Cytotoxicity (ADCC)[37]. Numerous preclinical and clinical studies demonstrated a critical role for Fc receptors suggesting that ADCC may be a major mechanism of action of several mAbs used in cancer immunotherapy (Table 2), including trastuzumab and cetuximab[38].

Trastuzumab is a humanized IgG1 antibody specific for human epidermal growth factor receptor 2 (Her2)[39], while cetuximab is a epidermal growth factor receptor (EGFR)-specific chimeric IgG1 antibody[40]. Both Her2 and EGFR are members of the ErbB receptor family of transmembrane tyrosine kinases, which are physiologically expressed on epithelial cells, but when overexpressed, are involved in tumorigenesis. Her2 is overexpressed in some adenocarcinomas of the lung, ovary, prostate and the gastrointestinal tract[41], while EGFR can be found in metastatic colorectal cancer, metastatic non-small cell lung cancer, and head and neck cancer[42]. The Fab region of both trastuzumab and cetuximab binds tumor receptors, blocking the interaction with ligand, inhibiting receptor dimerization and cell growth signals transduction, with a final downregulation and internalization[43]. These two antibodies, bearing a human IgG1 Fc, are able to efficiently recruit immune cells to carry out the antibody indirect mechanisms of action, inducing ADCC (Figure 2).

	Target	Status	Reference or clinical trial#
MAb			
Rituximab	CD20	FDA approved for non-Hodgkin's lymphoma	Cartron et al. (42)
		Phase I relapsed indolent B-cell non-Hodgkin	NCT02384954
		Lymphoma, combined with ALT-803[a]	
		Combination with matrix metalloproteases inhibitor in pre-clinical models	Romee et al. (51)
Obinutuzumab	CD20	FDA approved for chronic lymphocytic leukemia	Goede et al. (52)
Hul4.18K322A	GD2	Phase I neuroblastoma, melanoma,	NCT01576692
		Osteosarcoma, ewing sarcoma	NCT00743496
		Phase II neuroblastoma	NCT01857934
Hu3F8	GD2	Phase I GD2+ tumors	NCT01419834
		Phase I high-risk neuroblastoma and GD2+ solid	NCT01662804
		Tumors, combined with IL2	
		Phase I refractory high-risk neuroblastoma, combined with GM-CSF	NCT01757626
Dinituximab	GD2	FDA approved for high-risk neuroblastoma, combined with IL2 and GM-CSF	Yu et al. (53)
Trastuzumab	HER2	FDA approved for HER2+ breast cancer and HER2+ metastatic gastric adenocarcinoma	Junttila et al. (54)
Cetuximab	EGFR	FDA approved for metastatic colorectal cancer and head and neck cancer	Messersmith and Ahnen (55)
IMMUNOCYTOKINE			
Rituximab-RLI[b]	CD20	Tested for human B lymphoma in SCID mouse	Vincent et al. (56)
c.60C3-RLI[c]	GD2	Tested in mouse GD2+ cell lines EL4 (subcutaneous) and NXS2 (metastatic)-grafted mouse models	Vincent et al. (57)
Hul4.18-IL2	GD2	Completed phase II refractory neuroblastoma	Delgado et al. (58), Shusterman et al. (59)
KM2812	PSMA	Tested in human prostate cancer cell LNCaP-xenografted mouse model	Sugimoto et al. (60)
BI-SPECIFIC ANTIBODY AND SINGLE CHAIN VARIABLE FRAGMENT			
AFM13	CD30/CD16	Phase II relapsed Hodgkin lymphoma	NCT02321592
$(CD20)_2$xCD16	CD20/CD16	Tested in humanized mouse grafted with autologous human B cells	Glorius et al. (61)

[a]ALT-803 is a fusion protein consisting of mutated IL15 and IL15Rα/Fc complex.

[b]RLI (IL15Rα-linker-IL15) is a fusion protein linking the NH_2-terminal domains of IL15Rα to IL15 through a 20-amino acid linker.

[c]c.60C3 is a chimeric anti-GD2 mAb.

Table 2: Representative tumor-antigen targeting monoclonal antibodies an immunocytokines functioning through ADCC. The table is a selected (not complete) list of therapeutic mAbs that are capable of inducing ADCC (from [44]).

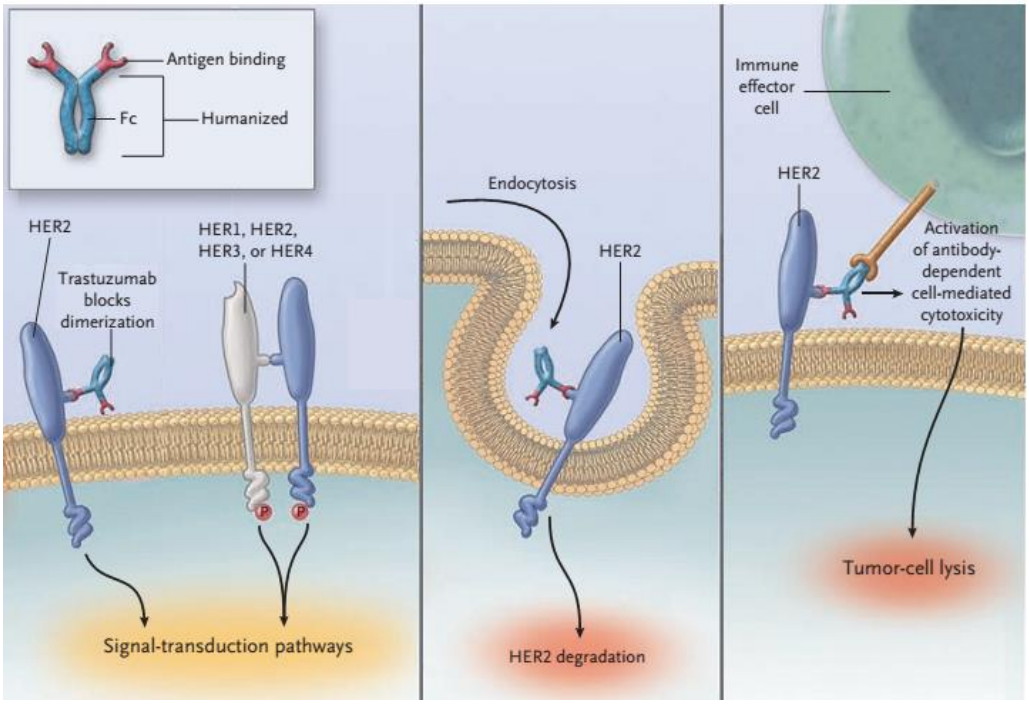

Figure 2: Mechanism of action of trastuzumab and cetuximab. Binding of trastuzumab to Her2 inhibits receptor dimerization and cell growth signals transduction (*left*), leading to downregulation through internalization (*middle*). Trastuzumab can recruit Fc-expressing immune effector cells, activating ADCC and leading to tumor cell killing (*right*). Cetuximab exerts its therapeutic effect using similar mechanisms upon binding EGFR (adapted from [39]).

Indeed, Fc region of mAbs bind to the extracellular portion of Fcγ receptors (FcγRs), which are expressed with different patterns by various cell types of the immune system, namely monocytes, macrophages, neutrophils and NK cells.

FcγRs can be divided in activating and inhibitory receptors. Inhibitory receptors are endowed with an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their intracytoplasmatic domain[45]; the major inhibitory receptor is CD32a (FcgRIIa). By contrast, activating receptors possess a cytoplasmatic immunoreceptor tyrosine-based activation motif (ITAM), which can be either encoded directly or gained by association with a common ITAM γ-chain[45]. CD64a (FcγRIa) is the activating receptor expressed by macrophages, dendritic cells, neutrophils and eosinophils, and binds antibody Fc region with high affinity. CD16a (FcγRIIIa) is expressed by NK cells, dendritic cells, macrophages and mast cells, binds antibody Fc region with low affinity and is required for ADCC[46].

The binding of antibodies to tumor cells enables their recognition and destruction by FcγRs-expressing immune effectors[47]. Cross-linking of the FcγRs extracellular domain results in the tyrosine phosphorylation of the ITAM, and activation of signal transduction pathways that lead to NK cell degranulation, release of perforin and granzyme B, cytokine secretion, and finally tumor cell lysis[48]. Thus, IgG1 mAbs are able to engage the immune system through the interaction of the Fc domain with FcγRs, which thus act as a connection between humoral and cellular immune response.

Numerous preclinical models and clinical studies strongly support the role of FcγR-mediated effector functions in mAbs cancer therapy, and the correlation between mAb binding affinity to FcγR and efficacy. Indeed, studies involving breast carcinoma xenograft models demonstrated that the anti-tumor response to trastuzumab was reduced in KO mice not expressing activating FcR; additionally, the use of antibodies engineered to disrupt Fc binding to FcγR indicated the fundamental role of Fc interaction with FcR for mAbs to exert efficacy[49]. Moreover, it has been reported that certain FcγR polymorphism can determine the clinical efficacy of trastuzumab[50] and cetuximab[51]. For example, CD16 is characterized by the valine (V)/phenylalanine (F) polymorphism at position 158, which determine a high (V158) or low (F158) affinity for the Fc, with a corresponding higher or lower induction of ADCC activity, respectively[52]. Patients bearing CD16-158V/V genotype showed improved response rates and prolonged overall survival when treated with trastuzumab and cetuximab, supporting the hypothesis that FcγR-mediated ADCC plays an important role in clinical effect of these mAbs[50,51]. Taken together, these studies indicate that the recognition of Fc receptor, and thus the triggering of ADCC, is an essential component of *in vivo* anti-tumor efficacy of trastuzumab and cetuximab.

Optimization of antibody interaction with CD16a (FcγRIIIa)

Since ADCC is suggested as a key contributor to the *in vivo* antitumor activity of many mAb therapies, increasing their binding affinity to FcγRs and thus enhancing their ability to mediate ADCC, is expected to markedly improve clinical efficacy of therapeutic antibodies. The interaction between CD16 and mAbs has been optimized by engineering the Fc region of mAbs, either through amino acid substitutions in the Fc portion or modification of Fc-linked glycosylation[53].

The nature of oligosaccharides attached to the Asn297 of CH2 domain of the IgG heavy chains, is crucial for FcRs binding (Figure 3). The removal of a fucose at this particular site induces conformational changes that selectively increase the binding affinity for CD16, with consequent

enhancement of ADCC efficacy[54,55]. A rationale for increased affinity may be the reduction or absence of steric inhibition at the receptor-ligand interface, exposing regions that are otherwise not accessible to interaction with CD16. On the other hand, non-glycosylated CD16 has the same binding affinity for fucosylated as well as non-fucosylated IgG Fc[56], supporting the finding that also carbohydrate interactions of Fc and FcR are important for enhanced binding[57]. Fucose-lacking antibodies are produced using Lec13 cell line, a variant of the Chinese hamster ovary cell line (CHO), which is unable to add the fucose but provides IgG with a glycosylation pattern that is otherwise similar to that found in normal CHO cell lines and in human serum[55,58]. An alternative approach to modify antibodies fucosylation is the overexpression or knockout of key enzymes involved in the production of CH2-attached oligosaccharides[59]. Clinical trials are already evaluating non-fucosylated antibodies for therapy[60,61], such as the defucosylated anti-CCR4 humanized monoclonal antibody that received the first approval in Japan[62].

Protein-engineered antibodies are obtained by the substitution of specific amino acids in the protein backbone, and in particular in the hinge or CH2 regions of antibodies. Several antibody variants showed a significantly enhanced binding affinity for CD16 and effector functions[63,64]. By combining single-amino acid variants, double and triple variants can be obtained. In particular, antibodies harboring the S239D-I332E double amino acid substitution were shown to trigger stronger NK cell-mediated ADCC[59,63].

Introducing modifications at both the fucose level and the amino acid sequence, combined glyco- and protein-engineered antibodies were obtained. Depending on the modification used in combined approaches, different effects on immune cells were acquired, including the enhancement of CD16 binding, the triggering of NK-cell mediated ADCC, or the selective binding to different FcγRs[55,65]. Taken together, these studies demonstrated that the modification of antibody Fc is a promising approach to improve ADCC activity of therapeutic antibodies, producing molecules endowed with a unique FcγR binding and selectivity profile, and resulting in a tailored spectra of effector mechanisms. The full length Fc-engineered antibodies used in this thesis project were generated based on the V-regions of trastuzumab and the constant regions of a IgG1 antibody, either in the wild type format or in a modified form bearing two amino acid substitutions to enhance binding to CD16 (S239D/I332E, named V90 variant)[63]. The production of these protein-engineered molecules in Lec13 or in wild type CHO cells allowed to obtain antibodies with different levels of fucosylation[65] (Figure 4). Similar antibodies were derived from rituximab, which targets CD20 instead of Her2, and were used as controls.

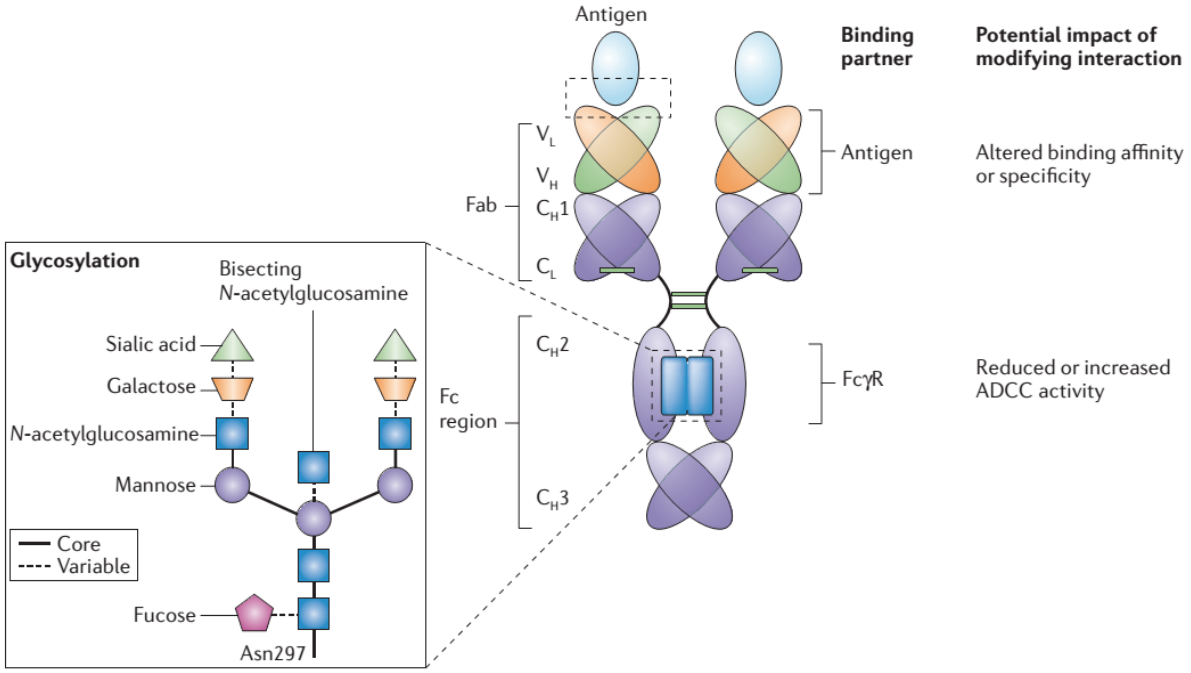

Figure 3: Antibody structure and sites for engineering. IgG antibodies are composed by a Fab region, which mediates antigen binding, and a Fc region, which interacts with FcγRs expressed by effector cells and triggers ADCC. Effector functions require the presence of a glycan moiety (dark blue) in the Fc-region, and are crucially influenced by its structure. The N-linked glycan that is attached to the conserved asparagine (Asn) residue at position 297 comprises a core structure of N-acetylglucosamine and mannose, plus additional carbohydrate residues that can vary, including fucose, galactose, sialic acid and bisecting N-acetylglucosamine (adapted from [66]).

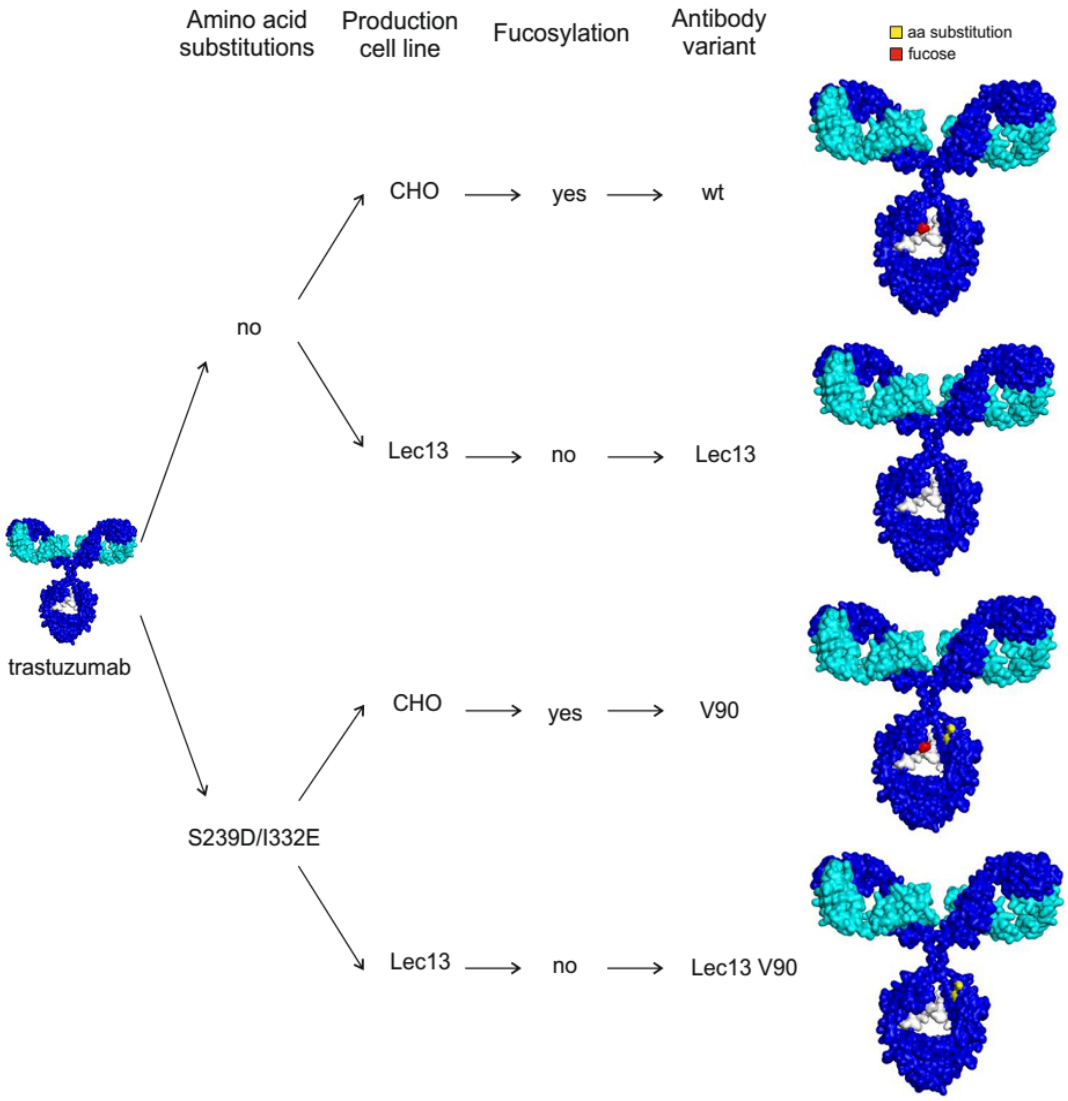

Figure 4: Schematic representation of engineered antibodies. Amino acid substitutions were introduced in the protein backbone of trastuzumab. Variants lacking Fc fucosylation were produced in Lec13 cells, while variants containing fucose in the Fc bound carbohydrates were expressed in CHO cells.

Bispecific antibodies and recombinant immunoligands

Besides Fc engineering, other strategies to enhance effector cell recruitment and activation are available. Advances in antibody engineering have generated novel antibody constructs by assembling different parts of native antibodies, and thus obtaining molecules that have two, three or even four binding sites, which simultaneously can target different epitopes on tumor as well as on immune effector cells (Figure 5)[66].

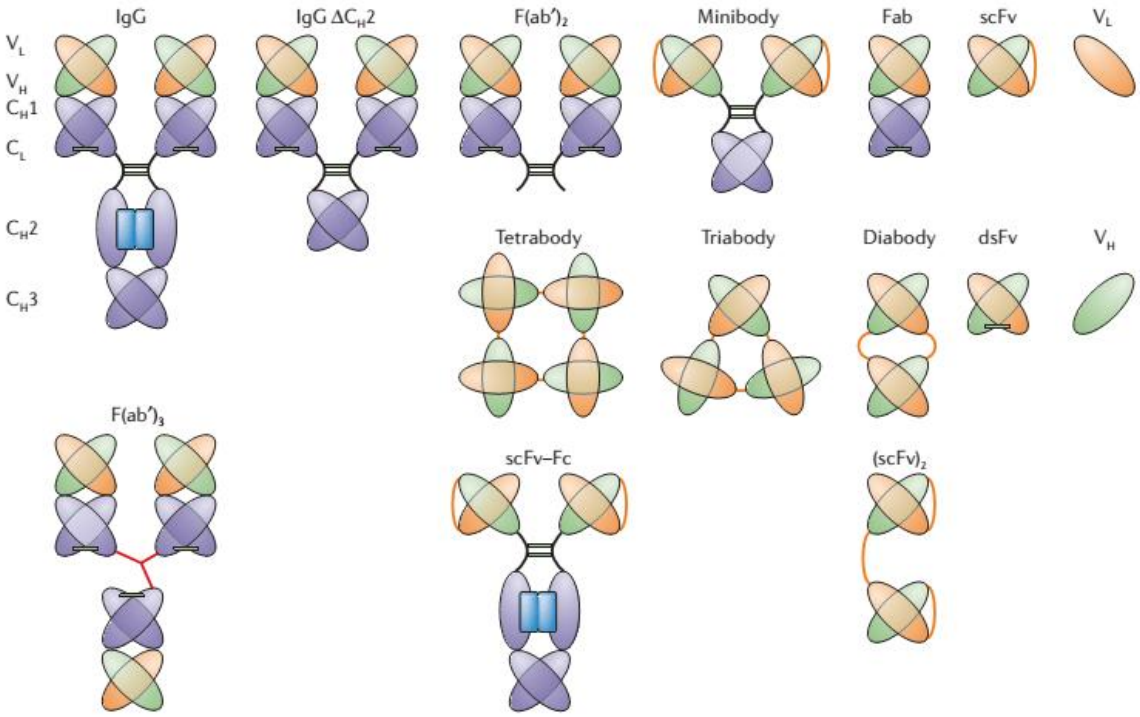

Figure 5: Representative antibody formats. The modular domain architecture of immunoglobulins has been exploited to create a growing range of alternative antibody formats. The building block that is most frequently used to create novel antibody formats is the single-chain variable (V)-domain antibody fragment (scFv), which comprises V domains from the heavy and light chain (VH and VL domain), joined by a peptide linker (adapted from [66]).

Different formats of genetically-engineered bispecific antibody-derivatives have been designed, for example diabodies, tribodies, minibodies and single chain diabodies[66,67]. Several of these non-conventional antibodies have been shown to have equivalent or superior potency as compared to

their IgG counterparts, and some are being evaluated in clinical trials[68]. Bispecific antibodies (BsAb) combine two binding specificities, one directed against a tumor-associated antigen, and the other directed against a trigger molecule on effector cells. Thereby, these antibody-variants with immunomodulatory functions are able to activate and enhance effector cells cytotoxicity, by binding to cytotoxic trigger molecules, such as CD3 and CD16[69]. Thus, they efficiently recruit cytotoxic effector cells to the tumor site and activate them to mediate the elimination of the cancer cells via redirected lysis[68]. The engagement of CD3 allows the triggering of $CD3^+$ T cells, which cannot be directly recruited by the conventional IgG antibodies, as they usually do not express Fc receptors. An example of a bispecific antibody already evaluated in clinical trials is Blinatumomab, which is directed against both CD3 and CD19, a tumor-associated antigen expressed by B cell malignancies. It proved to have high efficacy in tumor eradication and improved patients survival, thus obtaining the FDA approval for the treatment of refractory acute lymphoblastic leukemia[68].

Recombinant immunoligands are bispecific fusion proteins consisting of a single-chain fragment variable (scFv) specific for a tumor-associated antigen fused to the C-terminus of a ligand for an activating receptor, such as ULBP-2 or B7 homolog 6 (B7-H6), which are ligands of the NKG2D and NKp30 activating NK cell receptors, respectively (Figure 6). In a recent report, Kellner *et al.* demonstrated that similar immunoligands bearing the scFv of the 7D8 anti-CD20 antibody fused to NKG2D and NKp30 ligands (designated as ULBP2:7D8 and B7-H6:7D8), were able to bind to target cells and to mimic an induced-self phenotype, which efficiently triggered NK cells to kill lymphoma and leukemia cells[70,71]. Indeed, by mimicking an induced-self phenotype, such recombinant immunoligands convey a danger signal, trigger effector cell cytotoxicity and enhance ADCC[72].

Compared to mAbs, the reduced size favors tumor penetration, and the lack of the Fc domain avoids retention on non-activating Fc receptors or on Fc receptors expressed by non-cytotoxic cells[72].

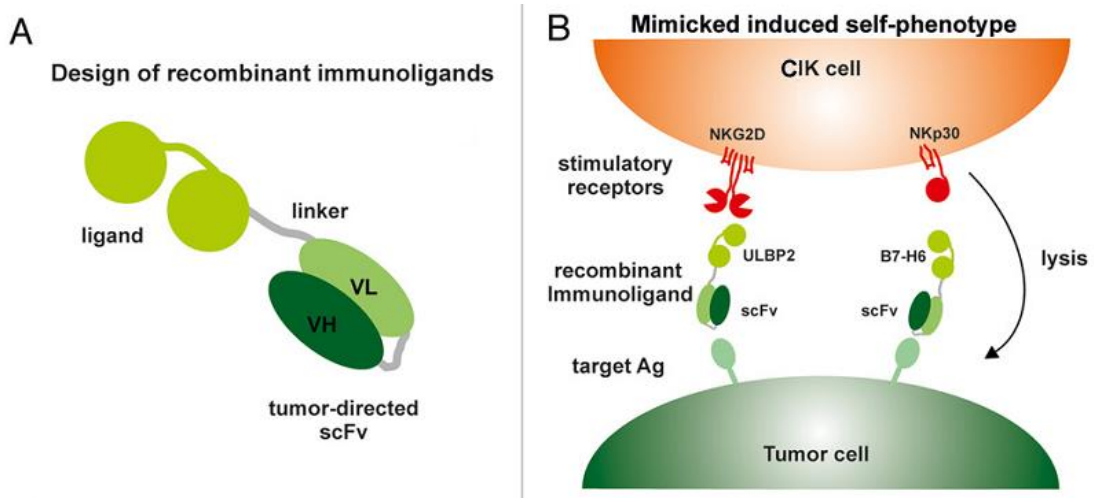

Figure 6: Enhancing CIK cell cytotoxicity by recombinant immunoligands. (A) Tumor-directed recombinant ligands are bispecific fusion proteins consisting of a scFv specific for a tumor-associated antigen, fused to the C-terminus of a ligand (e.g., ULBP2) for an activating NK cell receptor (e.g., NKG2D). (B) Binding a tumor-associated Ag via their scFv moiety, recombinant immunoligands mimick an "induced-self" phenotype, thereby facilitating the recognition of cancer cells by NK cells and inducing NK cell-mediated cytotoxic functions (adapted from [72]).

For the purposes of this study, we used a bispecific antibody in the tandem scFv format, which monovalently binds and targets CD3 and Her2, respectively[73]. This molecule consists of a CD3-directed scFv-fragment that is C-terminally fused to a Her2-specific scFv (Figure 7 A). A similar molecule targeting CD20, instead of Her2, served as a control. The resulting fusion proteins were designated as Her2xCD3 and CD20xCD3, respectively.

To generate recombinant immunoligands, the extracellular domains of B7-H6 and ULBP-2 were genetically fused to a Her2-specific scFv derived from the humanized antibody humAb4D5-8[74], and endowed with a 6 x His-tag to allow purification. The resulting fusion proteins were designated as B7H6xHer2 and ULBP2xHer2, respectively (Figure 7 B).

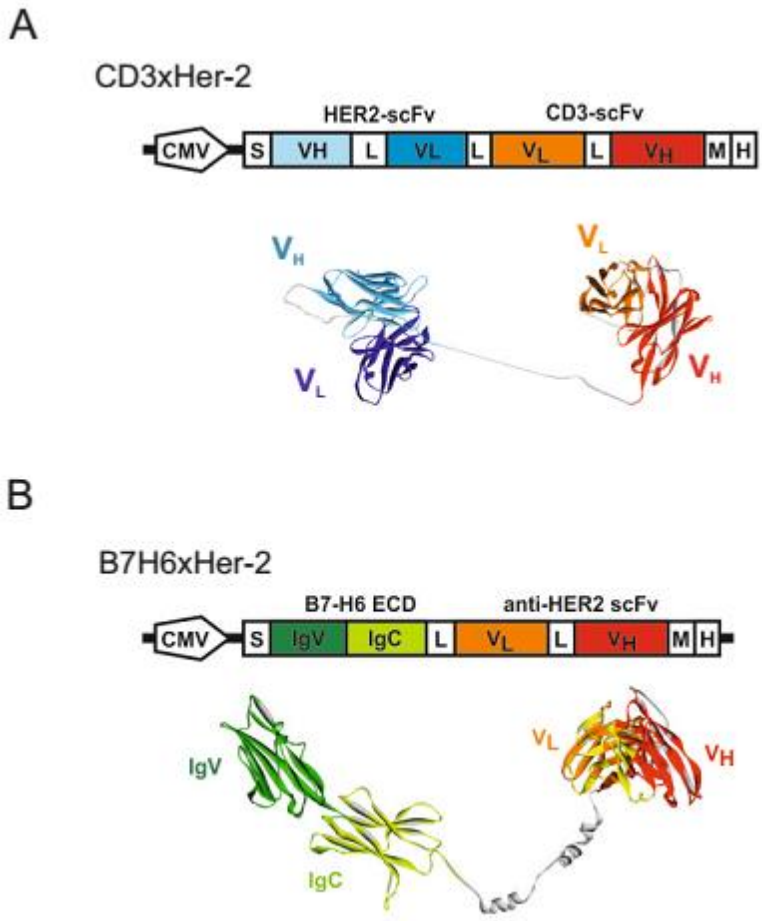

Figure 7: Schematic representation of bispecific antibodies. (A) CD3xHer2 molecule is composed by two scFv targeting CD3 and Her2, respectively. (B) The extracellular domain (ECD) of B7-H6 is genetically fused to a Her2-specific scFv. CMV indicates the cytomegalovirus immediate early promoter.

AIM OF THE STUDY

Adoptive cell immunotherapy is a very promising approach for the treatment of cancer patients. While several approaches have proven highly efficient at preclinical level and encouraging results have been collected in some clinical settings, numerous difficulties still limit the effective translation of ACT in clinics. In most cases, the expansion of large amounts of antitumor immune effectors, their persistence *in vivo*, selective tumor killing, restriction to specific human MHC alleles and safety, are still crucial and open issues. Very promising results have been provided from studies on CIK cells, as they present unique biological and functional characteristics that may favorably affect their clinical translation.

The aim of this study was to characterize CIK cells more thoroughly at both a phenotypical and a functional level. In particular, our attention focused on the expression of CD16 and its relevance as a potential inducer of ADCC by CIK cells when combined with clinical-grade antibodies. Moreover, different strategies for the improvement of CIK cell antitumor activity were assessed, including the combination with engineered antibodies and recombinant immunoligands targeting other activating receptors different from CD16.

MATERIALS AND METHODS

Generation and characterization of CIK cells

CIK cells were obtained from healthy donors as previously reported by other groups[18–20]. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by means of Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation. The monocytes were depleted by adhesion to tissue culture flask for 90 minutes. The non-adherent cells were resuspended and cultured at a density of 1 to $2x10^6$ cells/ml in complete medium consisting of RPMI 1640 (Euroclone), 10% heat-inactivated FBS (Gibco), 1% Ultraglutamine, 1% Hepes buffer, 1% penicillin/streptomycin (Lonza) at 37°C, 5% CO_2. At day 0, medium was supplemented with rhIFN-γ (PeproTech) at 1000 U/ml. After 24 hours of incubation, anti-CD3 mAb (OKT-3, Ortho Biotech Inc) at 50 ng/ml and rhIL-2 (Proleukin, Novartis) at 300 IU/ml were added to the culture medium. Every 2-3 days medium was replenished by adding fresh rhIL-2 at 450 IU/ml. CIK cells phenotype was analysed using multi-colour flow cytometry. Briefly, cells were harvested between day 7 and 28 and counted with a Nebauer chamber; $0.5x10^6$ cells were washed with staining buffer (PBS 1% FBS) and centrifuged to obtain a pellet. Staining was performed in a final volume of 50 µl, for 30 minutes at 4°C, using the following antibodies: CD3-BV510 (clone UCHT1), CD8-BV421 (clone RPA-T8), CD4-APC-H7 (clone RPA-T4), CD28-PE-Cy™7 (clone CD28.2), TCRα/β-FITC (clone T10B9.1A-31), TCRγ/δ-BV421 (clone B1), CD11a-FITC (clone G43-25B) from BD Bioscience; CD56-PE (clone HCD56), NKG2D-APC (clone 1D11), CD16a-FITC (clone 3G8), NKp30-APC (clone P30-15), NKp44-APC (clone P44-8) from BioLegend; NKp46-APC (clone 9E2) from Miltenyi Biotec. For CD16 staining, cells were pre-incubated with 2.5 µg/10^6 cells of Human BD Fc Block (BD Pharmingen) to avoid non-specific Fc Receptor-mediated antibody binding. Flow cytometry analysis was performed either on FACScalibur or LSRII, using CellQuest software (BD Bioscience). Data analyses were performed using FlowJo software (Treestar).

Depletion of NK cells

Where reported, NK cells were removed from bulk cultures by immunomagnetic depletion. CIK cells were harvested at day 14 to 21, and stained with an APC-conjugated anti-NKp46 antibody (Miltenyi Biotec) in a final volume of 200 µl, for 20 minutes at 4°C. Cells were then washed, mixed with anti-APC microbeads (Miltenyi Biotec), and incubated for 15 minutes on ice. MS MACS Columns and

MACS Separator from Miltenyi Biotec were used according to the manufacturer instructions. Unlabeled cell fraction, containing NKp46$^-$ CIK cells, and bead-coated cells, containing NKp46$^+$ NK cells, were collected separately, washed with PBS and stained with fluorochrome-conjugated antibodies to verify purity of fractions.

Cell lines and cultures

The following cell lines were used in this study: K562 human erythroleukemia cells; Raji human B-cell lymphoma cells; HCT-15, HT29 and LoVo human colorectal adenocarcinoma cells; PC-3 human prostate cancer cells; Panc human pancreatic carcinoma cells; MKN-45 human gastric cancer cells; H2452 and H28 human mesothelioma cells; SKOV-3, IGROV-1, A2780 and OVCAR-3 human ovarian carcinoma cells; MDA-MB-231 and MDA-MB-468, human breast adenocarcinoma cells; pdOVCA37, pdOVCA39, pdOVCA46, pdOVCA49bis, pdOVCA52, pdOVCA57, pdOVCA66, primary ovarian cancer, which were obtained from ascitic fluids of ovarian cancer patients and were kindly donated from Dr. Indraccolo. Cells were maintained in DMEM (Panc, MDA-MB-231 and MDA-MB-468) or RPMI 1640 (all the other cell lines) medium (Euroclone) supplemented with 10% heat-inactivated FBS (Gibco), 1% Ultraglutamine, 1% Hepes buffer, 1% penicillin/streptomycin (all Lonza). For HCT-15, HT29, LoVo, H2452, H28, SKOV-3, IGROV-1, A2780 and OVCAR-3 cell lines the RPMI medium was additionally supplemented with 1% Sodium pyruvate (Lonza). All cell lines were grown in 25, 75 or 175 cm^2 flasks (Falcon, Becton Dickinson) and under standard conditions (5% CO_2 and 37 °C). All cell lines were authenticated by STR sequences analysis.

Assessment of HER2 and EGFR expression

The expression of Her2 and EGFR was assessed on cell lines by flow cytometry using as primary antibodies the humanized anti-Her2 monoclonal IgG1 antibody trastuzumab (Roche) and the chimeric mouse-human anti-EGFR monoclonal IgG1 antibody cetuximab (MerckSerono), respectively, and a PE-conjugated anti-IgG1 antibody (Miltenyi Biotec) as secondary antibody.

Cytotoxicity assays

Cytotoxic activity of CIK cells was assessed using a standard 4-hours ^{51}Cr-release assay. A total of 10^6 target cells were labelled with 100 µCi ^{51}Cr for 1 hour at 37°C, washed twice with culture medium. Depending on the experiment, target cells were incubated for 30 min with 10 µg of trastuzumab or cetuximab, or scaling concentrations of engineered antibodies or bispecific immunoligands, ranging from 10 µg/ml to 3.2 ng/ml. For blocking experiments, effector cells were incubated for 30 min with 10 µg anti-CD16 blocking antibody. Target and effector cells were then plated at the indicated E/T ratio in a 96-well U-bottom plate in a total volume of 200 µl. After a 4-h incubation at 37°C, 30 µl supernatant was removed from each well and transferred on a scintillation plate (Perkin Elmer), and measured using a Top Count gamma counter (Perkin Elmer). Each test was performed in triplicate. The results are expressed as the percentage of lysis, which is calculated as follows: % Specific Lysis = (experimental release – spontaneous release)/(maximal release–spontaneous release) x 100. Spontaneous and maximal release were obtained by incubating target cells in medium alone or in PBS 2% SDS, respectively.

Trasduction of tumor cells with luciferase gene

To visualize tumor cells in vivo by bioluminescence imaging, SKOV-3 and IGROV-1 cells were transduced with the Luciferase enzyme gene, using a lentiviral vector coding for luciferase (LV-LUX)[75] (Figure 8). Cells were harvested and resuspended in 1 ml complete medium with concentrated LV-LUX. Cells were incubated O.N. at 37°C in the presence of the virus, then the supernatant containing the virions was discarded and fresh medium was added. Seventy-two hours after infection, cells were tested for the luciferase activity. The cells were collected, resuspended in 50 µL PBS and plated in a 96-well black plate (Nunc). 50 µL of D-Luciferin (0.3 mg/ml, Caliper) was then added and the photons emitted by the positive Lux cells were read by using IVIS Lumina II (Caliper).

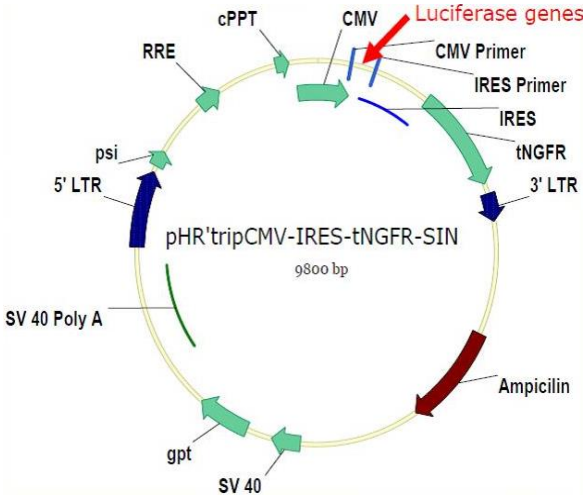

Figure 8: LV-LUX vector used for tumor cell transduction. The Fluc gene is driven by a cytomegalovirus (CMV) promoter and followed by the truncated nerve growth factor (tNGFR) gene, separated by an internal ribosomal entry site (IRES). Tumor cells were transduced and assessed for luciferase expression by BLI.

In vivo studies

On day 0, female NOD/SCID common γ chain knockout (NSG, Charles River) mice at 6 weeks of age were injected intraperitoneally with tumor cells (10^6 SKOV-3 or IGROV-1 cells), and randomly assigned to four experimental groups. The first group received the combined treatment of 1 mg trastuzumab or 1.5 mg cetuximab on day 3, 6, 9 with 10^7 CIK cells on day 4, 7, 10 after tumor injection. The other three experimental groups were used as control: mice received PBS or only the mAbs or only CIK cells, at the same doses and on the same days of the combined treatment. Tumor growth was monitored weekly by BLI under isoflurane gas anesthesia. Ten to fifteen min before imaging, each mouse received a single i.p. injection of 150 mg/kg of D-luciferin (Xenogen/Caliper) in PBS and was positioned properly to obtain ventral images within the imaging chamber of IVIS Lumina II (Xenogen/Caliper).

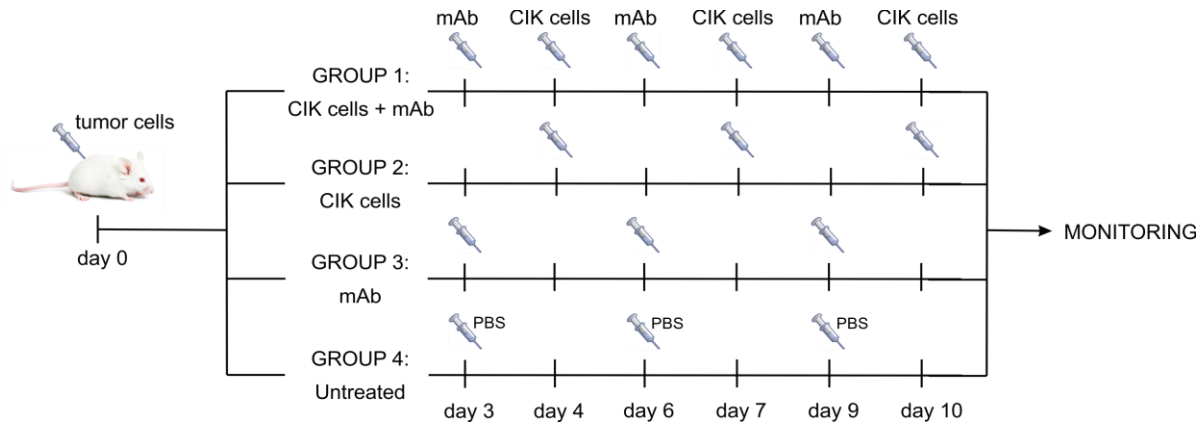

Figure 9: Schedule of mice treatment. On day 0, mice received intraperitoneally $1x10^6$ SKOV-3 or IGROV-1 cells. Depending on the experimental group, treatments consisted of a combination of 1 mg trastuzumab or 1.5 mg cetuximab on day 3, 6, 9 and $1x10^7$ CIK cells on day 4, 7, 10, or mAbs or CIK cells only at the same doses and on the same days of the combined treatment. The untreated group received three injections of PBS. Tumor growth was monitored weekly by BLI and mice survival was recorded.

Production and purification of antibody derivatives

Recombinant immunoligands were transiently expressed in Lenti-X 293 T cells (Takara Bio Europe / Clontech), which were maintained in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. For this purpose, 10 µg of the respective expression vectors were transfected using the calcium phosphate procedure including 5mM chloroquine. After 9 h, the transfection medium was replaced by fresh culture medium. Tissue culture supernatants were collected twice in a week. The bispecific antibody was captured on CaptureSelect™ IgG-CH1 affinity matrix (Life Technologies). The 6 x His-tagged immunoligands were dialyzed at 4°C in a buffer containing 50 mmol/l NaH_2PO_4, 300 mmol/l NaCl, 10 mmol/l imidazole, pH 8.0, and then purified by affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen). The proteins were then extensively dialysed against phosphate-buffered saline (PBS; Invitrogen) and stored at 4°C until use.

Antibody derivatives were analysed by SDS-PAGE under reducing and non-reducing conditions, according to standard procedures. Purity and concentrations of the purified proteins were estimated against a standard curve of BSA by Coomassie staining (colloidal Coomassie brilliant blue G250 solution (Carl Roth GmbH)) or determined by quantitative capillary electrophoresis using an

Experion Pro260 Automated Electrophoresis System (Bio-Rad), according to the manufacturer's instructions.

Statistics

Results were analyzed for statistical significance by using paired or unpaired Student *t* test, as appropriate (***P<0.001, **P<0.01, *P<.05). Mice survival was compared using log-rank survival statistics. Histograms represent mean values ± standard deviation. In scatter-plot graphs, symbols indicate different samples or assays, and horizontal bars represent means ± standard deviation. Statistical analysis was performed using GraphPad Prism 4.0 software.

Study approval

Anonymized human buffy coats were obtained from the Blood Bank of Padova Hospital, and donors provided their written informed consents to participate in this study. Procedures involving animals and their care were in conformity with institutional guidelines that comply with national and international laws and policies (D.L. 26/2014 and subsequent implementing circulars), and the experimental protocol (Autorizzazione n. 1143/2015-PR) was approved by the Italian Ministry of Health.

RESULTS

Characterization of CIK cell phenotype and antitumor activity

CIK cells were obtained from PBMCs of healthy donors by the timed addition of IFNγ, anti-CD3 (OKT3) antibody and IL-2, as previously reported[18,20] and described in the Material and Methods section. After 3 weeks of expansion, the resulting effector cell population comprised about 40% of cells co-expressing CD3 and CD56, while NK cells not only did not expand under this culture conditions, but progressively declined (Figure 10 A). Multi-colour cytometry analysis of the $CD3^+CD56^+$ subpopulation was carried out throughout the culture period to evaluate the expression of different markers. Results essentially confirmed the already reported phenotype[21,23–25] (Figure 10). In particular, the CD8 component of $CD3^+CD56^+$ CIK cells expanded reaching 82.7±4.2% of positivity at day 28, whereas the CD4 subset progressively decreased to 8.9±1.0%. A similar trend was observed for the TCR-expressing subpopulation: $TCR\alpha/\beta^+$ CIK cells increased during culture from 63.3±19.5% to 83.7±7.9%; in contrast, $TCR\gamma/\delta^+$ $CD3^+CD56^+$ cells decreased from 29.2±17.7% to 11.9±4.1%. At the end of culture almost 100% of cells expressed NKG2D (96.1±2.0%), while both CD28 (22.8±12.9%) and CD11 (94.2±5.0%) did not underwent substantial modifications (Figure 10 B).

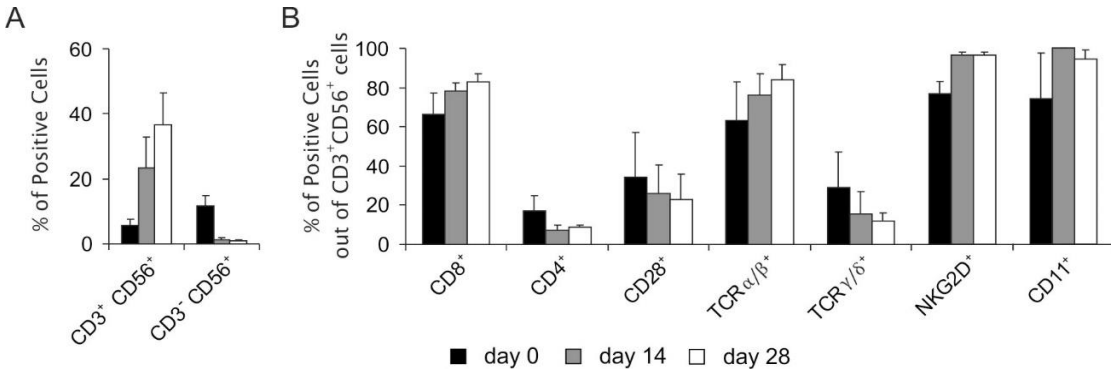

Figure 10: Phenotypic characterization of CIK cells. CIK cells were generated in vitro and analyzed for their phenotype by flow cytometry throughout the culture period. In the figure, histograms refer to three distinct time points, namely day 0 (black), day 14 (grey) and day 28 (white). (A) Percentage of CD3+CD56+ CIK cells and CD3-CD56+ NK cells in the bulk cultures at the reported time points. (B) Expression of different cell surface markers on CD3+CD56+ cells at the reported time points, as assessed by flow cytometry analysis. Results show the mean expression ± SD of 5 to 15 independent experiments performed on PBMCs and related cultures from distinct donors.

Next, the functional activity of CIK cells was evaluated against various tumor cells. CIK cells were assessed for lytic activity between day 14 and 21 of the culture, by a standard 4-hour ^{51}Cr-release assay using a broad range of tumor cell lines of different histotypes as target cells. Overall, CIK cells showed a dose-dependent cytotoxicity against all tumor cell lines tested, which however demonstrated a variable susceptibility to killing (Figure 11 and Figure 12). K562 cells were used as a positive control, as they have been already widely described as susceptible to NK-like killing[20,76]. Indeed and as expected, CIK cells were highly cytotoxic against K562 cells (58.1±11.2% at an E/T ratio of 25:1; Figure 11). A high antitumor activity against colorectal cancer cells was also observed, with a specific lysis of HCT-15, HT29 and LoVo cells of 63.0±15.8%, 26.8±13.6% and 32.5±12.0%, respectively, at an E/T ratio of 25:1. CIK cells also killed effectively prostate cancer cells (PC-3), with a specific lysis of 40.0±12.6% at the higher E/T ratio tested. The MKN-45 gastric cancer cells and the H2452 mesothelioma cells were less susceptible to killing, with a specific lysis of 18.5±12.0% and 14.7±7.4%, respectively. Breast cancer cell lines (MDA-MB-231 and MDA-MB-468) showed low susceptibility to CIK cell killing, with a specific lysis of 11.1±7.9% and 8.6±4.1%, respectively (Figure 11).

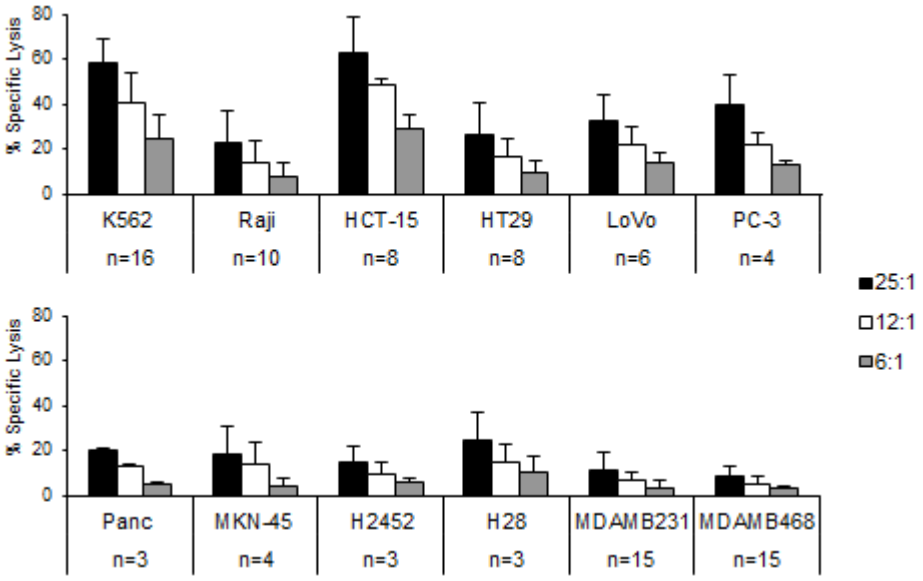

Figure 11: CIK cell cytotoxicity against different tumor cell lines. CIK cell lytic activity was measured between day 14 and 21 of the culture by ^{51}Cr-release assay. Several tumor cell lines of different histotypes were tested as target cells at an E/T ratio of 25:1 (black columns), 12:1 (white columns) and 6:1 (grey columns). Results show the mean specific lysis ± SD, while n indicates the number of independent experiments performed.

Taking advantage of the availability of a number of primary cell lines derived from ovarian cancer patients, we were prompted to assess CIK cell cytotoxicity against both primary samples and *in vitro* stabilized ovarian cancer cell lines. Overall, ovarian carcinoma cells generally showed a good susceptibility to CIK-mediated lysis (Figure 12). Among ovarian cancer cell lines, A2780 cells were the most susceptible to CIK cell killing, with a specific lysis of 40.3±13.0%, whereas values for IGROV-1 and OVCAR-3 cells were 29.0±13.5% and 16.4±10.1% (in all cases, at the higher E/T ratio). SKOV-3 cells were the less sensitive targets, with a cytotoxicity of 8.3±4.2% at the same E/T ratio (Figure 12 A). When challenged against seven different primary ovarian cancer samples, susceptibility to CIK cell cytotoxicity was always detected, albeit at variable levels. The most sensitive cells were pdOVCA37, while the less susceptible were pdOVCA66, with a specific lysis of 33.3±15.7% and 5.3±4.0%, respectively (Figure 12 B).

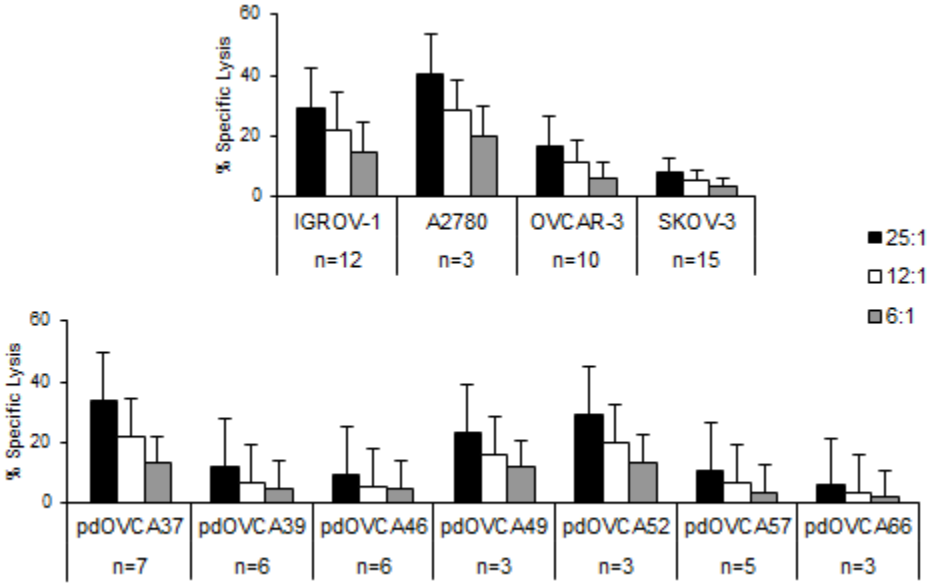

Figure 12: CIK cell cytotoxicity against ovarian cancer cells. CIK cells were challenged against *in vitro* stabilized ovarian cancer cell lines (left) and primary samples from ovarian cancer patients (right). CIK cell lytic activity was measured between day 14 and 21 of the culture by ^{51}Cr-release assay. Assays were carried out at an E/T ratio of 25:1 (black columns), 12:1 (white columns) and 6:1 (grey columns). Results show the mean specific lysis ± SD, while n indicates the number of independent experiments performed.

Assessment of CD16 expression on CIK cells

To assess CIK cell functionality and perform cytotoxicity assays against such a high number of different tumor cell lines, we expanded CIK cells from 97 different healthy donors. This high number of samples allowed us to evaluate CIK cell phenotype thoroughly. In particular, our attention focused on CD16 expression, because we repeatedly observed results that were discordant from those reported in literature. Overall, we studied in depth 60 cultures by flow cytometry using the 3G8 antibody clone, which had been already used by others[21,29], to assess CD16 expression. In our large set of samples, a remarkable expression of the CD16 receptor was detected; indeed, a mean of 16.0±13.3% (range 2.3-54.2%) of $CD3^+CD56^+$ cells co-expressed CD16 (Figure 13 A). The CD16-expressing cells were clearly detectable within the $CD3^+CD56^+$ CIK population, as they formed a defined and distinct subset in the dot plot graphs (Figure 13 B). CD16 expression in CIK cells was donor-dependent and characterized by a marked variability among donors, who were arbitrarily classified as “low” (<5%), “intermediate” (>5% and <25%), and “high” (>25%), according to the percentage of CD16 expression within their $CD3^+CD56^+$ populations (Figure 13 B). Interestingly, the fraction of CIK cells expressing the CD16 receptor remained stable during the entire period of culture, notwithstanding the enormous expansion of the $CD3^+CD56^+$ population. Indeed, 18 samples were analysed weekly by flow cytometry, and the expression of CD16 was compared at different time points within each sample, but no statistically significant differences were found (Figure 13 C).

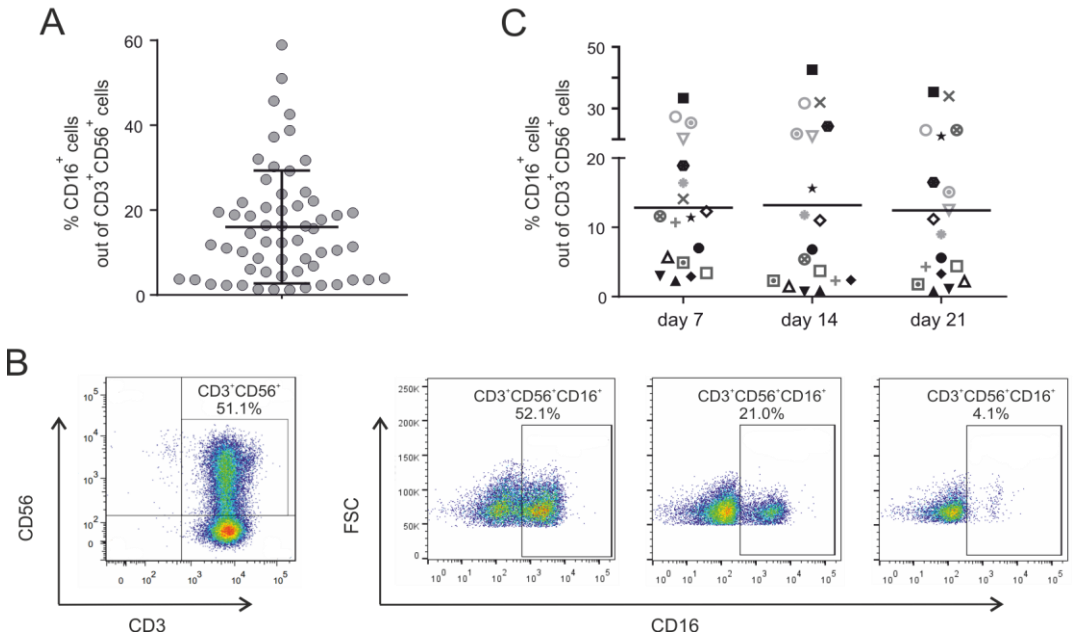

Figure 13: CD16 expression on CIK cells. CIK cells were analysed for CD3, CD56 and CD16 co-expression by flow cytometry. (A) CD16 expression was determined on samples obtained from different donors (n=60). The figure reports the individual values (grey circles) detected in the cultures between the second and third week in vitro. Average and standard deviations are also shown. (B) Gating strategy on $CD3^+CD56^+$ CIK cells for the evaluation of CD16 expression on double-positive cells. Bulk cultures were first gated for $CD3^+$ and $CD56^+$ co-espression to identify the CIK subset (*left panel*), followed by analysis of CD16 expression (*right panels*). The right panels show triple-positive CIK cells from three representative donors who were arbitrarily stratified as having a high (> 25%), intermediate (> 5% and < 25%) or low (< 5%) expression of CD16. (C) Analysis of the $CD3^+CD56^+CD16^+$ component at different time points of culture showed no statistically significant variations along the entire period of in vitro expansion. The donors (n=18) are indicated with different symbols.

Assessment of HER2 and EGFR expression on target cell lines

Chromium release assays showed the capability of CIK cells to kill a broad range of tumor histotypes. The observation that CIK cells express CD16 led us to investigate whether this receptor could mediate ADCC in CIK cells, as it occurs in NK cells. In fact, we hypothesized that CIK cell antitumor activity could be improved by combination with monoclonal antibodies, such as trastuzumab and cetuximab.

First of all, monoclonal antibodies were used as primary antibodies to evaluate Her2 and EGFR expression on target cells lines. K562 and Raji cells, both of hematological origin, were negative for

both Her2 and EGFR, as well as negative were A2780, pdOVCA49, pdOVCA57 and pdOVCA66 ovarian cancer cells. pdOVCA39 and pdOVCA52 were slightly positive for EGFR, whereas IGROV-1, OVCAR-3, pdOVCA37 and pdOVCA46 showed a higher expression level of this receptor. SKOV-3 were the only cells expressing both Her2 and EGFR among all cell lines tested. Both MDA-MB-231 and MDA-MB-468 breast cancer cell lines expressed high levels of EGFR, but resulted negative for Her2 being triple-negative tumors (Figure 14).

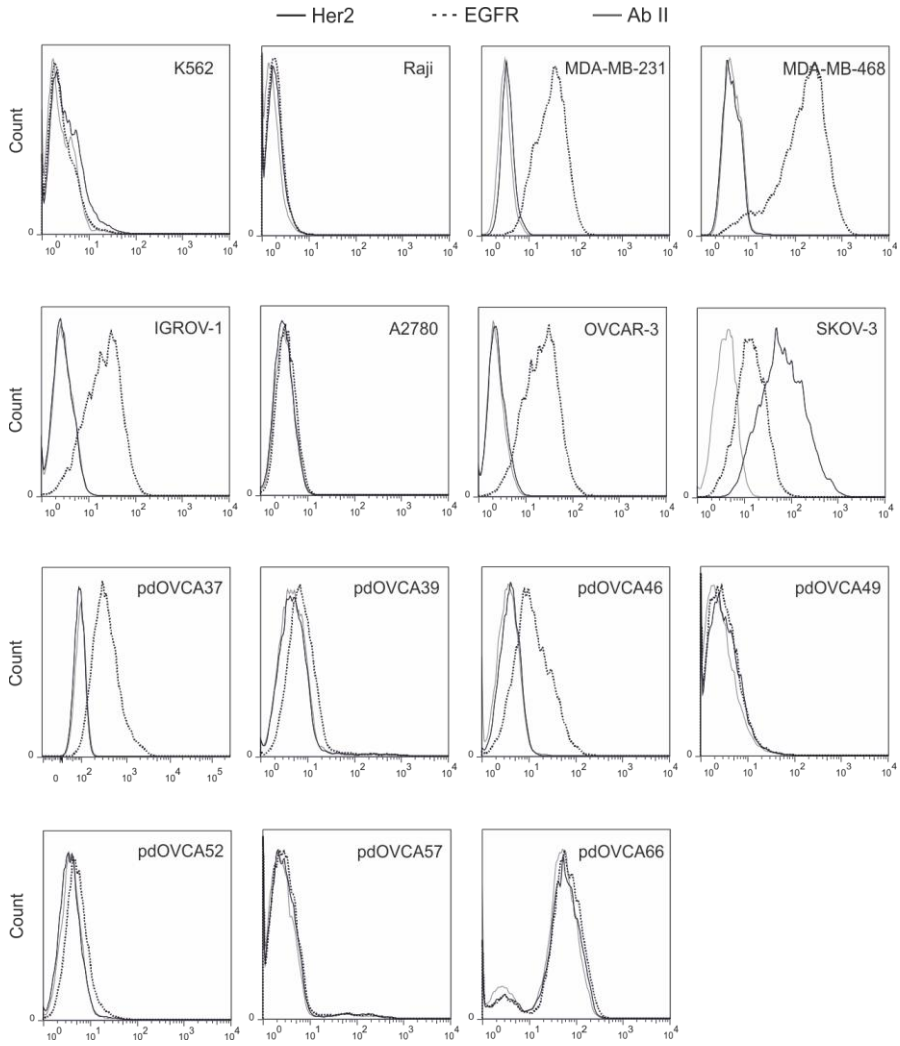

Figure 14: Her2 and EGFR expression on target cells. Target cells were analyzed for Her2 and EGFR expression by flow cytometry analysis. Fluorochrome-conjugated secondary antibody alone (Ab II) served as negative control. Results are representative of 5 experiments.

Evaluation of CIK cell Antibody-Dependent Cell-mediated Cytotoxicity (ADCC)

Based on previous results, we decided to study CIK cell cytotoxic activity using two ovarian (SKOV-3 and IGROV-1) and two breast (MDA-MB-231 and MDA-MB-468) cancer cell lines, as target cells. Thus, tumor cells were left untreated or pre-incubated for 30 minutes with 10 μg of trastuzumab or cetuximab, and used as targets in a standard chromium release assay.

Consistently with the receptor expression on target cells, a significant antigen-specific increase of cytotoxicity was observed when therapeutic antibodies were added to the assay (Figure 15). Indeed, both trastuzumab and cetuximab led to a significant enhancement of cytotoxicity against SKOV-3 cells, which express both receptors, and increased basal specific lysis (9.1±5.2%) to 35.4±13.8% and to 16.1±3.9%, respectively (Figure 15 A). On the other hand, only cetuximab increased CIK cell lytic activity toward IGROV-1, MDA-MB-231, and MDA-MB-468, as EGFR only is expressed on these cells; in this case, basal specific lysis (35.3±14.2%, 19.7±12.1% and 13.0±12.3%, respectively) increased to 59.0±7.7%, 45.2±14.4% and 41.3±21.3%, respectively (Figure 15 B-D). Importantly, trastuzumab did not increase the killing against Her2 negative target cell lines (Figure 15 B-D), thus suggesting that the enhanced cytotoxic activity was indeed mediated by the engagement of CD16 and subsequent ADCC.

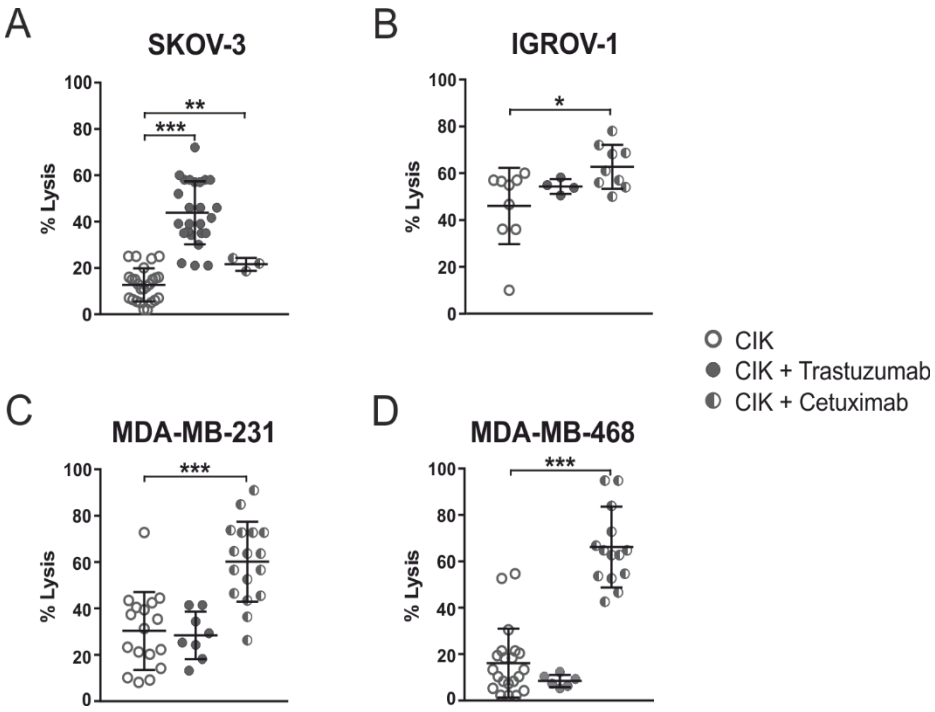

Figure 15: Monoclonal antibodies enhance CIK cells cytotoxicity. CIK cells were challenged in a standard cytotoxicity assay against SKOV-3 (A), IGROV-1 (B), MDA-MB-231 (C) and MDA-MB-468 (D) tumor cell lines. Lytic activity against each cell line was measured using bulk CIK cells as effector cells in the absence or presence of 10 μg of trastuzumab or cetuximab. Data shown are the percentage of specific lysis at an effector/target ratio of 100:1, with the assays carried out when CIK cell cultures were at day 21. The symbols refer to individual CIK cell cultures from different donors, and bars indicate mean values ± SD. Data were analyzed by Student's t-test (*** or §§§, $P \leq 0.001$; **, ## or §§, $P \leq 0.01$; *, # or §, $P \leq 0.05$; not statistically significant ($P > 0.05$) if not indicated).

CD16 blocking assay

To formally prove that the increase in cytotoxic activity was truly linked to the engagement of CD16 and the triggering of subsequent ADCC phenomena, CIK cells were pretreated with a mouse anti-human CD16 blocking antibody to prevent the specific interaction between CD16 and the Fc region of therapeutic mAbs. CD16-masking completely abrogated the additional CIK cell lytic activity ascribable to the addition of trastuzumab or cetuximab, leading the cytotoxicity to return to the basal levels observed with untreated CIK cells (Figure 16). In particular, the cytotoxicity against SKOV-3, which was enhanced by the addition of trastuzumab, reduced from 35.4±13.8% to 19.8±11.2% with the addition of anti-CD16 antibody, this latter value being not significantly different

from basal specific lysis (Figure 16 A). Similarly, masking of CD16 led to a specific lysis reduction to 28.3±15.3%, 24.5±5.8% and 28.3±7.1% for cetuximab-treated IGROV-1, MDA-MB-231, and MDA-MB-468, respectively, as the interaction between the receptor and mAb is prevented (Figure 16 B-C). As a control, CIK cells were pretreated with an irrelevant mouse IgG1 isotype antibody, in the presence or absence of mAbs, to rule out potential interferences in the tests (Figure 16). In all cases, the addition of such control antibody nor reduced the increased activity mediated by trastuzumab or cetuximab, nor interfered with basal CIK cell functions.

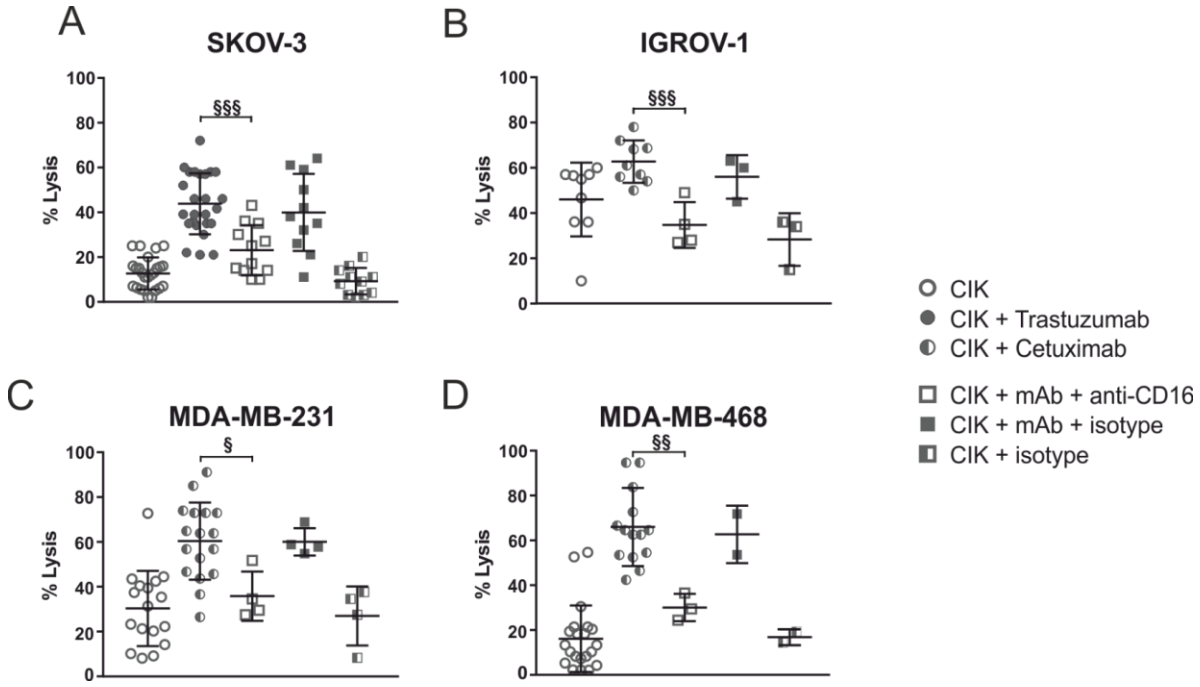

Figure 16: Blocking CD16 reduces cytotoxicity. CIK cells were challenged in a standard cytotoxicity assay against SKOV-3 (A), IGROV-1 (B), MDA-MB-231 (C) and MDA-MB-468 (D) tumor cell lines. Lytic activity against each cell line was measured using bulk CIK cells as effector cells in the absence or presence of 10 µg of trastuzumab or adding an anti-CD16 blocking antibody to the corresponding specific mAb, namely trastuzumab in A and cetuximab in B, C and D. An isotype antibody served as negative control. Data shown are the percentage of specific lysis at an effector/target ratio of 100:1, with the assays carried out when CIK cell cultures were at day 21. The symbols refer to individual CIK cell cultures from different donors, and bars indicate mean values ± SD. Data were analyzed by Student's t-test (*** or §§§, P ≤ 0.001; **, ## or §§, P ≤ 0.01; *, # or §, P ≤ 0.05; not statistically significant (P > 0.05) if not indicated).

As different levels of CD16 expression were detected on CIK cells obtained from different donors, we wanted to unveil whether cytotoxic activity was dependent on the amount of CD16 present on cells. To this aim, we performed cytotoxic assays by using CIK cells presenting different extent of CD16 expression. An antibody-mediated increase of the cytotoxic activity could be detected in all CIK cultures tested, irrespective of the levels of CD16 expression. Indeed, as shown in Figure 17, the cytotoxicity in the presence of the mAbs was statistically comparable between CIK populations with different CD16 expression levels, against both ovarian and breast cancer cell lines.

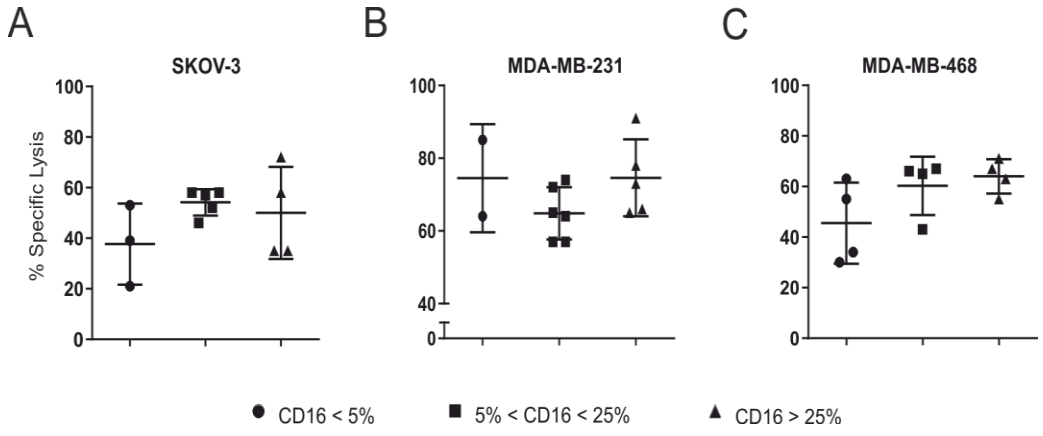

Figure 17: Correlation analysis between CD16 expression and ADCC activity. CIK cells samples were arbitrarily stratified as "low" (<5%), "intermediate" (>5% and <25%) and "high" (>25%), according to their CD16 expressing levels. The lytic activity of these subsets against SKOV-3 (A), MDA-MB-231 (B) and MDA-MB-468 (C) tumor cell lines was then measured in a standard cytotoxicity assay, in the presence of the corresponding specific mAb (trastuzumab in A, and cetuximab in B and C). The results are reported as the percentage of specific lysis at an E/T ratio of 100:1. Each symbol represents a different donor, and bars indicate mean values ± SD. Data were analyzed by Student t test. No statistical significant differences were found between different groups.

Depletion of NK cells and evaluation of killing

It is well known that CD16 is expressed by $CD3^{-}CD56^{+}$ NK cells, which also have the ability to kill tumor cells by ADCC [77]. NK cells were present in PBMCs at the beginning of *ex vivo* CIK cell expansion phase, while their percentage progressively decreased during culture (Figure 1 A). Therefore, to verify that the ADCC activity observed was not mainly due to the presence of a residual NK subpopulation but rather to the $CD3^{+}CD56^{+}CD16^{+}$ CIK cells, NK cells were removed from cultures by immunomagnetic depletion with a mAb against NKp46 [78,79]. This receptor is exclusively expressed by NK cells, but not by CIK cells[21,53,80]. Indeed, staining CIK cells with an NKp46-specific antibody, no expression was found on $CD3^{+}CD56^{+}$ cells (Figure 18 A). One example of NK depletion is reported in Figure 18 B, in which 10.5% of NK cells were present in bulk population. Immunomagnetic depletion allowed to removing almost completely NK cells from the culture, thus obtaining cell populations composed only by $CD3^{+}CD56^{+}$ CIK cells and $CD3^{+}CD56^{-}$ T cells.

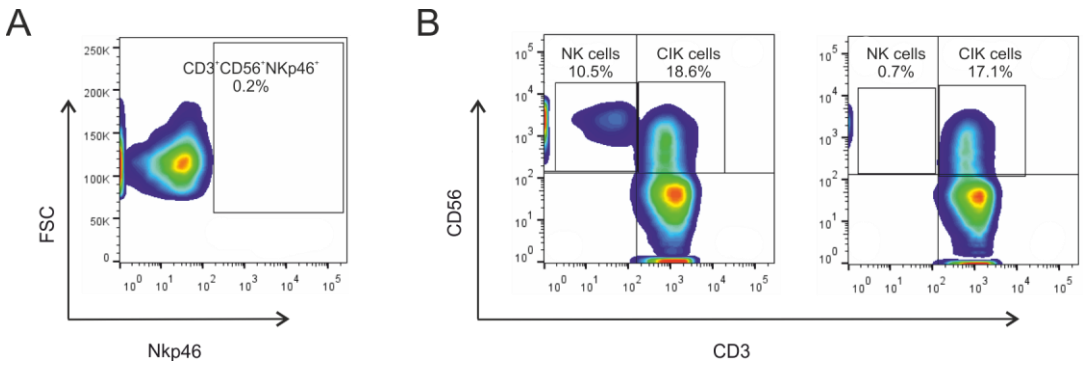

Figure 18: NK cell depletion from bulk CIK cell cultures. CIK cells were stained for CD3, CD56 and NKp46. (A) Flow cytometry analysis was performed in quadruple fluorescence to determine the absence of NKp46 expression on CD3+CD56+ cells. (B) NKp46+ NK cells were removed by magnetic beads depletion. The panels show one representative experiment out of 5 performed, where flow cytometry analysis was carried out before (*left*) and after (*right*) NK depletion.

Thus, bulk and NK-depleted CIK cells were challenged against target cell lines in a standard ^{51}Cr-release assay, in the presence or absence of the corresponding specific therapeutic mAb. Against all target cell lines tested, the cytotoxicity of NK-depleted and bulk CIK cells fully overlapped, both alone and in combination with the mAbs (Figure 19). The addition of mAbs to NK-depleted populations was still able to produce a significant increase of cytotoxicity. These results demonstrate

that the enhanced cytotoxicity induced by mAbs is associated with $CD3^+CD56^+CD16^+$ subpopulation in CIK cell cultures, confirming that CIK cells can perform ADCC by the engagement of CD16.

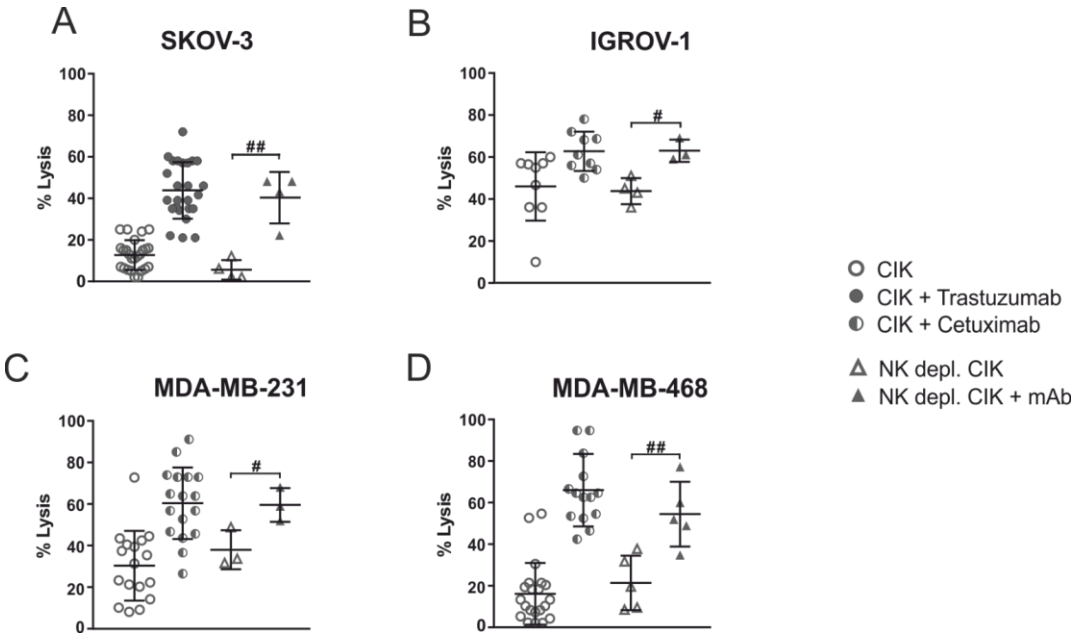

Figure 19: NK depletion does not affect CIK cell-mediated ADCC. CIK cells were challenged in a standard cytotoxicity assay against SKOV-3 (A), IGROV-1 (B), MDA-MB-231 (C) and MDA-MB-468 (D) tumor cell lines. Lytic activity against each cell line was measured using bulk CIK cells or NK-depleted CIK cells (NK depl. CIK) as effector cells in the absence or presence of the corresponding mAb, namely trastuzumab in A and cetuximab in B, C and D. Data shown are the percentage of specific lysis at an effector/target ratio of 100:1, with the assays carried out when CIK cell cultures were at day 21. The symbols refer to individual CIK cell cultures from different donors, and bars indicate mean values ± SD. Data were analyzed by Student's t-test (*** or §§§, $P \leq 0.001$; **, ## or §§, $P \leq 0.01$; *, # or §, $P \leq 0.05$; not statistically significant ($P > 0.05$) if not indicated).

In vivo therapeutic efficacy of CIK cells

The activity of CIK cells in combination with trastuzumab and cetuximab was evaluated in ovarian cancer xenograft models established in NSG mice. To follow tumor growth *in vivo* by bioluminescence imaging, tumor cells were transduced with a lentiviral vector coding for luciferase (LV-LUX), and were assessed for luciferase activity using BLI (Figure 20 A).

On day 0, female mice were injected intraperitoneally with $1x10^6$ SKOV-3-*luc* or IGROV-1-*luc* tumor cells. Then, animals were randomly assigned to control and experimental groups, and treated i.p. with CIK cells in combination or not with the mAbs, as described in the Material and Methods section. As shown in Figure 20 B, already three weeks after tumor injection there was a significant difference between the experimental groups. Untreated mice showed a very high tumor burden, whereas mice treated with CIK cells alone demonstrated a variable response to treatment. The best outcomes were achieved in mice receiving mAbs alone and in particular mAbs combined to CIK cells, as the tumor growth appeared to be more inhibited. As showed in Kaplan-Meier survival curves in Figure 20 C-D, the co-administration of CIK cells and mAbs produced long-term effects and significantly increased the survival of both SKOV-3 (Figure 20 C) and IGROV-1 (Figure 20 D) tumor-bearing mice, as compared to animals receiving CIK cells or mAb alone. In this setting, CIK cell monotherapy was not effective in improving the outcome of treated mice; conversely, when combined with trastuzumab or cetuximab CIK cells appeared even more efficient than the antibodies used alone, confirming the high therapeutic efficacy of the treatment.

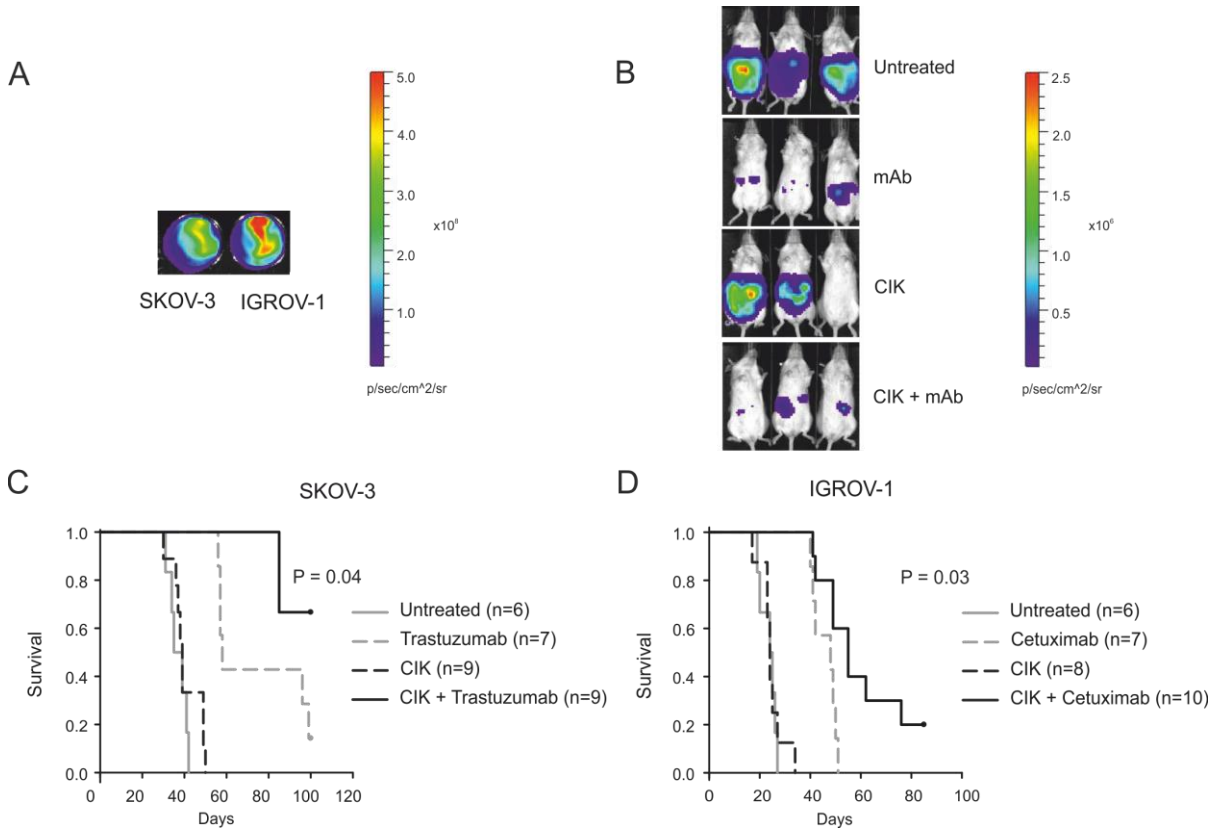

Figure 20: The in vivo combined approach with mAb and CIK cells improves the antitumor activity. (A) Assessment of Luciferase expression by SKOV-3 (*left well*) and IGROV-1 cells (*right well*) after lentiviral transduction using BLI. (B) Mice were injected i.p. with $1x10^6$ transduced tumor cells and were treated with three intraperitoneal doses of PBS only (untreated), the corresponding specific mAb only (1 mg/mouse of trastuzumab or 1.5 mg/mouse of cetuximab), 10^7 bulk CIK cells only, or both mAbs and CIK cells at the same doses of the other groups. Tumor growth was assessed weekly by BLI. The panel shows 3 representative mice for each group 3 weeks after tumor injection. (C-D) Mice survival was evaluated by Kaplan-Meier analysis. Combination therapy significantly increased survival compared to any other treatment, both in SKOV3 (C, P=0.04) and IGROV-1-injected mice (D, P=0.03).

Improvement of CIK cell cytotoxicity: Fc-engineered antibodies

As we demonstrated the expression of CD16 in CIK cells, and its ability to induce ADCC *in vitro* and to improve the therapeutic efficacy *in vivo*, we were prompted to investigate whether this function could be further improved. To evaluate whether an increased CD16 binding affinity could result in enhanced CIK cell cytolytic activity, different Fc-optimized versions of trastuzumab were used in

combination with CIK cells. Recombinant proteins were produced, purified by affinity chromatography and analyzed by SDS-PAGE with Coomassie staining to determine the protein concentration. SKOV-3 cells were used as target cells in the ^{51}Cr-release assays, and pre-incubated with the wild-type form of trastuzumab or with the engineered antibodies, in particular the glyco-engineered Lec13 (trastuzumab Lec13), the protein-engineered V90 (trastuzumab V90) or the both glyco- and protein-engineered antibody (trastuzumab Lec13 V90). The presence of the antibodies significantly enhanced CIK cell cytotoxicity, inducing a higher cell lysis against target cells as compared to the wild type antibody (Figure 21). At lower concentrations (0.0032 µg/ml), the double engineered trastuzumab Lec13 V90 triggered ADCC more efficiently in comparison to the other engineered antibodies trastuzumab Lec13 and trastuzumab V90. As expected, rituximab, which binds the CD20 molecule that is not expressed on SKOV-3 cells, was not able to induce target cell killing, either in the wild-type format or in the glyco-engineered form (Figure 21).

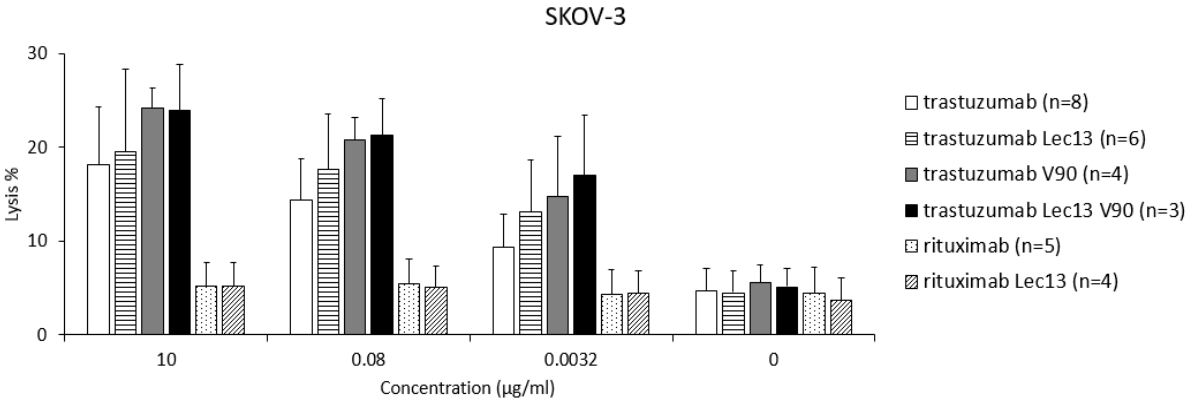

Figure 21: CIK cell cytotoxicity is enhanced by engineered antibodies. CIK cells were challenged in a standard cytotoxicity assay against SKOV-3 tumor cell line in combination with scaling concentrations of the wild-type form of trastuzumab (*white columns*), the glyco-engineered Lec13 (trastuzumab Lec13, *striped columns*), the protein-engineered V90 (trastuzumab V90, *grey columns*) or the both glyco- and protein-engineered antibody (trastuzumab Lec13 V90, *black columns*). Rituximab (*dotted columns*) and the glyco-engineered rituximab (*oblique striped columns*) served as negative controls. Data shown are the mean percentage ± SD of specific lysis at an effector/target ratio of 50:1, and the assays was carried out at day 21 of CIK cell cultures. Data were analyzed by one-way ANOVA, but were statistically significant ($P > 0.05$) only when compared to controls (rituximab and rituximab Lec13).

Improvement of CIK cell cytotoxicity: bispecific antibodies

To investigate whether CIK cell cytotoxicity could be enhanced through the engagement of receptors other than CD16, we decided to evaluate the expression of NKp30 on CIK cells by flow cytometry analysis. Figure 22 A shows one representative experiment in which CIK cells were stained with anti-NKp30 and an isotype control antibodies, showing a remarkable expression of the receptor on $CD3^+CD56^+$ cells. On the other hand, we had already assessed that NKG2D is expressed on almost all $CD3^+CD56^+$ cells (Figure 1 B).

Therefore, target cells were incubated with bispecific antibodies or immunoligands that target Her2 on target cells and CD3, NKG2D or NKp30 on CIK cells, and used in ^{51}Cr-release assays. The engagement of CD3 with the CD3xHer2 bispecific scFv produced an outstanding enhancement of killing, which resulted to be even higher than that obtained by trastuzumab. The increase of activity was antigen-specific, as the control CD3-specific bispecific scFv targeting CD20 was not able to induce target cell killing. On the contrary, the engagement of NKG2D and NKp30 did not produce a significant enhancement of activity, suggesting that the signals transduced through these molecules are not sufficient to enhance CIK cell cytotoxicity on their own (Figure 22 B).

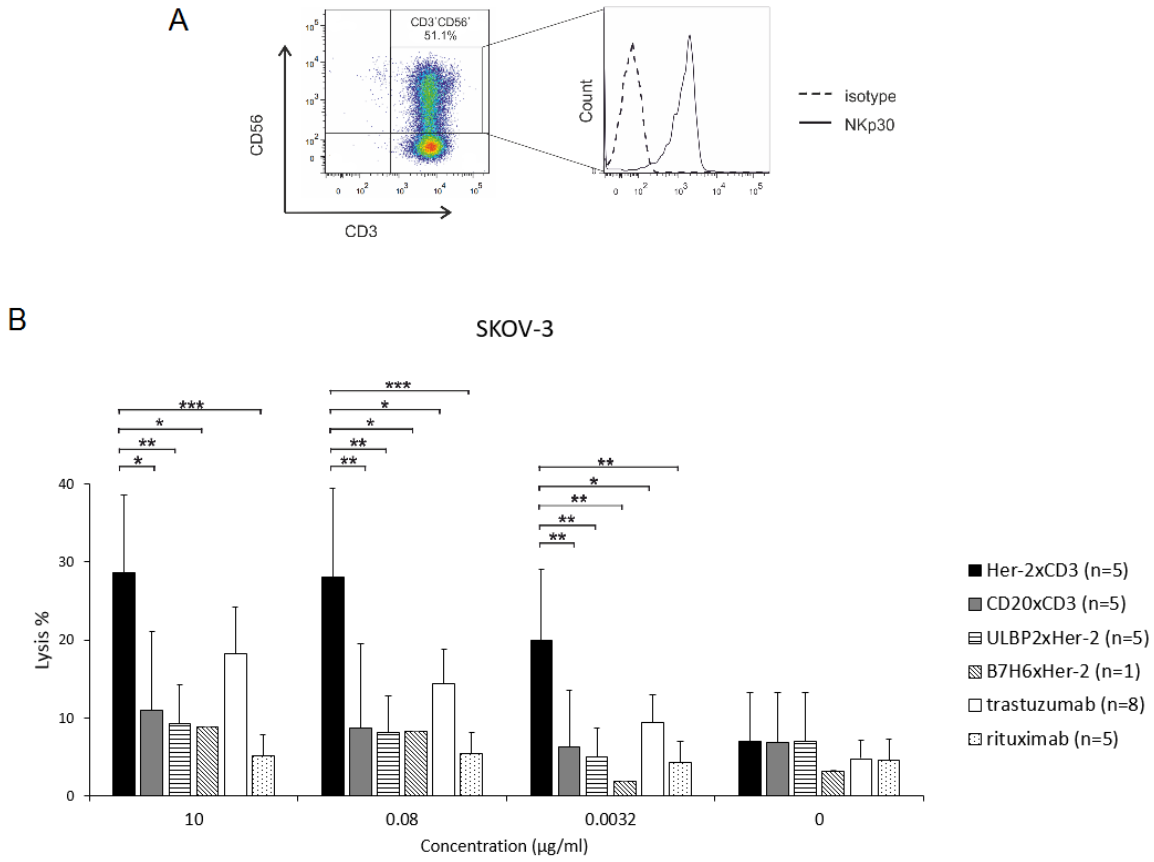

Figure 22: CIK cell cytotoxicity is enhanced by bispecific antibodies. CIK cells were analyzed for their NKp30 expression by flow cytometry. (A) Gating strategy on $CD3^+CD56^+$ CIK cells for the evaluation of NKp30 expression on double-positive cells. Bulk cultures were first gated for CD3 and CD56 co-espression to identify the CIK subset (*left panel*), followed by analysis of NKp30 expression (*right panel*). (B) Lytic activity of CIK cells was measured between day 14 and 21 of the culture by ^{51}Cr-release assay against the SKOV-3 tumor cell line, combining effector cells with scaling concentrations of Her2xCD3 (*black columns*), CD20xCD3 (*grey columns*), ULBP2xHer2 (*striped columns*), B7H6xHer2 (*oblique striped columns*) bispecific antibodies. Trastuxumab (*white columns*) and rituximab (*dotted columns*) served as controls. Data shown are the mean percentage ± SD of specific lysis at an effector/target ratio of 50:1. Data were analyzed by one-way ANOVA (*** or §§§, P ≤ 0.001; **, ## or §§, P ≤ 0.01; *, # or §, P ≤ 0.05; not statistically significant (P > 0.05) if not indicated).

DISCUSSION

Recent years have witnessed a renewed interest for immunotherapy in cancer, mainly due to the successes of checkpoint inhibitors[81], and ACT with CAR-redirected T cells[10]. These latter, in particular in hematological malignancies, outperformed previous strategies involving the infusion of *ex vivo* expanded TIL[11] or NK cells[12]. However, the genetic manipulation required for the obtainment of CAR-modified T cells and the extremely high costs for their production represent a strong limitation to the wide clinical diffusion of this approach. These obstacles could be overcome by using CIK cells, a population of effector cells obtained from PBMCs after *ex vivo* stimulation, which show unique and interesting features supporting their role as a promising tool for ACT approaches.

In this study, CIK cells were obtained from healthy donor PBMCs according to standard protocols[18–20]. Phenotype of the $CD3^+CD56^+$ subpopulation was evaluated by cytometry analysis and results essentially confirmed the already reported phenotype[21,23–25]. In particular, $CD3^+CD56^+$ CIK cells are mostly $CD8^+$, and express a functional TCR that is responsive to specific MHC-dependent stimuli but not directly involved in tumor cell killing[24]. On the other hand, CIK cells are characterized by high levels of NKG2D that is considered the main mediator of their antitumor activity, as it binds MICA/B and members of the ULBP family, leading to target cell recognition and lysis[25]. Moreover, we detected high levels of CD11, which is a requisite for target cell binding and lysis[24].

Furthermore, we characterized the functional activity of CIK cells against 23 different tumor samples comprising both primary and tumor cell line specimens derived from a broad range of different histotypes, and demonstrated a high antitumor potential for CIK cells. To carry out functional assays against such a large number of target cells, we expanded 97 different CIK cell cultures and for 60 of these we had the possibility to carry out a thorough analysis of the phenotype. To the best of our knowledge, this is the first report with such a high number of donors tested. Flow cytometry results highlighted a remarkable and donor-dependent expression of CD16 on $CD3^+CD56^+$ cells. In this regard, there is not a full agreement about the expression of CD16 on CIK cells, since some groups reported that CIK cells do not express this receptor[20,21,82], while others detected its expression at some extent, but did not further investigated such issue[80,83]. Pievani *et al*. reported that CIK cells present a dual functional ability to kill tumors, through either T cell-like TCR-dependent or NK cell-like NKG2D-mediated mechanisms[24]. Based on our phenotypical observations, we were prompted to investigate the potential functional relevance of CD16 in improving the CIK cells intrinsic cytotoxic

activity, when combined with antitumor therapeutic mAbs to exert ADCC phenomena. Indeed, it has been widely accepted that ADCC plays an essential role in mediating the antitumor effects of therapeutic mAbs routinely used in the treatment of cancer, such as trastuzumab and cetuximab[43,84]. The combination of mAbs with CIK cells produced a significant antigen-specific increase of cytotoxicity that was consistent with Her2 and EGFR expression on target cells. The direct involvement of CD16 was demonstrated by using a blocking monoclonal antibody against the CD16, which was able to bring about the cytotoxicity to return to the basal levels, thus indicating that the CD16 receptor is functional and directly involved in the ADCC. An important observation is the CIK cell ability to increase the cytotoxic activity irrespective to the levels of CD16 expressed. This finding suggest that even a small fraction of $CD16^+$ CIK cells can exert a relevant ADCC activity, likely due to the recycling potentialities of the effectors, and leads to a significant enhancement of the whole population cytotoxicity. Translated into a potential clinical application, this aspect could represent a critical issue, since patients generating CIK cell cultures with even a low expression of CD16 might strongly benefit from CIK and mAbs combination therapy.

Another relevant aspect is that most of the CIK cell studies reported in literature, including clinical trials of adoptive immunotherapy, used bulk-expanded CIK cultures where NK cells were still present as contaminants. To exclude that the ADCC activity observed was due to the presence of a residual NK subpopulation, NK cells were removed from cultures by immunomagnetic depletion with a mAb against NKp46[78,79]. Performing a depletion for NKp46, instead of a positive selection using CD5, which is not expressed by NK cells[85], allowed us to obtain "untouched" CIK cells, thus preventing unwanted nonspecific effector cell stimulation in the subsequent cytotoxicity assays. Analysis of lytic activity confirmed that the ADCC phenomena observed are accountable to the $CD3^+CD56^+CD16^+$ subpopulation, confirming for the first time that CIK cells can induce ADCC by the engagement of CD16. This result was further supported by *in vivo* experiments using human ovarian carcinoma xenografts in NSG mice, in which the co-administration of CIK cells and mAbs significantly increased the survival of treated mice. The inefficacy of the treatment with CIK cells as a single agent is consistent with our *in vitro* results, and mostly with reports from other groups where SKOV-3 cells have been described to be resistant to CIK cell-mediated killing[83,86,87]. Nevertheless, when treated with the combination of CIK cells and mAbs, even these resistant cells become responsive to therapy.

Overall, we advance that the combination of adoptive CIK cell therapy with clinical-grade mAbs can be proposed as a very promising strategy to improve the therapeutic approaches against different

types of tumors. Indeed, several clinical studies demonstrated the feasibility and high tolerability of CIK cell infusions[36], and moreover trastuzumab and cetuximab are well established and widely used in clinical practice; therefore, no particular safety and clinical concerns should be raised for this combination approach.

This novel strategy we advance could address one of the critical issues of current adoptive immunotherapy approaches, i.e. the rapid obtainment of sufficient numbers of targeted effector cells to be administered. Indeed, CIK cells are easy to produce and do not require genetic manipulations for expansion; moreover, the relevant enhancement of CIK antitumor potential mediated by mAb combination could allow to reduce the total cell dose and the number of infusions required for treatment, with positive implications for patients with limited *ex vivo* expansion rates of CIK cells[17]. Furthermore, the finding of no correlation between the expression level of CD16 on CIK cells and the cytotoxicity outcome when combined with mAbs, further broadens the number of patients that could potentially benefit from this therapy.

Moreover, the results reported in this work open additional opportunities to further improve CIK cell antitumor activity. Indeed, the combination of CIK cells with Fc-engineered antibodies demonstrated that tumor cell killing depends on an antigen-specific recruitment of CIK cells, and that a higher binding affinity between CD16 and mAbs is capable of leading to an increased cytotoxicity. Another promising strategy to improve CIK cell antitumor activity relies on the combination with bispecific antibodies, which have the potential to activate and trigger effector cells also through the engagement of receptors other than CD16, such as CD3 and NKp30. Importantly, when using bispecific antibodies, concomitant engagement of both the triggering molecule on CIK cells, and the tumor antigen on target cells is necessary to induce CIK cell cytotoxicity, thus assuring a safety mechanism for unwanted non-specific activation.

In conclusion, we demonstrated for the first time that CIK cells express CD16 in a donor-dependent manner, and that the concurrent administration of therapeutic mAbs, such as trastuzumab or cetuximab, leads to a significant improvement of their antitumor activity, triggering a potent ADCC activity. Additionally, the modification of the Fc region of mAbs increases their binding affinity to CD16 and enhances CIK cell cytotoxicity. Moreover, these cells can also be triggered by the engagement of alternative activating receptors, producing a further enhancement of antitumor activity. Taken together, the results produced in this study describe a novel function of CIK cells, which confirm their outstanding potential as a promising tool for ACT approaches. These data lead us to envisage new perspectives for adoptive immunotherapy where antigen-specific retargeting of

T cells can be achieved by the simple combination of non antigen-specific effector cells and tumor-specific mAbs, already widely employed in clinical practice.

ABBREVIATIONS

ACT	Adoptive cell transfer
ADCC	Antibody-Dependent Cell-mediated Cytotoxicity
B7-H6	B7 homolog 6
BLI	Bioluminescence imaging
BsAb	Bispecific Antibody
CAR	Chimeric antigen receptor
CH1 or 2	Constant domain 1 or 2 of the Heavy chain
CIK	Cytokine Induced Killer cells
E/T ratio	Effector/Target ratio
EGFR	Human epidermal growth factor 1
Fab region	Fragment antigen-binding region
Fc region	Fragment crystallizable region
FcγR	Fcγ receptor
GVHD	Graft versus host disease
Her-2	Human epidermal growth factor 2
i.p.	Intraperitoneal
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IL-2	Interleukin-2
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
KO	Knockout
luc	Luciferase gene
mAbs	Monoclonal Antibodies
MHC	Major Histocompatibility Complex
NKG2D	Natural-Killer group 2 member D
NSG	NOD/SCID common γ chain knockout
PBMCs	Pheripheral Blood Mononuclear Cells
scFv	Single chain fragment variable
ULBP	UL-16 binding protein

BIBLIOGRAPHY

1. Rosenberg, S. A. & Restifo, N. P. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science (80-.).* **348,** 62–68 (2015).
2. Rosenberg, S. A. IL-2: The First Effective Immunotherapy for Human Cancer. *J. Immunol.* **192,** 5451–5458 (2014).
3. Tarhini, A. a, Gogas, H. & Kirkwood, J. M. IFN-α in the treatment of melanoma. *J. Immunol.* **189,** 3789–93 (2012).
4. Mocellin, S., Mandruzzato, S., Bronte, V., Lise, M. & Nitti, D. Review Part I : Vaccines for solid tumours. **5,** 681–689 (2004).
5. McMahon, B., Bruden, D., Petersen, K. & Et, A. Antibody Levels and Protection after Hepatitis B Vaccination : Results of a 15-year follow-up. *Ann. Intern. Med.* **142,** 333–341 (2005).
6. Toh, Z. Q. *et al.* Reduced dose human papillomavirus vaccination: An update of the current state-of-the-art. *Vaccine* **33,** 5042–5050 (2015).
7. Vajdy, M. *et al.* Mucosal adjuvants and delivery systems for protein-, DNA- and RNA-based vaccines. *Immunol. Cell Biol.* **82,** 617–627 (2004).
8. Weide, B., Garbe, C., Rammensee, H. G. & Pascolo, S. Plasmid DNA- and messenger RNA-based anti-cancer vaccination. *Immunol. Lett.* **115,** 33–42 (2008).
9. Adams, G. P. & Weiner, L. M. Monoclonal antibody therapy of cancer. *Nat. Biotechnol.* **23,** 1147–1157 (2005).
10. June, C. H., Riddell, S. R. & Schumacher, T. N. Adoptive cellular therapy: A race to the finish line. *Sci. Transl. Med.* **7,** 280ps7–280ps7 (2015).
11. Feldman, S. A., Assadipour, Y., Kriley, I., Goff, S. L. & Rosenberg, S. A. Adoptive Cell Therapy—Tumor-Infiltrating Lymphocytes, T-Cell Receptors, and Chimeric Antigen Receptors. *Semin. Oncol.* **42,** 626–639 (2015).
12. Lim, O., Jung, M. Y., Hwang, Y. K. & Shin, E.-C. Present and Future of Allogeneic Natural Killer Cell Therapy. *Front. Immunol.* **6,** 286 (2015).
13. Lafont, V. *et al.* Plasticity of γδ T Cells: Impact on the Anti-Tumor Response. *Front. Immunol.* **5,** 622 (2014).
14. Giraudo, L. *et al.* Cytokine-induced killer cells as immunotherapy for solid tumors : current evidences and perspectives. *Immunotherapy* (2015).
15. Pittari, G., Filippini, P., Gentilcore, G., Grivel, J.-C. & Rutella, S. Revving up Natural Killer Cells

and Cytokine-Induced Killer Cells Against Hematological Malignancies. *Front. Immunol.* **6,** 10–20 (2015).

16. Aranda, F. *et al.* Trial Watch: Adoptive cell transfer for oncological indications. *Oncoimmunology* **4,** e1046673 (2015).
17. Sangiolo, D. Cytokine induced killer cells as promising immunotherapy for solid tumors. *J. Cancer* **2,** 363–368 (2011).
18. Schmidt-Wolf, I. G., Negrin, R. S., Kiem, H. P., Blume, K. G. & Weissman, I. L. Use of a SCID mouse/human lymphoma model to evaluate cytokine-induced killer cells with potent antitumor cell activity. *J. Exp. Med.* **174,** 139–149 (1991).
19. Introna, M. *et al.* Rapid and massive expansion of cord blood-derived cytokine-induced killer cells: an innovative proposal for the treatment of leukemia relapse after cord blood transplantation. *Bone Marrow Transplant.* **38,** 621–627 (2006).
20. Lu, P. H. & Negrin, R. S. A novel population of expanded human CD3+CD56+ cells derived from T cells with potent in vivo antitumor activity in mice with severe combined immunodeficiency. *J. Immunol.* **153,** 1687–1696 (1994).
21. Franceschetti, M. *et al.* Cytokine-induced killer cells are terminallydifferentiated activated CD8 cytotoxic T-EMRA lymphocytes. *Exp. Hematol.* **37,** 616–628 (2009).
22. Sangiolo, D. *et al.* Alloreactivity and anti-tumor activity segregate within two distinct subsets of cytokine-induced killer (CIK) cells: Implications for their infusion across major HLA barriers. *Int. Immunol.* **20,** 841–848 (2008).
23. Schmidt-Wolf, I. G. *et al.* Phenotypic characterization and identification of effector cells involved in tumor cell recognition of cytokine-induced killer cells. *Exp. Hematol.* **21,** 1673–9 (1993).
24. Pievani, A. *et al.* Dual-functional capability of CD3 +CD56 + CIK cells, a T-cell subset that acquires NK function and retains TCR-mediated specific cytotoxicity. *Blood* **118,** 3301–3310 (2011).
25. Verneris, M. R., Karami, M., Baker, J., Jayaswal, A. & Negrin, R. S. Role of NKG2D signaling in the cytotoxicity of activated and expanded CD8+ T cells. *Blood* **103,** 3065–3072 (2004).
26. Jamieson, A. M. *et al.* The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity* **17,** 19–29 (2002).
27. Diefenbach, a, Jamieson, a M., Liu, S. D., Shastri, N. & Raulet, D. H. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat.*

Immunol. **1,** 119–126 (2000).

28. Bauer, S. *et al.* Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* **285,** 727–729 (1999).
29. Karimi, M. *et al.* Silencing Human NKG2D, DAP10, and DAP12 Reduces Cytotoxicity of Activated CD8+ T Cells and NK Cells. *J. Immunol.* **175,** 7819–7828 (2005).
30. Verneris, M. R. *et al.* Engineering hematopoietic grafts: purified allogeneic hematopoietic stem cells plus expanded CD8+ NK-T cells in the treatment of lymphoma. *Biol. Blood Marrow Transplant.* **7,** 532–542 (2001).
31. Yang, Y. G., Dey, B. R., Sergio, J. J., Pearson, D. a & Sykes, M. Donor-derived interferon gamma is required for inhibition of acute graft-versus-host disease by interleukin 12. *J. Clin. Invest.* **102,** 2126–2135 (1998).
32. Baker, J., Verneris, M. R., Ito, M., Shizuru, J. a. & Negrin, R. S. Expansion of cytolytic CD8+ natural killer T cells with limited capacity for graft-versus-host disease induction due to interferon γ production. *Blood* **97,** 2923–2931 (2001).
33. Nishimura, R. *et al.* In vivo trafficking and survival of cytokine-induced killer cells resulting in minimal GVHD with retention of antitumor activity. *Blood* **112,** 2563–2574 (2008).
34. Edinger, M. *et al.* Revealing lymphoma growth and the efficacy of immune cell therapies using in vivo bioluminescence imaging. *Blood* **101,** 640–648 (2003).
35. Alvarnas, J. C., Linn, Y. C., Hope, E. G. & Negrin, R. S. Expansion of cytotoxic CD3+ CD56+ cells from peripheral blood progenitor cells of patients undergoing autologous hematopoietic cell transplantation. *Biol. Blood Marrow Transplant.* **7,** 216–222 (2001).
36. Schmeel, L. C., Schmeel, F. C., Coch, C. & Schmidt-Wolf, I. G. H. Cytokine-induced killer (CIK) cells in cancer immunotherapy: report of the international registry on CIK cells (IRCC). *J. Cancer Res. Clin. Oncol.* 839–849 (2014). doi:10.1007/s00432-014-1864-3
37. Scott, A. M., Wolchok, J. D. & Old, L. J. Antibody therapy of cancer. *Nat. Rev. Cancer* **12,** 278–87 (2012).
38. Weiner, L. M., Surana, R. & Wang, S. Antibodies and cancer therapy: versatile platforms for cancer immunotherapy. **10,** 317–327 (2010).
39. Hudis, C. A. Trastuzumab — Mechanism of Action and Use in Clinical Practice. *N. Engl. J. Med.* **357,** 39–51 (2007).
40. Galizia, G. *et al.* Cetuximab, a chimeric human mouse anti-epidermal growth factor receptor monoclonal antibody, in the treatment of human colorectal cancer. *Oncogene* **26,** 3654–3660

(2007).

41. Chen, J.-S., Lan, K. & Hung, M.-C. Strategies to target HER2/neu overexpression for cancer therapy. *Drug Resist. Updat.* **6,** 129–36 (2003).
42. Ciardiello, F. & Tortora, G. EGFR antagonists in cancer treatment. *N. Engl. J. Med.* **358,** 1160–1174 (2008).
43. Peipp, M., Dechant, M. & Valerius, T. Effector mechanisms of therapeutic antibodies against ErbB receptors. *Curr. Opin. Immunol.* **20,** 436–443 (2008).
44. Wang, W. NK cell-mediated antibody-dependent cellular cytotoxicity in cancer immunotherapy. *Front. Immunol.* **6,** (2015).
45. Ravetch, J. V & Bolland, S. IgG Fc Receptors. *Annu. Rev. Immunol.* **19,** 275–290 (2001).
46. Lanier, L. L., Ruitenberg, J. J. & Phillips, J. H. Functional and biochemical analysis of CD16 antigen on natural killer cells and granulocytes. *J. Immunol.* **141,** 3478–3485 (1988).
47. Nimmerjahn, F. & Ravetch, J. V. Antibodies, Fc receptors and cancer. *Curr. Opin. Immunol.* **19,** 239–245 (2007).
48. Smyth, M. J. *et al.* Activation of NK cell cytotoxicity. *Mol. Immunol.* **42,** 501–510 (2005).
49. Clynes, R. a, Towers, T. L., Presta, L. G. & Ravetch, J. V. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. *Nat. Med.* **6,** 443–446 (2000).
50. Musolino, A. *et al.* Immunoglobulin G Fragment C Receptor Polymorphisms and Clinical Efficacy of Trastuzumab-Based Therapy in Patients With HER-2/neu-Positive Metastatic Breast Cancer. *J. Clin. Oncol.* **26,** 1789–1796 (2008).
51. Bibeau, F. *et al.* Impact of Fc RIIa-Fc RIIIa Polymorphisms and KRAS Mutations on the Clinical Outcome of Patients With Metastatic Colorectal Cancer Treated With Cetuximab Plus Irinotecan. *J. Clin. Oncol.* **27,** 1122–1129 (2009).
52. van Sorge, N. M., van der Pol, W.-L. & van de Winkel, J. G. J. FcgammaR polymorphisms: Implications for function, disease susceptibility and immunotherapy. *Tissue Antigens* **61,** 189–202 (2003).
53. Seidel, U. J. E., Schlegel, P. & Lang, P. Natural Killer Cell Mediated Antibody-Dependent Cellular Cytotoxicity in Tumor Immunotherapy with Therapeutic Antibodies. *Front. Immunol.* **4,** 1–8 (2013).
54. Umaña, P., Jean-Mairet, J., Moudry, R., Amstutz, H. & Bailey, J. E. Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat. Biotechnol.* **17,** 176–180 (1999).

55. Shields, R. L. *et al.* Lack of Fucose on Human IgG1 N-Linked Oligosaccharide Improves Binding to Human Fcgamma RIII and Antibody-dependent Cellular Toxicity. *J. Biol. Chem.* **277,** 26733–26740 (2002).

56. Caaveiro, J. M. M., Kiyoshi, M. & Tsumoto, K. Structural analysis of Fc/FcγR complexes: a blueprint for antibody design. *Immunol. Rev.* **268,** 201–221 (2015).

57. Ferrara, C., Stuart, F., Sondermann, P., Brunker, P. & Umana, P. The Carbohydrate at Fc RIIIa Asn-162: an element required for high affinity binding to non-fucosylated IgG glycoforms. *J. Biol. Chem.* **281,** 5032–5036 (2006).

58. Patnaik, S. K. & Stanley, P. Lectin-resistant CHO glycosylation mutants. *Methods Enzymol.* **416,** 159–82 (2006).

59. Kellner, C., Derer, S., Valerius, T. & Peipp, M. Boosting ADCC and CDC activity by Fc engineering and evaluation of antibody effector functions. *Methods* **65,** 105–113 (2014).

60. Paz-Ares, L. G. *et al.* Phase I pharmacokinetic and pharmacodynamic dose-escalation study of RG7160 (GA201), the first glycoengineered monoclonal antibody against the epidermal growth factor receptor, in patients with advanced solid tumors. *J. Clin. Oncol.* **29,** 3783–90 (2011).

61. Salles, G. *et al.* Phase 1 study results of the type II glycoengineered humanized anti-CD20 monoclonal antibody obinutuzumab (GA101) in B-cell lymphoma patients. *Blood* **119,** 5126–5132 (2012).

62. Ishida, T. *et al.* Defucosylated anti-CCR4 monoclonal antibody (KW-0761) for relapsed adult T-cell leukemia-lymphoma: A multicenter phase II study. *J. Clin. Oncol.* **30,** 837–842 (2012).

63. Lazar, G. a *et al.* Engineered antibody Fc variants with enhanced effector function. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 4005–4010 (2006).

64. Richards, J. O. *et al.* Optimization of antibody binding to FcgammaRIIa enhances macrophage phagocytosis of tumor cells. *Mol. Cancer Ther.* **7,** 2517–2527 (2008).

65. Repp, R. *et al.* Combined Fc-protein- and Fc-glyco-engineering of scFv-Fc fusion proteins synergistically enhances CD16a binding but does not further enhance NK-cell mediated ADCC. *J. Immunol. Methods* **373,** 67–78 (2011).

66. Carter, P. J. Potent antibody therapeutics by design. *Nat. Rev. Immunol.* **6,** 343–357 (2006).

67. Peipp, M. & Valerius, T. Bispecific antibodies targeting cancer cells. *Biochem. Soc. Trans.* **30,** 507–511 (2002).

68. May, C., Sapra, P. & Gerber, H. P. Advances in bispecific biotherapeutics for the treatment of

cancer. *Biochem. Pharmacol.* **84,** 1105–1112 (2012).

69. Bruenke, J. *et al.* Effective lysis of lymphoma cells with a stabilised bispecific single-chain Fv antibody against CD19 and Fc??RIII (CD16). *British Journal of Haematology* **130,** 218–228 (2005).
70. Kellner, C. *et al.* Mimicking an Induced Self Phenotype by Coating Lymphomas with the NKp30 Ligand B7-H6 Promotes NK Cell Cytotoxicity. *J. Immunol.* **189,** 5037–5046 (2012).
71. Kellner, C. *et al.* Fusion proteins between ligands for NKG2D and CD20-directed single-chain variable fragments sensitize lymphoma cells for natural killer cell-mediated lysis and enhance antibody-dependent cellular cytotoxicity. *Leukemia* **26,** 830–834 (2012).
72. Kellner, C., Gramatzki, M. & Peipp, M. Promoting natural killer cell functions by recombinant immunoligands mimicking an induced self phenotype. *Oncoimmunology* **2,** e24481 (2013).
73. Oberg, H.-H. *et al.* Novel Bispecific Antibodies Increase T-Cell Cytotoxicity against Pancreatic Cancer Cells. *Cancer Res.* **74,** 1349–1360 (2014).
74. Peipp, M. *et al.* HER2-specific immunoligands engaging NKp30 or NKp80 trigger NK-cell-mediated lysis of tumor cells and enhance antibody-dependent cell-mediated cytotoxicity. *Oncotarget* **6,** 7–9 (2015).
75. Keyaerts, M. *et al.* Dynamic bioluminescence imaging for quantitative tumour burden assessment using IV or IP administration of d-luciferin: effect on intensity, time kinetics and repeatability of photon emission. *Eur. J. Nucl. Med. Mol. Imaging* **35,** 999–1007 (2008).
76. Ortaldo, J. R., Winkler-Pickett, R. T., Yagita, H. & Young, H. a. Comparative studies of CD3- and CD3+ CD56+ cells: examination of morphology, functions, T cell receptor rearrangement, and pore-forming protein expression. *Cell. Immunol.* **136,** 486–495 (1991).
77. Trinchieri, G. Biology of natural killer cells. *Adv. Immunol.* **47,** 187–376 (1989).
78. Bottino, C., Biassoni, R., Millo, R., Moretta, L. & Moretta, a. The human natural cytotoxicity receptors (NCR) that induce HLA class I-independent NK cell triggering. *Hum. Immunol.* **61,** 1–6 (2000).
79. Sivori, S. *et al.* P46, a Novel Natural Killer Cell-Specific Surface Molecule That Mediates Cell Activation. *J. Exp. Med.* **186,** 1129–1136 (1997).
80. Bonanno, G. *et al.* Thymoglobulin, interferon-γ and interleukin-2 efficiently expand cytokine-induced killer (CIK) cells in clinical-grade cultures. *J. Transl. Med.* **8,** 129 (2010).
81. Topalian, S. L., Drake, C. G. & Pardoll, D. M. Perspective Immune Checkpoint Blockade : A Common Denominator Approach to Cancer Therapy. *Cancer Cell* **27,** 450–461 (2015).

82. Schmidt-Wolf, G. D., Negrin, R. S. & Schmidt-Wolf, I. G. Activated T cells and cytokine-induced CD3+CD56+ killer cells. *Ann. Hematol.* **74,** 51–56 (1997).

83. Thorne, S. H., Negrin, R. S. & Contag, C. H. Synergistic antitumor effects of immune cell-viral biotherapy. *Science* **311,** 1780–1784 (2006).

84. Dalle, S., Thieblemont, C., Thomas, L. & Dumontet, C. Monoclonal antibodies in Clinical Oncology. *Anticancer. Agents Med. Chem.* **8,** 523–532 (2008).

85. Ishiyama, T. *et al.* The presence of CD5low+ NK cells in normal controls and patients with pulmonary tuberculosis. *Immunol. Lett.* **37,** 139–144 (1993).

86. Scheffold, C., Kornacker, M., Scheffold, Y. C., Contag, C. H. & Negrin, R. S. Visualization of Effective Tumor Targeting by CD8 + Natural Killer T Cells Redirected with Bispecific Antibody F (ab ') 2 Visualization of Effective Tumor Targeting by CD8 ⁺ Natural Killer T Cells. *Cancer Res.* **62,** 5785–5791 (2002).

87. Yoon, S. H. *et al.* Transfer of Her-2/neu specificity into Cytokine-Induced Killer (CIK) cells with RNA encoding Chimeric Immune Receptor (CIR). *J. Clin. Immunol.* **29,** 806–814 (2009).