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Design, characterization and preclinical validation of a combinatorial CAR-based immunotherapy against colorectal cancer with HER2 amplification.

Candidate

Dott. Marco Cortese

Tutor

Prof. Enzo Medico

Coordinator

Prof. Enzo Medico

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1. Abstract

ACT based on CAR-T cells has led to successful treatment of some hematological malignancies, but it remains extremely challenging for solid tumors, mostly because of “on-target off-tumor” toxicity, as observed in the case of anti-HER2 CAR-T cells treatment of CRC with HER2 amplification. To enable ACT against HER2amp CRC, was therefore considered a combinatorial strategy based on the synNotch-based artificial regulatory network. A synthetic Notch receptor was employed in which the extracellular domain is an anti-HER2 scFv and the intracellular domain contains the GAL4VP64 artificial transcription factor. Engagement of the anti-HER2 domain by target cells drives GAL4VP64 cleavage and translocation to the nucleus, where it drives expression of a CAR under a GAL4UAS. In this way, only cells co-expressing both HER2 and the CAR target are killed. As a CRC-specific CAR target CEA was selected. CEA expression is restricted to the digestive tract and is increased in cancer. As effector cells for the system, was selected the natural killer cell line NK-92. NK-92 cells transduced with the two lentiviral vectors encoding HER2-synNotch and inducible CEA-CAR were repeatedly sorted in the OFF and ON state to select those with the best CAR induction after synNotch engagement. Subsequently, cloning of sorted cells led to identification of an optimally responsive clone (5F). *In vitro*, the 5F clone displayed selective cytotoxicity against HER2amp/CEA+ CRC cells, with minimal killing activity against HER2amp/CEA- cells, or against HER2-/CEA+ cells. Additional assays on 3D organoids highlighted better recruitment and infiltration by clone 5F respect to NK-92 WT cells, only in HER2amp models. *In vivo*, the clone 5F significantly impaired tumor growth in two different HER2amp CRC models. To further improve survival, tumor penetration and *in vivo* efficacy of the NK-92-5F clone, a more complex system was built in which HER2-synNotch engagement drives not only expression of the CEA-CAR but also of IL-2. 5F-IL-2 cells displayed a further increase of cytotoxicity *in vitro*, also at a particularly low effector:target ratio (1:50). *In vivo*, 5F-IL-2 cells drastically increased survival of mice carrying HER2 amp CRC xenografts with respect to the parental 5F clone. Moreover, a MSLN synNotch/CEA CAR was also exploited in order to prove the adaptability of the synNotch system. The observed selective efficacy both *in vitro* and *in vivo* of the HER2-synNotch/CEA-CAR system, and its future evolutions, opens a perspective for possible clinical applications in cases of HER2amp CRC displaying primary or secondary resistance to HER2/EGFR blockade.

2. Introduction

2.1. Cancer immunotherapy

2.1.1 Cancer and the immune system

The immune system protects the integrity of an organism by defending it from infectious and other external or "non-self" intruders. The discovery of tumor antigens, the finding that tumors frequently contain lymphocytes, and the consideration that a significant immune infiltrate into the tumor correlates with a favorable prognosis bolstered the concept of immunological surveillance[1], i.e. the idea that host defense may prevent tumor development by establishing tumor immunity[2]. Subsequently, this concept has steered to the cancer immune-editing hypothesis[3], that can schematically be described with three sequential phases: elimination, equilibrium, and escape. In the first phase, before becoming clinically detectable, altered cells are killed and removed by both innate and adaptive immune responses[4]. If some cells escape elimination, the adaptive immune system may be able to limit their growth until equilibrium is reached. Nonetheless, constant immune selective pressure on genetically unstable tumor cells may result in variants that are no longer recognized by immune effectors because tumor cells can build immunosuppressive strategies at their site, such as downregulation of critical surface proteins, secretion of immunosuppressive cytokines, and/or recruitment of Treg cells. As a consequence, tumor cells enter in the escape phase and become clinically visible. The substantial investigation that followed these findings resulted in a profound understanding of the mechanisms regulating T and B cell antigen detection, activation, and function. Furthermore, the idea of harnessing the immune system's potential for clinical benefit was raised.

Cancer immunotherapy aims to boost the immune response against the tumor[5], thereby reflecting a completely distinct and alternative option for treating cancer: enhancing the immune system instead of focusing on the tumor.

There are different types of cancer immunotherapy:

1) The use of monoclonal antibodies (MAbs)/Checkpoint inhibitors, able to recognize and bind to specific antigens on the cancer cell surface. Antibodies are naturally present in human blood and contribute to the fight against external pathogens. Through complement activation, antibody-dependent cell mediated cytotoxicity

(ADCC), MAb-induced cytotoxicity and T cell redirection, MAb therapy can imitate natural antibodies, stimulating and assisting the immune system to target cancer. In other circumstances, MAbs are used to disrupt signaling from surface molecules crucial in the maintenance of the malignant phenotype. The results of this type of therapy include tumor cell growth inhibition and/or induction of apoptosis, as well as tumor angiogenesis inhibition. Finally, the most used and promising antibodies are utilized to inhibit the action of immunological checkpoint molecules present on the tumor surface, reverting T cell anergy and exhaustion[6].

2) Adoptive cell therapy (ACT) consists in the *ex vivo* manipulation and *in vivo* re-infusion of T cells, with the aim to potentiate the immune system response against the tumor. There are different categories of ACT, the most successful are:

2.1) Chimeric Antigen Receptor (CAR) based-adoptive cell therapies. Cells can be genetically modified with a chimeric receptor during the ex vivo phase[78]. Based on gene transfer technology, this kind of therapy allows patients' T lymphocytes to be redirected, focusing them to tumor antigens and boosting their functional capabilities to overcome barriers established by tumor cells and their microenvironment[9]. There are different classes of CAR based therapies, the most recent are:

2.1.1) Combinatorial CAR strategies. In recent years, numerous studies have been published in which a range of alternative therapies have been presented to improve CAR therapies efficacy, to enhance the control of the cytotoxic response and to overcome the adverse effects of the CAR approach, including safety issues such as "on-target off-tumor" toxicities and cytokine release syndrome[10].

3) Vaccine strategies. To protect against infectious diseases, most vaccinations are created from weakened or harmless forms of the disease-causing agent. Similarly, anti-cancer vaccinations are designed to detect proteins expressed particularly by cancer cells, enabling the immune system to recognize and kill the tumor[11].

4) Cytokine treatment. Cytokines are a class of small proteins that play a crucial role in immune system stimulation. They influence the balance of humoral and cell-based immune responses, as well as the maturation, proliferation, and responsiveness of specific immune cell types. Interferons and interleukins are naturally occurring cytokines that can be supplied exogenously as proteins or by cell/gene transfer[12].

2.1.2 Checkpoint inhibitors

Immune checkpoint inhibitors (ICIs) have transformed tumor therapy. While chemotherapy and radiotherapy are still the standard of care for the majority of cancer types, ICIs are now becoming first-line therapies for a variety of solid and liquid cancers. ICIs function by loosening the inhibitory brakes on T cells, resulting in immune system activation and productive anticancer immunological responses [13].

In the recent decade significant improvements have been seen in cancer immunotherapy, with blocking antibodies targeting immune inhibitory receptors such as the cytotoxic T lymphocyte-associated protein 4 (CTLA-4), the inhibitory receptor programmed cell death 1 (PD-1), and programmed death-ligand 1 (PD-L1), the most extensively used immunotherapeutic drugs. Antibodies directed against these molecules are already FDA-approved for a variety of cancers, while several antibodies and small compounds targeting further immune checkpoints, including LAG3, TIGIT, TIM3, B7H3, CD39, CD73, adenosine A2A receptor, and CD47, are in clinical development [14]. After activation, T cells express CTLA-4 which has a greater affinity for CD80/86 than the co-stimulatory molecule CD28. As a result, CTLA-4 excludes CD28 from the binding both preventing the co-stimulatory signals from APCs, and also delivering negative signals into T cells, limiting cytolytic activity. CTLA-4 is a good target molecule for immunological oncology since it is constitutively produced on Treg[15]. PD-1 through the binding to PD-L1 instead inhibits signaling downstream of the T-cell Receptor (TCR); the expression of PD-L1 in both, normal and tumor tissues, indicates that this pathway functions as a check on the immune response and might be exploited to boost anti-tumor immunity[16].

ICIs targeting three distinct molecules have been licensed for use in humans by the US Food and Drug Administration (FDA). Ipilimumab, an anti-CTLA-4 antibody, was the first to be approved for the treatment of metastatic melanoma[17]. The second class of ICIs are antibodies that block PD-1 on T cells, and consequently the interactions with its ligands PD-L1 and PD-L2, to prevent active T cell responses [18]. Currently, the FDA has approved two anti-PD-1 antibodies, pembrolizumab and nivolumab, which have been first approved to treat advanced-stage melanomas and even a variety of cancer types[19]. Finally, antibodies against PD-L1 represent the third class of FDA-approved ICIs. Atezolizumab, durvalumab, and avelumab are three anti-PD-L1 antibodies that are predominantly used to treat urothelial carcinoma, non-small-cell

lung cancer (NSCLC), and Merkel cell carcinoma[20]. Anti-PD-1 and anti-PD-L1 antibodies have outperformed anti-CTLA-4 antibodies in clinical trials due to a combination of higher clinical activity and tolerability[21]. Even though many patients achieve dramatic tumor reduction in response to ICIs, most malignancies do not react. Some of the patients who initially respond unfortunately acquire resistance and undergo tumor relapse[22].

T cells have been the linchpins of ICI therapy for years, anyway ICIs can stimulate even other cells of innate and adaptive immunity, all of which work in concert to orchestrate a successful response against malignancies. Changes in the immune response caused by ICIs have been reported both within tumors and in peripheral organs such as draining lymph nodes and peripheral blood. Indeed, peripheral immune responses are crucial in attaining positive therapeutic results. More studies are needed to completely understand the mechanism of resistance and to predict patient response to ICI therapy[23].

2.1.3 Adoptive cell therapy

Given the immune system's inherent ability to recognize tumors and the inverse relationship between immune response efficacy and disease malignancy, therapeutic approaches focused on increasing cytotoxic lymphocyte activation against cancer antigens have been investigated and developed. ACT is a highly personalized cancer treatment that involves delivering anticancer immune cells to the patient[24] (Fig. A). ACT provides several advantages in cancer immunotherapy, including the active *in vivo* proliferation of administered immune effectors, which enables extended anti-cancer efficacy.

The aim of the ACT has been to redirect patient T cell response based on the establishment of the immunological surveillance concept, tumor immunity, and immunoediting hypothesis. Rosenberg and colleagues pioneered this novel approach in cancer immunotherapy, focusing their research on improving procedures aimed at enhancing the activity of Tumor Infiltrated Lymphocytes (TIL)[25]. TILs were productively expanded and activated ex vivo before being re-infused into lymphodepleted patients, with the aim of repopulating the immune system with a suitable number of cells with natural anticancer activity. The observations originated in this study, *i.e.* that TILs can mediate durable and complete regression of cancer in patients with metastatic

melanoma, led to considerable interest in TILs, which were challenged for the treatment of several cancer types. Nevertheless, their application in tumors different from melanomas highlighted the emergence of some obstacles[26].

After that, many other immune cell types were explored as effector cells in adoptive immunotherapies strategies. Cytokine Induced Killer (CIK) cells, *in vitro*-expanded T lymphocytes with a CD3+CD56+ phenotype, have broad antitumor efficacy against both solid and hematologic cancers[27]. The ready availability of huge volumes of expanded CIK cells, their MHC-unrestricted tumor killing, the high growth rate, the potential effectiveness against various tumor types, and low costs are all factors supporting its simple clinical translation. CIK cells have been used in numerous clinical trials to treat both hematological and solid cancers. Intriguing future perspectives and challenges for combination with alternative immunotherapy techniques, targeted treatments, or even conventional chemotherapy are being investigated[28]. Another source are the natural killer (NK) cells. NK cells are a lymphoid population that, unlike T and B lymphocytes, does not express clonally distributed antigen receptors. NK cells are normally limited to the peripheral circulation, spleen, and bone marrow, but they can move to inflamed tissues in response to various chemoattractant[29] .There are various sources from which NK cells can be recovered or derived, namely peripheral blood mononuclear cells, cord blood, immortalized cell lines, hematopoietic stem and progenitor cells (HSPCs) and induced pluripotent stem cells (iPSCs)[30]. Autologous NK cells can be withdrawn from the patient peripheral blood, and they are the favorite source to use because do not cause Graft-versus Host disease (GvHD) but unfortunately their application in patient is limited, due to low availability and their dysfunctional phenotype frequently found in cancer, marked by altered gene expression profile and reduced cytotoxic function[31]. The NK-92 cells, the first NK cell-based immunotherapy approved for clinical development by the US Food and Drug Administration (FDA), are a homogenous, immortalized NK lymphoma cell line that can be expanded ex vivo to attain a high cell number[32]. Since NK-92 cells lack expression of most KIRs, they are less likely to become blocked from immunosuppressive signals, making them appealing for cell therapy application. Moreover, the NK-92 cell line does not share the dangerousness of T cells and could be a valid option to have safer immunotherapy effectors. Another advantage with respect to primary T or NK cells is that the use of NK-92 permits to have a ready "off

the shelf" therapy with lower production costs. Indeed, high production costs are one of the most critical points for CAR-T cell therapy clinical applicability[33]. However, due to their tumor origin, irradiation of NK-92-derived cell products is required for safety reasons prior to patient administration, which can have a negative impact on their long-term *in vivo* persistence and overall therapeutic potential. Although the first findings are promising, further research about the safety and efficacy of NK-92 cells in larger patient populations and for other indications will help narrow the range of potential clinical applications for these cells[34]. iPSCs are an appealing source for NK cells given their clonal growth and high expansion capacity, as well as their ability to differentiate *in vitro*, allowing for the manufacturing of large numbers of homogeneous NK cell products. One possible limitation is that iPSCs may have DNA methylation signals that match their somatic tissue of origin. Despite this, a rising number of genetically modified iPSC-NK cell candidates are appearing in preclinical investigations, giving the rationale for clinical trials[35]. Allogenic primary NK cells can be isolated from either peripheral blood or umbilical cord blood (CB-NK cells). CB-NK cells are available frozen from blood banks, whereas PB-NK cells require healthy donor apheresis and donor-specific collection[36]. All of these NK cells sources have been found to have advantages and downsides for adoptive cell therapy applications. The variety of different possible sources allows great flexibility in the choice of therapeutic methods, with platforms that can be specifically adapted for each patient and disease indication. Recent studies are focusing on further types of immune cells such as macrophages and dendritic cells, indicating that the entire repertoire of immunity-related cells, especially present in the tumor microenvironment, may play a fundamental role in the immune response against the tumor[37,38]. To address the limitations of adoptive cell-based treatment and broaden its application in cancer, different gene modification steps have been considered: T/NK cells were engineered to detect a specific target with different methods[5]. Engineered T/NK cells represent a significant advantage and opportunity that has contributed to the expansion of immunotherapy in the oncology scenario. First of all, T cells were genetically programmed to target specific tumor antigens through the introduction of genes encoding a specific TCR[39]. In melanoma patients, the first clinical success of ACT was achieved using engineered T cells with the exogenous TCR targeting NY-ESO1[40,41]. A similar approach has been shown to be effective in patients with myeloma[42], cholangiocarcinoma, and colorectal cancer[43].

Nonetheless, the identification and selection of a TCR with requested properties remains a difficult step, and - more importantly - the requirement of antigen presentation by the HLA/MHC system represents the major limitation of this therapeutic strategy, as the downregulation of such molecules is a fundamental mechanism of immune escape operated by the tumor.

Synthetic Biology techniques were used to address these limitations. To create artificial biological systems for research and medical applications, synthetic biology integrates components of biotechnology, genetic engineering, molecular biology, and computer science. Chimeric Antigen Receptors (CARs) have been developed in this context[44].

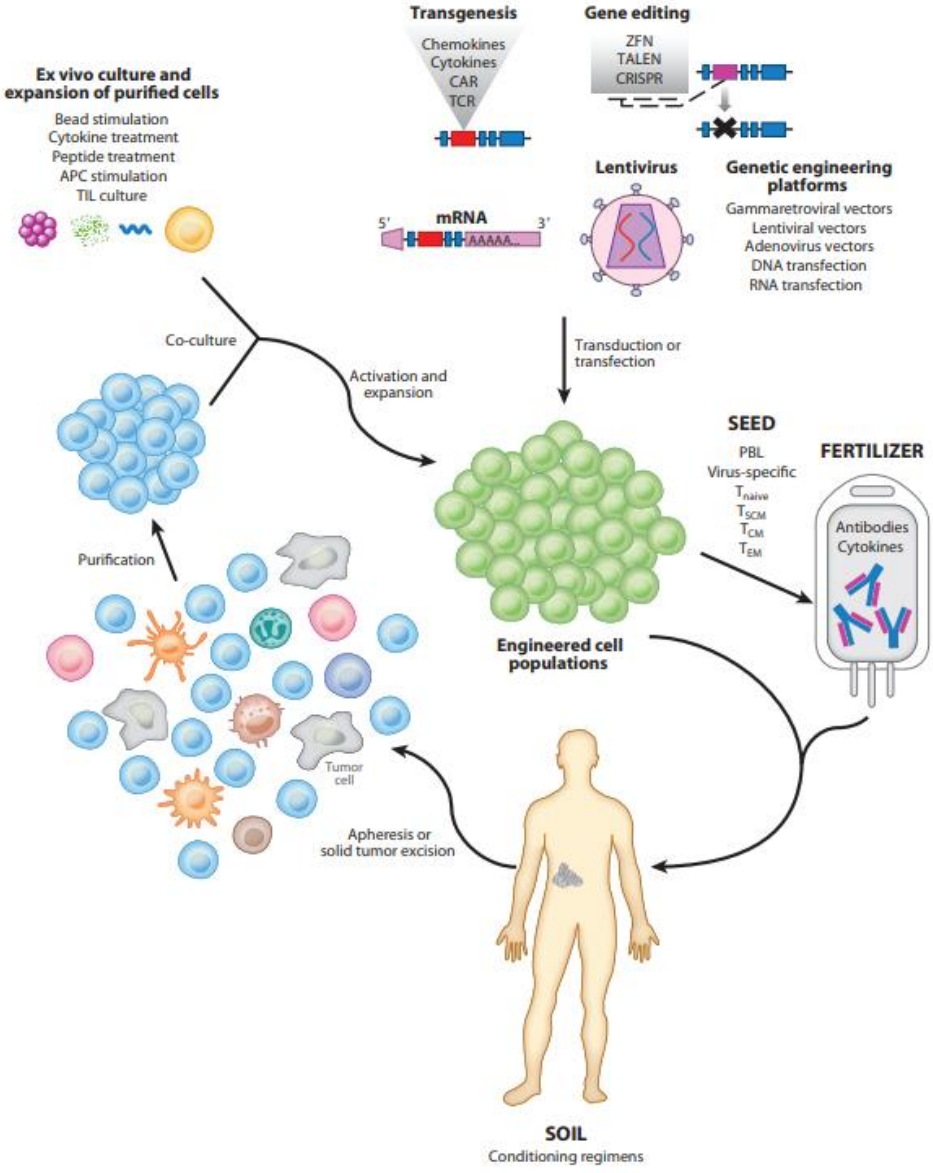

Figure A. Adoptive Cell Therapy (ACT) scheme. Schematic representation of the ACT process (modified from Maus et al., 2014)

2.1.4 CAR-based adoptive immunotherapy

Chimeric Antigen Receptor are recombinant receptors that recognize antigens expressed on the target cell's surface. Because of the contact with their antigen, they activate intracellular signals that lead to T cell killing activity[45]. Therefore, the immune system's specificity, function, and cytotoxic reaction are redirected. CARs are composed by an extracellular part generated from an antibody's binding site or a natural receptor ligand, and an intracellular portion derived from the TCR complex's signal domain; the two components are joined by a spacer[46] (Fig. B). Effective CARs incorporate T cell co-stimulation domains and provide a wide range of functional advantages, by directly soliciting specific co-stimulatory pathways[47].

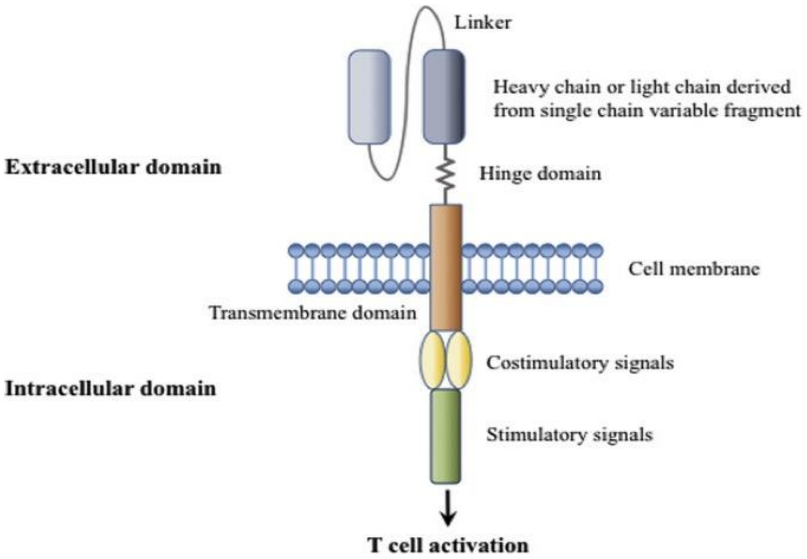

Figure B. CAR structure. Schematic representation of a Chimeric Antigen Receptor (modified from Han et al., 2017)

What makes CARs so intriguing is their ability to overcome some inherent limitations of TCR function. One advantage is the ability to avoid the issue of central tolerance. CARs are potentially applicable to all patients, regardless of their Human Leukocyte Antigen (HLA) haplotypes, because they do not require the HLA for antigen recognition. Furthermore, they are not affected by the absence or downregulation of MHC molecules on the surface of cancer cells, which is a strategy often used by tumor cells to elude the immune system[48]. Another advantage is the CAR's ability to detect any type of molecule as long as it is exposed on the cellular surface. TCR, on the other hand, can only bind a little peptide provided by an Antigen Presenting Cell (APC).

Finally, it was established that the intracellular signal induced by CAR causes a greater and long-lasting cytotoxic response than TCR. However, CARs have drawbacks as compared to TCRs: the main is that the antigen must be produced at a high concentration on the surface of cancer cells in order to compensate for the low avidity of the antibody moiety incorporated in the CAR itself[49]. The development of modified T cells expressing CD19 CARs represented a significant milestone in cell therapy; this therapeutic strategy, together with checkpoint blockade therapy, has changed cancer immunotherapy[50]. CARs have been tweaked over time to induce cytotoxic responses of varying potency. Three generations of CARs have been developed, with differences in the domains that compose the molecule's intracellular part[51] (Fig. C). The 1° generation of CARs includes in the intracellular part the ζ chain derived from CD3 or CD8. These molecules are part of the TCR complex. The first generation of CARs are able to activate lymphocytes, but not to generate a durable and intense cytotoxic response[52]. The 2° generation of CARs even owns in the intracellular region a costimulatory domain derived from CD28, or 4-1BB/CD137, or ICOS, or OX-40, or DAP10 in addition to the ζ chain. Compared to first-generation, second-generation CARs elicit a powerful cytotoxic response, which is accompanied by the cytokine release required to stimulate clonal proliferation of activated cells, and thus resulting in the reaction's requisite persistence. The 3° generation of CARs comprises two costimulatory domains instead of a single one in the intracellular region: CD28 *plus* the 4-1BB/CD137, or OX-40, or Lck. These CARs, designed as an upgrade on the second generation, elicit a powerful immunological response. Nonetheless, the high potency acquired by activating numerous costimulatory domains raises the possibility of uncontrollable collateral consequences[53]. Initially, patient-derived T cells were used to achieve therapeutic success using genetically modified T cell therapies. The rationale for autologous usage is to avoid the host being attacked by modified T cells and/or the host rejecting the therapeutic T cells. Nevertheless, some issues associated with the phenotypic and functional variability of each individual patient should be considered as well. The ability of modified T cells to move to tumor locations, expand, and mediate effector functions that destroy cancer cells is central to the ACT's anti-tumor actions. Which type and stage of differentiation must be the ideal immune cell to manipulate is still a matter of discussion. Recently, however, T cells have lost approval due to the serious side effects they can cause after infusion in the patient to

the detriment of NK cells which are much safer and more controllable in the immune response against cancer, in particular in solid tumor settings[10,54]. Another field currently under great expansion is the possibility to use synthetic cells, such as Induced Pluripotent Stem Cell (iPSC) in order to derive T lymphocytes or NK cells and thus overcome the risk of GVHD and to have a better histocompatibility[55]. At the current state of the art, to define the optimal T/NK cell product for adoptive cell therapy remains a challenge that will require careful phenotypic and biological characterization, taking in account also manufacturing and economic practicalities[56]. Therapies with CAR-immune cells, engineered in transient or stable way, against a variety of tumor types, have been applied in clinical trials in phase one, two, or three[57]. The majority of these studies describe the targeting of hematological tumors, and in particular the application of CAR T cells against CD19 or CD20 for the treatment of acute lymphoblastic leukemia (ALL)[58], chronic lymphocytic leukemia (CLL)[59] and non-Hodgkin lymphomas (NHL)[60]. Clinical trials lead to exceptional results, with total remission of the treated tumors[61]. These successful results prompted the FDA approval of four drugs for ALL, CLL, NHL targeting CD19 (Kymriah, Yescarta Tecartus and Breyanzi) and recently two for the multiple myeloma targeting BCMA (Abecma and Carvykti)[62]. On the other hand, the application of CAR immune cells in the context of solid tumors remains a major issue that requires additional research from the bench to the bed. CAR-immune cell trials for solid tumors have been carried out for every type of cancer in different organs, targeting over 40 antigens[63] (Fig. C). Many of these showed minimal clinical efficacy, a lack of growth, and long-term *in vivo* persistence. Despite the variable outcomes, several novel antigens are being investigated at various phases of clinical development in many other tumor types. The major difficulties rose in the application of CAR-immune cells to solid tumors and are related to the right antigen selection, trafficking problems, the presence of immunosuppressive microenvironment and the occurrence of side effects[64].

Organ	Cancer Type	Targeted Antigens
brain/CNS	brain	CD133, HER2, PSMA
	glioma	B7-H3, CD147, EGFR, EGFRvIII, EphA2, GD2, HER2, IL13Rα2, MUC1, CD133
	glioblastoma	B7-H3, ligands of chlorotoxin, EGFRvIII, HER2, IL13Rα2, NKG2D-Ligands, PD-L1
	primitive neuroectodermal tumor	B7-H3
	choroid plexus carcinoma	B7-H3
	pineoblastoma	B7-H3
	CNS tumor	B7-H3, EGFR806, HER2
	ependymoma	B7-H3
	medulloblastoma	B7-H3, NKG2D-Ligands
several organs	rhabdoid tumor	B7-H3, EGFR, GPC3
	Rhabdomyosarcoma	B7-H3, EGFR, GPC3
	desmoplastic small round cell tumor	B7-H3, EGFR
	sarcoma	GD2, HER2, NKG2D-Ligands, CD133, MUC1, CD117
	adenocarcinoma	CEA
	solid tumors	B7-H3, CEA, claudin 18.2, EGFR, EGFR family member, GD2, GPC3, HER2, Lewis Y, mesothelin, MUC1, MUC16ecto, TnMuc1, Nectin4, ROR2
pancreas	pancreatic	CD70, CD133, CEA, claudin 18.2, EGFR, EpCAM, HER2, mesothelin, MUC1, Nectin4, NKG2D-Ligands, PSCA, ROR2, EGFRvIII
	pancreatic ductal adenocarcinoma	claudin 18.2, mesothelin, TnMuc1
liver	liver	CD133, CEA, EGFR, EpCAM, GPC3, MG7, NKG2D-Ligands
	HCC (hepatocellular carcinoma)	AFP/HLA-A2, CD147, GPC3, MUC1, NKG2D-Ligands, c-MET, PD-L1
	hepatoblastoma	B7-H3, EGFR
	hepatoma	several
	gall bladder carcinoma	EGFR
	cholangiocarcinoma	EGFR, HER2, MUC1
lung	lung	CEA, EGFR, HER2, mesothelin, Lewis Y, PSCA, MUC1, PD-L1, CD80/86, MAGE-A1, MAGE-A4, GD2
	small cell lung cancer	DLL3
	mesothelioma	FAP, mesothelin
	lung squamous cell carcinoma	GPC3
	NSCLC	EGFR, mesothelin, MUC1, TnMuc1, Nectin4, PD-L1, ROR1, CD80/86
uterus/cervix	ovarian	CD70, CD133, CEA, EGFR, FBP, HER2, mesothelin, TnMuc1, Nectin4, NKG2D-Ligands
	cervical	mesothelin, GD2, PSMA, MUC1, mesothelin
	fallopian tube	mesothelin, TnMuc1
breast	breast	CD44v6, CD70, CD133, CEA, c-MET, EpCAM, HER2, mesothelin, Muc1 (cleaved from), Nectin4, GD2
	TNBC	c-MET, mesothelin, MUC1, TnMuc1, NKG2D-Ligands, ROR1
colon	colorectal	CD133, CEA, EGFR, HER2, MUC1, NKG2D-Ligands
	colon	EpCAM, HER2, NKG2D-Ligands
kidney	renal	CD70, EGFR, VEGFR2, ROR2, AXL
	neuroblastoma	B7-H3, CD171, EGFR, GD2, PSMA
	wilms tumor	B7-H3, EGFR, GPC3
stomach	gastric	CD44v6, CEA, claudin 18.2, EGFR, EpCAM, HER2, MUC1, NKG2D-Ligands, PSCA, ROR2
prostate	prostate	CD44v6, EpCAM, NKG2D-Ligands, PSCA, PSMA
head/neck	esophageal	EpCAM, HER2, MUC1
	nasopharyngeal	EpCAM, LMP1, NKG2D-Ligands
	SCCHN	ErbB dimers, HER2
	salivary gland	HER2
	thyroid cancer	ICAM1
skin	melanoma	B7-H3, CD20, CD70, c-MET, GD2, gp100/HLA-A2, IL13Rα2, VEGFR2
bladder	bladder	HER2, Nectin4, NKG2D-Ligands, ROR2, PSMA, FBP
soft tissue	synovial sarcoma	B7-H3, EGFR
	clear cell sarcoma	B7-H3, EGFR
	soft tissue sarcoma	B7-H3, EGFR, GPC3, ROR2
bone	osteosarcoma	B7-H3, EGFR, GD2
	ewing sarcoma	B7-H3, EGFR
abdomen	peritoneal	CEA, EpCAM, mesothelin
eye	retinoblastoma	B7-H3, EGFR
	uveal melanoma	GD2
ovary/testis	germ cell tumor	B7-H3, EGFR, GPC3
peripheral nerves	malignant peripheral nerve sheath tumor	B7-H3, EGFR

Figure C. CAR in clinic. Table representing CAR therapies in clinic (modified from Schaft et al., 2020)

The emergence of side effects in a subset of treated patients has been reported specifically during CAR-T applications[65]. Adverse events associated with T cell-based therapy can be immediate, delayed, mild, or severe, and they can last for the whole lifespan of the genetically modified T-cell, even, leading to patient death[66]. The main reason of this toxicity is CAR-T cell 'on-target/off-tumor' activity: when the antigen is not tumor specific, an immune response raging against the target express in non-tumor cells can occur, leading to organ failure[67]. Another common side effect of CAR T cell infusion is the emergence of systemic, over-powered, and uncontrolled immune activation, also known as Cytokine Release Syndrome (CRS)[68]. Clinical and physiological abnormalities such as hemophagocytic lymphohistiocytosis (HLH) or macrophage activation syndrome (MAS) are frequently associated with CRS. Neurological toxicity has also been reported[69]. A further unfavorable consequence has been highlighted: Tumor Lysis Syndrome (TLS), a collection of metabolic issues caused by the killing of a significant number of tumor cells[70]. In addition, active effector cell administration in the host can result in an abnormal immune response, such as Graft Versus Host Disease (GVHD), which occurs when donor cells attack the host tissue systematically[71], or as an immunoreaction against the CAR itself, because the host immune system recognizes the CAR as a foreign protein[72]. Finally, insertional oncogenesis is a possibility using lentiviral vector to engineered immune cells. This problem is still theoretical since examples of proto-oncogene activation have not been recorded. Nevertheless, this risk is inherent in the operation and must be considered[73].

2.1.5 Combinatorial CAR strategies

Despite the evident success of several clinical trials, the CAR therapeutic application requires refinement because there are still questions to be answered, mechanisms to be fully understood, and challenges to be resolved[74].

Four major topics of discussion are open. The first is connected to which immune cell recipient is the best for the CAR. A balance between the different immune cell choices is currently missing, and this is an active research area. The second point relates to the selection of the antigen suitable to be targeted in solid tumors. In this regard, neoantigens and new patient-specific mutant proteins generated by the tumor's high mutation rate are becoming increasingly appealing[75,76]. The third is concerned with the safety of therapeutic procedures and the possibility of side effects. The fourth category

includes both primary and acquired resistance occurrences[77]. Primary resistance can be attributed to restricted T/NK cell trafficking and migration within the tumor. Another source of primary resistance could be a poor rate of effector cell proliferation and persistence in the circulation, as in the case of the NK-92 effector cells, which must be irradiated prior to infusion. Moreover, the tumor microenvironment's strong immunosuppressive condition, particularly in solid tumors, is a significant limit that determines the ineffectiveness of CAR-T cell therapy[11]. Furthermore, regulatory T cells (Treg), MDSC, and TAMs produce cytokines such as IL-4, IL-10, and TGF-, which suppress T-cell function even further. Concerning secondary resistance, the key issue is antigen escape, namely the tumor's downregulation of the antigen targeted by the CAR, resulting in a lack of T/NK cell cytotoxic response.

In the last years a variety of different strategies have been proposed to get over the cited problems as safety and resistance. Here below the major investigated routes are listed (Fig. D).

CARs under inducible promoters: This approach entails the ability to modulate CAR expression in order to control and, if necessary, minimize and/or eliminate the cytotoxic reaction. To achieve tissue and/or time-dependent expression of the CAR, inducible synthetic promoters activated by exogenous added drug or particular micro-environmental conditions were incorporated in the expression cassette[78].

Bi-specific CARs: This strategy is based on a more complex CAR, which owns an extracellular region with two binding domains. The molecule functions according to the [OR] Boolean logic: the T/NK-cell cytotoxic activity is fully activated when the CAR recognizes the antigen "A" or the antigen "B". This approach can deal with the problem of secondary resistance caused by antigen escape[79].

Chimeric Costimulatory Receptor system: The CAR molecule is essentially ineffective in this system: upon engagement with the target antigen "A," it can only elicit a sub-threshold response. When a second chimeric molecule, the Chimeric Costimulatory Receptor (CCR), supplies the co-stimulatory elements required to fully activate the T/NK cell response, CAR becomes fully active; this happens when the CCR connects with a second target antigen "B". An effective immune response is thus triggered only in the presence of the two particular antigens (A and B). If the expression of the antigens targeted by the CAR and the CCR is a characteristic of transformed cells, this method can be used to address 'on target/off tumor' effects[80].

Synthetic Notch inducible CAR/molecules: This strategy entails creating a circuit in which a synthetic receptor, after interacting with a specific antigen "A," induces CAR/molecule expression. After binding with a second antigen "B" the CAR triggers a cytotoxic response. CAR expression and the subsequent T cell response occur exclusively in the presence of the two specific antigens (“A” and “B”). As with the CCR/CAR system, the 'on target/off tumor' effect is controlled when the co-expression of A and B antigens is unique to the tumor lesion and not present in normal tissue. Roybal and colleagues verified this system by employing a synNotch, a synthetic receptor composed of an external portion capable of recognizing a specific antigen and an intracellular portion constituted by a transcriptional factor. When the synNotch receptor binds to a specific antigen, a proteolytic event occurs, resulting in the release of a transcription factor (TF) into the cytoplasm. This TF promotes CAR/molecules expression. In this scenario, T cells were obtained using a circuit in which the synNotch identified the GFP expressed on the target cell's membrane, the TF was GAL4-VP64, and the CAR was direct against CD19. Only cells expressing both GFP and CD19 were targeted by the cytotoxic response[81].

Inhibitory CAR system: This approach was set up as an alternative to the previously mentioned strategies for reducing 'on target/off tumor' effects. It follows the [NOT] Boolean logic. In addition to the authentic CAR that recognizes antigen "A," a second synthetic receptor, an inhibitory CAR (iCAR), that recognizes antigen "B," is expressed in effector cells. Domains originating from immunological inhibitory receptors such as programmed death-1 (PD-1) or cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) are found in the iCAR intracellular region. When antigen "B" is present, iCAR limits CAR signaling; however, if antigen "A" but not antigen "B" is expressed on the cell to be targeted, CAR killing capability is fully unleashed. Achieving specific recognition via negative discrimination of non-cancer signals is especially suitable for preventing, rather than treating, the effects of insufficient T/NK cell specificity[82].

Dissociated CAR system: This system was designed to strictly control CAR expression and is based on a CAR composed of two distinct subunits: the antigen binding domain is expressed on one subunit (subunit 1) and the intracellular signaling domain is expressed on a second protein (subunit 2). A dimerization module is present in both subunits and can be activated by a small molecule. The small molecule's binding to

subunit 1 results in heterodimerization with subunit 2, which mediates the formation of a fully functional CAR[83].

CAR and suicide gene: In the case of severe toxicity, the ability to permanently disable CAR function and decrease CAR T cells may be required. This can be accomplished by expressing a suicide gene in modified T cells that is activated when an external drug is administered. Systems based on the herpes virus-derived thymidine kinase (HSV-TK) or the inducible human caspase 9 gene (iCasp9) have both been successfully used[84,85].

CAR and cytokine release/induction: The approach to condition tumor site with favorable cytokines has been pursued in order to overcome the primary resistance to CAR-T/NK therapy caused by the existence of a hostile tumor microenvironment. The production of cytokines such as IL-2, IL-12, and IL15 enhances the response to malignant cells and mediates higher tumor killing via the recruitment and activation of additional immune system effectors, promotes T cell proliferation and prevent exhaustion[86,87]. A particularly intriguing option for achieving exogenous cytokine expression is based on the employment of CAR T cells redirected for universal cytokine killing (TRUCK) or armored CAR. CAR-T cells are modified to express a transgenic cytokine via an extra cassette. This promoter is responsive to nuclear factor of activated T cells (NFAT), a transcriptional factor increased in immune cells when they are actively killing. Thus, interaction of the CAR by its target generates intracellular reactions that result in the activation of NFAT, which activates cytokine production[88]. Besides cytokines, even further useful molecules can be expressed using armored T cells[89].

ACT and checkpoint inhibitors: Given that all of the factors that physiologically induce anergy have the potential to dampen the therapeutic activity of the CAR-based approach, administering CAR T/NK cells while simultaneously inhibiting the signals generated by immune checkpoint molecules offers the opportunity to increase the antitumor effects. One possibility is to employ antibodies blocking CTLA-4, or PD-1, or the PD ligand (PD-L1) *plus* CAR-T strategy[90,91].

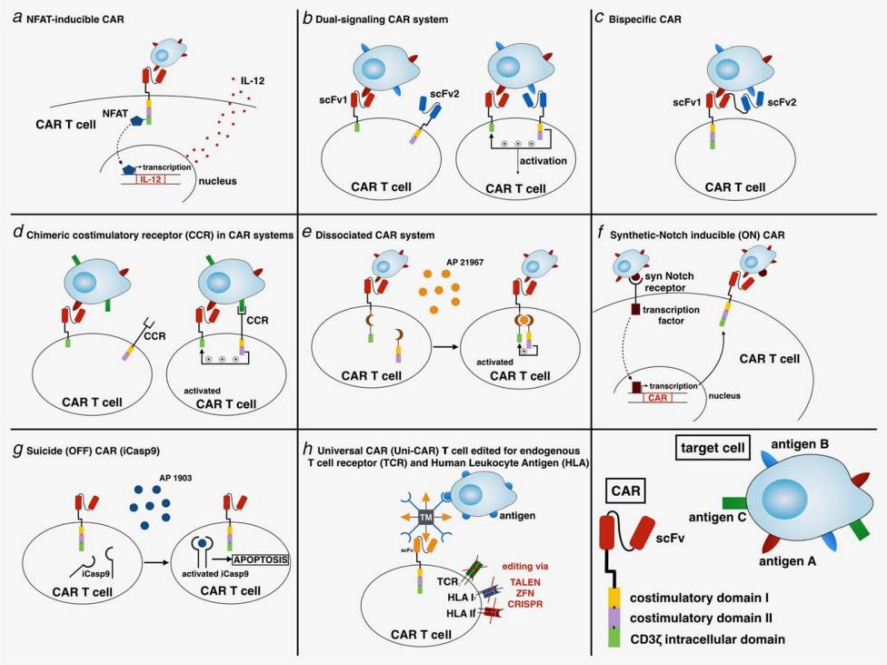

Figure D. Combinatorial CAR strategies. Schematic representation of combinatorial CAR strategies (modified from Schubert et al., 2018)

2.2. Immunotherapy in colorectal cancer

2.2.1 Checkpoint blockade in CRC

According to Global Cancer Observatory, more than 1.8 million new instances of colorectal cancer (CRC) were diagnosed globally in 2020, accounting for 10% of all malignancies. Rates are likely to rise globally, with more than 2.2 million additional CRC cases expected by 2030. CRC is the third most frequent type of cancer. Despite therapy improvements, the 5-year survival rate for patients with metastatic CRC (mCRC) is only 10-15%[92]. The mainstay of first-line therapy for advanced CRC is chemotherapy, which can be combined with an anti-vascular endothelial growth factor (VEGF) or anti-epidermal growth factor receptor (EGFR) antibody, based on specific genetic tumor features; however, most tumors progress within one year[93].

Immune checkpoint inhibitors have recently shown promising results in patients with CRC and other solid cancers that are mismatch repair defective (dMMR). The molecular abnormality causes large amounts of frame-shift mutations, which are detectable as variations in the length of short segments of DNA (microsatellites), a condition known as microsatellite instability (MSI). Because of the high frequency of mutations, there are numerous opportunities for new peptide sequences

(neoantigens) to be presented by tumor cell HLA molecules to cytotoxic T cells with receptors capable of recognizing these distinct antigens[94]. High expression of cell surface inhibitory checkpoint molecules that downregulate the immune response (e.g., PD-1/PD-L1, and CTLA-4) is frequent in the T-cell-infiltrated microenvironment of microsatellite instability-high (MSI-H)/dMMR tumors. As a result, the inhibition of PD-1/PD-L1 and CD80/CTLA-4 interactions promote T-cell proliferation and activation. Long-lasting responses of MSI-H/dMMR tumors to checkpoint inhibitors resulted in accelerated FDA approval of two PD-1 inhibitors, nivolumab (with or without low-dose ipilimumab) and pembrolizumab, for MSIH/dMMR mCRC after chemotherapy progression[95]. To date, three ICIs-based regimens, pembrolizumab and nivolumab +/- ipilimumab for first- and subsequent-line setting[96] and dostarlimab[97] for second- or subsequent treatment line, have shown a significant clinical benefit for patients with dMMR/MSI-H mCRC.

However, a better understanding of the immune-mediated behavior of both mCRC subtypes, and many immunotherapeutic agents is needed to target the intrinsic vulnerabilities of microsatellite unstable tumors and overcome primary immunotherapy resistance in microsatellite stable tumors[98].

2.2.2 Cell-based adoptive immunotherapy in CRC

In recent years, cell-based immunotherapy has produced encouraging results in some CRC patients, particularly those with high MSI or poor mismatch repair[99]. CRC tumors include many altered proteins because of genetic changes, which may emerge as new targets identified by the immune system. The MSI phenotype is characterized by the presence of a large number of TIL. The introduction of immunomodulators has altered the landscape of CRC treatment. The understanding of the complicated link between the immune system and cancer has led to significant development in tumor immunotherapy[100]. In several fields, T-cell immunotherapy is now a valuable and more effective alternative to standard cytotoxic medicines. Some patients with clinically aggressive tumors have already benefited from immunotherapy, however cell-based immunotherapy in CRC faces obstacles due to partial responsiveness to immune checkpoint inhibitors[101].

TILs, PBMC, NK cells, and IPSC have been used as treatments for metastatic CRC in recent decades, with ambiguous results due to management difficulties, such as

patient heterogenicity. Recently, based on strong results with CAR-T strategies against hematological tumors, some breakthroughs occurred in CAR-T cell treatment for CRC. Carcinoembryonic antigen (CEA), Mesothelin (MSLN), Guanylyl cyclase C (GUCY2C), epithelial cell adhesion molecule (EpCAM), Human epidermal growth factor receptor-2 (HER2) Doublecortin-like kinase 1 (DCLK1) are in clinical trial evaluation as target for CAR-T cell treatment in CRC[102]. Even though these clinical trials are still in the early phases, CAR-T cells have become one of the most researched and promising cancer treatments[103]. This therapy, however, has some difficulties that limit its clinical application. Furthermore, CAR-T cell therapy can result in a number of harmful side effects, the most common is the CRS[8]. A glaring example was the case of a patient with advanced CRC who experienced a severe adverse reaction after being treated with ERBB2-targeting CAR-T cells. This patient had received adoptive transfer of T cells designed to express ERBB2-targeted CARs containing CD28, 4-1BB, and CD3 signaling moieties; after 15 minutes from T cell infusion, exhibited respiratory distress and died 5 days later[66].

To effectively address these serious side effects, it is crucial to improve the CAR-T cells therapy for CRC treatment.

2.3. Rationale for a combinatorial CAR strategy against HER2amp CRC

2.3.1 Frequency

Human epidermal growth factor receptor 2 (HER2) is a proto-oncogene which encodes for a tyrosine kinase member of the epidermal growth factor receptor (HER/EGFR/ERBB) family. HER2 cannot directly bind ligands, therefore its activation results from heterodimerization with another ERBB member or by homodimerization when HER2 concentration is high, for instance in cancer. HER2 regulates cell proliferation, differentiation, and migration via a variety of signaling pathways, including mitogen-activated protein kinase/extracellular signal-regulated kinases (MAPK/ERK) and phosphoinositide 3 kinase (PI3K)/Akt mammalian target of rapamycin (mTOR). HER2 mutations include gene amplification and missense mutations, which frequently result in protein overexpression and can be considered founder events in carcinogenesis and tumor growth[104]. HER2 status is being assessed

in breast and gastric cancers to identify patients who are candidates for anti-HER2 therapy. However, HER2 mutations have been found in a variety of other solid tumors, including colorectal cancer[105]. HER2 overexpression is found in 5-6% of CRC patients (100.000 new cases every year worldwide), with somatic HER2 gene changes, including amplifications, found in 7% of patients. HER2 mutations in colonic epithelial cells have been demonstrated to promote HER2 signaling pathway activation, enhance independent cell proliferation, and potentially gain resistance to EGFR-targeted therapy, resulting in a poor prognosis for patients[106]. Multiple studies have indicated that HER2 can be effectively addressed in metastatic CRC settings[107], providing solid rationale for HER2-targeted therapy in CRC in clinical practice.

2.3.2 Current therapies and their limitations

Anti-HER2 antibodies (e.g., trastuzumab, pertuzumab) are currently used to treat HER2-positive (i.e., score 3+ by IHC or 2+/in situ hybridization (ISH)-positive) breast and gastric cancer[108,109]. HER2 emerged as a negative predictive biomarker for CRC, since HER2 amplification or overexpression was linked to a lack of response to anti-EGFR therapy[110]. In this context the first clinical trials in mCRC investigated the combination of trastuzumab with chemotherapy. The role of FOLFOX combined with trastuzumab in the treatment of HER2-positive mCRC was evaluated as second- or third-line therapy. With a median duration of response of 4.5 months, 24% of the patients had a partial (PR) or complete response (CR)[111]. Another phase II study assessed the combination of trastuzumab and irinotecan in HER2-positive mCRC patients who had previously received one line of therapy. 71% of the patients had objective responses recorded, that were maintained for at least 6 months[112]. The HERACLES-A multicenter study examined the combination of trastuzumab and lapatinib in patients with KRAS exon-2 wild-type (WT) and HER2 amplification and/or overexpression mCRCs, resistant to standard treatment[113] (Fig. E).

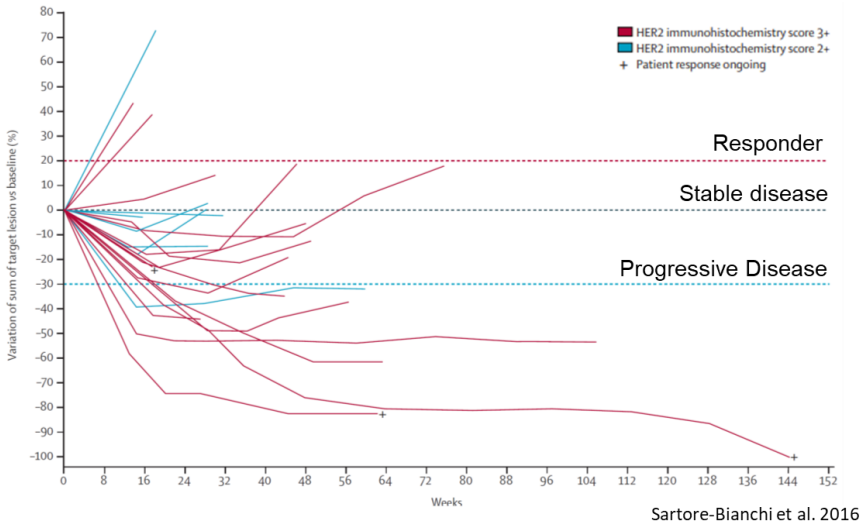

Figure E. HER2 amplified patient response to Lapatinib + Trastuzumab treatment. Individual lines represent the percentage change in target tumor burden from treatment start (day 0) to the day of objective disease progression, based on serial assessment every 8 weeks. Dashed lines show a 30% reduction (light blue) or a 20% increase (red) from baseline. Crosses denote patients who were responding at the time of data cut-off (modified from Sartore-Bianchi et al., 2016)

After 6.7 years of follow-up, the overall response rate (ORR) was 28%, with 1 CR and 8 PRs, a disease control rate (DCR) of 69%, a median progression-free survival (mPFS) of 4.7 months, and a median overall survival (OS) of 10 months for 32 treated patients (Fig. F). A grade 3 decrease in left ventricular ejection fraction was reported by 6% of the patients, while fatigue was reported by 16% of the patients[114].

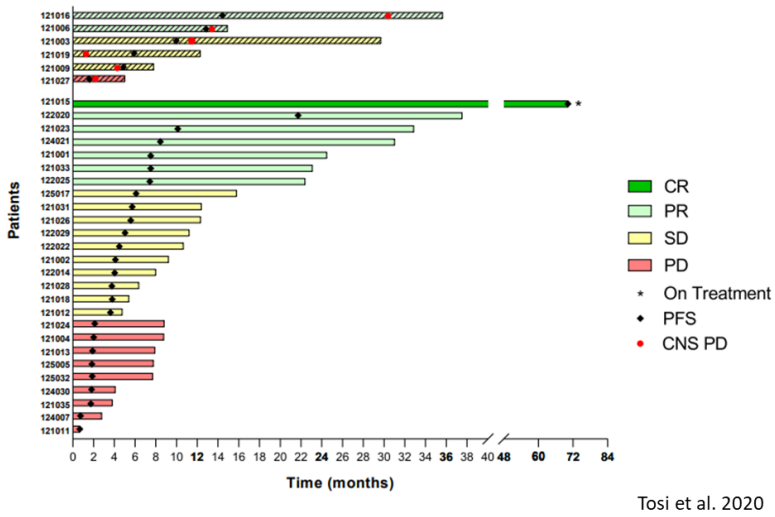

Figure F. HER2 amplified patient survival after Lapatinib+Trastuzumab treatment. Swimmer plot regarding progression-free survival (PFS) and overall survival (OS) of HER2-Positive patients treated with trastuzumab and lapatinib (modified from Tosi et al., 2020)

HERACLES-B, on the other hand, investigated the combination of pertuzumab and the antibody-drug conjugate trastuzumab emtansine (TDM-1) in RAS and BRAF WT and HER2-positive mCRCs, resistant to standard therapies. Even though the treatment was well tolerated, the primary endpoint was not met[115].

In MyPathway phase II trial, patients with HER2-amplified mCRC received trastuzumab and pertuzumab together: one patient reached a CR, while 30% received a PR. The treatment was well-tolerated, with G1 or 2 diarrhea, fatigue, and nausea being the most common adverse events. When compared to the KRAS WT population, patients with a KRAS mutation had significantly shorter PFS and OS[116].

Trastuzumab deruxtecan (T-DXd), a humanized anti-HER2 antibody conjugate to the topoisomerase I inhibitor, has been approved by the US Food and Drug Administration (FDA) for HER2-low (i.e., score 1+ or 2+/ISH-negative) breast cancers[117], and it is currently under investigation in other types of tumors, also in CRC. The T-Dxd was tested in the phase II DESTINY-CRC01 trial. This antibody-drug conjugate was tested in patients with HER2-positive RAS-BRAF WT mCRC who had progressed on two or more lines of treatment, even including patients who had previously received different anti-HER2 agents. Only patients with HER2 3+ in IHC showed a response[118]. In 9.3% of the patients, pulmonary toxicity in the form of interstitial lung disease and pneumonitis was observed. Due to grade 5 lung toxicity, two patients died. Surprisingly, trastuzumab deruxtecan was also effective in patients with RAS mutations[119]. Furthermore, many trials with anti HER2 drug in combination with other tyrosine kinase inhibitors such as tucatinib, pyrotinib, neratinib were investigated with promising results but with frequent adverse events[120]. While anti-HER2 therapy in CRC is still awaiting approval, many early trials are ongoing with some promising results. Unfortunately, drug resistance occurs: resistance to trastuzumab, which has recently been explained as one of the possible mechanisms in HER2-positive gastric cancer, by vessel destabilization and activation of the glycolytic pathway inducing 6-phosphofructo-2-kinase (PFKFB3), remains a major challenge[121]. HER2-activating mutations are also associated with tumors with high microsatellite instability, which has been observed in CRC[122]. Indeed, with the advancement of immunotherapy, and the approval of pembrolizumab by the FDA, it has become a game-changing treatment option for unresectable or mCRC in patients with MSI-H or dMMR[96]. Despite positive

results in some phase I trials, only a small proportion of CRC patients responded to immune checkpoint therapy in metastatic settings, and all of these tumors were MSI-H/dMMR with a high tumor mutation burden. While tumor mutational burden has been linked to immune checkpoint response rates in other tumor types, such as melanoma and non-small-cell lung cancer, the underlying mechanism remains unknown, though it could be related to immune cell reactivity, increasing T-cell infiltration[123]. Despite encouraging results with both HER2 target therapy and checkpoint inhibitors, there is an unmet clinical need for more than half of the HER2 amplified metastatic CRC cases, most notably for drug resistance caused by the HER2 downstream pathway mutation. The CAR based ACT could overcome these limitations because it is not affected by pathway mutations, but only by the presence of the antigen on the cell surface. Besides, combinatorial strategies as the synNotch regulatory network system, may also avoid the toxic issues associated with current therapies and CAR-T treatment.

2.4. Detailed features and advantages of the synNotch system

A fundamental goal in the emerging fields of synthetic biology and cell engineering is to be able to rationally change which extracellular antigen a cell recognizes, as well as the resulting cellular response. Customized cell sensing/response pathways would be extremely useful in the engineering of therapeutic cells, allowing them to autonomously sense user-specified disease or injury signals and precisely deploy therapeutic or repair functions[7].

Customized cell sensing/response behaviors could also be used to report on cell connectivity and environmental conditions. Novel cell-cell communication channels may also allow for the creation of multicellular assemblies, the self-organization of which is governed by specific cell-cell signaling networks. Morsut and colleagues investigated synthetic pathways in which input and output can be flexibly altered in a modular fashion[124]. Furthermore, such synthetic pathways would be ideal for operating orthogonally from endogenous pathways and from one another, allowing for combinatorial input integration with minimal crosstalk. The Notch receptor was selected as the foundation for this system. When the Notch receptor interacts with its ligands, Delta family proteins found on the surface of partner cell, intramembrane

proteolysis occurs: sequential proteolysis by a disintegrin-metalloproteinase (ADAM) and the gamma-secretase complex[125]. The intracellular fragment of Notch is released when the receptor is cleaved. The Notch intracellular domain is a transcriptional regulator which can only function once it is released from the membrane and enters the nucleus, where it activates target genes involved in cell-cell signaling during development[126]. Previous research has shown that the intracellular domain of Notch can be replaced with an artificial transcription factor (e.g., Gal4-VP64) to create a Notch activity reporter[127]. Notch activation's physical mechanism has also been studied, and it has been discovered that the extracellular domain of Notch can be replaced by other domains[128]. Notch signaling's simple mechanism has also inspired the development of novel proteolytically induced receptors and reporter systems[129] (Fig. G).

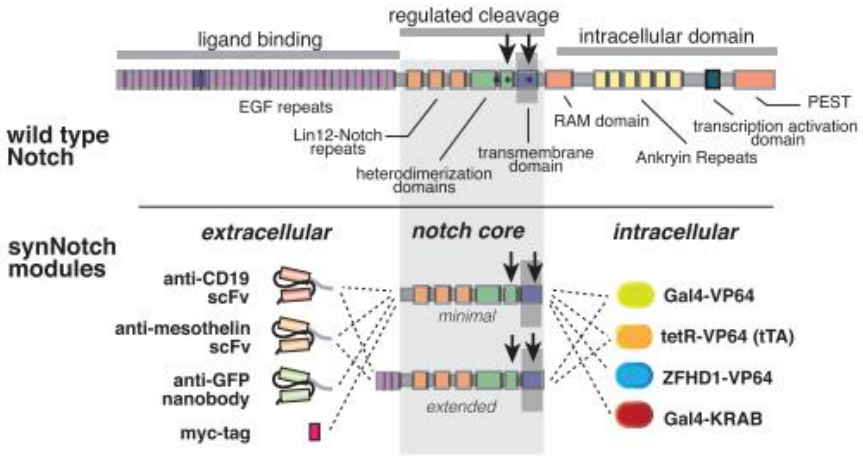

Figure G. SynNotch structure. Schematic representation of a SynNotch molecular structure (modified from Morsut et al., 2016)

Because of the modularity of Notch receptors, Roybal et al. investigated whether the Notch receptor could be used as a platform to generate synthetic signaling pathways that customized both sensing and response. They showed that, by swapping the extracellular recognition domain of these receptors, they can customize input sensing, including the use of antibody-based domains (e.g., single-chain antibodies or nanobodies) to detect a wide range of user-specified cell-surface proteins, such as disease antigens. Simultaneously, by swapping the intracellular transcription domain and providing specific downstream effector target genes, they can link these novel inputs to customized responses. The resulting synthetic Notch (synNotch) receptors

only retain the native Notch's minimal transmembrane core domain, which controls proteolysis. SynNotch receptors could be used in a variety of cells, including immune cells and neurons. They can spatially direct the induction of complex responses such as differentiation and pattern formation by using synNotch pathways. It was shown that as long as synNotch receptors have distinct intracellular and extracellular domains, they function orthogonally to one another because they share no common signaling intermediates. As a result, multiple synNotch pathways can be used to engineer complex combinatorial sensing circuits in the same cell. The adaptability of SynNotch receptors in engineering novel cell behaviors makes them useful tools for building therapeutic cells[130], driving the formation of complex multicellular patterns, or modulating or reporting on cellular behavior in a complex *in vivo* system. With their customizable input/output function, SynNotch receptors add a powerful and flexible sensing capability to the mammalian synthetic biology toolbox[131] (Fig. H).

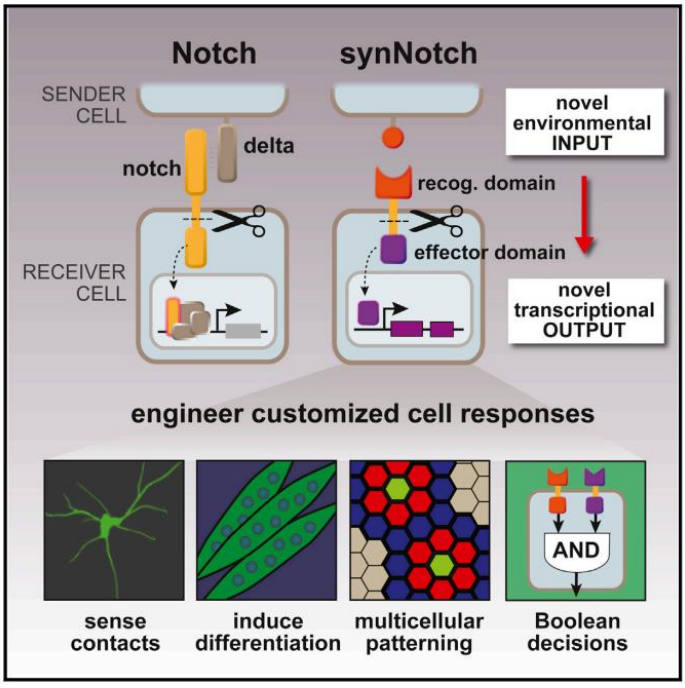

Figure H. SynNotch functions. SynNotch functions schematic representation (modified from Morsut et al., 2016)

In recent years, numerous studies have been presented regarding a range of alternative therapies to improve CAR therapies efficacy, to better control the cytotoxic response and to overcome the adverse effects of the CAR approach, including safety issues such as "on-target off-tumor" toxicities and cytokine release syndrome[10].

The synNotch regulatory network strategy creates a circuit in which a synthetic receptor, after interacting with a specific antigen "A" induces CAR/molecule expression. After binding with a second antigen "B", the CAR triggers a cytotoxic response. CAR expression, and the subsequent T cell response, occur exclusively in the presence of the two specific antigens (“A” and “B”, Fig. I). The 'on target/off tumor' effect can be controlled when the co-expression of A and B antigens is unique to the tumor lesion and not detected in normal tissue. Roybal and colleagues verified this system by employing a synNotch, a synthetic receptor constituted of an external portion capable of recognizing a surfaceGFP (sGFP) and an intracellular portion composed of a transcriptional factor (TF): the GAL4VP64. When the synNotch receptor binds the sGFP, a proteolytic event occurs, resulting in the GAL4VP64 release into the cytoplasm. This TF promoted an anti-CD19 CARexpression[81].

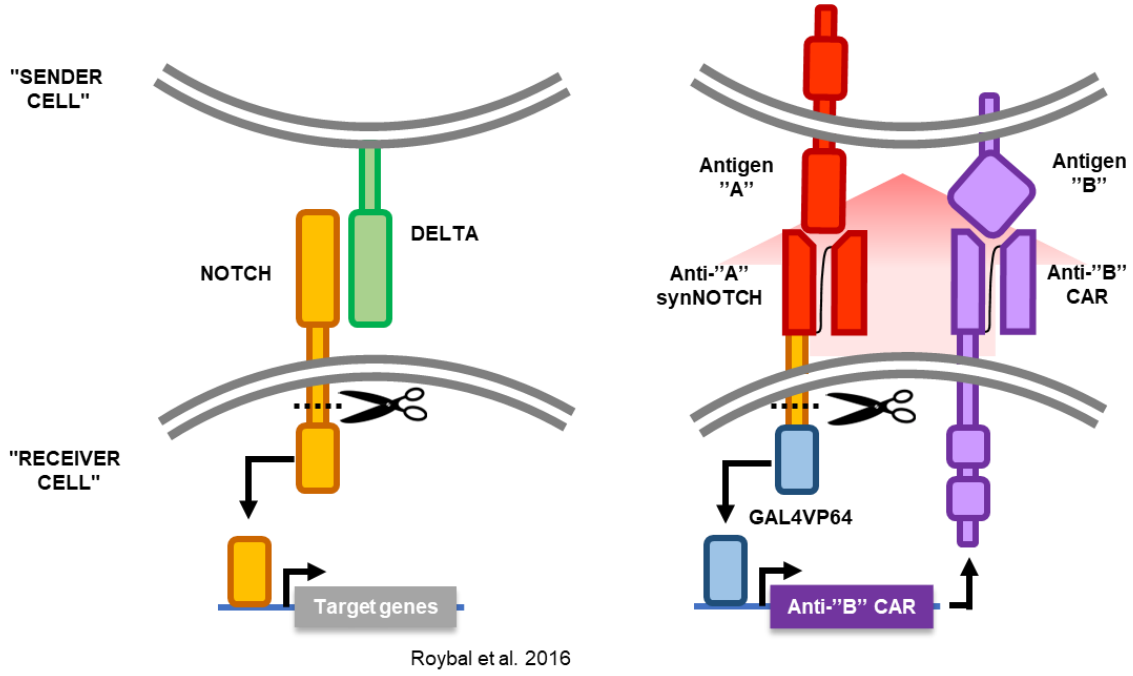

Figure I. Anti-“A” synNotch GAL4 anti-“B” CAR system. Anti-“A” SynNotch GAL4 anti-“B” CAR system schematic representation (modified from Roybal et al., 2016)

When compared to the multiple CAR approach, combinatorial control over T cell activation with the synNotch-CAR AND-gate has several advantages. Importantly, the synNotch receptor does not directly activate T cells, but it is completely independent of CAR/TCR signaling. As a result, activating the synNotch receptor does not cause any damage to the synNotch antigen-bearing tissue; it simply results in the priming response of inducing CAR expression.

The majority of engineered T cell strategies have focused on identifying and targeting a single tumor-specific antigen using a CAR or engineered TCR. As a result, the lack of tumor-specific antigens has limited this approach. Although there are numerous tumor-associated antigens, only a small number of them are tumor-specific, almost only in liquid tumors. Instead, solid tumors overexpress antigens that could be targeted, but they are also expressed at lower levels in other bystander tissues. The synNotch/CAR robust AND-gate dual antigen detection now opens the door to tumors being targeted based on combinatorial antigen signatures[132,133]. Multiple antigens are far more likely to provide greater discriminatory power between tumor and normal tissues[134]. To attack a specific type of cancer cell more precisely, it may be possible to target both a disease-associated antigen and a tissue-specific (normal) antigen. Tissue-specific antigen detection by synNotch receptors could limit therapeutic immune cell priming to specific tissues. This is a novel way for synNotch receptors to improve therapeutic efficacy while decreasing systemic toxicity[135].

SynNotch receptors could be used to restrict the expression of this class of CARs to the disease site and to avoid off-target tissue. SynNotch receptors expand the landscape of targetable antigens for CARs and may facilitate the use of CARs that were previously reported to be toxic[136]. SynNotch receptors are a versatile and potent platform for not only localizing therapeutic immune cell activity, but also for developing combinatorial antigen-sensing capabilities that improve any therapeutic cell's ability to recognize diseased target tissues with high precision and specificity. Although further developments are needed, the versatility and modularity of the synNotch receptor system could be used to program therapeutic cells to perform a wide range of combinatorial logical decisions beyond dual antigen sensing.

3. Aim of the study

In the last few years many studies highlighted HER2 amplification and overexpression (HER2amp) as an effective target for colorectal cancer treatment. In particular, a combined HER2/EGFR-targeted treatment (Lapatinib + Trastuzumab) was found to induce tumor regression in almost 50% of patients carrying advanced metastatic HER2amp CRC[113]. Despite such positive results, two major unmet clinical needs remain: primary resistance to HER2/EGFR therapy, and limited duration of the response, due to acquisition of resistance-promoting mutations[110,118]. Consequently, a sizeable fraction of HER2-amplified CRCs still needs alternative effective therapies. In this perspective, one of the most promising strategies to overcome the above limits is adoptive cell immuno-therapy. ACT is a highly personalized therapy that involves administration to the patient of immune cells engineered to acquire selective anticancer activity by transduction with a Chimeric Antigen Receptor. CAR-killer cells successfully killed hematological tumors, like B-cell leukemia[58]. However, ACT remains extremely challenging in solid tumors, mostly because the identified CARs target antigens also expressed by normal tissues, leading to occasionally fatal adverse reactions, also with HER2-directed CARs[66].

The main aim of this study is to set up and validate a combinatorial antigen-targeting approach based on the synNotch system, to engineer killer cells against HER2amp CRC without directing them also against normal tissues.

To reach this objective, the study is articulated in the following intermediate aims:

(i) To identify an optimal second antigen to be combined with HER2, through bioinformatic analyses of mRNA expression data from public repositories and in-house patient-derived CRC models.

(ii) To preliminary validate the synNotch system by using a synNotch receptor against an artificial antigen (surface GFP) and testing induction of a CAR against a second antigen driven by the synNotch engagement[81].

(iii) to obtain the final HER2 synNotch / “antigen B” CAR constructs and functionally validate them in a model cell line, verifying that the HER2 synNotch is activated only by HER2-overexpressing target cells and not by cells expressing normal HER2 levels. This can be achieved by engineering the synNotch using a low affinity anti-HER2 scFv.

(iv) To obtain natural killer effectors engineered with the synNotch/CAR system. In particular engineering the NK-92 a lymphoma natural killer cell line, IL-2 dependent, that do not express inhibitory receptor, that has an high anti-tumor intrinsic activity and that are clinically approved if irradiated before injection[137]. All these characteristics should make the NK-92 cell lines a valid immune cell model for immunotherapy strategies.

(v) To validate *in vitro* and *in vivo* the specific activity of engineered NK-92 effectors against CRC carrying HER2 amplification. Biologic activity, including cytotoxic activity and tumor invasion of synNotch/CAR effector cells will be evaluated against human cancer cell lines and organoids characterized by different levels of surface antigens expression. Specificity and selectivity of the system will be assessed by testing engineered NK-92 cell killing against cancer cells engineered to obtained variable levels of target antigens on their surface. Safety issues related to 'on target-off tumor' activities will be evaluated testing multiple cell models, while prediction of the inflammatory responses will be done analyzing the release of specific cytokines by NK-92, before and after the interaction with the target cells. *In vivo* efficacy will be tested in different HER2amp CRC models.

(vi) To further evolve the synNotch/CAR system for improved activity, survival, tumor penetration, and efficacy and to evaluate additional targets for the synNotch component.

4. Materials and methods

4.1. Normal tissues, CRC and NK-92 mRNA expression data analysis

RNA-seq expression data of normal tissues/cell lines were selected and downloaded from the Entrez Gene Database, composed by RNA-seq of tissue samples from 95 human individuals representing 27 different tissues in order to determine tissue-specificity of all protein-coding genes[138] (https://www.ncbi.nlm.nih.gov/gene/2064, https://www.ncbi.nlm.nih.gov/gene/1048) and from the ProteinAtlas database composed by the sum of two different sources: (i) the HPA RNA-seq data analyzing 1055 cell lines, 51 human tissues and 18 blood cell types, (ii) GTEx RNA-seq data analyzing 36 human tissues (https://www.proteinatlas.org/ENSG00000141736-ERBB2/tissue, https://www.proteinatlas.org/ENSG00000105388-CEACAM5/tissue).

For the Protein expression (score), ProteinAtlas utilized tissue microarrays from 144 individuals corresponding to 44 different normal tissue types.

RNA seq / microarray expression data of CRC cell lines / PDX were selected and downloaded from (i) The Cancer Genome Atlas (TCGA): the dataset included 450 CRC PDX; (ii) from the Candiolo Cancer Institute database composed by 602 RNA-seq samples[139] / 515 microarray samples[140] CRC PDX and by 119 RNA-seq samples CRC cell lines (Manuscript in preparation).

NK-92 RNA was extracted, quantified, sequenced, and analyzed as previously describe[139]. 134 NK cell related genes were downloaded from Immport (https://www.immport.org/resource)

Expression values, calculated in counts per million (CPM), were plotted after addition of 1 CPM (to avoid zero values) and log2 transformation.

4.2. Cell lines and Patient Derived Organoids cultures

SKBR3 human breast adenocarcinoma cells, LS180 human colorectal adenocarcinoma cells, K562 human chronic myeloid leukemia in blast crisis cells, 293T human kidney cells, Jurkat acute T cell leukemia cells, OVCAR-3 human ovarian adenocarcinoma, SW1116 human colorectal adenocarcinoma were purchased by American Type Culture Collection (ATCC/LGC Standards Srl, Manassas, USA).

NCI-H508, NCI-H508 HER2-cDNA human colorectal adenocarcinoma and DIFI, DIFI HER2-cDNA human colorectal adenocarcinoma were gently given by the Trusolino Laboratory of the Candiolo Cancer Institute[141].

NK-92 human malignant non-Hodgkins's lymphoma cells purchased by the Leibniz Institute DSMZ (Braunschweig, DEU)

CRC0080 colorectal cancer cells, CRC0186 colorectal cancer cells were derived from Patient Derived Xenograft (PDX) obtained from patient tumor tissue in the FPO-IRCCS Candiolo[142].

SNU-254 human colorectal adenocarcinoma was purchased by Korean Cell Line Bank (Seul, South Korea)

Cells were maintained at 37 °C in 5% CO2 in recommended media: Roswell Park Memorial Institute (RPMI-1640) medium for NCI-H508, K562, Jurkat and T cells (for T cells with IL-2 100 U/ml), OVCAR-3, SNU-254; Dulbecco Modified Eagle Medium (DMEM) medium for SKBR3 cells; Minimum Essential Medium Eagle (MEM) medium for LS180 cells; Iscove Modified Dulbecco Medium (IMDM) for 293T cells; Ham's F12 medium (F12) for DIFI cells; Dulbecco Modified Eagle Medium Nutrient Mixture F12 (DMEM/12) for CRC0080, CRC0186 and SW1116 cells (all media are from Sigma Life Science and GIBCO Thermo Fisher), supplemented with 10% of Fetal Bovine Serum (FBS, Euroclone SpA), 1% of L-glutamine (SigmaAldrich), 1% Penicillin-Streptomycin (SigmaAldrich) and only for CRC0186 also Rock Inhibitor 10 uM (Y-27632, Selleckem) CRC0080 and CRC0186 Patient Derived Organoids (PDO) were culture with DMEM/F12 supplemented with B-27 supplement (1X) (Thermo Fischer), N-2 supplement (1X) (Thermo Fischer), N-acetylcysteine (1mM) (Thermo Fischer), 1% Penicillin-Streptomycin, 1% and EGF (20 ng/ml). Alpha Minimum Essential Medium Eagle (alpha MEM) for NK-92 cells supplemented with 12,5% of Fetal Bovine Serum (FBS, Euroclone SpA), 12,5% of Horse Serum (HS, Euroclone SpA), 1% of L-glutamine and 1% Penicillin-Streptomycin

All the cell lines were cultured in Petri dishes for adherent cells or flask for suspension cells (only for CRC0186 se used Collagen I coated petri dishes) and split when confluent. All the cell cultures were tested for mycoplasma contamination.

4.3. Lentiviral vectors construction

EGFP ligand, LAG16 SynNotch (anti-GFP), 4D5-5 HER2 synNotch, 5xGAL4 UAS BFP PGK Cherry, and 5xGAL4 UAS lentiviral vectors were purchased by Addgene.
MSLN CAR lentiviral vector was purchased by Creative Biolabs.
CEA CAR BW431/26 (composed by: Anti-CEA scFv mAb BW431/26 - IgG4 hinge - CD28 TM domain – CD28 domain – CD3z domain), GAL4 CEA CAR, GAL4 IL-2 CMV Cherry, MSLN synNotch (composed by: MSLN scFv derived from Creative Biolab MSLN CAR T2A iCAS9 – EGF repeat – SynNotch part from HER2 synNotch), LUC CMV DT lentiviral vectors were designed in house but purchased by Vector Builder.
EFS GAL4VP64 PGK PURO lentiviral vector were designed and cloned in house.

4.4. Lentiviral vectors preparation

The third-generation lentiviruses (EGFP ligand, LAG16 SynNotch (anti-GFP), 4D5-5 HER2 synNotch, 5xGAL4 UAS BFP PGK mCherry, 5xGAL4 UAS, MSLN CAR.
CEA CAR BW431/26, GAL4 CEA CAR, GAL4 IL-2 CMV Cherry, MSLN synNotch, LUC CMV DT, GAL4 MSLN CAR, GAL4VP64 PGK PURO stocks production was obtained mediating 293T cells calcium phosphate transient transfection. The 293T were seeded $5x10^6$ in p15 petri dishes. The day after, 37,5 µg of the transfer vector, 16,26 µg of the packaging plasmids pMDLg/pRRE, 6,25 µg of the plasmid pRSV.REV and 9 µg of the vesicular stomatitis virus (VSV) envelope plasmid pMD2. Plasmids solution (bring to volume final volume of 1125ul with TE and distilled water) was added in a solution of 125 µl CaCl2 2,5 M and 1250 µl of HBS 2X (for the formation of Ca3(PO4)4 particle). After 16 hrs from transfection medium was changed and then added 1 mM/L Na butyric acid (Sigma). 30 hrs later the supernatants with the viral particle were purified and concentrated mediating ultracentrifugation. Determination of the viral p24 antigen concentration was done by HIV-1 p24 Core profile ELISA (Perkin-Elmer Life Science, Inc.).

4.5. Generation of genetically modified target cells lines

(HCT116 / SKBR3 / LS180 / CRC0080 / CRC0186) sGFP: the cells were plated 50.000 cells/well in 6 well plates in complete medium. After 24 hrs, the medium was replaced and the cells were transduced with 1 ul of concentrate EGFP ligand lentivirus, with

polybrene (8µg/ml). Identical procedures were also performed for the LUC CMV DT lentivirus.

4.6. Transduction and sorting of synNotchs and CEA CAR NK-92 cells

NK-92 cells were plated 100.000 to 300.000 cells/well in 24 well plates in K562 conditioned medium, Human IL-2 1000 U/ml (cat num: 130-097-743, Miltenyi Biotec), BX795 (Catalog code: tlrl-bx7, Invivogen), protamine sulfate (cat. num: P3369, Sigma) and HER2 synNotch concentrated virus (P24 236 ng/ul); plates were centrifugate 1000 g for 1 hr; after 24 hrs of incubation, the medium was changed and new virus was added, plates were again centrifugate 1000g for 1 hr and after 24 hrs the cells were washed and resuspended in culture medium. HER2 synNotch expression was confirmed by FACS analysis.

These cells were sorted with MoFlo ASTRIOS EQ ™ Cell Sorter (Beckman Coulter, Brea,CA) for two times for HER2 synNotch positivity (ab anti myc-tag (9B11) Mouse mAb #2276, Cell signaling). HER2 synNotch positive NK-92 were transduced with GAL4 CEA CAR with the same procedure of the HER2 synNotch lentivirus. The HER2 synNotch CEA-CAR population was tested for CEA CAR induction after co-culture with HER2 normal and HER2 amplified cell lines. To reduce the basal CEA CAR induction, a first negative sorting was performed in which only the cells negative for the CEA CAR (Alexa Fluor® 647 AffiniPure F(ab')2 Fragment Goat Anti-Human IgG, H+L antibody, Jackson ImmunoResearch) were selected after a co-culture with HER2-normal target cells. Subsequently, to increase the CEA CAR induction a second positive sorting was performed in which only CEA CAR positive cells were selected, after a co-culture with HER2 amplified target cells. Simultaneously, the population was cloned. The clones were tested for CEA CAR basal expression and CEA CAR induction after co-culture. The best clones were chosen (See results).

The 5F clone was transduced with the GAL4 IL-2 PGK mCherry lentivirus with the same procedure of HER2 synNotch. Then the 5F clone GAL4 IL-2 PGK mCherry population was sorted once for mCherry positivity.

The same procedure of HER2 synNotch transduction was used to transduce NK-92 with MSLN synNotch lentivirus. MSLN synNotch expression was confirmed by FACS analysis. These cells were sorted for once for MSLN synNotch positivity (ab anti myc-

tag (9B11) Mouse mAb #2276, Cell signaling). MSLN synNotch positive NK-92 were transduced with GAL4 CEA CAR with the same procedure of the HER2 synNotch lentivirus.

The MSLN synNotch/CEA-CAR population was tested for CEA CAR induction after co-culture with MSLN normal and MSLN high cell lines. To increase the CEA CAR induction a positive sorting was performed in which only CEA CAR positive cells were selected, after a co-culture with MSLN high target cells. Simultaneously, the population was cloned. The clones were tested for CEA CAR basal expression and CEA CAR induction after co-culture. The best clones were chosen (See results).

4.7. Irradiation of NK-92 cells

CAR-NK-92 and NK-92 cells naïve were collected by centrifugation, counted, washed, resuspended in fresh growth medium, and irradiated with 5 Gray (Gy) (RAD GIL, Gilardoni S.p.a.) For *in vitro* proliferation, cells were irradiated with 5 Gy, washed, resuspended in fresh growth medium, and cultured for up to 7 days. Proliferation was analyzed by counting viable cells every day using trypan blue exclusion. For cytotoxicity assays and *in vivo* experiments, cells were irradiated with 5 Gy and used directly.

4.8. Flow cytometry analysis

Cell lines were washed twice with cold Phosphate Buffer Saline (PBS, Sigma), mechanically detached with 1 mM of PBS EDTA and resuspended at the final concentration of 100.000 cells/100 µl in PBS-1% BSA plus 1 µl of anti-HER2 (PE Mouse Anti-Human HER-2/neu Clone NEU24.7) or anti-CEA Ab (PE, CD66abce Antibody, anti-human REAaffinity Clone REA876) for 30 min on ice. For all the HER2 and CEA expression analysis MFI was calculated on the total cell population. The phenotype of synNotch/CAR-transduced or control NK-92 were analyzed by standard flow cytometric assays. CARs expression was detected using 1 µl of mAb specific for the IgG1/CH2CH3 spacer, Alexa Fluor® 647 AffiniPure F(ab')2 Fragment Goat Anti-Human IgG, H+L antibody (Jackson ImmunoResearch). For the surface marker analysis on NK-92 and were used the following antibodies: 1 µl of anti-CD56 (PE/APCvio770 Mouse Anti-Human, Clone MY31) anti-CD3 (VioBlue mouse anti-human Clone BW264/56), anti-NKG2D (PE/APC Mouse Anti-Human Clone 1D11),

anti-DNAM1 (APC mouse anti-human clone DX11), anti-NKp46 (Viobright515 mouse anti-human clone 9E2), anti-NKp30 (PEvio615 anti-human clone REA823), anti-NKp44 (PEvio770 anti-human clone REA1163), anti-CD8 (APC Mouse Anti-Human, Clone RPA-T8), anti-CD4 Ab (APCvio770 mouse anti-human clone M-T466). Abs all from Miltenyi. After 30 min of incubation on ice all the cells were washed with cold PBS, co-stained with DAPI (1µl of 1 µg/ml working solution, Sigma-Aldrich) for 5 min at 4 °C and analyzed by the flow cytometry (Cyan ADP, Beckman Coulter s.r.l.) using Summit 4.3 software (Dako). The fluorescence signal derived from the Isotype control or without Ab was set as threshold ($0<MFI>10^1$).

4.9. CEA CAR induction experiments

The CEA CAR induction in NK-92 engineered cells were assessed *in vitro* against HER2/MSLN amp/high and HER2/MSLN normal CEA positive/negative cancer cell lines; by flow cytometry analysis. The CEA CAR induction was determined in co-cultures of target cells (30.000 cells/well in a 24 well plate) with expanded engineered NK-92 cells or WT NK-92 at 1:3 effectors/target ratio for 24h for CRC0080/CRC0186 or SW1116 / 48h for LS180/SKBR3/SNU254/OVCA3, in culture medium with at 37 °C, 5% CO2.

4.10. *In vitro* cytotoxicity assays

The tumor-killing abilities of NK-92 engineered cells and WT NK-92 cell were assessed *in vitro* against HER2 amplified CEA negative, HER2 amplified CEA positive, HER2 positive CEA positive cancer cell lines; all by bioluminescent cell viability essay. In all the case, the immune-mediated killing was determined in co-cultures of target cells (10000 cells/well in a 96 well plate) with expanded engineered NK-92 cells or WT NK-92 at various effectors/target ratios (1:1, 1:5, 1:10, 1:20 and 1:50) for 48 hours for short term assays and 96h for long term assays, in culture medium with at 37 °C, 5% CO2, evaluating cell viability by Cell Titer-Glo Luminescent Cell Viability Assay (Promega) and Luciferase Assays (D-Luciferin Firefly potassium salt, PerkinElmer Part Number #122799). The chemiluminescence was detected with Spark 10M (Tecan).
The same procedure was executed for the MSLN synNotch/CEA CAR strategy.

4.11. Cytokine secretion assays

Engineered NK-92 cell and WT NK-92 cell were incubated with HER2 and CEA targets cell line (24 well plate, E/T 1:3) for 48 hrs in NK-92 cell-culture medium as described in the cytotoxic *in vitro* assay. Thereafter, supernatants from the co-cultures and from wells with NK-92 cell were collected. IFN-γ, Granzyme B and lL-2 production were analyzed with Human IFN-gamma Quantikine ELISA Kit (Catalog #: DIF50, R&Dsystem), Human Granzyme B ELISA Kit (Catalog # BMS2027-2, Thermo Fisher) Human IL-2 Quantikine ELISA Kit (Catalog # D2050, R&D system).

4.12. Degranulation Assay

Degranulation of 5F clone NK-92 and parental NK-92 cells was induced upon interaction with target cells at 1:1 ratio for 24 h at 37 °C and was assessed by measuring the surface expression of CD107a with an anti-CD107a detection antibody (PE anti-human clone REA792, Miltenyi Biotec). Effector cells without targets were used for negative control respectively.

4.13. Invasion assays

CRC0080 or CRC0186 derived organoids were plated in Matrigel Matrix (Product Number: 356234, Corning) domes in 8-well glass-bottom chamber slides (Falcon). After 48-72 hours organoids were overnight labeled with NucBlue (NucBlue™ Live Ready Probes™ Reagent) directly in the culture chamber slide wells. Then CRC0080 or CRC0186-derived organoids were co-cultured with NK-92 Clone 5F or paired NK-92 WT/HER2 synNotch cells stained with PKH26 dye at an E:T ratio of 2:1 in culture medium in the presence of IL-2 (100 U/mL). After 48 hrs of co-culture, NK-92 cells were removed, and organoids were fixed with PAF solution 4% for 10 minutes and covered with mounting medium, to be observed using a TCS SPE Leica microscope. Image acquisition was performed by maintaining the same laser power, gain, offset, and magnification (20x). Maximum intensity projections for each analyzed organoid were generated with LAS X Software (Leica) to quantify NK-92 cell recruitment and infiltration. Images of the total PKH26 red fluorescence area present either at the boundary or inside the organoids were analyzed using ImageJ software.

The other invasion experiment was performed by Transwell assay (Product Number: 3422, Corning). At the bottom of the culture wells target cells were plated at 70%

confluency in 700 ul. The migration chambers were coated with 50ul of Matrigel Matrix (Product number: 356234, Corning) to cover the 8um pore membrane, then the NK-92 engineered or not stained with Pkh26 dye were plated on it (SigmaAldrich) in 500 ul. After 48h the cells present in the bottom well were analyzed, counting the Pkh26 positive NK-92 cells that cross the Matrigel and the pore membrane. The fold increase was calculated with respect to the wells with only the NK-92 cells on the migration chamber (NK-92 WT / HER2 synNotch / 5F clone) and no target cells on the bottom chamber.

4.14. *In vivo* experiments

The antitumor activity and the CEA CAR inducibility of NK-92 clone 5F/ NK-92 clone 5F GAL4 IL-2 and controls NK-92 WT, NK-92 HER2 synNotch was evaluated using CRC xenograft models in immunodeficient mice. *In vivo* experiments received approval by the competent committee and internal review board (auth. N° 225/2021-PR). CRC xenografts were established in 6- to 7-week-old NOD/SCID (Charles River Laboratories, SRL) male mice by subcutaneous injection with $1x10^6$ cells or PDX implantation obtained from two CRC [CRC0080 PDX, CRC0186 PDO/cell line]. For the CEA CAR induction experiments the mice were injected with $5x10^6$ NK-92 CLONE 5F pre-marked with PKH26 dye (SigmaAldrich) directly into the tumor mass (volume around 200 mm^3) and were sacrificed after three days from injection, the tumors were explanted and analyzed by IF. In the efficacy experiments when tumors were approximatively 20 mm^3 in volume (after one/two week from inoculation/implantation), mice were infused TV twice a week with $5x10^6$ irradiated NK-92 clone 5F or controls NK-92 WT, NK-92 HER2 synNotch and not irradiated NK-92 clone 5F and NK-92 clone 5F GAL4 IL-2 resuspended in PBS (150 ul), for a total of six infusions. Mice injected with PBS only were used as controls. Treatment and control cohorts included 6/8 mice each for the all the models. In both the efficacy experiments with mice were sacrificed at the endpoint (tumor volume > 1000 mm^3). Mice were monitored daily for possible toxicities, while tumor growth was measured two times per week with manual caliper. Tumor volume was calculated by the following formula: V ¼ 4/3 x p x (a/2)2 x (b/2), where a is the length and b is the width of the tumor.

4.15. Immunofluorescence analysis

CRC0080 xenografts explant were analyzed by IF. Samples (5-mm thick) were cut from OCT-fixed, mounted on slides, and treated as per standard IF procedures. Fixing the slice with Zinc solution for 10 min, permeabilization with PBS-Triton 0,1% for 10 min, saturation with 1% PBS-BSA RT 60 min, incubation with primary antibody in humid chamber overnight 4°C (Alexa Fluor® 647 AffiniPure F(ab')2 Fragment Goat Anti-Human IgG, H+L antibody, Jackson Immunoresearch), DAPI (D9542, Sigma) staining 5 min RT, tissue sections were mounted on glass slides with mounting solution Mowiol and visualized with a TCS SPE Leica microscope (10x) and analyzed with LAS X Software (Leica).

4.16. Statistical analysis

Average, standard deviation (SD) and standard error of the mean (SEM) were calculated using Microsoft Office Excel 2010 software (Microsoft Corporation, Redmond, Washington) and GraphPad Prism 9. Statistical significance was determined using a two-tailed Student's t test, One way ANOVA test, Two-way ANOVA test. All experiments were repeated at least two times. Figures show one representative experiment.

5. Results

5.1. Identification of CEA as an optimal target for combinatorial CAR approaches against HER2-amplified CRC.

To identify an optimal second antigen to be combined with HER2 in the synNotch/CAR system, a set of antigens already validated as CAR targets in CRC was considered: carcinoembryonic antigen (CEA), encoded by the CEACAM5 gene[143]; epithelial cell adhesion molecule (EPCAM), encoded by the EPCAM gene[144], prominin 1 (CD133), encoded by the PROM1 gene[145], mucin 1 (MUC1), encoded by the MUC1 gene[146] and epidermal growth factor receptor (EGFR), encoded by the EGFR gene (NCT03542799). Initially, mRNA expression of the candidates was evaluated in normal tissues on Entrez Gene database[138]. The analysis showed that only CEA was specifically expressed at high levels in the colon, with very low levels in esophagus and stomach. Indeed, CEA expression cannot be detected in most normal adult tissues, except in the gastrointestinal tract at a low level restricted to the apical surface of the epithelial cell membranes facing the lumen, which is invisible to immune cells[147]. Instead, all the other antigens, despite being used as CAR targets given their high levels in the CRC, were not colon-specific but could be found also at high levels in many other normal tissues, including duodenum, esophagus, kidney, lung, skin, small intestine, and stomach, that also express HER2 at significant levels (Fig. 1). The expression of CAR targets in normal tissues raises the risk of “on-target off-tumor” toxicity. CEA, instead, carrying a “safe” profile could be the optimal candidate to associate with HER2 in a combinatorial immunotherapy strategy.

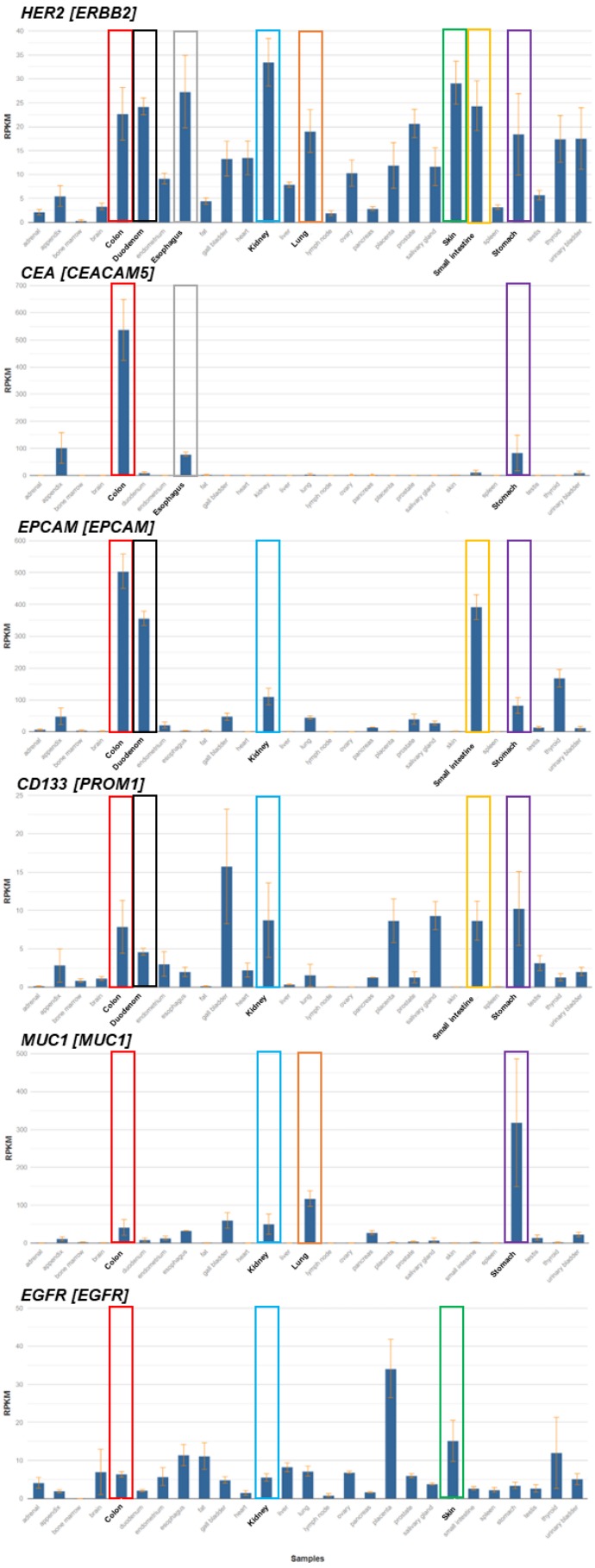

Figure 1. HER2, CEA, EPCAM, CD133, MUC1 and EGFR mRNA expression in normal tissue from Entrez Gene database. The six-bar graphs represent the mRNA expression of HER2, CEA, EPCAM, CD133, MUC1 and EGFR respectively. The colored boxes highlight organs in which the already validated CRC CAR targets are highly express and that also express HER2 at significant level (RPKM = Reads Per Kilobase of transcript per Million reads mapped).

To evaluate more in detail HER2 and CEA expression in normal tissue, their mRNA and protein expressions were also assessed on the ProteinAtlas database[148,149]. The analysis confirmed that HER2 expression was physiologically high in almost all the tissues and that CEA expression is physiologically high only in the colon, with low levels in the proximal digestive tract, in the bone marrow and lymphoid tissues (Fig. 2). The expression of HER2 and CEA could be high in normal tissues but not to the level of HER2 amplification and CEA overexpression in cancers. These data confirmed that a CAR-based ACT against a HER2 could be very dangerous paving the way to "on-target off-tumor" toxicities on organs far from the tumor site[66]. Instead, the combination of HER2 amplification and CEA could be a suitable option in the contest of combinatorial immunotherapy strategies in CRC, provided that the HER2 synNotch is activated only in the presence of extremely high levels of HER2, typical of HER2amp CRC cells.

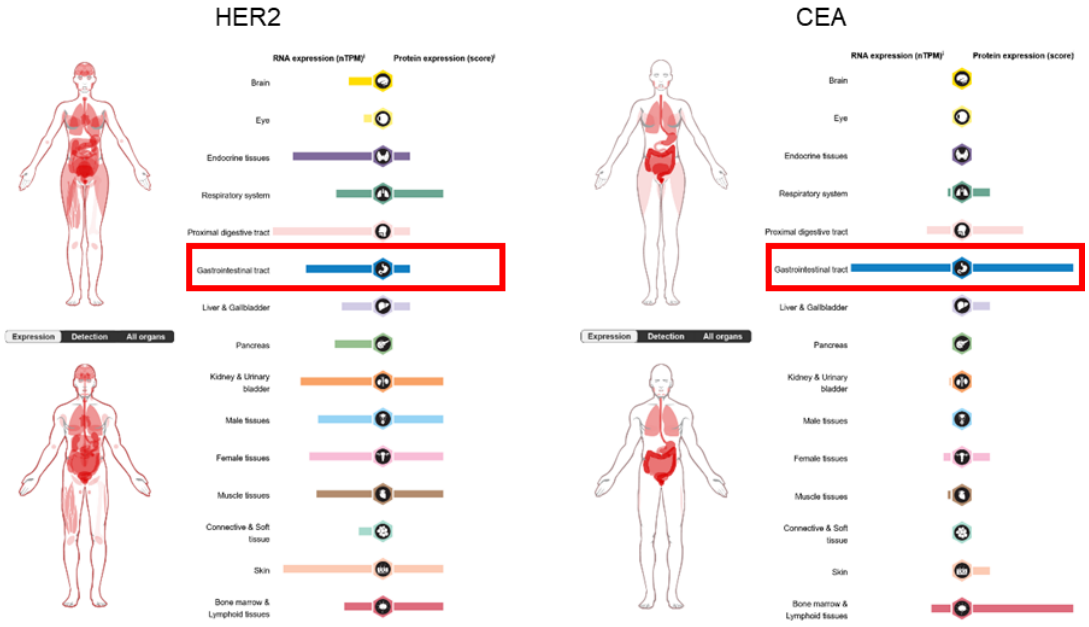

Figure 2. HER2 and CEA mRNA and protein expression in normal tissue from ProteinAtlas database. The two images represent the mRNA and protein expression of HER2 and CEA respectively in normal tissues; the red rectangles highlight the gastrointestinal tract mRNA and protein expression of HER2 and CEA.

Although CEA was found in the normal colon and at low level in other normal tissues, it is consistently overexpressed in many cancers[150]. To investigate the presence of high levels of CEA in HER2amp CRC, and to identify suitable patient-derived models for preclinical testing of a possible HER2-synNotch CEA-CAR based therapy of CRC, mRNA expression of HER2 and CEA was evaluated in 450 CRC samples from TCGA[151], 119 CRC cell lines (Manuscript in preparation), and 602 CRC PDXs[139]. As shown in Figure 3, HER2 was found to be overexpressed in 1-3% of the cases. Notably, all HER2-overexpressing samples also express very high levels of CEA.

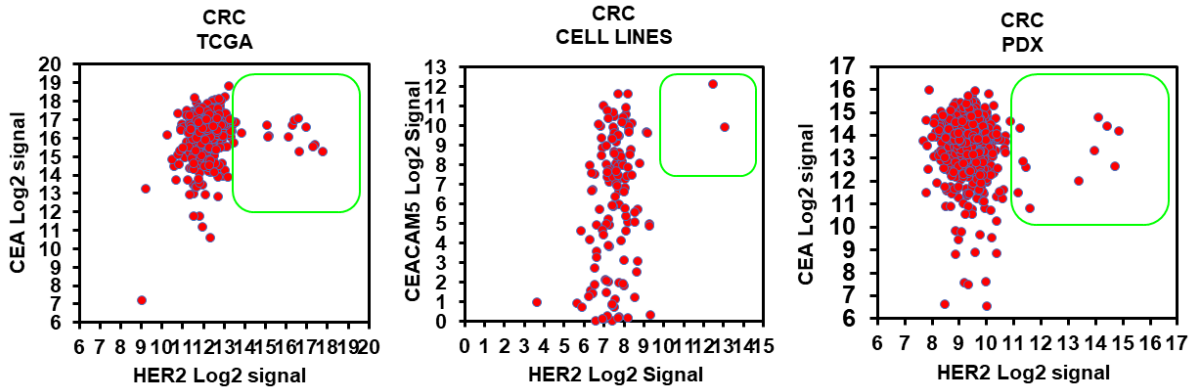

Figure 3. HER2 and CEA mRNA expression in TCGA samples, CRC cell lines and CRC PDX. In the three dot-plot CEA mRNA expression is show in y-axis and HER2 mRNA expression is show in x-axis. Green box highlighted HER2 amplified CEA highly expressing samples.

HER2 and CEA surface protein expression was further confirmed in selected cell lines by flow cytometry. In particular, the HER2-amplified breast cancer cell line SKBR3 was selected as positive control for HER2 overexpression[152] and negative control for CEA expression. Three CRC cell lines were also selected, all positive for CEA expression: LS180 with normal HER2 levels, and the two PDX-derived cell lines CRC0080 and CRC0186, both carrying HER2 amplification and overexpression[142] (Fig. 4).

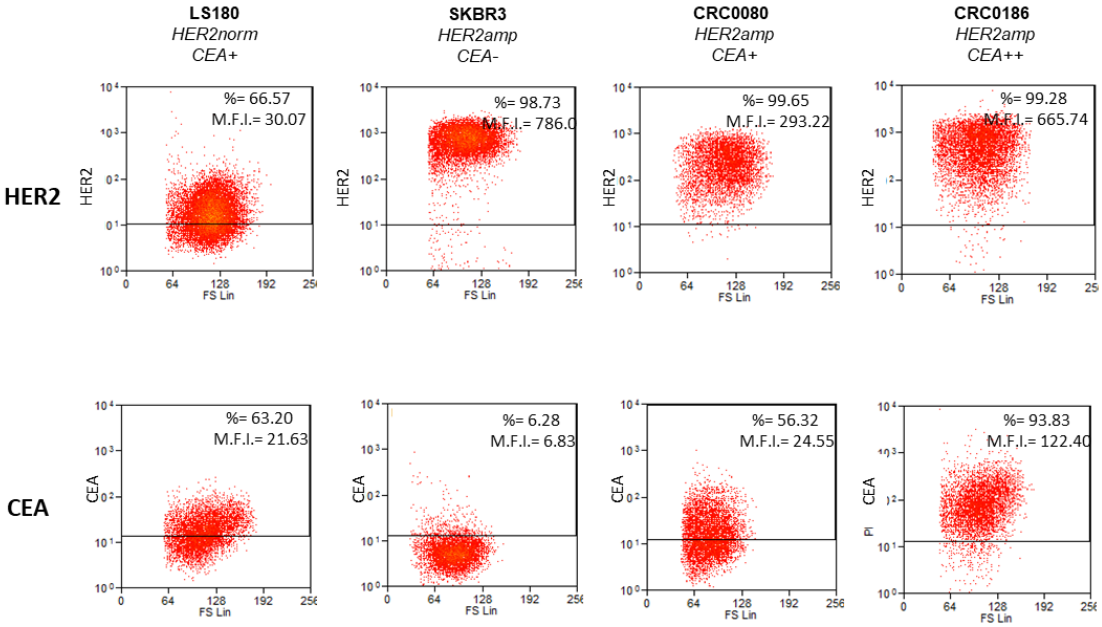

Figure 4. Flow cytometry analysis of HER2 and CEA surface protein expression in selected cell lines. Representative flow cytometry dot-plots showing on the y-axis HER2 (top row) and CEA (bottom row) surface expression in different cell lines used as models; y-axis= log10 expression, x-axis= Forward Scatter. %= percent of cell above the depicted positivity threshold; M.F.I. = Mean Fluorescent Intensity (of all displayed cells).

To generate additional, tightly controlled models of HER2 overexpression vs normal expression in the same cell line, the DIFI and H508 cells, expressing normal HER2 levels, were transduced with a lentiviral vector carrying a constitutive HER2 expression cassette. In this way, for each cell line, two versions with different levels of HER2 (normal vs overexpressed) were obtained[141] (Fig. 5).

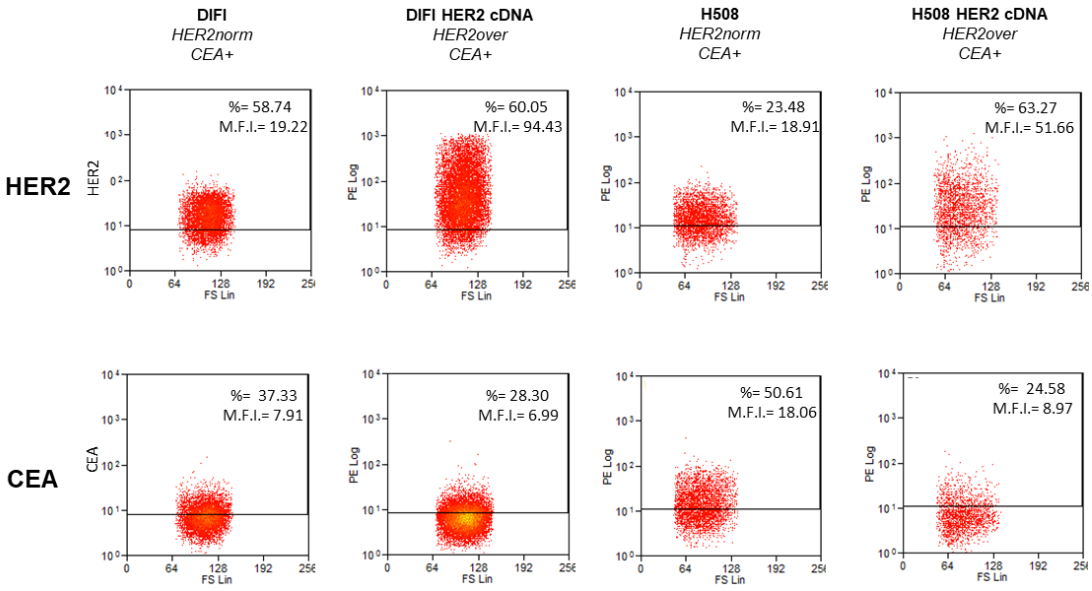

Figure 5. Flow cytometry analysis of HER2 and CEA surface protein expression in engineered cell lines. Representative flow cytometry dot-plots showing on the y-axis HER2 (top row) and CEA (bottom row) surface expression in different cell lines used as models; y-axis= log10 expression, x-axis= Forward Scatter. %= percent of cell above the depicted positivity threshold; M.F.I. = Mean Fluorescent Intensity (of all displayed cells).

In light of the expression data of HER2 and CEA in normal tissues and in CRC, the synNotch-CAR system was built using HER2 as antigen “A” for the synNotch, and CEA as antigen “B” for the CAR.

5.2. Initial set-up of the SynNotch system in Jurkat cells

Initially the synNotch system was assembled in Jurkat cells, that in part recapitulates the immune cell behavior, before moving to the final effector cells: the NK-92.

The CEA-CAR was cloned in a lentiviral vector downstream the inducible promoter GAL4-UAS-minCMV, this sequence is recognized by the transcription factor GAL4VP64. To easily test the inducibility of CEA-CAR under this inducible promoter, another vector was generated that constitutively expresses the transcription factor GAL4VP64 under the control of an EFS promoter (Fig. 6).

Jurkat cells were simultaneously transduced with GAL4-CEA-CAR vectors plus -or not- EFS-GAL4VP64. Then, CEA-CAR expression was evaluated by FACS analysis. Around 70% of CEA CAR positive cells were observed, but with 30% of CEA CAR basal expression in cells transduced only with the GAL4 CEA CAR vector, suggesting a partial basal activity of the promoter 5x GAL4 UAS mCMV (Fig. 6).

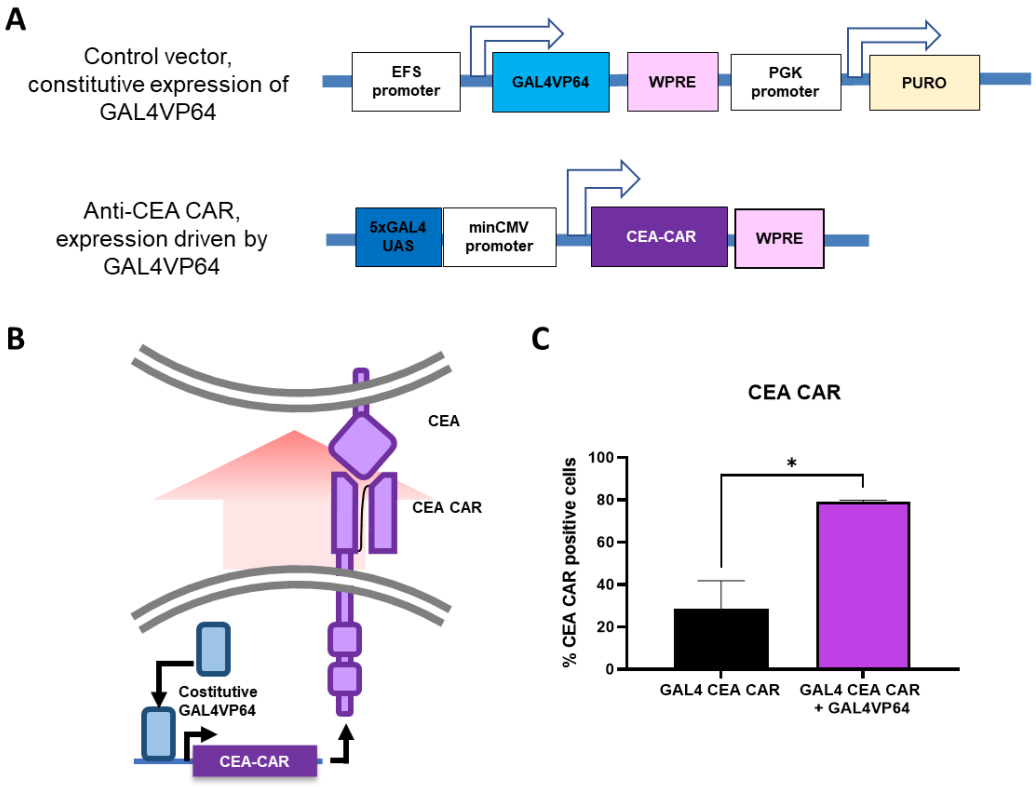

Figure 6. CEA CAR induction by the constitutive transcription factor GAL4VP64. A. Schematic representation of the GAL4 CEA CAR and GAL4VP64 lentiviral vectors (EFS= elongation factor 1α short, PGK= Human phosphoglycerate kinase promoter, GAL4VP64 = transcription factor, WPRE= Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element, minCMV= minimal cytomegalovirus promoter, PURO = Puromycin resistance element). **B**. In this system the cells were transduced with the transcription factor under a constitutive promoter and so could induce expression of the CEA CAR. Grey lines represent the plasma membrane. **C**. The bar graph shows the percentage of CEA CAR-positive Jurkat cells, in the absence or presence of constitutively expressed GAL4VP64 (data obtained by flow cytometry analysis; bars: Standard Deviations). Statistical significance was calculated by T-test. Stars indicate P values: * = pvalue < 0.05.

Subsequently, CAR induction by synNotch was tested, initially using a synNotch (LAG16-SynNotch), recognizing the artificial antigen green fluorescent protein (GFP). As target cells, the HCT116 CRC cell line was used, either wild-type (WT, negative control) or transduced with a lentiviral vector encoding an outer membrane-exposed Green Fluorescent Protein, named surface GFP (sGFP). Jurkat cells already transduced with the GAL4 CEA CAR were further transduced with the LAG16-SynNotch vector and subsequently co-cultured with target HCT116 cells, either WT or

overexpressing sGFP. Indeed, significantly higher levels of CEA CAR were observed by flow cytometry after co-culture with HCT116 sGFP respect to controls (Fig. 7). However, some CEA CAR expression (~35% positive cells) was also observed negative control conditions, indicating a certain degree of basal activity of either the synNotch construct or of the GAL4-driven promoter.

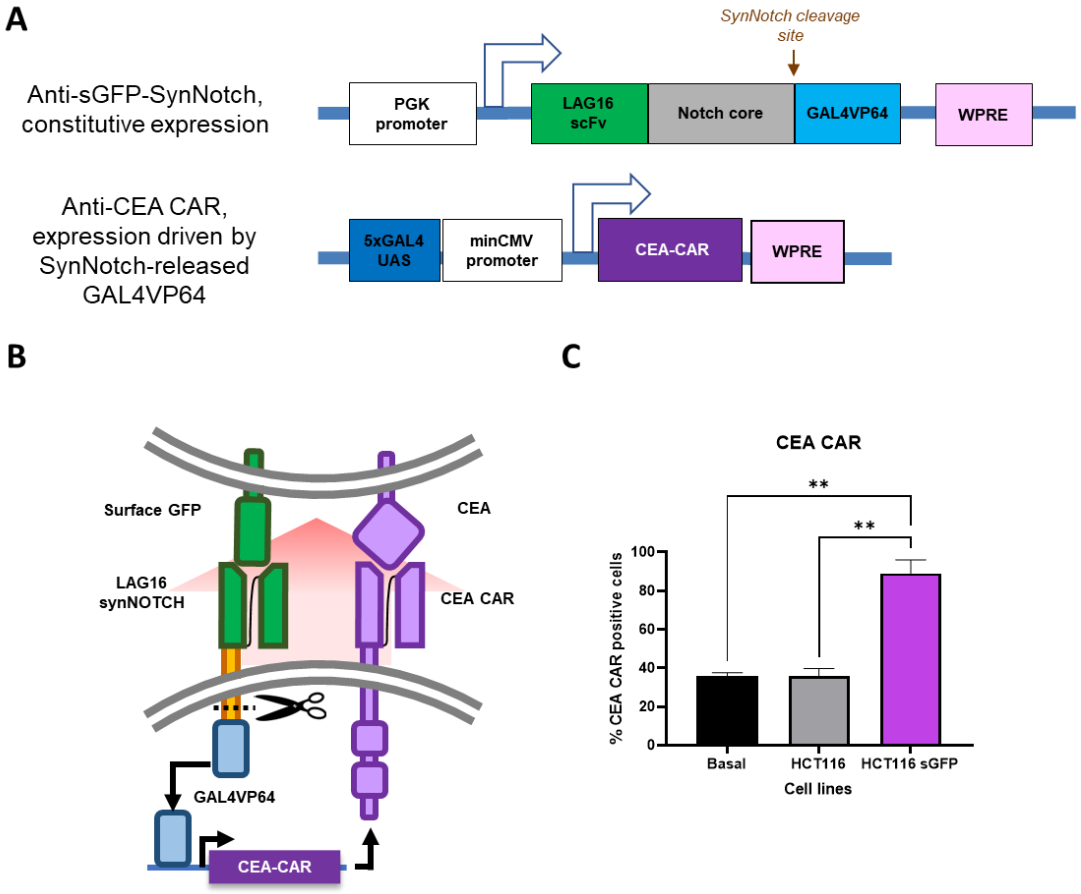

Figure 7. CEA CAR induction by the LAG16 synNotch after coculture with target cells. A. Schematic representation of the Lag16 synNotch and GAL4 CEA CAR lentiviral vectors (PGK= Human phosphoglycerate kinase promoter, GAL4VP64 = transcription factor, WPRE = Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element, minCMV= minimal cytomegalovirus promoter). **B.** The Notch receptor is extracellularly modified to recognize a different antigen with respect to the wild type and intracellularly modified by swapping the intracellular transcription domain and providing specific downstream effector target genes. In this system a synthetic Notch receptor that, upon binding to a first antigen "A" (surfaceGFP) on the surface of target cells, is cleaved in the intracellular portion so that a transcription factor domain (GAL4VP64) reaches the nucleus and induces the expression of a CAR against the second antigen "B" (CEA). Grey lines represent the plasma membrane. **C.** The bar graph shows the percentage of CEA CAR positive Jurkat cells induced by the LAG16 (anti-sGFP) synNotch after the co-culture with HCT116, data obtained by flow cytometry analysis (Bars: Standard Deviations. Statistical significance was calculated by One-way Anova. Stars indicated P values: ** = pvalue < 0.01).

After the positive preliminary results obtained with the constitutive GAL4VP64 and the LAG16 synNotch, the vector combination for a HER2 synNotch/CEA CAR system was assembled for further experiments (Fig. 8A-B).

The HER2-synNotch selected for the experiments was the 4D5-5 HER2 synNotch, incorporating extracellularly the 4D5-5 single chain Fragment variable (scFv) derived from the trastuzumab antibody, and intracellularly the GAL4VP64 transactivator[81]. Indeed, the 4D5-5 scFv has lower affinity for HER2 respect to trastuzumab, because of an inserted mutation in the original trastuzumab variable fragment sequence[153]. Thus, synNotch engagement and cleavage was expected to occur only after contact with HER2-overexpressing target cells.

Jurkat cells were double-transduced with the HER2-synNotch and the GAL4 CEA CAR, and co-cultured with different target cells: SKBR3 (breast cancer, HER2 amplified and overexpressed, CEA negative), CRC LS180 (HER2 normal, CEA positive) and CRC0080 (HER2 amplified and overexpressed, CEA positive). CEA CAR expression, assessed by flow cytometry, was basally low (<10% positive cells) after co-culture LS180 cells, and significantly higher (20-35% positive cells) in the presence of HER2-overexpressing cells (Fig. 8C). These results indicated good specificity of the system, but limited CAR induction by HER2-overexpressing target cells.

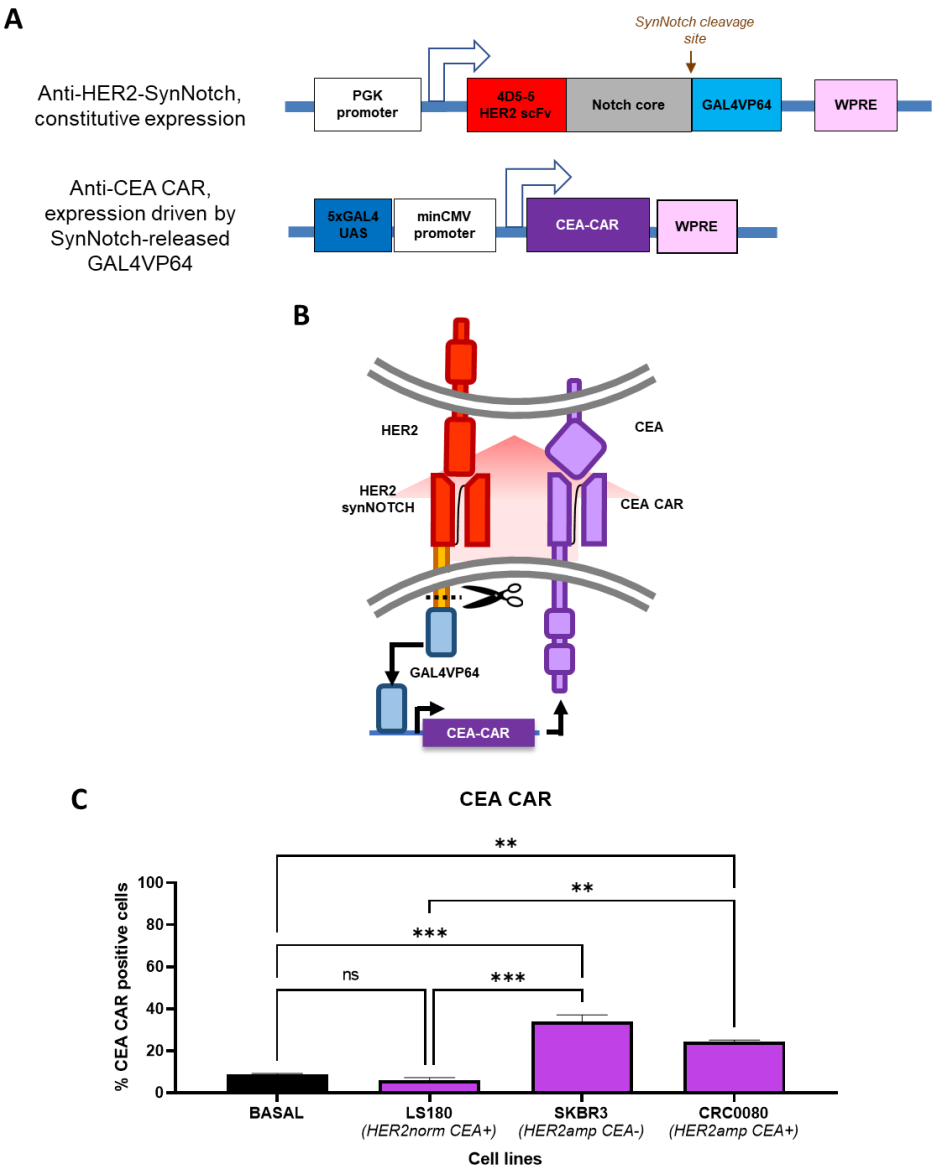

Figure 8. CEA CAR induction by the HER2 synNotch after the binding with target cells. A. Schematic representation of the HER2 synNotch and GAL4 CEA CAR lentiviral vectors (PGK= Human phosphoglycerate kinase promoter, GAL4VP64 = transcription factor, WPRE = Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element, minCMV= minimal cytomegalovirus promoter) **B**. The Notch receptor is extracellularly modified to recognize a different antigen respect to the wild type and intracellularly modified by swapping the intracellular transcription domain and providing specific downstream effector target genes. In this system a synthetic Notch receptor that, upon binding to a first antigen "A" (HER2) on the surface of target cells, is cleaved in the intracellular portion so that a transcription factor domain (GAL4VP64) reaches the nucleus and induces the expression of a CAR against the second antigen "B" (CEA). **C**. The bar graph shows the percentage of CEA CAR positive Jurkat cell induced by the HER2 synNotch after the co-culture with HER2 normal expressing or HER2 amplified cell, data obtained by flow cytometry analysis (Bars: Standard Deviations. Statistical significance was calculated by One-way Anova. Stars indicated P values: * = pvalue < 0.05, ** = pvalue < 0.01, *** = pvalue < 0.001).

To evaluate possible functional consequences of the observed CEA CAR induction, the level of CD69 was assessed by flow cytometry. CD69 is a marker of lymphocyte activation and was measured on HER2 synNotch/CEA CAR Jurkat cells, before and after co-culture with CRC0080 cells. These target cells express high levels of both HER2 and CEA, and were expected to induce CEA CAR expression, and also CAR activation. Indeed, around 35% of Jurkat cells became positive to CD69, highlighting a specific activation mediated by engagement of the CEA CAR with the CEA antigen (Fig. 9).

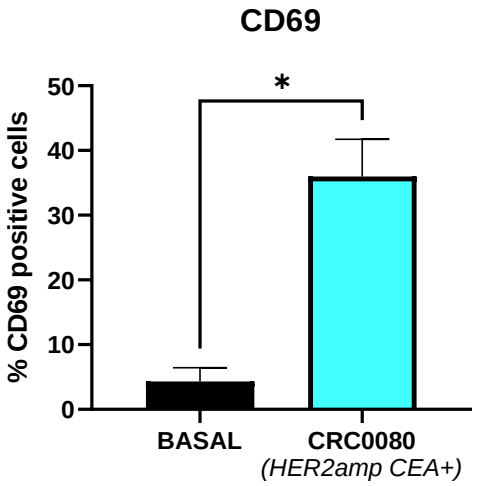

Figure 9. CD69 induction by the HER2 synNotch/ CEA CAR Jurkat cells after the binding with CRC0080. The bar graph shows the percentage of CD69 positive cells induced by the CEA CAR binding with target cells, data obtained by flow cytometry analysis (Bars: Standard Deviations. Statistical significance was calculated by T-test Stars indicated P values: * = pvalue < 0.05).

5.3. Generation, cloning and characterization of HER2 synNotch/ CEA CAR expressing NK-92 cells.

After the successful preliminary tests in Jurkat cells, the HER2 synNotch/CEA CAR system was moved to a clinically validated immune cell effector, the NK-92 natural killer cell line[154]. NK-92 cells were first, transduced with the HER2 synNotch. To overcome the limited efficiency of NK-92 transduction with lentiviral vectors, HER2 synNotch-positive cells were sorted twice, finally obtaining 90% of positive cell (Fig. 10A).

The sorted population was then transduced with the GAL4-driven CEA CAR vector. The transduced population was subjected to one sorting for negative CEA-CAR expression after co-culture with HER2-normal cells, to eliminate cells that express the CEA CAR also in the absence of HER2-overexpressing target cells (Fig. 10B). Subsequently, cells were further sorted for positive expression of CEA CAR after co-culture with HER2-overexpressing cells. The “double-sorted” population displayed

low/negative basal expression of CEA CAR, also when exposed to target cells expressing HER2 at normal levels, and significant CEA CAR expression after contact with positive control cells carrying HER2 amplification and overexpression (Fig. 10C).

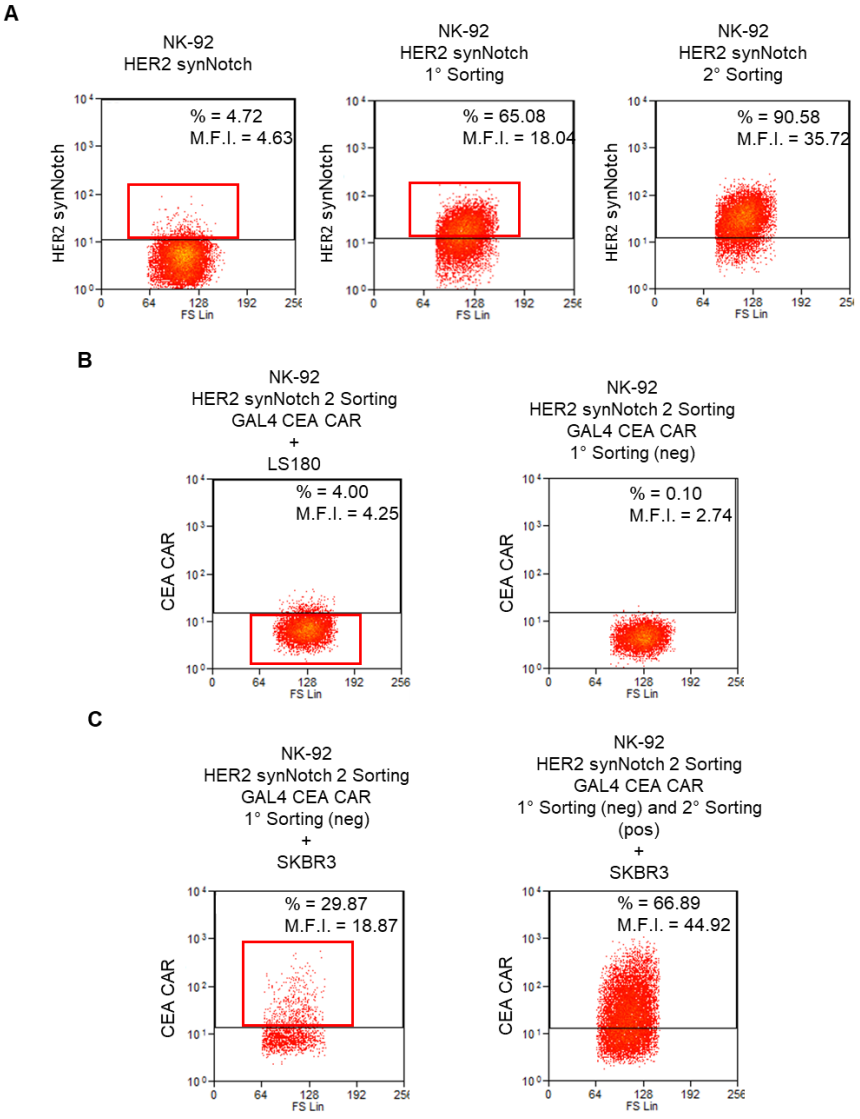

Figure 10. NK-92 HER2 synNotch and CEA CAR sorting procedures. A. Representative flow-cytometry analysis of HER2 syn Notch expression in NK-92 before and one HER2 synNotch positive sorting. **B**. Representative flow-cytometry analysis of CEA CAR basal expression in NK-92 HER2 synNotch 2 sorting GAL4 CEA CAR after co-culture with a HER2 normal expressing cells, before and after a CEA CAR negative sorting procedure. **C**. Representative flow-cytometry analysis of CEA CAR induction in NK-92 HER2 synNotch 2 sorting GAL4 CEA CAR 1 sorting (neg) after co-culture with HER2 amplified cells, before and after the CEA CAR positive sorting procedure. Red boxes represent the selected populations. y-axis= log10 expression, x-axis= Forward Scatter. %= percent of cell above the depicted positivity threshold; M.F.I. = Mean Fluorescent Intensity (of all displayed cells).

CEA CAR induction was reassessed in detail after expansion of the sorted population (Fig. 11). The very low/negative basal CEA CAR expression (0-10%) after co-culture with HER2-normal control cells was confirmed. A massive CEA CAR induction was observed after co-culture with the HER2-amplified, CEA-negative SKBR3 breast cancer cell line. The CEA CAR was also substantially induced upon incubation with the HER2-amplified CRC0080 and CRC0186 colorectal cancer cell lines, obtaining 25-30% of CEA CAR-positive NK-92 cells. The lower induction in CRC models could be due to either lower HER2 expression or to target cell killing that reduces the contact time with surface HER2.

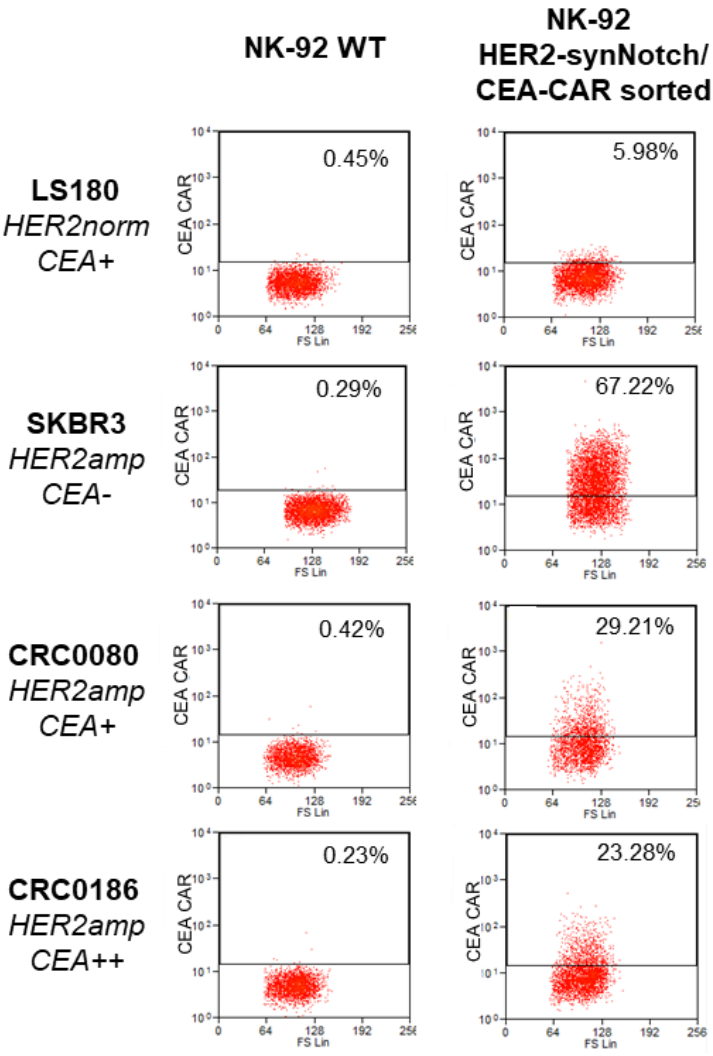

Figure 11. CEA CAR induction in NK-92 HER2 synNotch GAL4 CEA CAR sorted population after co-culture with different target cells. Representative flow-cytometry analysis of CEA CAR induction in NK-92 HER2 synNotch GAL4 CEA CAR after co-culture with HER2 negative, normal, and amplified cells (Numbers in the square are percentage of the CEA CAR positive cells).

To standardize and further increase CAR induction, eleven clones were generated from the final HER2-synNotch/CEA-CAR NK-92 sorted population, and initially selected seven clones displaying low basal CEA CAR expression (less than 5% positive cells) (Fig. 12).

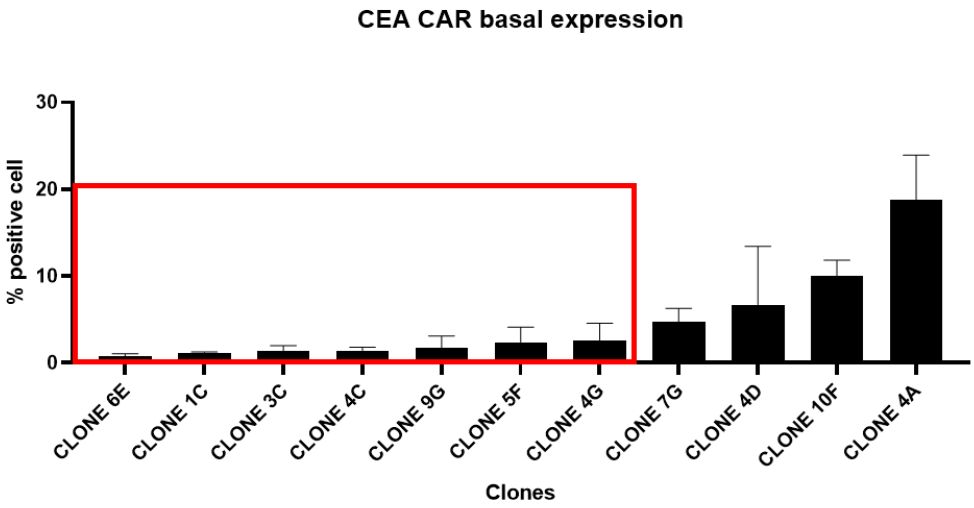

Figure 12. CEA CAR basal expression in all the clones. Flow-cytometry analysis of CEA CAR basal expression in each generated clone; the red box indicates the clones with the lowest CEA CAR basal expression, selected for the subsequent induction experiments against the HER2 amplified cell line (Bars: Standard Deviations).

These seven clones were then tested for CEA CAR induction by co-culture with SKBR3 cells, leading to identification of three clones with high CEA CAR induction (Fig. 13).

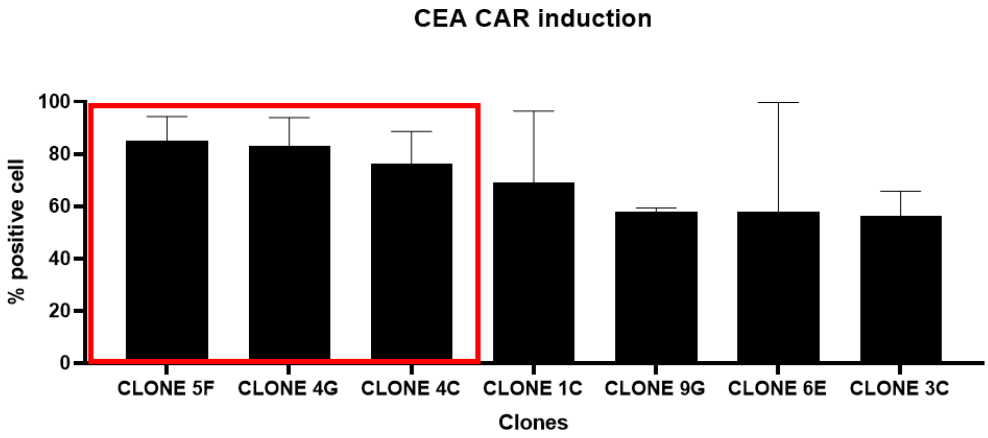

Figure 13. CEA CAR induction in clones after co-culture with SKBR3. Flow-cytometry analysis of CEA CAR induction in the selected clones after the co-culture with the SKBR3; the red box indicates the clones with the higher CEA CAR induction, selected for the subsequent induction experiment against all the target cells (Bars: Standard Deviations).

The 5F, 4C and 4G clones were subsequently expanded and further characterized, confirming low basal CAR expression (0-5%) and high CAR induction after co-culture with different target cell lines (up to 80-90% with SKBR3). Finally, the 5F clone was selected for further experiments, in view of its superior induction performances (Fig. 14).

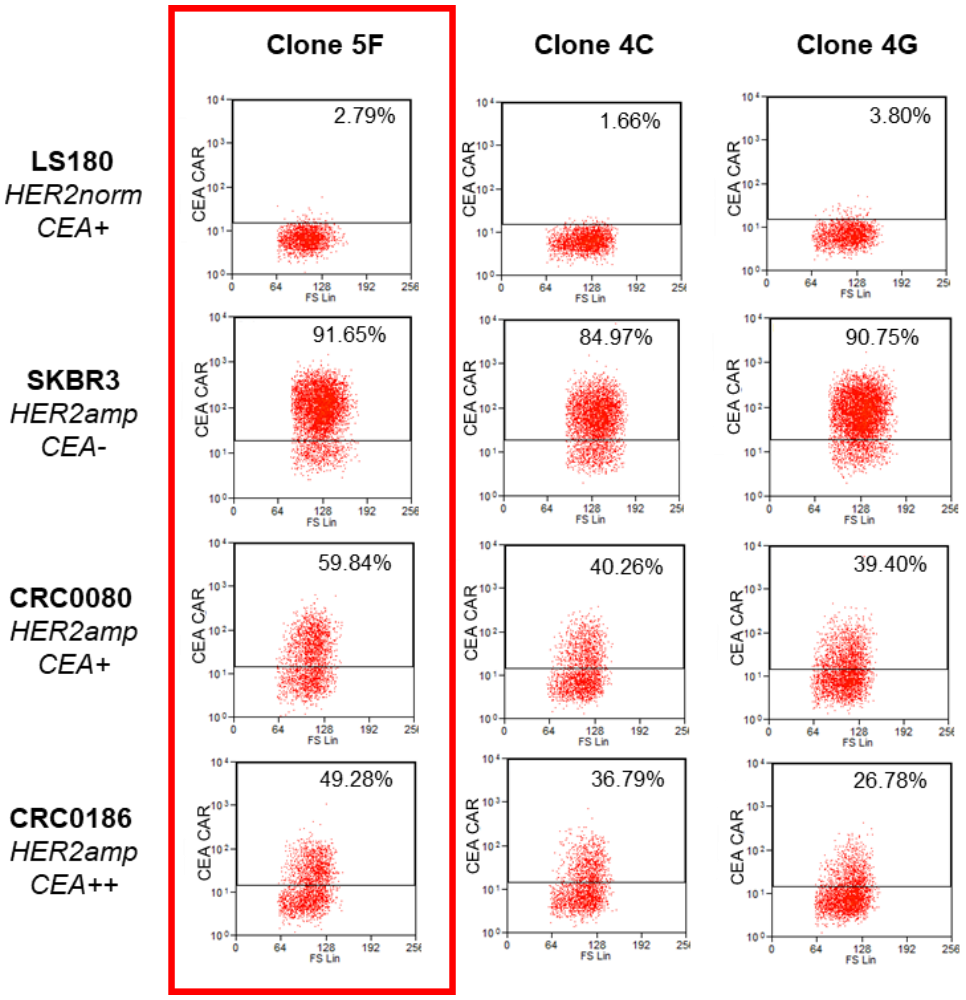

Figure 14. CEA CAR induction in the best 3 clones after co-culture with different target cells. Representative flow-cytometry analysis of CEA CAR induction after co-culture with HER2 negative, normal, and amplified cells; the red box indicates the clone with the higher CEA CAR induction, selected for the subsequent killing experiment against all the target cells (Numbers in the square are percentage of the CEA CAR positive cells).

In the following image the summary scheme of all the sorting and cloning procedures is shown (Fig. 15).

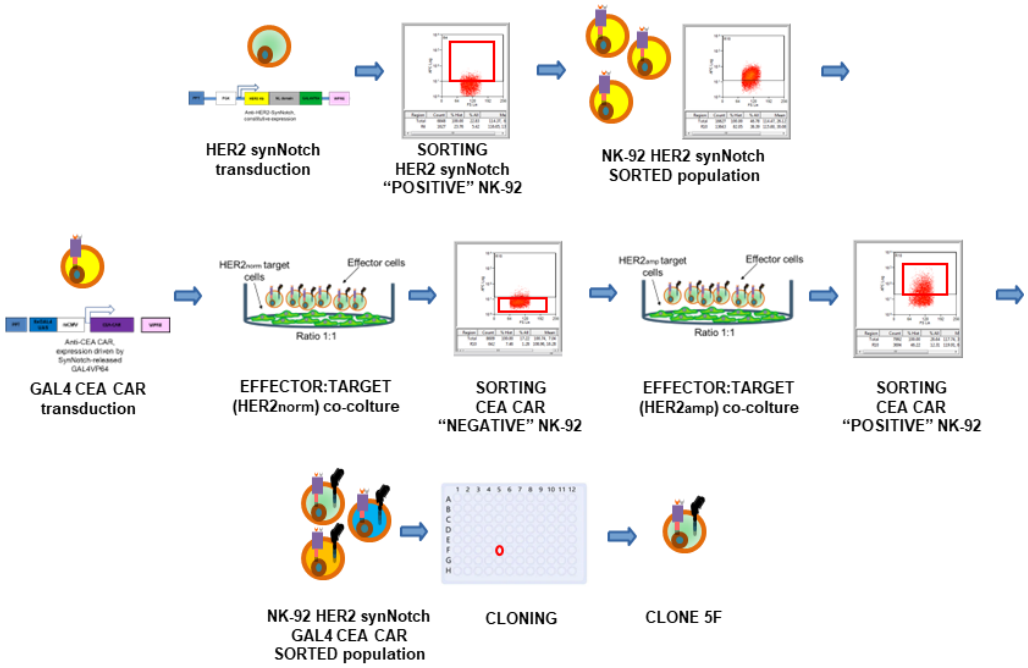

Figure 15. Representative summary scheme of the workflow follows with the NK-92. Firstly, the NK-92 were infected with the HER2 synNotch lentiviral vector, then the NK-92 HER2-synNotch that was initially sorted twice for HER2 synNotch to increase the HER2 synNotch-positive population. After that it was transduced with a vector expressing the GAL4 CEA CAR. The HER2-synNotch/CEA-CAR population was subjected to one sorting for negative CEA-CAR expression after the co-culture with HER2-normal CRC cells and to one sorting for positive CEA CAR expression after the co-culture with HER2 amplified cells. Finally, the sorted population was cloned, generating different clones.

To verify if the 5F clone maintained the NK-92 typical features, immunophenotyping was performed by flow cytometry. Indeed, the 5F clone displayed no major differences in the levels of the NK markers CD56, NKG2D, NKp30, NKp46 and NKp44, with the DNAM-1 receptor expressed by a lower fraction of cells. Moreover a 134-gene signature related to NK cells downloaded from IMMPORT[155] was evaluated. Apparently, no major differences in gene expression are observed between the 5F clone and the WT NK-92 cells. Overall, these results indicates that the 5F clone does not showed a significant genetic drift respect to the NK-92 WT and that the clone should behave similarly to parental NK-92 cells in the absence of HER2/CEA-expressing target cells (Fig 16)

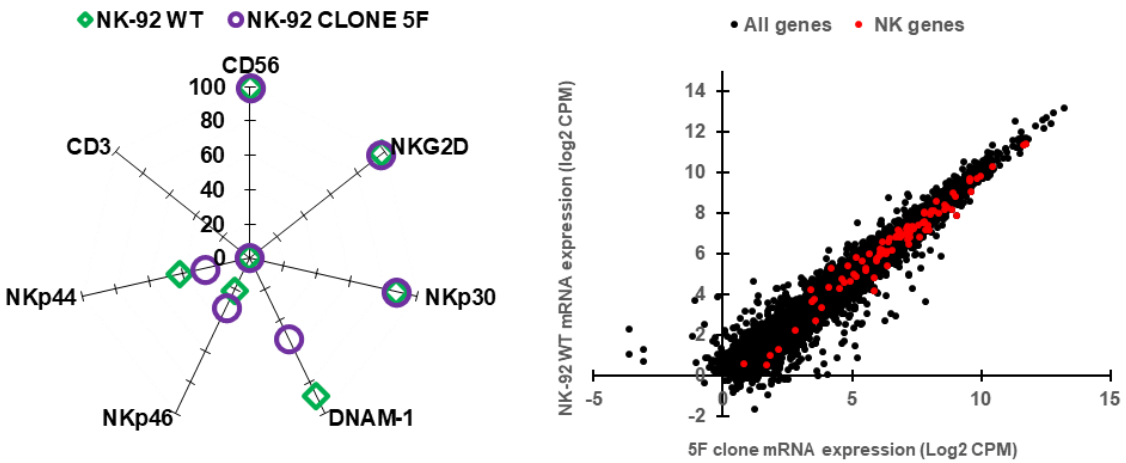

Figure 16. 5F clone vs NK-92 WT immunophenotyping. Representative flow-cytometry analysis of the major NK/T cell antigen markers on the NK-92 WT and on the 5F clone (Left panel). The dot-plot represents the correlation between NK-92 WT and the 5F clone mRNA all gene expression (black dots), the red dots represent the correlation between the 5F clone gene expression and the NK signature gene (Right panel).

To compare the kinetics and extent of CEA CAR induction and repression between the 5F clone and the sorted population, time-course co-culture experiments were performed using SKBR3 as target cells. Indeed, the 5F clone showed a better CEA CAR induction / decay profile, with higher and faster induction: at twelve hours it was already possible to detect CEA CAR induction in the 5F clone, but not in the sorted population. At the same time when SKBR3 cells were removed, after 48 hours of co-culture the CEA CAR expression was completely abrogated in the 5F clone, while in the sorted population a fraction of cells remained positive. Residual CAR expression after detachment from target cells could be very dangerous in the clinical context because the effectors could also target normal tissues; all effectors should become CEA CAR negative when detached from target cells, in order to reduce the possibility of “on-target off-tumor” toxicity (Fig. 17).

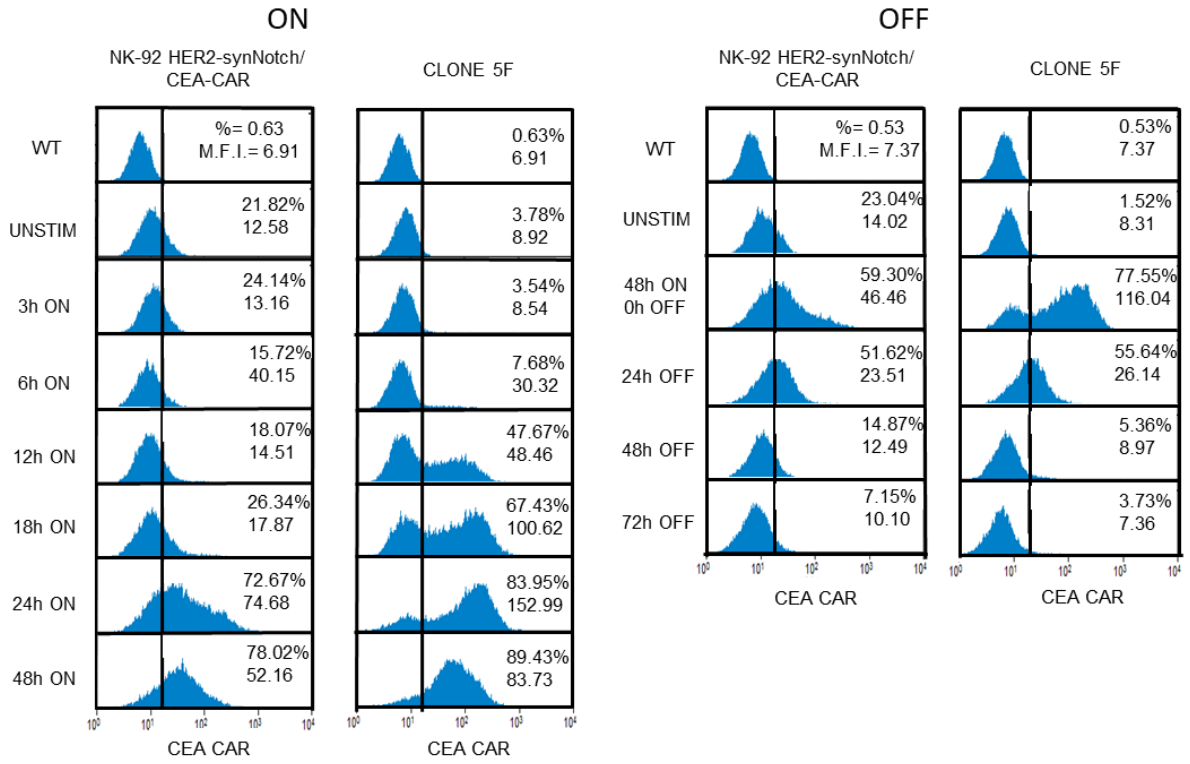

Figure 17. CEA CAR induction dynamics. Representative flow-cytometry analysis of CEA CAR induction and decay after co-culture with HER2 amplified cells at different time points between the sorted population and the 5F clone; the left part “ON” represent the induction of the CEA CAR during time, the right part “OFF” represent the decay of the CEA CAR expression after the removal of the target cells, during time (3h ON= 3hours stimulation with SKBR3, 24h OFF= 24hours from the removal of SKBR3, UNSTIM= not stimulated with SKBR3, %= percent of cell above the depicted positivity threshold; M.F.I. = Mean Fluorescent Intensity (of all displayed cells).

The 5F clone showed a higher induction respect to the sorted population, also when co-cultured with DIFI and H508 cells artificially overexpressing HER2, confirming higher specificity and sensitivity of the clone respect to the sorted population (Fig. 18).

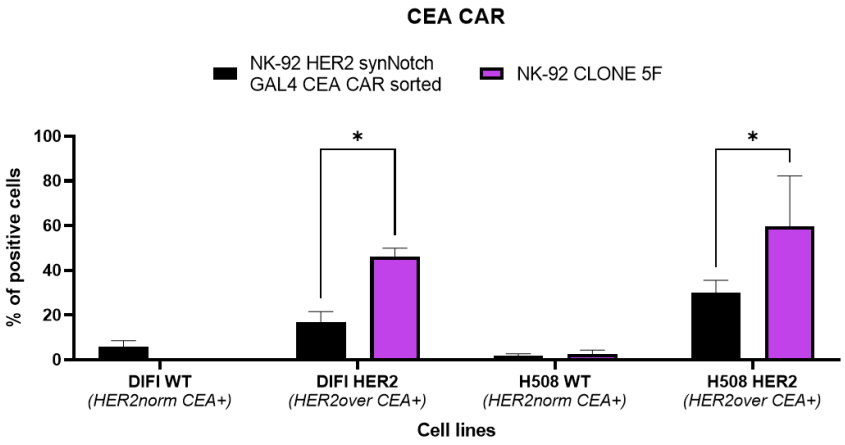

Figure 18. CEA CAR induction in 5F clones against engineered cell lines. The bar graph shows the percentage of CEA CAR positive NK-92 cells induced by the HER2 synNotch after the co-culture with HER2 normal expressing or HER2 overexpressing cells, data obtained by flow cytometry analysis (Bars: Standard Deviations. Statistical significance was calculated by Two-way Anova. Stars indicated P values: ns= no significance, * = pvalue < 0.05).

In light of possible future treatments of human patients, for which NK-92 cells have to be irradiated[137], NK-92 irradiation was optimized to achieve a complete proliferative block without compromising short-term viability (Fig. 19).

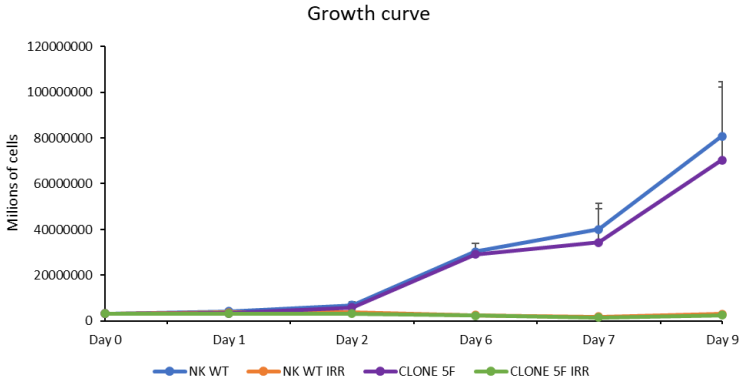

Figure 19. Irradiated NK-92 cell growth curve. Growth curve analysis (vitality analysis) effectuated by CTG of the NK-92 effector cells irradiated or not. CTG measurements were performed at days 0,1,2,6,7,9 (IRR= irradiated, Bars: Standard Deviation).

Then the CEA CAR induction was investigated in the 5F clone also after irradiation (Fig. 20). CAR induction after co-culture with HER2-amplified CRC cells was not significantly reduced. Only in the presence of SKBR3 the extent of CAR induction was slightly but significantly reduced.

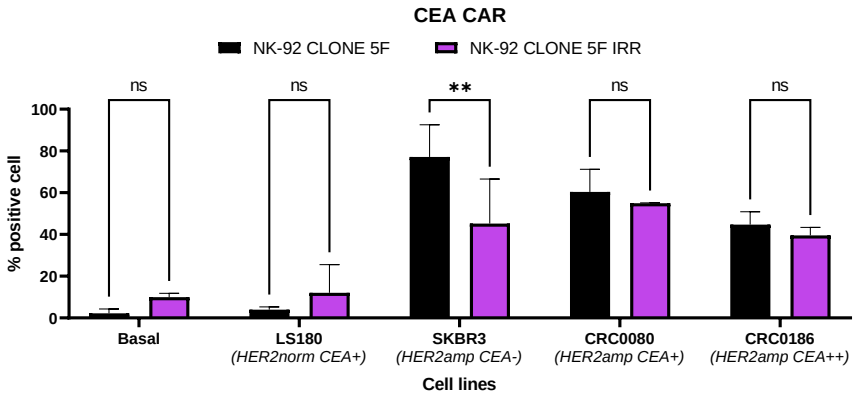

Figure 20. CEA CAR induction in 5F clones after irradiation. The bar graph shows the percentage of CEA CAR positive 5F clone irradiated or not irradiated, after the co-culture with HER2 normal expressing or HER2 amplified cells, data obtained by flow cytometry analysis (Bars: Standard Deviations. Statistical significance was calculated by Two-way Anova. Stars indicated P values: ns= no significance, ** = pvalue < 0.01).

5.4. NK-92 5F clone biologic activity *in vitro* and *in vivo.*

The killing activity of the 5F clone was tested by ATP assays and Luciferase assays. For the luciferase assays, target cells were previously infected with a Luciferase-expressing vector. The assays showed consistent and significant cytotoxicity of the 5F clone compared to the controls (NK-92 WT and NK-92 HER2 synNotch), only against HER2amp/CEA+ CRC models, at all effector:target ratios, independently of effector irradiation (Fig. 21). In the case of HER2amp/CEA- SKBR3 control target cells, low killing with no significant difference between the 5F clone and control effectors was observed. Also, HER2normal/CEA+ CRC cells (LS180) were not preferentially killed by the 5F clone. However, in this case, higher basal activity of NK-92 controls was observed, indicating higher sensitivity of LS180 cells to NK-92 killing.

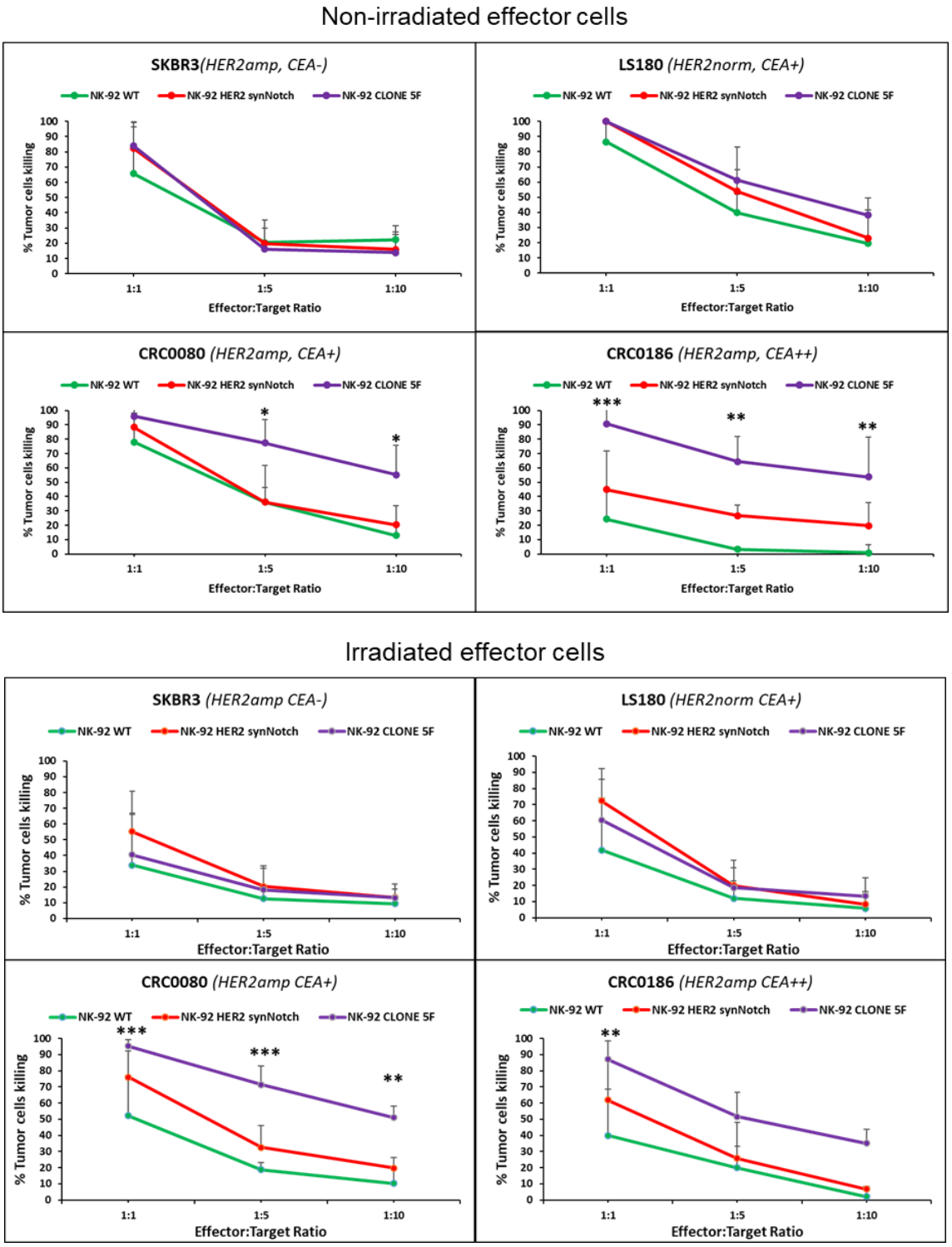

Figure 21. Cytotoxic activity of the 5F clones in not irradiated or irradiated setting against human cancer cells. 5F clone specific killing activity at different Effector:Target ratio against SKBR3, LS180, CRC0080 and CRC0186 without effectors irradiation (top panels) or with effectors irradiation (bottom panels) after 48h of co-culture. (Bars: Standard Deviations. Statistical significance between NK-92 WT and 5F clone was calculated by Two-way Anova. Stars indicated P values: * = P≤0.05; ** = P≤0.01; *** = P≤0.001).

As an additional readout of NK-92 cell activation and killing, degranulation upon interaction with target cells was evaluated by measuring the surface expression of CD107a[156] and the release in the supernatant of Interferon- γ (IFN-γ)[157] and Granzyme B[158] (Fig. 22). Massive degranulation and CD107a increase by 5F cells were observed only upon co-culture with HER2amp/CEA+ CRC cells; similar results were achieved also with irradiated effectors. Overall, these data showed that the NK-92 5F clone expressing HER2-synNotch and CEA-CAR is selectively activated only by HER2amp/CEA+ CRC cells.

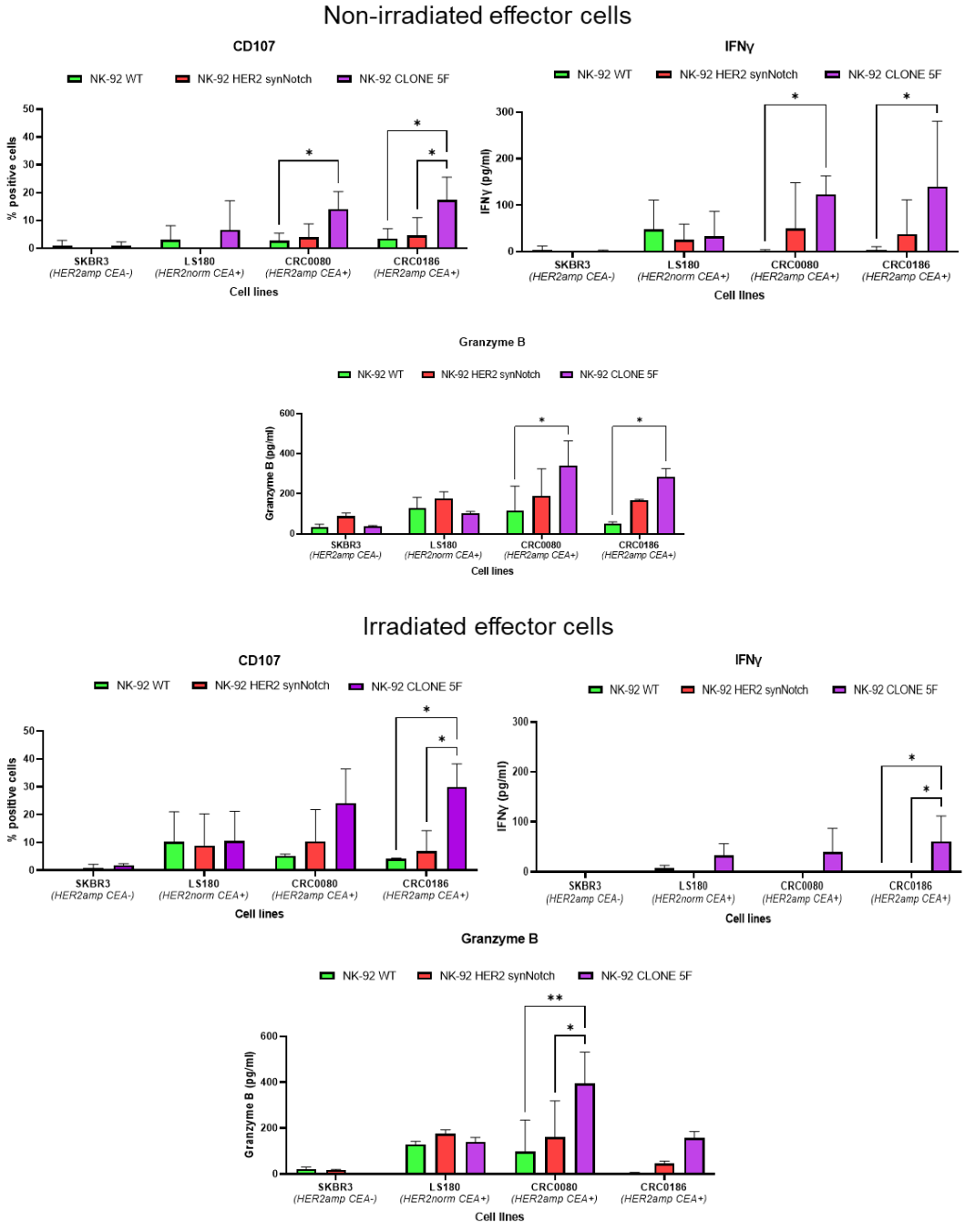

Figure 22. IFN-γ, Granzyme B release and CD107 expression by not irradiated or irradiated 5F clone. 5F clone specific cytokine release (ELISA assays) / degranulation (FACS analysis) against SKBR3, LS180, CRC0080 and CRC0186 without effectors irradiation (top panels) or with effectors irradiation (bottom panels). (Bars: Standard Deviations. Statistical significance was calculated by Two-way Anova. Stars indicated P values: * = P≤0.05; ** = P≤0.01)

To be fully active *in vivo*, effector cells need to be able to efficiently perform tumor homing and penetration. Indeed, higher homing of the 5F clone with respect to controls was observed by transwell assays when HER2amp/CEA+ CRC cells were used as attractors (Fig. 23). Moreover, specific homing and penetration by the 5F clone with respect to control NK-92 cells were observed by immunofluorescence upon co-culture with HER2amp/CEA+ CRC organoids (Fig. 24).

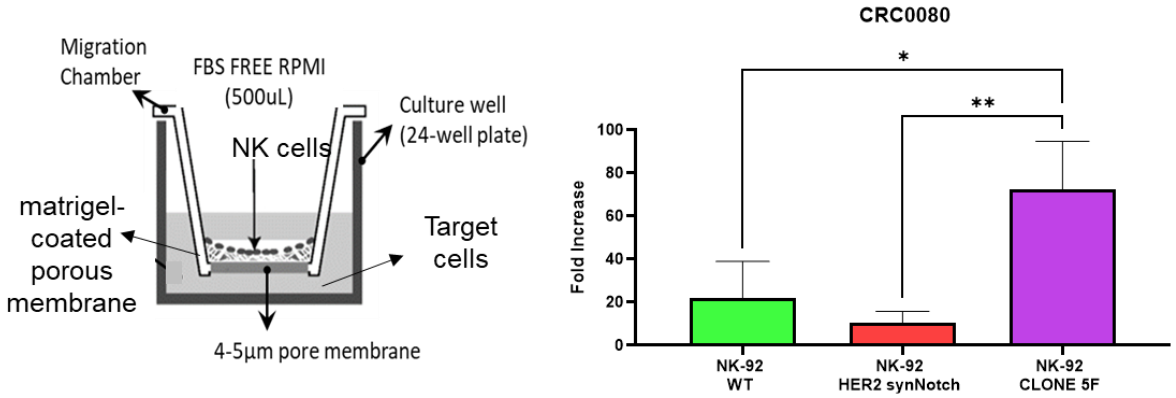

Figure 23. Invasion assays mediating transwell analysis on CRC0080. On the left there is the schematic representation of the transwell assays. On the right there is the bar graph representing the quantification of the transwell assay obtained counting and calculating the fold increase in the diverse condition of the Pkh26 positive NK-92 cells that cross the Matrigel and the pore membrane (Bars: Standard deviation. Statistical significance was calculated by Two-way Anova. Stars indicated P values: *= P≤0.05; **= P≤0.01).

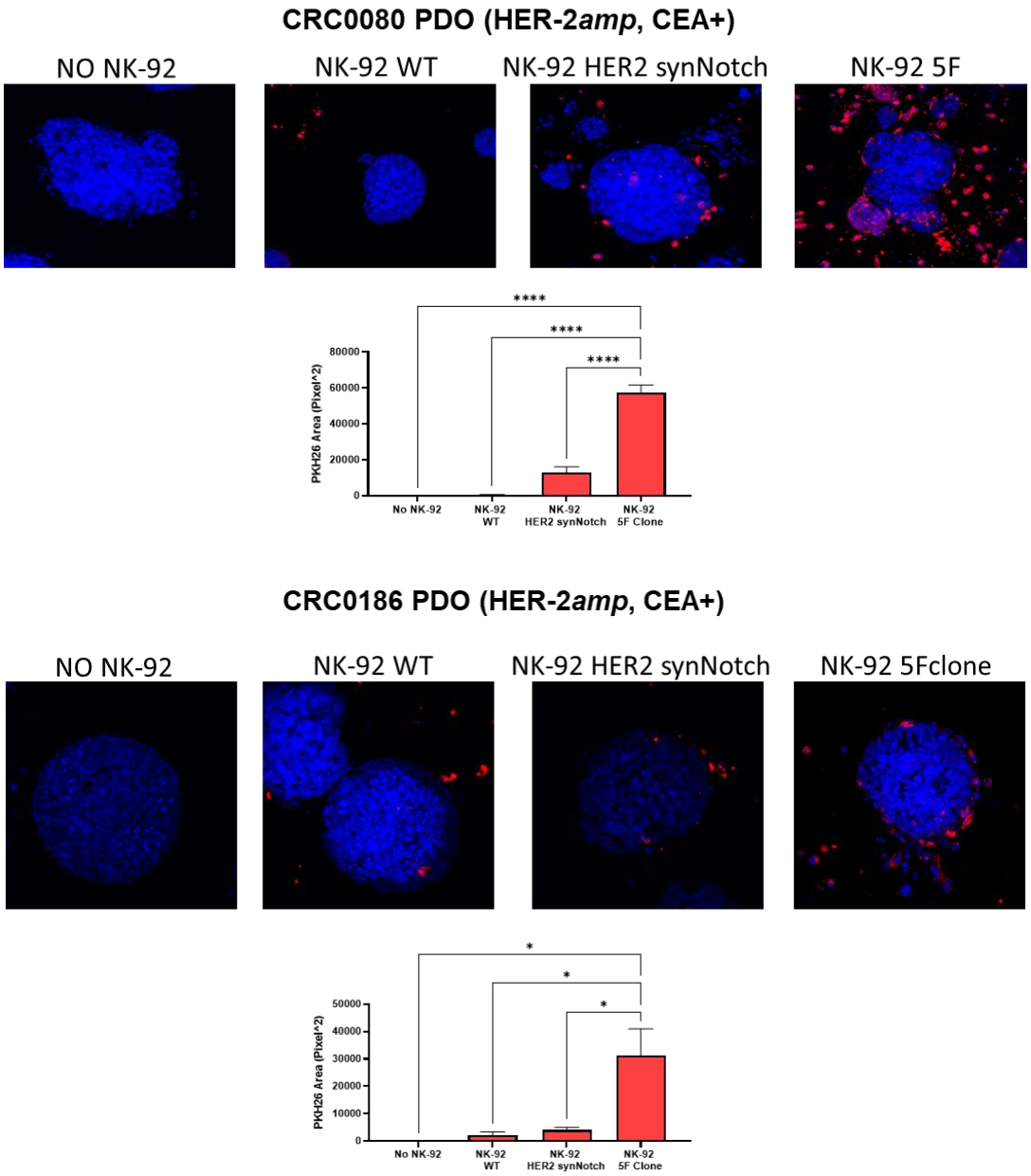

Figure 24. Invasion assays on CRC0080 and CRC0186 PDO. Representative pictures of organoids derived from CRC0080 (top panel) and CRC0186 (bottom panel) primary colon carcinoma cells grown in a three-dimensional matrix in the presence of effector cells for 3 days. Cancer cells were stained with NucBlue (Blue signal), and NK-92 effector cells were stained with PHK26 (Red signal). Confocal microscopy images taken after 48hours of co-culture. Magnification, 20x; scale bars, 75μm. Under the fluorescent images both for the CRC0080 and the CRC0186 there is the quantification of NK-92 effector cells infiltration into the derived organoids and the Matrigel analyzing the red fluorescence PKH26 area (px2, Bars: Standard deviation. Statistical significance was calculated by Two-way Anova. Stars indicated P values: *= P≤0.05; ****= P≤0.0001).

After confirming the specific activity of the 5F clone *in vitro*, the *in vivo* activity was assessed using mouse xenografts of the HER2amp/CEA+ CRC0080 and CRC0186 models. Initially, *in vivo* induction of the CEA CAR by injection of the 5F clone directly into HER2-amplified xenografts was assessed by immunofluorescence. After 72 hours from injection, CEA CAR expression was observed in the majority of the intratumorally detected 5F cells (Fig. 25). These data demonstrated that when 5F cells get in contact with HER2amp target cells *in vivo*, CEA CAR expression is induced.

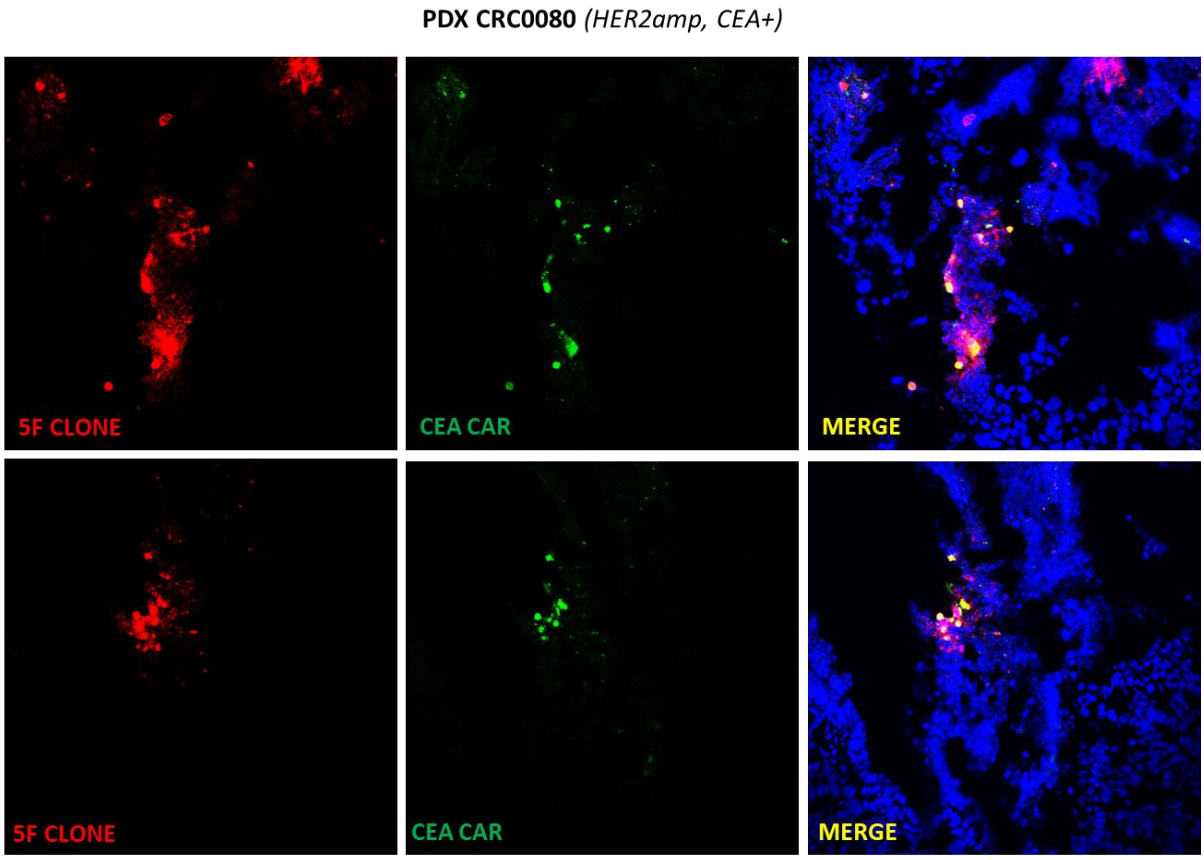

Figure 25. Immunofluorescent analysis of the CEA CAR induction *in vivo*. Representative immunofluorescent pictures of 5F CEA CAR induction on the CRC0080 tumor slice; the tumor was explanted 3 days after the intratumoral inoculation of the 5F clone cells (red= pkh26 labeled 5f clone cells, green= CEA CAR antibody, blue= NucBlue labeled cancer cells). Magnification, 10x; scale bars, 75μm.

Subsequently, the 5F clone was tested for *in vivo* antitumor activity against the two HER2amp/CEA+ CRC model xenografts, treating mice with 5x10^6 irradiated cells twice a week for three weeks, for a total of six injections. In both models, the 5F clone induced a significant and sustained reduction of tumor growth. Control effectors (NK-

92 WT and HER2 synNotch only) displayed only marginal, not significant activity (Fig. 26). Overall, these results showed that the NK-92 5F clone is specifically effective against HER2-amplified and CEA-positive CRC models both *in vitro* and *in vivo*.

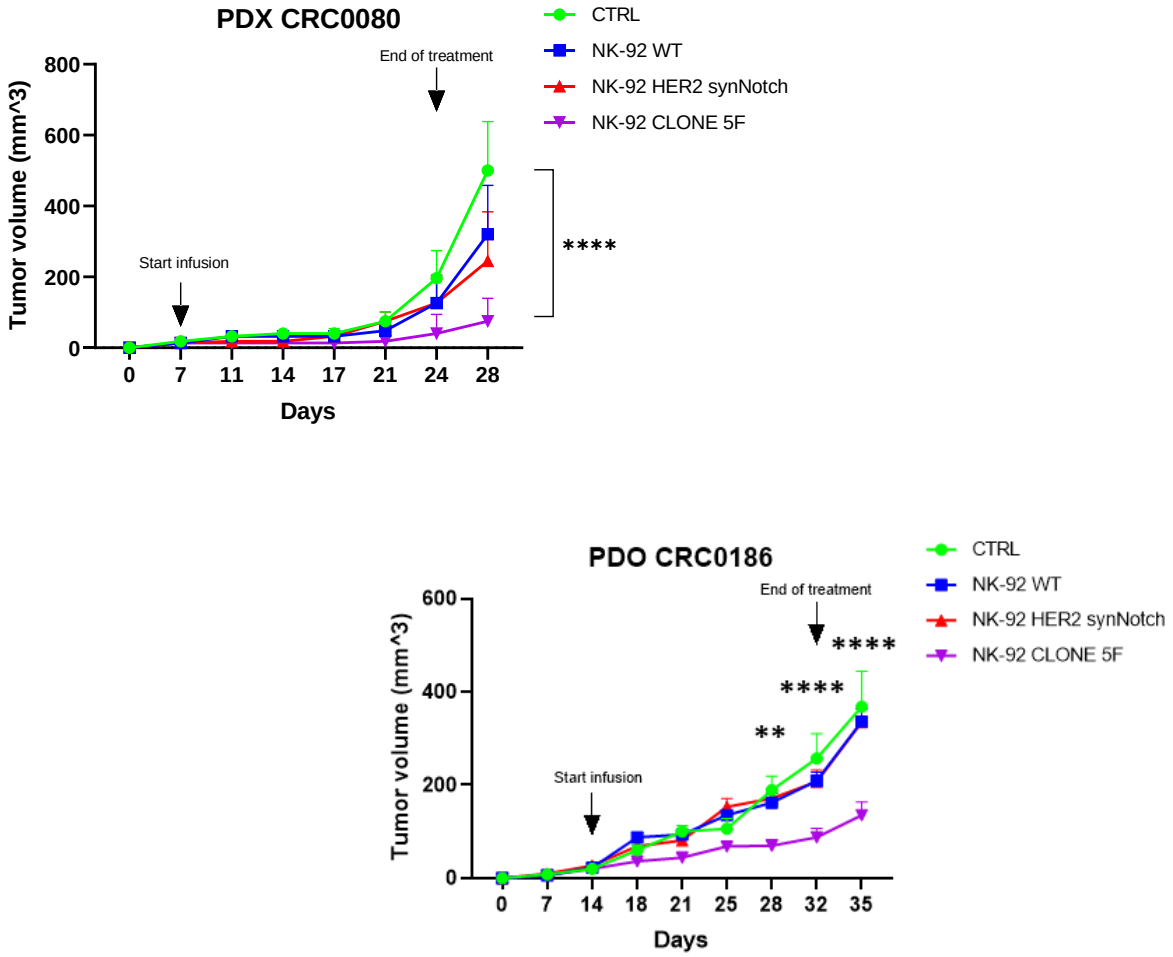

Figure 26. 5F clone therapeutic activity in experimental HER2 amplified tumors. CRC0080 PDX tumor growth in NOD-SCID mice treated with $5x10^6$ NK-92 effector cells by intravenous injection on days 7, 11,14,17,21 and 24 (Top panel). CRC0186 PDO tumor growth in NOD-SCID mice treated with $5x10^6$ NK-92 effector cells by intravenous injection on days 14,18,21,25,28 and 32 (Bottom panel) (CTRL: PBS; Bars: SEM. Statistical significance between Vehicle and 5F clone was calculated by Two-way Anova. Stars indicated P values: *= P≤0.05, **= P≤0.01, ***= P≤0.001, ****=P≤0.000).

5.5. Further improvement of NK-92 5F clone activity

CAR-based adoptive immunotherapy of solid tumors faces two major challenges: (i) low persistence of the effectors in the patient: in some cases, immune cell exhaustion is reached shortly after the infusion[159]; and (ii) inadequate efficacy, mostly due to low penetration of the effectors inside the tumor[160]. From the clinical application

standpoint, NK-92 cells have an intrinsically low persistence, because they have to be irradiated. However, in preclinical experimental conditions, they can serve as a useful model platform to explore strategies to increase *in vivo* persistence or tumor penetration, using non-irradiated effectors. The 5F clone was therefore exploited as a model to assess if transduction with further GAL4-driven expression cassettes could lead to improvements in persistence and efficacy. Considering that NK-92 cells are strongly dependent on IL2 for their proliferation, survival, and activation[32], IL-2 was included as an additional GAL4-driven module.

The 5F clone was transduced with a GAL4-driven IL-2 vector, also providing constitutive mCherry fluorescent protein expression. In this way, upon engagement of the HER2-synNotch, in addition to CEA-CAR expression, also IL-2 is induced and secreted. The secreted IL-2 can then exert autocrine and paracrine activities (Fig. 27).

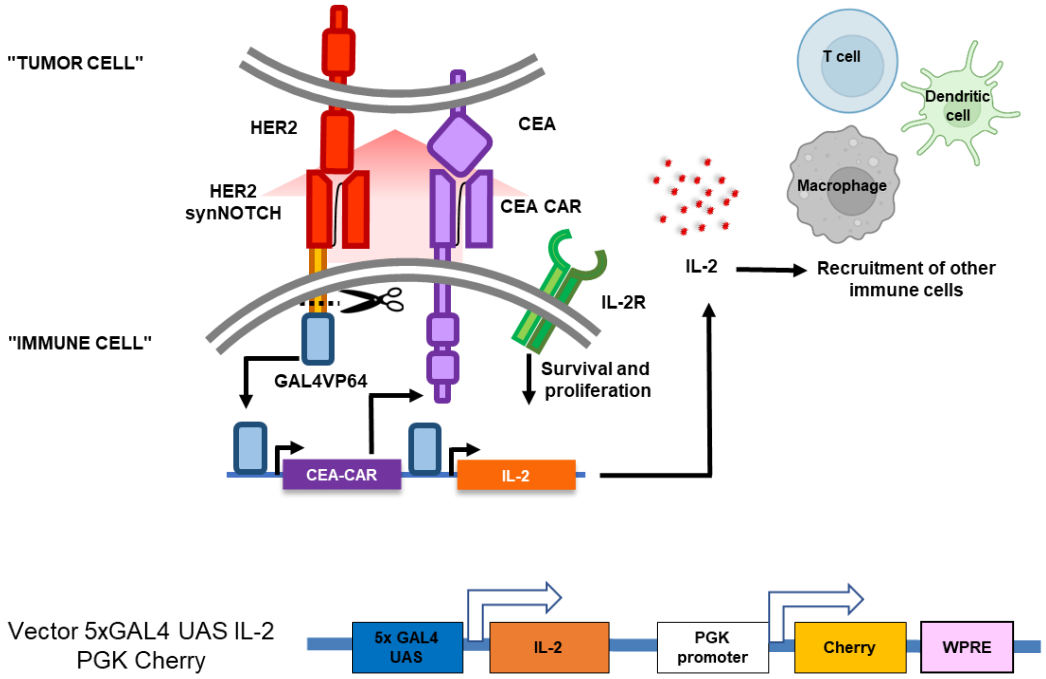

Figure 27. Schematic representation of the HER2 synNotch GAL4 CEA CAR GAL4 IL-2 system and the GAL4 IL-2 lentiviral vector. In this improved system the synthetic Notch receptor that, beyond the CEA CAR expression also induced the cytokine IL-2, that released in the tumoral microenvironment will acts both with an autocrine loop on the NK-92 itself boosting them and in a paracrine way by attracting other anti-tumoral immune cells to the tumor site. Grey lines represent the plasma membrane (IL-2R= IL-2 receptor) (Top panel). Schematic representation of the GAL4 IL-2 lentiviral vectors (PGK= Human phosphoglycerate kinase promoter, WPRE = Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element) (Bottom panel).

After transduction with the GAL4 IL-2 vector, 5F cells were sorted for mCherry expression, increasing the mCherry-positive population (and so the cells potentially expressing IL-2) from 2% to 75% (Fig. 28).

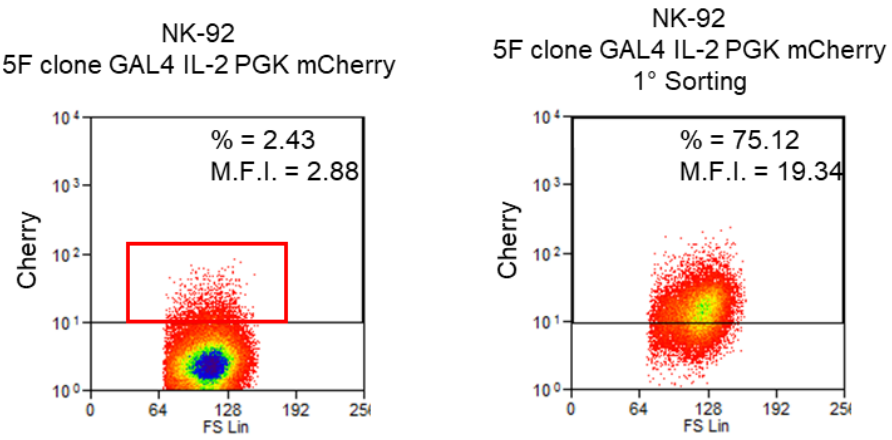

Figure 28. Clone 5F GAL4 IL-2 positive sorting for cherry. Representative flow-cytometry analysis of Cherry expression in the 5F clone after Cherry one positive sorting. The red box represents the selected population. y-axis= log10 expression, x-axis= Forward Scatter. %= percent of cell above the depicted positivity threshold; M.F.I. = Mean Fluorescent Intensity (of all displayed cells).

ELISA assays were performed to assess IL-2 release in the supernatants before and after target cells engagement (Fig. 29). The assays showed high and selective IL-2 release only when effector cells were co-cultured with HER2 target cells; IL2 inductions were detectably lower when the effectors were irradiated.

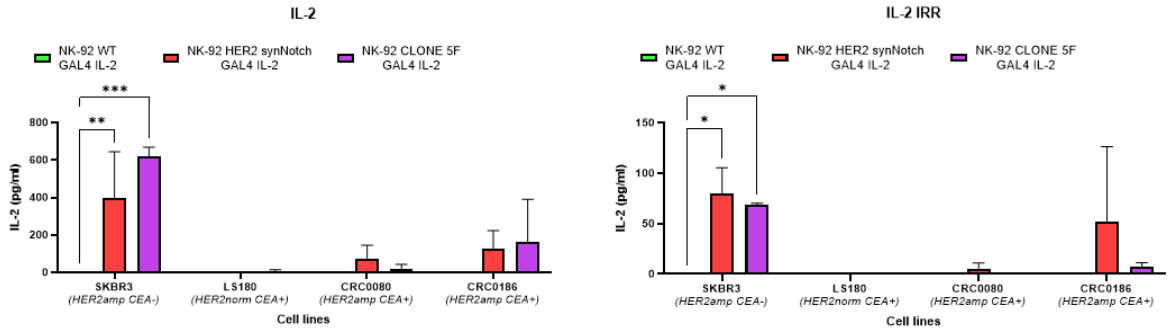

Figure 29. IL-2 ELISA assays. ELISA assays show 5F GAL4 IL-2 population specific IL-2 release against SKBR3, LS180, CRC0080 and CRC0186 without effectors irradiation (Left graph) or with effectors irradiation (Right graph). (Bars: Standard Deviations. Statistical significance was calculated by Two-way Anova. Stars indicated P values: * =P≤0.05; ** =P≤0.01, *** =P≤0.001).

In luciferase-based killing assays, 5F-IL-2 cells displayed higher cytotoxic activity with respect to parental 5F, also at lower effector: target ratio (1:10, 1:20, 1:50) (Fig. 30). Unfortunately, with irradiated effector cells, there was a reduction in the cytotoxic activity ratio between 5F clone and 5F-IL-2 (Fig. 30).

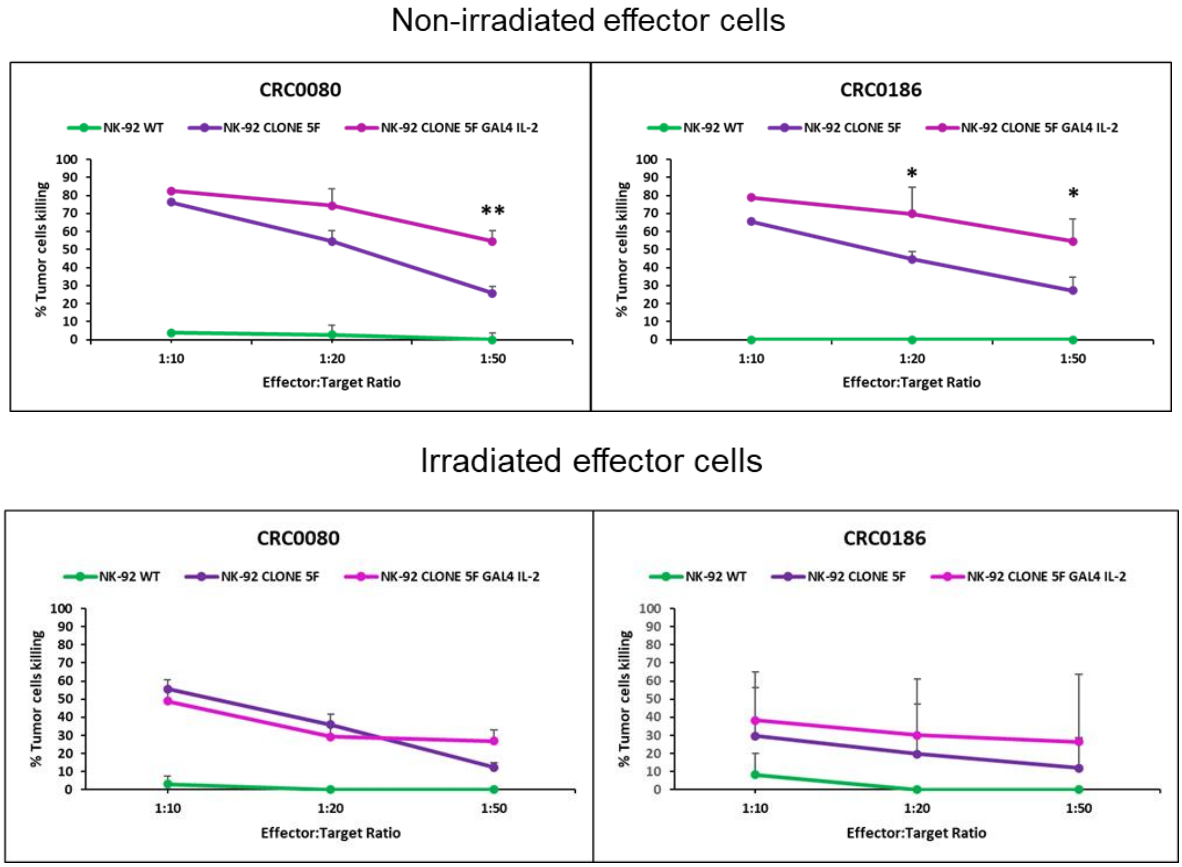

Figure 30. 5F GAL4 IL-2 cells vs 5Fclone cytotoxic activity against HER2 amplified cells. 5F GAL4 IL-2 vs 5F clone specific killing activity at low Effector:Target ratio against CRC0080 and CRC0186 without effectors irradiation (top panels) or with effectors irradiation (bottom panels) after 96h of co-culture. (Bars: Standard Deviations. Statistical significance between 5F GAL4 IL-2 cells and 5F clone was calculated by Two-way Anova. Stars indicated P values: * = P≤0.05; ** = P≤0.01).

5F-IL-2 cells were subsequently tested for *in vivo* activity, without irradiation, against a HER2amp/CEA+ xenograft model, exploring long-term survival rather than short-term effects. 5F-IL2 effectors induced a detectable and significant increase in survival with respect to controls and parental 5F, highlighting a likely increased persistence and efficacy *in vivo*; further analyses are required to confirm this hypothesis (Fig. 31).

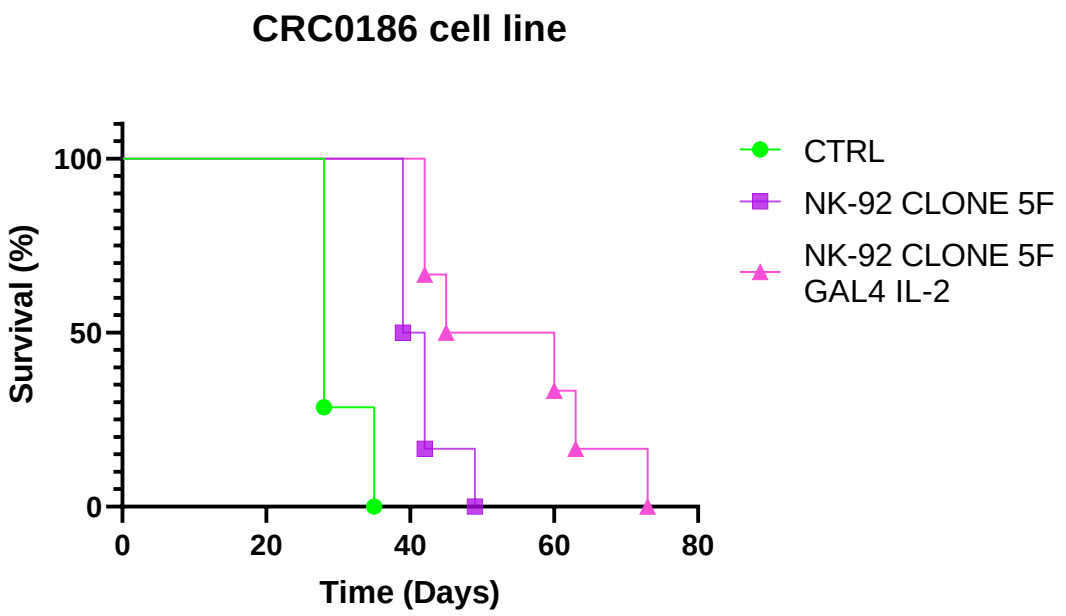

Figure 31. *In vivo* mice survival treated with 5F GAL4 IL-2. The Kaplan-Meier represents the overall survival of NOD-SCID mice injected with CRC0186 cell line and treated with $5x10^6$ NK-92 effector cells by intravenous injection on days 7, 11,14,17,21 and 24. (CTRL: PBS).

5.6. Mesothelin as an additional synNotch receptor target

To exploit the extreme versatility of the synNotch system, an alternative synNotch receptor, recognizing Mesothelin (MSLN), was developed. MSLN is a glycosylphosphatidylinositol-linked membrane glycoprotein present at relatively low levels in mesothelial cells of the pleura, peritoneum, and pericardium of healthy individuals. However, MSLN is overexpressed in several different cancers, including mesotheliomas, stomach cancer, prostate cancer, pancreatic cancer, lung cancer, cholangiocarcinoma, breast cancer, as well as ovarian cancer[161]. For this reason, it has already been considered a good target for CAR-based therapy[162]. Nevertheless, like for HER2, basal expression of certain normal tissues poses the problem of on-target off-tumor side effects. Recently, MSLN overexpression was also recognized as a poor prognosis factor in CRC[163]. To investigate the concomitant presence of high levels of MSLN and CEA in CRC, and to identify suitable patient-derived models for preclinical testing of a possible MSLN synNotch CEA CAR based therapy of CRC, mRNA expression of MSLN and CEA was evaluated in 450 CRC samples from TCGA[151], 119 CRC cell lines (Manuscript in preparation), and 515 CRC PDXs[140] (Fig. 32).

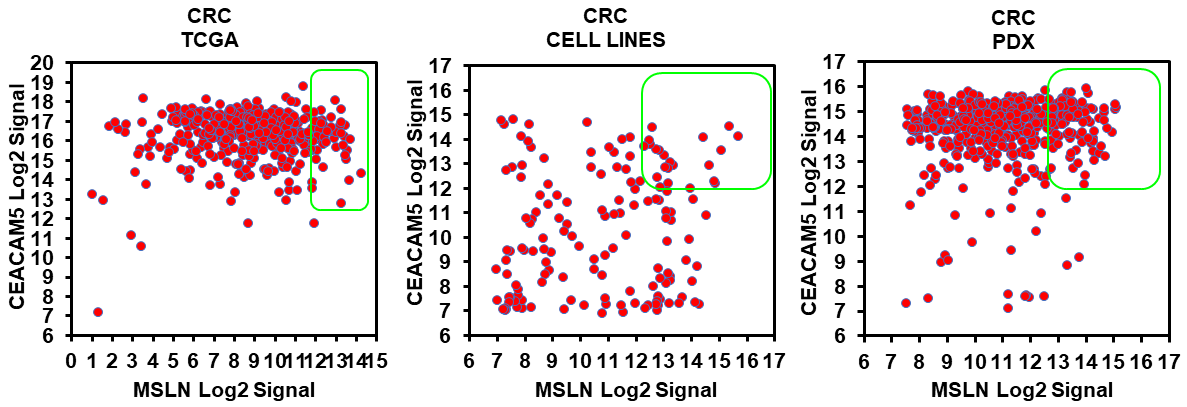

Figure 32. MSLN and CEA mRNA expression in TCGA samples, CRC cell lines and PDX. In the three dot-plot CEA mRNA expression is show in y-axis and MSLN mRNA expression is show in x-axis. Green box highlighted MSLN high and CEA high expressing samples.

Different cell lines, with different levels of MSLN and CEA were selected. Protein expression of MSLN and CEA was also confirmed by flow cytometry analysis. In particular, the MSLN-amplified ovarian cancer cell line OVCAR3 was selected as positive control for MSLN overexpression[164] and negative control for CEA expression. Three CRC cell lines were also selected, all positive for CEA expression: SNU283 (MSLN-negative), and SW1116 and LS180 (both MSLN-high; Fig. 33)

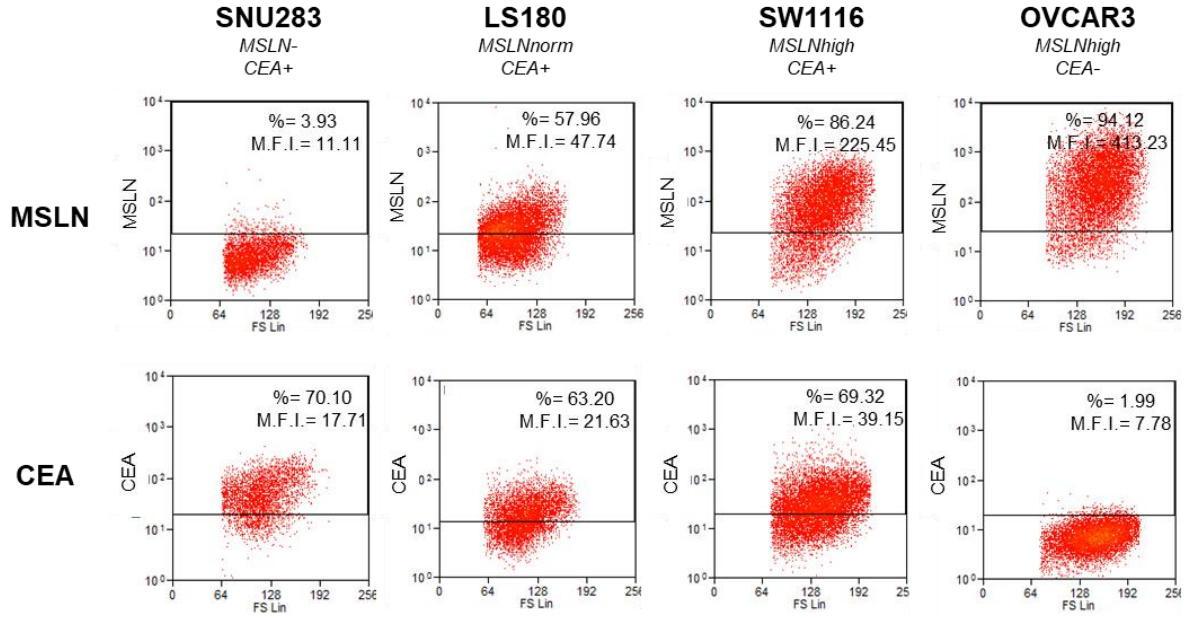

Figure 33. Cell lines MSLN and CEA surface protein expression by FACS analysis. Representative flow cytometry dot-plots showing on the y-axis MSLN (top row) and CEA (bottom row) surface expression in different cell lines used as models; y-axis= log10 expression, x-axis= Forward Scatter. %= percent of cell above the depicted positivity threshold; M.F.I. = Mean Fluorescent Intensity (of all displayed cells).

The MSLN synNotch was designed with the same transmembrane and intracellular structure of the HER2 synNotch (synNotch CORE+GAL4VP64) but the anti-HER2 scFv was substituted with an anti-MSLN scFv. Moreover, an EGF repeat was inserted between the MSLN scFv and the synNotch CORE (Fig. 34); this sequence was used to avoid high basal synNotch activation[124].

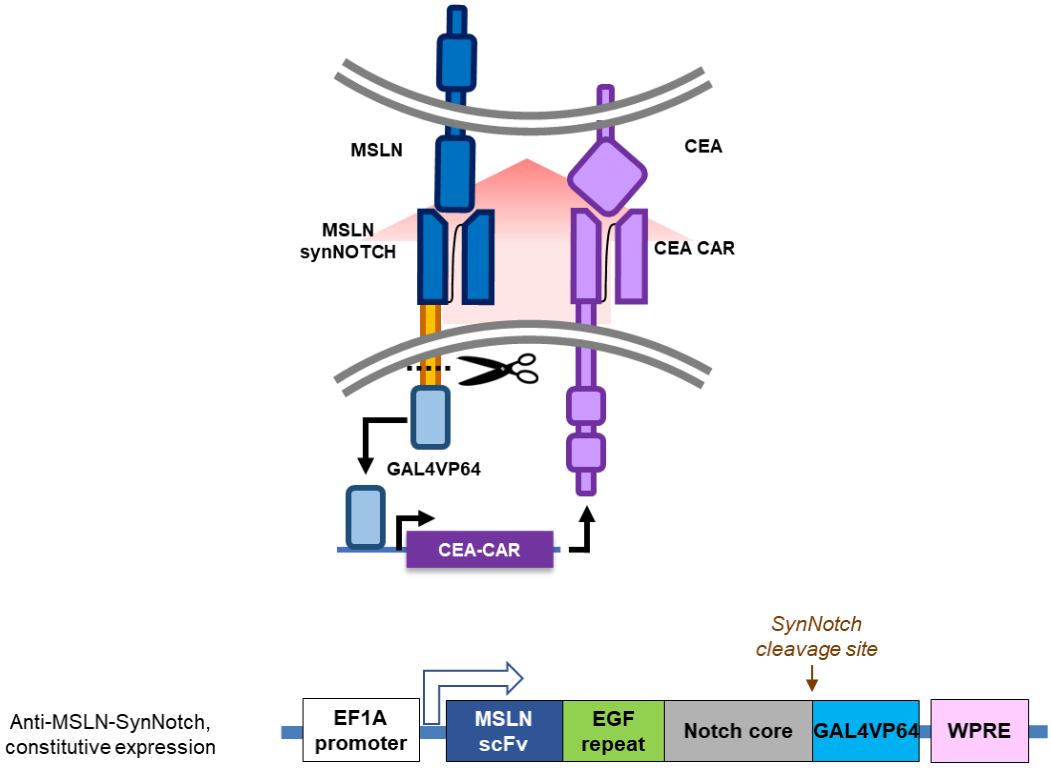

Figure 34. Schematic representation of the MSLN synNotch GAL4 CEA CAR system. In this system a synthetic Notch receptor that, upon binding to a first antigen "A" (MSLN) on the surface of target cells, is cleaved in the intracellular portion so that a transcription factor domain (GAL4VP64) reaches the nucleus and induces the expression of a CAR against the second antigen "B" (CEA) (Top panel). On the bottom the schematic representation of the MSLN synNotch lentiviral vector is reported (EF1A= eukaryotic translation elongation factor 1 α promoter, Ab= antibody, GAL4VP64 = transcription factor, WPRE = (WHP) Posttranscriptional Regulatory Element)

Jurkat cells were transduced with the MSLN-synNotch and the GAL4 CEA CAR and cultured in the absence or presence of LS180 target cells (CRC, MSLNhigh and CEA+). CEA CAR expression, assessed by flow cytometry, was basally low (<10% positive cells) and significantly higher (40% positive cells) in the presence of LS180 cells (Fig. 35). To evaluate the functional consequences of CEA CAR induction and engagement, CD69 expression was assessed by flow cytometry on MSLN synNotch/CEA CAR Jurkat cells, before and after co-culture with LS180 cells. Indeed, around 65% of Jurkat cells became positive to CD69 (with 20% of CD69 positive cells basally), highlighting a specific activation mediated by engagement of the newly induced CEA CAR with its antigen (Fig. 35). Overall, these results confirmed good specificity and inducibility of the MSLN synNotch /CEA CAR system, with margins for improvement by sorting and cloning the transduced effectors.

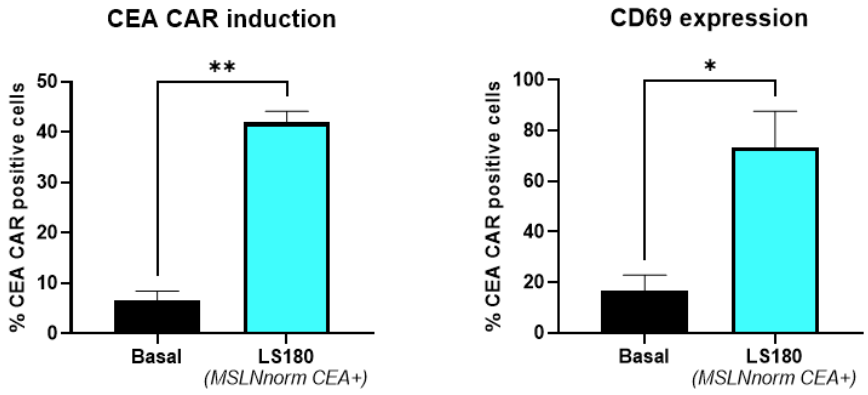

Figure 35. CEA CAR induction and CD69 expression after co-culture with LS180. Left graph: The bar graph shows the percentage of CEA CAR positive Jurkat cell induced by the MLSN synNotch after the co-culture with MSLN expressing cell. Data were obtained by flow cytometry analysis. Right graph: The bar graph shows the percentage of CD69 positive cells induced by the CEA CAR binding with target cells, data obtained by flow cytometry analysis (Bars: Standard Deviations. Statistical significance was calculated by T-test Stars indicated P values: * = pvalue < 0.05).

Subsequently, NK-92 cells were transduced with the MSLN synNotch lentiviral vector and sorted, finally obtaining 87% of synNotch-positive cells (Fig. 36A). The sorted population was then transduced with the GAL4-driven CEA CAR vector and subjected to one sorting for positive CEA-CAR expression after co-culture with MSLN-high target cells (Fig. 36B).

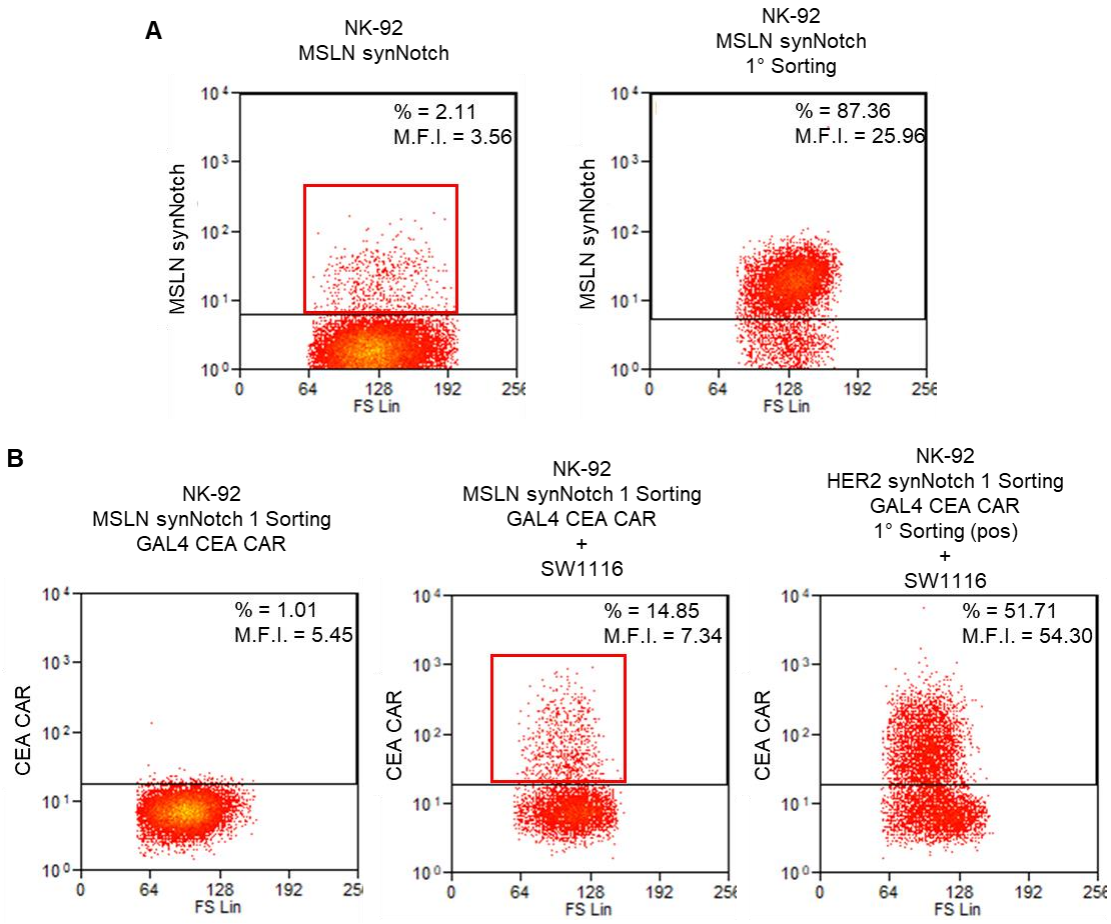

Figure 36. NK-92 MLSN synNotch and CEA CAR sorting procedures. A. Representative flow-cytometry analysis of MLSN synNotch expression in NK-92 before and after one MSLN synNotch positive sorting. **B**. Representative flow-cytometry analysis of CEA CAR induction in NK-92 MLSN synNotch one sorting GAL4 CEA CAR after co-culture with MLSN high expressing cells, before and after the CEA CAR positive sorting procedure. Red boxes represent the selected populations. y-axis= log10 expression, x-axis= Forward Scatter. %= percent of cell above the depicted positivity threshold; M.F.I. = Mean Fluorescent Intensity (of all displayed cells).

CEA CAR induction was assessed in detail after expansion of the sorted population (Fig. 37). A massive CEA CAR induction was observed after co-culture with the MSLN-amplified, CEA-negative OVCAR3 ovarian cancer cell line. The CEA CAR was also substantially induced upon incubation with the MSLN-high, CEA+ SW1116 colorectal cancer cells, obtaining 55-60% of CEA CAR-positive NK-92 cells.

The lower induction in CRC models could be due to either lower MSLN expression or to target cell killing that reduces the contact time with surface MSLN. Indeed, a significant fraction of the sorted NK-92 population was basally positive for CEA CAR expression (20-25%), also after co-culture with MSLN-low control cells.

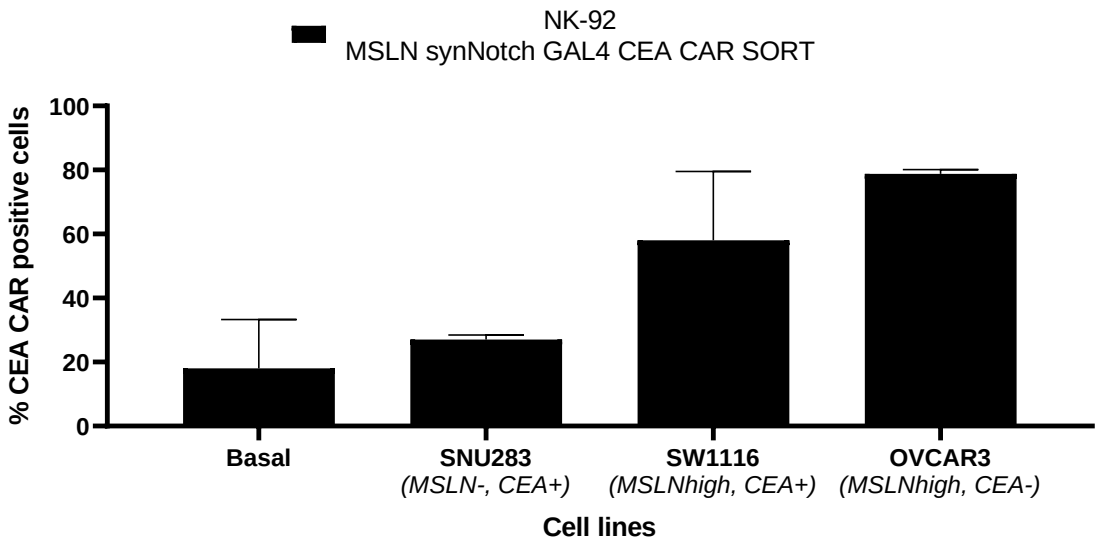

Figure 37. CEA CAR induction in the sorted population and in the clones. The bar graph shows the percentage of CEA CAR positive NK-92 MSLN synNotch GAL4 CEA CAR sorted population cell induced by the MSLN synNotch after the co-culture with MSLN normal expressing or overexpressing cells, data obtained by flow cytometry analysis (Bars: Standard Deviations)

To avoid CEA CAR basal expression, three clones were generated from the MSLN-synNotch/CEA-CAR NK-92 sorted population, displaying low basal CEA CAR expression (0-10%) and high induction (70-80%) after co-culture with MSLN-high target cells (Fig. 38). Finally, the 2C8 clone was selected as the one with the lowest basal and highest induction (80%).

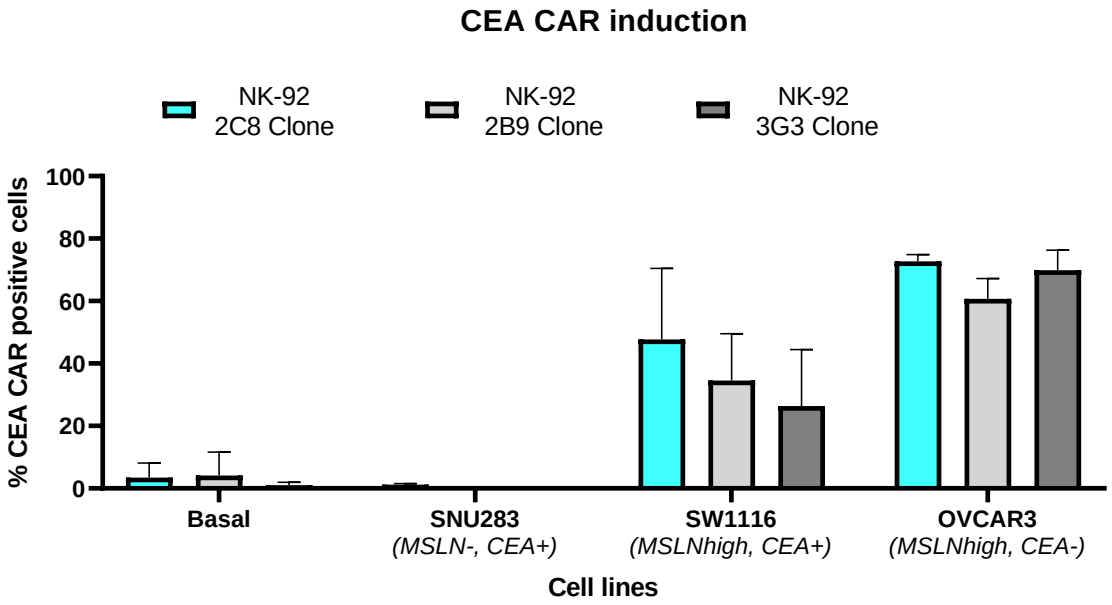

Figure 38. CEA CAR induction in the sorted population and in the clones. The bar graph shows the percentage of CEA CAR positive from 3 different clones induced by the MSLN synNotch after the co-culture with MSLN normal expressing or HER2 overexpressing cells, data obtained by flow cytometry analysis (Bars: Standard Deviations)

The killing activity of the 2C8 clone was tested by ATP assays. The assays showed consistent and significant cytotoxicity of the 2C8 clone compared to controls (NK-92 WT and NK-92 MSLN synNotch), only against MSLNhigh/CEA+ CRC models, at all effector:target ratios, independently of effector irradiation (Fig. 39). In the case of MSLN-/CEA+ SNU283 control target cells, low killing with no significant difference between the 2C8 clone and control effectors was observed. Also, MSLNhigh/CEA- ovarian cancer cells (OVCAR3) were not preferentially killed by the 2C8 clone. However, in this case, higher basal activity of NK-92 controls was observed, indicating higher sensitivity of OVCAR3 cells to NK-92 killing.
The promising results *in vitro* indicated the potential of the MSLN synNotch/CEA CAR strategy also for future *in vivo* studies.

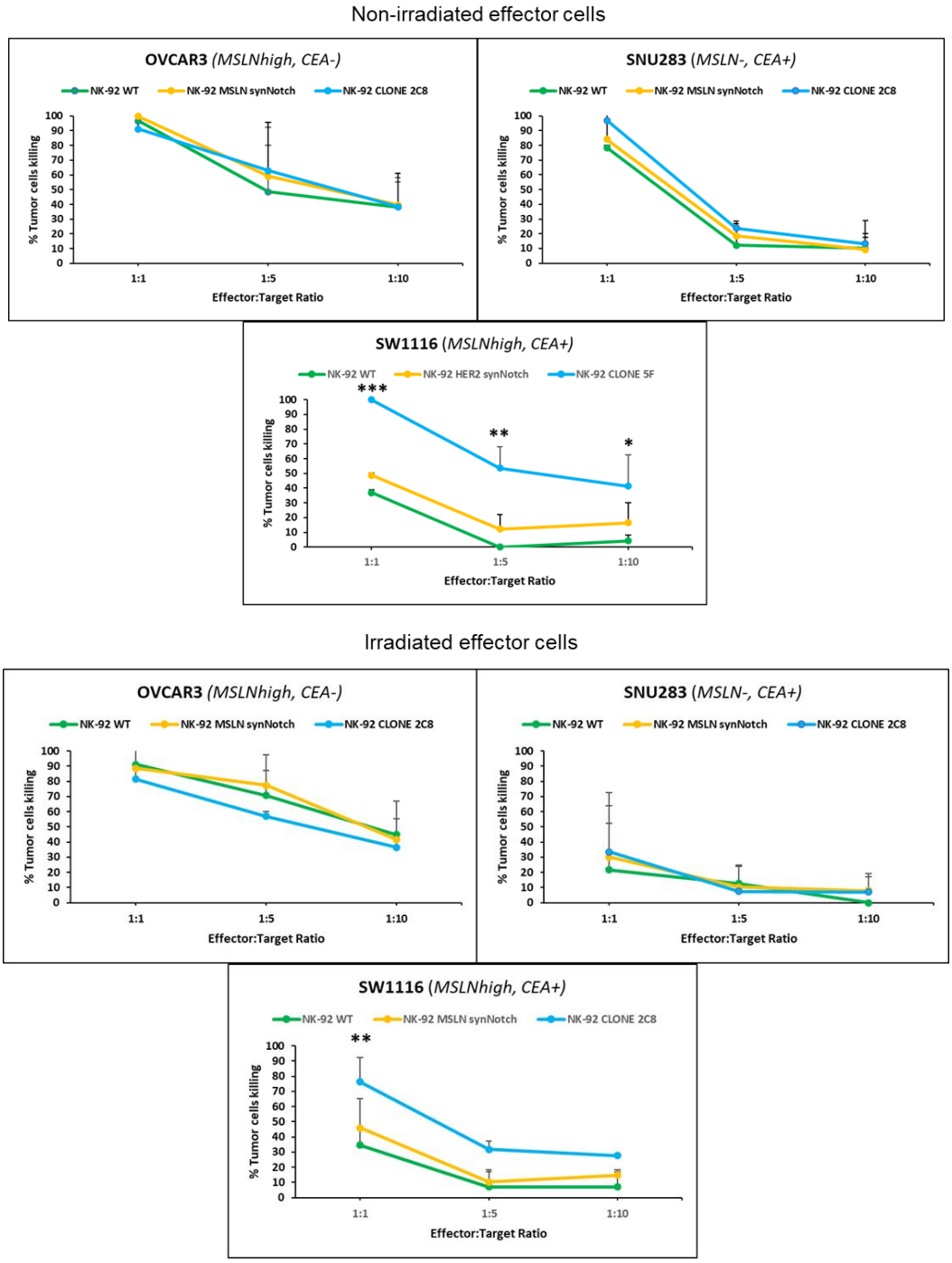

Figure 39. *In vitro* 2C8 clone killing activity in not irradiated and irradiated setting. 2C8 clone specific killing activity at different Effector:Target ratio against OVCAR3, SNU283 and SW1116 without effectors irradiation (top panels) or with effectors irradiation (bottom panels) after 48h of co-culture. (Bars: Standard Deviations. Statistical significance between NK-92 WT and 5F clone was calculated by Two-way Anova. Stars indicated P values: * = P≤0.05; ** = P≤0.01; *** = P≤0.001)

6. Discussion

This study presents a novel combinatorial immunotherapy approach based on immune cell redirection against HER2-amplified, CEA-positive CRC. HER2 overexpression is detected in 5-6% of CRC patients (100.000 new cases every year worldwide) with somatic HER2 gene amplification, resulting in activation of the HER2 signaling pathway, resistance to EGFR-targeted therapy, and poor prognosis[106]. Multiple studies have shown that metastatic HER2amp CRC can be successfully treated with a combined therapy targeting HER2 and EGFR[113]. Despite these encouraging results, two major unmet clinical needs remain: (i) primary resistance to HER2/EGFR therapy, occurring in almost half of the cases, and (ii) limited duration of the response, due to acquisition of resistance-promoting mutations. As a consequence, a sizeable fraction of HER2-amplified CRCs needs alternative therapies. In this perspective, one of the most promising strategies to overcome the above limits is CAR-based adoptive cell therapy. While CAR-cell adoptive transfer demonstrated remarkable efficacy in the treatment of B-cell malignancies, its applications in solid tumors have yet to yield impressive results[60]. In this context, one important issue to address is the lack of specific cancer targets, which makes eliminating tumor cells without harming healthy tissues very challenging. A promising option for solid tumor treatment with CAR-based ACT relies on identification of two tumor-associated target antigens instead of one, so that only cancer cells but not normal tissues co-express both at high levels. The main aim of this study was therefore to set up and validate a combinatorial antigen-targeting approach based on the synNotch system, to engineer killer cells against HER2amp CRC without directing them also against normal tissues, known to express HER2 at potentially critical levels.

To identify an optimal second target to be combined with HER2 in the synNotch/CAR system, a set of antigens already validated as CAR targets in CRC was considered. Among all, CEA emerged as the best candidate. CEA is highly expressed in carcinomas as well as in normal gastrointestinal tract, but it is virtually absent in all other normal tissues of the human body[150,165]. CEA is a strong prognostic biomarker in patients with colorectal cancer who underwent surgical resection and adjuvant chemotherapy[166]. Elevated CEA levels at the time of new diagnosis of colorectal cancer is associated with poor prognosis[167]. Its measurement is the most sensitive

detector of liver metastasis from colorectal cancers that can be resected surgically[168]. Based on its expression profile, several trials targeted CEA for ACT were employed. A phase I trial (NCT02349724) was conducted to evaluate the safety and efficacy of anti-CEA CAR-T cells in CEA positive refractory mCRC patients[143,169]. Seven of ten patients obtained stable disease, without significant CAR-related toxicity. However, highly active CEA-directed investigational therapeutics have been reported to be toxic, causing severe colitis[170]. A phase I trial, CAR T-cells targeting CEA were first tested in three patients with mCRC[171]. One patient displayed an objective response at lung and liver metastases. Unfortunately, all three patients experienced severe colitis. In another study no objective clinical responses were observed. Engraftment was short-lived with a rapid decline of systemic CAR T cells within 14 days. Patients in the treated cohort had transient, acute respiratory toxicity which, in combination with lack of prolonged CAR T cell persistence, resulted in the premature closure of the trial[172]. Despite several studies being in phase one clinical trial some reservations remain given the side effects, as “on-target off-tumors”, associated with strategies based on constitutive CARs[173].

The bioinformatic analysis pointed out that, even if individual expression of HER2 and CEA is not completely restricted to tumor cells, only CRCs carrying HER2 amplification overexpress both HER2 and CEA at the optimal levels required for combinatorial targeting. In this way, the 'on-target/off-tumor' toxicity is kept under control, because the synNotch system allows CEA CAR expression only in the presence of HER2 amplification, which is absent in a physiological state.

Two recent works also explored the synNotch/CAR system in solid tumors. Hernandez-Lopez and colleagues built an HER2 synNotch with low affinity for the antigen and a GAL4- inducible HER2 CAR with high affinity for the antigen[133]. Indeed, such circuit relies on high HER2 expression by all target cells for optimal efficacy. It is known that HER2 expression levels can vary within the tumor, also in the context of HER2 amplification[174–176]. Hyrenius-Wittesten and colleagues chose HER2 as CAR target, driven by an anti ALPPL2 synNotch, again requiring homogeneously high HER2 levels within the tumor[132].The choice of the CEA CAR presented in this thesis, targeting a lineage antigen expressed by the vast majority of cancer cells, should lead to increased efficacy and reduced probability of escape.

The preliminary series of experiments in Jurkat cells confirmed that the synNotch receptor, when engaged with its cognate antigen, correctly drives expression of the target CAR under the GAL4 promoter, although with some basal activity and suboptimal induction levels. This could be due either to intrinsic limits of the vector system, or to random integration in unfavorable genome regions. In the second case, sorting and cloning of transduced effectors could lead to substantial improvements of the expression dynamic range. Indeed, sorting and cloning of synNotch/CAR-transduced NK-92 cells led to undetectable CEA CAR basal expression and to its robust induction upon engagement of the HER2 synNotch. These results indicate that using this system in primary T or NK cells could be problematic, considering the need for at least one sorting round and the short ex vivo lifetime of such effectors.

The synNotch/CAR NK-92 system, being based on an immortalized cell line, allowed complex handling procedures, such as multiple transductions, sorting, and cloning, leading to very high and specific CAR induction in the 5F clone only when co-cultured with HER2amp target cells. In addition, the system's ability to completely abolish CAR expression within just 24-48 hours after removal of HER2amp target cells indicate excellent safety in a future clinical setting: CEA CAR-expressing cells moving away from the tumor do not maintain CEA-CAR expression, thereby lowering the risk of "on-target off-tumor" toxicities. As mentioned, being the NK-92 a human lymphoma-derived cell line, irradiation before *in vivo* application is mandatory and compromises persistence. Indeed, we also observed that irradiation reduces *in vitro* degranulation and release of IFN-g. However, it did not significantly impair NK-92 short-term viability and target killing both *in vitro* and *in vivo,* suggesting potential clinical applicability under GMP procedures. Surely, irradiated NK-92 effectors cannot be considered as a “living drug” able to expand and persist in patients. This limit however is counterbalanced by the greatly increased availability as an “off-the-shelf” product, easy to expand and potentially usable also in an allogeneic setting, as already demonstrated in multiple clinical studies[34,177]. Therefore, future more appealing strategies, that permit to avoid irradiation, could involve transduction and sorting of iPSC, followed by their differentiation into mature T or NK effectors. iPSCs are a new attractive source for NK cells given their clonal growth and high expansion capacity, as well as their ability to differentiate *in vitro*, allowing for the manufacturing of large numbers of homogeneous NK cell products. Indeed, a rising number of genetically

modified iPSC-NK cell candidates are appearing in preclinical investigations, giving the rationale for clinical trials[35,178].

The 5F clone showed specific functional activation and target killing only when co-cultured with HER2amp/CEA+ CRC cells, while sparing CEA+ CRC cells expressing physiologically high HER2 levels in the absence of amplification and extreme overexpression. This behavior is most likely a consequence of the cell sorting criteria: NK-92 were selected to not express the CAR when exposed to cells which express HER2 at high -but not extremely high- levels and to exhibit extreme CAR induction when exposed to HER2amp cells. The Transwell and 3D organoid experiments showed increased homing and penetration of the 5F clone with respect to NK-92 WT, again only when exposed to HER2amp/CEA+ CRC cells.

Active migration of the 5F clone towards HER2-overexpressing cells is unlikely to be mediated by the HER2-synNotch because it requires cell-cell engagement and mechanical stretching for activation. More likely, when 5F cells randomly encounter their target cells and get fully activated by concomitant synNotch and CAR engagement, they degranulate and kill target cells, leading to the release of chemotactic factors attracting additional 5F cells.

As a control, the NK-92 population only transduced with the HER2 synNotch, but not with the CEA CAR, is very interesting, both for the *in vitro* and *in vivo* experiments, to investigate if the physical engagement of the synNotch, stabilizing NK-target cell interaction, could lead to an increase in NK-92 killing activity even in the absence of the CAR. The results presented here show that synNotch engagement in the absence of CAR induction is not sufficient to significantly increase NK-92 activity. A second important control was the challenge of the 5F clone with HER2amp, CEA- target cells. In fact, it was proved that the sole CAR expression without engagement with the target antigen could promote a certain degree of effector activation, leading to exhaustion in the case of T cells[179]. This phenomenon was not observed in the functional activation experiments against HER2amp/CEA- target cells; probably because the CAR intracellular costimulatory domain was T cell specific and not NK specific. Exploring NK related CAR intracellular costimulatory domain as the NKG2D intracellular portion could be a very fascinating field of investigation for the future. 5F clone activity was found to be specific, and far more potent than NK-92 cells lacking the combinatorial system, even if a basal cytotoxic activity was measured in all the experiments. This

background activity was related to the presence of the NK-92 cell line's intrinsic anti-tumor activity.

In vivo, the 5F clone successfully controlled growth of HER2amp/CEA+ CRC xenografts, although it did not cause overt tumor regression. Notably, the basal killing activity of naïve NK-92 cells observed *in vitro* did not lead to any significant impairment of xenograft growth. This is not surprising, considering the poor homing of naïve NK-92 and of NK-92 HER2 synNotch *in vitro*, and further confirms the need for CAR expression to achieve proper effector activity. Mice treated with the 5F clone showed no signs of pain, and no organ toxicity was observed during gross autopsies (data not shown). In future studies, it will be critical to optimize the frequency of infusions and the number of infused effectors to achieve the best therapeutic outcome with limited toxicity.

Even if the 5F clone exhibited interesting homing and penetration capabilities *in vitro* and remarkable tumor growth reduction *in vivo*, it did not cause tumor regression; indeed, common criticisms to CAR-based strategies against solid tumors, aside from toxicity, include limited CAR-cell ability to invade tumors, low efficacy, and low persistence of effector cells in the bloodstream. In recent years, a major causal role for these issues has been attributed to the tumor microenvironment (TME). In this context, critical TME features include (i) physical barriers to immune cell penetration into the tumor, (ii) upregulated checkpoint ligands, (iii) a pro-tumor stromal niche, (iv) abundant immunosuppressive and pro-metastatic soluble factors, and (v) modulated expression of chemokines to preferentially recruit immunosuppressive leukocytes[180]. Knowledge about these features is currently leading to the development of CAR-T/NK cells that respond to TME elements or modify them to improve efficacy. To this aim, a possible strategy is to remodel the tumor-cellular composition and phenotype to reverse the immunosuppressive cell niche. CAR-T/NK cells have been genetically modified to secrete cytokines or other soluble factors that induce TME remodeling in a paracrine or endocrine manner. Indeed, several studies demonstrated the advantages of CAR based strategies involving co-expression of immunostimulatory cytokines. For example, Markley's group showed that co-expressing the CAR and immunostimulatory cytokines could significantly improve CAR-T cell proliferation, survival, and effector function. T cells that constitutively co-express a CD19-targeting CAR plus IL-2, IL-7, IL-15, or IL-21, have been shown to achieve better *in vivo* tumor

control[181] additionally, CAR-T cells engineered to secrete IL-12 or IL-18 have been shown in syngeneic mouse models to remodel the TME by reprogramming TAMs to an M1 phenotype and decreasing the presence of MDSCs and Tregs[182,183]. Along the same line, the 5F clone was transduced with a GAL4-driven IL-2 expression cassette, to release IL-2 after HER2 synNotch engagement, in addition to inducing CEA CAR expression. In this manner, IL-2 could perform an autocrine function by promoting NK-92 proliferation and survival, and a paracrine function by creating an inflammatory anti-tumoral microenvironment and potentially recruiting other anti-tumoral immune cells. The 5F GAL4 IL-2 cells demonstrated superior cytotoxic effect *in vitro*, and a significant improvement of *in vivo* activity, with increased survival of xenografted mice. To detect these improvements, we had to use non-irradiated cells, in a context therefore not directly amenable to clinical application. However, these results provide a proof of concept of the potential improvements obtainable using multiple synNotch-driven constructs. Considering that the *in vivo* experiment has been performed in immunocompromised mice, the observed superiority was most likely due to IL-2 activity on NK-92 cells themselves. Possible additional paracrine effects on the innate immune cells still present in the host mice remain to be explored also with irradiated NK-92 effectors.

It should be noted that constitutive overexpression of immunostimulatory cytokines, as previously described, can lead to unwanted side effects and increased toxicity. The strategy of rendering IL-2 expression dependent on synNotch engagement adds a further element of safety, by increasing effector function only in the target tissue.

To extend the applicability of the synNotch-CAR approach to additional subsets of CRC, an alternative to HER2 as a synNotch target was considered, maintaining CEA as the target of the synNotch-driven CAR to restrict effector activity to GI tissues. MSLN was chosen because it is a known CAR target for other tissues[184], but it is also highly expressed in a subset of CRCs, and its overexpression is associated with CRC aggressiveness and poor prognosis[163]. Indeed, the MSLN synNotch/CEA CAR clone 2C8 efficiently and specifically killed MSLNhigh/CEA+ CRC cells *in vitro*, warranting a follow-up with future *in vivo* experiments.

7. Conclusions and perspectives

In conclusion, this work showed the design, characterization, and preclinical validation of a combinatorial CAR-based immunotherapy strategy against colorectal cancer with HER2 amplification. The chosen approach for combinatorial targeting, based on the synNotch/CAR system has demonstrated excellent performance and flexibility, making it ideal for targeting different antigen combinations. The system was further improved by engineering HER2 synNotch/CEA CAR cells with synNotch-driven inducible IL-2 release. Flexibility of the synNotch system was demonstrated by changing the synNotch target, and so the combination of the antigens, from HER2/CEA to MSLN/CEA. This study potentially offers a future treatment option in the contest of HER2amp CRC, using the HER2 synNotch/CEA CAR strategy in CRC resistant to targeted therapy and in addition to HER2/EGFR targeted treatment in responsive cases.

8. References

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