Universitat Autònoma de Barcelona

Redirection of T cells as immunotherapeutic approach against HER2-positive breast cancer

Irene Rius Ruiz

Universitat Autònoma de Barcelona

Facultat de Medicina

Departament de Bioquímica i Biologia Molecular

Programa de Doctorat en Bioquímica, Biologia Molecular i Biomedicina

Redirection of T cells as immunotherapeutic approach against HER2-positive breast cancer

Thesis presented by Irene Rius Ruiz for the degree of Doctor of Philosophy (PhD) in Biochemistry, Molecular Biology and Biomedicine by Universitat Autònoma de Barcelona

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Director i Tutor

Dr. Joaquín Arribas López

PhD candidate

Irene Rius Ruiz

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SUMMARY

Despite the success of anti-HER2 therapies, the appearance of primary and acquired resistances is limiting the antitumor potential of the current treatments for HER2-positive breast cancer. Moreover, the expression of the tumour-associated antigen HER2 in normal epithelial tissues may cause undesired side effects upon anti-HER2 treatments. Therefore, there is a clinical need to develop more effective and safer treatments against HER2-driven tumours.

Immunotherapy is one of the most promising field in cancer treatment. One of the strategies to activate the immune system against tumours is the redirection of T cells via bispecific antibodies or CAR T technology. Despite the potential of these therapeutic strategies, to date, only CAR Ts and TCBs targeting certain haematological malignancies have been approved to treat patients.

One of the main hurdles in the development of CAR Ts and TCBs for solid tumours is the scarcity of tumour-specific antigens. Because of this shortage, CAR Ts and TCBs have been directed against tumour-associated antigens, which are also expressed in normal tissues, albeit at lower levels. These CAR Ts and TCBs have caused serious or even fatal toxicities because of their “on-target off-tumour” effects on normal tissues.

In this thesis, we demonstrate that p95HER2, a truncated form of HER2, is a *bona fide* tumour-specific antigen, expressed by a subset of HER2-positive breast cancer patients.

Moreover, we prove that the redirection of T lymphocytes via p95HER2-TCB or p95HER2 CAR Ts could be a safe therapeutic option against breast cancer, in contrast to other immune therapies targeting tumour-associated antigens.

RESUMEN

A pesar del éxito de las terapias contra HER2, la aparición de resistencias primarias y adquiridas limita el potencial antitumoral de los tratamientos actuales contra el cáncer de mama HER2-positivo. Además, la expresión de HER2 en tejidos epiteliales normales puede conllevar la aparición de efectos secundarios post-tratamiento. Por todo ello, hay una necesidad de desarrollar tratamientos más seguros y eficaces contra tumores HER2-positivos.

La inmunoterapia constituye un nuevo grupo de terapias que han revolucionado el campo del cáncer. Una de las estrategias para activar el sistema inmunitario contra tumores consiste en la redirección de células T a través de anticuerpos biespecíficos o de CAR Ts. A pesar del potencial de estas nuevas estrategias terapéuticas, hasta ahora, solo se han aprobado CAR Ts y TCBs para pacientes con enfermedades hematológicas.

Uno de los principales problemas en el desarrollo de CAR Ts y TCBs dirigidos a tumores sólidos es la falta de antígenos realmente específicos de tumor. Debido a esta falta, la mayoría de CAR Ts y TCBs desarrollados hasta ahora van dirigidos a antígenos asociados a tumor, los cuales también se encuentran en tejido sano, aunque a niveles mas bajos. Estos CAR Ts y TCBs han causado graves efectos secundarios o incluso toxicidades letales debido a la falta de especificidad de tejido tumoral.

En esta tesis, demostramos que p95HER2, una forma truncada de HER2, es un antígeno específico de tumor, expresado en un grupo de pacientes de cáncer de mama HER2-positivo.

Además, proponemos p95HER2-TCB y p95HER2 CAR Ts como estrategias terapéuticas inmunes que podrían suponer una opción más segura que las inmunoterapias que se dirigen a antígenos asociados a tumor.

ABBREVIATIONS

A488	AlexaFluor 488
Ab	Antibody
ACT	Adoptive T cell transfer
ADC	Antibody-drug conjugate
ADCC	Antibody-dependent cellular cytotoxicity
AIDS	Acquired Immune Deficiency Syndrome
ALL	Acute lymphoblastic leukaemia
ANOVA	Analysis of variance
APC	Antigen-presenting cells
BBB	Blood brain barrier
BC	Breast cancer
BiTE	Bi-specific T-cell engagers
BRCA-1	Breast cancer gene 1
BSA	Bovine serum albumin
bsAb	Bispecific antibody
CAR	Chimeric antigen receptor
CAR Ts	Chimeric antigen receptor T cells
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD45	Cluster of differentiation 45
CD8	Cluster of differentiation 8

CEA	Carcinoembryonic antigen
CMV	Cytomegalovirus
CRS	Cytokine release syndrome
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
Cy5.5	Cyanine 5.5
DC	Dendritic cells
DMEM	Dulbecco's minimal essential medium
Dnase	Deoxyribonuclease
ECL	Enhanced chemiluminiscence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Human epidermal growth factor receptor-1
ER	Receptor of oestrogen
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment crystallisable
FDA	U.S. Food and Drug Administration
FISH	Fluorescence in situ hybridization
GvHD	Graft-versus host disease
HAMA	Human anti-mouse antibody
HER	Human epidermal growth factor receptors

HER2	Human epidermal growth factor receptor-2
HER3	Human epidermal growth factor receptor-3
HER4	Human epidermal growth factor receptor-4
HIV	Human Immunodeficiency Virus
HR	Hormone receptor
HSC	Hematopoietic stem cell
Hygro	Hygromycin
i.p	Intraperitoneal administration
i.v	Intravenous administration
IDO	Indoleamine 2,3-dioxygenase
IgG1	Immunoglobulin G1
IHC	Immunohistochemistry
IL-2	Interleukin 2
IRGs	Immune-related genes
IVIS	In vivo imaging system
MAGE-3	Melanoma antigen E
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complexes
mTOR	Mammalian target of rapamycin
NEC	Normal epithelial cells
NFAT	Nuclear factor of activated T-cell

NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHL	Non-Hodking lymphoma
NK	Natural killer
NOD/SCID	Non-obese diabetic/Severe combined immunodeficiency
NSCLC	Non-small cell lung cancer
NSG	NOD scid gamma
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
pCR	Pathological complete response
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PD-L2	Programmed death-ligand 2
PDX	Patient-derived xenografts
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	Protein kinase C
PLC	Phospholipase
PR	Receptor of progesterone
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homolog
RPMI	Roswell Park Memorial Institute medium
scFv	Single-chain variable fragment

TAA	Tumour-associated antigens
TCB	T cell bispecific antibody
T_{CM}	T central memory
TCR	T cell receptor
Tem	T effector memory
TILs	Tumour-infiltrating lymphocytes
TKI	Tyrosine kinase inhibitor
TMB	Tumour mutation burden
TME	Tumour microenvironment
T_N	T naïve
TNBC	Triple-negative breast cancer
TRAC	T-cell receptor α constant
TSA	Tumour-specific antigen
T_{SCM}	T stem central memory
T_{TE}	T terminal effector
UTD	Untransduced T cell

INTRODUCTION

1. Breast cancer

Breast cancer (BC) is the most common cancer and the leading cause of cancer-related mortality in woman worldwide, accounting for 626,679 deaths in 2018 (Bray et al., 2018). Environmental factors, such as diet and lifestyle, may have an impact on BC incidence, as observed in the increase of Japanese women suffering breast cancer when adopting a western lifestyle (Iqbal et al., 2015). Moreover, hormonal factors, obesity, pregnancy and lactation history may also affect BC incidence (Kroemer et al., 2015). In addition, hereditary BC is responsible for the approximately 10% of all BC cases, mainly due to defects on *breast cancer* gene (*BRCA-1*) (Cancer Genome Atlas Network, 2012).

1.1. Subtypes

BC is a heterogeneous disease that can be divided into four major intrinsic biological subtypes: luminal A, luminal B, human epidermal growth factor receptor-2 (HER2)-positive and triple negative (Cancer Genome Atlas Network, 2012; Goldhirsch et al., 2011; Van't Veer et al., 2002; Perou et al., 2000). Each subtype is characterised by different risk factors, therapeutic response, disease progression, and preferential organ sites of metastases (Reis-Filho & Pusztai, 2011; Weigelt & Reis-Filho, 2009). Tumours are classified into these subtypes using few biomarkers analysed by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). These biomarkers are the hormone receptors of oestrogen (ER) and progesterone (PR), excess levels of HER2 protein, and/or extra copies of the *HER2* gene and the presence of Ki67 (Wolff et al., 2013; Hammond et al., 2010) (see **Table 1**).

Luminal A tumours are the most common subtype, representing 50-60% of all BCs. They are characterised by high levels of ER and/or PR and low levels of the proliferation marker Ki67. Patients with these characteristics have a good prognosis with a relapse rate significantly lower than the other subtypes. Still, recurrence is common in bone and treatment is mainly based on hormonal therapy (Haque et al., 2012; Kennecke et al., 2010).

Luminal B tumours comprise the 15-20% of all BCs and have a more aggressive phenotype and worse prognosis than luminal-A (Creighton, 2012). They are defined as ER^+ with high Ki67 and some are also $HER2^+$. The current treatment consists on hormonal therapy plus chemotherapy and additional anti-HER2 therapy in HER2-positive cases (Esserman et al., 2009). Bone, brain and lung are the preferable sites of metastases (Kennecke et al., 2010).

HER2-postive subtype accounts for the 15-20% of BCs. These tumours are characterised by a high expression of *HER2* gene and other genes involved in the HER2 pathway and/or HER2 amplicon, followed by an ER- profile. With the introduction of anti-HER2 therapies 20 years ago, such as the monoclonal antibody trastuzumab, the prognosis of this group of patients improved notably (Wuerstlein & Harbeck, 2017; Baselga et al., 2012). However, toxicities associated with the treatment are an important issue and the emergence of primary and acquire resistances also limits the antitumor potential. Consequently, patients frequently relapse, presenting metastasis mainly in brain (Pondé, Lambertini & de Azambuja, 2016; Kennecke et al., 2010).

Triple-negative breast cancer (TNBC) is the most heterogeneous group and it accounts for 15-20% of all BCs. TNBC is also the most aggressive type and as its name implies, lacks of ER, PR and HER2. Of note, 75% of BRCA-1 mutated patients are included in this group. The current treatment is limited to chemotherapy although PARP inhibitors are in advanced clinical development (Sonnenblick et al., 2015). This subtype of tumours together with the HER2-positive, are associated with the highest rates of metastasis, especially to brain and lung (Kennecke et al., 2010; Heitz et al., 2009).

Subtype	Incidence	Biomarkers	Treatment	Metastasis	Prognosis
Luminal A	50-60%	ER+ and/or PR+, Ki67 low, HER2-	Endocrine therapy	Bone	
Luminal B	15-20%	ER+ and/or PR+, Ki67 high, HER2-	Endocrine therapy ± chemotherapy	Bone	
		ER+ and/or PR+, HER2+	Endocrine therapy + anti-HER2 therapy + chemotherapy	Bone, brain, lung	
HER2-positive	15-20%	ER-, PR-, HER2+	Anti-HER2 therapy + chemotherapy	Brain, bone	
Triple negative	15-20%	ER-, PR-, HER2-	Chemotherapy	Brain, lung	

Table 1: Histological subtypes of breast cancer

BC classification currently used in the clinical practise. Prognosis before treatment is indicated. ER: Estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2. Adapted from (Goldhirsch et al., 2011).

Nowadays, gene expression signatures using high-throughput technologies have been developed not only to improve our understanding on BC, but also for clinical practise. For instance, PAM50, MammaPrint or Onco*type* DX are different assays that estimate the risk of recurrence of each individual patient and they provide prognostic information (Kwa, Makris & Esteva, 2017). On the other hand, additional genomic studies have given rise to new intrinsic molecular BC subtypes, such as claudin-low, previously included in the triple-negative subtype (Prat & Perou, 2011). Furthermore, a possible sub-stratification of HER2-positive breast cancer patients may be implemented in the future to avoid the use of chemotherapy in certain patients (Prat et al., 2019), although more studies are still required to validate this concept.

This thesis is focused on HER2-positive BC subtype.

1.2. HER2-positive BC

1.2.1. HER family

HER2 is a member of the human epidermal growth factor receptors (HER) family of membrane tyrosine-kinases receptors, known as ERBB family, which also includes EGFR, HER3 and HER4. The generic structure of the HER receptors comprise an extracellular ligand-binding domain, a transmembrane domain and an intracellular kinase domain. HER receptors are activated by several ligands, including epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) for EGFR, and neuroregulins for HER3 and HER4. Ligand-binding causes receptor dimerization, activation of the kinase domains, auto-phosphorylation and initiation of downstream signalling. As an exception, HER2 does not bind to any of the HER ligands and is constitutively in a conformation suitable for dimerization. On the other hand, HER3 has an impaired kinase domain but is capable of ligand binding and hetero-dimerization (Arteaga & Engelman, 2014).

The dimerization and activation of HER kinases initiates a variety of signalling pathways, principally the mitogen-activated protein kinase (MAPK), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and protein kinase C (PKC) pathways, resulting in cell proliferation, differentiation and survival. Homo-dimerization of HER4 or EGFR, as well as their respective hetero-dimerization with HER3, leads to relatively weak signalling. However, the heterodimers containing HER2 exhibit prolonged activation of downstream signalling pathways. The most active heterodimer is that composed of HER2 and HER3 (Arteaga & Engelman, 2014; Baselga & Swain, 2009)(**Figure 1**).

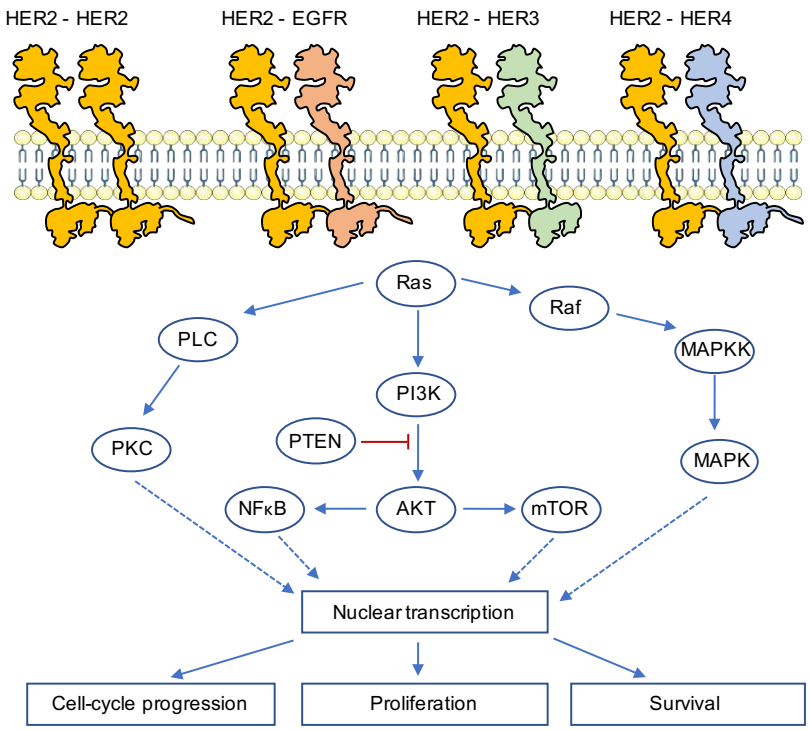


Figure 1: Main transduction pathways regulated by the four HER family members—EGFR, HER2, HER3, and HER4.

HER2 is the preferred dimerization partner for other HER receptors. Receptor phosphorylation results in activation of the indicated downstream signalling pathways. Consequently, different nuclear factors are recruited and modulate the transcription of different genes involved in cell-cycle progression, proliferation and survival. EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; PLC, phospholipase C; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; NFκB, nuclear factor κB; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase. Image adapted from (Fornaro et al., 2011).

1.2.2. HER2 and p95HER2

HER2 gene amplification is associated with a shorter disease-free survival. In fact, evidence suggests that HER2 amplification is an early event in human breast tumorigenesis and the HER2 status is maintained during progression to invasive disease, nodal and distant metastasis (Park et al., 2006).

HER2 overexpression has also been reported in 10-30% gastric cancers (Yano et al., 2004), 20-30% of ovarian cancers (Bartlett et al., 1996) and around 6% of non-small cell lung cancer (NSCLC) (Chen et al., 2014). In all cases, HER2 overexpression has also been correlated with a poorer outcome (Liu et al., 2018; Iqbal & Iqbal, 2014).

Certain HER2-positive tumours also express carboxyl terminal fragments of HER2, collectively known as p95HER2 fragments. Truncated p95HER2 receptors are generated by protease cleavage or by alternative initiation of translation of HER2. In the first case, the metalloprotease ADAM10 sheds the extracellular domain of HER2, generating an extracellular domain detectable in serum (sHER2) and a 95-100 kDa p95HER2 fragment anchored to the membrane (Gingras et al., 2017; Arribas et al., 2011). On the other hand, alternative initiation of translation from the AUG codons located in the 611 position generates a p95HER2 fragment of 100-115 kDa (611-CTF) (Anido et al., 2006). This work mainly focuses on p95HER2 fragment of 100-115 kDa, from now on, uniquely referred to as p95HER2.

p95HER2 is a constitutively active because of its ability to form homodimers maintained by intermolecular disulphide bonds. In fact, transgenic mice expressing this fragment developed tumours that, compared with tumours driven by full-length HER2, were more aggressive and invasive (Pedersen et al., 2009). As is the case of several active oncogenes, high levels of p95HER2 may lead to a senescent phenotype, characterised by a cell cycle arrest and a distinct secretory phenotype (Angelini et al., 2013).

Despite the short extracellular domain (41 amino acids), monoclonal antibodies against p95HER2 have been generated (Sperinde et al., 2010; Parra-Palau et al., 2010). This was possible because of the different three-dimensional structures of the extracellular juxtamembrane region in the p95HER2 fragment and in the full-length HER2. Consequently, the N-terminus of p95HER2 contains epitopes that are not accessible in full-length HER2 (Arribas et al., 2011).

The use of monoclonal antibodies to determine p95HER2 expression in human tumour samples revealed that about 30% HER2-positive breast tumours express p95HER2. Around the 80% of tumours expressing p95HER2 belong to the HER2-positive subtype and just one third of these tumours are also ER+ (Parra-Palau et al., 2010).

Of note, the expression of p95HER2 has also been detected in lung cancer (Cappuzzo et al., 2012) and at high levels in endometrial cancers (Hossler et al., 2018; Growdon et al., 2015). In fact, recent data in metastatic endometrial cancer have shown a significant increase of p95HER2:HER2 ratio compared to primary tumour samples (Hossler et al., 2018).

Several studies have shown that BC tumours expressing p95HER2 have an aggressive behaviour and tend to be resistant to trastuzumab (Sperinde et al., 2010; Scaltriti et al., 2007; Saez et al., 2006). However, when chemotherapy was combined with trastuzumab, p95HER2 expression was associated with a better pathological complete response (pCR) (Sperinde et al., 2018; Loibl et al., 2014; Loibl et al., 2011). Further studies from our laboratory have shown that chemotherapy induce a HER2 stabilization that may sensitize p95HER2 positive cells (Parra-Palau et al., 2014). In addition, Scaltriti and colleagues have shown that there is a positive correlation between HER2 and p95HER2 expression, although HER2 is a stronger predictor of response to trastuzumab and/or lapatinib compared to p95HER2 (Scaltriti et al., 2015).

1.2.3. Targeted therapies for HER2-positive BC

In early-stage HER2-positive BC, the current therapeutic regimen consists of neoadjuvant treatment with a combination of chemotherapy and anti-HER2 targeted therapy, mainly trastuzumab (described below). This is followed by surgery, radiotherapy and another twelve-month of HER2-targeted therapy. Endocrine adjuvant therapy may also be applied depending on the ER status (Wuerstlein & Harbeck, 2017). Around 90% of patients with stage I HER2-positive BC have excellent 7-year disease-free survival rates (Baselga et al., 2017). Unfortunately, a great number of patients are diagnosed at advanced stages, suffering a substantially worse prognosis. Moreover, despite the efficacy of trastuzumab, some patients do not respond to therapy. To treat these resistant tumours, several novel HER2-targeting agents have been approved: pertuzumab, trastuzumab-emtansine (T-DM1), lapatinib and neratinib (Baselga et al., 2017) (**Figure 2** and **Table 2**).

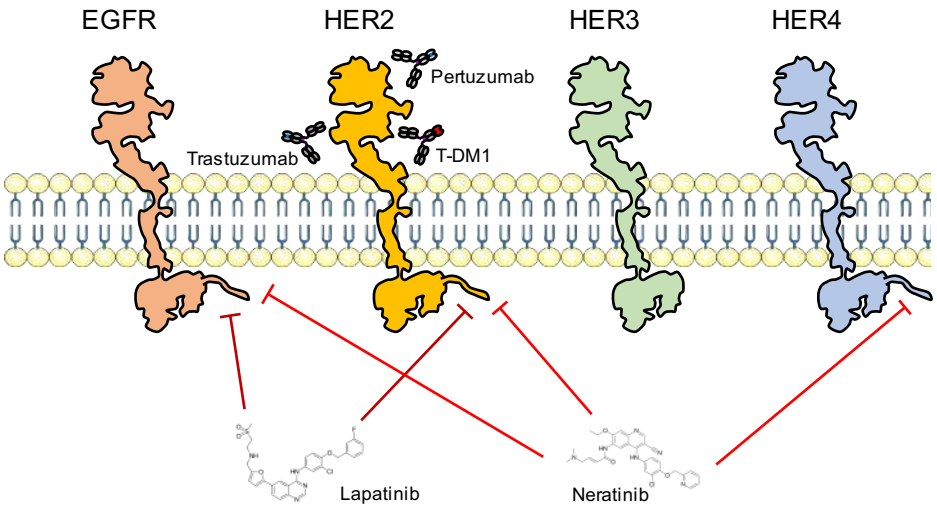


Figure 2: HER2-targeted agents approved for the treatment of BC.
The monoclonal antibodies trastuzumab, pertuzumab and T-DM1 target different extracellular domains of HER2. The tyrosine kinase inhibitors (TKIs) lapatinib and neratinib impair the intracellular kinase domains of EGFR, HER2 (for lapatinib) in addition to HER4 (for neratinib). Image adapted from (Baselga et al., 2017).

Trastuzumab, also known as Herceptin, is a humanized monoclonal antibody that binds to the extracellular portion of HER2 (Schmitz & Ferguson, 2009). It was the first HER2-targeted agent introduced in the clinic and it remains a key component of the most effective treatments against HER2-positive BC (National Comprehensive Cancer Network, 2017). Two main mechanisms of action have been proposed for trastuzumab: (i) suppression of HER2 signalling and (ii) initiation of an antibody-mediated immune response. Trastuzumab inhibits HER2 signalling by destabilizing HER2 heterodimers or by inducing internalization of HER2 receptors (Hudis, 2007). Importantly, trastuzumab does not recognize p95HER2 fragments (Arribas et al., 2011; Anido et al., 2006), which could explain the resistance observed in p95HER2-positive patients treated with trastuzumab as monotherapy (Sperinde et al., 2010; Saez et al., 2006). Xenograft experiments have confirmed the contribution of the immune system to the effect of trastuzumab through the Fc-gamma receptor, which induce antibody-dependent cellular cytotoxicity (ADCC). Therefore, immunosuppression may compromise the effect of trastuzumab (Park et al., 2010; Clynes et al., 2000).

Pertuzumab is another humanized monoclonal antibody against HER2 that binds to a different epitope than trastuzumab, preventing the formation of HER2-HER3 heterodimers. For this reason, it has shown effectivity in combination with trastuzumab, by the so-called dual-blockade (Baselga et al., 2010; Nahta, Hung & Esteva, 2004). In fact, it constitutes the first line of treatment, in combination with trastuzumab and chemotherapy, for metastatic BC patients (Swain et al., 2015; Baselga et al., 2012).

Trastuzumab-emtansine, also known as T-DM1, is an antibody-drug conjugate (ADC). This type of drugs allows the targeted delivery of cytotoxic compounds to the tumour, increasing efficiency and reducing toxicity. T-DM1 contains a derivative of maytansine (DM1), a potent cytotoxic agent, linked to trastuzumab. Upon binding to HER2, T-DM1 is internalized into tumour cells (Lambert & Morris, 2017). Currently, it constitutes the second line of treatment for metastatic BC patients (Diéras et al., 2017).

Drug	Structure	Line of treatment
Trastuzumab	Monoclonal antibody	1st line of treatment early and metastatic BC
Pertuzumab	Monoclonal antibody	1st line of treatment metastatic BC
T-DM1	ADC	2nd line of treatment metastatic BC
Lapatinib	TKI	3rd line of treatment metastatic BC
Neratinib	TKI	1st line of treatment early BC with high risk of recurrence

Table 2: Structure and line of treatment of the different anti-HER2 targeted drugs
The current anti-HER2 drugs consist in antibodies or tyrosine kinase inhibitors. Each drug is used at different lines of treatment of early or metastatic breast cancer. BC: Breast Cancer; ADC: antibody-drug conjugate; TKI: tyrosine kinase inhibitor

Lapatinib is a small-molecule tyrosine kinase inhibitor (TKI) that targets the catalytic activity of HER2 and EGFR. Despite showing clinical activity in HER2-positive metastatic patients, it is more toxic and less active than trastuzumab (Gelmon et al., 2015). Its use is reserved for the latest lines of treatment or for brain metastasis due to its penetration through the blood brain barrier (BBB) (Pivot et al., 2015).

Neratinib is an irreversible pan-tyrosine kinase inhibitor of HER2, EGFR and HER4. Due to its ability to prevent recurrence, neratinib has been recently approved as extended adjuvant therapy for patients with early-stage HR-positive, HER2-positive BC with high risk of relapse (Dhillon, 2019; Chan et al., 2016).

The introduction of these new anti-HER2 therapies has notably improved the prognosis of HER2-positive BC patients. However, some patients relapse, mainly developing brain metastasis (Pondé, Lambertini & de Azambuja, 2016; Kennecke et al., 2010).

1.2.3.1. Targeted therapies for brain metastasis in HER2-positive BC

Approximately 20-40% HER2-positive metastatic BC patients suffer from brain metastases, with a higher incidence on ER-negative patients. Brain metastases have a distinct biology and consequently, brain metastatic patients have a very poor prognosis (Gong et al., 2017). The current treatment is based on radiotherapy and surgery, supported by TKIs plus chemotherapeutic agents (Larionov, 2018). Trastuzumab has a limited effect on brain metastasis due to its poor permeability through the blood-brain barrier (BBB) (Hudis, 2007). In contrast, the small-molecules TKIs, penetrate much better because of its size and chemical properties (Morikawa et al., 2014). Clinical trials combining lapatinib and/or chemotherapeutic agents have demonstrated a substantial effect, although there is a still a limited clinical efficacy (Pivot et al., 2015). Recent studies from a phase II clinical trial showed that neratinib has a benefit when used in combination with chemotherapy (Freedman et al., 2019).

The development of therapeutic agents that modulate the immune system, known as immunotherapy, has revolutionized cancer treatment. Immunotherapy has proven to be an effective strategy for certain tumours and it may represent an alternative for the emerging resistances (Martin-Liberal et al., 2017; Palucka & Coussens, 2016).

2. The immune system and cancer

Tumour cells are closely connected to tumour microenvironment (TME), which contributes to the evolution and progression of the disease. Immune cells are one of the main components of the TME, which is also composed of endothelial cells and fibroblasts (Balkwill, Capasso & Hagemann, 2012). However, the role of the immune system in tumour progression has been questioned for decades. Only recently has become clear the concept of immunosurveillance, which postulates that tumours evolve and progress in an uncontrolled way only when malignant cells manage to escape from the immune recognition (Weinberg, 2013). Multiple studies comparing immunocompromised versus immunocompetent mice have shown higher ratios of tumour formation on mice with an impaired immune system, suggesting an active role of the immune compartment in cancer prevention (Shankaran et al., 2001). Human patients with an impaired immune system have confirmed the results observed in immunocompromised mouse models. For instance, patients with the Human Immunodeficiency Virus (HIV) and suffering the Acquired Immune Deficiency Syndrome (AIDS) frequently develop a variety of tumours triggered by viral infections (Engels et al., 2008; Frisch, Biggar & Goedert, 2000). Similarly, increased rates of cancer have been documented in patients undergoing organ transplantation, due to the use of immunosuppressors to prevent organ rejection (Euvrard, Kanitakis & Claudy, 2003; Birkeland et al., 1995).

2.1. The immune recognition

In healthy individuals, anticancer immune responses constantly occur to eliminate tumour cells. They are initiated by antigen-presenting cells (APCs), mainly dendritic cells (DC), which capture and process tumour antigens, and present them to T lymphocytes either at tumour sites or in the lymph nodes (Chen & Mellman, 2013) (**Figure 3**). Moreover, activated T cells are capable to recognize tumour antigens that are displayed on major histocompatibility complexes (MHCs) of malignant cells and proceed to destroy them (Schumacher & Schreiber, 2015).

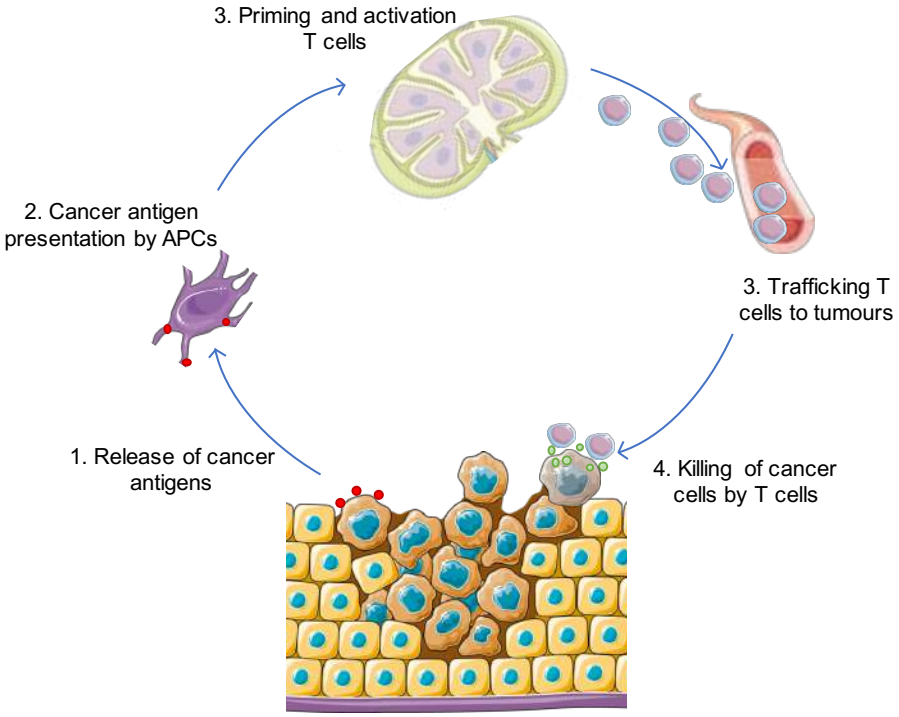


Figure 3: T cells are the main mediators of the anticancer immune recognition.
Tumours release antigens that are captured by APCs and presented to T cells. T effector cells migrate through blood vessels to the tumour site where the recognition of TCR and tumour antigen takes place through MHC class I, triggering to the cytotoxic effect of T cells and the subsequent lysate of tumour cell. Adapted from (Chen & Mellman, 2013).

2.1.1. Tumour antigens

Tumour cells tend to express unique proteins or overexpress certain molecules that healthy cells do not. The immune system takes advantage of these differences to discriminate cancer cells from healthy cells (Weinberg, 2013). There are two types of tumour antigens: tumour-specific antigens (TSA) and tumour-associated antigens (TAA).

Tumour-specific antigens (TSA) are antigens that uniquely exist in tumour cells; they are not present in healthy cells. TSA may originate from viral infections or mutations in DNA (*i.e* p53, Ras, EGFR) (**Table 3**). As compared with self-antigens, these novel proteins have not induced immune tolerance during development (Weinberg, 2013).

In fact, neoepitopes that are created by tumour-specific DNA alterations, also called neoantigens, are required for certain immunotherapies to be efficacious. However, a large fraction of mutations found in human tumours are not shared between patients. Therefore, deep-sequencing technologies are required to identify putative neoantigens that could predict T cell reactivity from each patient (Schumacher & Schreiber, 2015). In this thesis, we show that p95HER2 is a TSA and we explore its use as a safe target for two different immunotherapeutic strategies.

Tumour-associated antigens (TAA), as its name suggests, are antigens overexpressed in tumour cells but that are also present in healthy tissues. This group of TAA include HER2, carcinoembryonic antigen (CEA) or melanoma antigen E 3 (MAGE-3) (Hammarström, 1999; Gaugler et al., 1994) (**Table 3**). Therapeutic strategies that target TAAs may cause major side effects on healthy tissues due to the lack of tumour specificity. In fact, cardiotoxicity may be a serious side effect in BC patients because of the treatments directed to the HER2, which is also expressed in cardiomyocytes (Mehta et al., 2018; Junttila et al., 2014; Morgan et al., 2010).

Tumour-associated antigens		**Tumour-specific antigens**	
Protein	Human Tumour	Protein	Human Tumour
CEA	Colon cancer	**Bcr-Abl translocation**	CML
MAGE-3	Melanoma, NSCLC, liver carcinoma	**EGFRvIII**	Glioblastoma
PSA	Prostate cancer		
HER2	Breast, ovarian, NSCLC		

Table 3: Tumour-associated and tumour-specific antigens and the corresponding human tumour that they are present in. CEA: Carcinoembryonic antigen; MAGE-3: Melanoma antigen E 3; PSA: prostate-specific antigen; NSCLC: Non-small-cell lung cancer; CML: Chronic myelogenous leukemia.

Once dendritic cells have processed tumour antigens, they load them on major histocompatibility class (MHC) I or II molecules and present them to T cells in lymph nodes. This results in the priming and activation of effector T cells, which will traffic from lymph nodes to the tumour site, generating a specific anticancer immune response (Chen & Mellman, 2013) (**Figure 3**). Unfortunately, cancer patients have this immune recognition process impaired at some point, by different immune escape mechanisms.

2.2. Immune escape mechanisms in BC

Tumours thrive by using several factors and mechanisms to evade the immune system (Chen & Mellman, 2017; Marincola et al., 1999). The alteration of tumour antigen presentation machinery and the expression and/or secretion of immunosuppressive molecules or receptors are two general mechanisms of immune escape found in BC (Bates et al., 2018).

2.2.1. Alteration of tumour antigen presentation

Tumour cells decrease or even completely shut down the expression of tumour-specific antigens or TAAs, thus becoming invisible to the immune system. However, in some instances, cancer cells cannot stop displaying TSA or TAA because these tumour antigens may be essential drivers of tumour progression. In these cases, cancer cells down-modulate the synthesis of MHC class I proteins. In fact, decrease or loss of MHC I expression is associated with a more oncogenic potential and therefore, poor prognosis in a variety of tumours (Restifo et al., 1996). Importantly, the total absence of MHC I is much more infrequent because natural killer (NK) cells recognize cells without MHC I as defective and efficiently kill them (Mocikat et al., 2003). Mutations affecting the immune synapse between MHC complex and the epitopes have also been identified in some tumours (Kreiter et al., 2015).

2.2.2. Immunosuppressive molecules

Cancer cells can also actively express molecules that inhibit several components of the immune system. In BC, the most well-characterized immune evasion mechanisms include expression of immunosuppressive ligands, such as Programmed Death-ligand 1 (PD-L1), and the release of suppressive factors, such as interleukin 10 (IL-10), transforming growth factor beta (TGF-β) and indoleamine 2,3-dioxygenase (IDO) (Bates et al., 2018).

2.2.2.1. Immune checkpoint receptors

Inhibitory immune receptors or immune checkpoints are expressed on the surface of activated T cells to maintain immune response under control by inhibiting T cell activity when it is no longer needed. However, immune checkpoints also interact with immunosuppressive ligands used by tumour cells to suppress antitumor T cell activity (Topalian, Drake & Pardoll, 2015) (**Figure 4**).

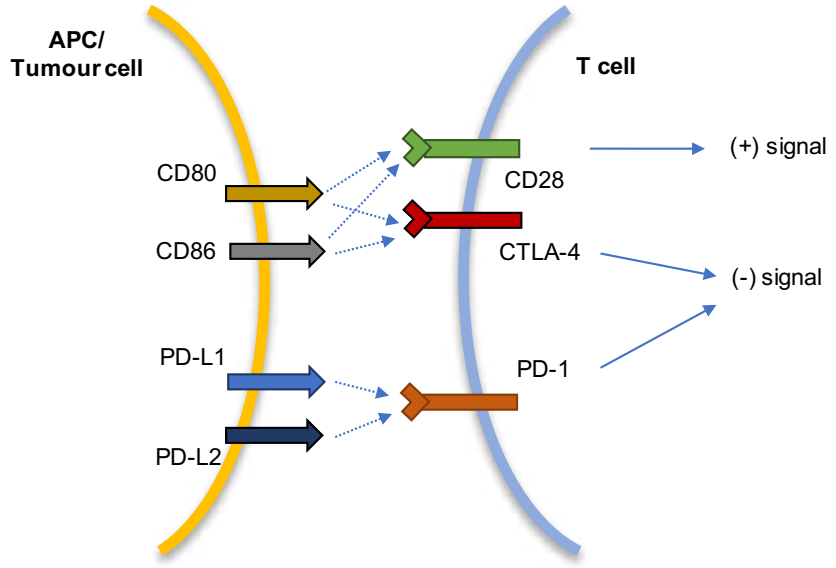


Figure 4: Immune checkpoint receptors and the interactions with its ligands.
The two defined ligands for PD-1, PD-L1 and PD-L2, mediate an inhibitory (-) signal on T cells. The two known ligands for CTLA-4 are CD80 and CD86, which also bind the co-stimulatory receptor CD28 on T cells inducing an activating (+) signal. Adapted from (Topalian, Drake & Pardoll, 2015).

In BC, immune checkpoint regulators are very frequently expressed, contributing to resistance to immunotherapy. Programmed cell death protein-1 (PD-1) and cytotoxic T-lymphocyte associated protein-4 (CTLA-4) are the two most studied checkpoint molecules so far (Topalian, Drake & Pardoll, 2015). CTLA-4 is known to be an early checkpoint, because of its rapid up-regulation upon T cell activation, competing with the co-stimulatory molecule CD28 for the binding of the shared ligands CD80 and CD86, reducing T cell proliferation and survival (**Figure 4**). On the other hand, PD-1 is primarily expressed on activated T cells receiving sustained antigen stimulation (late checkpoint). Its main ligands are PD-L1 and PD-L2, whose interaction with PD-1 inhibits downstream TCR signalling by phosphorylating several components of the pathway (**Figure 4**) (Chen & Mellman, 2017).

2.3. Immune biomarkers in BC

Different studies have shown that human BCs contain tumour-infiltrating lymphocytes (TILs), mainly composed by T cells and in a lower proportion B and NK cells (Salgado et al., 2014). In fact, efforts have focused on the molecular reclassification of specific BC subtypes taking into account immunity-related transcriptomic signatures. As an example, HER2-positive tumours have been subdivided into three clusters, one of which is enriched in immune-related genes (IRGs). Importantly, this subset of patients correlates with an improved disease outcome (Staaf et al., 2010).

The percentage of TILs in each BC subtype varies notably. Luminal tumours are the least infiltrated, with approximately 10% of TILs. HER2-enriched tumours harbour 15% of TILs and TNBC, the highest immune infiltrated subtype, contains 20-25% of TILs (Kroemer et al., 2015).

In the last 5 years, it has become clear that the presence of TILs at diagnosis is a prognostic factor in HER2-enriched and TNBC therapeutic setting (Denkert et al., 2015; Adams et al., 2014), experiencing the largest benefit patients with lymphocyte-predominant cancer (LPBC), defined as having TILs in ≥50% of the tumour (Loibl et al., 2014). Specifically, stromal TILs are a more robust prognostic factor than intra-tumour TILs, probably because the latter are less frequent and harder to discern (Dieci et al., 2015).

Because of the good prognostic value of TILs in the HER2-enriched subtype, strategies to boost the T cell infiltration seem to be a reasonable therapeutic approach. In this thesis, we explore two immunotherapeutic strategies that redirect T lymphocytes to the tumour site in a subset of HER2- positive BC.

3. Cancer immunotherapies

Activating the immune system for cancer therapy has long been a goal in immunology and oncology. In the last decade, remarkable advances in this field have progressively changed the paradigm of cancer care and marked the beginning of a cancer immunotherapy revolution (Mellman, Coukos & Dranoff, 2011).

In the last year, the FDA has granted approvals for 10 immunotherapeutic agents, including two chimeric antigen receptor T cells (CAR Ts), eight anti-PD-1 and anti-PD-L1 agents and one bispecific antibody (bsAb) (Locke et al., 2017; Grupp et al., 2013; Topalian et al., 2012; Topp et al., 2011).

Among the different strategies to enhance the antitumor effect of the immune system, two major groups are included. One approach takes advantage of an already existing immune reaction and pursues to boost the immune recognition. The other strategy consists in redirecting T cells into the tumour site, which tends to be immune-deprived. Depending on tumour nature, one or even both strategies can be applied.

3.1. Boosting an existing immune response

3.1.1. Check-point inhibitors

Cancer cells have the ability to activate different immune checkpoint pathways and induce an immunosuppressive environment (**Figure 4**) (Topalian, Drake & Pardoll, 2015). Immune checkpoint blockade, mainly through monoclonal antibodies against PD-1/PD-L1 and CTLA-4, have resulted in durable responses across many tumour types, leading to a number of FDA-approved agents and many others in clinical development (Tang et al., 2018). However, a large number of patients do not respond, so predictive biomarkers of response are still required (Martin-Liberal et al., 2017). So far, tumour mutation burden (TMB) and inflammatory biomarkers, such as T-cell inflamed profile and PD-L1 expression, appear to be the best predictors of response (Cristescu et al., 2018; Miao et al., 2018).

3.1.2. Co-stimulatory agonists

Full T cell activation and proliferation is a complex process that requires a primary initiating signal (signal 1) in addition to secondary co-stimulatory receptor engagement (signal 2) and cytokine receptor stimulation (signal 3) (**Figure 5**) (Kershaw, Westwood & Darcy, 2013).

Several co-stimulatory receptors have been identified, including CD28, 4-1BB and OX-40. They are attractive targets for cancer therapy because their stimulation by their specific ligands increases antitumor immune responses (Melero et al., 1997; Harding et al., 1992). Agonist antibodies have been developed for several co-stimulatory receptor targets and are currently under clinical development. To date, 4-1BB is the most advanced, with agonists already in phase II trials. As monotherapies, most co-stimulatory agents tend to have limited antitumor activity, so combination with other immune therapies seems to be a promising approach (Mayes, Hance & Hoos, 2018). In fact, Claus and colleagues have recently showed an enhanced antitumor response when combining a 4-1BBL agonist with a T-cell bispecific antibody (Claus et al., 2019).

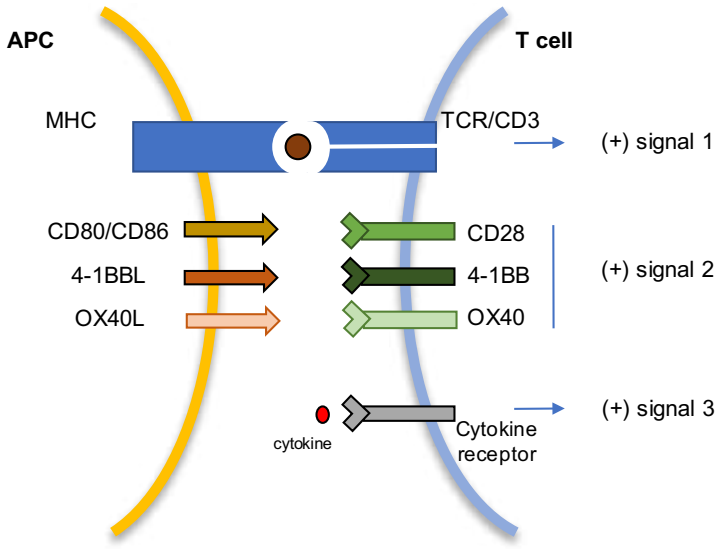


Figure 5: Full T cell activation requires three signals.

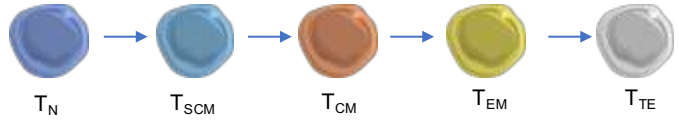
Activation of T cells is initiated by the interaction of its TCR with peptides displayed by MHC molecules on the surface of APCs, transmitting a primary activating signal, known as signal 1. This signal alone is insufficient to fully activate T cells, which require co-stimulation that is mediated by several co-stimulatory receptors (CD28, 4-1BB, OX40) with its corresponding ligands. Further enhancing of T cell activation is provided by cytokines through the engagement of cytokine receptors on T cells (signal 3). Adapted from (Kershaw, Westwood & Darcy, 2013).

3.2. Redirection of T lymphocytes

3.2.1. T lymphocytes subsets

The intrinsic properties related to the differentiation state of T cells are crucial to the success of several immunotherapies (Sadelain, Rivière & Riddell, 2017). T cell differentiation begins when mature T cells are released from the thymus into peripheral blood as naïve T cells. Following antigen recognition and co-stimulatory signals (**Figure 5**), naïve T cells clonally expand into effectors, and a small proportion of these will develop into memory T cells, capable of persisting in the long term (**Figure 6**) (Lugli & Gattinoni, 2015a; Zhang & Bevan, 2011). It is believed that each T cell differentiation state is associated with different epigenetic patterns (Akondy et al., 2017).

While naïve T cells are a fairly homogeneous population, memory T cells are highly heterogeneous (**Figure 6**). Memory T cells are divided into central memory and effector memory T cells, based on the migratory capacity and immediacy of effector functions. Central memory T cells act as reservoir of memory T cells in a stem cell manner (Lanzavecchia & Sallusto, 2002). An additional subset of T lymphocytes are the terminally differentiated cells, known as terminal effectors. They are abundant in CD8 but rare in CD4 lymphocytes, displaying effector functions and being defective in proliferative and survival capacities (Chattopadhyay et al., 2009; Brenchley et al., 2003).



	T_N	T_{SCM}	T_{CM}	T_{EM}	T_{TE}
CD45RO	-	-	+	+	-
CD45RA	+	+	-	-	+
CCR7	+	+	+	-	-
CD28	+	+	+	-	-
CD57	-	-	-	+/-	+
CD95	-	+	+	+	+

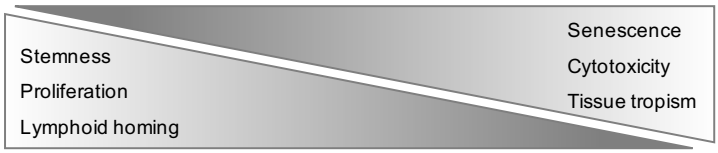


Figure 6: Differentiation state of peripheral T cells in humans

Expression of multiple surface markers on T cells, such as CD45RO, CD45RA, CCR7, CD28, CD57 and CD95 allows to identify several T cell subsets, differing on stemness state, proliferation ratio and tissue homing. T_N: naïve T cell; T_{SCM}: stem cell memory T cell; T_{CM}: central memory T cell; T_{EM}: effector memory T cell; T_{TE}: terminal effector T cell. Adapted from (Lugli & Gattinoni, 2015a).

So far, the most appropriate T cell subset for T cell redirection remains unknown. In this thesis, we explore which T cell subsets could be appropriate for T cell redirection through bispecific antibodies.

3.2.2. Bispecific antibodies

Bispecific antibodies (bsAbs) are engineered molecules designed to recognize two different antigens. There are many formats, differing in manufacturing, valency, Fc-mediated functions and *in vivo* half-life (**Figure 7**). The efficacy of each format may vary depending on the characteristics of the targets (Labrijn et al., 2019; Kontermann & Brinkmann, 2015).

T cell bispecific antibodies or TCBs is the largest group of bsAbs. Their mechanism of action consists in engaging T cells with malignant cells and form an immune synapse. The binding of TCBs to the T cells, mainly through CD3ε domain, induces TCR activation and triggers the release of granzyme and perforin, which eventually lysate the target cell. Therefore, TCBs resemble the mechanism of TCR-peptide-loaded MHC interaction, including the sequence of events, molecular composition and activation of signalling pathways (Offner et al., 2006). Importantly, TCBs stand out for bypassing MHC restriction and causing activation independent of the TCR specificity. It has been reported that the activity of TCBs depends on the properties of the tumour antigen *i.e* mobility in the membrane (Pfosser et al., 1999). In addition, the dimensions of the tumour antigen and the distance to the target cell membrane are known to be critical for a proper cytotoxic effect of the TCBs (Li et al., 2017; Bluemel et al., 2010).

On the other hand, prompted by the clinical success of antibodies against the immune checkpoints, bsAbs targeting these and other negative immune receptors are emerging, such as PD-1 x CTLA-4 bsAb (Labrijn et al., 2019). In addition, targets that positively regulate T cells, such as OX-40 and 4-1BB are also being targeted by bsAbs (Claus et al., 2019).

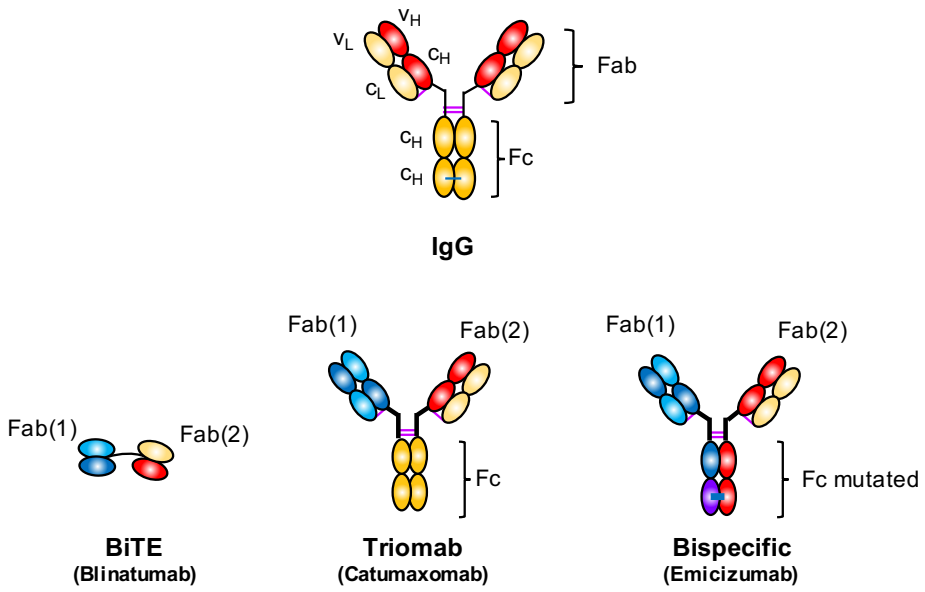


Figure 7: Schematic representation of various bispecific antibody formats

The upper line depicts a general immunoglobulin G as an example. The bottom line shows different formats of bsAb: BiTE, Triomab and Bispecific.

So far, two bsAbs have been approved: blinatumomab and emicizumab (**Figure 7**). The first one is a BiTE TCB against the antigen CD19 for the treatment of non-Hodking lymphoma (NHL) and acute lymphoblastic leukaemia (ALL) patients (Gökbuget et al., 2018). On the other hand, emicizumab is a bispecific IgG used as an enzyme-cofactor replacement in haemophilia A (Oldenburg et al., 2017). Despite the recent withdrawal of catumoxomab in 2017, a TCB targeting EpCAM for the treatment of malignant ascites, there is an increasing interest in developing TCBs for cancer treatment. In fact, there are more than 43 TCBs currently in clinical development (Labrijn et al., 2019). Of note, tumour targets being targeted by TCBs for the treatment of haematological malignancies are commonly expressed on normal B cells, but depletion of these cells can be tolerated without inducing severe adverse effects. By contrast, many solid tumours antigens, such as HER2, CEA or EpCAM, are expressed at low levels in critical normal tissues. Therefore, TCBs designed against these tumour-associated antigens may induce severe adverse events (Junttila et al., 2014). Nevertheless, around 14 TCBs against TAA in solid tumours are being evaluated in the clinic, including HER2, EpCAM or CEA (Labrijn et al., 2019).

To avoid severe adverse events due to the lack of tumour specificity, choosing a genuinely tumour-specific antigen is an obvious alternative. In this thesis, we develop a bispecific TCB against p95HER2. Moreover, we demonstrate that this therapeutic agent not only induce a potent antitumor effect but also bypass the usual side effects observed in other immune therapies targeting tumour-associated antigens.

3.2.3. Chimera-antigen receptor T (CAR T) cells

Adoptive T cell transfer (ACT) is opening new avenues in cancer immunotherapy, involving the infusion of autologous lymphocytes previously cultured *ex vivo*. Three forms of ACTs are being developed so far, including tumour-infiltrating lymphocytes (TILs), T cell receptor (TCR) T cells and CAR T cells (Rosenberg et al., 2008).

TIL therapy induce durable responses in melanoma, but its applicability is dependent on the existence of enough TILs in patients and the immunogenicity of the tumours (Verdegaal et al., 2016).

Gene-engineered T cell technology has been applied to generate TCRs or CARs. TCR T cell immunotherapy relays on TCR recognition of peptides bound to MHC-complexes in the surface of tumour cells. TCRs holding an enhanced affinity for different peptides-MHCs are being developed, with efforts focused on the recognition of tumour neoantigens (June, Riddell & Schumacher, 2015). Finally, CAR T therapy is the first form of gene transfer therapy that received the commercial approval by FDA (June et al., 2018). For the purpose of this thesis, we are going to describe this type of ACT in depth.

CARs are synthetic receptors that combine the antigen-binding domain of a single-chain variable fragment (scFv) from an antibody with intracellular signalling motifs capable of T cell activation (Eshhar et al., 1993). In contrast to TCRs, CARs only recognize antigens present on the cell surface and this recognition occurs independently of the MHC. Thus, it potentially permits the treatment of tumours that have acquired defects on antigen presentation as mechanisms of immune evasion (Khong & Restifo, 2002).

Depending on their complexity, several engineered CARs have been described (**Figure 8**). First generation CARs harbour CD3ζ unit as the intracellular signalling domain. Second generation CARs contain an additional co-stimulatory signalling domain, mainly CD28 or 4-1BB. Each co-stimulatory domain imparts different functional and metabolic profiles. CD28-based CARs mainly promote T cell proliferation with a self-limited persistence, while 4-1BB-based CARs induce a less potent effect but support a greater T cell persistence (Kawalekar et al., 2016; Zhao et al., 2015). The signalling domain of third generation CARs contains two co-stimulatory signals. Fourth-generation CARs, the so-called TRUCKs, combine second generation CARs with factors that enhance antitumor activity, such as cytokines, co-stimulatory ligands or enzymes that degrade the matrix of solid tumours (Hartmann et al., 2017).

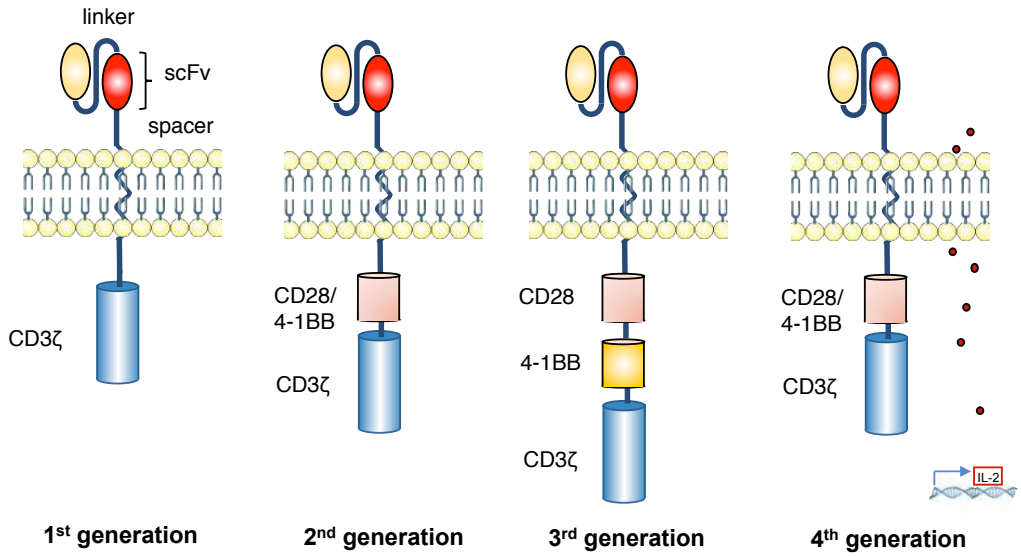


Figure 8: CAR T cell designs

All CAR T designs contain a single chain variable fragment (scFv) that recognize an antigen domain and a signalling domain that provides 'signal 1' to activate T cells. A co-stimulatory signalling domain that provides 'signal 2' is added in second-generation CARs, and in third-generation CARs two co-stimulatory signalling domains are included. Fourth-generation CARs, the so-called TRUCKs, combine second generation CARs with factors that enhance antitumor activity, such as cytokines (signal 3).

Two CAR Ts have gained commercial approval by the regulatory medical agencies for treating ALL and NHL patients: Kymriah and Yescarta. Both CAR Ts target CD19 but differ in the co-stimulatory signal. Nowadays, there are more than 250 clinical trials testing CAR Ts. However, the majority are focused on haematological malignancies, reflecting the limited success in solid tumours (June et al., 2018).

CAR T cell therapies for solid tumours raise several challenges: finding a right tumour antigen, the ability to expand in an often-immunosuppressive TME and avoiding adverse side effects. CAR Ts targeting HER2 have led to fatal toxicity, presumably because of the expression of the antigen in healthy tissues (Morgan et al., 2010). Currently, lower affinity HER2 CAR Ts together with lower doses are being investigated in HER2-positive sarcoma patients (Ahmed et al., 2015). So far, clinical trials of CARs targeting other TAA in solid tumours, including mesothelin, CEA and the GD2, have induced minimal antitumor activity when major toxicities were reduced (June et al., 2018).

In this thesis, we also propose p95HER2 CAR Ts as a novel therapeutic strategy against a subset of HER2-positve breast cancer.

HYPOTHESIS & OBJECTIVES

Hypothesis

The appearance of primary and acquired resistances is limiting the antitumor potential of the current anti-HER2 treatments. Based on the need to develop novel therapeutic approaches, we hypothesized that redirection of T cells via p95HER2 is an effective and safe way to treat a subset of HER2-positive tumours.

Objectives

1. Evaluation of p95HER2 in normal tissues.
2. Generation and characterization of a bispecific antibody against p95HER2 (p95HER2-TCB).
3. Assessment of the antitumor effect of p95HER2-TCB *in vitro.*
4. Evaluation of the effectivity of p95HER2-TCB on cells expressing basal levels of HER2.
5. Establishment of a p95HER2 threshold required for effective treatment.
6. Evaluation of the efficacy of p95HER2-TCB *in vivo.*
7. Study the antitumor potential of p95HER2-TCB on intracranial lesions.
8. Development of CAR T cells against p95HER2.
9. Assessment of the antitumor effect of p95HER2 CAR Ts *in vitro.*

MATERIALS & METHODS

Cell lines

MCF7 (#HTB-22) were purchased from ATCC and maintained in Dulbecco's minimal essential medium:F12 (DMEM:F12) (1:1) (#21331-046, Gibco-LifeTechnologies), supplemented with 10% fetal bovine serum (FBS) (#10270-106, Gibco-LifeTechnologies) and 1% L-glutamine (#X0550-100, Invitrogen). **MCF7p95HER2** cells were generated by lentiviral transduction of pTRIPZ-p95HER2 vector, generated by a modification of the pTRIPZ vector (Open Biosystems, Thermo Scientific) to allow p95HER2 overexpression, and pTRIPZ-shp21. pTRIPZ-shp21 was generated by cloning shp21 sequence from pGIPZ CDKN1A shRNA from Open Biosystems-Thermo Scientific (RHS4430-200281172 clone V3LHS-322234) into pTRIPZ vector. Cells were supplemented with 1 µg/ml puromycin (#P7255, Sigma-Aldrich) and 200 µg/ml Geneticin G-418 (#11811-031, Gibco-LifeTechnologies). **MCF7 HER2** were generated by retroviral transduction of pBabe-hygro HER2 vector and supplemented with 100 µg/ml hygromycin (#10687-010, Gibco-LifeTechnologies).

MCF10A (#CRL-10317) were purchased from ATCC and maintained in DMEM:F12 supplemented with 10% FBS, 1% L-glutamine, 9 µg/ml of insulin (#i9278, Sigma-Aldrich), 0,5 µg/ml hydrocortisone (#H0888, Sigma-Aldrich) and 20 ng/ml of EGF (#E9644, Sigma-Aldrich). **MCF10Ap95HER2** were generated by lentiviral transduction of pLEX-p95HER2 and retroviral transduction of pQCXIH-empty. **MCF10A HER2** were generated by lentiviral transduction of pLEX-empty and retroviral transduction of pQCXIH-HER2. **MCF10A HER2-M611A** were generated by retroviral transduction of pQCXIH-HER2 previously mutated in M611A by site-directed mutagenesis. All MCF10A transfectants were supplemented with 100 µg/ml hygromycin and 1 µg/ml puromycin.

MKN45 (#CRL-1739) were purchased from ATCC and maintained in RPMI 1640 (#61870-044, Gibco-LifeTechnologies) supplemented with 10% FBS and 1% L-glutamine.

Jurkat-Lucia NFAT (#SL0032) cells were purchased from Signosis and maintained in in RPMI 1640 and supplemented with 10% FBS and 1% L-glutamine.

Patient-derived xenografts (PDXs)

Human breast tumours used in this work were from biopsies or surgical resections at Vall d'Hebron University Hospital, Barcelona, and were obtained following institutional guidelines. The institutional review boards (IRBs) at Vall d'Hebron Hospital provided approval for this study in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients who provided tissue and/or blood.

For BC PDXs, fragments of patient samples were implanted into the fat pad of NOD.CB17-Prkdcscid (NOD/SCID) (#SM-NOD-5S-F, Janvier) or NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) (#005557, Charles River Laboratories) mice. 1 μM of β-estradiol (#E8875-1G, Sigma-Aldrich) was added to drinking water. Tumour xenografts were measured with callipers 2 times a week, and tumour volume was determined using the formula: (length x width^2) x (π/6). Mouse body weight was monitored once a week.

Western blot

Primary antibodies recognizing HER2 (α-C-terminalHER2) (CB11, #MU134-UCE, BioGenex), human p95HER2 (α-p95HER2) (32H2, in-house), human GAPDH (#Ab128915, Abcam) were used. As secondary antibodies, we used ECL rabbit IgG, HRP-linked whole antibody (from donkey, #NA934-1ML, Amersham GE Healthcare) and ECL mouse IgG, HRP-linked whole antibody (from sheep) (#NA931, Amersham GE Healthcare).

Immunohistochemical analysis

PDXs or human breast cancer sample tissues were fixed in 4% formaldehyde (#2529311315, Panreac) buffered to pH=7 (stabilized with methanol) for 24 hours and then paraffin-embedded (FFPE). Tissue sections of 4 µm thickness were mounted on positively charged glass slides and immunostained with α-HER2 (#A0485, Agilent Technologies), α-p95HER2 (32H2, in-house), α-cytokeratin (#M3515, Dako) and α-CD8 (#5937248001, ventana, Roche). A certified pathologist, R.F., evaluated p95HER2 expression by histoscore (H-score), a semi-quantitative assessment of intensity of staining (graded as: 0, non-staining; 1, weak; 2, median; or 3, strong, using adjacent normal tissue as reference) and the percentage of positive cells. R.F. also calculated the percentages of cytokeratin and CD8 positive cells. Tissue microarrays, MNO1021 and ab178227, were purchased from Biomax and Abcam, respectively and immunostained as tissue sections.

PBMCs, CD4 and CD8 isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from pre-analyzed buffy coats obtained from healthy donors through the Blood and Tissue Bank of Catalonia (Banc de Sang i Teixits) or peripheral blood from patients from Vall d'Hebron Hospital. Blood was diluted with 1xPBS(L0615-500, VWR) at 2:1 blood:PBS ratio, and transferred to 50 ml tubes with Ficoll-Plus-Paque (#70-1440-02, GE Healthcare) at 4:3 blood:ficoll ratio, followed by a centrifuge of 400g with no breaks for 30 minutes.

The interphase layer was collected and incubated with Red Blood Cell Lysis Buffer (#00-4333-57, eBioscience) for 4 minutes at RT. Cells were centrifuged at 400g for 5 minutes, washed twice with 1xPBS and counted. CD4 and CD8 cells were purified from PBMCs using specific beads (CD4 #130-045-101, CD8 #130-045-201, Miltenyi biotec), following manufacturer's protocol.

T cell subsets isolation

CD4 and CD8 previously purified were stained with 1/300 hCD8 PE-Cy7 (#344712), 1/300 hCD4 BV421 (#317434), 1/1000 CD45RO-PE (#304205) and 1/100 CCR7-BV650 (#353233), all from BioLegend for 30 minutes. Zombie Aqua (#423101, Biolegend) was used as a viability marker at 1:1000 dilution Then, cells were cell sorted according to the gating strategy showed in Figure 13. Recovered T cell subsets were counted and used straight away for co-culture experiments.

Viability assays and T cell activation

Tumours derived from PDXs were excised and digested as described above. Cell suspension was plated in 40 µl dots of Matrigel (#354234, Corning) in 48-well flat plates. Once organotypic cell cultures were established, organoids were digested to single cells. Then, cells were labelled with CFSE (#C34554, Invitrogen) according to the manufacturer's protocol.

CFSE-labelled cells (derived from PDXs or cell lines) were co-cultured with PBMCs from healthy donors or matched patients at the indicated T:E ratio in the presence of TCB or vehicle in 96-well U bottom plates. After 48-72 hours of incubation, the mixture of cells was washed with 1xPBS and re-suspended in 1xPBS, 2.5 mM EDTA, 1% BSA, and 5% horse serum for 20 minutes. Then, cells were stained with hCD8 PE-Cy7 (#344712), hCD4 BV421 (#317434), hCD25 APC (#302610) all from BioLegend at 1:300 dilution. Zombie Aqua (#423101, Biolegend) was used as a viability marker at 1:1000 dilution. CFSE positive cells were counted and the percentage of corresponding leukocytes measured on LSR Fortessa (BD Bioscience) and analysed with FlowJo software.

Profiling humanized mice

Blood from humanized mice (50 µl) was extracted, washed twice with 1xPBS and incubated twice with red blood cell lysis buffer following manufacturer's protocol. Then, cells were incubated with 1xPBS, 2.5 mM EDTA, 1% BSA, and 5% horse serum for 20 minutes. Then, cells were stained with a myeloid or lymphoid panel for 30 minutes. The myeloid panel contained: 1/300 hCD45-PE (#304205), 1/300 mCD45-A488 (#103122), 1/500 CD11b-APC (#101211), 1/300 CD15-BV421 (#323039) and 1/100 CD14-Percpcy5.5 (#325621). The lymphoid panel included 1/300 hCD45-PE (#304205), 1/300 mCD45-A488 (#103122), 1/400 hCD3-APC (#300412), 1/100 CD56-Percpcy5.5 (#362505) and 1/500 CD19-APC.Cy7 (#302217), all from Biolegend. Zombie Aqua (#423101, Biolegend) was used as a viability marker at 1:1000 dilution.

Humanized xenograft models

NSG mice were injected orthotopically with 3 x 10^6 MCF7p95HER2/HER2/ parental cells or implanted with approximately 3 x 3 mm tumour fragments. Once tumour volume reached 200 mm^3, animals were intraperitoneally (i.p.) injected with 10^7 PBMCs obtained from healthy donors or matched patients. After 48 hours, animals were treated bi-weekly with 1 mg/kg of p95HER2-TCB, non-targeting TCB, or vehicle intravenously (i.v.). In the case of MCF7p95HER2 cells, mice were maintained in the presence of doxycycline (1g/L) in the drinking water.

To obtain immunodeficient mice with a reconstituted human immune system, HSC-$CD34^+$ cells were purified from human cord blood obtained through the Blood and Tissue Bank of Catalonia. These cells were i.v injected into five-week-old NSG mice previously treated i.p. with 15 mg/kg of busulfan to ablate the hematopoietic system of the mouse. After 4-5 months, the percentages of circulating human CD45 cells were determined, and mice containing > 30% hCD45 in peripheral blood were orthotopically implanted with approximately 3 x 3 mm tumour fragments. Once tumours reached 200 mm^3, animals were randomized and treated bi-weekly with 1 mg/kg of p95HER2-TCB or vehicle (i.v.).

At the end of the experiment, mouse blood and tumours were analysed. Tumours were cut into small pieces and digested in 300 U/ml Collagenase IA and 1 µg/ml of DNAse I in RPMI medium. After 1 hour of incubation at 37ºC with 80 rpm shaking, the mixture was filtered through 100 µm strainers (#352360, Corning). Red blood cell lysis was performed in digested tumours and blood samples. After a wash with PBS, the cells from tumour and blood were resuspended in PBS, 2.5 mM EDTA, 1% BSA, and 5% horse serum for 20 minutes. Then, cells were stained with hCD45 PE (#304008); mCD45 AF488, (#103122); hCD3 PerCP-Cy5.5 (#300430); hCD8 PE-Cy7; hCD4 BV421; hCD25 APC; all used at 1:300 dilution. Zombie Aqua (#423101, Biolegend) was used as a viability marker at a 1:1000 dilution. Samples were acquired on LSR Fortessa and analyzed with FlowJo software. Box plots show the percentage of the indicated live leukocytes ($CD45^+$ or $CD8^+$). Lower and higher whiskers indicate 10th and 90th percentiles, respectively; lower and higher edges of the box indicate 25th and 75th percentiles, respectively; the inner line in the box indicates 50th percentile.

Quantitative p95HER2 assay (VeraTag)

p95HER2 was quantified using the VeraTag platform and a monoclonal antibody against p95HER2 that does not recognize full-length HER2, as previously described in (Sperinde et al., 2010).

Jurkat-luciferase assay

PDXs tumours were excised and cut into the smallest pieces possible, incubated for 1 hour with 300 U/ml Collagenase 1A and 1 µg/ml of DNAse I in RPMI medium. After 1 hour of incubation at 37ºC with 80 rpm shaking, the mixture was filtered through 100 µm strainers (#352360, Corning), incubated in red blood cell lysis buffer 5 minutes and cell counted (Vi-Cell counter, Beckman). Single target cells were co-cultured with effector Jurkat-Lucia cells at a 1:5 T:E ratio. Luciferase signal was measured using Luciferase Assay System (#E1501, Promega) and following manufacturer's protocol.

Intracranial tumour assay

MCF7p95HER2 cells were transduced with the lentiviral vector pLENTI-CMV-V5-Luc Blast (MCF7 p95HER2/luc cells) and selected with Blasticidin (#ant-bl-1, Ibian Technologies). Then, cells were stereotactically inoculated in the brains of NSG mice humanized with HSC-CD34^{+} cells as described above, in the presence of doxycycline (1 g/L) in the drinking water. The rate of tumour growth was monitored by in vivo bioluminescence imaging with the IVIS-200 imaging system from Xenogen (PerkinElmer).

CAR vector design and production

Vector plasmids coding for p95HER2 CARs (hp95HER2, p95HER2 (II) and p95HER2 (III)) were synthesized and cloned into pMSGV-1 retroviral vector (Genscript, Netherlands). Then, stocks of p95HER2 CARs, HER2 CAR and Empty CAR retrovirus were produced. Briefly, 0,7 µg of envelope plasmid (RD-114) and 1.5 µg of transfer plasmid (p95HER2, HER2, Empty CARs in pMSGV-1) were co-transfected in GP-293 cells (#631458, Clontech). After 2 and 3 days, cell supernatant containing retrovirus particles was collected and store at -80C for future transductions.

Transduction and expansion of CAR T cells

PBMCs were stimulated with 10 ng/ul of α-CD3 (OKT3) (#16-0037-85, Thermo-Fisher) and 300 U/ml IL-2 (#703892-4, Novartis) for 48 hours before transduction. Then, cell supernatant containing retroviral particles was thawed and centrifuge in retronectin (#T100A, Takara)-coated 6-well plates for 2 hours at 2000g. Next, 2 x 10^{6} stimulated PBMCs were added on top and centrifuged for 10 minutes at 400g. After 5 days, CAR expression and cytotoxic assays were performed. Untransduced T cells (UTD) were transduced with empty CAR retrovirus.

CAR expression analyses

0,2 x 10^{6} CAR Ts were washed twice with 1xPBS and re-suspended in 1xPBS, 2.5 mM EDTA, 1% BSA, and 5% horse serum for 20 minutes.

Then, cells were stained with 1/20 Biotin anti-IgG (#115-065-072, Jackson ImmunoResearch) for 30 minutes and washed twice with 1xPBS. APC-Streptavidin antibody (#405207, Biolegend) at 1/150 and 1/300 anti-CD3-PE (#300408, Biolegend) were added for 30 minutes. Zombie Aqua (#423101, Biolegend) was used as a viability marker at 1:1000 dilution. CAR expression was measured on FACSCelesta (BD Bioscience) and analysed with FlowJo software.

CAR T cytotoxic assay

CFSE-labelled MCF10A p95HER2/empty cells were co-cultured with CAR T cells at the indicated E: CAR T ratio in 96-well flat bottom plates. After 48 hours of incubation, the mixture of cells was washed with 1xPBS and re-suspended in 1xPBS, 2.5 mM EDTA, 1% BSA, and 5% horse serum for 20 minutes. Then, cells were stained with hCD8 PE-Cy7 (#344712), hCD4 BV421 (#317434) and hCD25 APC (#302610), all from BioLegend at 1:300 dilution. Zombie Aqua (#423101, Biolegend) was used as a viability marker at 1:1000 dilution. CFSE positive cells and CAR T activation were counted on LSR Fortessa (BD Bioscience) and analysed with FlowJo software.

Screening α-p95HER2 antibodies

0,2 x 10^6 MCF10A cells expressing p95HER2, HER2, HER2-M611A or wild type were trypsinized (#25300096, Life Technologies), washed with 1xPBS and re-suspended in 1xPBS, 2.5 mM EDTA, 1% BSA, and 5% horse serum for 20 minutes. Then, cells were stained with increasing doses of α-p95HER2 antibodies (in-house) up to 2 µg/ml for 30 minutes. Cells were washed twice with 1xPBS and incubated with 1/500 anti-mouse IgG-A488 (#A11001, Invitrogen) for 30 minutes. Propidium iodine (#81845, Sigma-Aldrich) at 2 µg/ml was used as a viability marker. Mean fluorescence intensity as a read out of antibody-binding was measured on FACSCelesta (BD Bioscience) and analysed with FlowJo software.

Statistics

GraphPad Prism 6.0 was used for statistical analyses. For *in vitro* experiments, comparisons between two groups were made by Student's *t* test. For *in vivo* experiments, we used two-way ANOVA with subsequent Bonferroni correction. We did not use statistical methods to predetermine sample size in animal studies, but we did make efforts to achieve the scientific goals using the minimum number of animals. A sample size of three to seven mice per group was chosen on the basis of our previous experience.

RESULTS

1. Anti-p95HER2 antibody exclusively recognizes human p95HER2

Approximately 30% of HER2-positive BC express p95HER2, a truncated form of HER2 (Arribas et al., 2011). Our laboratory and others have developed antibodies that recognize p95HER2 but not full-length HER2 (Parra-Palau et al., 2010; Sperinde et al., 2010). In particular, our group has developed and characterised a monoclonal anti-human p95HER2 antibody (α-p95HER2) (Parra-Palau et al., 2010) (**Figure 9A**). The epitope recognized by the α-p95HER2 antibody, PIWKFPD, is located 34 amino acids from the plasma membrane (**Figure 9B**).

It is currently unknown if the α-p95HER2 antibody recognizes the mouse p95HER2. The human and mouse sequences only differ in one amino acid in the epitope region (PIWKYPD) (**Figure 9B**). In order to determine the relevance of this change, we transiently overexpressed human or mouse p95HER2 into HEK239 cells. Western blot analyses showed that mouse p95HER2 is not recognized by the α-p95HER2 antibody (**Figure 9C**), suggesting that a single amino acid change is essential for proper recognition. These results indicate that, similarly to trastuzumab (Schwall et al., 2003), the side effects caused by the α-p95HER2 antibody, or therapeutic antibodies derived from it, cannot be evaluated in mouse models.

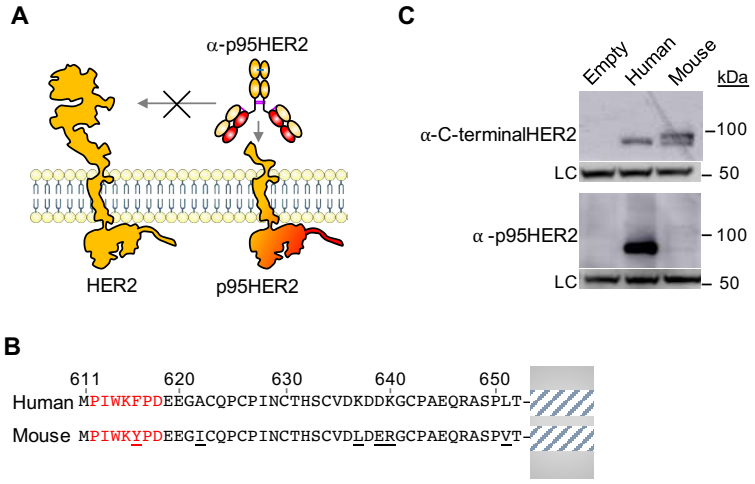


Figure 9: Recognition of the α-p95HER2 antibody to human and mouse p95HER2.

(A) Schematic illustrating the specificity of the α-p95HER2 antibody used in this study. (**B**) Sequences of the extracellular fragment of human p95HER2 and its murine homolog. Differences in the amino acid sequence are underlined. (**C**) HEK293 cells were transfected with empty vector or the same vector encoding mouse or human p95HER2. Expression of p95HER2 was analyzed by Western blot with an antibody against the C-terminus of HER2, which recognizes an epitope conserved in human and mouse, or α-p95HER2. α-tubulin staining is shown as loading control (LC).

2. p95HER2 is not expressed in normal tissues

The expression of p95HER2 in normal tissues has not been analysed yet. To address this issue, we used tissue microarrays containing samples from 36 normal adult human tissues. Immunohistochemical analysis of the expression of p95HER2 using the α-p95HER2 antibody revealed that p95HER2 was not detected in any normal human tissue (**Figure 10**). In contrast, and as previously reported (Pressl, Cordon-Cardo & Slamon, 1990), full-length HER2 could be readily detected in the epithelia of skin, gastrointestinal, respiratory, reproductive and urinary tracts (**Figure 10**). These results show that p95HER2 is likely a bona fide tumour-specific antigen.

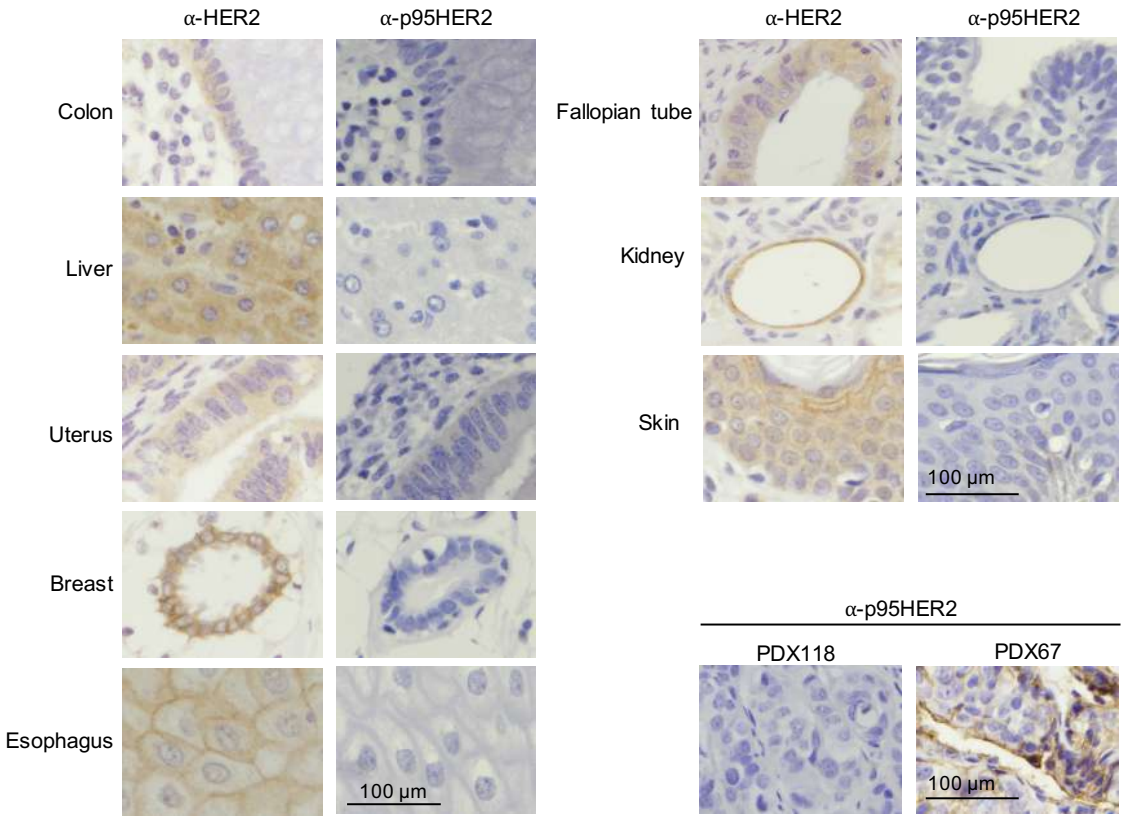


Figure 10: Expression of p95HER2 and HER2 in normal tissues.

Representative immunohistochemical analyses of the expression of HER2 and p95HER2 in the indicated epithelia using tissue microarrays containing normal adult human tissues. Patient-derived xenograft (PDX) 118 and 67 were used as negative and positive controls of p95HER2.

3. Development of T cell bispecific antibody against p95HER2

3.1. Generation and characterization of bispecific antibodies against p95HER2

Our previous results showed that p95HER2 is a tumour-specific antigen. Thus, we hypothesised that it could be used as a safe therapeutic target. In collaboration with Roche, we developed two T-cell bispecific antibodies (TCBs) (**Figure 11**) using the α-p95HER2 antibody described before (**Figure 9**). The 1:1 TCB format consists of an asymmetric two-armed immunoglobulin G1 (IgG1) that binds monovalently to both CD3ε and the tumour antigen p95HER2. In contrast, the 2:1 TCB binds bivalently to p95HER2 and monovalently to CD3ε, increasing the avidity of the antibody. Both TCBs have a low affinity for CD3ε when tumour target is not recognised (Bacac et al., 2018), which disfavours T cell binding and activation in the absence of binding to p95HER2. In addition, the fragment crystallisable (Fc) region of p95HER2-TCBs was engineered to avoid binding to Fcγ receptor by introducing P329G LALA mutations (Bacac et al., 2018; Bacac et al., 2016).

To elucidate which format of p95HER2-TCB had a higher efficacy, we co-cultured MCF7 cells expressing p95HER2 with PBMCs and different concentrations of TCBs (**Figure 11B**). Nontargeting-TCB, a 2:1 bispecific antibody that does not recognize any known target, was used as a control. At a low concentration of TCBs (1 nM), the 2:1 format had a great cytotoxic effect, inducing around 80% of the cell-death. In contrast, the 1:1 format induced a clear cytotoxic effect at 10 nM. The activation of CD4 and CD8 cells, measured in parallel by means of CD25 positivity, were higher when using the 2:1 TCB (**Figure 11C**). These results indicate a higher cytotoxic effect of 2:1 p95HER2-TCB compared with the 1:1 format. For this reason, we used the 2:1 p95HER2-TCB as the unique p95HER2-TCB.

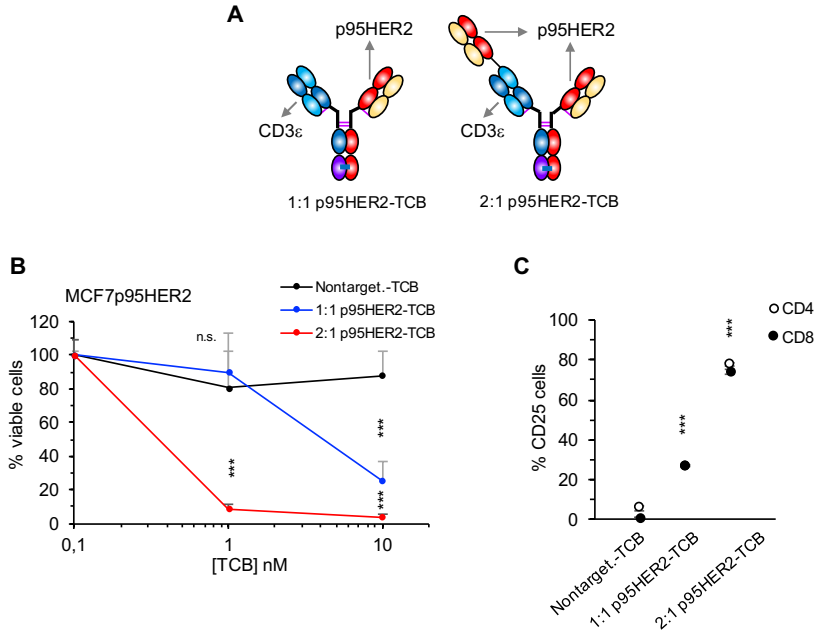


Figure 11: Characterization of 1:1 and 2:1 p95HER2-TCBs

(A) Schematic showing the structure of 1:1 and 2:1 p95HER2-TCBs**. (B,C)** MCF7 expressing p95HER2 were incubated with PBMCs and different concentrations of TCBs for 48h. **(B)** Viability of MCF7p95HER2 cells and **(C)** expression of the activation marker CD25 on CD4 and CD8 cells at 10 nM was measured by flow cytometry. N=3 expressed as means ± SD. **P*<0.05, ***P*<0.01, ****P*<0.001 two-tailed *t* test. In all cases we compared the group treated with nontarget.-TCB with the group treated with p95HER2-TCB (1:1 or 2:1).

3.2. Cytotoxicity induced by T cells subsets through TCBs

It has been described that both CD4 and CD8 subpopulations are activated by TCBs (Seckinger et al., 2017; Bacac et al., 2016). Indeed, in our *in vitro* setting, we detected a similar CD4 and CD8 activation when co-culturing tumour cells with TCBs (**Figure 11C**). The cytotoxic effect of both CD4 and CD8 upon TCB treatment has also been addressed (Seckinger et al., 2017; Ishiguro et al., 2017) although the role of naïve and memory cells remains poorly explored.

In the laboratory, we have several TCBs available, including the HER2-TCB and the CEACAM5-TCB. Both TCBs have the same format as the p95HER2-TCB; they bind the very same CD3ε epitope. Thus, we considered them to be good models to elucidate which T cell subsets are triggering a cytotoxic effect upon TCB treatment (**Figure 12A**); p95HER2-TCB was prioritized for other experiments.

To analyse individual T cell subpopulations, we isolated PBMCs from healthy donors and positively purified CD4 or CD8 cells. As target cells, we used MKN45 or MCF10A HER2 cells, which overexpress CEA and HER2 respectively (**Figure 12A**). CD4 and CD8 cells induced a similar cytotoxic effect when using several doses of CEACAM5-TCB or Target:Effector ratios (**Figure 12B, C**). Similarly, HER2-TCB also induced cytotoxicity through both CD4 and CD8 subpopulations (**Figure 12D**).

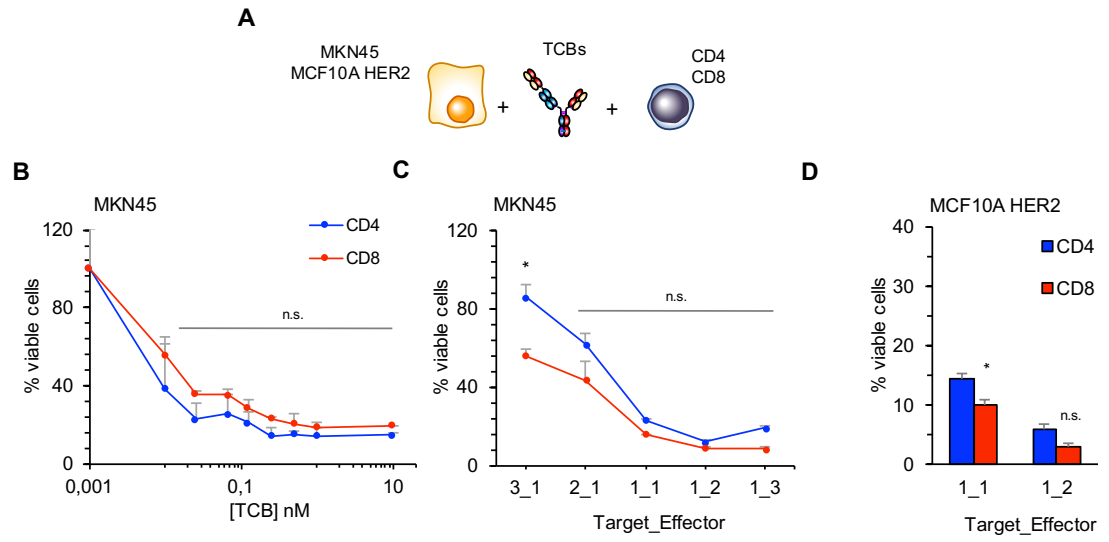


Figure 12: Effect of CD4 and CD8 cells upon TCB treatment.

(A) Schematic drawing illustrating the co-culture experiments. **(B)** MKN45 cells were co-cultured with CD4 or CD8 cells at 1:1 T:E ratio with several doses of CEACAM5-TCB and viability was assessed at 72h. **(C)** MKN45 cells were co-cultured with CD4 or CD8 cells at different T:E ratios and 0,5 nM of CEACAM5-TCB and viability was assessed at 72h. **(D)** MCF10A HER2 cells were co-cultured with CD4 or CD8 at 1 nM of HER2-TCB and viability was assessed at 72h. All results were measured by flow cytometry. N=3 expressed as means ± SD. Results were normalised to non-treated cells. Statistics compare CD4 with CD8 treated groups. $P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ two-tailed *t* test.

To shed light on which T cell subsets within the CD4 and CD8 populations induce cell death through TCBs, we sorted naïve, central memory (CM), effector memory (EM) and terminal effector (TE) cells from two healthy donors (**Figure 13**). We used anti-CD45RO and anti-CCR7 antibodies to discriminate the different populations as described in (Restifo, 2014). As expected, each donor had a different T cell subset distribution (**Figure 13**). Moreover, terminal effector markers were only detected on CD8 cells, as previously reported (Lugli & Gattinoni, 2015). After sorting the indicated populations, T cells were co-cultured with MCF10A HER2 cells and HER2-TCB. At 1:1 ratio, central memory CD4 cells and terminal effector CD8 cells did not induce a significant cell death. Unexpectedly, naïve cells induced a similar response as the rest of the T cell subsets (**Figure 14A**). When we increased the T:E ratio to 1:2, all the T cell subsets triggered cytotoxicity, although central memory CD4 and effector CD8 did it at less extent (**Figure 14B**). These results suggest that almost all subtypes of T cells may contribute to killing by TCBs.

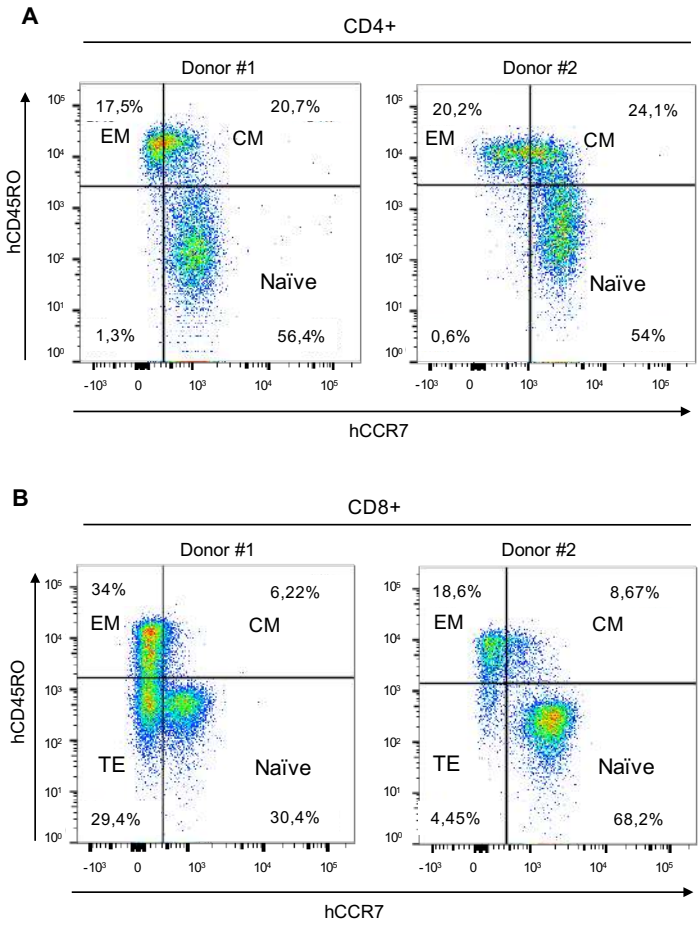


Figure 13: Flow cytometry strategy for sorting T cell subsets.

(A, B) Gating strategy applied to **(A)** CD4 or **(B)** CD8 cells in two donors, using CD45RO and CCR7 antibodies. Central Memory (CM), effector memory (EM), terminal effector (TE).

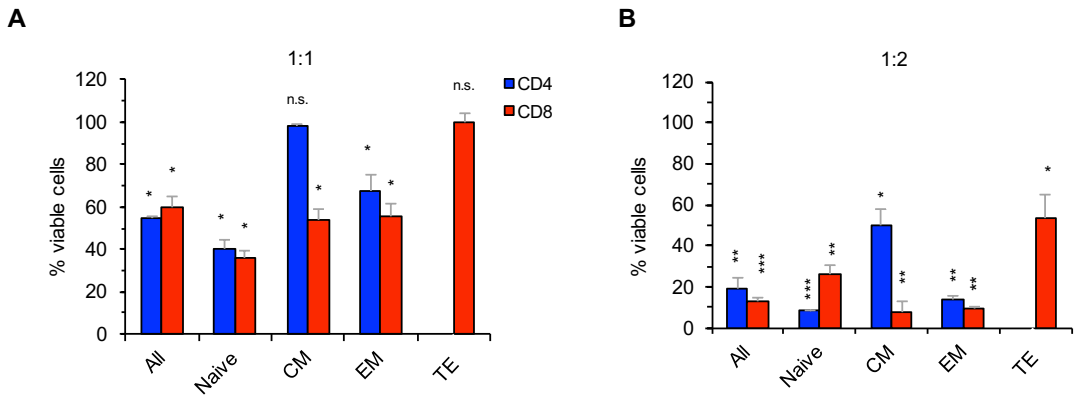


Figure 14: Cytotoxic effect of T cell subsets upon TCB treatment.

(A, B) MCF10A HER2 cells were co-cultured with 1 nM of HER2-TCB and the indicated T cells at T:E **(A)** 1:1 ratio or **(B)** 1:2 ratio for 72h. Viable cell were analysed by flow cytometry. The data was normalised to non-treated cells. Representative results obtained from donor #1. N=3 expressed as means ± SD. Statistics compare non-treated cells with the indicated groups. $P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ two-tailed *t* test.

3.3. Characterization of humanized mice models

The increasing interest in immunotherapy research has prompted the development of complex mice models enabling the engraftment of human immune cells (Zitvogel et al., 2016); the so-called humanized mice. There are several strategies to generate humanized mice, including the injection of human hematopoietic stem cells (HSC-CD34^{+}) or human PBMCs on immunocompromised mice, such as NOD scid gamma (NSG). Depending on the question being addressed, one model may be more suitable than the other (De La Rochere et al., 2018).

In this thesis, we have established the humanized mouse models mentioned above to assess the efficacy of p95HER2-TCB *in vivo*.

3.3.1. Fully humanized mice model recapitulates human haematopoiesis

In this work, we generated fully humanized mice as shown in **Figure 15**. Briefly, HSC-CD34$^+$ cells were purified from human cord blood and injected into five-week old NSG mice previously treated with busulfan to ablate the remaining murine hematopoietic system. After 20-40 weeks, the percentages of several hematopoietic lineages of circulating human immune cells were determined by flow cytometry under the gating strategy shown in **Figure 16** and **17**. Of note, whole blood from three healthy donors was used as a control. In both 20 and 40 weeks old fully humanized mice, 80% of leucocytes were lymphocytes (**Figure 18A**). Interestingly, the percentage of T cells was higher in elder mice (**Figure 18B**). Despite the presence of myeloid cells in fully humanized mice, the percentage was much lower than in humans and, within this population, the majority were monocytes (**Figure 18C**).

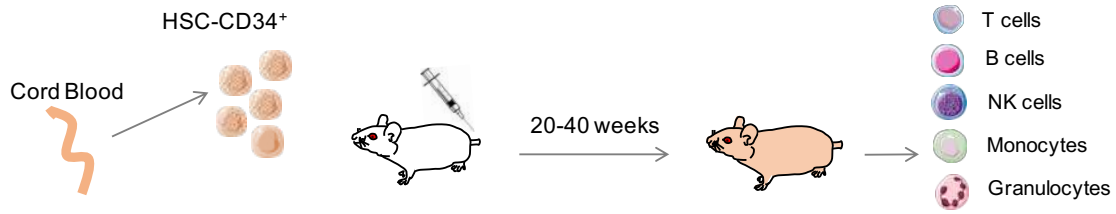


Figure 15: Schematic drawing illustrating the humanization of mice with CD34$^+$ hematopoietic stems cells. HSC-CD34$^+$ cells were purified from human cord blood and injected into five-week old NSG mice. After 20-40 weeks, the percentages of several linages of circulating human immune cells were determined.

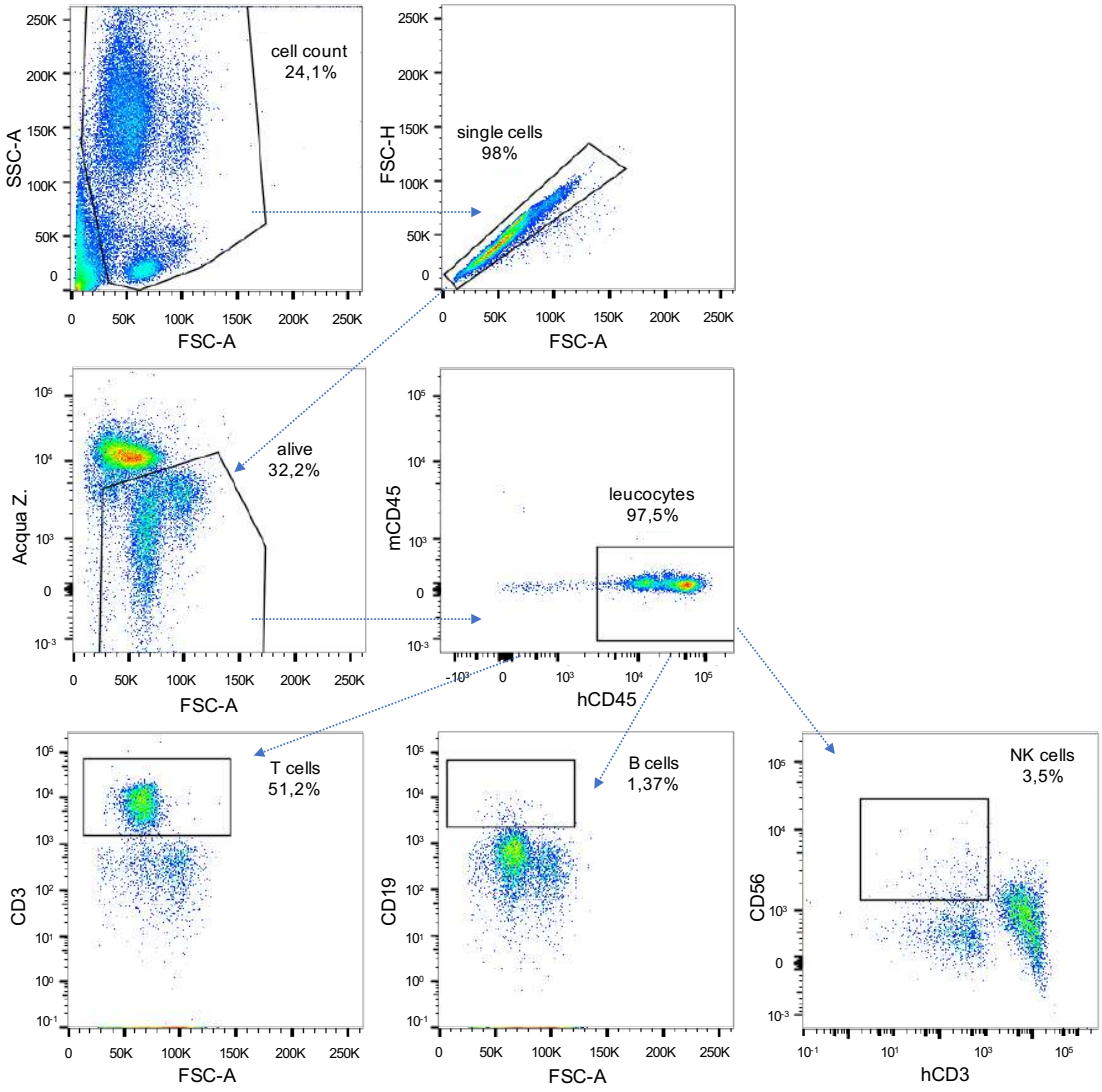


Figure 16: Representative flow cytometry analyses of lymphocytes in blood of a healthy human donor. Gating strategy followed: **leucocytes**: hCD45^{+}; **T cells**: hCD45^{+}, hCD3+; **B cells**: hCD45^{+}, hCD19^{+}; **NK cells**: hCD45^{+}, hCD3^{-}, hCD56^{+}.

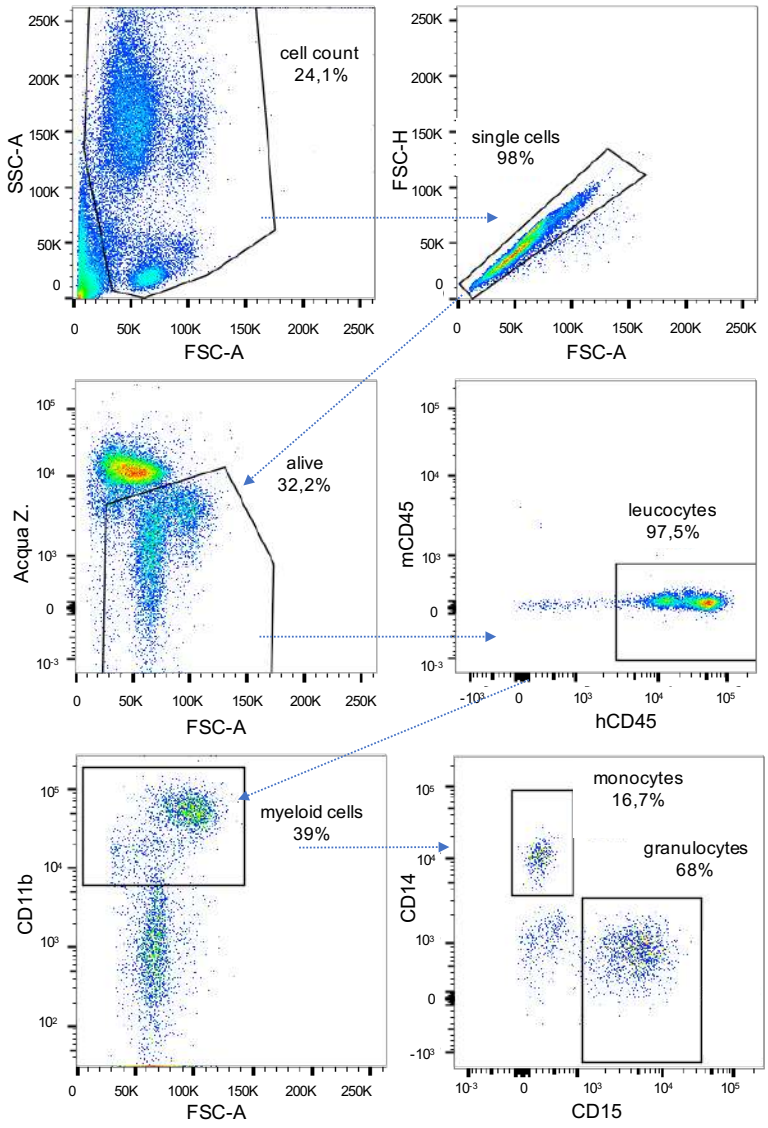


Figure 17: Representative flow cytometry analyses of myeloid cells in blood of a healthy human donor. Gating strategy followed: **leucocytes**: hCD45^{+}; **myeloid cells**: hCD45^{+}, CD11b^{+}; **monocytes**: hCD45^{+}, CD11b^{+}, hCD14^{+}; **granulocytes**: hCD45^{+}, CD11b^{+}, hCD15^{+}.

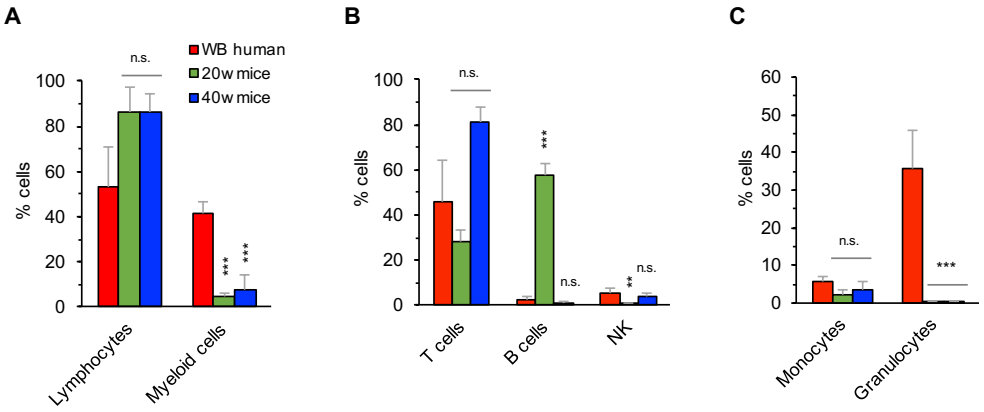


Figure 18: Human immune cell populations on fully humanized mice.

(A) Percentage of lymphocytes and myeloid cells. Whole blood (WB) from three healthy donors is shown in red as a reference. Data from 20 weeks and 40 weeks old fully humanized mice are plotted in green and blue. **(B)** Percentage of the different subsets of lymphocytes (T, B and NK cells). **(C)** Percentage of myeloid populations (monocytes and granulocytes). All percentages are referred to total alive leucocytes. N= 3 expressed as mean ± SD. Statistics compare WB with the indicated groups. $P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ two-tailed *t* test.

These results confirm that mice humanized with HSC-CD34^{+} produce several human immune cell lineages, although the percentage of myeloid cells is much lower than in humans. In order to assess the efficacy of p95HER2-TCB *in vivo*, BC PDX engraftment was the next step. Of note, in the laboratory we have experienced different rates of engraftment between different types of cancer PDXs (**Table 4**). In general, BC PDXs engraft poorly compared to colon cancer PDXs. This information limits our use of HER2-postive BC PDXs on fully humanized mice models.

Cancer Type	Subtype	PDX	% Engraftment
Breast Cancer	TNBC	PDX284	66,7
	TNBC	PDX384	50,0
	HER2+	PDX118	72,7
	HER2+	PDX173	15,0
	HER2+	PDX251-G	13,3
	HER2+	PDX67	0,0
Colon Cancer		PDX48	92,7
		PDX50	86,2
		PDX63	96,2
		PDX64	98,3
		PDX66	88,0

Table 4: Percentage of tumour engraftment on fully humanized mice

Each indicated PDX was implanted in at least five fully humanized mice. The percentage of engraftment was evaluated up to three months post-implantation.

3.3.2. PBMCs-humanized mice model exclusively produces T cells

Another methodology to assay immune therapies *in vivo* consists in injecting PBMCs into immunocompromised NSG mice carrying xenografted tumour cells. This model is known to have a very rapid T cell expansion (Zitvogel et al., 2016). To confirm this assumption, we proceeded to generate mice humanized with PBMCs as shown in **Figure 19**.

Briefly, PBMCs were purified from whole blood or buffy coats and injected into NSG mice carrying human tumours. After 2 weeks, the percentages of several lineages of circulating human immune cells were determined by flow cytometry under the gating strategy shown in **Figures 16** and **17**. Of note, whole blood from three healthy donors was used as a control.

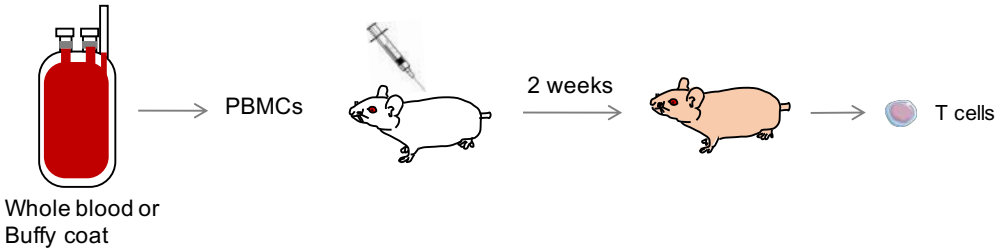


Figure 19: Schematic drawing illustrating humanization of mice with PBMCs

PBMCs were purified from whole blood or buffy coats and injected into NSG mice. After 2 weeks, the percentages of several linages of circulating human immune cells were determined by flow cytometry.

Mice humanized with PBMCs had approximately 95% of human lymphocytes from the total human leucocyte population (**Figure 20A**). From those lymphocytes, close to 100% of cells were T cells (**Figure 20B**). As expected, no myeloid cells engrafted in this model (**Figure 20A, C**).

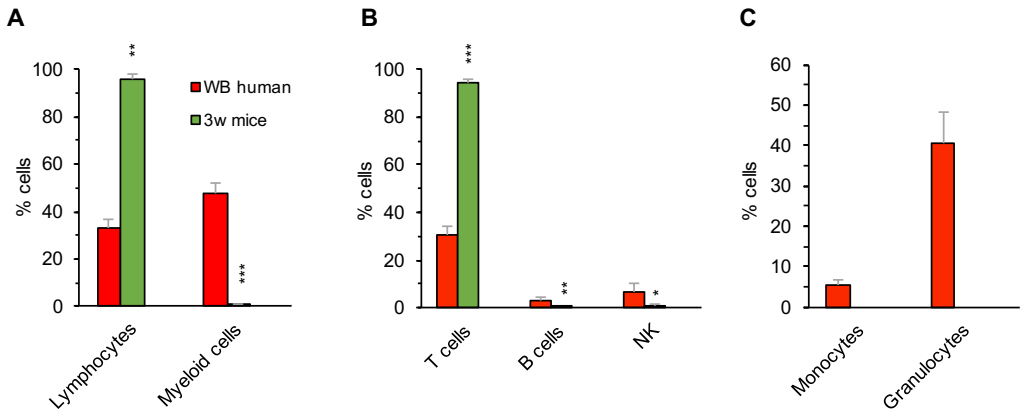


Figure 20: Human immune cells populations on PBMCs humanized mice

(A) Percentage of lymphocytes and myeloid cells. Whole blood (WB) from three healthy donors is shown in red as a reference. Data from 2 weeks post-humanization is plotted in green. **(B)** Percentage of the different subsets of lymphocytes (T, B and NK cells). **(C)** Percentage of myeloid populations (monocytes and granulocytes). All percentages are referred to total alive leucocytes. N= 3 expressed as mean ± SD. Statistics compare WB with 3w mice. $P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ two-tailed *t* test.

These results confirm that humanization with PBMCs model exclusively produce human T cells on immunocompromised mice. Since T cells are the main immune cell population required for the activity of TCBs, this model is also suitable for our work.

3.4. Antitumor effect of p95HER2-TCB on MCF7p95HER2 cells *in vivo*

As described above, transplantation of PBMCs into immunocompromised mice carrying xenografted tumour cells is a suitable model to assess the activity of p95HER2-TCB.

In our first *in vivo* approach, we orthotopically engrafted MCF7p95HER2 cells and once mean tumour volume reached 200 mm^3, we injected PBMCs obtained from a healthy donor and started the treatment (**Figure 21A**) with p95HER2-TCB or as controls nontargeting-TCB or vehicle. p95HER2-TCB had a very potent antitumor effect after three doses of treatment (**Figure 21B**). We also observed that the levels of humanization, defined as circulating cells positive for human CD45, were similar between treated groups at endpoint of the experiment (**Figure 21C**). In fact, supporting the efficacy and specificity of the p95HER2-TCB, we did not detect human lymphocyte engraftment in the outlier mouse bearing a tumour that showed a growth rate similar to those of mice treated with vehicle or nontargeting-TCB (**Figure 22**).

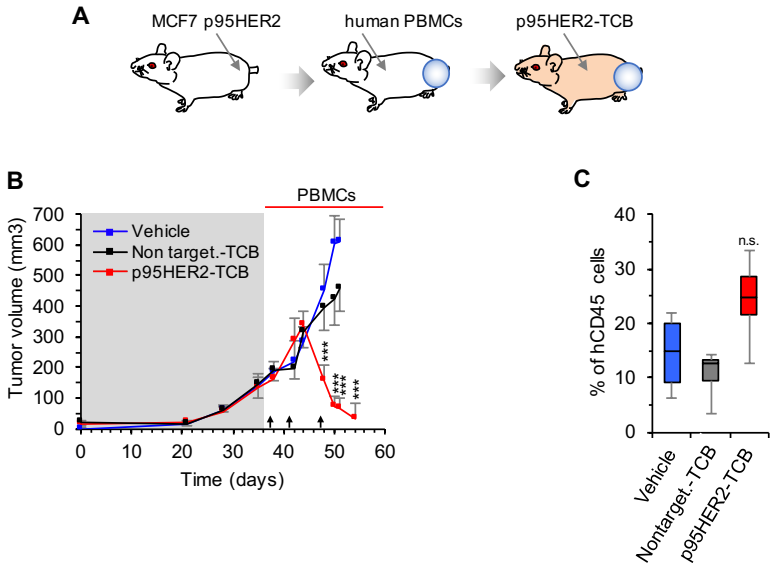


Figure 21: Effect of p95HER2-TCB on orthotopic tumour growth *in vivo*.
(A) Schematic drawing illustrating the orthotopic implantation of MCF7p95HER2 cells and humanization of mice with PBMCs. **(B)** Mice were treated with vehicle or 1 mg/kg of nontargeting-TCB or p95HER2-TCB (arrows). Tumour volumes, expressed as means ± SD are shown, n≥ 7 per group. **(C)** Percentage of circulating human CD45, relative to total leukocytes, at the end of the experiment shown in B. * P<0.05, ** P<0.01, *** P<0.001 **(B)** two-way ANOVA and Bonferroni correction, **(C)** two-tailed *t* test.

On the other hand, the percentage of tumour cells, measured by positivity against an α-cytokeratin antibody, was significantly lower (P<0.001) after treatment with the p95HER2-TCB (**Figure 23A**). This indicates that the measured volumes likely overestimate tumour burden in p95HER2-TCB-treated mice. At the end of the experiment, the percentages of circulating human CD8 cells were similar in the three groups of mice (**Figure 23B**, Blood). In contrast, tumours in p95HER2-TCB treated group contained a higher percentage of CD8 lymphocytes (**Figure 23C**, Tumour), indicating T cell recruitment by p95HER2-TCB.

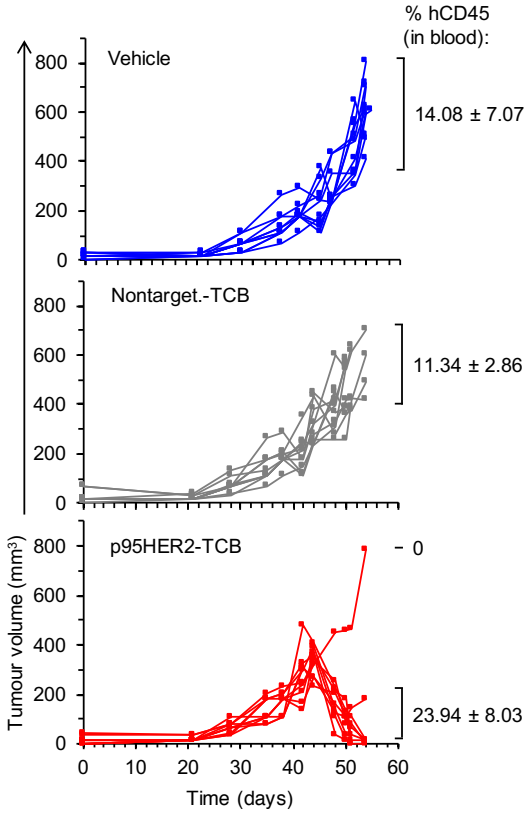


Figure 22: Growth curves of individual tumours shown in Figure 21.

The percentages of circulating human CD45 cells, relative to total leucocytes, at the end of the experiment, shown as means ± SD.

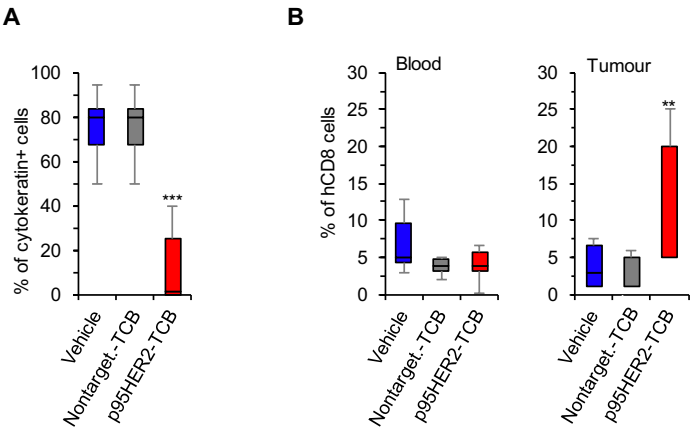


Figure 23: Parameters analysed at the end of the experiment shown in Figure 21.

(A) Percentages of cells positive for cytokeratin were quantified in tumours by immunohistochemistry. **(B)** Percentages of circulating (Blood) and intra-tumour (Tumour) human CD8 cells at the end of the experiment. Percentages in blood were calculated by flow cytometry and are relative to total leucocytes. Percentages in tumours were calculated by immunohistochemistry and are relative to tumour cells. **P*<0.05, ***P*<0.01, ****P*<0.001 two-tailed *t* test. In all cases we compared the group treated with vehicle with the group treated with p95HER2-TCB.

These results show the strong antitumor effect of p95HER2-TCB on p95HER2-expressing BC cell line xenograft. Next, we pursued to evaluate the toxicity of p95HER2-TCB on normal tissues.

3.5. p95HER2-TCB has no effect on cells expressing normal levels of HER2

The expression in normal tissues of HER2 is likely the reason for the side effects observed with targeted therapies against HER2 (van der Stegen et al., 2013; Morgan et al., 2010). To address possible side effects on normal tissues induced by p95HER2-TCB, we directly compared the redirection of T cells via p95HER2 and HER2. To this end, we used a trastuzumab-based HER2-TCB with a 2:1 structure identical to that of p95HER2-TCB.

3.5.1. Comparison between HER2-TCB and p95HER2-TCB: *in vitro* assays

To address possible effects of p95HER2-TCB on normal tissues *in vitro*, we used normal epithelial cells (NEC) derived from reduction mammoplasties, which are known to express HER2 (**Figure 10**, Breast). In line with the basal expression of HER2 shown in Figure 10, HER2-TCB, but not p95HER2-TCB, bound to the surface of NEC (**Figure 24A**). Accordingly, co-culture experiments showed that HER2-TCB, but not p95HER2-TCB, induced the activation of cytotoxic lymphocytes and induced cell death (**Figure 24B, C**). Thus, p95HER2-TCB does not affect non-transformed cells, whereas HER2-TCB induces their cytotoxic destruction.

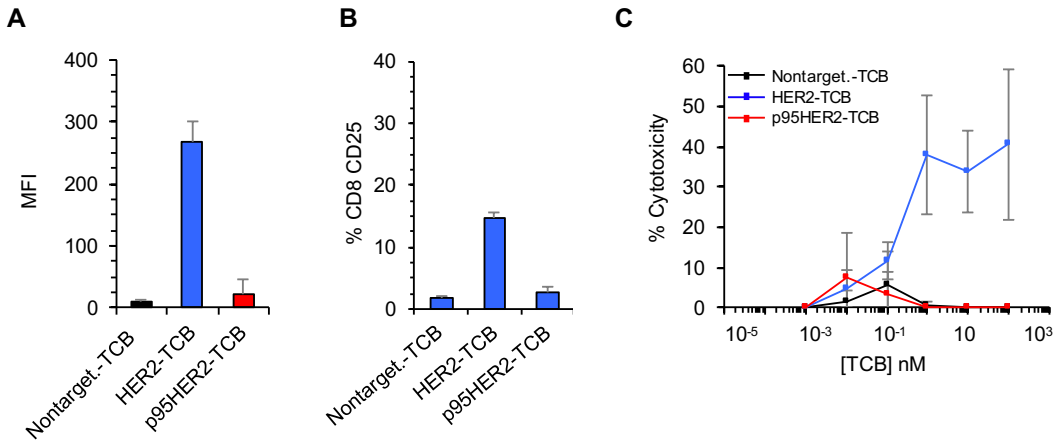


Figure 24: Effect of HER2-TCB and p95HER2-TCB on normal epithelial cells (NEC)

(A) Median fluorescence intensity (MFI) of binding of the indicated TCBs to NEC. **(B, C)** NEC were incubated with PBMCs at T:E ratio 1:10 and different concentrations of TCBs for 48h. **(B)** Then, the expression of the activation marker CD25 on CD8 cells at 10 nM of TCB was measured by flow cytometry and **(C)** cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release. N=3 expressed as means ± SD.

3.5.2. Comparison between HER2-TCB and p95HER2-TCB: *in vivo* assays

To further examine the apparent lack of toxicity of p95HER2-TCB, we engrafted parental MCF7 or MCF7 cells overexpressing HER2 in immunocompromised mice (**Figure 25A, B**). MCF7 cells are considered to express levels of HER2 similar to those expressed by normal epithelial cells (Subik et al., 2010). Once mean tumour volumes reached 200 mm^3, we injected PBMCs obtained from a healthy donor and started the treatment. In line with the previous results *in vitro*, HER2-TCB, but not p95HER2-TCB, induced regression of tumours generated by parental MCF7 cells (**Figure 25C, E**). Levels of humanization, defined as circulating cells positive for human CD45, were similar between treated groups at endpoint of the experiment (**Figure 25D, F**). Of note, the efficacy of HER2-TCB on MCF7 parental cells and on the same cells overexpressing HER2 was similar (**Figure 25E**). This result suggests that HER2-TCB is not able to discriminate low HER2 expressing cells (MCF7 parental) from high HER2 expressing cells (MCF7 HER2). Consequently, p95HER2-TCB could represent a novel means to discriminate healthy from tumour tissue, in contrast to HER2-TCB.

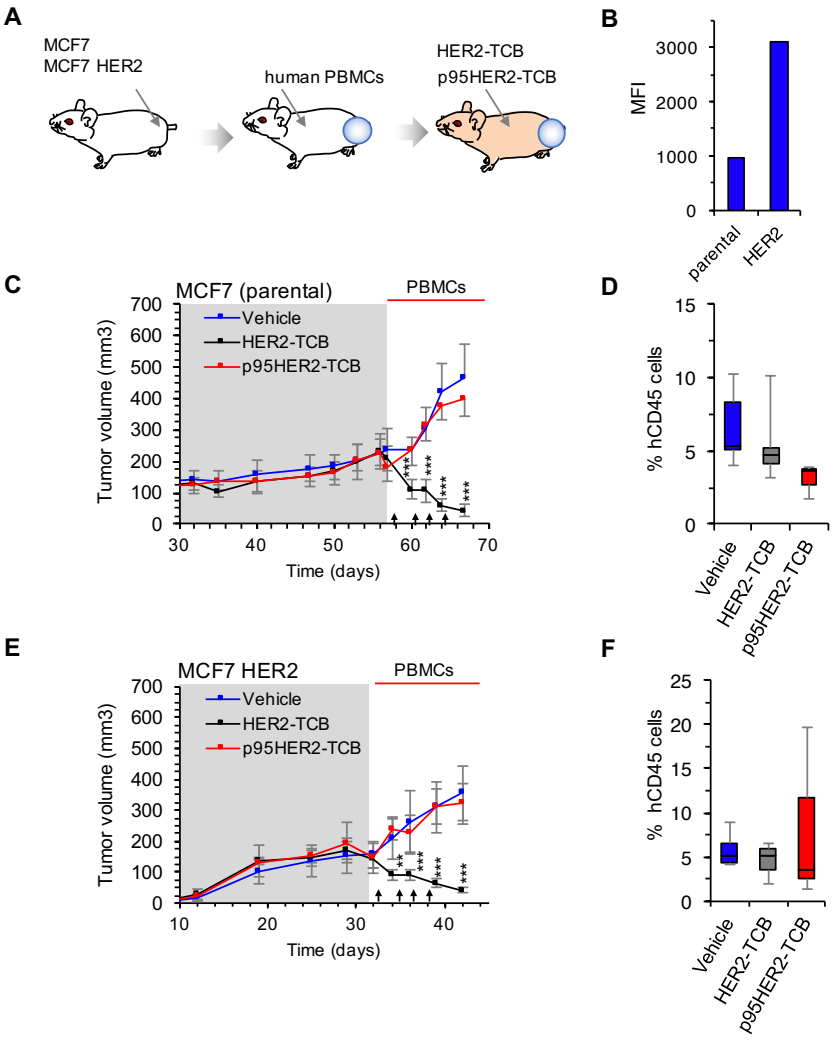


Figure 25: Effect of p95HER2-TCB and HER2-TCB on MCF7 cells and on MCF7 cells transfected with HER2.

(A) Schematic drawing illustrating humanization of mice with PBMCs and orthotopic implantation of MCF7 parental or the same cells transfected with a vector encoding HER2. **(B)** Median Fluorescence intensity of binding of trastuzumab to parental MCF7 cells or MCF7-HER2, analysed by flow cytometry. **(C)** NSG mice (n ≥ 6 per group) were injected with parental MCF7 cells and humanized with PBMCs. Mice were treated with vehicle, 1 mg/kg of HER2-TCB or 1 mg/kg p95HER2-TCB (arrows). **(D)** Percentage of circulating human CD45 cells at endpoint of C. **(E)** NSG mice (n ≥ 6 per group) were injected with MCF7 HER2 cells, humanized, and treated as in C. **(F)** Percentage of circulating human CD45 cells at endpoint of E. ***P*<0.01, ****P*<0.001, two-way ANOVA and Bonferroni correction.

3.6. Defining a threshold of effectivity for p95HER2-TCB

Next, we explored the levels of p95HER2 necessary to trigger the activation of lymphocytes mediated by p95HER2-TCB. To do so, we used a panel of HER2-positive PDXs, some of which express p95HER2.

Firstly, we compared the levels of p95HER2 in the original tumour and in the corresponding PDXs by histoscore (H-score). We detected that the expression of p95HER2 in samples from original tumours and the corresponding PDXs was similar (**Table 5**: IHC columns and **Figure 26A-B**). Thus, during expansion in immunocompromised mice, expression of p95HER2 by tumour cells remained largely invariable.

In addition, we assessed more in depth the levels of p95HER2 in the PDXs in a quantitative IHC-based assay, known as VeraTag (Sperinde et al., 2010). This method analyses the relative fluorescence/ tumour area (RF/mm^2) of p95HER2 staining. The results showed a positive correlation between the H-score and RF/mm^2 (**Table 5**: VeraTag columns, **Figure 26C**).

	Original tumour				PDX				
	IHC				IHC				VeraTag
	1+	2+	3+	HS	1+	2+	3+	HS	RF/mm^2
PDX67	10	20	50	200	50	25	25	175	40,93
PDX118	5	5	5	30	10	10	5	45	8,79
PDX515	20	55	20	190	50	25	25	175	48,09
PDX173	15	60	25	150	5	25	70	265	24,68
PDX251G	25	45	25	185	10	40	50	240	28,02
PDX251	20	45	35	215	5	35	60	255	35,43
PDX144	25	25	40	195	40	25	15	145	24,03
PDX284	<1	0	0	<1	0	0	0	0	
PDX347	0	0	0	0	0	0	0	0	

Table 5: Quantification of the expression of p95HER2 in different PDXs.
Expression of p95HER2 in the original tumours and the corresponding PDXs determined by H-score (HS) or a quantitative IHC-based assay (VeraTag). Numbers in the columns labelled “1+,” “2+,” and “3+” are the percentages of positive cells in each intensity category.

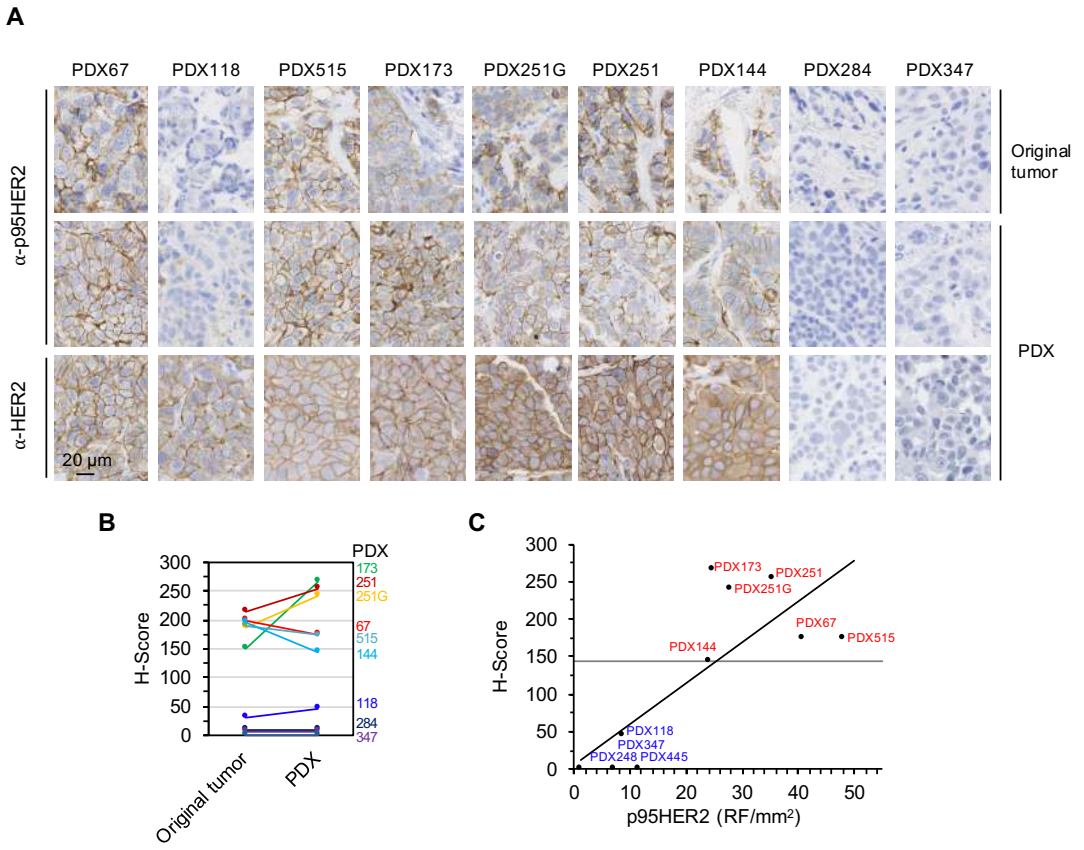


Figure 26: Expression of p95HER2 in different PDXs and the corresponding original tumour.
(A) Representative images of the immunohistochemical analysis of the samples in Table 5. **(B)** H-Score evolution between the original tumour and the corresponding PDX **(C)** H-scores were plotted against the amount of p95HER2 determined using VeraTag assay. In red and blue are PDXs that scored positive and negative, respectively, for the activation of T cells mediated by p95HER2-TCB (Figure 27).

Secondly, we quantified the activation of T cells induced by p95HER2-TCB in the battery of PDXs available (**Figure 26**). To do so, we used Jurkat T cells expressing an NFAT-driven reporter of TCR activation coupled to luciferase (**Figure 27A**). Using two PDXs previously characterized as high (PDX67) and low (PDX118) expressors of p95HER2 (**Figure 26**) (Parra-Palau et al., 2014), we showed that increasing concentrations of p95HER2-TCB resulted in TCR activation just in co-cultures with high p95HER2-expressing cells (**Figure 27B**). A similar analysis was performed on 8 additional HER2-positive PDXs and, as controls, on 2 PDXs established from TNBCs (PDX284 and 347) (**Figure 27C**).

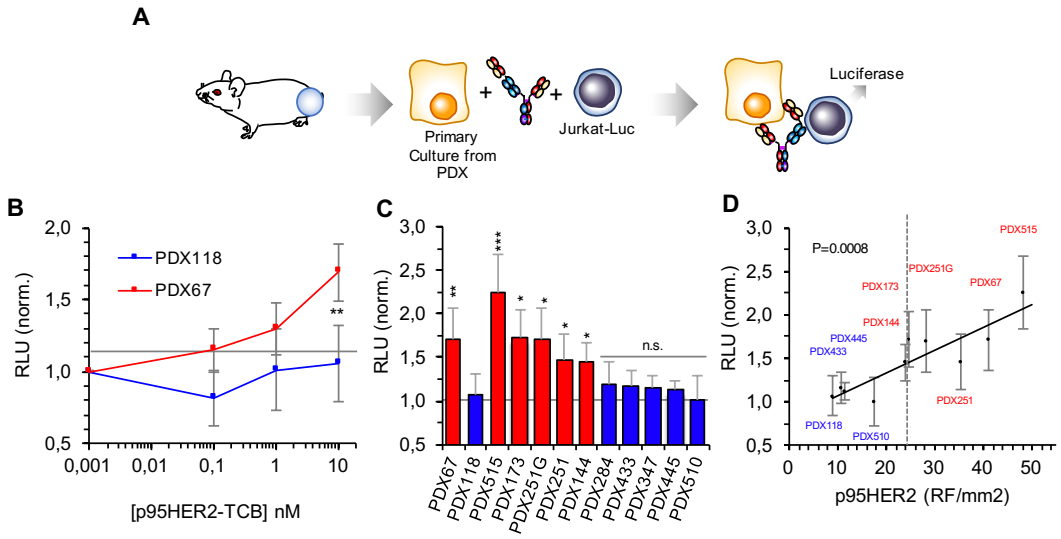


Figure 27: Effect of p95HER2 expression on the efficacy of p95HER2-TCB

(A) Schematic drawing illustrating the co-culture experiments. **(B)** Cells from PDXs 118 and 67 (negative and positive for p95HER2, respectively) were co-cultured in the presence of the indicated concentrations of p95HER2-TCB, with Jurkat T cells expressing a luciferase-based reporter for the activation of the T cell receptor. Relative luminescence units (RLU) were assessed and normalized to nontargeting-TCB, n = 6 expressed as means ± SD. **(C)** Cultures from the indicated PDXs were analysed as in B with 10 nM p95HER2-TCB. Statistics compare the results obtained with the different cultures with those obtained with the p95HER2-negative PDX118, n ≥ 3 expressed as means ± SD. **(D)** Results obtained in C were plotted against the amount of p95HER2 determined using VeraTag assay. **(B, C)** * P<0.05, ** P<0.01, *** P<0.001 two-tailed *t* test.

There was a positive correlation between the activation of the TCR induced by the p95HER2-TCB and the amount of p95HER2 determined by the quantitative IHC-based assay VeraTag (**Figure 27D**). These results allowed us to establish a threshold of p95HER2 expression above which p95HER2-TCB induces activation of the TCR: ~24 RF/mm^2, as judged by the VeraTag assay, and H-Score ~145, as judged by immunohistochemistry (**Figure 27D and Table 5**).

3.7. p95HER2-TCB induces antitumor effect only on p95HER2-positive PDXs *in vivo*

To confirm the p95HER2 threshold that induces p95HER2-TCB activation, we performed *in vivo* experiments using several PDXs previously analysed (**Table 5, Figure 26**). Importantly, PDXs are considered more powerful predictors of therapeutic response than cell lines because the resemble more faithfully human tumour heterogeneity (Byrne et al., 2017), also observed in **Figure 26**. Moreover, PDXs express natural levels of p95HER2, in contrast to the MCF7p95HER2 cell line.

The previous *in vivo* experiments involved the transplantation of PBMCs from healthy donors (**Figure 21B, 25C,E**). However, a caveat of this model is the possible allogeneic reaction of the T lymphocytes against the tumour cells because of class I histocompatibility complex mismatch (MHC I) (Zitvogel et al., 2016). Thus, the degree of histocompatibility may determine the degree of tumour rejection in this experimental system.

In the case of PDX173, a p95HER2-positive PDX (**Figure 27**), we had the opportunity to use PBMCs from the very same patient (matched), avoiding a possible allogeneic reaction.

Using these autologous PBMCs, we observed a clear antitumor effect of the p95HER2-TCB on PDX173 *in vivo* (**Figure 28**, matched, middle panel). Furthermore, PDX173 cells were established as organotypic cell cultures *in vitro* and further co-cultured as single cells with matched PBMCs, reproducing the results showed *in vivo* (**Figure 28**, matched, left panel).

In parallel, we also used PBMCs from an unrelated healthy donor and showed that, also in this experimental system, p95HER2-TCB had also a clear antitumor effect (**Figure 28**, non-matched), despite the fact that these allogeneic PBMCs had some cytotoxic effect on vehicle-treated PDX173, both *in vitro* and *in vivo* (**Figure 28**, compare matched and non-matched PBMCs in assays with vehicle). Levels of humanization, defined as circulating cells positive for human CD45, were similar between treated groups at endpoint of the experiment (**Figure 28**, right panels).

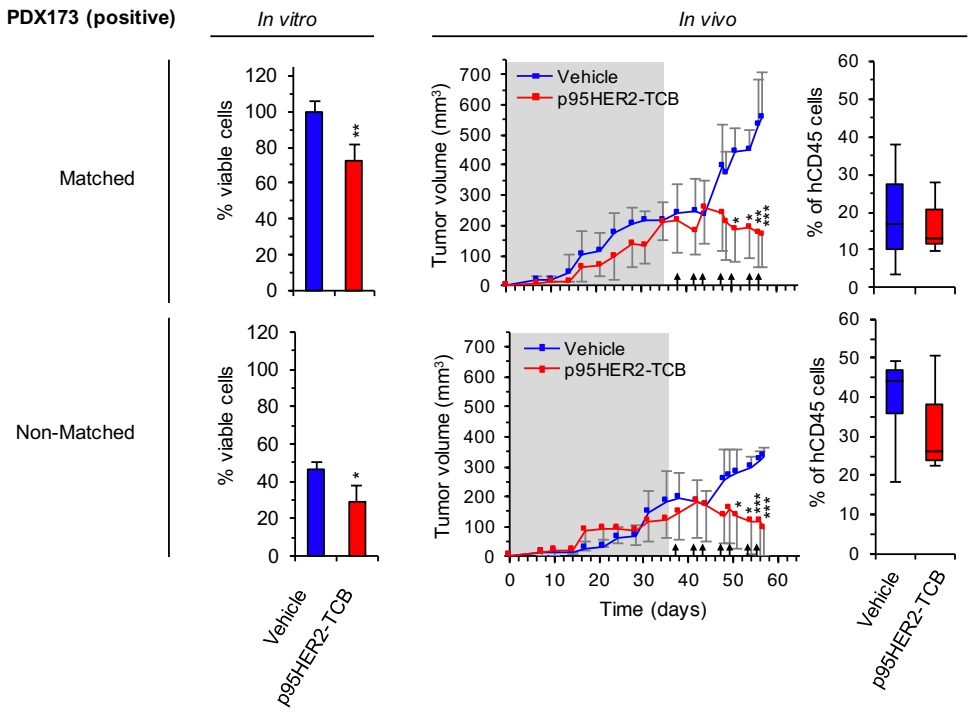


Figure 28: Effect of p95HER2-TCB on p95HER2-positive PDX173 using different donors of PBMCs. ***In vitro***, Primary cultures from PDX173 were incubated with PBMCs from the same patient (matched) or a healthy volunteer (non-matched) at ratio T:E 1:10, and treated with vehicle or 50 nM p95HER2-TCB for 72 hours. Viable PDX173 cells were counted by flow cytometry and represented as percentage of viable cells. N= 3 expressed as mean ± SD. ***In vivo***, NSG mice carrying PDX173 were transferred with matched or non-matched PBMCs. Mice were treated with vehicle or 1 mg/kg of p95HER2-TCB (arrows). Means ± SD are shown, n ≥ 3 per group. At the end of the experiment, the percentages of circulating human CD45 cells, relative to total leukocytes, were determined. * P<0.05, ** P<0.01, *** P<0.001, (middle) two-way ANOVA and Bonferroni correction, (left) two-tailed t test.

As HSC-CD34^{+}-based model reconstitutes more faithfully the human immune system (Zitvogel et al., 2016) than the PBMCs model, we performed an additional *in vivo* experiment to test the efficacy of p95HER2-TCB on PDX173 in fully humanized mice (**Figure 29A**). Although the PDX173 engrafted in a low percentage of fully humanized mice (**Table 4**), we successfully detected an impairment of tumour growth in the p95HER2-TCB treated-group (**Figure 29B, C**). The treatment also promoted the infiltration of CD8 lymphocytes on the tumour site and their activation (**Figure 29D**).

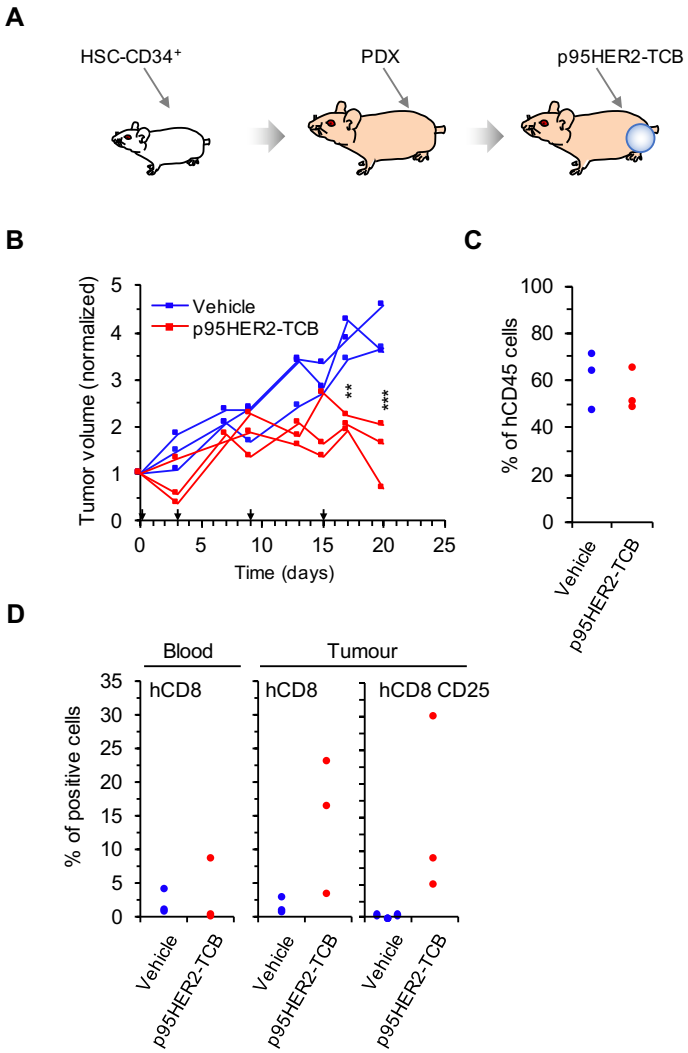


Figure 29: Effect of p95HER2-TCB on PDX173 using the HSC-CD34$^+$ humanised mice model.
(A) Schematic drawing illustrating the in vivo experiment shown in B-D. **(B)** HSC-CD34$^+$ cells obtained from human cord blood were injected into five-week-old NSG mice. After 5 months, PDX173 was implanted and when tumours reached 200 mm^3, mice were treated with vehicle or 1mg/kg p95HER2-TCB. Tumour volume was monitored and normalized to the volume at day 0 of treatment. * P<0.05, ** P<0.01, *** P<0.001, two-way ANOVA and Bonferroni correction. **(C)** Percentages of circulating human CD45 cells, relative to total leukocytes, from mice monitored in B. **(D)** Percentages of circulating (blood, relative to total leukocytes) and intra-tumour (tumour) human CD8 cells at the end of the experiment shown in B. The expression of the activation marker CD25 was also analysed in intra-tumour CD8 lymphocytes

In summary, PDX173, a tumour that was predicted to respond against p95HER2-TCB, was efficiently targeted by the bispecific antibody. Moreover, we proved the antitumor activity of p95HER2-TCB against PDX173 using different mouse models, including PBMCs model with matched or non-matched lymphocytes, and the fully humanized mice model.

To further test the p95HER2 threshold that induces p95HER2-TCB activation, we analysed the effect of p95HER2-TCB in two additional HER2-positive PDXs, 251G and 445. PDX251G expressed p95HER2 levels above the threshold necessary for the activation of the TCR mediated by p95HER2-TCB (**Figure 27**). On the other hand, PDX445 expressed p95HER2 levels below the threshold established. In both cases, we used PBMCs from healthy donors to humanize mice since previously we shown that it is a suitable model to test the efficacy of p95HER2-TCB despite of the alloreactivity (**Figure 28**).

As expected, we observed a robust antitumor effect of p95HER2-TCB in the p95HER2-positive PDX251G (**Figure 30**, *in vivo*). PDX251G cells were also established as organotypic cell cultures *in vitro* and further co-cultured as single cells with PBMCs, reproducing the antitumor effect showed *in vivo* (**Figure 30**, *in vitro*).

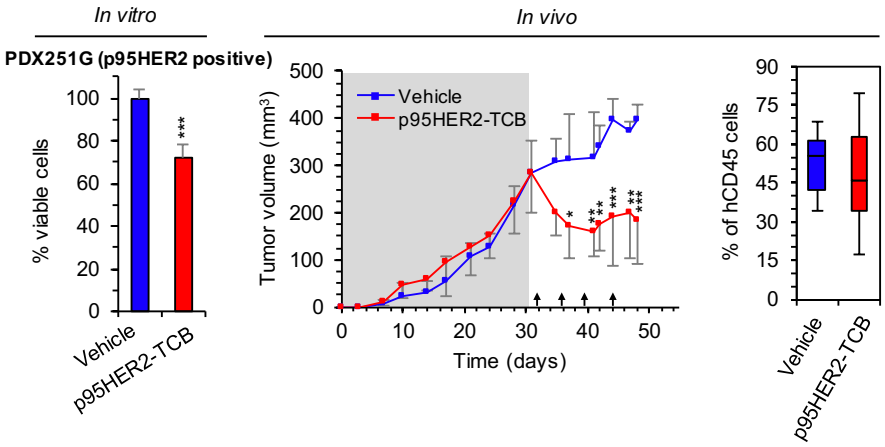


Figure 30: Effect of p95HER2-TCB on the growth of the p95HER2-positive PDX251G

In vitro, Primary cultures from PDX251G (p95HER2-positive) were incubated with PBMCs from a healthy volunteer at ratio T:E 1:10 and treated with vehicle or 50 nM of p95HER2-TCB. PDX251G cells were counted by flow cytometry and represented as percentage of viable cells. **In vivo**, NSG mice carrying PDX251G were transferred with PBMCs. Mice were treated with vehicle or 1 mg/kg of p95HER2-TCB (arrows). Means ± SD are shown, n ≥ 3 per group. At the end of the experiment, the percentages of circulating human CD45 cells, relative to total leukocytes, were determined. * P<0.05, **P<0.01, ***P<0.001. (left) two-tailed t test, (middle) two-way ANOVA and Bonferroni correction.

On the other hand, we did not observe an antitumor effect of p95HER2-TCB both *in vivo* and *in vitro* when using the p95HER2-negative PDX445 (**Figure 31**).

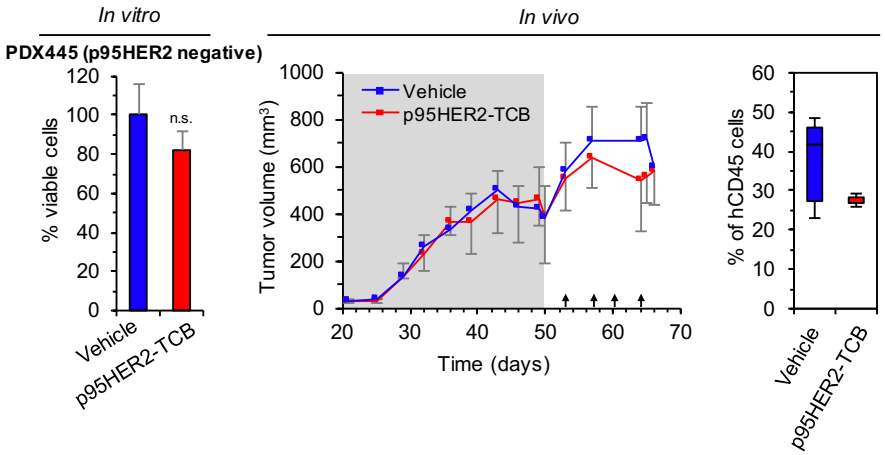


Figure 31: Effect of p95HER2-TCB on the growth of the p95HER2-negative PDX445

In vitro, Primary cultures from PDX445 (p95HER2 negative) were incubated with PBMCs from a healthy volunteer and treated with vehicle or 50 nM of p95HER2-TCB. PDX445 cells were counted by flow cytometry and represented as percentage of viable cells. **In vivo**, NSG mice carrying PDX445 were transferred with PBMCs. Mice were treated with vehicle or 1 mg/kg of p95HER2-TCB (arrows). Means ± SD are shown, n ≥ 3 per group. At the end of the experiment, the percentages of circulating human CD45 cells, relative to total leukocytes, were determined. * P<0.05, **P<0.01, ***P<0.001. (left) two-tailed t test, (middle) two-way ANOVA and Bonferroni correction.

These results confirmed that the quantitative analysis of p95HER2 expression is useful to predict the antitumor activity of p95HER2-TCB, because treatment was only effective in the PDX with p95HER2 expression above the defined threshold.

3.8. Antitumor effect of p95HER2-TCB on intracranial lesions

Current therapies targeting HER2, such as trastuzumab, are effective against extracranial HER2-positive tumours, but their efficacy against brain metastases remains limited (Kodack et al., 2015). Therefore, there is a need to develop drugs for patients with HER2-positive brain lesions.

To test the efficacy of p95HER2-TCB on brain lesions, we used MCF7p95HER2 cells transfected with a luciferase reporter. These cells were inoculated into the brains of fully humanized mice followed by the indicated treatment (**Figure 32A**). Bioluminescence monitoring showed efficacy of p95HER2-TCB against the intracranial tumours (**Figure 32B-E**). Moreover, we showed that the tumours were inside the brain (**Figure 32D**), being p95HER2-TCB able to cross the BBB.

In summary, this data reinforces the possibility of using TCBs against malignant brain lesions due to its potential to cross the BBB, in contrast to trastuzumab.

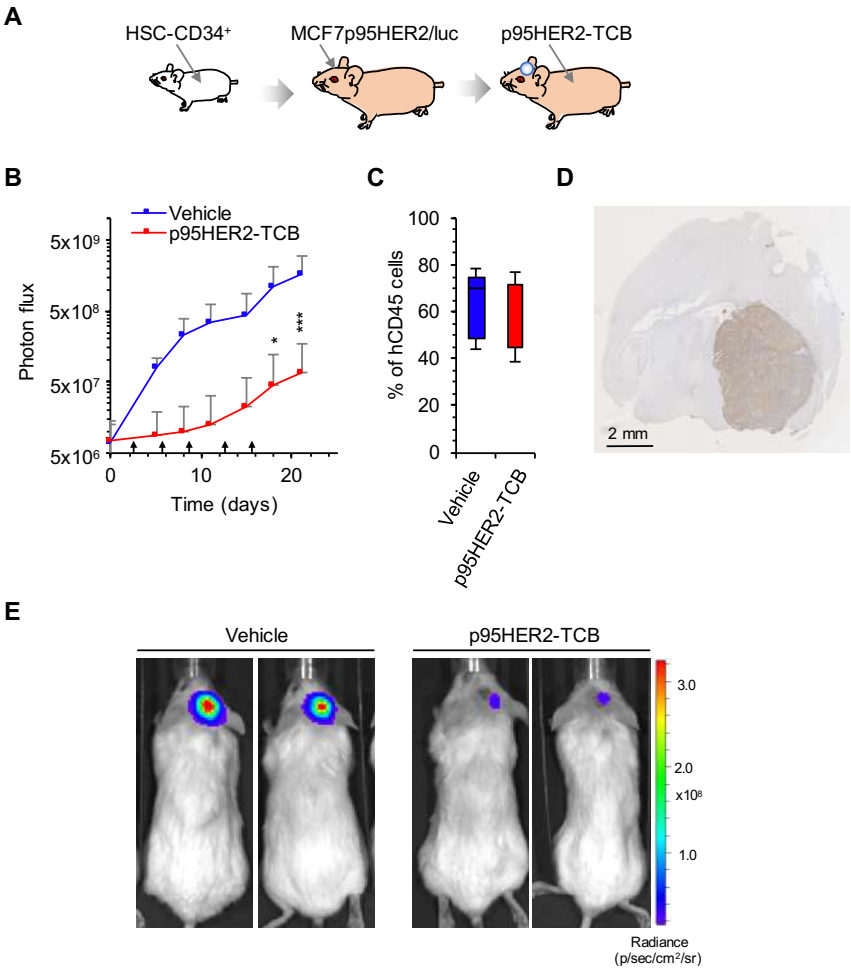


Figure 32: Effect of p95HER2-TCB on intracranial tumour growth *in vivo*.

(A) Schematic drawing illustrating humanization of mice with HSC-CD34$^+$ cells and intracranial injection of MCF7 p95HER2/luciferase cells. **(B)** Intracranial tumour growth monitored by assessing bioluminescence, expressed as means ± SD; n≥ 4 per group. **(C)** Percentages of circulating human CD45 cells, relative to total leukocytes, were determined 5 months after injection of HSC-CD34$^+$ cells into NSG mice. **(D)** Representative immunohistochemistry image showing a horizontal section of the brain stained with anti-cytokeratin of a vehicle-treated mouse. **(E)** Representative images of the tumour growth monitored by assessing the bioluminescence. * P<0.05, ** P<0.01, *** P<0.001, (I) two-way ANOVA and Bonferroni correction.

4. Development of CAR T cells against p95HER2

A different immunotherapeutic strategy to redirect T cell against the tumour site consists in the generation of CAR T cells. Chimeric antigen receptors or CARs are synthetic receptors that fuse an antigen-binding domain of a single-chain variable fragment (scFv) from a monoclonal antibody to the intracellular signalling motifs capable of T cell activation (Eshhar et al., 1993). In the last part of this thesis, we pursued the generation of p95HER2 CAR Ts.

4.1. A humanized p95HER2 CAR T exclusively induce cytotoxicity on p95HER2 expressing cells

Briefly, we generated retroviral vectors containing the scFv of the α-p95HER2 antibody (**Figure 9**), fused to the signalling domain of CD3ζ and to the co-stimulatory domain of CD28 (**Figure 33**). Of note, in collaboration with Roche, we humanized the scFv, as it has been reported that non-humanized CAR T cells may induce human anti-mouse antibody (HAMA) responses (Maus et al., 2013). As a positive control, we used a CD28-based CAR targeting full-length HER2.

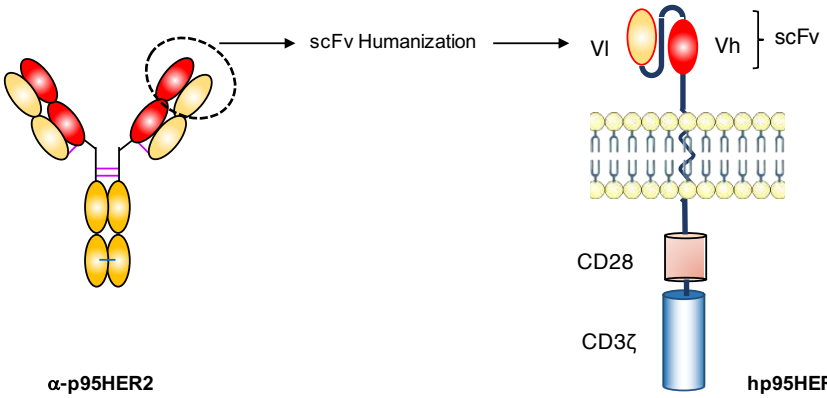


Figure 33: Schematic illustrating the origin of the scFv used for the humanized (h) p95HER2 CAR T. hp95HER2 CAR contains the scFv from the α-p95HER2 humanized, a transmembrane domain and a CD28 co-stimulatory domain fused to CD3ζ.

Next, we activated and transduced PBMCs from three healthy donors with the indicated CAR constructs (**Figure 34A**). At day 5 post-transduction, we analysed CAR expression on T cells. HER2 CAR and humanized p95HER2 CAR (hp95HER2) were efficiently expressed on the surface of T cells, although the expression of the humanized p95HER2 CAR was slightly lower (**Figure 34B**).

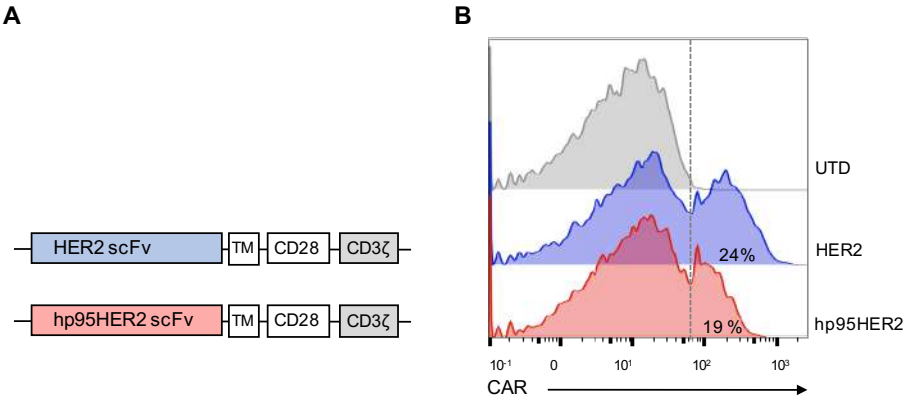


Figure 34: Design and expression of hp95HER2 CAR T.

(A) Schematic representation of the chimeric receptors containing an scFv that binds to full-length HER2 (blue) or p95HER2 (red). **(B)** Surface expression of the indicated CARs in A on T cells at day 5 post-transduction; percentage of positive-CAR T from total T cells are indicated. UTD: untransduced T cells. Representative analyses from one PBMCs healthy donor.

Then, we assessed the cytotoxic effect of CAR T cells on MCF10A cells either expressing p95HER2 or not. To do so, we co-cultured the indicated cell line with different ratios of CAR T cells for 48 hours (**Figure 35A**). The results showed that humanized p95HER2 CAR T induced cell-death on MCF10Ap95HER2, although the efficacy was evident at high ratios of Target: CAR T cells (**Figure 35B**). Importantly, hp95HER2 CAR Ts did not induce any cytotoxicity on cells that do not express p95HER2 (MCF10A) (**Figure 35D**). As expected, HER2 CAR Ts had a cytotoxic effect on either MCF10A expressing or not p95HER2, due to the recognition of full-length HER2 expressed at basal levels (Subik et al., 2010). In line with the cytotoxicity results, hp95HER2 CAR T activation was detected only at 1:5 Target: CAR T ratio on MCF10Ap95HER2 (**Figure 35C-E**).

We speculated that the humanization process may have impaired the affinity of the scFv and consequently, the potential antitumor effect of the hp95HER2 CAR T. Unfortunately, the non-humanized p95HER2 CAR version could not be expressed in the cell membrane, being retained in the cytosol for unknown reasons (data not shown). Thus, we could not compare the effect of humanization on the affinity for p95HER2.

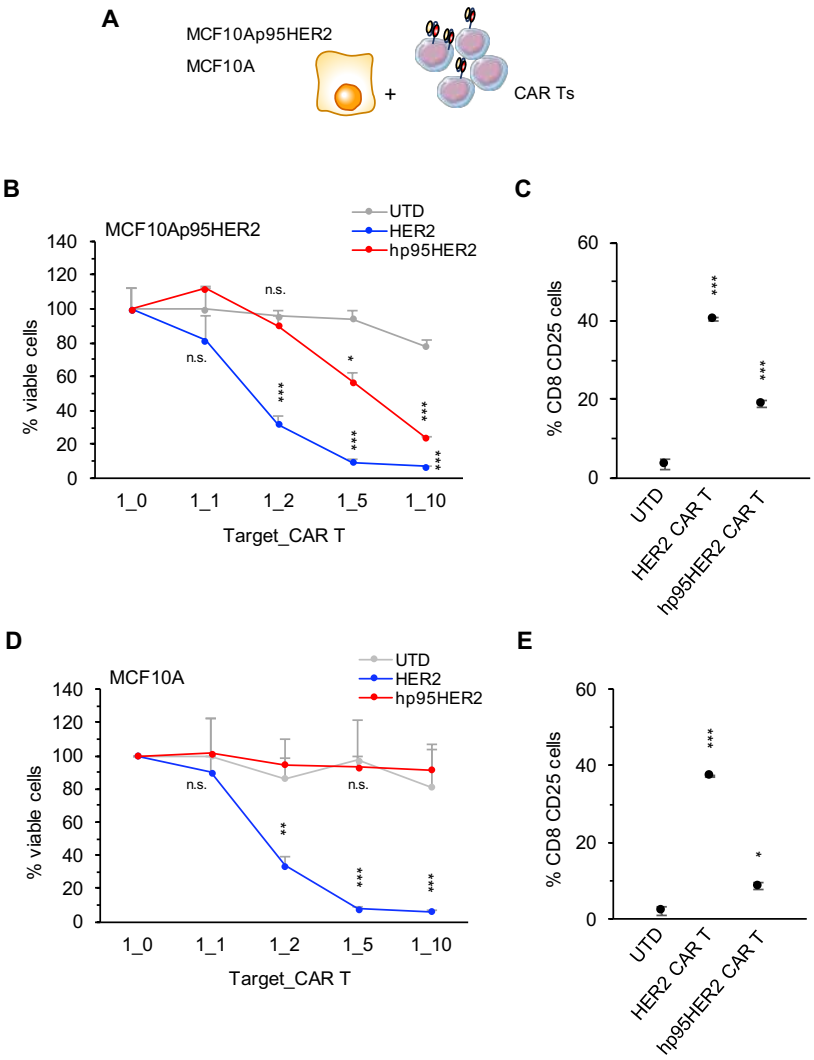


Figure 35: Cytotoxicity of indicated CAR T cells on MCF10A cells expressing or not p95HER2.
(A) Schematic drawing illustrating the co-culture experiments. **(B)** MCF10Ap95HER2 cells were co-cultured with CAR T cells at the indicated ratios. At 48h, viable target cells were assessed by flow cytometry **(C)** CAR T cells from B at 1:5 Target:CAR T ratio were stained with CD4 or CD8 and CD25 at endpoint of the experiment and analysed by flow cytometry. **(D)** MCF10A cells were co-cultured with CAR T cells at the indicated ratios. At 48h, viable target cells were assessed by flow cytometry. **(E)** CAR T cells from D at 1:5 Target:CAR T ratio were stained with CD4 or CD8 and CD25 at endpoint of the experiment and analysed by flow cytometry. N=3 expressed as means ± SD. Representative analyses from one PBMCs healthy donor. Statistics compare UTD with HER2 or hp95HER2 CAR Ts. $P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ two-tailed *t* test. UTD: untransduced T cells.

4.2. Generation of additional p95HER2-CAR T cells

The previous data using hp95HER2 CAR T showed effectivity on MCF10Ap95HER2 but only at relatively high ratios of Target:CAR T. In order to increase the potency of p95HER2 CAR Ts, we used scFv from additional anti-p95HER2 antibodies previously generated in the laboratory.

4.2.1. Screening of anti-p95HER2 antibodies

We focused on four antibodies, the α-p95HER2s (II), (III), (IV) and (V), which showed detectable binding to p95HER2 in preliminary experiments (Parra-Palau et al., 2010). To select the more suitable for the subsequent generation of p95HER2 CAR Ts, we performed binding assays on different cell lines.

On one hand, we used MCF10A cells expressing p95HER2 to assess the affinities of the α-p95HER2 antibodies. The α-p95HER2 antibody previously described was used as a control. Of note, α-p95HER2 (II) and (III) antibodies bound to p95HER2 similarly as α-p95HER2 (**Figure 36A**).

Despite the clear preference for binding cells expressing p95HER2, α-p95HER2 (II) and (III) antibodies also bound to HER2-overexpressing MCF10A cells (**Figure 36B**). This residual binding could be due to the interaction with the full-length HER2 receptor or, alternatively, to a low expression of p95HER2 in cells overexpressing HER2. Because p95HER2 is synthesised from the mRNA encoding HER2 through alternative initiation of translation from the AUG codon encoding methionine-611(Pedersen et al., 2009), we analysed the binding of α-p95HER2 antibodies to cells expressing HER2 with a methionine-to-alanine mutation in position 611 (**Figure 36C**). Indeed, the results showed that the binding of α-p95HER2 antibodies to cells overexpressing HER2 is largely due to the generation of p95HER2 synthesised by alternative initiation of translation from methionine-611 (**Figure 36B-C**). In parallel, we also proved that α-p95HER2 (II) and (III) antibodies did not bind to full-length HER2 expressed at basal levels using MCF10A wild type cell line (**Figure 36D**).

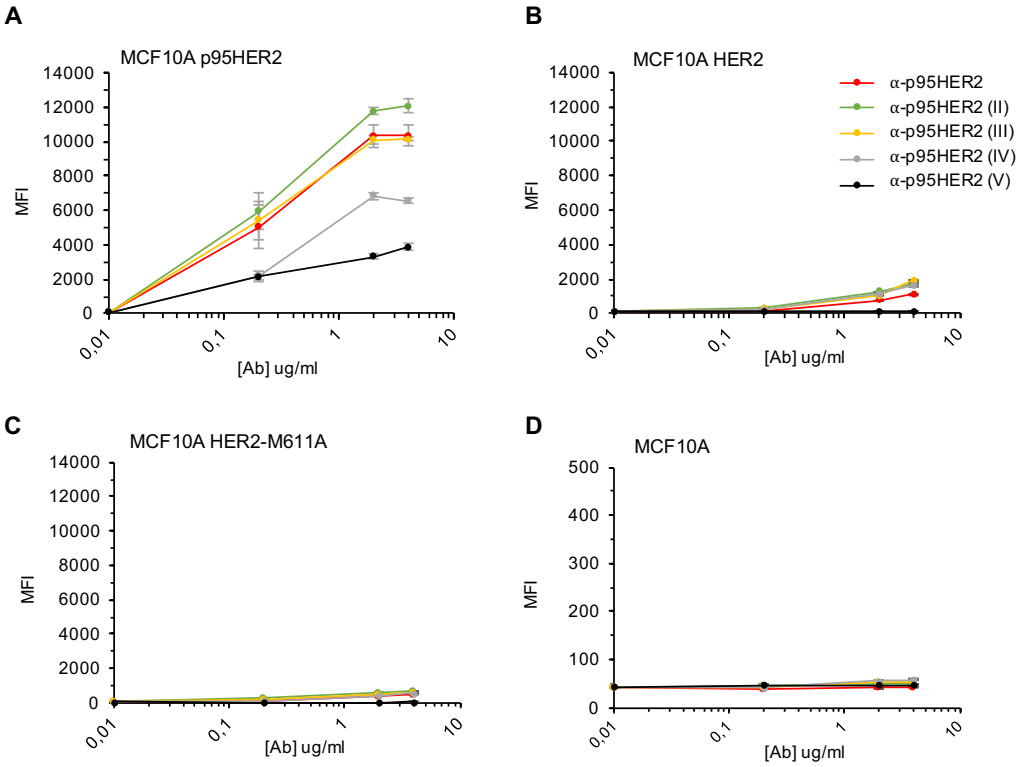


Figure 36: Median fluorescence intensity (MFI) of the binding of α-p95HER2 antibodies to different MCF10A cells

(A) MCF10A expressing p95HER2, **(B)** MCF10A overexpressing HER2, **(C)** MCF10A overexpressing HER2 with a mutation in the methionine-611 and **(D)** MCF10A wild type were stained with the indicated α-p95HER2 antibodies. Then, mouse secondary antibody was added and results were analysed by flow cytometry. N= 3 expressed as means ± SD.

These results indicated that α-p95HER2 (II) and (III) antibodies were good candidates, due to its high affinity for p95HER2 and low affinity for full-length HER2.

4.2.2. Additional p95HER2-CAR T induce a potent effect on p95HER2-expressing cells

As a result of the previous binding assay (**Figure 36**), we sequenced the scFv from the hybridomas that produced α-p95HER2 (II) and (III) antibodies. Then, we generated retroviral vectors containing the scFv of the α-p95HER2 antibodies (II) or (III), fused to the signalling domain of CD3ζ and to the costimulatory domain of CD28 (**Figure 37**). As previously indicated, we used the CD28-based CAR targeting full-length HER2 as a positive control.

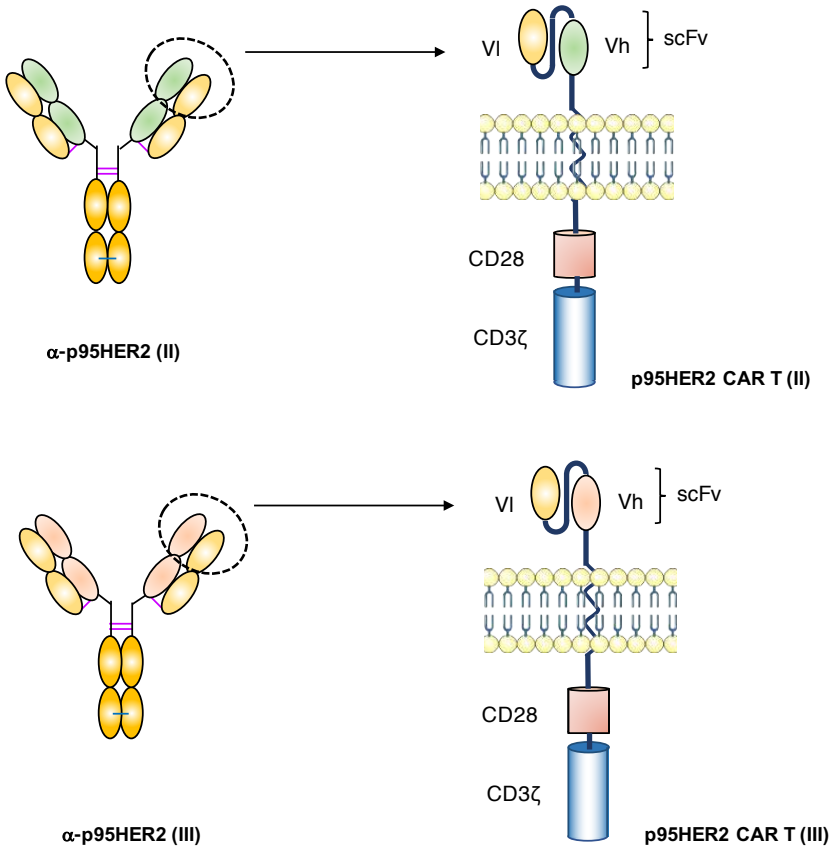


Figure 37: Schematic illustrating the origin of the scFvs used for p95HER2 CAR T (II) and (III)
p95HER2 CAR (II) and (III) contain the scFv from the α-p95HER2 (II) and (III) respectively, a transmembrane domain and a CD28 co-stimulatory domain fused to CD3ζ.

Next, we activated and transduced PBMCs from two healthy donors with the indicated CAR constructs (**Figure 38A**) and analysed CAR expression on T cells. All CARs were efficiently expressed on the surface of T cells, being the p95HER2 (II) CAR the one that was expressed at higher levels (**Figure 38B**).

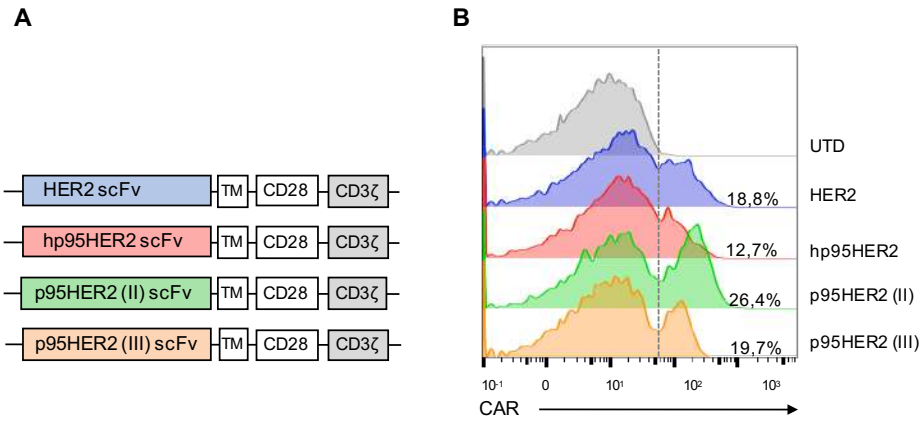


Figure 38: Design and expression of additional p95HER2 CAR Ts.

(A) Schematic representation of the chimeric receptors containing an scFv that binds to full-length HER2 (blue) or p95HER2 (red, green and orange). **(B)** Surface expression of the indicated CARs in A on T cells at day 5 post-transduction; Percentages of CAR T-positive cells from total T cells are indicated. UTD: untransduced T cells. Representative analyses from one PBMCs healthy donor.

Then, we assessed the cytotoxic effect of CAR T cells on MCF10A cells expressing p95HER2 (**Figure 39A**). We observed that p95HER2 CAR Ts (II) and (III) induced a significant cytotoxic effect at low Target:CAR T ratios, similarly to HER2 CAR T (**Figure 39B**). As observed in **Figure 35**, hp95HER2 CAR T only induced a clear cytotoxicity at higher ratios. In line with the cytotoxic results, p95HER2 CAR Ts (II) and (III) and HER2 CAR Ts were the most activated at 1:5 Target: CAR T ratio on MCF10A p95HER2 cells (**Figure 39C**).

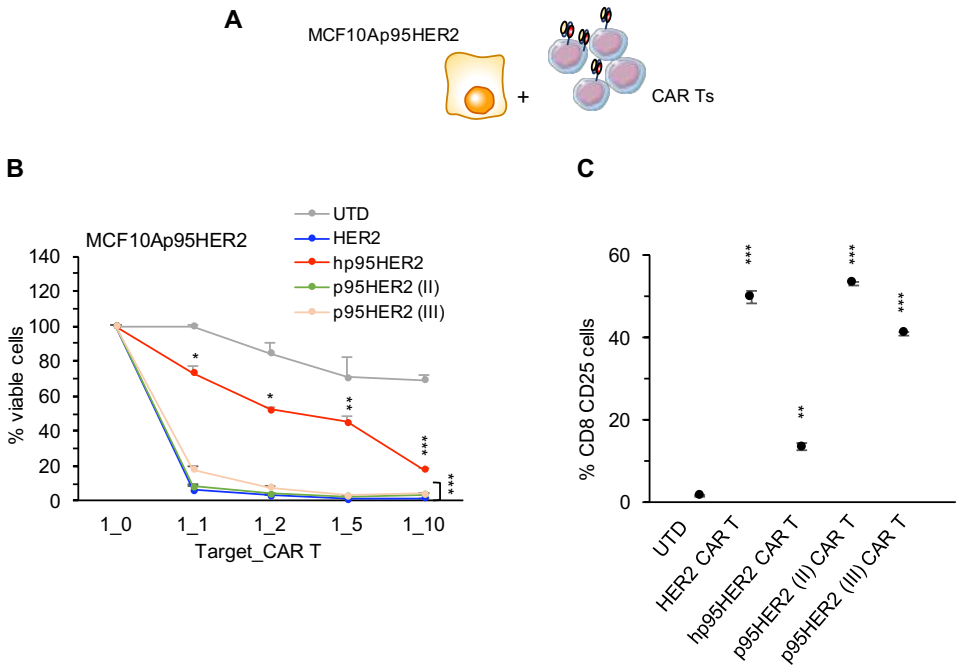


Figure 39: Cytotoxicity of the indicated CAR T cells on MCF10A cells expressing p95HER2.
(A) Schematic drawing illustrating the co-culture experiments. **(B)** MCF10A p95HER2 cells were co-cultured with CAR T cells at the indicated ratios. At 48h, viable target cells were assessed by flow cytometry **(C)** CAR T cells from B at 1:5 Target:CAR T ratio were stained with CD4 or CD8 and CD25 at endpoint of the experiment and analysed by flow cytometry. N=3 expressed as means ± SD. Representative analyses from one PBMCs healthy donor. Statistics compare UTD with the indicated CAR Ts. $P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ two-tailed *t* test. UTD: untransduced T cells.

In summary, we were able to generate CAR Ts directed against p95HER2. On one hand, we developed a humanized p95HER2 CAR T that induced a mild cytotoxicity. On the other hand, we generated two independent p95HER2 CAR Ts, derived from α-p95HER2 (II) and (III), that triggered a much potent cytotoxicity on p95HER2 expressing cells. Undoubtedly, the next steps will be to humanized the scFvs and assess the activity of this humanized CAR Ts on cells expressing or not p95HER2.

Collectively, in this thesis we prove that p95HER2 is a *bona fide* TSA expressed by a subset of HER2-positive BC patients. Moreover, we show that the redirection of T lymphocytes via p95HER2 could be a safe and effective therapeutic option, in contrast to other immune therapies targeting tumour-associated antigens.

DISCUSSION

The use of anti-HER2 therapies over the last 20 years has notably improved the prognosis of HER2-positive BC patients (Wuerstlein & Harbeck, 2017; Baselga et al., 2012). However, the appearance of primary and acquired resistances limits the antitumor efficacy of current treatments. Consequently, patients frequently relapse, mainly developing brain metastasis (Pondé, Lambertini & de Azambuja, 2016; Kennecke et al., 2010). Therefore, new therapeutic strategies are still necessary to improve treatments.

Another shortcoming of anti-HER2 therapies are the toxicities due to their 'on-target off-tumour' effects. As previously described and confirmed in this thesis, HER2 is expressed in several normal epithelial tissues and in some mesodermal tissues, such as cardiac tissue (Pressl, Cordon-Cardo & Slamon, 1990b). In fact, drugs targeting HER2, such as the monoclonal antibody trastuzumab or the TKI lapatinib, can induce cardiotoxicity by inhibiting HER2 expressed by cardiomyocytes, particularly in combination with chemotherapy (Valachis et al., 2013). The expression in normal tissues is also the likely reason for the serious side effects caused by CARs and TCBs targeting HER2 (van der Stegen et al., 2013; Morgan et al., 2010). To avoid these undesired effects, doses of CAR T cells against HER2 have been lowered, from 10^{10} (Morgan et al., 2010) to 10^{8} cells (Ahmed et al., 2015). Although these doses seem to be safe (Wermke et al., 2017; Ahmed et al., 2015; Wuellner et al., 2015), they may compromise the antitumor effect of the treatment.

In this thesis, we demonstrated that p95HER2, a truncated form of HER2, is a *bona fide* tumour-specific antigen (TSA) (**Figure 10**), expressed by a subset of HER2-positive BC patients (**Figure 26**). Therefore, redirection of T lymphocytes via p95HER2 could be a safe and effective therapeutic option, in contrast to immune therapies targeting HER2.

1. The design of p95HER2-TCB

In the first part of this work, we characterized two T-cell bispecific antibodies (TCBs), one that binds monovalently to p95HER2 and the other that comprises a bivalent p95HER2-targeting arm. As expected, the second format generates an enhanced antitumor response as a result of the increased binding avidity (**Figure 11**), also reported in other studies (Bacac et al., 2016). These TCBs contain an Fc domain that confers a higher stability in plasma compared with other TCB formats *i.e* BiTEs, which lack an Fc region (Lowe et al., 2011). However, the bivalent format it is produced at lower yields (Birch & Racher, 2006), making its production limiting. The Fc-mediated effector functions of these TCBs are suppressed by the introduction of P329G LALA mutations, which minimize off-target toxicity by the Fcγ receptor (Bacac et al., 2018; Bacac et al., 2016; Woodle et al., 1999).

An additional advantage of p95HER2 as a target of TCBs is its short extracellular domain. The peptide recognized by the p95HER2-TCB is located only 34 amino acids from the plasma membrane (Parra-Palau et al., 2010); this proximity likely facilitates T cell synapse formation and, as a consequence, tumour cell lysis (Li et al., 2017b).

2. Cytotoxicity induced by T cells subsets through TCBs

We showed here that TCBs are capable of inducing a cytotoxic effect through different T cell subsets. The unexpected ability of CD4 cells to effectively kill target cells has also been shown by other groups (**Figure 12**) (Seckinger et al., 2017; Bacac et al., 2016). However, the mechanisms of cell-death induction upon TCB treatment are not well understood. Theoretically, activation of CD4 lymphocytes leads to the secretion of cytokines and the subsequent recruitment other immune cells.

In contrast, CD8 lymphocytes elicit direct cytotoxicity, releasing granzyme B and perforin as well as cytokines (Abbas, Lichtman & Pillai, 2014). Given these different traits between CD4 and CD8 lymphocytes, it is unexpected to detect a similar tumour cell-death induction upon TCB activation (**Figure 12**). We concluded that in our *in vitro* setting, TCBs induce such a T cell activation that CD4 lymphocytes achieve a similar cytotoxic phenotype as CD8 lymphocytes. Indeed, it has been described that CD4 lymphocytes may differentiate into a cytotoxic phenotype and release granzyme B and perforin under certain conditions (Takeuchi & Saito, 2017; Quezada et al., 2010; Appay et al., 2002). In future experiments, it would be interesting to further characterize the mechanisms behind these observations.

Compared with their naïve counterparts, memory T cells are known to have less stringent requirements for activation and a more rapid effector response (Pennock et al., 2013). Thus, we hypothesized that naïve T cells would not be as potent cell-death inducers upon TCB treatment as memory T cells. Nevertheless, in our *in vitro* setting, both subtypes induced a similar cytotoxic effect (**Figure 14**), suggesting that TCBs induce such a potent effect that it induced the activation of T cells regardless its stage of differentiation, naïve or memory. Of note, we observed an impaired cytotoxicity by terminal differentiated CD8 cells, presumably because their exhausted phenotype. These results suggest that the different T cell subset distribution between patients (**Figure 13**), being naïve T cells less frequent in older people (Saule et al., 2006), would not affect the antitumor potential of TCBs. However, a predominant exhausted circulating T cell phenotype would indicate a possible decrease of TCB potency in patients. Importantly, more studies would be needed to further prove this hypothesis, such as *in vivo* experiments exclusively employing each T cell subset.

In addition, other groups have demonstrated that TCBs also activate T regs, which inhibit T cell proliferation and cytotoxic antitumor effect (Duell et al., 2017). Even though we have not address this issue directly, CD4 lymphocytes induced a similar cytotoxicity to CD8 lymphocytes upon TCB induction, suggesting a minor role of Tregs in our *in vitro* setting. Nevertheless, it would be interesting to isolate Tregs from PBMCs and assess its role upon p95HER2-TCB induction.

3. Humanized mice models and p95HER2-TCB

Humanized mice models have allowed us to assess the effect of p95HER2-TCB on cultured cells and in PDXs *in vivo*. We have shown that both the PBMC model and the fully humanized model are suitable to assess the efficacy of p95HER2-TCB (**Figure 18, 20**). In the fully humanized mice model, the graft-versus host disease (GvHD) is greatly decreased, compared with the PBMC model (Byrne et al., 2017). With this model, we could have achieved longer periods of treatment and thus, we could have tested durability of the tumour regression. However, in our experimental setting, HER2-positve breast cancer PDXs engrafted very poorly compared to other tumour types (**Table 4**), limiting the use of this model (**Figure 29**). The short but sufficient therapeutic window of the PBMC model did allow us to gauge the antitumor effect of p95HER2-TCB (**Figure 21, 25, 28, 30-31**).

As an aside, it is currently unclear how allogeneic reactions affect experimental models aimed to reconstitute the interplay between tumour and immune cells (Byrne et al., 2017). In our analyses, we included MHC-matched and non-matched lymphocytes and confirmed that allogeneic PBMCs may reject tumours. Thus, using non-matched lymphocytes can complicate the interpretation of some experiments, although p95HER2-TCB had a clear effect on p95HER2-positive cells, irrespectively of the source of PBMCs (**Figure 28**).

4. p95HER2-TCB and safety

Our results fully support the hypothesis that p95HER2 is a safe target for the redirection of T cells against tumour cells. p95HER2 was not detected in healthy tissues by IHC (**Figure 10**). In addition, we did not detect any effect of p95HER2-TCB on cells with low HER2 expression (**Figure 25C**) or even on HER2-amplified, p95HER2-negative tumours (**Figure 25E, 31**). These results greatly reinforce the conclusion that p95HER2 is a TSA in contrast to the tumour-associated antigen HER2.

The α-p95HER2 antibody described in this thesis, recognized the corresponding mouse p95HER2 sequence with very low affinity (**Figure 9**), despite a single amino acid change in the epitope. Thus, possible side effects detected in healthy mouse tissues could not be evaluated. Of note, the p95HER2 sequence of cynomolgus monkey is closer to humans, being the epitope of α-p95HER2 antibody fully conserved. Therefore, the on-target off-tumour toxicities could be evaluated *in vivo* in this animal model.

5. p95HER2-TCB and intracranial lesions

One serious limitation of the current anti-HER2 drugs is their lack of efficacy against brain metastases. In the case of trastuzumab, this inefficacy is attributed to its inefficient penetration through the blood brain barrier (BBB) (Kodack et al., 2015). A BiTE targeting CD3 and EGFRvIII had promising therapeutic results in mice harbouring intracerebral gliomas (Choi et al., 2013), suggesting the possible penetration of TCBs through BBB. However, the mechanism of passage across the BBB remains unknown (Razpotnik et al., 2017).

In our *in vivo* setting, p95HER2-TCB effectively impaired the growth of intracranially implanted cells, indicating that cytotoxic lymphocytes can be redirected against brain metastases. Additional experiments *in vitro* performed in the laboratory also indicated that p95HER2-TCB transports lymphocytes to reach intracranial tumours (data not shown). However, given the limitations of simulating BBB *in vitro*, this hypothesis should be tested in the future, for instance, using syngeneic immunocompetent mice models. Moreover, a retrospective analysis of the p95HER2 positivity on brain metastasis of HER2-positive BC patients would be essential, which has not been conducted yet.

6. TCBs efficacy and TILs status

T cell infiltration is variable in the subset of HER2-positive tumours, being present in approximately 20% of the cases (Kroemer et al., 2015). It has been reported that TCBs are capable of activating intra-tumour T-cells in different carcinomas (Schreiner et al., 2016). Therefore, it seems reasonable to believe that highly infiltrated tumours could respond better to TCBs. Building on this idea, humans TILs typically have memory features (Gros et al., 2014) and our *in vitro* data suggest that this phenotype can be efficiently engaged and further activated by TCBs. Nevertheless, the therapeutic efficacy of TCBs may be subjected to the dysfunctional state of T cells, where exhausted T cells may hamper the TCB potency (Labrijn et al., 2019).

Immune-desert BC tumours, which lack an immune compartment, are the ones with worse prognosis (Chen & Mellman, 2017; Kroemer et al., 2015). The mechanism of action of TCBs consist in redirecting T lymphocytes to the tumour site and thus, inducing an antitumor response. Therefore, TCBs could offer a therapeutic option even in low T cell infiltrated tumours. In fact, in our *in vivo* studies, PDXs tumours are poorly infiltrated (**Figure 29D**, tumour) presumably because of the loss of immune cells when human tumour biopsies are engrafted in mice. Even in these conditions, p95HER2-TCB had a potent antitumor effect, suggesting that its potency may be independent on the TILs status. Nevertheless, it would be interesting to assess the immune status of p95HER2-positive human tumours to further validate this hypothesis.

7. An alternative redirection of T cells: p95HER2 CAR Ts

In the last part of this thesis, we developed CAR T cells, an alternative immunotherapeutic agent that also redirects T lymphocytes to the tumour site. CAR T cells can mediate long-term durable remissions in B cell malignancies, as proved in CAR Ts targeting CD19 (June et al., 2018). However, using CARs against solid tumours is still challenging for multiple reasons, being a key factor the lack of specific targetable tumour antigens (Klebanoff, Rosenberg & Restifo, 2016). In this thesis, we have shown the successful generation of second generation CAR Ts against p95HER2 positive BC.

On one hand, we generated a humanized CAR T against p95HER2. It has been reported that non-humanized CAR T cells may induce human anti-mouse antibody (HAMA) responses which could limit the persistence of the CAR T cells or cause allergic sensitization (Maus et al., 2013). To generate the humanized CAR, we used a humanized version of the scFv from the α-p95HER2 antibody, fused to a CD28 signalling domain. Our results indicate that humanized p95HER2-CAR Ts have a specific cytotoxic effect on p95HER2-positive cells (**Figure 35B**). However, the affinity of human p95HER2 CAR T cells is considerably lower compared with a HER2 CAR T (**Figure 35B**). In fact, the HER2 CAR T induces a very effective cytotoxic effect on target cells with low levels of HER2 (**Figure 35D*)***, suggesting its possible potent effect on healthy cells. We speculated that the humanization process might have impaired the affinity of the scFv and consequently, the potential antitumor effect of the CAR T. However, this hypothesis could not be proved because the non-humanized CAR version could not be expressed in the cell membrane, being retained in the cytosol for unknown reasons (data not shown).

To overcome these difficulties, we produced p95HER2-CAR Ts using scFvs from two additional α-p95HER2 antibodies, the α-p95HER2 (II) and the α-p95HER2 (III) antibodies. Both CAR Ts had a more potent effect on target cells expressing p95HER2 (**Figure 39**). Certainly, the next step will be to humanize theses scFv versions and generate new humanized CAR T cells to avoid HAMA responses (Maus et al., 2013). Then, the most cytotoxic and specific humanized CAR T versions will be chosen for further *in vitro* and *in vivo* studies. On the basis of the future results, we are planning to run a phase one clinical study on p95HER2 positive BC patients.

7.1. Generation of CAR Ts

Several groups have focused on defining which T cell populations are more appropriate for CAR T cells generation. So far, the 1:1 ratio of CD4:CD8 CAR T cells seems to achieve the maximum antitumor response, although alternative ratios are still being studied (June et al., 2018). Regarding the T cell differentiation state, accumulating data suggests that engineering naïve T cells and/or central memory T lymphocytes, which are the less differentiated T cells, provides a higher CAR T cell persistence *in vivo* (Srivastava & Riddell, 2018; Turtle et al., 2016; Berger et al., 2008). In our preliminary data, we exclusively used CAR T derived from PBMCs, thus, for future experiments, generating p95HER2 CAR Ts from different T cell subsets (**Figure 13**) will be required to find more potent and persistent p95HER2 CAR Ts.

CARs are typically transduced using retroviral or lentiviral vectors (Lim & June, 2017), which integrate randomly in the genome of T cells. As a consequence, CAR gene expression is highly variable, owing to position effects and variations in vector copy number. Nevertheless, CAR T cells engineered in this manner are capable of tumor eradication in preclinical models, but they frequently display high tonic signaling and accelerated exhaustion, leading to lack of persistence of CAR Ts (Ellis, 2005). To overcome this hurdle, Eyquem and colleagues have engineered a strategy to insert CAR genes into the T-cell receptor α constant (TRAC) locus. These TRAC-CAR T cells have shown reduced constitutive antigen-independent CAR signaling, delayed antigen-dependent differentiation and exhaustion, resulting in increased therapeutic potency (Eyquem et al., 2017). Therefore, introducing this technique in our p95HER2 CAR Ts will be a promising approach to generate more potent and persistent CAR Ts.

7.2. CAR Ts and side effects

CAR T therapy is associated with unique toxicities, being cytokine release syndrome (CRS) and neurotoxicity the most commonly observed side effects. CRS consists in a systemic inflammation caused by the activation of immune cells and the release of cytokines (Neelapu et al., 2018). The mechanisms behind CAR T cell-mediated CRS are poorly understood, in part because the field lacks informative animal models to study it (June et al., 2018). Recently, several studies addressing this issue have unveiled possible mouse models to assess the CAR T cell-mediated CRS (Norelli et al., 2018; Giavridis et al., 2018). These mouse models are capable to reconstitute the human immune cells that trigger CRS, mainly though monocytes and macrophages. In future experiments, we could implement these mouse models in our laboratory to further assess p95HER2 CAR T cell toxicity.

8. TCBs *versus* CAR Ts

The two approaches for T cell redirection, TCBs and CAR T cells, have demonstrated effectivity in patients with haematological malignancies. In contrast, they have not shown efficacy against solid tumours in the clinic. An immunosuppressive TME, inefficient extravasation to the tumour site, along with a lack of T cell persistence may hamper the antitumor potential of TCBs and CAR T cells against solid malignancies (Slaney et al., 2018; June et al., 2018).

Several studies confirmed that both platforms induce serial killing by T cells, with one T cell able to kill several target cells (Davenport et al., 2015; Hoffmann et al., 2005). Studies comparing TCBs and CARs suggest that the cytokines secreted upon T cell activation also contribute to the lysis of antigen-negative tumour cells, inducing the so-called bystander effect (Ross et al., 2017; Lanitis et al., 2012), although these results differ depending on the tumour antigen (Henderson et al., 2013).

Theoretical differences between the CAR- and TCB-based treatments approaches exist regarding tumour recurrence. CAR T cells may be able to persist and provide tumour-reactive T cells against recurring lesions, even before they become evident (June, Riddell & Schumacher, 2015). In contrast, without continuous administration of TCBs, recurrent tumours may regrow (Kontermann & Brinkmann, 2015). Nevertheless, TCBs treatment has the possibility of being stopped at will if side effects become intolerable. With the same objective, inducible death of CAR T cells by the so-called ‘suicide switches’ have also been developed to control engineered T cell persistence (Di Stasi et al., 2011).

Few studies directly compared the functional efficacy of the two strategies related to their antitumor effect. Stone and colleagues observed that CAR T cells were more potent than BiTEs against cancers expressing low levels of antigens (Stone et al., 2012). However, additional studies using different *in vivo* models found opposite results to Stone and colleagues, complicating the right comparison between both strategies with the current preclinical models (Slaney et al., 2018). Therefore, it is still not clear which T cell strategy induces a more potent antitumor effect.

9. Patients eligible for anti-p95HER2 therapy

We believe that patients HER2/p95HER2 positive and that they are resistant to the current anti-HER2 treatment, could benefit from these novel immunotherapeutic agents. O'Rourke and colleagues have shown that CAR T cells targeting EGFRvIII are capable of crossing the BBB in a phase one clinical trial (O'Rourke et al., 2017). Thus, p95HER2 CAR Ts could also represent an alternative therapy to treat breast cancer patients with brain metastasis, which is the most prominent metastatic site in HER2-positive breast cancer patients.

In summary, p95HER2 is a *bona fide* tumour-specific antigen expressed by a subset of HER2-positive breast cancers. Redirecting T cells towards p95HER2 (by TCBs or CAR Ts) is likely a safe and effective antitumor immune therapy.

CONCLUSIONS

- p95HER2 is a tumour-specific antigen, in contrast to full-length HER2.

- p95HER2-TCB has a strong antitumor effect exclusively on p95HER2-expressing breast cancer cells, both *in vitro* and *in vivo*.

- TCBs induce cytotoxicity through naïve and memory T cells *in vitro*.

- Contrary to HER2-TCB, p95HER2-TCB has no toxicity on normal tissues.

- Intracranial lesions p95HER2-postive can be targeted by p95HER2-TCB.

- A humanized p95HER2 CAR T exclusively induce cell-death on p95HER2-expressing cells.

Altogether, this thesis proves that that redirection of T lymphocytes via p95HER2 could be a safe and effective therapeutic option, in contrast to other immune therapies targeting tumour-associated antigens.

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