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Kamal Asgarov

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THESE

Pour l'obtention du Diplôme de Doctorat de l'Université de Franche-Comté Spécialité :
Sciences de la Vie et de la Santé

**Development of more precise and efficient antibodies for
cancer targeting: Membrane associated form specific anti-
mesothelin antibodies and CAR as an example.**

Soutenue le 13 décembre 2016 par

ASGAROV Kamal

Sous la direction du Pr Christophe BORG et Dr Bernard ROYER

Membres du Jury :

Directeur de thèse : Docteur Bernard ROYER

Co-directeur de thèse : Professeur Christophe BORG

Rapporteurs : Professeur Mohamed Hebbar

Professeur Jan de Mey

Examineurs : Professeur Robin FAHREUS

Docteur Andy CLARK

Docteur John Wijdenes

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ABBREVIATIONS

A

ADC : Antibody-drug conjugate

ADCC : antibody dependent cell cytotoxicity

ADP : adenosine diphosphate

APC : Antigen Presentating Cell

B

BV : brentuximab-vedotin

C

CAR : chimeric antigen receptor

CD- : cell differentiation antigen

CDC : complement dependent cytotoxicity

CDR : complementarity-determining region

CH : heavy chain constant domain

CLL : chronic lymphocytic leukemia

CM : central memory

CTLA4 : cytotoxic T lymphocyte antigen 4

D

DNA : deoxyribonucleic acid

DLT : dose-limiting toxicity

DTH : delayed-type hypersensitivity

E

EF2 : elongation factor 2

EGF : epithelial growth factor

EGFR : epithelial growth factor receptor

ELISA : enzyme linked cell sorbent assay

EM : effector memory

EMA : European Medicines Agency

F

FACS : fluorescence-activated cell sorting

Fab : fragment antigen-binding

Fc : Fragment crystallizable

FcR : Fc binding receptor

FDA : United states food and drug administration

Fv : Fragment variable

G

GM-CSF : granulocyte-macrophage colony-stimulating factor

GPI : glycosyl-phosphatidyl-inositol

H

HSV-tk : Herpes simplex virus thymidine kinase

HLA : human leucocyte antigen

I

iCasp9 : inducible caspase 9

IFN- γ : interferon gamma

IgG1 : Immunoglobulin G1

I κ B : nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor

IKK : I κ B kinase

IL- : interleukin

L

LAG3 : lymphocyte activation gene 3 protein

LB : lysogeny broth medium

M

mAb : monoclonal antibody

MACS : magnetic-activated cell sorting

MHC : major histocompatibility complex

MMAE : Monomethyl auristatin E

MPF: megakaryocyte-potentiating factor

MPM : malignant pleural mesothelioma

MTD : maximum tolerated dose

N

NF- κ B : nuclear factor kappa-light-chain-enhancer of activated B cells

NK : natural killer

NPT : 2,4,6-trinitrophenyl

P

PCR : polymerase chain reaction

PD-1 : programmed cell death protein 1

PD-L1 : programmed cell death protein 1 ligand 1

PD-L2 : programmed cell death protein 1 ligand 2

PE : pseudomonas exotoxin

PE 24 : pseudomonas exotoxin 24 kDa

PEG : poly-ethylene glycol

PI-PLC : GPI specific phospholipase C

PKC : protein kinase C

PLC : phospholipase C

PVDF : Polyvinylidene difluoride

R

RI : recombinant immunotoxin

RNA: ribonucleic acid

RT-PCR : reverse transcription PCR

S

Sc-Fv : single chain fragment variable

SDS : sodium dodecylsulfate

SMRP : soluble mesothelin related peptides

SPECT-CT : single photon emission computed tomography-computed tomography

T

TACE : TNF- α converting enzyme

TCR : T cell receptor

TIM3 : T cell membrane protein 3

TNF- α : tumor necrosis factor alpha

TME : tumor micro-environment

Treg : regulator T lymphocyte

V

VEGF : vascular endothelial growth factor

VEGFR : vascular endothelial growth factor receptor

VH : variable domain of heavy chain

VHH : nanobodies; specific antibodies comprising only heavy chain variable domain

VL : variable domain of light chain

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ABSTRACT

Antibody based immune treatment is a promising component of cancer therapy. To date there are more than 30 approved monoclonal antibodies for cancer therapy. More than 350 antibodies are also in different phases of clinical development. Mesothelin is one of the most promising targets for immunotherapy. It is present at relatively low levels in mesothelial cells of the pleura, peritoneum and pericardium of healthy individuals, but is highly expressed in a number of different cancers, including mesotheliomas, stomach cancers, squamous cell carcinomas, as well as prostate, pancreatic, lung, and ovarian cancers. Mesothelin is a glycosylphosphatidylinositol (GPI)-linked glycoprotein synthesized as a 69 kDa precursor and proteolytically processed into a 30 kDa NH₂-terminal secreted form (formerly referred to as Megakaryocyte Potentiating Factor (MPF)) and a 40 kDa membrane-bound form. Besides that it can be cleaved by a protease leading to the production of a soluble, shedded, form of mesothelin. It has already been shown that this soluble form of mesothelin acts as a ligand and neutralizes the mesothelin targeting therapeutic antibodies. Therefore antibodies could not reach cancer cells and remained inefficient. In our work we decided to develop discriminating antibodies specific to a membrane associated form so as to overcome the antagonism produced by soluble forms of mesothelin. To this aim we used a novel method of mouse immunization, in which we first tolerized the mouse with soluble mesothelin before immunization with mesothelin expressing cells. By using phage display technology we obtained nearly 150 mesothelin recognizing clones in 34 VH-CDR3 families, among which we identified only 2 families that bind membrane mesothelin with high affinity and do not recognize any other soluble form of mesothelin. Here we suggest that this Fab can be effective candidates to be used for mesothelin expressing cancer therapy being allowed to pass through the soluble mesothelin barrier. To show their efficacy for therapeutic use we constructed a CAR with the sc-Fv of a membrane-form discriminating antibody.

INTRODUCTION

Cancer and immunotherapy

Cancer is the second cause of death worldwide, after cardio-vascular diseases and represents a significant public health problem. A great majority of patients are diagnosed at an advanced stage, requiring the use of heavy therapies such as radiotherapy and chemotherapy. Unfortunately, these therapies lack specificity and are therefore very toxic. The development of targeted therapies, more effective and less toxic has long been a major challenge in cancer research. The progressive understanding of the molecular mechanisms involved in tumor progression has given place to the recent development of new therapeutic approaches targeting signal transduction, tumor angiogenesis, cell cycle or inducing tumor apoptosis.

Cancer treatment has been evolving in the past decades from the era of chemotherapy and radiotherapy to the development of targeted immune based therapies.

Immunotherapy is a treatment strategy which uses different biological or chemical agents as a vector to target a specific signaling pathway (monoclonal antibodies) or as agents modulating established cancer immunity. These include:

- Monoclonal antibodies
- Blockade of immune checkpoint inhibitors
- Cancer vaccines

I. FDA approved monoclonal antibodies for cancer treatment

Monoclonal therapeutic antibodies (mAbs) are the molecules which target specific antigens to inhibit cancer development. After the identification of specific cancer markers expressed on cell surface, thanks to their high specificity, antibodies were engineered to target these proteins and eliminate the cancer cells. The use of mAbs as therapeutic tools for cancer treatment started in the early 1980s. Antibodies were first produced in murine species and engineered by hybridoma technology for targeting hematologic malignancies. Although murine antibodies revealed successful results, the human immune system developed rapidly an immunogenic response leading to the production of anti-murine antibodies. By the development of antibody engineering tools, chimeric, humanized and also full human antibodies were constructed to avoid the undesirable reactivity of the human immune system. Antibodies or their engineered forms (Fab, sc-Fv, etc.) were also modified by conjugating them with several drugs or toxins to increase their therapeutic efficacy. During the last years, after the discovery of inhibitory proteins which block the human T cell activation and produce the depletion of immune response, another application of mAbs was also implemented in therapy by targeting these molecules. Currently, there are more than 30 therapeutic antibodies and their derivate for targeting both hematologic and solid tumors approved by US food and drug administration (FDA), European Medicines Agency (EMA), and a number of other national regulatory agencies for clinical treatments as well as more than 350 mAbs in different phases of clinical trials.

a. Structure

The most used form of antibodies in therapeutics is IgG1. An IgG1 molecule consists of two identical heavy and two identical light chains. The heavy chain contains three constants (CH1, CH2, CH3) and a variable domain (VH), while the light chain includes only one constant (CL) and one variable (VL) domains. Each variable domain consists of three complementarity-determining regions (CDR1, CDR2 and CDR3). CH2-CH3 represents together a Fc (constant fragment) portion and CH1-VH and CL-VL represents a Fab portion of an antibody. Heavy chains attached to each other and to light chains with the number of disulfide bonds. Research has shown that the specificity of an antibody depends on its variable domains, especially on the sequence of VH-CDR3 (Figure 1).

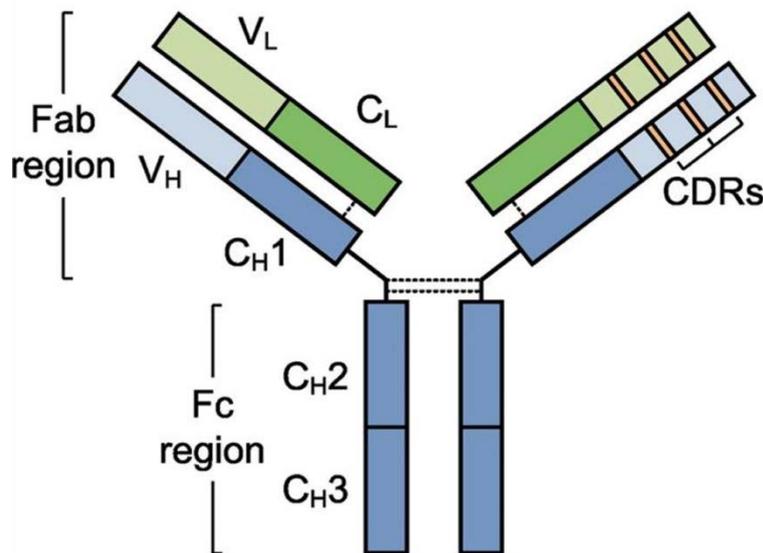


Figure 1. Antibody structure (Vincent J et al. 2011).

Even if the variable fragment (Fv) is necessary for antigen recognition, the constant fragment (Fc) of antibodies serves to exhibit biological effector functionality by interacting with complement and Fc-receptors expressed on different blood cells.

Monoclonal antibodies are classified according to their production and engineering methods (Figure 2):

- Full murine antibody: non-engineered and produced in murine species and containing only murine IgG1 constant and variable regions.
- Full human antibody: Engineered and obtained from human cells and only human IgG1 constant and variable regions.
- Chimeric antibody: Variable fragments are produced in murine species and linked to human constant fragment.
- Humanized antibody: It is the most advanced format of chimeric antibody. It contains only CDRs from murine and the rest belongs to a human antibody.

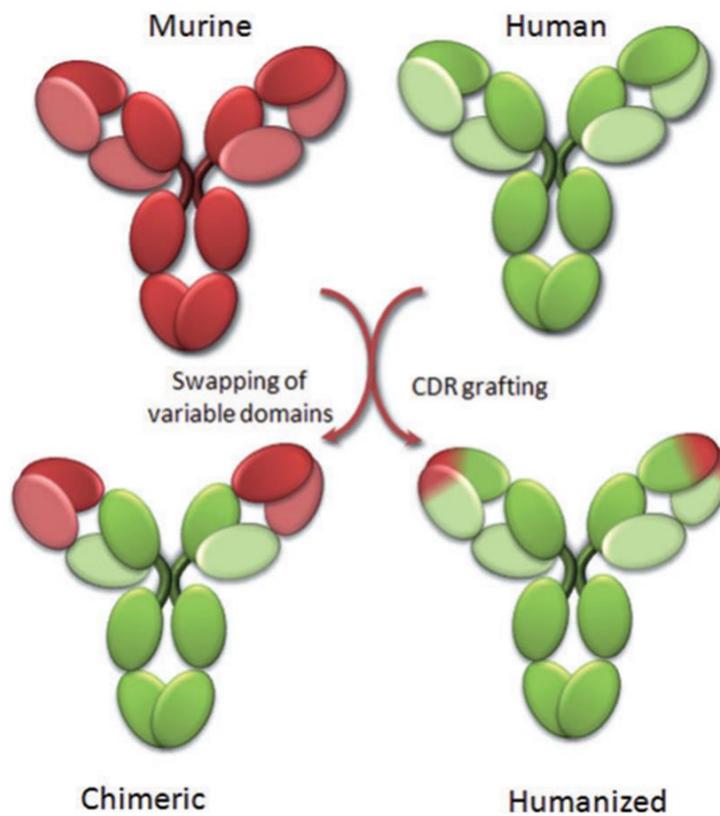


Figure 2. Chimeric and humanized antibodies (*Patrick Chames et al., 2009*).

Murine sequences are depicted in red and human sequences in green, using light colors for light chain and dark colors for heavy chains.

Administration of a therapeutic antibody can lead to an anti-antibody response (AAR). Consequently, to resolve this problem, a certain type of antibodies has been engineered to have a much similar structure to human antibodies. In 1984 *Boulianne et al.* have demonstrated the feasibility of functional chimeric mouse/human antibody (1). Then by substitution of CDR regions of human IgG1 with the same region of antigen specific animal antibodies humanized antibodies have also been developed. The similar affinity of the humanized antibodies has been observed comparing them to their animal homologues (2,3). In 1991 *Padlan EA* has shown that the humanized antibodies are less antigenic (4). *William Ying et al.* have published a review of reported AAR to murine, mouse–human chimeric, and humanized antibodies (5). They have demonstrated that it is very rare for a murine mAb to show negligible immunogenicity, and that it is very common for nearly the 100% of patients to produce human anti-mouse antibodies (HAMA). However, in contrast they have found that patients treated with chimeric antibodies produce 40% human anti-chimeric antibodies (HACA). This response has been diminished to only 9% with humanized antibodies. Another study has also confirmed by administering the mouse anti-human TNF antibody and its humanized homolog to healthy voluntaries that the humanized antibody reduces the immunogenicity by nearly 90 % (6).

b. Antibody engineering approaches

1. Hybridoma technology

To produce the antibodies for therapeutic aims, animals are immunized by different methods with antigens in accordance to the desired antibody characteristics. *Kohler and Milstein* have demonstrated in 1975 that the cell lines made by the fusion of mouse myeloma cells and mouse spleen cells from an immunized animal can produce appropriate antibodies. The authors of this work which described a method of antibody production based on hybridoma technology received the Nobel Prize in Physiology or Medicine in 1984. Hybridoma technology is the most used method to produce antibodies from an immunized animal. In this method the extracted B cells from the spleen or lymph nodes of an animal are

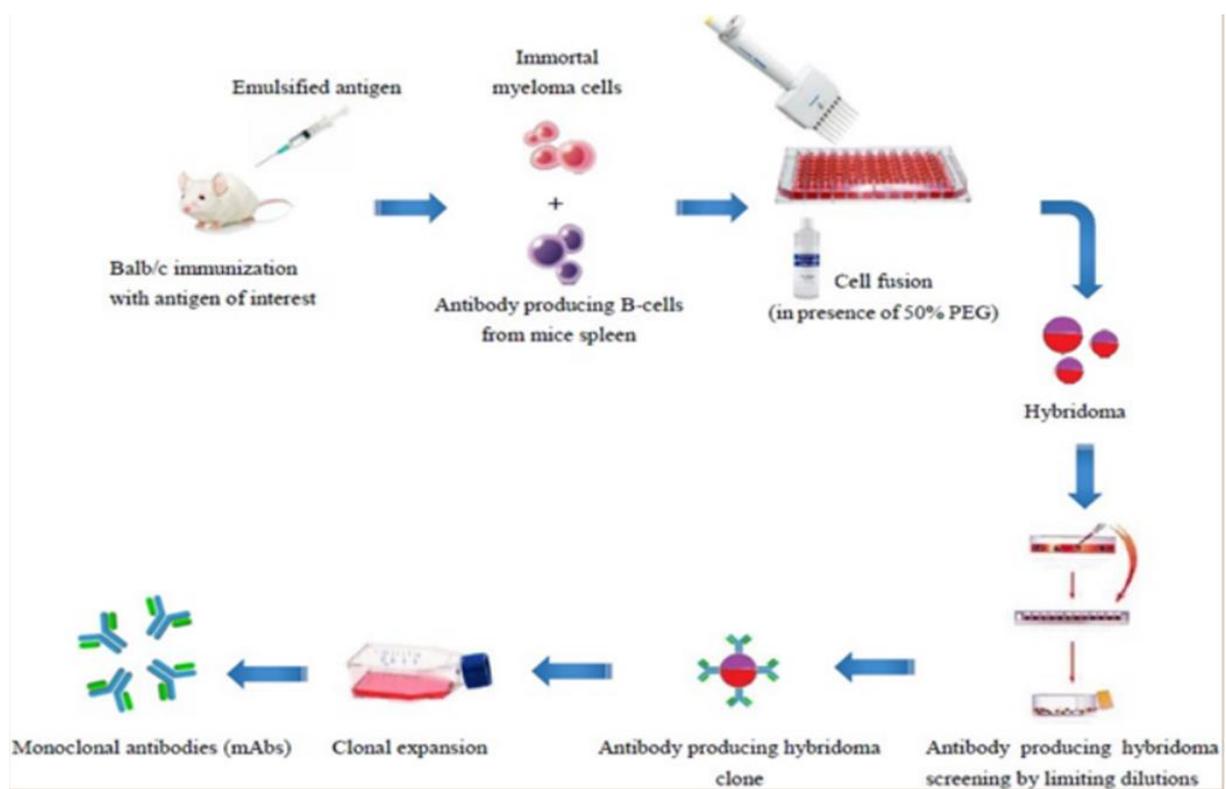


Figure 3. Schematic illustration of hybridoma technology (Abdullah Farhan et al. 2016).

fused with immortalized myeloma cells by using polyethylene-glycol (PEG) as a fusion

promoting agent to produce hybridoma cells. Myeloma cell lines selected in this technology are deficient for a hypoxanthine-guanine phosphoribosyl-transferase (HGPRT-) enzyme which participates in the neo-synthesis of nucleotides. Because of this molecular deficiency, myeloma cells use another pathway of nucleotide neo-synthesis which can be blocked using a chemical agent (most commonly aminopterin and thymidine). The fusion of a myeloma cell with a spleen cell enables them to acquire a functional HGPRT pathway. Also a medium containing aminopterin select myeloma cells that had actually been acquired by fusion (complementation) HGPRT pathway and which are therefore capable of activating the alternative pathway of nucleotide production (7). These cells are distributed in cell culture plates as single cell colonies and they are let to produce the antibodies. Antibody production and the presence of a specific candidate can be screened by different techniques such as enzyme linked cell sorbent assay (ELISA) and analytical tools like fluorescence-activated cell sorting (FACS) to find the target-specific antibodies (Figure 3).

The possibility to produce full human antibody by generating hybridoma cells from human lymphocytes has also been investigated (8,9). Antigen-specific human B cells are rarely present in circulation, and display low fusion efficiency. *Steinitz et al.* showed that the infection and transformation of B cells by Epstein-Barr virus (EBV) substantially increases the fusion efficiency and development of mAbs (10). EBV transformation of B cells isolated from peripheral blood of two individuals with asymptomatic human immunodeficiency virus type 1 (HIV-1) has been successfully used for the production of human mAbs against HIV-1 (11).

Even though hybridoma technology allows producing murine antibodies and regarding the poor reproducibility related to hybridoma cell cultures, this technology could not be

successfully used for other animals. Moreover, immunization with proteins which have a high similarity with its murine homologue results in the generation of low-affinity or non-specific antibodies. Altogether, these limitations necessitated development of new antibody producing technologies.

2. Phage display

The development in molecular biological applications in antibody production conducted to use phage display technology to generate a diverse number of antibodies. In 1985 *Smith* demonstrated that proteins, together with cloned antigens can be displayed on the phage surface (12). Then *Mccafferty et al.* have shown the possibility of expression of the

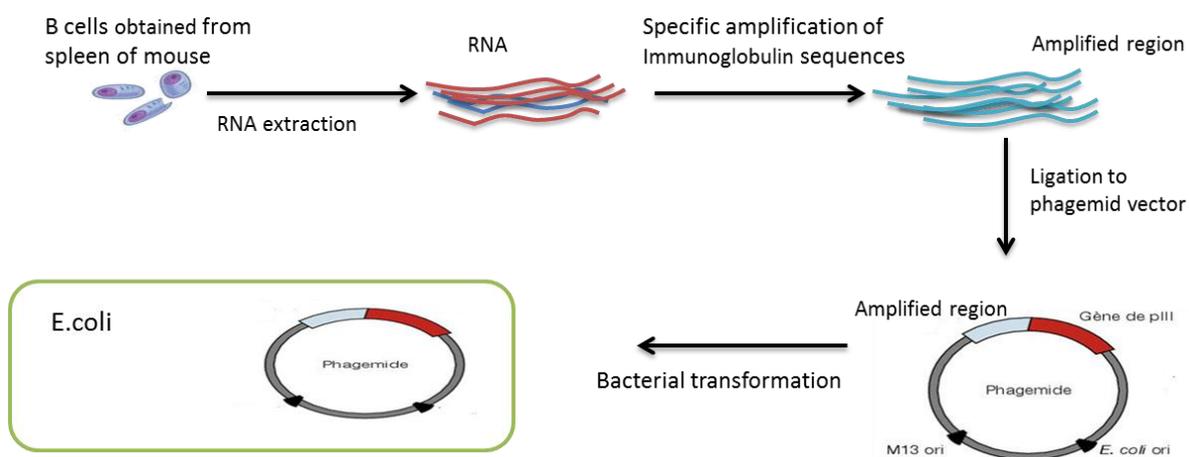


Figure 4. Phage library construction.

B cells are obtained from mouse spleen, total RNA is extracted and converted to cDNA by reverse transcription. Immunoglobulin sequences are specifically amplified by PCR. To obtain the phage library, amplified sequences are cloned to phagemid vector and transformed to E.coli cells.

immunoglobulin variable genes on phage surfaces after the extraction and amplification from hybridomas or directly from splenocytes or B cells using the polymerase chain reaction, and cloning them into expression vectors (13). They have also described affinity chromatography for specific phage isolation. One year later the same team prepared the phage library from

peripheral blood lymphocytes (PBLs) of unimmunized donors by using the VH and VL genes and showed the use of this library for isolation of antigen or hapten specific antibodies (14). Preparation and use of phage libraries from seropositive individuals has also been demonstrated in the early 1990s (15).

To produce antibodies by phage display technology, in the first step, RNA extracted from murine splenocytes or human peripheral blood lymphocytes is converted to DNA by reverse-transcription reaction. Then the antibody coding sequences are amplified by using specific primers and cloned into phage vector to create a library. After the transformation of phage library to E.coli, several panning (selection) rounds are performed to select specific

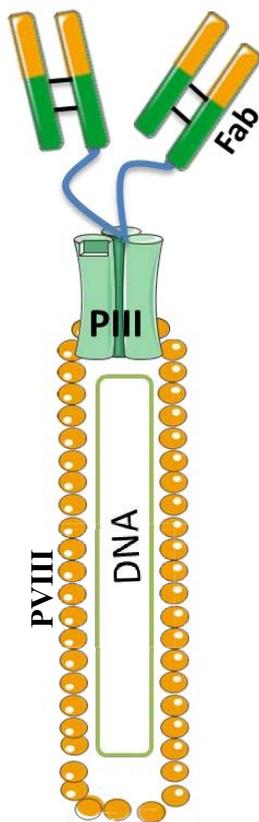


Figure 5. Schematic illustration of filamentous bacteriophage M13.

phages. Selected phages are eluted by using acid shock or enzymatic cleavage and then retransformed to E.coli strain. Single colonies are isolated by spreading the bacteria onto LB-agar plate. Finally to identify specific mAbs or antibody fragments, supernatant of monoclonal bacterial culture is screened by using ELISA or FACS (Figure 4) (16).

Phages are viruses which infect bacteria and could replicate its DNA inside the bacteria. M13 filamentous phage which has the advantage to multiply at extremely high titers (10^{12} - 10^{13} pfu/ml) and possesses a simple DNA genome is commonly used for surface displaying of banks of random peptides and antibodies (17,18) (Figure 5). Its genome is a simple circular DNA strand of about 6.4 kb

coding for 10 proteins. Protein pIII, pVI, pVII, pVIII and pIX form the structure of the capsid. The pIII protein is anchored to the extremity of phage with its C-terminal domain, and its N-terminal domain is responsible for the attachment to the pili of *E. coli*. pII, pV and pX are involved in replication of the phage DNA, while the pI and pIV form a channel in the membrane of *E. coli*, allowing the release of newly produced phage particles (19). The number of copies of protein pVIII is variable and serves to form the capsid of phage. The size of the viral tube consisting of the pVIII protein is variable and can be extended depending on the length of the DNA to be packaged. This flexibility allows the insertion of large fragments of exogenous DNA into the viral genome and renders M13 ideal for displaying antibodies or peptides. Another advantage of M13 phage is that it does not induce lysis of bacteria which differs it from other phages (17).

Generally, pIII and pVIII proteins are used for the presentation of peptides or antibody fragments by M13 phage. The pVIII protein is the most abundant, but, it may present only short size peptides (6-8 amino acids) due to the size restrictions imposed by the geometric assembly constraints of pVIII subunits to form the capsid (20). Displaying multiple copies of an antigen could also result in the selection of low affinity clones (21). In contrast, pIII protein is presented with only 5 copies on the phage surface which allows to display high molecular weight peptides and to select the clones with the highest affinity (22).

Although pIII allows to present high molecular weight peptides, a full length IgG expression in *E. coli* is generally problematic (23). As a matter of fact, IgG expression and isolation in *E. coli* has been demonstrated by some groups investigating phage display technology, it was not described clearly (24–26). Alternatively, smaller antibody variants as Fabs, sc-Fvs, etc. can be successfully displayed by pIII (Figure 6). The advantage of these

small fragments is that, they can target the occluded epitopes on the cell surface which otherwise are not accessible by full antibodies.

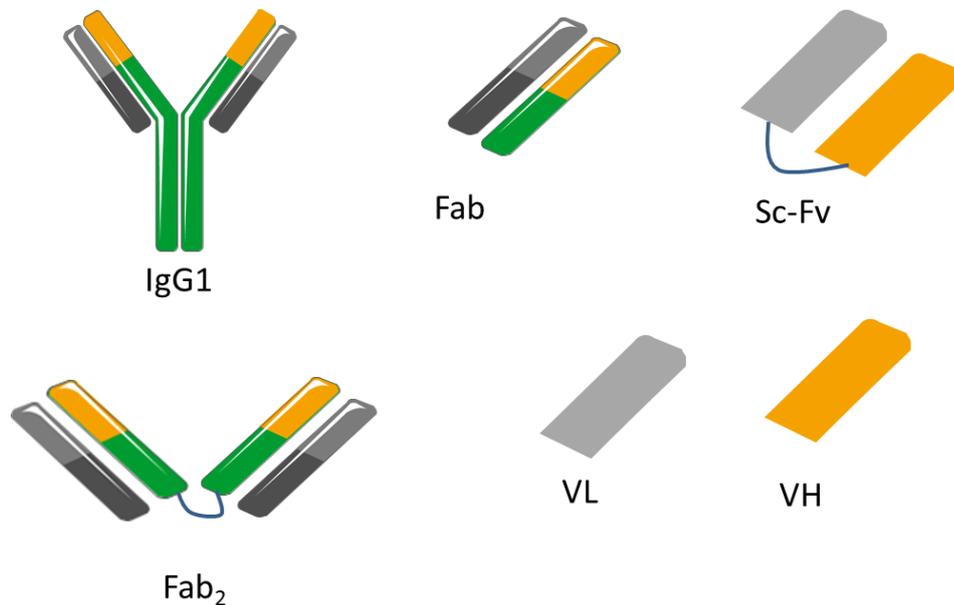


Figure 6. Different fragments of antibodies.

IgG1: full antibody immunoglobulin 1; Fab: fragment antigen binding; Sc-Fv: single chain variable fragment; VL: variable light chain; VH: variable heavy chain.

The main aim of phage display is to obtain libraries containing the largest diversity of antibody or antibody fragments. The probability of selecting molecules of interest with a high affinity is proportional to the size of the library, as well as the diversity of the molecular repertoire. In general, libraries are composed of 10^7 - 10^8 clones (27), and they could be divided into four main types which include (28):

- Naive antibody libraries: Genetic information coding for antibodies obtained from non-immunized animal B cells or splenocytes. *Marks et al.* have constructed for

the first time the naïve libraries from human peripheral blood B cells with 10^7 clones (14).

- Immunized antibody libraries: Libraries constructed from the genetic materials obtained from different animals which are selectively immunized with a desired antigen or from seropositive humans (29–32).
- Synthetic antibody libraries: Construction of synthetic libraries involves rearranging VH and VL gene segments *in vitro* and introducing artificial complementarity determining region (33–35).
- In-vivo recombined antibody libraries: Phage vectors obtained from a synthetic library were transfected to cells and the phage population produced from this primary library was then allowed to infect bacteria. This method allowed the exchange between different VH-VL vectors to create more diverse libraries. Additionally, these libraries have been shown to have the diversity of 10^{10} and 10^{11} clones (36,37).

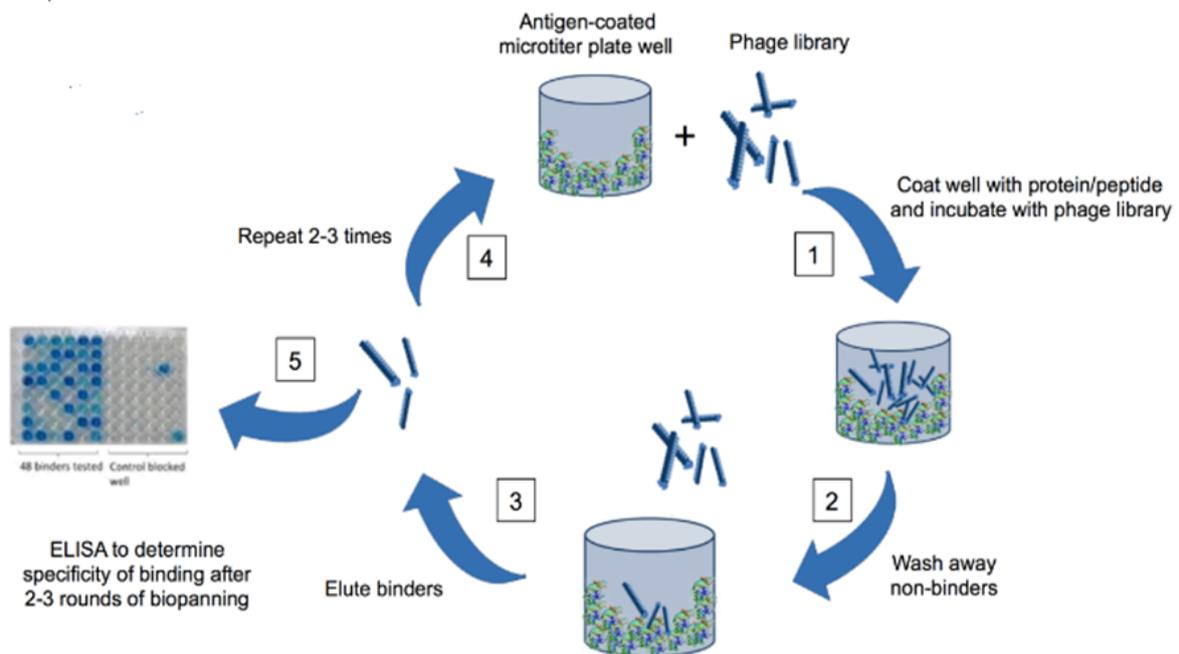


Figure 7. Phage panning and screening on ELISA (<http://axiomxinc.com>)

The next step of this technology is dedicated to the selection of high affinity antibodies using several selection rounds. The principle of affinity selection is based on the reversible bond between an antibody library and its target. The recombinant phages were thus selected for their ability to bind to a selected target. The selections are often made using the purified molecule of interest, and are generally composed of the following steps: i) fixing of the target phage, ii) washing to eliminate the less specific phage, iii) eluting of specific phages iv) and finally infecting *E. coli* bacteria where the selected phages will be amplified. The repetition of these binding-elution steps allows to highly enrich the proportion of high affinity phages to target. Usually 2-3 rounds of selection in increasing stringency conditions are required to

isolate high affinity antibodies for the target (38) (Figure 7).

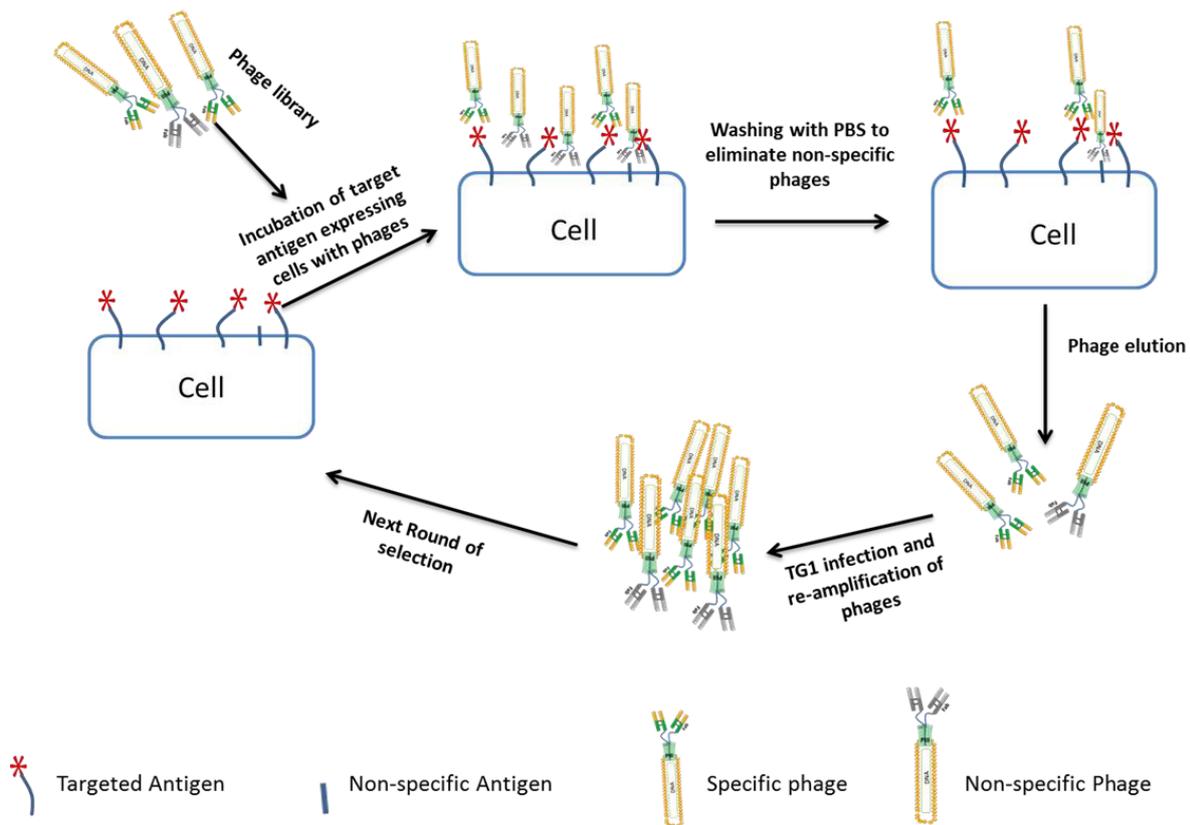


Figure 8. Selection of phages by antigen expressing cells.

Targeted antigen expressing cells are incubated with antibody phage library. Non-binding phages are eliminated by several wash steps and then binder phages are eluted. *E. coli* cells are infected with eluted phage and amplified. Then the next round of selection is started. 2-3 rounds are necessary to identify specific binders.

Selection strategies are usually dependent on the desired characteristics of the antibody. For therapeutic uses, antibodies must recognize the naturally expressed form of antigens on cell surfaces. Selection rounds can be performed on the targeted antigen expressing cells to identify natural-form binders (39) (figure 8). Cell selection method could be performed successfully to select cell surface antigens targeting phages.

Zhu et al. have described for the first time the therapeutic use of an antibody obtained from phage display (40). They demonstrated that two antibodies obtained from a single-chain

antibody phage display library constructed from spleen cells of mice immunized with a soluble form of a human vascular endothelial growth factor (VEGF) receptor, could block VEGF binding to kinase insert domain-containing receptor (KDR). In vitro cell culture assays showed that these antibodies could significantly suppress the mitogenic response of human umbilical vein endothelial cells to recombinant human VEGF in a dose-dependent manner, and reduce VEGF-dependent cell proliferation by 60% and 40% (41). An *in vivo* analysis of these recombinant antibodies in a rat cornea angiogenesis model revealed that both of the two antibodies could suppress the development of new corneal vessels (42).

Recently another antibody engineering approach was also developed to generate antibodies from isolated single B cells which allowed to study the expressed antibody repertoire in humans (43). Based on surface marker expression, individual cells at different stages of human B cell development are isolated by fluorescence-activated cell sorting. For each cell Ig heavy and corresponding Ig light chain gene transcripts are amplified and cloned into eukaryotic expression vectors to produce monoclonal human antibodies of the same specificity *in vitro*. This method of cloning and express human monoclonal antibodies is unbiased, highly efficient, requires only small cell numbers and the recombinant antibodies allow direct conclusions on the frequency of specific human B cells in a diverse repertoire.

c. Mechanism of action

In therapy, antibodies are used as full monoclonal antibodies or conjugated with different drugs, immunotoxins, and cytokines. Therapeutic function of antibodies depends on the specificity of the paratope leading to antigen recognition and neutralizing or agonistic properties. The nature of the Fc fragment, or its vectorization using antibody-drug conjugation technology might also be used to develop a functional mAb-based therapy.

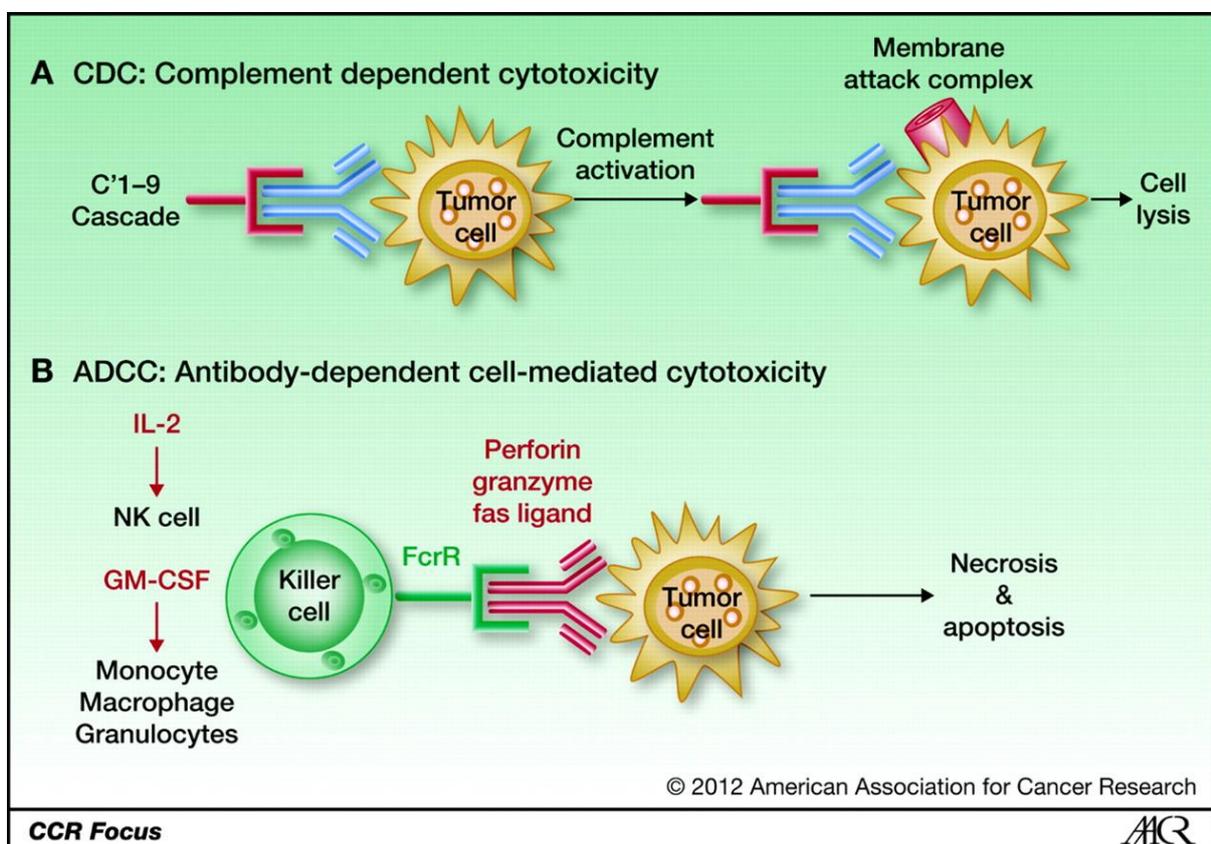


Figure 9. Mechanism of CDC and ADCC (Katherine K. Matthay et al. Clin Cancer Res 2012;18:2740-2753).

The mAb binds to the receptor and initiates the complement cascade, which results in the formation of a membrane attack complex that makes a hole within the cell membrane, causing cell lysis and death. B, ADCC: The Fc fragment of the monoclonal antibody binds the Fc receptors on monocytes, macrophages, granulocytes, and natural killer cells (NK). These cells in turn engulf the bound tumor cell and destroy it. Natural killer cells secrete cytokines that lead to cell death. GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin.

1. ADCC and CDC

Antibody dependent cell cytotoxicity (ADCC) represents the killing of target cells by effector cells that are activated by binding to the Fc portion of the monoclonal antibody molecule (44) (figure 6). Monocytes, macrophages granulocytes and natural killer (NK) cells express Fc-receptors (FcR) which bind to the Fc part of the antibody and trigger the cellular activation. Activated effector cells can make the synapse with the tumor cell and eliminate it.

NK cell cytotoxic activity was linked to cancer risk in 2000 by *Imai et al.* (45). They have demonstrated after the 11 year of follow-up survey of 3625 healthy persons that the in 154 cases cancer was developed and the low cytotoxic activity of peripheral blood cells was associated with a high cancer risk. NK cell activation is controlled by the balance of inhibitory (KIR family receptors) and activator signals triggered by NKG2D, DNAM-1, and 2B4 and the natural cytotoxicity receptors including NKp46, NKp44, and NKp30 (46–49).

Additionally, Fc receptors play a crucial role for an antibody dependent cellular cytotoxicity of NK cells. The human Fc γ R family contains six known members in three subgroups, including Fc γ RI (CD64), Fc γ RIIa,b,c (CD32a,b,c) and Fc γ RIIIa,b (CD16a,b), expressed by various effector cells of the immune system, including macrophages, neutrophils, dendritic cells and natural killer (NK) cells (50). These cells are recruited by antibodies and then the synapses are created with targeted cells, these synapses lead to the release of perforin/granzyme and the establishment of the Fas/FasL interaction, both leading to apoptosis of the target cells. Except Fc γ RIIb receptor which recognizes human IgG1 and IgG3 with low affinity, all of the other Fc γ Rs are activator receptors (51,52). Fc γ RI (CD64) binds to human IgG1 and IgG3 with high affinity, the Fc γ RIIa binds human IgG1, IgG2, and IgG3. Fc γ RIIIb is expressed only by neutrophils and may play a role in neutrophil activation.

CD56dim CD16+ NK cells highly express an activator low affinity FcγRIIIa (CD16) receptor which mediates ADCC and do not express inhibitory receptor FcγRIIb. Activation of FcγRIIIa depends on the affinity to the Fc part of an antibody. Recognition of the Fc part of an antibody by FcγRIIIa triggers a strong activating signal which impairs the inhibitory signals and results in both cytotoxicity and the cytokine secretion (53,54). *Hubert et al.* have demonstrated the role of FcγR in ADCC by using a mouse model. They showed that the cytotoxicity of a Tn antigen-specific chimeric mAb against murine breast cancer was diminished in FcγR deficient mice (55). Another study has also confirmed the role of inhibitory and activator FcγR in ADCC. The inhibitory receptor FcγRIIb deficient mice showed much more ADCC after the treatment by trastuzumab and Herceptin (both anti-Her2 mAbs) when compared with mice deficient for activator receptors (56).

The principal and most encountered problem with full, unconjugated, antibodies is their low affinity to Fcγ receptors (FcγR) to generate ADCC (57). The polymorph variant of FcγRIIIa which is expressed in 20 % of the population and contains a valine at position 158 instead of phenylalanine in 80 % of the population has been identified (58,59). A 5 times higher affinity to IgG1 Fc of FcγRIIIa-158V compared with FcγRIIIa-158F that leads to a more efficient *in vivo* ADCC in peripheral blood mononuclear cells (PBMCs) or purified NKs. *Musolino et al.* demonstrated with fifty-four consecutive patients with Her2/neu-amplified breast cancer receiving trastuzumab (anti-Her2 antibody) plus taxane that PBMCs with FcγRIIIa-158V/V or/and FcγRIIIa-131H/H genotypes had a significantly higher trastuzumab-mediated cytotoxicity than PBMCs harboring different genotypes (60).

Monoclonal antibodies are used mostly in second line therapies in patients who have already received several cycles of chemotherapeutic agents which hamper their immune

systems; their effector immune cells are therefore not really functional. For this reasons, several clinical trials are performed in order to use the mAbs in first line therapies with or without the combination with chemo-or radio-therapy (61–67). This hypothesis could be associated with the confusing results of several studies using rituximab (anti-CD20 mAb) to evaluate the role of the polymorphism of FcyRIIIa or FcyRIIb. *Cartron et al.* have demonstrated in 49 patients having received rituximab for a previously untreated follicular non-Hodgkin lymphoma that the FcyRIIIa-158V/V genotype was correlated to a better rituximab efficacy (58). Interestingly all of other studies on first line administration of rituximab agreed that the FcyRIIIa-158V/V genotype was the single favorable parameter associated with clinical and molecular responses (59,68–70). In contrast these results have not been confirmed in second line studies (71–73). *Prochazka et al.* with a large cohort of 102 patients have demonstrated that the FcyRIIIA receptor genotype does not influence an outcome in patients with follicular lymphoma treated with risk-adapted immune-chemotherapy. Similar confusing results in favor of first line therapy which shows considerable high ADCC with FcyRIIIa-158V/V genotype have been published for transtuzumab, cetuximab (anti-EGFR mAb) and anti-GD2 antibodies (74,75).

It has also been reported that the glycosylation of the CH2 domain (Asn 297) of the Fc portion has a negative effect on ADCC (76). The presence of fucose residues in the carbohydrate part has been shown to decrease the affinity of an antibody to the FcRyIII, therefore impairing ADCC efficiency (77,78). Glycosylation of antibodies has been demonstrated as a result of the method of production, especially depending on an antibody producing cell line (79).

Another limitation for a ADCC reaction is the competing effect by naturally occurring IgGs in human blood for the binding on FcγR receptors. A study to evaluate a role of naturally occurring IgGs on inhibition of fully human monoclonal IgG1 against epithelial cell adhesion molecule (Ep-CAM) and trastuzumab induced ADCC has been conducted (80). The results showed that the adding of the human serum with physiological levels of IgG1 inhibits the ADCC induced by those therapeutic antibodies and could be reversed by eliminating the IgG1 by affinity chromatography.

The Fc part of antibodies can also enhance the complement dependent cell death (CDC) by binding the C1q. C1q binding triggers the complement cascade which leads to formation of membrane attack complex (C5b to C9) at the surface of target cell, as a result of the classical pathway of complement activation. All therapeutic mAbs couldn't activate the complement cascade, because it has been already shown that to induce CDC, an antibody must bind an epitope near the cell surface. This mechanism of cytotoxicity has been largely studied for anti-CD20 antibodies. Cragg et al. showed that the complement depletion using cobra venom factor markedly reduced the efficacy of rituximab and 1F5 (anti-CD20 mAb) in 2 lymphoma xenograft models (81). Another study has also demonstrated that the anti-CD20 antibody ofatumumab induces considerably higher levels of CDC (82). Based on a higher CDC activity novel types of anti-CD20 mAbs were developed and they showed to be more performant in '*in vitro*' assays (83). Using murine models, antibodies which induce ADCC and CDC at the same time have been shown to be more suitable for cancer treatment (84).

d. Pharmacokinetics of antibodies

Pharmacokinetic properties of mAbs differ markedly from those of non-antibody-type drugs, and these properties can have important clinical implications (85). Antibodies and endogenous immunoglobulins are protected from degradation by binding to the neonatal Fc-receptor (FcRn), which explains their long elimination half-lives (57). FcRn was discovered as a responsible of the transmission of maternal antibodies from mother to pup (86,87). In addition, FcRn protects IgG from degradation, thereby explaining the long half-life of this class of antibody in the serum (88,89). Because of the β 2microglobulin association the structure of FcRn, is related to MHC class I (90). FcRn binds the Fc portion of IgGs. A major site of expression of FcRn is vascular endothelial cells where FcRn functions to extend the serum persistence of IgG by recycling internalized IgG back to the surface. *Akilesh et al* demonstrated by using FcRn deficient mice that in addition to its expression in the vascular endothelium of several organs, FcRn was highly expressed in bone marrow-derived cells and professional APCs in different tissues. Experiments using bone marrow chimeras showed that FcRn expression in these cells acted to significantly extend the half-life of serum IgG indicating that in addition to the vascular endothelium, bone marrow-derived phagocytic cells are a major site of IgG homeostasis (91,92). Another study confirmed the expression of FcRn in monocytes, intestinal macrophages, and dendritic cells (93). FcRn bound human IgG at different pHs which indicates that IgG enter cells through non-specific, fluid-phase pinocytosis (94). Endocytosed IgG is trafficked along the endosomal pathway and encounters FcRn in the early endosome where the acidic conditions favors a productive IgG-FcRn interaction (95).

With the aim of increasing the serum half-life of the therapeutic IgGs several studies have been performed. *Ghetie et al.* have used the random mutagenesis of Thr252, Thr254, and Thr256 followed by selection using bacteriophage displays and obtained the mutant of FcRn with the highest affinity and a significantly longer serum half-life than the wild type fragment. (96). Another study showed that the mutagenesis of His-436-Ala decreased the activity of the Fc hinge fragments in both '*in vivo*' and '*in vitro*' assays (97). The several mutations in the Fc portion of antibodies also suggested the better binding to FcRn and resulted with an increased half-life (98). *Ulrich et al* have also described a variable number of tandem repeats polymorphism influencing the transcriptional activity of the neonatal FcRn promoter (99).

e. Targets for therapeutic antibodies

The first and the most important step to generate an antibody for therapeutic aims is the choice of the target antigen. The expression or the functional pathway of the antigen must be restricted for tumor cells. For unconjugated antibodies, it is desirable to target cell surface antigens which are not internalized when they bind antibody. In contrast internalization is desirable for the antibodies conjugated with toxins or drugs (100).

Different antigens and cellular pathways for approved antibodies for clinic use described below (Table 1):

1. Cell differentiation (CD) antigens:

The therapeutic objective here is to identify an antigen whose expression is selectively distributed in the organism in order to specifically target a tissue or a cell while controlling the specific distribution of the mAb in the whole organism. An example of such development is illustrated by anti-CD20 mAb.

CD20 is a cell differentiation antigen, expressed from early pre-B cells to later in differentiation, but it is absent on terminally differentiated plasma cells. Rituximab was the first chimeric antibody approved for therapeutic use which targets CD20 (101). It is active in a variety of human lymphomas and chronic lymphocytic leukemia (66,102,103). Rituximab induces cell death through ADCC and CDC (104). *McLaughlin et al.* have published the results of 166 patients with relapsed low grade or follicular lymphoma with rituximab of which 48 % responded (105). Rituximab can induce CDC and ADCC as well as direct programmed cell death (106,107). Even if it is widely used for lymphoma therapy alone or in combination regimens mainly for relapsed and refractory lymphomas (108,109), the efficacy

Agent	Target(s)	FDA-approved indication(s)
Ado-trastuzumab emtansine (Kadcyla)	HER2	<ul style="list-style-type: none"> Breast cancer (HER2+)
Alemtuzumab (Campath)	CD52	<ul style="list-style-type: none"> B-cell chronic lymphocytic leukemia
Atezolizumab (Tecentriq)	PD-L1	<ul style="list-style-type: none"> Urothelial carcinoma
Bevacizumab (Avastin)	VEGF ligand	<ul style="list-style-type: none"> Cervical cancer, Colorectal cancer, Fallopian tube cancer, Glioblastoma, Non-small cell lung cancer, Ovarian cancer, Peritoneal cancer, Renal cell carcinoma
Brentuximab vedotin (Adcetris)	CD30	<ul style="list-style-type: none"> Hodgkin lymphoma, Anaplastic large cell lymphoma
Cetuximab (Erbix)	EGFR (HER1/ERBB1)	<ul style="list-style-type: none"> Colorectal cancer (KRAS wild type), Squamous cell cancer of the head and neck
Daratumumab (Darzalex)	CD38	<ul style="list-style-type: none"> Multiple myeloma
Denosumab (Xgeva)	RANKL	<ul style="list-style-type: none"> Giant cell tumor of the bone
Dinutuximab (Unituxin)	B4GALNT1 (GD2)	<ul style="list-style-type: none"> Pediatric neuroblastoma
Elotuzumab (Empliciti)	SLAMF7	<ul style="list-style-type: none"> Multiple myeloma
Ibritumomab tiuxetan (Zevalin)	CD20	<ul style="list-style-type: none"> Non-Hodgkin's lymphoma
Ipilimumab (Yervoy)	CTLA-4	<ul style="list-style-type: none"> Melanoma
Necitumumab (Portrazza)	EGFR	<ul style="list-style-type: none"> Squamous non-small cell lung cancer
Nivolumab (Opdivo)	PD-1	<ul style="list-style-type: none"> Hodgkin lymphoma, Melanoma, Non-small cell lung cancer, Renal cell carcinoma
Obinutuzumab (Gazyva)	CD20	<ul style="list-style-type: none"> Chronic lymphocytic leukemia, Follicular lymphoma
Ofatumumab (Arzerra, HuMax-CD20)	CD20	<ul style="list-style-type: none"> Chronic lymphocytic leukemia
Panitumumab (Vectibix)	EGFR	<ul style="list-style-type: none"> Colorectal cancer (KRAS wild type)
Pembrolizumab (Keytruda)	PD-1	<ul style="list-style-type: none"> Melanoma, Non-small cell lung cancer (PD-L1+), Head and neck squamous cell carcinoma
Pertuzumab (Perjeta)	HER2	<ul style="list-style-type: none"> Breast cancer (HER2+)
Ramucirumab (Cyramza)	VEGFR2	<ul style="list-style-type: none"> Colorectal cancer, Gastric cancer or Gastroesophageal junction (GEJ) adenocarcinoma, Non-small cell lung cancer
Rituximab (Rituxan, Mabthera)	CD20	<ul style="list-style-type: none"> Non-Hodgkin's lymphoma, Chronic lymphocytic leukemia, Rheumatoid arthritis, Granulomatosis with polyangiitis
Tositumomab (Bexxar)	CD20	<ul style="list-style-type: none"> Non-Hodgkin's lymphoma
Trastuzumab (Herceptin)	HER2	<ul style="list-style-type: none"> Breast cancer (HER2+), Gastric cancer (HER2+)

Table 1. FDA approved monoclonal antibodies for cancer therapy (www.mycancergenome.org).

of rituximab is modest and often variable especially when used for CLL treatment which results with an objective response rates ranged between 25% and 35% (110,111). Second generation mAbs designed as humanized or fully human with unmodified Fc domain and compared with rituximab in clinical trials. Among them, Ocrelizumab showed higher ADCC and lower CDC and ofatumumab showed higher CDC and less immunogenicity as compared with rituximab (112–114). The third generation humanized mAbs are modified mAbs in the Fc region. The Fc domain was engineered with the glycol or protein. The main goal of this modification is to improve the therapeutic efficacy in all patients; particularly among patients in which the expression with low affinity version of the Fc receptor is found on their effector immune cells (115). More recently other three, tositumomab, ofatumomab, obinutuzomab, anti-CD20 targeting antibodies have also been approved for treatment of diverse range of B-cell malignancies.

Other cell differentiation antigens targeted by alemtuzumab and daratumumab are CD52 and CD38 respectively. Like rituximab, these antibodies are effective in hematological malignancies (116–119).

2. Growth factors:

Family of growth factor receptors and ligands, because of their large expression in tumor cells are the promising targets for the treatment of solid cancers by monoclonal antibodies.

Epithelial growth factor receptors (EGFR) and vascular endothelial growth factor receptors (VEGFR) and their ligands have already been targeted by monoclonal antibodies which were approved for clinical use.

It was demonstrated in the late 1980's and the early 1990's that the EGF family proteins are highly expressed in solid malignancies and correlates with a poor prognosis

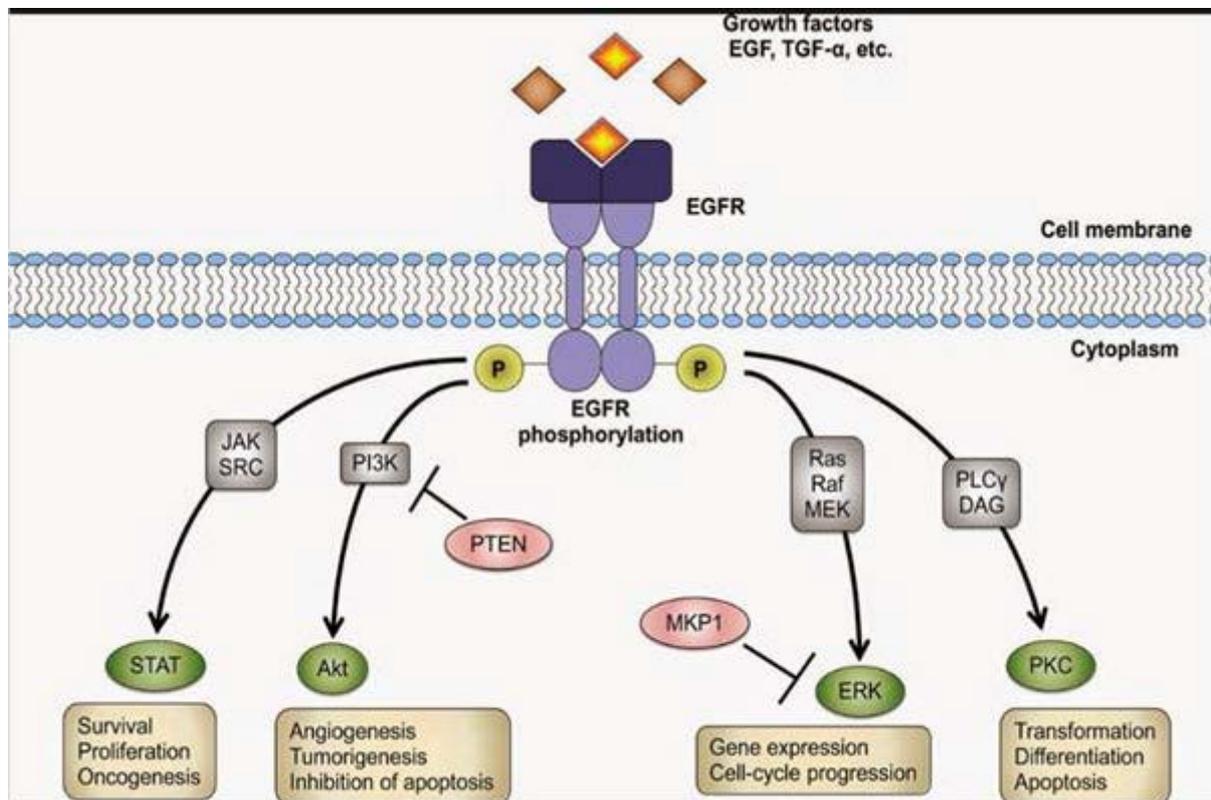


Figure 10. EGFR signaling pathway (Morten Bjerklund).

Binding to its ligand activates EGF receptor and leads to auto phosphorylation. This activation triggers different signaling pathways which increase cell proliferation, angiogenesis, and abolishes apoptosis.

(120,121), which makes them attractive candidates for antibody targeting. Activation of EGF receptors leads to different signaling pathways which increase cell proliferation and impair tumor cell apoptosis. It has also been demonstrated that the activation of EGFR by binding its ligand triggers tumorigenic processes, as cell survival, cell cycle progression, angiogenesis and inhibition of apoptosis (122,123) (Figure 10). Cetuximab is the first approved chimeric antibody for EGFR inhibition. Panitumumab is another fully human antibody which impairs EGFR signaling and thus leading to cell death. Panitumumab has been shown to be effective for colorectal cancer patients either as a single agent or combined with chemotherapy (124–

126). The role of KRAS status have been investigated to compare the panitumumab monotherapy to best supportive care by detecting KRAS mutations in tumor sections collected in a phase III mCRC trial. The results of this study suggested that the wild type (WT) KRAS patients had a longer overall survival (127). Similar results have also been obtained for the cetuximab treatment in favor of non-mutated KRAS status (128). Several clinical studies have also confirmed that the patients whose tumors were KRAS wild type, respond to these anti-EGFR antibodies, but in some cases an acquired resistance to cetuximab have also been observed. (129–132). To identify the mechanisms of acquired cetuximab resistance, *Montagut et al.* established cetuximab-resistant cells from the highly sensitive human mCRC cell line DiFi (which is wild-type for KRAS, BRAF and PI3K and has an amplification of EGFR). These cells were treated and rendered resistant to cetuximab. Without detecting neither the mutations in KRAS, BRAF nor PIK3CA or loss of PTEN expression, they identified an EGFR ectodomain mutation (S492R) in cetuximab resistant cells. Furthermore, they have also identified this mutation within two of ten subjects with disease progression after the cetuximab treatment. They also showed that the subject with cetuximab resistance harboring the S492R mutation responded to treatment with panitumumab (133).

Another EGF receptor overexpressed in breast and gastric cancers is HER2/EGFR2. It is targeted by trastuzumab and pertuzumab. Neutralizing antibodies targeting other variants of EGFRs are in clinical evaluation stages (134,135). The results of two trials that compared adjuvant chemotherapy with or without concurrent trastuzumab in women with surgically removed HER2-positive breast cancer demonstrated that trastuzumab combined with paclitaxel after doxorubicin and cyclophosphamide treatment improves outcomes (136). Several studies have confirmed the efficacy of trastuzumab or pertuzumab against HER2

overexpressing invasive breast cancers in both locoregional and advanced breast cancers (137,138). Although HER2 overexpressing cancers are sensitive to the antibody treatment, but soluble form of HER2 has been shown as an indicator of poor prognosis in HER2+ metastatic breast cancer and impairs the antibody efficacy (139,140).

VEGF receptors and ligands are also targeted by therapeutic antibodies in solid tumors. This family of proteins has an essential role in tumor angiogenesis. Therefore blocking the ligand or the receptor of VEGFs alters the tumor progression. The most utilized anti-VEGF antibody is bevacizumab (avastin) which neutralizes VEGF and impairs its binding to the receptor as well as triggering a signaling pathway of angiogenesis. Since it has no direct effect in tumor eradication, it is combined with different chemotherapies to obtain the better results (61,62,141,142). In a clinical trial conducted with 813 patients with previously untreated metastatic colorectal cancer it has been demonstrated that the addition of bevacizumab to fluorouracil-based combination chemotherapy results in statistically significant and clinically meaningful improvement in survival among patients with metastatic colorectal cancer (143). The study on 1,401 patients with metastatic colorectal cancer has revealed that the addition of bevacizumab to oxaliplatin-based chemotherapy significantly improved PFS in first-line therapy (144).

3. Immune-checkpoint molecules

A better understanding of the role of the immune system provides great insight into tumor microenvironment (TME) hence enabling the development of novel and highly promising immune modulators (145). Effector T cells (CD8+) can be activated and they can play a crucial role in TME for cancer cell elimination. Activation of T cells passes through the recognition of the antigen presented by major histocompatibility complex (MHC) class I by

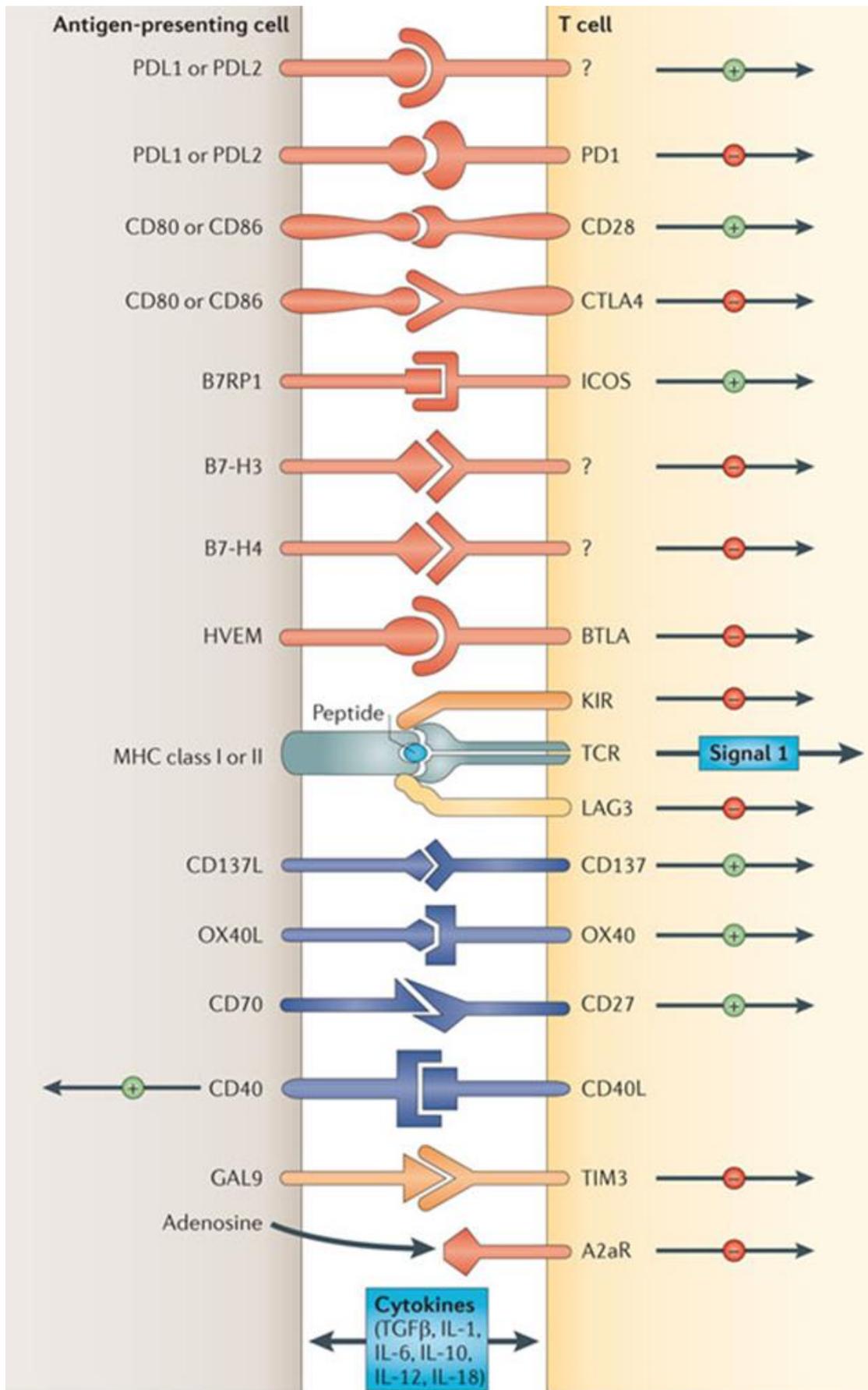


Figure 11. Immune check-point regulation (Pardoll et al. 2012).

its T

cell receptors (TCR) but also needs a second costimulatory signal modulated by B7 family members CD28 or CD80 (146–149). or There are also inhibitory molecules expressed by tumor cells to impair the T cell activation (150). Tumor cell death depends on the modulation of these inhibitor and activator molecules (Figure 11 and 12). The checkpoint molecules, CTLA-4, programmed death-1 (PD1), and its ligand (PD-L1), etc. are the inhibitory signals

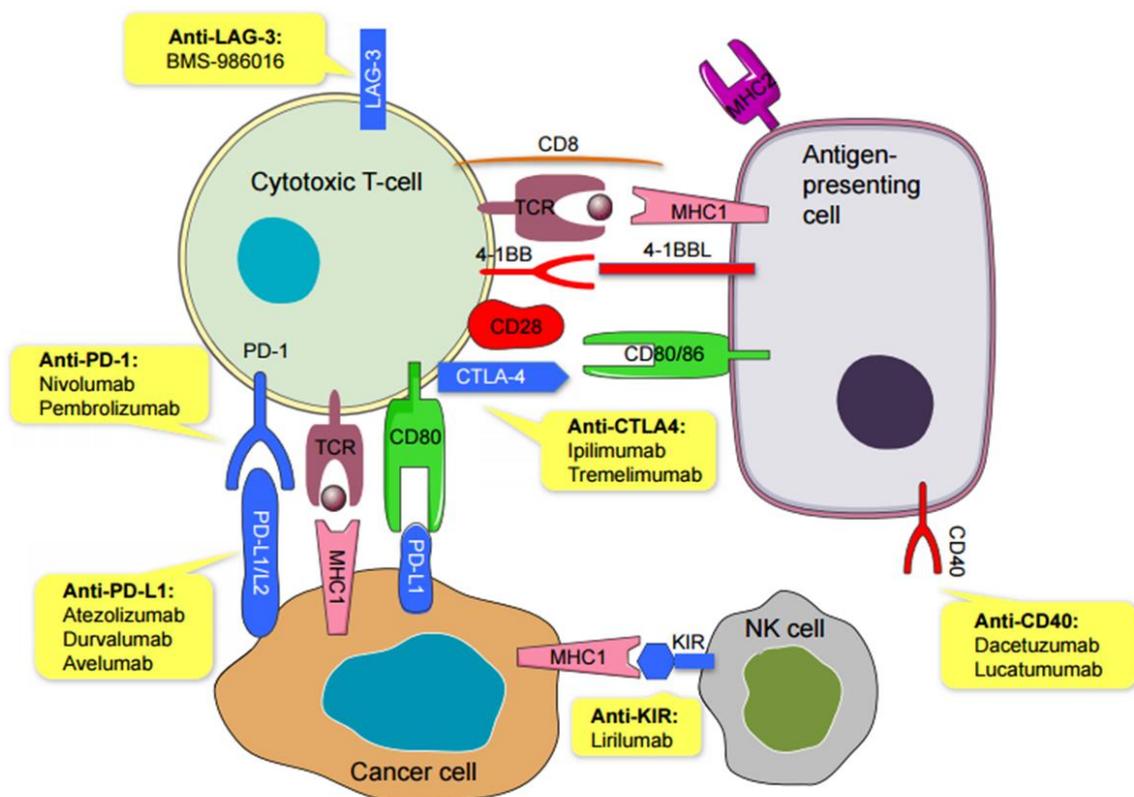


Figure 12. Interactions of different immune checkpoints between T-cells, APCs, and cancer cells that can be exploited in cancer therapeutics (El-Osta et al., 2016).

Abbreviations: APC, antigen-presenting cell; KIR, killer immunoglobulin like receptor; LAG-3, lymphocyte activation gene-3; PD-1, programmed death-1; PD-L1, programmed death ligand 1; MHC1, major histocompatibility complex 1; NK, natural killer; TCR, T-cell receptor; 4-1BB, CD137; 4-1BBL, 4-1BB ligand.

that serve to cancer cells to evade the immune system (151). Even if the activation signal by TCR and the co-stimulation receptors (145,145,152–154) are present, interaction between the ligand and receptor of checkpoint molecules can generate negative feedback signals that

dampen the immune response. Therefore the blockade of checkpoint molecules by monoclonal antibodies is a great avenue for cancer immunotherapy.

CTLA4 is the first immune-checkpoint receptor targeted by therapeutic antibodies. It regulates early T cell activation and it is a co-inhibitor of CD28. CD28 binds to its ligand CD80 or CD86 and strongly amplifies the activator signal of T cells received after MHC class I and TCR interaction. CTLA4 binds to the same ligands stronger than CD28, and acting as a competitor antagonist which impairs the T cell activation (155–158). The principal role of CTLA4 has been described to be the down-modulation of T helper cell activity and enhancement of regulatory T (159,160) cells which have been described as a poor prognostic for cancer progression (161,162). These characteristics of CTLA4 made it to become a crucial target for immune therapy. CTLA4 blockade has been shown to enhance the immunologic response, which ultimately can induce tumor regression. Also in the case of poorly immunogenic tumors, in a murine model which does not induce substantial endogenous immune responses, the combination of a granulocyte–macrophage colony-stimulating factor (GM-CSF)-transduced cellular vaccine and a CTLA4 antibody could induce a strong enough immune response to slow tumor growth and in some cases eliminate established tumors (163). Ipilimumab, an anti-CTLA4 antibody showed in clinical trials showed survival benefits for melanoma patients and as a matter of fact it was approved by FDA in 2010 for clinical use (164).

PD1 is another immune-checkpoint receptor expressed in the cell surface of T-cells. PD1 expression is induced when the cells are activated (165). PD1 can bind two ligands (PD-L1 and PD-L2) expressed on cancer cells. The interaction between PD1 and its ligands blockades the T cell activation by recruiting the phosphatase SHP2 (166). PD1 is also highly

expressed on Treg cells, where it may enhance their proliferation after binding to its ligand. Blockade of the PD1 pathway may also enhance anti-tumor immune responses by diminishing the number and/or suppressive activity of intra-tumoral Treg cells.

PD-L1 is expressed in melanoma, ovarian, lung and many other cancer cells (167–169). PD-L1 expression was also observed in tumor infiltrating myeloid cells as myeloid derived suppressor cells which correlates with a poor prognosis of tumors. PD-L1 expression is regulated by different factors in different tumors. In some tumors like glioblastoma, the PD-L1 expression appears as a result of an oncogenic pathway activation which is called innate immune resistance (170). The alternative mechanism (adaptive immune resistance) for PDL1 upregulation on tumors is the response of tumor cells to interferons (IFNs)- predominantly $IFN\gamma$ (171–173). Interestingly, the interaction between PD-L1 and CD80 (in this case expressed as a receptor on T cells) has been shown to induce an inhibitory signaling pathway in T cells (174).

Anti-PD1 and anti PD-L1 antibodies have been demonstrated to be effective for various cancers (152,154,168,175). The mechanisms of the blockade with anti-PD1 and anti-PD-L1 antibodies are different, because even though anti-PD1 antibodies block both PD-L1/PD1 and PD-L1/CD80 interactions, they could not target PD-L1/CD80 interaction. However anti-PD-L1 antibodies neutralize the PD-L1/CD80 and PD-L1/PD1 interactions but not the binding of PD-L2 to PD1.

Lymphocyte activation gene 3 (LAG3; also known as CD223), 2B4 (also known as CD244), B and T lymphocyte attenuator (BTLA; also known as CD272), T cell membrane protein 3 (TIM3; also known as HAVcr2), adenosine A2a receptor (A2aR) and the family of killer inhibitory receptors, B7 family inhibitory ligands — in particular B7-H3 (also known as

CD276) and B7-H4 (also known as B7-S1, B7x and VCTN1) have been identified as immune-checkpoint blockaders in animal models and are targeted by monoclonal antibodies which are under clinical investigations (176–182).

f. Antibody-drug conjugates

Antibody-drug conjugates (ADC) combine the selectivity of an antibody and the toxicity of chemotherapy. An antibody portion of ADC binds selectively with the target cell and is internalized by endocytosis. Then the cytotoxic drug kills the target cell. Antigen selection is important for ADCs; i) it must be highly expressed and has to be selective for

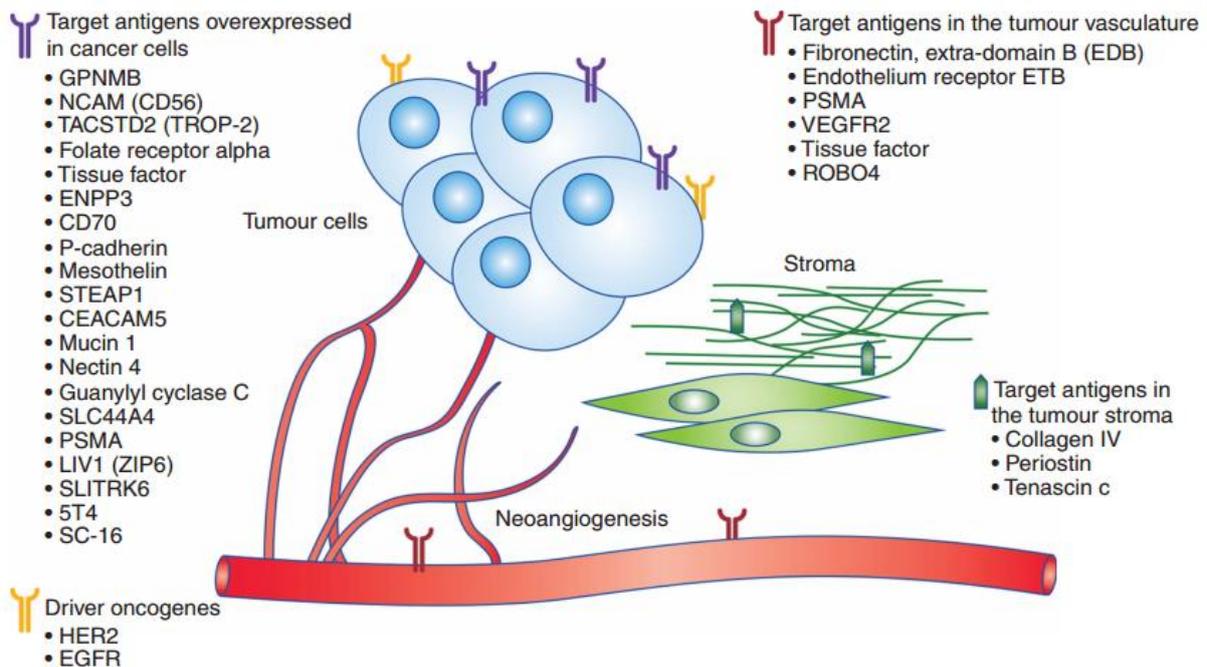


Figure 13. Antigen targets for ADCs in solid tumors. (www.bjcancer.com)

tumor cells. It must have a limited expression in normal tissues, ii) it could be internalized after binding to ADC for a successful delivering of the toxic agent, iii) it must not be shed or downregulated after the ADC treatment. There are more than 25 antigens investigated to be targeted by ADCs in different phases on clinical trials (Figure 13). These include i) overexpressed targets on cancer cells as NCAM, CD70, Mesothelin, CEACAM5, Mucin 1, etc. ii) Antigens acting in angiogenesis; PSMA, ROBO4, VEGFR2, etc. iii) antigens presented in tumor stroma; collagen IV, periostin, etc (Figure 10).

Two main groups of drugs, microtubule inhibitors and DNA-damaging drugs are used for ADC development. The linkage between the antibody and the drug is also important, because it must be stable and must not be cleaved in patients' blood till the delivery of ADC to the target cell. It must as well comprise a cleavage site for lysosomal enzymes for the release of drug once ADC is internalized.

ADCs were firstly designed as therapeutic agents for second line therapy in solid tumors or hematological malignancies to circumvent resistance to chemotherapy. To date, only two ADCs are approved by the FDA for cancer therapy, T-DM1 and brentuximab-vedotin (BV). T-DM1 is composed of trastuzumab, a humanized IgG1 anti-HER-2 antibody linked with a stable non-cleavable linker to the maytansinoid DM1, a potent tubulin inhibitor (183). T-DM1 has been approved in the second line setting by the FDA for HER-2-positive patients who had previously received treatment with trastuzumab and taxane chemotherapy. It has also been investigated in clinical trials as a single agent (184,185).

Brentuximab vedotin comprises an anti-CD30 mAb connected to the highly potent tubulin inhibitor Monomethyl auristatin E (MMAE) (186). It targets CD30, a tumor necrosis factor (TNF) family member and triggers cell death once it binds with the antigen. BV has gained approval for the treatment of patients with relapsed or refractory CD30+ Hodgkin lymphoma following autologous stem cell transplant (ASCT) or patients not legible for ASCT who have failed at least two other chemotherapy treatments (187,188). BV has also been approved for patients with anaplastic large cell lymphoma (ALCL) as a second line treatment (189,190).

II. CAR T cell clinical trials

a. Introduction

Several studies have already showed that the presence of immune cells in tumor micro-environment is not sufficient for their activation and functionality. This phenomenon can be linked with the expression of check point inhibitors by cancer cells. Over the past years, a new therapy has been developed to be useful in the absence of efficacy of therapeutic antibodies or when the intra-tumoral lymphocytic infiltrate is absent. The objective was to re-program *ex vivo* the patient's immune system and restore the ability of T cells to recognize and destroy tumor cells. This immunotherapy based on the use of cytotoxic effector functions of immune cells is called adoptive T cell therapy. Adoptive T cell therapy has become a promising immunotherapy for cancer treatment. Identification of tumor cell specific antigens expressed on cell surface permitted to target these cells by biologically engineered chimeric T cell receptors (CAR). First CARs are genetically modified T cell receptors comprising: a single chain of the monoclonal antibody which targets cell surface antigen and a CD3 zeta chain serving for T cell activation. The last generations of CARs were also engineered to express the intracellular domains of costimulatory receptors as CD28 and 4-1BB. CAR recognizes the antigen by antibody domain and the activation generated by CD3 zeta. Lentiviral and retroviral vectors are generally engineered for CAR expression and used to infect T cells. T cells expressing the enough copies of CARs could be activated when they bind to their target. Once a CAR T cell is activated, it undergoes clonal expansion and triggers a signalization pathway to express several pro-inflammatory cytokines. By the force of this activation they can easily eliminate target cells. The main advantage of CAR compared to natural T cell receptors is that the antigen recognized by CAR does not need to be presented

by a major histocompatibility complex (MHC) on antigen presenting cells (APCs) which allows them to be effective even in the presence of the low copies of antigen in cell surface.

b. Functionality and acting mechanism

Establishment of the immunologic synapse between CAR T cells and target cells following antigen recognition results in the elimination of target cells and pro-inflammatory cytokine expression IFN γ , IL2, and TNF. Several studies showed that the effector T cells were essentially CD8⁺ cells. Activation of CAR T triggers the degranulation of CD8⁺ cells and results in granzyme perforin expression.

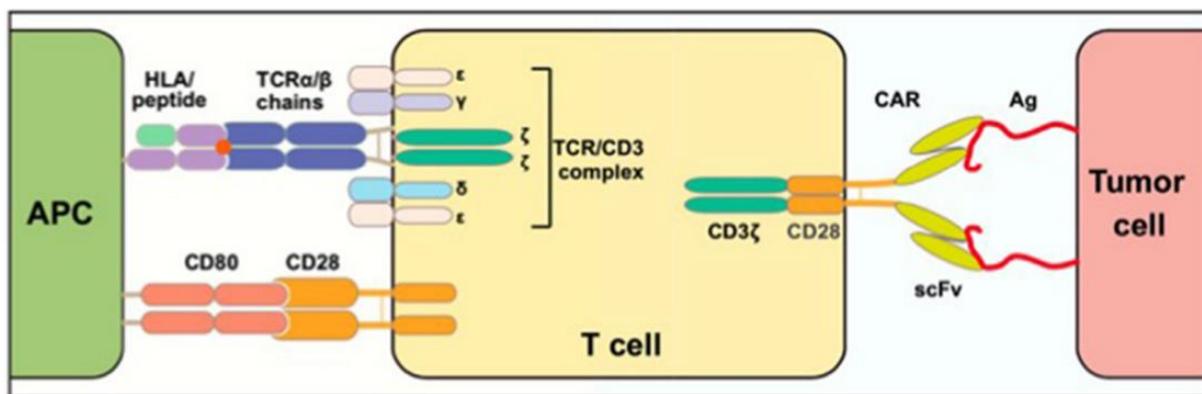


Figure 14. Antigen binding with normal T cell receptor and chimeric antigen receptor (Kroemer *et al.* 2015).

Left: structure of the T cell receptor (TCR) – hetero-dimer associated with the CD3 complex and co-stimulatory CD28 receptor. Right: prototype of a second generation chimeric antigen receptor (CAR) combining the scFv for antigen recognition, the cytoplasmic domain CD3 for activation and the cytoplasmic portion of CD28 to improve the functions and persistent of T cells.

Phillip K. *et al.* demonstrated the critical role of IFN γ and perforin expression for CAR T cell efficacy (191). The importance of IFN γ and perforin expression was also confirmed by several other studies (192–194). Neeson P. *et al.* conducted a research using the lewis Y antigen (LeY-T) directed-CAR T cells in order to determine if CAR T cells generate

functional CD8⁺ T cells with effector (EM) and central (CM) memory-like phenotype. They demonstrated that after transduction and expansion culture of peripheral blood mononuclear cells from normal donors or multiple myeloma patients, CD8⁺ LeY-T cells polarized to EM- and CM-like phenotype (195). The effects of signaling via the endogenous T-cell receptor or CAR on killing kinetics were studied by *Alexander J. et al.* (196). They used a transgenic mouse (designated CAR.OT-I), in which CD8⁺ T cells co-expressed the clonogenic OT-I TCR, recognizing the H-2Kb-presented ovalbumin peptide SIINFEKL, and an scFv specific for human HER2. T cells obtained from that mouse co-incubated with SIINFEKL-pulsed or HER2 expressing cell lines and the kinetics of cell death went under research. They found that engagement via CAR or TCR did not affect cell death kinetics. They have also demonstrated that the CAR T cells can sequentially kill multiple targets as cytotoxic lymphocytes and natural killer cells (NK and CD8⁺ T cells) (197–199).

c. Different generations

There are three generations of CARs, characterized by an accelerating increase in the activity of co-stimulation via the addition of co-stimulation signals.

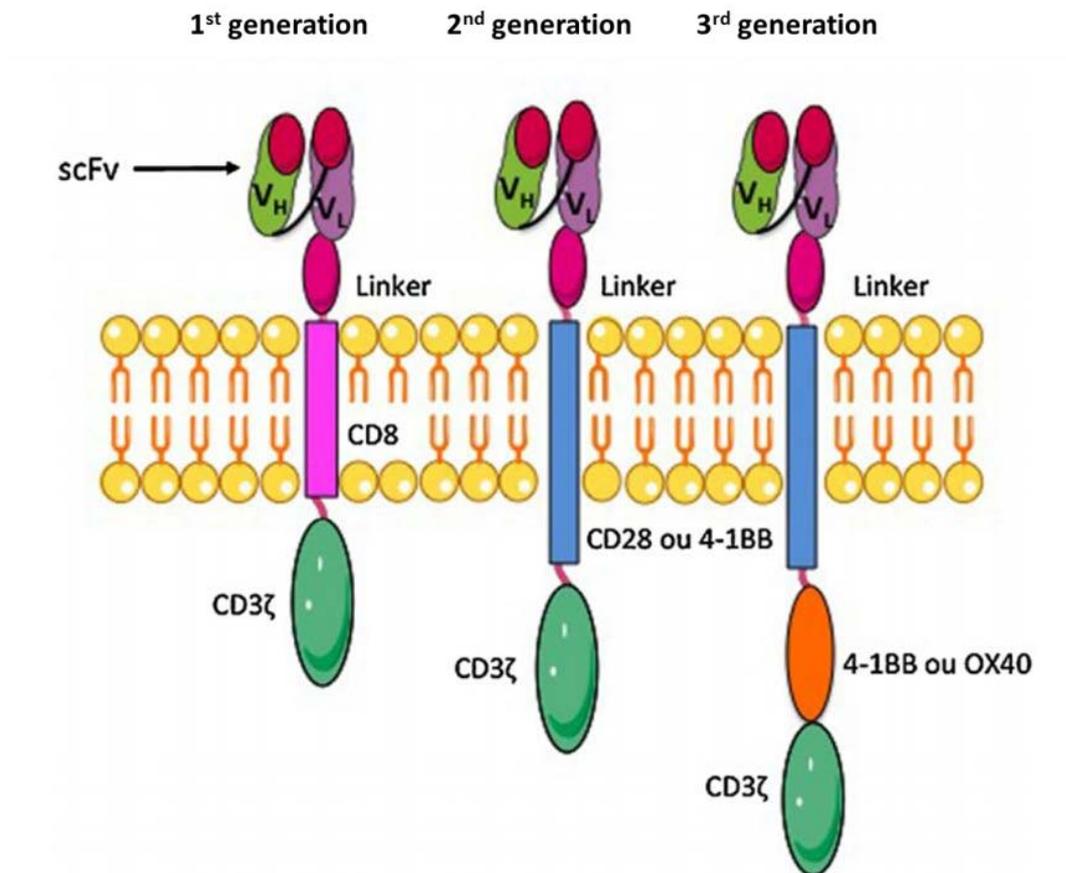


Figure 15. Structure of different generations of chimeric antigen receptors (Kroemer et al. 2015).

First generation chimeric antigen receptor (CAR): cytoplasmic part consists of CD3 zeta linked to an inert transmembrane domain (CD8). Second generation CARs: the transmembrane domain now consists of a signaling module (4-1BB or CD28) linked to the cytoplasmic CD3 zeta. Third generation CAR: a second signaling module fused to the first and linked to the CD3 zeta.

In 1993 *Eshhar et al.* are the first to report the use of CAR in a T cell. This CAR consisted on the extracellular binding domain (scFv) specific to 2,4,6-trinitrophenyl (NPT)

and was merged either to the intracellular domain of FcR or to the intracellular domain of CD3 chain (200). Thus, the first CARs or T-body comprises: an extracellular domain sc-Fv derived from a murine antibody; a neutral transmembrane domain (CD8, CD4, CD25 or CD16) and a signaling module, CD3 or FcR.

First-generation CARs have had a limited clinical impact in the treatment of neuroblastoma, lymphoma or prostate cancer (201–203). The activation of genetically modified T cells was only transient and the production of cytokines such as IL-2 was suboptimal to prevent the establishment of a strong and durable anti-tumor response.

Second generation CARs have two signaling modules, one for activation and the other for co-stimulation. Improved persistence of T cells by a CAR dual signaling was confirmed in patients treated with T cells transduced either with a CD28/CD3 or with only the CD3 chain (204). There are many possible configurations of second-generation CARs. A comprehensive comparison of the efficiency of different combinations should be studied.

A third generation of CARs, combining two costimulatory signals and an activation signal, has been described. T lymphocytes expressing such configuration in some mouse models suggested having a greater anti-tumor efficacy (205). A first clinical study, using a specific CAR CD20 in patients with non-Hodgkin lymphoma and having the following configuration CD28 / 4-1BB / CD3, has reported encouraging responses. Progression-free survival was 12 and 24 months respectively for 2 of 4 patients included in the trial (206).

d. Clinical trials

To date there are more than 100 CAR T cell clinical trials registered at clinicaltrials.gov (the list of all clinical trials to May 2016 are presented in Annexe 1.). Most of them are performed in United States and China. Only a few number of CAR T cell trials have been held in Europe, Japan and Australia. A high proportion of these trials are targeting hematological malignancies. More than 50 percent of these trials have been held on CD19 positive malignancies by using CD19 targeting CAR T cells with different signalization domain combinations (207–211).

The team of June, M.D. of the University of Pennsylvania has developed a CAR targeting CD19 comprising a chain with intracytoplasmic signaling elements of CD3 and 4-1BB (CD137). The first patient treated was the subject of a publication in 2011 (212). They used the lentiviral vector for transduction of transgene (CAR). The adoptive transfer of approximately 1.5×10^5 cells per kilogram of body weight was performed to the patient with refractory chronic lymphocytic leukemia (CLL). Interleukin-2 was administered to allow the expansion of transgenic T cells. A 1000-fold of expansion of genetically modified T cells was observed and engineered cells persisted at high levels for 6 months in the blood and bone marrow and continued to express the chimeric antigen receptor. A complete remission was reported 10 months after the first injection. The treatment of 2 children with acute leukemia B was then reported in 2014 (213). These encouraging results permitted to include a total of 30 children and adults in a phase II study for anti CD19 CAR treatment (214). The authors reported that the complete remission was achieved in 27 patients (90%), including 2 patients with blinatumomab-refractory disease and 15 who had undergone stem-cell transplantation. CD19-directed chimeric antigen receptor cells proliferated in vivo and were detectable in the

blood, bone marrow, and cerebrospinal fluid of patients who had a response. Sustained remission was achieved with a 6-month event-free survival rate of 67%, and an overall survival rate of 78%. At 6 months, the probability that a patient would have a persistence of CTL019 was 68% and the probability that a patient would have relapse-free B-cell aplasia was 73%.

CAR T cells targeting solid tumors as ovarian, pancreas, liver and prostate cancer antigens have also been developed and their in vitro efficacy has already been confirmed (215–217). The results of phase I studies targeting Muc I, PSMA, Mesothelin and CEA by CAR T cells in solid tumor have already been reported (218–221). A phase I study using CEA targeting CAR T cells was conducted in six patients, including four patients with more than 10 liver metastases (221). One patient remained alive with stable disease at 23 months following CAR-T cell treatment, and 5 patients died of progressive disease.

III Mesothelin as a target for cancer therapy

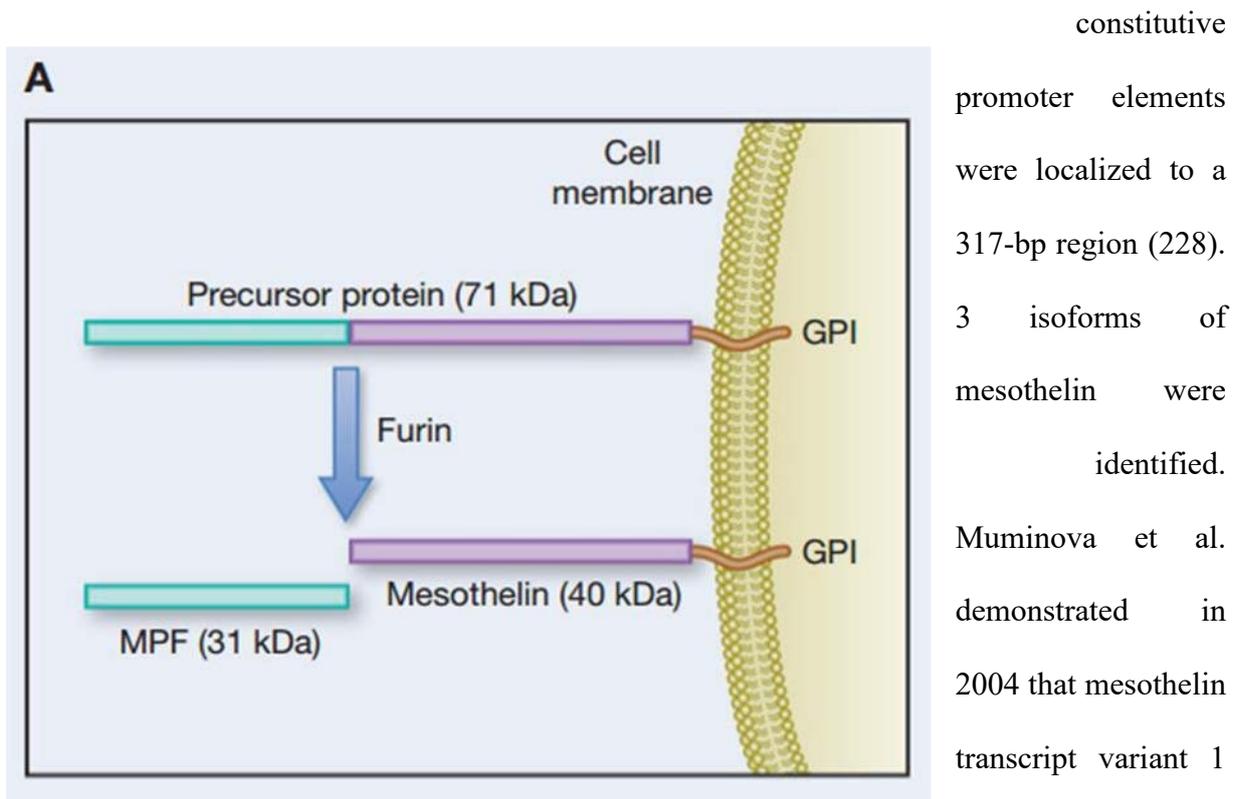
a. Discovery

Mesothelin was first reported in 1992 by Ira Pastan et al. as a target of an antibody reacting with ovarian cancer cells named mAb K1(222,223). This antibody was generated by hybridoma technology by immunizing mice with normal liver or kidney cells before treating them with cyclophosphamide to kill the B cells activated by this immunization. Then these mice were re-immunized with human ovarian carcinoma cells (OVCAR-3) to obtain ovarian cancer specific antibodies. After the initial screening using immunofluorescence on OVCAR-3 cells, and secondary screening using immunohistochemistry on cryostat sections of normal human tissues and human tumors, the K1 clone was identified. The antigen of monoclonal antibody K1 was found in many ovarian non-mucinous tumors, as well as in squamous tumors of the esophagus, and cervical cancer in immunohistochemistry analysis. The antigen expression was limited on mesothelia of the peritoneal, pleural and pericardial cavities, and a little expression was observed on cells of the fallopian tubes and tonsils. Subsequent studies showed the recognition of malignant mesotheliomas, squamous cell carcinomas of the esophagus and cervix by K1 mab (224,225). This antigen was confused at first with OC125, but it was rapidly identified as a new target and was named mesothelin because of its highly expression by mesothelial cells. Mesothelin expression was rapidly confirmed in different cancers, as non-mucinous ovarian carcinomas, cancers of the pancreas, lung, stomach, bile ducts, and triple-negative breast cancer (226,227).

b. Structure and function

1. Structure:

The gene of mesothelin contains an 1884-bp open reading frame encoded by 15 exons of chromosome 16. The promoter of mesothelin does not contain a TATA box and other regulatory elements which are commonly found in mammalian promoters. Minimal



Pastan I, Hassan R. *Cancer Res.* 2014 Jun 1;74(11):2907–12.

Figure 16. Structure of mesothelin.

Mesothelin is synthesized as a precursor of 71 kDa and cleaved to 31 kDa secreted protein MPF and 40 kDa membrane anchored protein mesothelin.

expressed by both normal and tumor cells (229). The expression of mesothelin gene produces a precursor of 71 kDa protein, which will be cleaved to 31 kDa secreted protein macrophage promoting factor (MPF) and 40 kDa membrane anchored protein mesothelin (230) (Figure 13). In our study, we were interested in the 40 kDa membrane attached mesothelin protein.

constitutive promoter elements were localized to a 317-bp region (228). 3 isoforms of mesothelin were identified. Muminova et al. demonstrated in 2004 that mesothelin transcript variant 1 represents the predominant mature mRNA species

2. *Biological function*

Although mesothelin is largely investigated as a clinical target, its function isn't clearly known. It has already been shown that the mesothelin gene is not required for a normal mouse development and reproduction. According to *Bera and Pastan*, mesothelin gene inactivated mice develop normally, without any anatomical or histological abnormalities (231).

According to *Servais et al.* the overexpression of mesothelin in human and murine malignant pleural mesothelioma (MPM) cells induced the expression of MMP9 and promoted cell invasion. They have also described the correlation of mesothelin and MMP9 in MPM patients (232). This mechanism was confirmed in breast cancer cells by overexpressing mesothelin in MCF7 cell line (233). Mesothelin overexpression induced MMP9 expression and invasive capacity of MCF7 cells. Inhibition of the ERK1/2 pathway reduced significantly the MMP9 expression as well as invasive capacity of cells.

Bharadwaj et al. have also examined the effect of mesothelin on cancer cell proliferation, cell cycle progression, and signal transduction pathways by using mesothelin-overexpressed pancreatic cancer cell lines (MIA and PaCa-2), and mesothelin targeting siRNA (siMSLN) transfected BXPC3 cell line (234). Mesothelin overexpression correlated with increased cell proliferation in transfected cells, by contrast, siMSLN transfected BXPC3 cells proliferated slower than non-transfected cells. They showed that the cell proliferation related to mesothelin overexpression passed through Jak/Stat3 pathway. Small interfering RNA against Stat3 significantly reduced the MIA-MSLN cell cycle progression with a concomitant decrease in cyclin E expression. All this suggested that the activation of the transcription factor Stat3 by mesothelin results in an enhanced expression of cyclin E and

cyclin E/cyclin-dependent kinase 2 complex formation to promote the cell cycle. In 2011, the same team demonstrated another mechanism for the induction of pancreatic cancer cell proliferation by mesothelin (235). In stably mesothelin overexpressing pancreas cancer cell lines (MIA-MSLN and Panc1-MSLN) a higher IL-6 production was observed. Silencing MSLN by small interfering RNA, and blocking the activation of nuclear factor-kappaB (NF- κ B) with IKK inhibitor wedelolactone significantly reduced IL-6 levels, also reduced cell proliferation, cell cycle progression and induced apoptosis with a significant decrease of c-myc/bcl-2. Their data suggested that MSLN-activated NF- κ B induces an elevated IL-6 expression, which acts as a growth factor to support pancreas cancer cell survival/proliferation through a novel auto/paracrine IL-6/sIL-6R trans-signaling. Then they also published another research work and evaluated their previous hypothesis by testing a large spectrum of pancreas cancer cell lines and suggested a MSLN-Akt-NF- κ B-IL-6-Mcl-1 survival axis that may be operative in cells, and might as well help cancer cells' survival in the highly inflammatory milieu evident in pancreas cancer (236).

The role of p53 in mesothelin induced proliferation of cells has also been studied. The effects of mesothelin overexpression or silencing on pancreas cancer cells according to their p53 status (muted or not) were studied by *Zheg et al.* (237). They showed that mesothelin promotes proliferation and decreases apoptosis by p53 dependent manner in p53 wild type cells, and p53-independent manner in p53 muted cells.

However, the variety of studies were performed to explore the role of mesothelin in different cancers, it could not be clearly described. Further research is warranted to clarify the mechanisms and effects of mesothelin expression.

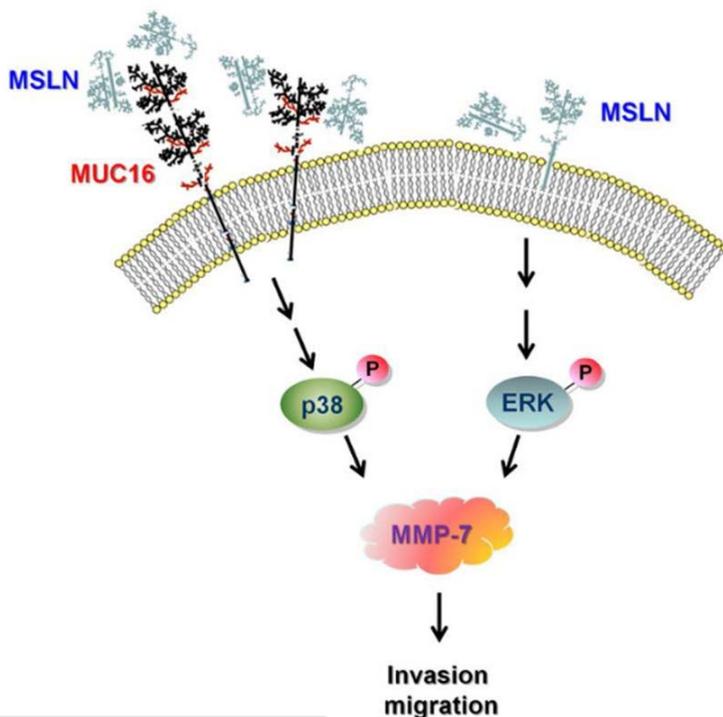
3. Mesothelin and CA125 interaction

The only identified protein which mesothelin can bind in ovarian and pancreatic cancers and in mesothelioma is CA125 (238–241). CA125 is a trans-membraneglycoprotein used as a tumor marker for epithelial ovarian cancers. CA125 is encoded by gene MUC16 and could be either soluble or attached to the cellular surface. CA125 has many molecular interactions such as with mesothelin and galectin. These interactions were believed to play a key role in the stimulation and the dissemination of ovarian and pancreatic cancer and also of

mesothelioma (242).

In advanced grade ovarian cancers CA125 and mesothelin expression correlates and their interaction not only promotes cell adhesion and but can also promote metastasis (241). By using truncated mutagenesis and alanine replacement techniques *Kaneko et al.* identified a binding site on mesothelin for CA125

(243). The binding of mesothelin to CA125 was evaluated on cancer cells by flow cytometry



Chen S-H et al. Sci Rep [Internet]. 2013 May 22 [cited 2016 May 2];3. <http://www.nature.com/articles/srep01870>

Figure 17. Mesothelin/CA125 interaction.

Mesothelin binds to MUC16 and induces cell migration and invasion by p38/MMP7 pathway. Mesothelin can also induce the cell motility directly by ERK/MMP7 pathway.

and enzyme-linked immunosorbent assay. They identified region (296–359) consisting of 64 amino acids at the N-terminal of cell surface mesothelin as the minimum fragment for

complete binding activity to CA125 which is required and sufficient for the interaction between mesothelin and CA125.

Gubbels et al. have also demonstrated under conditions that mimic the peritoneal environment that oxidation of the MUC16 glycans, removal of its N-linked oligosaccharides, and treatment of the mucin with wheat germ agglutinin and erythro-agglutinating phytohemagglutinin abrogates its binding to mesothelin. These observations suggested that at least a subset of the MUC16-associated N-glycans is required for binding to mesothelin (240).

The co-expression of mesothelin and CA125 has been shown to play a role in the invasion process and correlates with an unfavorable patient outcome in pancreatic ductal adenocarcinoma (244,245). It has already been reported by using human pancreatic carcinoma cells that the stimulation of the motility of pancreatic cancer cells passes through PI3K and P38 pathways (246,247). *Chen et al.* published in 2013, that the CA125 expression correlates with mesothelin expression in pancreatic cancers (239). They showed that mesothelin interacts with CA125 and promotes cell motility. In this study they have also demonstrated that the increased cell migration and invasion were dependent on Mesothelin/CA125 interaction which activates MMP7 via P38-MAPK dependent pathway (Figure 14).

c. Expression on different cancers

Mesothelin expression was firstly detected by measuring mRNA levels in several cancer types (248,249). The membrane expression of mesothelin was detected by using immunohistochemistry (250) (Figure 15). These analyses revealed the high levels of mesothelin expression especially in mesotheliomas, pancreas and ovarian cancers. Mesothelin has also been shown to be present in cholangiocarcinoma, hepatic and lung cancers (251,252).

1. Membrane expression

Nelson and Ordonez carried out an immunohistochemistry study on 55 mesotheliomas (44 epithelioid, 3 biphasic, and 8 sarcomatoid), 48 carcinomas of the lung (31 adenocarcinomas, 17 squamous carcinomas), and 86 non-pulmonary adenocarcinomas (14 ovary, 5 peritoneum, 9 endometrium, 11 pancreas, 4 stomach, 16 colon, 12 breast, 9 kidney, 4 thyroid, and 2 prostate) samples (253). Mesothelin expression was identified in 44 (100%) of the epithelioid mesotheliomas, 12 (39%) of the lung adenocarcinomas, and 42 (49%) of the non-pulmonary adenocarcinomas (14 [100%] ovary; 5 [100%] peritoneum; 6 [67%] endometrium; 10 [91%] pancreas; 2 [50%] stomach; 5 [31%] colon; in none [0] of the breast, kidney, thyroid, or prostate) and three (18%) of the squamous carcinomas of the lung, but

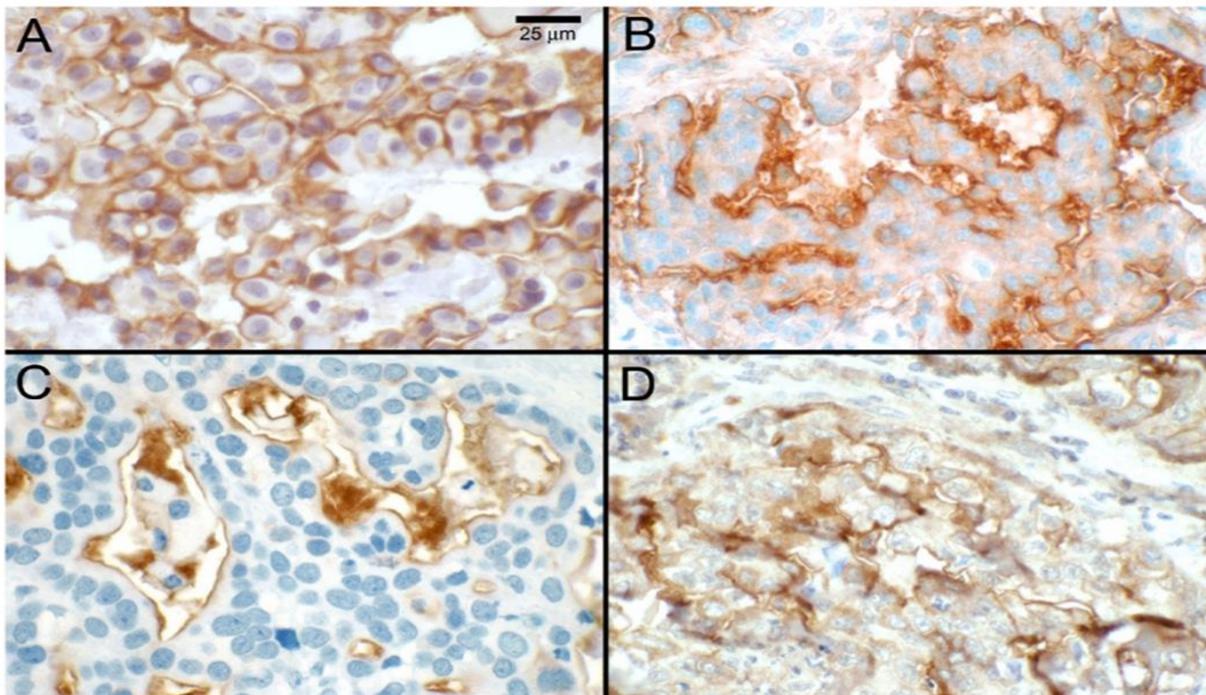


Figure 18. Mesothelin expression in human tumors (*Hassan R., et al., 2008*).

Mesothelin expression was detected by immunohistochemistry using monoclonal antibody (mAb) 5B2 in tissue specimens of patients with mesothelioma (A); ovarian cancer (B); pancreatic adenocarcinoma (C); and lung adenocarcinoma (D).

none of the sarcomatoid mesotheliomas,

Mesothelin expression in pancreas cancer was discovered by using a serial analysis of gene expression and it was confirmed by in situ hybridization for 4 resected primary pancreatic adenocarcinomas and reverse transcription PCR (RT-PCR) in 18 of 20 pancreatic cancer cell lines (254). It was also observed that the normal pancreatic tissues do not express mesothelin. *Joel et al.* have confirmed that the mesothelin expression could be detected by in situ hybridization, RT-PCR or immunohistochemistry in pancreas cancer (255)

The use of immunohistochemistry was revealed the mesothelin positivity in 34 of the 48 analyzed ovarian cancer cases by Hassan et al (256).

2. Soluble mesothelin

The natural presence of soluble mesothelin-related proteins (SMRP) has already been reported in patients' serum and extracellular fluid of tumors. Mesothelin could be released in treatment with phosphatidylinositol-specific phospholipase C which shows that the protein is attached to the cell membrane by glycosyl-phosphatidyl-inositol (GPI) anchor (257). Although mesothelin can be released from the cell membrane by phospholipase C, it seems that phospholipase C does not play a significant role in the physiological shedding process. Previously, *Sapede et al.* have shown that the production of SMRP could be related to abnormal splicing events leading to synthesis of a secreted protein or to an enzymatic cleavage from membrane-bound mesothelin. To test these hypotheses, they used a panel of mesothelioma cells established in culture from pleural effusions of malignant pleural mesothelioma patients. The expression of mesothelin-encoding RNA variants was screened by reverse transcription-polymerase chain reaction experiments. Protease involvement in

mesothelin cleavage from the cellular surface analyzed treating MPM cells with GM6001, a broad-spectrum MMP- and ADAM-family inhibitor. The GM6001 treatment significantly impaired the SMRP production by MPM cell lines, in favor of an enzymatic-mediated shedding process. In addition, a splice variant transcript of mesothelin (variant 3) was detected in these MPM cell lines, in accordance with the release of a secreted part of the protein (258). Later, in 2011, *Zhang et al.* have confirmed the protease implication. They found that the mesothelin sheddase activity is mediated by a TNF- α converting enzyme (TACE), a member of the matrix metalloproteinase, a disintegrin and metalloprotease family. They also showed that EGF and TIMP-3 act through TACE as endogenous regulators of mesothelin shedding (259).

Mesothelin is highly present in the serum of several types of cancer patients, as mesothelioma, ovarian and pancreas cancers (226,260,261). Recently mesothelin expression has also been observed in other types of cancers such as, lung adenocarcinoma, colon and gastrointestinal carcinomas (262–265). Even though, the effects of serum mesothelin levels have been largely described in patients, the induction and secretion mechanism of mesothelin still needs to be investigated.

d. Mesothelin as a therapeutic target

After the confirmation of a high mesothelin expression in mesothelioma, ovarian and pancreas cancers and its limited expression in normal tissues, mesothelin was considered as a good target for cancer therapy.

1. Therapeutic antibodies

The use of monoclonal antibodies against membrane targets could be a promising approach to increase the survival of patients suffering from several cancers. Different targeted therapies based on mabs have been developed against mesothelin. These include a chimeric mab anti-mesothelin MORAb-009(266), immunotoxin coupled mabs SS1P and RG7787 (267,268), and a drug conjugated anti-mesothelin mab BAY-94 9343 (269). Recently a new human single-domain antibody (SD1-hFc) has also been reported. This latest mab can elicit a potent anti-tumor activity by generating a complement-dependent cytotoxicity reaction targeting an epitope in mesothelin close to the cancer cell surface(270).

2. Recombinant Immunotoxins

Recombinant Immunotoxins (RIs) are a 38 kDa truncated portion of *Pseudomonas*

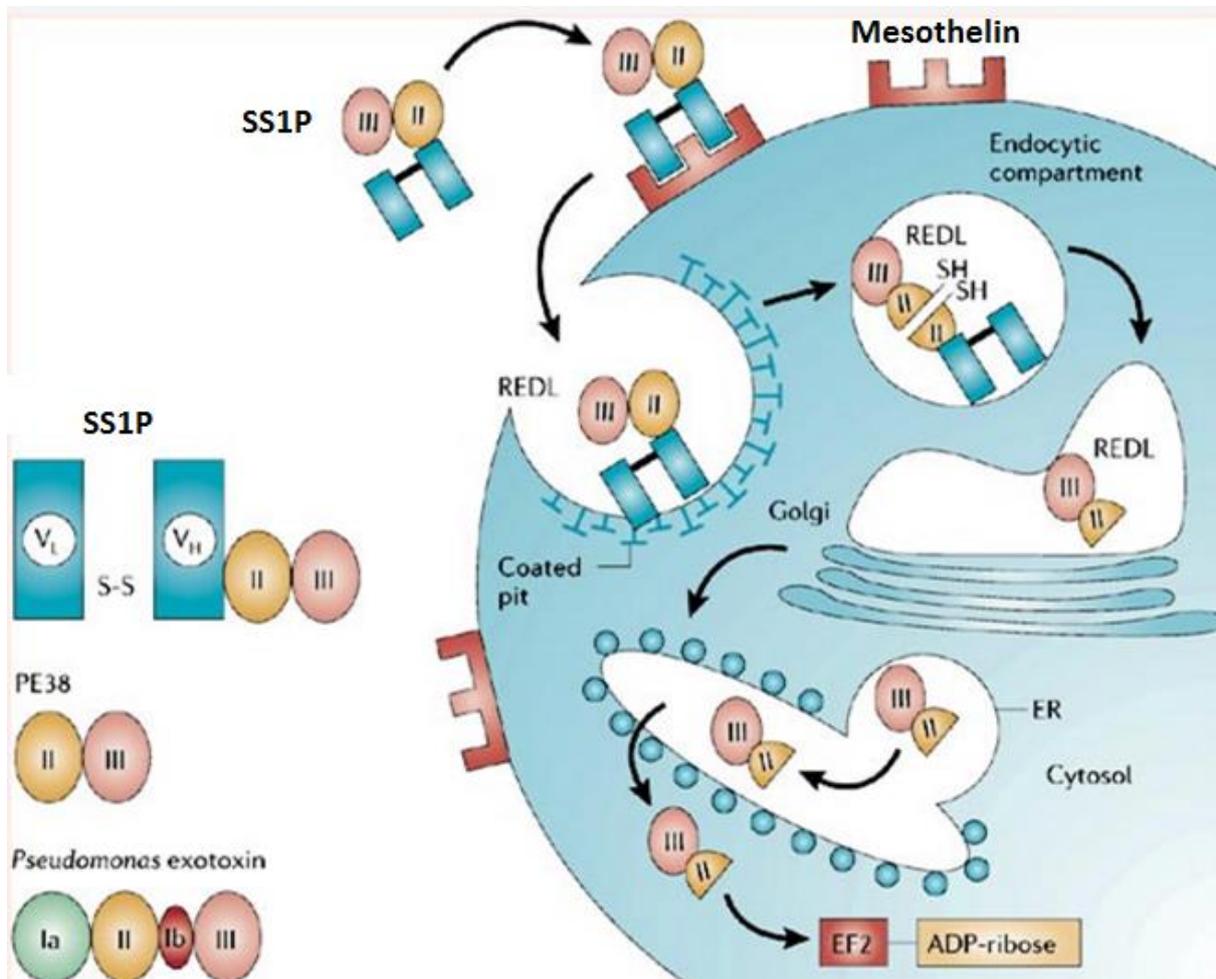


Figure 19. SS1P acting mechanism (Pastan I. et. Al 2006).

SS1P is composed of the variable region of the light (VL) and heavy (VH) chains of an anti-mesothelin antibody that is joined by a disulphide bond and connected to PE38 by a peptide bond. PE38 is a truncated portion of *Pseudomonas aeruginosa* exotoxin A (PE) that lacks domain Ia. SS1P binds to surface mesothelin and then enters the cell through clathrin-coated pits. In the endocytic compartment PE38 is cleaved into two fragments. The C-terminal fragment, which is composed of domain III and a portion of domain II, is transported to the endoplasmic reticulum (ER), presumably through the REDL sequence (REDL functions as a KDEL ER-targeting motif sequence for ER retrieval). From the ER the toxin translocates to the cytosol and adenosine diphosphate (ADP)-ribosylates elongation factor 2 (EF2). This modification causes the inhibition of protein synthesis and leads to cell death.

exotoxin (PE) coupled to sc-Fv or Fab portions of antibodies (271). The antibody portion of RIs acts to target specific antigen expressing cancer cells, and internalizes into the cell by

clathrin-coated pits domain III of PE38 catalyzes the adenosine diphosphate (ADP)-ribosylation and inactivation of elongation factor 2 (EF2), which leads to the inhibition of protein synthesis and cell death (Figure. 16) (272).

Thanks to its selective cytotoxicity, this methodology has been selected to target mesothelin-expressing cells. mAb K1 was ascribed to make an immunotoxin against mesothelin expressing cells and although mAb K1 was found to bind to mesothelin on cancer tissues, it could not function as an immunotoxin (273). Additional antibodies were then generated against mesothelin by hybridoma technology, but non of those with PE succeeded in killing mesothelin expressing cells. After several unsuccessful attempts by hybridoma technology, *Chowdhury et al.* used a phage display technology to generate the antibodies from cDNA immunized mice. The RNA used for phage display was extracted from spleens of mice (268). After the identification of mesothelin specific single chain (SS1), it was fused to Pseudomonas exotoxin and named SS1P. Its selective cytotoxicity was confirmed against mesothelin expressing cancer cells. It also produced regressions of mesothelin expressing tumors in subcutaneous tumor xenografts models in nude mice. This combination of selective cytotoxicity, high activity, and stability made the immunotoxin a good potential candidate for as a therapeutic agent.

The functionality of SS1P has also been validated against human gynecologic cancers grown in organotypic culture in vitro (274,275) and in 3D cultures (276). In addition protein kinase Inhibitor H89, Src kinase inhibitors and the protein kinase C (PKC) inhibitor enzastaurin were described to enhance the activity of SS1P against mesothelin expressing cells in vitro (277–279). Several preclinical studies have also been performed in vivo against xenograft models in mice by the combination of SS1P with different types of treatments.

It has been shown that the combination of Taxol and SS1P exerts a synergistic antitumor effect in animals but not in cell culture (280). Then it was confirmed that Taxol facilitates the immunotoxin uptake by the tumor cells in parallel with an associated reduction of soluble mesothelin in tumor extracellular fluid (281). The same synergy against tumor xenografts was also observed by using the combination of SS1P with another chemotherapeutic agent gemcitabine (282).

The effects of combination of SS1P with radiotherapy were also investigated. In the low-dose or high-dose radiation and SS1P combination studies, mice treated with the combination of radiation and SS1P had a statistically significant prolongation in time to tumor doubling or tripling compared with control, SS1P, or radiation alone (283).

Despite high mesothelin expression, several pancreas cancer cell types are resistant to the SS1P treatment. One of the reasons of the resistance to SS1P has been identified as high levels of expression of Insulin receptor. siRNA knockdown of the insulin receptor enhanced the cytotoxic action of native *Pseudomonas* exotoxin and enhanced SS1P toxicity on several human cell lines (284). Another reason was the low levels of expression of pro-apoptotic protein Bak. In *in vitro* studies, the combination of SS1P by increasing of Bak expression, with TRAIL agonists, and BH3-mimetic ABT-737 caused cell death on resistant cell lines (285,286).

Due to the positive results obtained in preclinical studies, Phase I study of SS1P was enhanced and SS1P was administered as a single agent. As a single agent by continuous infusion or bolus dosing, SS1P was well tolerated up to 25 microg/kg/d x10. Continuous infusion showed no significant advantage over bolus dosing, and further clinical development of SS1P went ahead by bolus dosing in combination with pemetrexed and cisplatin (287,288).

Combination with chemotherapy was safe, well-tolerated and exhibited significant antitumor activity among patients (289).

The clinical use of SS1P is limited by its propensity to induce neutralizing antibodies. Since SS1P is constituted of bacterial exotoxin, it is an immunogenic protein. Consequently, majority of patients develop neutralizing antibodies in 1 or 2 cycles. To date different methodologies are used to improve the efficiency of SS1P:

1) Depleting lymphocytes by chemical agents to diminish immune response to the bacterial toxin component of the immunotoxin. Bortezomib, a proteasome inhibitor, was used as treatment after several immunizations of mice by SS1P, thus eliminating all neutralizing antibodies, as well as pentostatin plus cyclophosphamide treatment (290,291). Based on these data, a study by Raffit Hassan's laboratory started in 2011 to study whether pentostatin plus cyclophosphamide can decrease the immunogenicity of SS1P in patients with chemo-refractory mesothelioma and allow repeated administration of SS1P or not (292). The preliminary results of this study are encouraging, three out of 10 threated patients showed a major regression.

2) By using the different mutated variants of immunotoxins to improve the activity and decrease the immunogenicity of SS1P. B-cell epitopes and protease-sensitive regions of PE38 were removed and, a truncated 24-kDa PE fragment (PE24) has been developed. PE24 was coupled genetically to SS1 and named RG7787. This immunotoxin showed a high cytotoxic activity against pancreatic cancer cell lines as well as primary cells from patients when combined to paclitaxel (285). Similar results were also obtained against mesothelin expressing triple negative breast cancer cell lines in xenograft tumor models (293). Recently, *in vivo* experiments in anti-drug antibodies by the elimination or mutations of T-cell epitopes

in recombinant immunotoxin was also published (294,295). Elimination of either T or B cell epitopes or both, did not affect the cytotoxic capacity of immunotoxins, and still remained active (296).

Another limitation for the clinical use of SS1P is the high concentration of mesothelin in patients' serum and the interstitial space of tumors (269,270). *Zhang et al.* highlighted that the administration of SS1P, combined with Taxol or other chemotherapeutic agents, decreased the concentration of shed mesothelin (280,299). They demonstrated that the synergy of SS1P with Taxol was due to the Taxol-induced fall in shedding antigen levels.

3. Chimeric-antibodies

In parallel to the SS1P development, Hassan et al. chimerized the same single chain (SS1) used for SS1P construction, with an human IgG1 constant fragment in order to use it in clinical applications (300). This chimeric antibody called MORAB-009 (amatuximab) bound selectively the mesothelin transfected human epidermoid carcinoma cells (A431-K5). MORAB-009 has been shown to be internalized by mesothelin expressing cells. Its capacity to induce antibody-dependent cellular cytotoxicity has also been confirmed on mesothelin-expressing ovarian carcinoma cells OVCAR-3, on pancreatic cancer cells, Panc 3.014, and on mesothelioma cells NCI-H226. MORAB-009 could also block the mesothelin induced cell adhesion via targeting specifically mesothelin-CA125/MUC16 interaction. The crystal structure of the complex between the mesothelin N-terminal fragment and Fab of MORAB-009 at 2.6 Å resolution revealed that both the monoclonal antibody and the cancer antigen CA-125 bind to the same region (266,301). The synergism to induce cell death in mouse models was also observed with combination of MORAB-009 and several chemotherapeutical

agents as gemcitabine and taxol. After the confirmation of the lack of toxicity in monkeys, MORAB-009 was used in clinical trials.

Two phase I studies were conducted in patients from the United States and Japan with advanced mesothelin-expressing cancers to determine its safety, dose-limiting toxicity (DLT), and maximum tolerated dose (MTD) (302,303). In these studies MORAb-009 was well tolerated, the MTD when administered weekly is conservatively set at 200 mg/m² and the pharmacokinetic profile of amatuximab in the Japanese population was similar to that seen in the United States .

Another phase I study was also conducted with 24 mesothelin expressing cancers patients to evaluate the serum CA-125 levels after amatuximab treatment (238). MORAb-009 administered as an intravenous infusion (12.5-400mg/m²) doses weekly with 2 weeks off before the next cycle. Increases in serum CA-125 levels were observed, most likely due to MORAb-009 inhibiting the binding of tumor shed CA-125 to mesothelin.

Because of its safety, a phase II clinical trial of amatuximab was conducted with pemetrexed and cisplatin combinations in advanced unresectable pleural mesothelioma (304). Overall, 89 patients were enrolled to study. A median of five cycles (range, 1-6) of combination treatment were administered, and 56 (63%) patients received amatuximab maintenance. However, combination therapy resulted in no overlapping toxicities, response rate (40%) was not higher than the result rates from another phase III study with cisplatin and pemetrexed alone (45,5 %) (305).

Biodistribution of MORAB-009 was investigated first in in vivo models (306). The tumor uptake in A431/K5 tumor was four times higher than that in A431 tumor, indicating

that the tumor uptake in A431/K5 was mesothelin mediated. The bio-distribution studies also revealed that the injected dose of mAb could be individualized, based on the tumor size or the blood level of the shed antigen. The bio-distribution of MORAB-009 has also been studied in patients with mesothelin expressing cancers using a single photon emission computed tomography-computed tomography (SPECT-CT) imaging (307). This study revealed that the antibody uptake was higher in mesothelioma than in pancreatic cancer.

4. Antibody-drug conjugates

Antibody-drug conjugates aim to take advantage of the specificity of monoclonal antibodies to deliver potent cytotoxic drugs selectively to antigen-expressing tumor cells (308). Three ADCs (DMOT4039A, MDX-1382 and BAY 94-9343) targeting mesothelin has been published (269,309). MDX-1382 coupled to duocarmycin, a DNA alkylating agent. Preclinical studies showed that MDX-1382 binds to cynomolgus monkey mesothelin with similar affinity and tissue distribution in humans and its intravenous administration of 0.4 #956;mole/kg was well tolerated in cynomolgus monkeys and did not result in overt clinical or histological signs of toxicity. But there is no available data for its clinical applications since 2009.

BAY 94-9343 (Anetumab ravtansine) is an ADC consisting of an anti-mesothelin antibody conjugated to DM4, a potential tubulin inhibitor (269). Using surface plasmon resonance, immunohistochemistry, flow cytometry, and fluorescence microscopy binding capacity of BAY 94-9343 to mesothelin were confirmed. In vitro BAY 94-9343 showed a selective cytotoxicity to mesothelin expressing cells on nanomolar ranges. After in vitro validation, its functionality revealed on xenograft tumor models derived from mesothelin expressing cancer patients. BAY 94-9343 treatment of mice resulted on complete response in

most of the mesothelin expressing tumors. Next to the preclinical validation of BAY 94-9343, several clinical trials were conducted to determine the maximum tolerable dose, or effects of BAY 94-9343 and chemotherapy combinations on advanced mesothelin expressing solid tumors. In phase I study aimed to determine the maximum tolerable dose of BAY 94-9343, it was administered IV on 2 schedules: every 21 days (q3w) in 45 patients (21 mesothelioma, 9 pancreatic, 5 breast, 4 ovarian cancer, 6 other) at doses ranging from 0.15 to 7.5 mg/kg and in an expansion cohort at 6.5 mg/kg (12 mesothelioma and 20 ovarian cancer); and weekly in 71 patients (31 Mesothelioma and 40 ovarian cancer) randomized to either 1.8 mg/kg or 2.2 mg/kg. Preliminary data with AR at 6.5 mg/kg q3w showed durable PRs in patients with advanced mesothelioma (310). Therefore a phase II trial was initiated to evaluate the efficacy and safety of anetumab ravtansine *versus* vinorelbine in patients with advanced or metastatic malignant pleural mesothelioma overexpressing mesothelin, and who have progressed on first-line platinum/pemetrexed-based chemotherapy. Approximately 183 patients will be randomized in a 2:1 ratio to receive anetumab ravtansine 6.5 mg/kg Q3W or vinorelbine 30 mg/m² QW. This trial is open and currently in accrual patients (311).

Another ADC targeting mesothelin is DMOT4039A (312). DMOT4039A is an antibody–drug conjugate composed of the anti-mesothelin mAb h7D9.v3 and the potent antimitotic agent, monomethyl auristatin E. DMOT4039A revealed an anti-proliferative activity against mesothelin-expressing cancer cells in vitro and a potent antitumor activity in xenograft cancer models expressing clinically relevant levels of mesothelin. Phase I study of DMOT4039A treatment in ovarian and pancreatic cancer patients revealed a tolerable safety profile and antitumor activity in both pancreatic and ovarian cancer, having a total of 6 patients confirmed partial responses (4 ovarian; 2 pancreatic) (313).

5. Vaccine anti-mesothelin

In phase I study of allogeneic GM-CSF-secreting pancreatic tumor vaccine induced a dose-dependent delayed-type hypersensitivity (DTH) response to autologous tumor cells in 3 out of 14 patients (314). It has then been reported that although, neither of the vaccinating pancreatic cancer cell lines expressed HLA-A2, A3, or A24, the consistent induction of CD8+ T cell responses to multiple HLA-A2, A3, and A24-restricted mesothelin epitopes exclusively in the three patients with vaccine-induced DTH responses (315). These results provided the first direct evidence that CD8 T cell responses can be generated via cross-presentation. In another study a H-2D(b)-restricted mesothelin peptide-specific cytotoxic T-lymphocyte (CTL) epitope (amino acid (aa) 406-414) has been identified, and the control of mesothelin expressing cells by the adoptive transfer of this peptide has been described (316).

It has also been shown that, a DNA vaccine encoding a single-chain trimer of HLA-A2 or linked to a human mesothelin peptide and vaccination with chimeric virus-like particles that contain human mesothelin generates anti-tumor effects against human mesothelin-expressing tumors (317–319). Inhibition of growth and metastasis of pancreatic cancer on human by the adenovirus 40 based vaccine was also described by *Yamasaki et al.* (320).

A *Listeria monocytogenes* strain was engineered to express human mesothelin (CRS-207) in order to use in mesothelin expressing cancers. A phase I study published in 2012 showed that the CRS-207 administration was safe and resulted in immune activation (321). Another clinical study performed on 90 pancreas cancer patients by combination of GVAX and CRS-207 revealed that the vaccination extends survival of patients, with minimal toxicity (322).

6. CAR anti-mesothelin

Mesothelin is a membrane target that allows immune-based therapy. Studies on genetically modified T cells with chimeric antigen receptors report a significant efficacy in different malignancies (323). Recently, CAR T cells have also been investigated for the treatment of solid tumors (324). Anti-mesothelin CAR T cells are also being currently investigated in clinical studies (325,326). *Beatty and al.* reported 2 cases from undergoing phase I study for 3rd generation (CD3- ζ and 4-1BB co-stimulatory domains containing) CAR T cells against mesothelin (216). In these 2 cases administered CAR T meso cells selectively targeted mesothelin expressing primary and metastatic tumors and elicited an antitumor immune response revealed by the development of novel anti-self-antibodies. Another CAR comprised of a human mesothelin-specific single-chain antibody variable fragment (P4 scFv) was coupled to T cell signaling domains which kills mesothelin-expressing tumors in vitro and in vivo was constructed and evaluated by *Lanitis et al* (327). According to *Moon et al.* CCR2 expression enhances the cytotoxic activity of mesothelin targeting CAR T cells (328). These findings support the development of mRNA CAR-based strategies for carcinoma and other solid tumors.

AIM of the thesis

Mesothelin is a GPI linked membrane protein. Besides that it can be cleaved by a protease and can produce a soluble, shedded form of mesothelin. It has already been showed that this soluble form of mesothelin acts as a ligand and neutralizes the mesothelin targeting therapeutic antibodies. Therefore antibodies could not reach to cancer cells and thus remain inefficient.

Considering the reasons described hereinabove, we seek to develop a discriminating antibody which targets selectively membrane-form of mesothelin.

First we aimed to show that a novel immunization method combining with phage display allows obtaining specific antibodies which recognize only the membrane form of mesothelin. Then our objective was to validate that this antibody does not recognize any other forms of mesothelin presented in cell culture, in patients' serum or in exosomes extracted from cell culture. After that our goal was to validate whether this antibody recognizes the mesothelin expressed naturally in different cell lines or not.

At last we decided to construct a CAR from the sequences of a discriminating antibody to validate its anticancer function on mesothelin expressing cells.

RESULTS

I. Article in submission



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Contributing Authors	Mr. Kamal ASGAROV , Jeremy Balland , Charline Tirole , Adeline Bouard , Virginie Mougey , Diana Ramos , Antonio Barosso , Vincent Zangiacomi , Marine Jary , Stefano Kim , Dr. Maria Gonzalez-Pajuelo , Bernard Royer , Hans de Haard , Andy Clark , John Wijdenes

I. A new anti-mesothelin antibody targets selectively membrane associated form

Asgarov Kamal^{1,2}, Balland Jeremy^{1,2}, Tirole Charline^{1,2}, Bouard Adeline^{1,2}, Mougey Virginie^{1,3}, Ramos Diana⁴, Barroso António⁴, Zangiacomì Vincent², Jary Marine⁵, Kim Stefano⁵, Pajuelo Maria⁴, Royer Bernard^{1,2,5}, de Haard Hans⁶, Clark Andy⁷, Wijdenes John⁷, Borg Christophe^{1,2,3,5}.

¹ INSERM unit 1098, University of Bourgogne-Franche-Comte, 8 rue du Docteur Jean-François-Xavier Girod BP 1937 25020 Besançon Cedex, France.

² ITAC Platform of Clinical Investigation center-Biotherapy, 3 bd Fleming, F-25030 Besançon Cedex, France.

³ Blood bank Bourgogne-Franche-comté

⁴ Fairjourney Biologics, Rua do Campo Alegre, 1021, Porto, Portugal.

⁵ J.Minjoz University Hospital, 3 bd Fleming, F-25030 Besançon Cedex, France.

⁶ arGEN-X BVBA, Zwijnaarde, Belgium

⁷ Mabdesigns, London, UK

Corresponding author: Christophe Borg, 8, rue du Docteur Jean-François-Xavier Girod BP 1937 25020 Besançon Cedex France. Tel : +33 3 81 66 93 21 ; fax : +33 3 81 66 97 08 ; email : christophe.borg@efs.sante.fr

Abstract

Mesothelin is a GPI anchored membrane protein which shows promise as a target for antibody directed anti-cancer therapy. High levels of soluble forms of the antigen represent a barrier to directing therapy to cellular targets. The ability to develop antibodies which can selectively discriminate between membrane-bound and soluble conformations of a specific protein and thus target only the membrane associated antigen is a substantive issue. We show the use of a tolerance protocol provides a route to such discrimination. Mice were tolerized with soluble mesothelin and a second round of immunizations was performed using mesothelin transfected P815 cells. RNA extracted from splenocytes was used in phage display to obtain mesothelin-specific antigen-binding fragments (Fabs) which were subsequently screened by flow cytometry and ELISA. This approach generated 147 different Fabs in 34 VH-CDR3 families. Utilising competition assays with soluble protein and mesothelin containing serum obtained from metastatic cancer patients 10 of these 34 VH-CDR3 families were found exclusively to bind to the membrane-associated form of mesothelin. Epitope mapping performed for the 1H7 clone showed that it does not recognize GPI anchor. VH-CDR3 sequence analysis of all Fabs showed significant differences between Fabs selective for the membrane associated form of the antigen and those which recognize both membrane bound and soluble forms. This work demonstrates the potential to generate an antibody specific to the membrane bound form of mesothelin. 1H7 offers potential for therapeutic application against mesothelin bearing tumors which would be largely unaffected by the presence of the soluble antigen.

Keywords

Mesothelin, therapeutic antibody, soluble mesothelin, membrane-specific antibody, phage display, serum mesothelin, competition assay, tolerance immunization.

INTRODUCTION

Mesothelin is a glycosylphosphatidylinositol (GPI)-linked glycoprotein synthesised as a 69 kDa precursor and proteolytically processed into a 30 kDa NH₂-terminal secreted form (formerly referred to as Megakaryocyte Potentiating Factor (MPF)) and a 40 kDa membrane-bound form [1]. Mesothelin is present at relatively low levels in mesothelial cells of the pleura, peritoneum and pericardium of healthy individuals, but is highly expressed in a number of different cancers, including mesotheliomas, stomach cancer, squamous cell carcinomas, prostate cancer, pancreatic cancer, lung cancer, and ovarian cancer [2–6]. In particular, it has been reported that a majority of serous carcinomas of the ovary and adenocarcinomas of the pancreas express high levels of mesothelin [7]. In addition, high levels of mesothelin have been detected in greater than 55% of lung cancers and greater than 70% ovarian cancers [5, 8]. The limited expression of mesothelin on normal cells makes it a viable target for tumour immunotherapy. Administration of antibodies against mesothelin has been proposed as a strategy for mesothelioma as well as lung, ovarian and pancreatic cancer.

Although mesothelin is considered as a membrane binding protein, soluble forms of mesothelin have also been reported in patients' serum and in the stroma of tumors, including malignant mesothelioma, ovarian cancers or highly metastatic cancers. Soluble mesothelin has been reported as a result of both alternative splicing and protease cleavage [9], [10]. Tumor necrosis factor- α converting enzyme (TACE), a member of the MMP/ADAM family, has been identified as a mesothelin sheddase [11].

Soluble receptors can be released in the tumoral microenvironment, especially when expressed on cancer cells and act as a decoy for therapeutic antibodies. It has previously been

shown that a GPI anchor can affect protein conformation, indeed the presence of a GPI anchor can result in sufficient conformational changes in the protein to be specifically targeted by antibodies, in contrast to their cleaved, non-GPI, forms [12–14].

The use of monoclonal antibodies against membrane targets has been shown to increase the survival of patients suffering from several cancers. Different targeted therapies based on mAbs have been developed against mesothelin. These include a chimeric mAb anti-mesothelin MORAb-009 [15], immunotoxin coupled mAbs SS1P and RG7787[16, 17], and a drug conjugated anti-mesothelin mAb BAY-94 9343 [18]. Recently a new human single-domain antibody (SD1-hFc) has also been described. That latest mAb is reported to elicit a potent anti-tumor activity by generating a complement-dependent cytotoxicity (CDC) reaction targeting an epitope in mesothelin close to the cancer cell surface [19]. Although these mAbs showed a therapeutic effect against mesothelin expressing tumors, a significant number of antibodies have failed to do so. One possible explanation for the lack of efficacy might be the presence of soluble mesothelin in the blood as well as in the extracellular space of tumors, which could reduce the efficacy of the therapeutic mAb by competition [20]. A recent study demonstrated that reduced shedding of surface mesothelin as a result of treatment with taxol improves the efficacy of mesothelin targeting recombinant immunotoxins [21]. Similarly, mutation of the protein responsible for mesothelin cleavage such that it is not shed results in improved efficacy of the immune-toxin SS1P [21].

Here we propose a new approach to overcome the impact of shed mesothelin on antibody based therapies. Our goal was to identify an antibody which exclusively targets the whole cell membrane-associated form of mesothelin without interfering with the soluble-form of the protein. Identifying antibodies which specifically recognize the membrane-bound mesothelin could have a significant therapeutic impact on patients' outcome. For this purpose

we used a new method to generate antibodies comprising: i) immunizing an animal with a soluble form of the antigen, ii) administering an agent that selectively kills rapidly dividing cells [22], iii) immunizing the animal with a membrane-bound form of the antigen, and iv) screening for an antibody which can bind to the second antigen without binding the first one.

Phage display technology was used to produce antibody fragments because of known advantages in specific epitope targeting. It has been shown as a promising technology to produce specific antibodies against targets on the cell surface [23]. Phage display libraries have also been reported as a strong alternative to hybridoma technology for antibody development with desired antigen binding characteristics [24]. Indeed, occluded epitopes on the cell surface may be difficult to access by full-size immunoglobulin G (IgG) antibodies produced by hybridoma technology. These epitopes can be targeted by small fragments of antibodies such as antibody binding fragment (Fab) and single chain variable fragment (scFv).

Here we showed that, from a total of 480 Fab clones obtained by phage display technology, 147 clones were identified as mesothelin specific, amongst which 116 clones bound to the native form of mesothelin on Hela cells. Following further analysis of these 116 antibodies, we identified the clone 1H7 which targets selectively the membrane bound form of mesothelin without binding shed forms of this antigen.

RESULTS

Production of mesothelin targeting Fabs:

To identify new antibodies which selectively target the whole cell membrane bound form of mesothelin, we hypothesized that there was a conformational difference between the membrane bound and soluble form of the common components of mesothelin, resulting in different conformational epitopes. A novel subtractive immunization was thus used to generate antibodies which only recognize epitopes selectively derived from membrane-bound proteins. To prevent mice from producing antibodies against soluble mesothelin antigens, mice were tolerized by immunizing them with the full-length recombinant mesothelin protein, and then treated with cyclophosphamide to kill the activated lymphocytes. In order to generate antibodies targeting membrane-associated mesothelin, these mice were subsequently immunized with mesothelin-transfected P815 cells (P815-Meso). After 4 cycles of immunizations, mice were sacrificed, total RNA was extracted from splenocytes, and 5 Fab libraries were created by using phage display technology (Figure 1A). To avoid crosslinking with antibodies recognizing other antigens on P815 cells, phage libraries were first selected, against mesothelin-expressing CHO cells (CHO-Meso). Then the second round of panning was performed against P815-Meso cells (Figure 1B). Non transfected CHO and P815 cells were also used as a control for panning to assess specific phage enrichment (data not shown). After two rounds of panning for each library, Fab containing periplasmic extractions of 96 single colony cultures were collected and screened on CHO-Meso cells. Overall 147 Fabs binding to mesothelin were identified.

In order to identify antibodies binding specifically to mesothelin on cancer cell membrane, we re-screened all positive Fabs able to recognize HeLa, a cervix adenocarcinoma

cell line which expresses naturally high levels of mesothelin. Only 116 of the 147 Fabs were identified as native form specific binders on Hela cells (figure 1C). Then all 147 Fabs were sequenced and their VH-CDR3 regions were determined for further analysis.

Regrouping Fabs in families following their VH-CDR3 similarities:

VH-CDR3 sequences of antibodies have already been reported as the most important region for epitope recognition [25–27]. On this basis, VH-CDR3 regions of all Fabs were aligned and associated in 34 families according to their amino acid sequence similarities. Fabs recognizing Hela cells were distributed in only 20 of 34 VH-CDR3 families (figure 1D). The screening data of Fab staining on CHO-Meso and Hela cells revealed that all clones of each family showed uniform staining on cells, suggesting that all of them recognized the same epitope (supplementary figure 1). On the basis of these data, only one clone of each family was selected for further testing.

Eliminating soluble mesothelin binding Fabs:

After the identification of natural form binders, the next screening was designed to eliminate Fabs which recognize soluble mesothelin. To compare their ability to bind either, only membrane or both membrane and soluble forms of mesothelin, the binding of Fabs to Hela cells was tested in competition with recombinant mesothelin protein. Fab concentration was adjusted by endpoint dilution assay. In order to establish the endpoint dilution, two-fold dilution series for each Fab were performed. These solutions were tested on Hela cells to identify the limited concentration (endpoint dilution) for which the Fab signal decreases in flow cytometry analysis (supplementary figure 2). To identify the Fabs which discriminate between membrane bound and soluble antigen, each Fab was incubated at its endpoint dilution for 30 minutes with 0.8 μ g of soluble mesothelin, before assessing the binding

capacity on HeLa cells. From flow cytometry analysis only 10 VH-CDR3 families were identified as partial or completely discriminating Fabs. In figure 2A, a representative staining on HeLa cells of Fabs 1H7, 3C2 and 3C1 is shown. 1H7 and 3C2 Fabs bound 69 and 71 percent on HeLa cells respectively. Incubation with 0.8 µg of mesothelin before staining did not alter the binding capacity of any of the selected Fabs, whilst the staining capacity of 3C1 decreased significantly. To confirm the results obtained by flow cytometry, a biotinylated mesothelin protein was attached to streptavidin-coated plate and used for the screening of discriminating and non-discriminating Fabs using direct enzyme-linked immunosorbent assay (ELISA). Only non-discriminating Fabs, 3C1 for example, could bind to mesothelin. In contrast, Fabs that don't bind soluble mesothelin were not able to detect mesothelin in ELISA assay (figure 2B).

These experiments confirmed that the methodology used for immunization allows us to identify discriminating FAB families. Next we sought to investigate if the soluble mesothelin-related peptides (SMRP) present in physiological fluids, specifically those present in mesothelin expressing cancer patients reduces the proportion of discriminating Fabs binding the antigen presented on the cell surface.

Competition tests with SMRP in patients' serum and cell supernatant:

High levels of SMRP is a prognostic factor for mesothelioma, ovarian and pancreatic cancers [28–32]. The presence of SMRP can also alter the binding capacity of mesothelin targeting therapeutic agents [20, 21]. On the basis of these data, we used the serum of 21 patients suffering from metastatic colon and pancreatic cancers in competition assays with selected Fabs. Serum concentration of mesothelin from the 21 patients and from two healthy volunteers (A and B) was first determined by ELISA assay (figure 3A). Mesothelin concentration was shown to be significantly higher in all of the 21 patients compared with

healthy donors. According to their mesothelin concentration, serum samples were pooled in two groups: high- (>20 ng/ml) and low-serum (<20 ng/ml) respectively. Hela cell derived supernatant containing 35ng/ml of mesothelin was also used as another source of SMRP (figure 3B). Competition assays were performed with high- or low-serum samples as well as with Hela supernatant. The samples were pre-incubated with selected Fabs for 30 minutes, and then added to Hela cells prior to flow cytometry analysis. Five families were revealed as discriminating Fabs against physiological mesothelin containing fluids. Figure 3C shows that the presence of high- and low-serum samples or Hela cell supernatant containing 35ng/ml of mesothelin did not significantly alter the binding capacity of representative discriminating Fabs (*e.g.* 1H7 and 3C2). In contrast, the binding ability of a competing Fab (*e.g.* 3C1) was shown to decrease in presence of high- or low-serum samples, and with concentrated Hela supernatant as well (24 vs. 9, 11 and 12 percent respectively; *cf.* figure 3C).

Mesothelin is anchored to cell membrane with GPI. GPI anchors with attached proteins are released from cells in two forms: in complexes with membrane lipids or exosomes [33]. For this reason, we investigated whether mesothelin resided in tumor-released exosomes. Exosomes were isolated from Hela cell supernatant by ultracentrifugation and mesothelin was quantified by ELISA assay (supplementary figure 3A). Mesothelin expression was confirmed by confocal microscopy showing a co-localization of the protein with the exosome specific marker CD63 (supplementary figure 3B). The binding capacity of Fabs was tested in competition with exosomes. In those experiments mesothelin expressing exosomes failed to prevent Fab binding to Hela cells (supplementary figure 3C).

Altogether, these experiments demonstrate that the discriminating Fabs can selectively target mesothelin expressed on the surface of cancer cells without being subject to the antagonist effects of SMRP present in physiological fluids.

1H7-hFc specifically binds mesothelin expressing cancer cells

We next sought to determine if our discriminating antibodies target mesothelin expressing cancer cells. For this purpose, we chose two Fabs, 1H7 as a discriminating antibody, for chimerization with human IgG1. Binding capacity of 1H7-hFc was then assessed on different cancer cell lines. We observed that 1H7-hFc bound HeLa and CHO-Meso, a cell line over-expressing human mesothelin, but not CHO cells. 1H7-hFc strongly bound breast cancer cell line MDA-MB231, but moderately recognized colon cancer cells HT29 while non-small cell lung cancer cells A549 were weakly bound by this clone. No binding could be observed for cervix cancer cells C33A which is a mesothelin negative cell line (figure 4). In order to assess if 1H7-hFc could recognize primary cell lines, we tested three different pancreatic cancer cells BesPac-C7, BesPac-C8 and BesPac-C12, obtained in our laboratory from ascites of pancreatic cancer patients. Flow cytometry analysis showed that 1H7-hFc bound strongly BesPac-C7 and BesPac-C12, and weakly BesPac-C8 cells. Mesothelin expression on these cells was confirmed by qRT-PCR and western blotting experiments (data not shown). These results demonstrated that 1H7-hFc selectively recognizes native mesothelin on different types of cancer cells.

1H7 does not recognize GPI anchor

Experiments presented above demonstrate that 1H7 is selective for the membrane associated conformation of the mesothelin protein and does not recognize other conformations such as the recombinant form or that contained within the physiological fluids from mesothelin expressing cancer patients. A new set of experiments were designed to investigate the role of the presence of GPI in epitope recognition by 1H7-Fc. To show the specificity of 1H7-hFc to mesothelin HeLa cells were treated with 1U and 10U of GPI specific

phospholipase C (PI-PLC) which cleaves the phosphor-glycerol bond and leaves protein from cell membrane with the GPI anchor attached [11]. Depletion of mesothelin expression on cell membrane was confirmed by cytometry analysis (figure 5A),

It remains possible that 1H7 is recognizing some component of the GPI anchor or a shared epitope between the GPI anchor and the protein. In order to decipher the role of the GPI-anchor in the epitope recognition of 1H7-hFc we assessed whether 1H7 might be a non-specific antibody to GPI. To eliminate this hypothesis PC3 and LN-CAP cells which do not express mesothelin but highly express another GPI anchored protein CD59 were stained by 1H7-hFc. The cells were highly positive for CD59 but not for 1H7-hFc which confirmed that 1H7 doesn't recognize the GPI anchor (figure 5B).

Second, we hypothesized that 1H7-hFc might bind a shared epitope between the GPI anchor and mesothelin. This hypothesis was also eliminated by using a FITC coupled pre-aerolysin protein, FLAER, which binds specifically to GPI [34]. We determined the saturation concentration of FLAER by staining Hela cells. Then, cells were incubated with 25, 50, and 100nM of FLAER and analyzed by flow cytometry. Relative median fluorescent intensities (RMFI) were 4.8, 5 and 5.2 for each concentration, respectively. 50nM was then selected as a saturation concentration (figure 5C) and Hela cells were incubated with 50nM of FLAER one hour before staining with 1H7-hFc. Flow cytometry analysis showed that the binding properties of 1H7 were not altered by the presence of FLAER (figure 5D). These results suggested that GPI isn't involved directly in the epitope recognition by 1H7.

Together, these experiments indicate that GPI has no direct role in the recognition of cancer cells by 1H7 and suggest that 1H7 recognizes a conformational epitope generated by molecular interactions between mesothelin and the GPI anchor.

VH-CDR3 sequence comparison:

Due to the differences in epitope recognition between discriminating and non-discriminating Fabs, we looked to see if there were consistent differences in their VH-CDR3 sequences. Regardless of epitope recognition, the VH-CDR3 length was between 10 to 16 amino acids for all Fabs. According to IMGT unique numbering and color menu for amino acids (329), all 105-117 positions of VH-CDR3 sequences were identified and amino acid frequencies were calculated for all positions of the CDR3 region (figure 6). At position 105 both the discriminating and non-discriminating sequences encoded frequently alanine, but 30% of discriminating sequences encoded threonine, inversely to leucine (10 %) and serine (10%) in non-discriminating sequences. At position 106, arginine was used frequently for both sequences. At positions 107 and 110 glycine was more frequent (50% and 60%, respectively) in discriminating sequences. Positively charged lysine, hydrophobic leucine, isoleucine and glycine were found more in discriminating sequences compared non-discriminating sequences at position 108 and 109. At position 113, only tryptophan (10% for both) and serine (20% and 10% respectively) were coded commonly for both discriminating and non-discriminating sequences, but aspartic acid (10%), methionine (10%), phenylalanine (10%), leucine (30%), and isoleucine (10%) were only present in discriminating sequences. At position 114 in discriminating sequences, proline (10%) and serine (10%) were observed, but not in non-discriminating sequences. The presence of tyrosine, phenylalanine, and glycine were common for both discriminating and non-discriminating sequences. No significant difference of frequencies of amino acids was noted at positions 115, 116 and 117.

Comparison of VH-CDR3 sequences of discriminating Fabs *versus* non-discriminating Fabs showed that the frequencies of amino acids at positions 107 to 114 were significantly

different in discriminating Fabs. Further research is warranted to confirm our data and determine the role of these amino acids in epitope recognition.

DISCUSSION

This work describes the generation of a novel anti-mesothelin antibody (1H7) resulting from a subtractive immunization protocol inducing tolerance to the soluble antigen followed by the construction of mouse phage display libraries. 1H7 binds preferentially to mesothelin expressing cells and does not compete with cleaved or recombinant mesothelin. It binds an epitope which appears to exist only when the protein is on the cell surface, and disappears in aqueous solutions.

Mesothelin has already been described in the literature as a good target for cancer therapy [36]. Several agents, including immunotoxin coupled mAbs, chimeric monoclonal antibodies, antibody drug conjugates, anti-mesothelin tumor vaccines and anti-mesothelin CAR are in various stages of development for the treatment of patients with mesothelin-expressing tumors [37–39]. A chimeric antibody (MORAb-009) and an immunotoxin coupled mAb (SS1P) constructed from the same murine SS1 scFv targeting mesothelin have already been developed and are currently being examined in clinical trials for mesothelioma and pancreatic cancers. The clinical use of SS1P is limited by its propensity to induce neutralizing antibodies. To date two different approaches have been used to improve the efficiency of SS1P: i) depletion of lymphocytes by chemical agents to diminish immune response to the bacterial toxin component of the immunotoxin [40], ii) use of mutated variants of immunotoxins to improve the activity and decrease the immunogenicity of SS1P [16]. A further limitation for clinical use of SS1P is the high concentration of mesothelin in patients' serum and interstitial space of tumors [41, 42]. Zhang *et al.* highlighted that the administration of SS1P combined with Taxol decreased the concentration of shed mesothelin [20]. They demonstrated that the synergy of SS1P with Taxol was due to the Taxol-induced

fall in shed antigen levels. Here we suggest that conjugating the antibody 1H7 with a less immunogenic toxin than SS1P might substantially enhance the efficacy of treatment by both reducing clearance of the conjugate and avoiding the issue of the therapeutic antibody binding to shed antigen.

The implication of the GPI anchor in conformational epitope recognition has been investigated in several studies. Bradley *et al.* showed by using de-lipidation of Thy-1 by different enzymes and constructing a recombinant Thy-1 without a GPI anchor, that the recognition of Thy-1 by the monoclonal antibodies K117, 5E10, and AS02 in western blots is abolished or greatly diminished [43]. However Thy-1 was detected in western blotting after exclusive partitioning into the insoluble phase. mAbs that bind to GPI anchored proteins such as VSG or gp23 on the surface of parasites do not react with the same proteins when they are in soluble form [44–46]. This suggests the existence of different conformational epitopes in soluble and membrane-associated forms of GPI anchored proteins which play a critical role in antibody binding. Changes in GPI-dependent antibody reactivity have also been observed in mammalian proteins; for example human CD59 antibodies whose reactivity decreases after GPI specific phospholipase D (GPI-PLD) treatment. This phenomenon has also been observed for human CD52 and Thy-1 proteins [47, 48]. The work described above shows that membrane associated mesothelin can be targeted specifically by monoclonal antibody fragments which do not recognize the soluble form.

Therapeutic antibody based anti-cancer approaches rely on the over-expression of the target antigen on cancer cells relative to the expression on normal tissues. The selectivity relies on this differential expression being sufficient to allow a killing action on the target cells without substantial damage to normal tissues.. Moreover, this Fab might be potential

candidate for converting its single chain to clinically relevant-molecules: i) combined with chemotherapy [49], ii) linking with an immunotoxin [50], or iii) being used alone through ADCC [51].

Mesothelin is a membrane target that allows immune-based therapy [39]. Studies on genetically modified T cells with chimeric antigen receptors (CAR) report significant efficacy in different malignancies [52]. Anti-mesothelin CAR T cells are also currently being investigated in clinical studies [53]. Finally, this membrane bound antigen specificity could be of interest for generating anti-mesothelin CAR T cells, using one of these discriminating Fabs.

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Competing interests

The authors declare that they have not competing interests.

MATERIALS AND METHODS

Patient samples

Blood samples from metastatic colon and pancreatic cancer patients were obtained from the oncology department of University Hospital of Besancon, France. Healthy donor blood samples were provided by the blood bank of Bourgogne-Franche-Comte.

Cell culture

CHO, P815, C33A, HT29, A549, PC3, LN-CAP and Hela cells were purchased from ATCC. CHO, C33A, HT29, A549, and Hela cells were cultured in DMEM-Glutamax (GIBCO) , PC3, LN-CAP and P815 in RPMI 1640 supplemented with 10% of heat inactivated fetal bovine serum ((FBS-HI)-Gibco), and 1% of Penicillin-Streptomycin (10,000 U/mL) (GIBCO). BesPac-C7, BesPac-C8, and BesPac-C12, were purified in our laboratory from ascites of pancreatic cancer patients donated as above and cultured in DMEM-Glutamax supplemented with 10% of heat inactivated fetal bovine serum. All cells were cultured in T175 Flasks at 37°C in a 5% CO₂ incubator and sub-cultured every 2 days when cells confluence reached 80-90% of the cell flask. P815 and CHO cells were transfected with mesothelin expressing PCMV6-XL4 vector (Invitrogen) to obtain CHO-Mesothelin and P815-Mesothelin cells.

Immunization of Mice

Four weeks old female BALB/c mice were firstly immunized intraperitoneally with 1 µg of full length mesothelin recombinant protein (RayBiotech, ref: 230-00043) and (in next two days) 1 mg of cyclophosphamide was injected intraperitoneally to each mouse. This procedure was performed 4 times at intervals of two weeks. 4 days after the last cyclophosphamide injection, mice were immunized intraperitoneally (3 times at intervals of

two weeks) with 10 million P815 cells transfected with mesothelin expressing vector. A further two weeks later an intravenous injection was performed with 1 million P815-Meso cells. 4 days after the last injection the mice were sacrificed and the spleens were collected.

RNA extraction and cDNA construction

All spleens were separately mechanically degraded and splenocytes were collected. RNA was extracted using Qiagen RNeasy® Midi Kit according to manufacturer's instructions. 40 µg of RNA was used to make the cDNA by using SuperScript III First Strand Synthesis System by Invitrogen (cat#18080-051).

Sub-library construction

PCR reactions were performed with primers to amplify specifically the VH-Ch1 and Vk-Ck coding regions of lymphocytes. The PCR amplifications were performed using the Expand High Fidelity System by Roche (cat# 4738268001) in 100 µl reaction volume using 6 µl of non-purified cDNA as template and 500nM of each combination of primers. After the migration and purification of PCR products, 2nd PCR reactions were performed, this time with restriction enzyme sites containing primers. After the second PCR all products for VH-Ch1 and Vk-Ck were pooled and digested with SfiI/NotI and ApaLI/AscI respectively. At the other side phage-mid pCB3 vector was digested with the same enzymes and ligation was performed separately to obtain two sub-libraries VH-CH1 and Vk-Ck. After the transformation of *E. coli* strain TG1 by electroporation, sub-libraries with the size of greater than 1E+08 colonies were created.

Library construction

After overnight culture midi-prep was performed for each of the sub-libraries. VH-Ch1 regions were cloned on the Vk-Ck sub-libraries after digestion by SfiI/NotI enzymes.

Transformation was performed with electroporation on *E. coli* strain TG1 and libraries with sizes of greater than 1E+08 colonies were obtained.

Panning of library

Phages were incubated with 5E+06 cells in solution for one hour at 4°C and then washed several times with 10% FBS/PBS. Bound phages were retrieved using trypsin (1 mg/ml) at room temperature. Protease activity was immediately neutralized by applying 16 mM protease inhibitor AEBSF. On the first round of screening the CHO-Mesothelin cells were used to select the phage libraries, on the second round the P815-Mesothelin cells were used to select the first round rescued phage.

Preparation of periplasmic extracts containing soluble Fabs

Soluble Fabs were produced from individual *E. coli* clones as described (14). Fab expression was induced by adding 1mM of isopropyl 1-thio-β-D-galactopyranoside. Periplasmic extracts containing soluble Fab (P.E.s) were extracted by a freeze/thaw cycle; bacteria were pelleted and frozen at -80°C for 2 hours and then thawed at room temperature and resuspended in 110 μl of PBS. The periplasmic extract (P.E.) containing the soluble Fab was collected after 30 min shaking at room temperature and centrifugation.

Flow cytometry and screening

20 μl of extracted Fabs were tested for binding to CHO-Mesothelin and to CHO-WT cells using a Flow cytometer BD Accuri™ C6 system. The binding of the soluble Fabs to CHO-Mesothelin and to CHO-WT cells (1E+05 cells) was detected with an anti c-myc antibody (Myc 9E10 Diaclone cat#855.313.020 and a goat anti-mouse IgG-FITC (Cappel MP cat#55526). Fab binding levels were determined by the percentage of positive cells signal and mean intensity levels in the FL1 channel detector. Each monoclonal Fab sample was also

tested on non-expressing cells, CHO-WT, as a control. The positive clones were then tested in the presence (0.8 µg/well) and absence of soluble recombinant human mesothelin (Raybiotech, cat#230-00043-50). Selected clones were also tested with the same method on HeLa cells to determine the natural form binders.

Human mesothelin fluorescein conjugated antibody (ref: FAB32652F) from Biotechne was used for positive control staining on mesothelin expressing cells. Anti CD59 antibody was purchased from Diaclone.

FLAER were purchased from Cedarlane labs and used according to the manufacturer's instructions.

Sequencing of antibody genes

Agar stabs were prepared from the panel of positive clones, by inoculating with 2 µl of stock culture flat-bottom 96-well plates containing LB Agar supplemented with Ampicillin at 100µg/ml and 2% Glucose, according to LGC Genomics recommendations. The variable heavy chain was sequenced with primer pelb3 and the variable light chain was sequenced with primer M13R. The amino acid sequences of the Fab variable domains obtained from LGC genomics were extracted using CLC Workbench or SIMPLE antibody extractor software. The Fabs were grouped in families based on the VH-CDR3 amino acid sequence homology.

Production and purification of chimeric monoclonal antibodies

1H7 was chimerized at RD Biotech (Besançon, France) with human IgG1 expressing mammalian vector and transfected to CHO cells. Cell culture supernatants were collected and antibody concentration was determined by RD Biotech.

ELISA

Binding of selected clones to soluble mesothelin was also assayed by ELISA. Wells of Maxisorp plates (Nunc, cat#456537) were coated with 100 ng of full length recombinant mesothelin protein in 100 μ l of PBS and incubated overnight at 4 °C. The wells were saturated with 200 μ l of 5% BSA solution for 2 hours. 100 μ l of PBS containing 20 μ l of extracted Fabs was applied to each well and incubated for 1 hour at room temperature. After washing several times with 1X washing buffer, bound Fabs were detected with biotinylated anti c-myc antibody and revealed with HRP coupled streptavidine for 30 minutes. After washing several times with 1X washing buffer 100 μ l of HRP substrate TMB was added to each well. Color development was stopped after 5 minutes with adding 100 μ l of 2N H₂SO₄ and the OD readings were taken at 450 nm.

To determine mesothelin quantity in samples an ELISA assay kit from Biolegend (cat: 438604) was used. ELISA was performed following manufacturer's instructions.

Exosome extraction

Exosomes were extracted from Hela cell culture. Cell culture was centrifuged at 300 xg for 10 mins to precipitate the cells, 2000 xg for 10 mins to pellet dead cells, 3000 xg for 30 mins to pellet cell debris and supernatant was collected. Then an ultracentrifugation was performed at 100000 xg for 70 mins to precipitate exosomes. Supplementary wash step with PBS 1X at 100000 xg for 70 mins was also performed to remove the contaminant proteins.

Exosomes were detected by confocal microscopy with CD63 staining and mesothelin concentration was determined by ELISA assay.

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Figures

Figure 1.

Generation of mouse anti-mesothelin FABs.

Figure 2.

Competition tests with soluble mesothelin protein.

Figure 3.

Competition tests with patients' sera and concentrated Hela supernatant.

Figure 4.

Test on other cancer cell lines.

Figure 5.

1h7-hFc does not bind to GPI.

Figure 6.

VH-CDR3 sequence analysis of competing and non-competing Fabs.

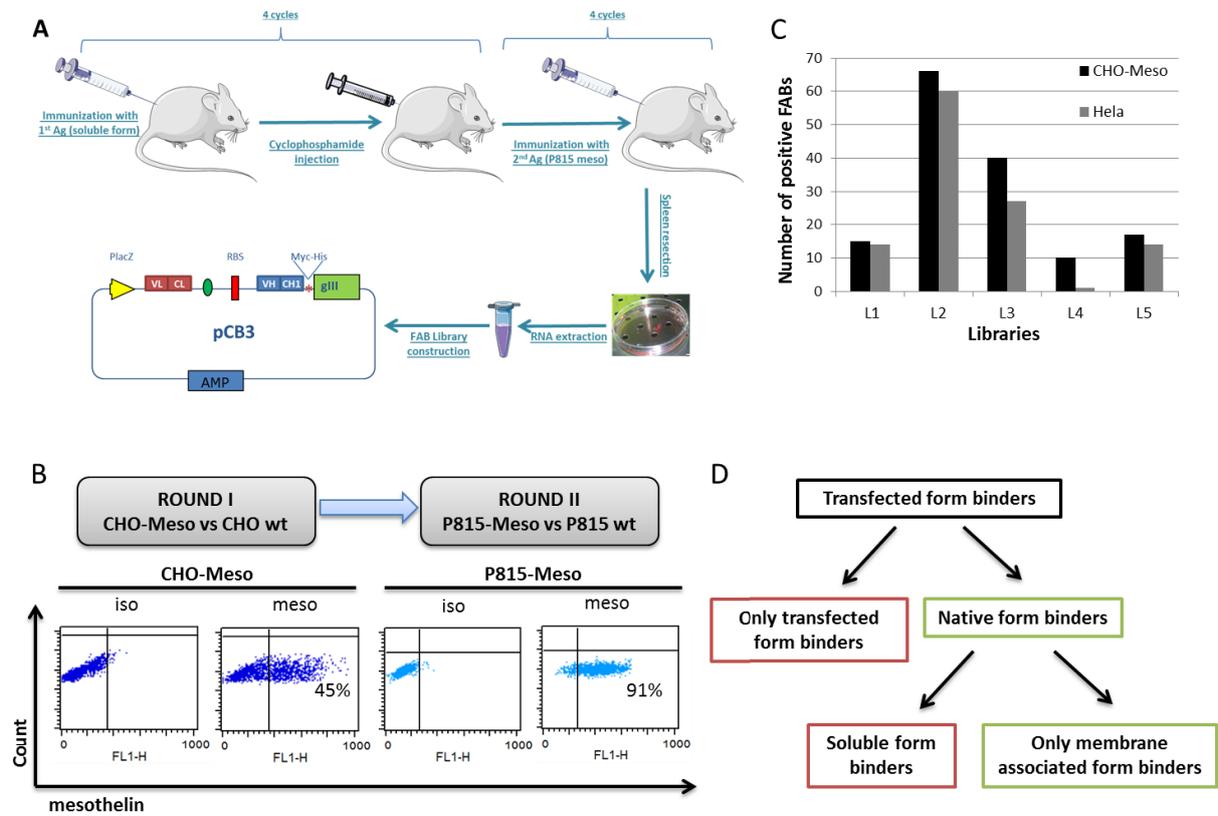


Figure 1. Generation of mouse anti-mesothelin Fabs

Figure 1. Generation of mouse anti-mesothelin Fabs.

5 mice were firstly tolerized with soluble form of mesothelin, then immunized with P815-Meso cells. After 4 cycles of immunization mice were sacrificed and spleens were resected. Total RNA was extracted from splenocytes and cDNA was produced. Vh-Ch1 and Vk-Ck sequences were amplified, cutted and inserted to pCB3 phage vector. (A). 2 rounds of panning were performed on libraries with CHO-Meso (45 %) vs CHO-WTcells (Round I) and P815-Meso (90 %) vs P815 wt cells (Round II) respectively (B). Number of positive FABs screened on CHO-Meso and Hela cells for each mouse phage display library (C). Study pathway to identify non-competing Fabs which bind only membrane associated form of mesothelin (D).

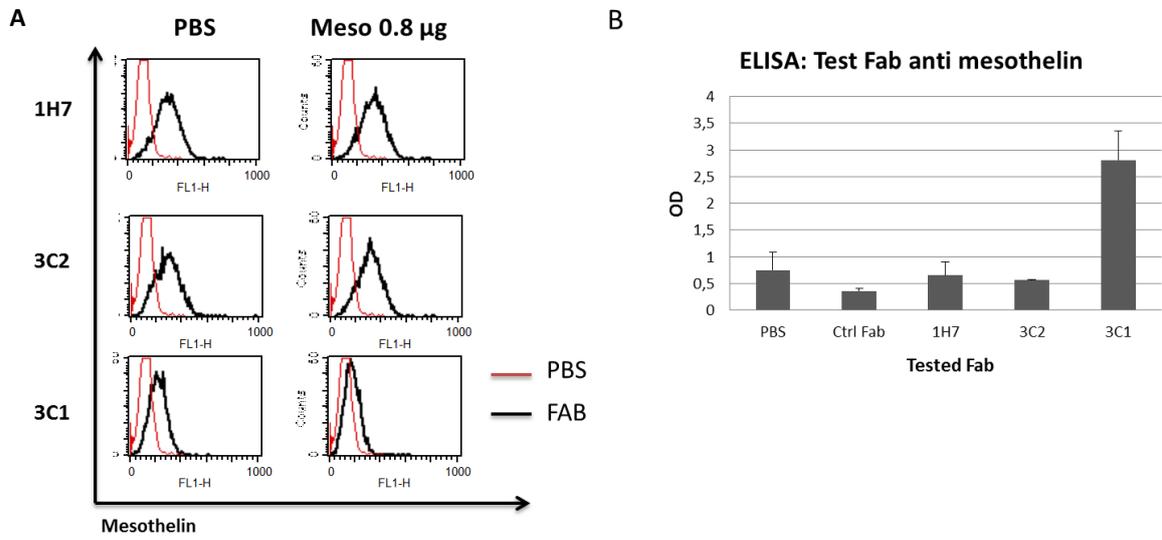


Figure 2. Competition tests with soluble recombinant mesothelin protein

Figure 2. Competition tests with soluble recombinant mesothelin protein.

Example of non-competing (3C2 and 1H7) and competing (3C1) Fabs with staining on HeLa cells with or without presence of 0.8 μg soluble mesothelin protein. (A). ELISA assay to detect the binding capacity of Fabs (3C1, 3C2 and 1H7) to soluble recombinant mesothelin protein (B).

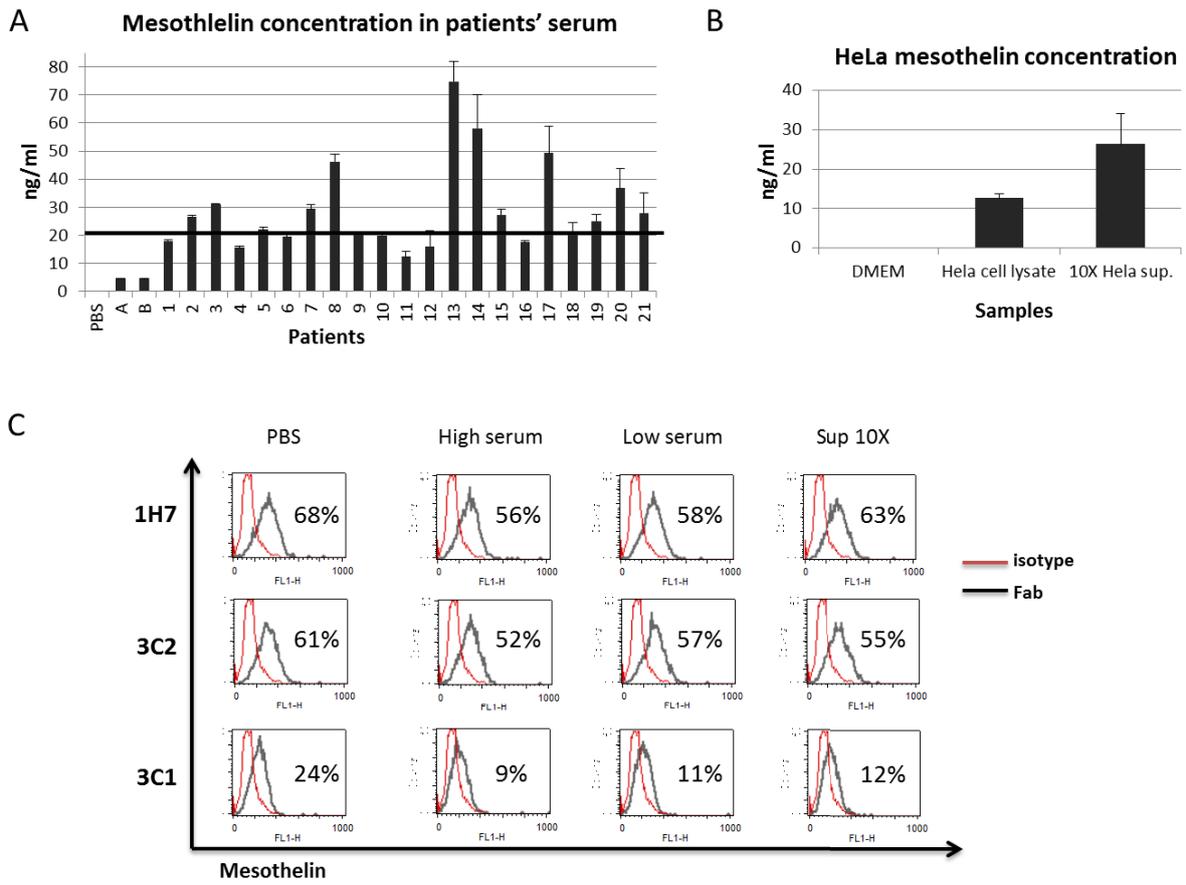


Figure 3. Competition tests with patients' sera and concentrated HeLa supernatant

Figure 3. Competition tests with patients' sera and concentrated Hela supernatant.

Mesothelin concentration in patients' serum determined by ELISA assay (1 to 21). 2 safe donors (A and B) and PBS was used as a negative control for mesothelin expression and ELISA specificity, respectively (A). Mesothelin concentration in 10X concentrated Hela supernatant with ELISA assay. Hela cell lysate was used as a mesothelin containing control. (B). Examples of staining on Hela cells with non-competing Fabs (1H7 and 3C2) and competing FAB (3C1) in presence of 10X concentrated Hela supernatant and patients' sera high and low (C).

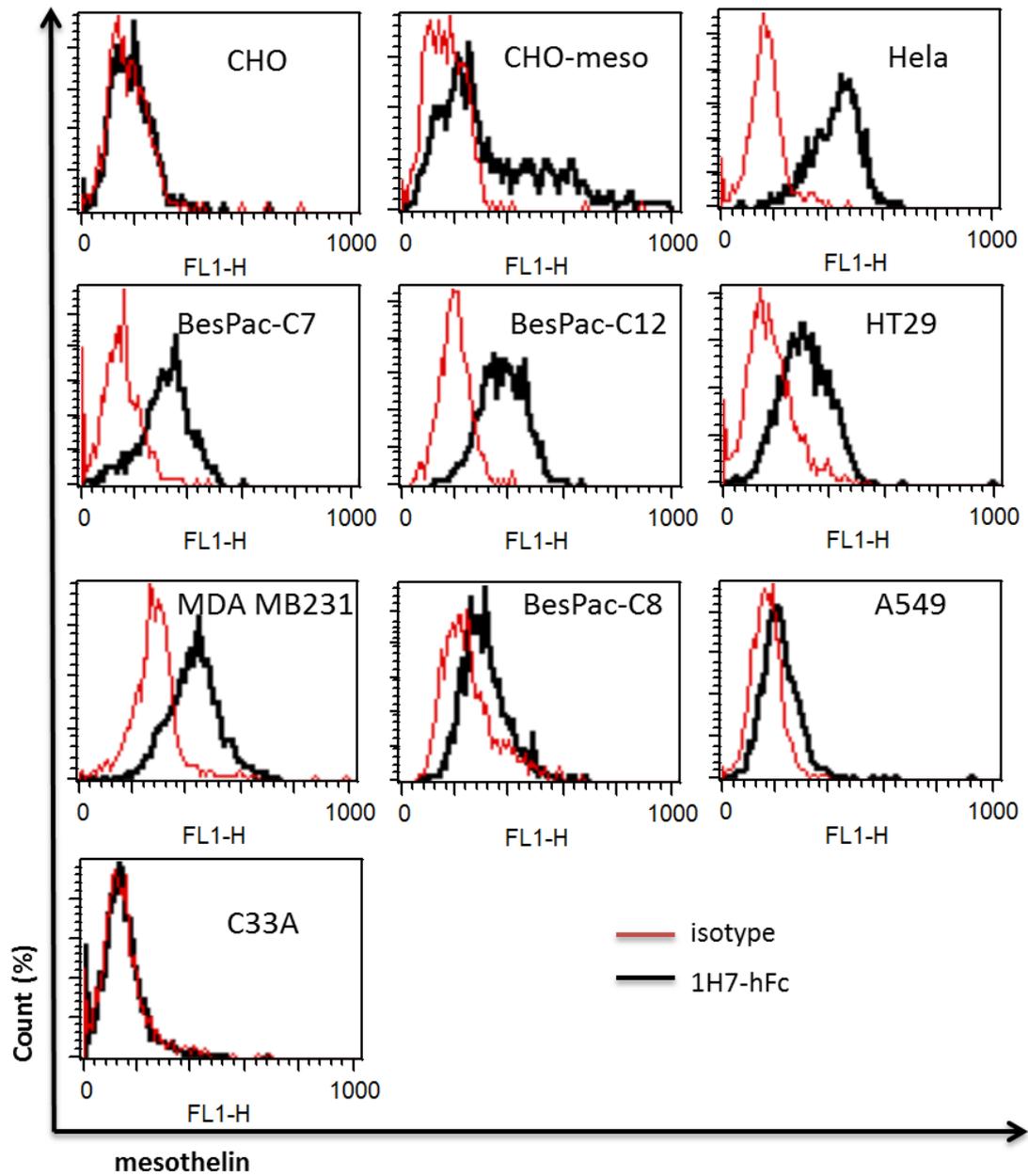


Figure 4. Test on other cancer cell lines

Figure 4. Test on other cancer cell lines.

FACS analysis of staining by 1H7-hFc on different cancer cells. CHO-Meso vs CHO-WT cells were used as a specificity control for 1H7-hFc.

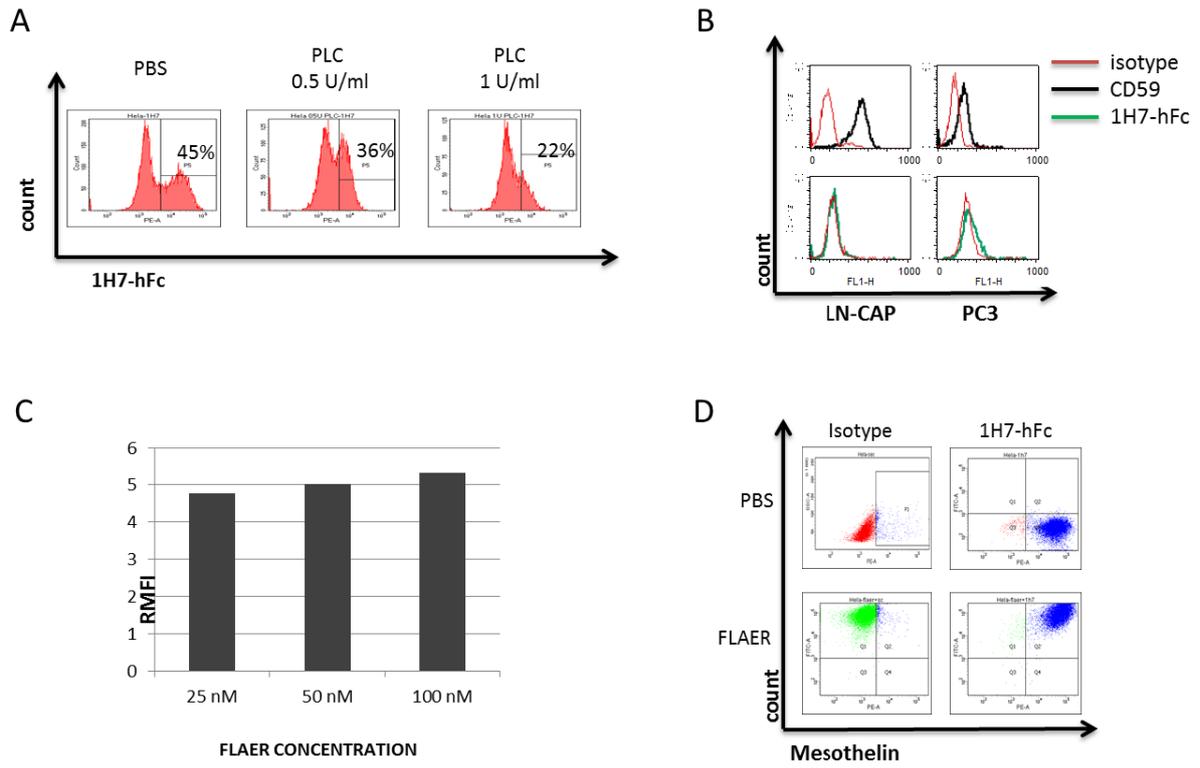


Figure 5. 1h7-hFc does not bind to GPI

Figure 5. 1h7-hFc does not bind to GPI.

Mesothelin staining by 1H7-hFc on Hela cells after treatment with 1 and 10 U/ml of PI-PLC for 1 hour (A). FACS analysis of PC3 and LN-Cap cells by CD59 and 1H7-hFc staining (B). RMFI on Hela cells by staining different concentrations (25, 50 and 100 nM) of FLAER (C). FACS analysis of staining on Hela cells by 1H7-hFc with or without the presence of 50 nm of FLAER (D).

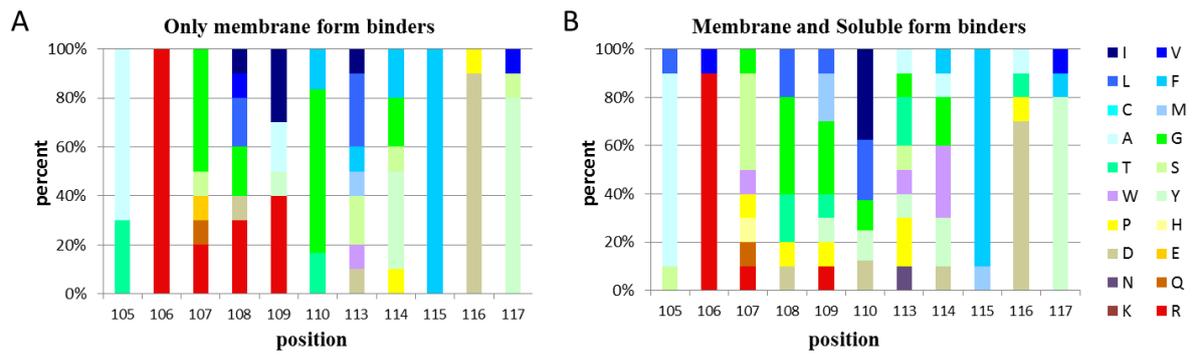


Figure 6. VH-CDR3 sequence analysis of competing and non-competing Fabs

Figure 6. VH-CDR3 sequence analysis of competing and non-competing Fabs.

The frequency of individual amino acids at the specific eleven positions of competing (n = 10) (A) and non-competing binders' (n = 10) (B) VH CDR3 sequences. The color menu for amino acids is according to IMGT . CDR3 positions are shown according to the IMGT unique numbering. Only membrane associated form binders (non-competing Fabs) in panel A, soluble form binders (competing Fabs) in panel B.

Supplementary data

Supplementary figure 1.

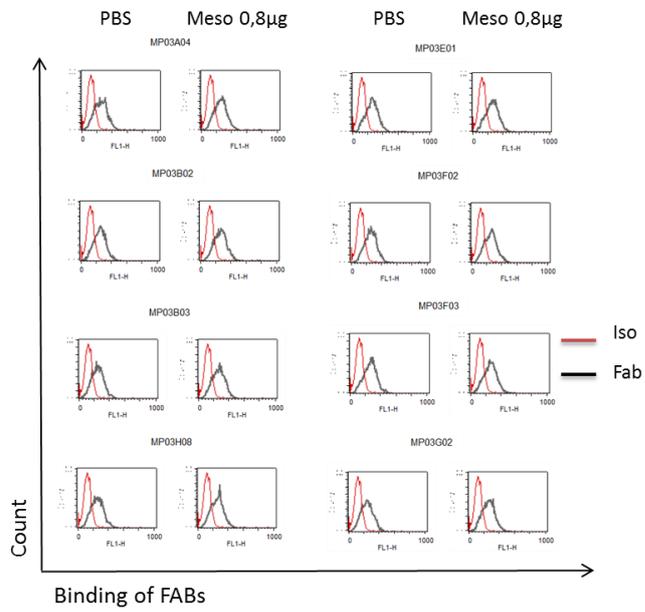
FACS with different clones of the same family on HeLa cells.

Supplementary figure 2.

Retained endpoint dilution and competition test with 0.8 μg of soluble mesothelin.

Supplementary figure 3.

Exosomes



Clone ID	Vh-CDR3 family	Staining on HeLa (%)	Staining on HeLa (%) competition with 0,8µg Meso
MP03A04	4	48.01	51.65
MP03B02	4	46.45	54.34
MP03B03	4	43.48	55.10
MP03H08	4	48.84	51.38
MP03E01	4	51.35	51.85
MP03F02	4	51.47	51.43
MP03F03	4	57.58	52.47
MP03G02	4	43.65	52.79

Supplementary Figure 1.

Supplementary figure 1.

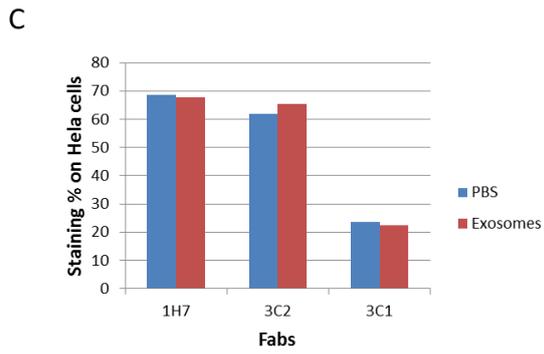
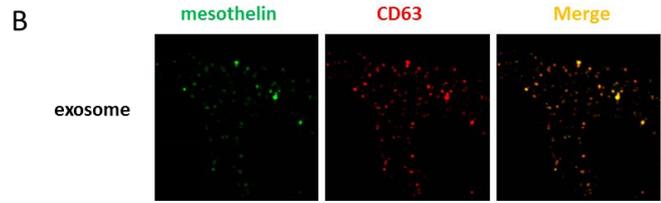
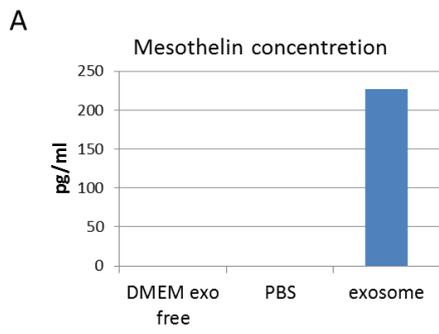
FACS analysis with different clones of the same family (family 4) with or without competition with 0.8 μg of soluble mesothelin on HeLa cells.

FAB Name	Family	Competition	Dilution retained	Staining on Hela (%)	Competition on Hela (%)
MP02B03 (1)	1	no	1/8	41,22	50,64
MP01F09 (2)	2	yes	1/2	37,37	3,36
MP03C11 (4)	4	no	1/4	37,23	33,58
MP01G04 (5)	5	no	1	24,63	20,47
MP01H07 (6)	6	no	1/4	68,64	69,7
MP03C02 (7)	7	no	1/8	61,8	67,15
MP03C01 (10)	10	yes	1/2	23,71	8,18
MP03G01 (11)	11	yes	1	23,93	3,26
MP02E11 (12)	12	no	1/4	30,63	42,95
MP03C04 (14)	14	yes	1	6,97	2,94
MP03G10 (21)	21	yes	1/8	24,64	1,42
MP05H10 (24)	24	no	1/64	42,14	43,48
MP03G08 (25)	25	no	1/16	47,41	48,98
MP03B05 (28)	28	yes	1/2	24,54	11,05
MP03A09 (29)	29	yes	1/2	29,79	8,16
MP05F09 (30)	30	yes	1/2	14,65	5,28
MP05D08 (31)	31	yes	1/2	26,75	0,78
MP03D04 (32)	32	Yes	1	8,57	1,51
MP04D10 (33)	33	no	1	2,31	12,84
MP05D01 (34)	34	no	1/2	13,4	13,78

Supplementary figure 2.

Supplementary figure 2.

Retained endpoint dilution and competition test with 0.8 μg of soluble mesothelin for all natural form binding Fab families.



Supplementary figure 3.

Supplementary figure 3. Exosomes

Mesothelin concentration of exosomes measured by ELISA (A). Mesothelin expression was localized on exosomes, also in high and low serum of patients' by staining on confocal microscopy. CD63 staining is used to localize exosomes (B) Competition test performed on Hela cells on presence of exosomes for Fabs..

II. Preliminary results obtained with CAR-mesothelin

CAR vector construction

To target mesothelin by the chimeric antigen receptor (CAR) expressing T cells a previously described discriminating antibody 3C02 was selected for its high binding capacity. This antibody has the capacity to recognize the conformational epitope presented in the cell surface attached mesothelin without interfering with any other soluble forms of mesothelin (figure 1A). The sequences of VH and VK chains of 3C02 was determined (figure 1B) and cloned to retroviral vector to generate a CD28-4-1BB-CD3zeta signaling 3rd generation CAR (figure 1C). This vector was also engineered to express a reporter gene dCD19. In the next step we prepared a packaging cell line (PG13) for stable expression of CAR-meso.

Preparation of CAR expressing PG13 cells (packaging cell line)

Retroviral CAR vector was first transfected to GP87 cells and then PG13 cells were infected with the cell free supernatant of these cells (figure 2A). dCD19 expression of infected cells was measured by flow cytometry (approximately 29 % of CD19 expression) and then incubated by anti-CD19 beads and were sorted positively by using magnetic cell sorting (MACS) (figure 2B). To confirm the CAR expression sorted cells were collected after 48 hours of incubation at 37 C and western blotting assay was performed to detect the CD3zeta expression (figure 2C). After the confirmation of CAR expression in high dCD19 expressing PG13 cells, these cells were cultivated for 48 hours and their supernatant was collected to make a CAR expressing virus stock. This stock was then used to infect human T lymphocytes to produce the CAR-meso T cells.

Characterization of CAR expression in T cells

At the next step PBMC extracted from healthy donors were activated by CD3/CD28 beads and cultivated in presence of 500U of IL2. At day 2, activated cells were infected with the cell free virus stocks obtained from PG13-CARmeso cells (figure 3A.). The CAR transduction efficiency was verified according to dCD19 expression 2 days after the infection. Activated both CD8⁺ and CD4⁺ T cells expressed only 5 % of dCD19 (figure 3B). To increase the amount of CAR expressing cells, transduced cells were sorted positively according to dCD19 expression at day 7 to activation. After the cell sorting dCD19 expression reached to nearly 43.8 % (figure 3C). Then the cells cultured in presence of 500U of IL2 till to obtain enough cells to evaluate their functionality. dCD19 expression was stable during the cell expansion.

CAR T cells could be activated by mesothelin expressing cells.

Next set of experiments were designed to investigate if CAR T cells could bind to mesothelin expressing cells and activated subsequently. Mesothelin expressing Hela and MKN45 cells were cultivated during 5 hours with the ratio of 1 target for 3 effector cells. Then the externalization of CD107a receptor which is an activation marker for CD8⁺ T cells was measured by flow cytometry. CARmeso T cells incubated with Hela or MKN45 showed respectively, 32.5 and 37.7 % staining for CD107a (figure 4). These results showed that the CARmeso transduced CD8⁺ T cells can bind to mesothelin expressing cells and trigger their activation. Then we sought to determine if CARmeso expressing cells were functional and can produce cytotoxicity versus mesothelin expressing cells.

CAR T cells can eliminate mesothelin expressing cells in 'in vitro' culture

To evaluate the cytotoxicity of CARmeso T cells, mesothelin expressing Hela, MKN45 and mesothelin negative CHO cells were co-incubated with effector cells during 3 days at initial ratio of 1:1. After 3 days a MTT assay was performed. The results of MTT test showed that the proliferation of mesothelin expressing cells was significantly diminished in presence of CARmeso T cells comparing to non-transduced T cells (figure 5A). In contrast mesothelin negative CHO cells showed similar proliferation in all conditions. These results suggested that CARmeso T cells could target specifically the mesothelin expressing cells and can induce the cell death.

Then to determine the precocious cytotoxicity of CARmeso transduced T cells, we incubated Hela, MKN45 and mesothelin negative P815 cells in the same conditions explained above for MTT test. After 24 hours of co-incubation targeted cells stained with Annexin-V and 7-AAD for determine the apoptosis. Living cell percentage (Annexin-V/7-AAD negative) was then calculated (figure 5B).

MATERIAL AND METHODS

Cell culture

MKN45, and HeLa cells cultured in Dulbecco's Modified Eagle Medium (DMEM-Glutamax) (GIBCO), and P815 in RPMI 1640 supplemented with 10% of heat inactivated fetal bovine serum ((FBS-HI)-Gibco), and 1% of Penicillin-Streptomycin (10,000 U/mL) (GIBCO).

Plasmid preparation

A retroviral vector was designed to express mesothelin scFv linked to CD28 (intramembranous region), 4-1BB (intracellular region) and CD3zeta (intracellular region). A truncated CD19 protein was linked also to chimeric antigen receptor (CAR) sequence with 2A sequence. Constructed plasmid has been synthesized by Eurofins Scientific.

Packaging cell line preparation

GP87 and PG13 cell lines were purchased from ATCC (American Type Culture Collection) and cultured in DMEM-Glutamax (GIBCO), supplemented with 10% of heat inactivated fetal bovine serum ((FBS-HI)-Gibco), and 1% of Penicillin-Streptomycin (10,000 U/mL) (GIBCO). PG13 cells expressing the CAR was generated by transiently transfecting GP87 cells with the plasmid encoding the retroviral vector CAR-dCD19 and subsequently infecting PG13 cells 3 days consecutively with cell-free virus stocks from the transfected GP87 cells. Infected PG13 cells (PG13-CARmeso) then sorted positively for dCD19 expression.

PBMC culture and transduction

PBMC was isolated from healthy donors' blood and activated with the beads anti-CD3/CD28 with the ratio of 1 bead for 2 cells after magnetic sorting obtained randomly from blood bank of Bourgogne-Franche-Comte. Activated lymphocytes were infected at day 2 by cell-free supernatant of PG13-CAR_{meso} cells. At day 5 the dCD19 expression was measured. At day 9 positive magnetic cell sorting (MACS) was performed for dCD19 expression according to manufacturer's instructions.

Flow cytometry

For membrane staining, cells were incubated with corresponding antibody combinations for 30 min at 4°C then washed with PBS (phosphate buffered saline). Ten thousands cells from each sample were evaluated for fluorescence detection using BD FACS Canto cytometer (Becton Dickinson, Le Pont de Claix, France) and analysed in FACS Diva software. Anti CD3, CD8, CD4, CD19 7-AAD, and anti-annexin-V-FITC (eBioscience) antibodies were used for cell staining.

CAR T CD8⁺ cell degranulation assay

CAR T CD8⁺ cells were co-cultured in the presence of target cells for 5 h at a 1:1 E:T ratio with GolgiStop (BD Biosciences) according to the manufacturer's protocol. Degranulation of CAR T CD8⁺ cells was monitored by flow cytometric analysis of CD107a (H4A3, BD Biosciences) expression.

MTT assay

Cell proliferation in vitro was analyzed with the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT). 10000 mesothelin expressing cells were incubated with 1:1 ratio of effector CAR cells in triplicates for 3 days in 96-well micro-plates containing 200 μ L of medium per well. Incubation of target cells with non-infected T cells was used as a negative control. For analysis, 10 μ L of MTT substrate (of a 5 mg/mL stock solution in phosphate-buffered saline) was added to each well, and the plates were let to standard tissue incubator conditions for an additional 2 hours. Cells were solubilized in 200 μ L of dimethyl sulfoxide, and colorimetric analyses were performed (wavelength, 570 nm).

Western blotting

Cellular proteins were extracted in laemmli 1X buffer by adding beta mercapto-ethanol and heated to 95 °C. They were separated by electrophoresis (SDS-PAGE) in a polyacrylamide gel in presence of sodium dodecylsulfate (SDS) according to their molecular weight, and then transferred to a polyvinylidene difluoride film (PVDF) for staining by specific antibody (CD3zeta and actin) and then detection by secondary anti-human antibody coupled to peroxidase was realized.

Figures

Figure 1.

Retroviral CAR vector construction .

Figure 2.

Packaging cell line preparation and validation.

Figure 3.

PBMC preparation and transduction

Figure 4.

Activation of CAR expressing T-cells by mesothelin
expressing cells

Figure 5.

'In vitro' validation of functionality of CAR T cells.

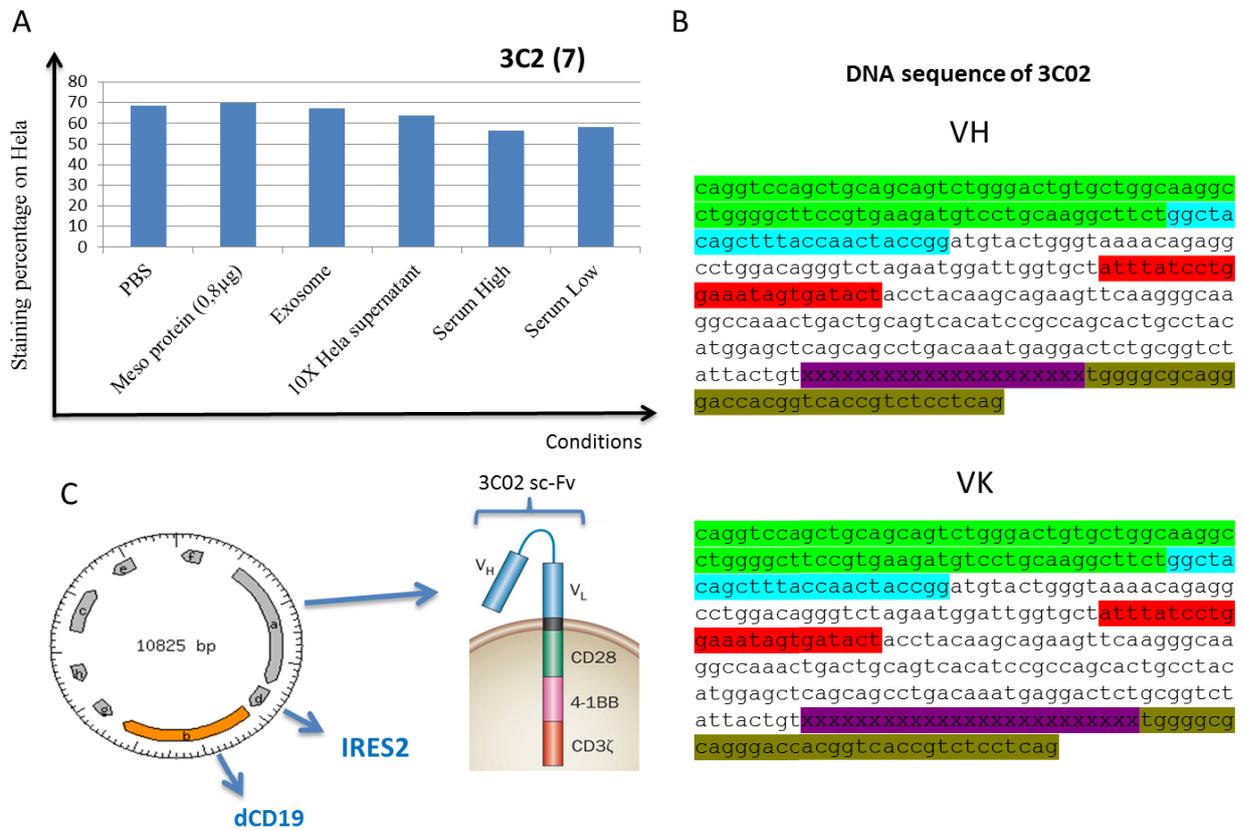


Figure 1. Retroviral CAR vector construction

Figure 1. Retroviral CAR vector construction .

A) Staining of 3C02 on Hela cells in presence of different mesothelin containing conditions. B) VH and VL (VK) sequence of 3C02 a membrane form discriminating anti-mesothelin antibody. **FR1; CDR1; CDR2; CDR3; FR4** sequences according to IMGT nomenclature. C) Construction of CAR-meso expressing retroviral vector.

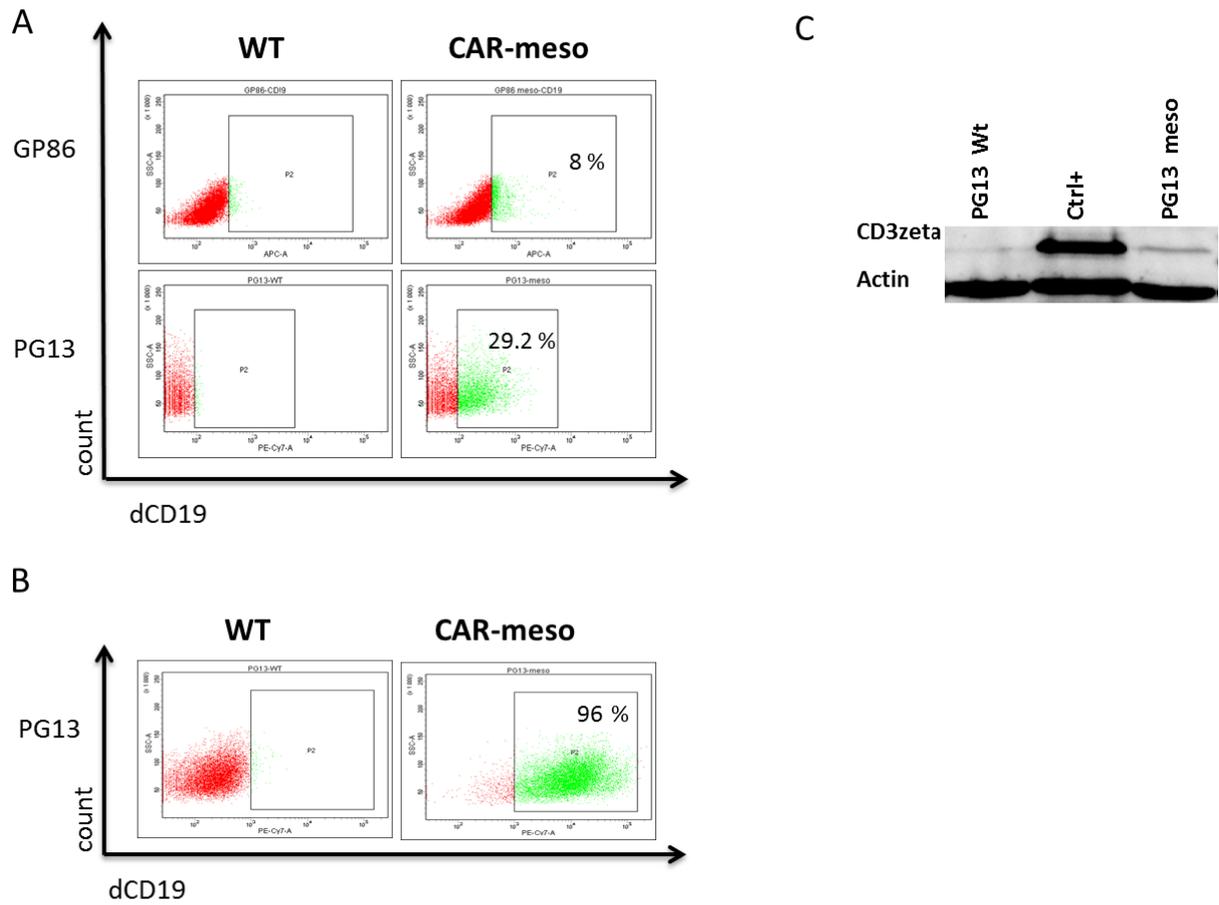


Figure 2. Packaging cell line preparation and validation

Figure 2. Packaging cell line preparation and validation.

A) Expression of dCD19 on GP86 cells after CAR transfection, and expression of dCD19 on PG13 cells after the transduction with cell free supernatant of transfected GP86 cells. B) dCD19 expression on CAR-meso transduced PG13 cells after magnetic activated cell sorting based on dCD19 expression. C) Expression of CD3 zeta in transduced and sorted PG13 cells in western blotting. Actin was used as a control domestic gene..

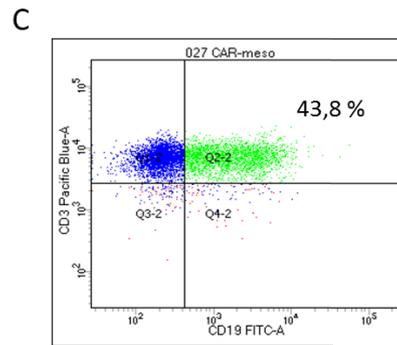
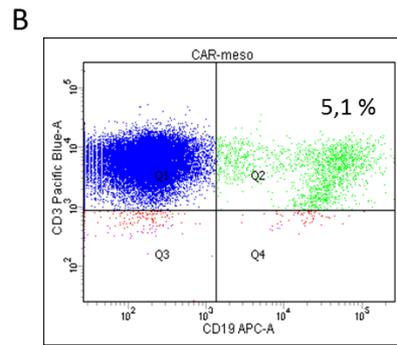
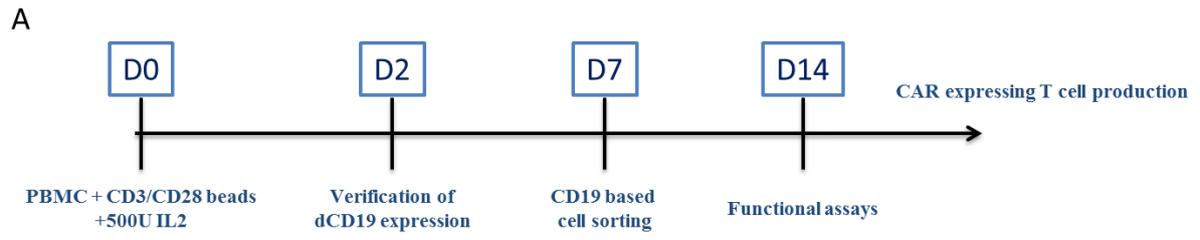


Figure 3. PBMC preparation and transduction

Figure 3. PBMC preparation and transduction.

A) Schematic representation of CAR meso expressing T cell production. B) dCD19 expression on T cells after 2 days of transduction with cell free supernatant of PG13 CARmeso cells. C) dCD19 expression after cell sorting with CD19 beads.

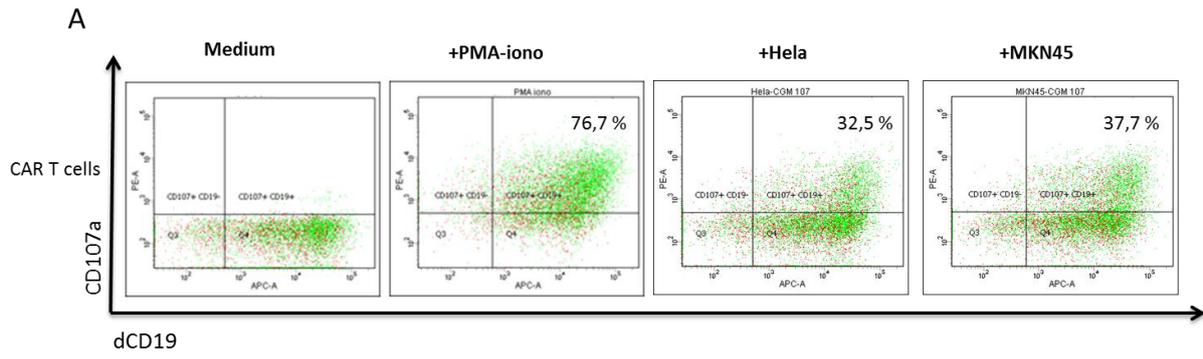


Figure 4. Activation of CAR expressing T-cells by mesothelin expressing cells.

Figure 4. Activation of CAR expressing T-cells by mesothelin expressing cells.

T CD8⁺ cell degranulation was determined by CD107a staining after 5 hours of co-incubation of Hela and MKN45 cells with CAR_{meso} expressing T cells. PMA-iono was used as a degranulation inducing agent for positive control.

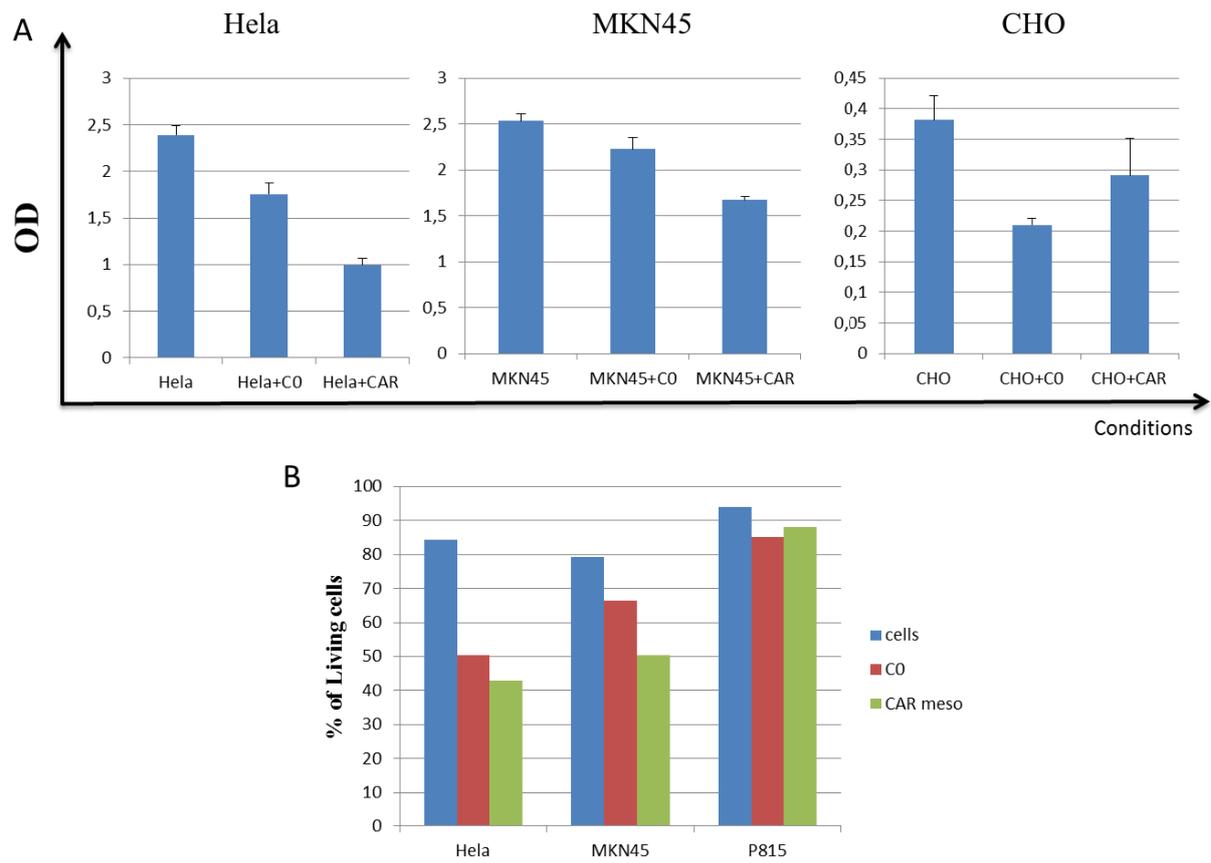


Figure 5. 'In vitro' validation of functionality of CAR T cells

Figure 5. 'In vitro' validation of functionality of CAR T cells.

A) Results of MTT test after 3 days of co-incubation of Hela, MKN45 and CHO cells with CARmeso T cells. All test realized in triplicates. B) Detection of living cell percentage (Annexin V/7AAD negative cells) of mesothelin expressing Hela, MKN45 and mesothelin negative P815 cells after 24 hours of incubation with CARmeso T cells. CAR: CARmeso T cells; T0: non-transduced T cells.

DISCUSSION AND PERSPECTIVES

In 2016, there are more than 30 approved monoclonal antibodies in clinical trials for cancer treatments.

The lack of efficacy in the treatment of solid tumors may be also the result of a number of factors:

a) Solid tumors are dense and poorly vascularized internally. The antibody molecule is large and may simply take too long to penetrate the tumor mass.

b) Tumors are heterogeneous and the antigen target may not be present on the surface of all cells within the tumor mass.

c) Tumor cells shed antigen from the cell surface, the soluble circulating antigen binds and neutralize the therapeutic antibody.

Factors a) and b) have been addressed by attaching to the therapeutic antibody a killing agent that acts in a localized but non-discriminatory way which is referred to as antibody conjugation. These agents include radio-isotopes of elements such as Yttrium, Iodine, Indium or Cobalt. A range of chemo-toxic agents have also been targeted in this way, including conventional cytotoxic drugs, toxins such as ricin and calicheamicin and pro-drugs of several forms. The localization to the tumor of a non-selective killing agent addresses both the issues of penetration and the heterogeneity of the tumor. Problems encountered with drug or immunotoxin linked antibodies are the immunogenicity generated in host by the conjugated molecules.

Tumor antigens (ACE, CA125), as well as CD125, CD130, TRAIL receptors, CD25, CD69 and many other antigens have been reported to shed soluble forms (330–335). When

antibodies are administered to target these antigens, it is believed that the shed antigen levels mentioned in factor c) can be high enough to present a significant problem and to interfere with the action of the antibody. Our work highlighted how to overcome this issue by developing antibodies discriminating the membrane form of mesothelin.

However, mesothelin expression was largely studied in ovarian cancer, pancreas cancer and mesothelioma. The development of a therapeutic monoclonal antibody also requires to determine the stability of its antigen expression during several biological processes such as epithelial-mesenchymal transition (EMT) and acquired resistance to chemotherapy (oxaliplatin). This issue was addressed in our laboratory since EMT and resistance to chemotherapy might be acquired by cancer cells during cancer progression and modulate mesothelin expression. We have also detected the presence of the soluble mesothelin in highly metastatic colon and pancreas cancer patients.

Mesothelin expression during epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a highly conserved cellular process that allows epithelial cells to convert to mesenchymal cells (336). Tumor cells, which undergo EMT, acquire capacities of proliferation, migration and resistance to apoptosis. By co-opting a program involved in embryonic development, carcinoma cells are able to invade normal tissues and to induce metastasis. EMT also confers to carcinoma cells, stem cell-like properties indispensable to clonal expansion and initiation of metastasis. Altogether, EMT is one of the major molecular mechanisms carried out during oncogenesis to enable cancer progression (337). However, there is no published data to date which shows the role of mesothelin in EMT, we seek to investigate the change of mesothelin expression during EMT. To investigate this issue, we selected a model of reversible EMT based on TNF- α and TGF-

β 1 treatment of the cell line A549. Decrease of epithelial cell marker, EpCAM expression confirmed the EMT induction. An immunophenotyping of A549 was performed using our chimeric antibody 1H7-hFc. In these experiments, we demonstrated that mesothelin expression was decreased by the induction of EMT, but remains detectable (figure 20).

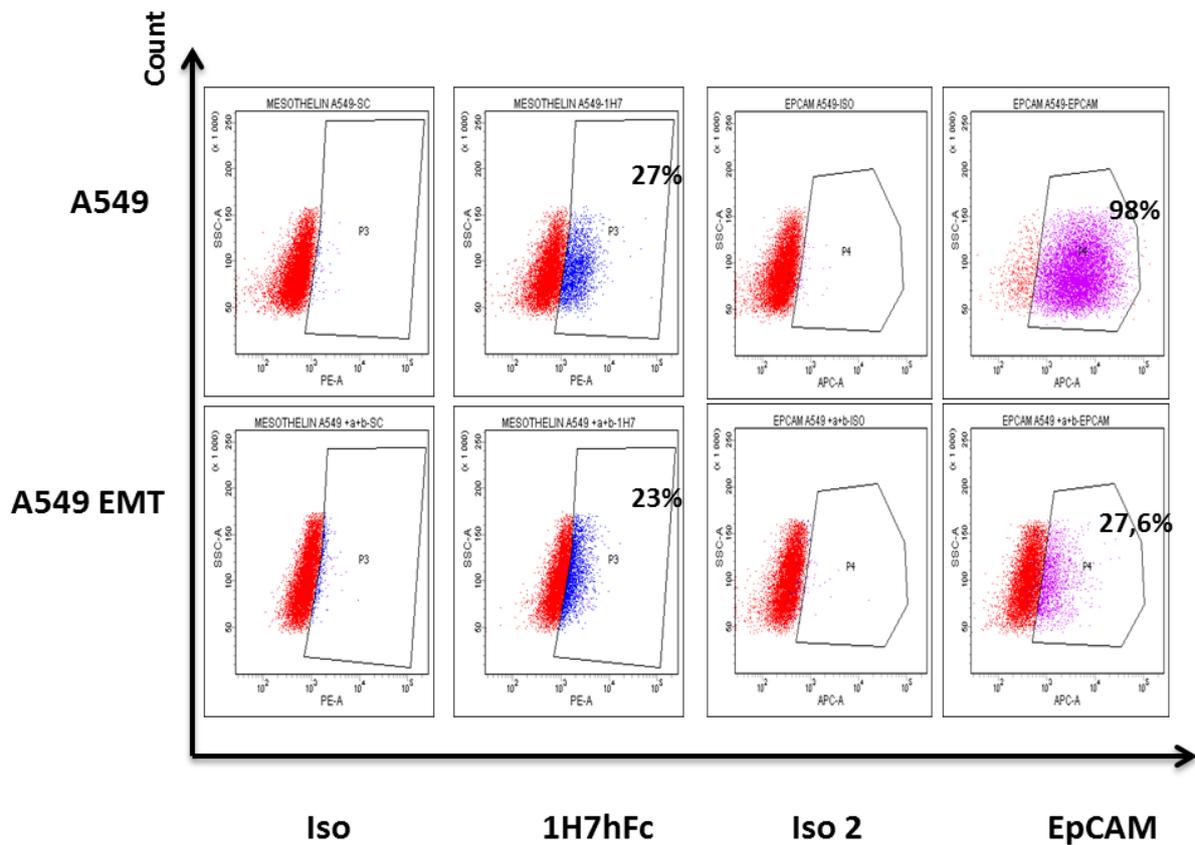


Figure 20. Mesothelin staining on A549 and A549 treated with TNF α +TGF β .

A549 EMT: A549 cells treated with 5ng/ml of TGF β and 20 ng/ml of TNF α within 5 days to induce EMT. 1H7-hFc was used to detect the mesothelin staining. EpCAM staining was performed to confirm the EMT induction.

Mesothelin expression on oxaliplatin resistant colon cancer cells

We have also demonstrated that mesothelin expression remains constant or increased in colon cancer cell lines after exposition to oxaliplatin. Colon cancer cell lines HT29, Colo-205 and Colo-320 were exposed to increasing doses of oxaliplatin and rendered resistant (Figure 21).

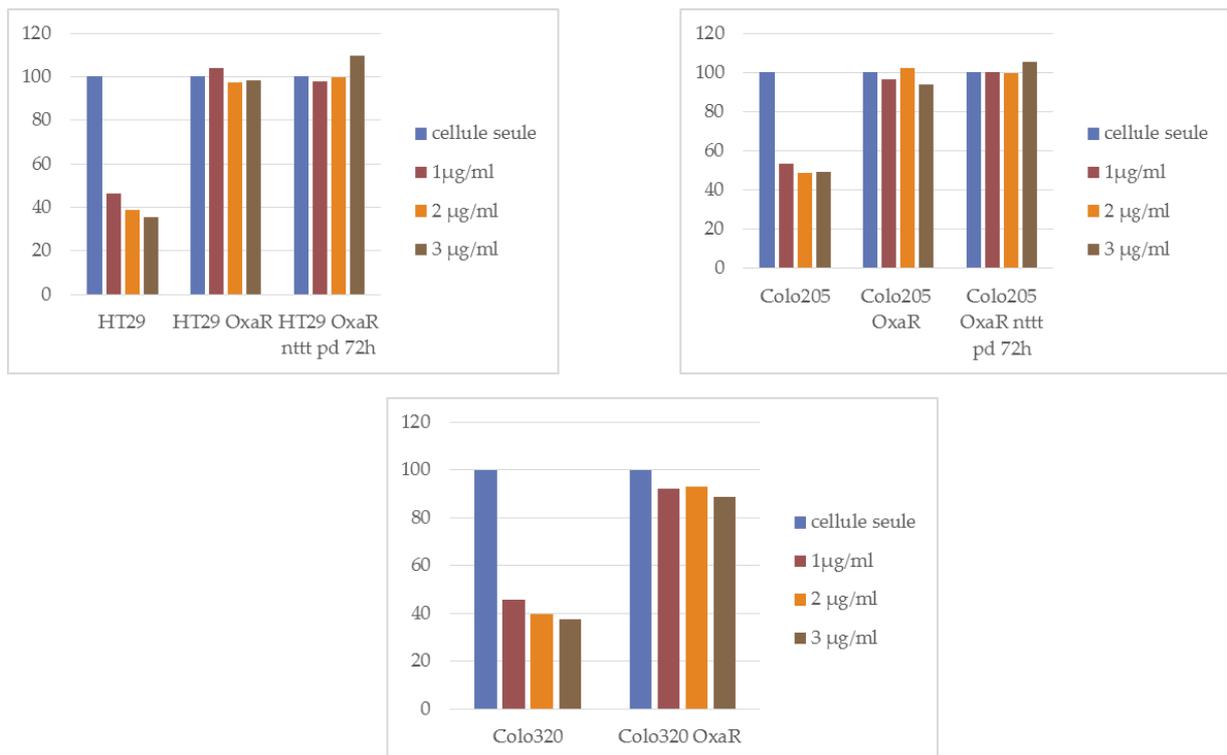


Figure 21. MTT assay after 3 days of exposition to different doses of oxaliplatin. OxaR: Oxaliplatin resistant cell lines; OxaR nttt pd 72h: resistant cells which were not treated with oxaliplatin during 72 hours before the test.

Then the expression of mesothelin was assessed by flow cytometry using our chimeric anti-mesothelin antibody 1H7 (Figure 22). Mesothelin expression in all tested cell lines did not change during the acquired resistance to oxaliplatin which suggested that the mesothelin could be a good candidate to target for the patients who develop the chemo-resistance.

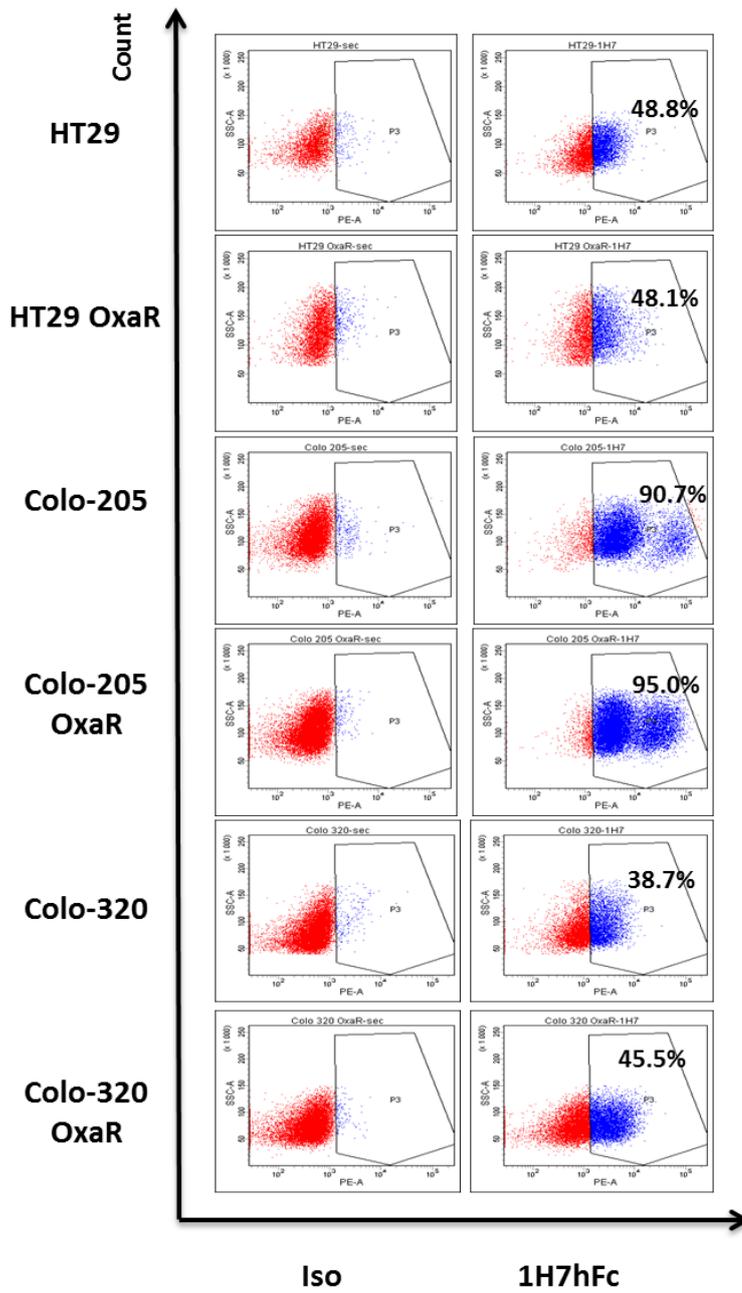


Figure 22. Mesothelin expression on colon cancer cells.

Mesothelin expression was detected by 1H7-hFc on colon cancer cell lines and their oxali-resistant variants.

Soluble mesothelin in the serum of colon and pancreas cancer patients

As it was already described in **Results section** in our laboratory we obtained the mesothelin expressing primary cell lines from the ascites of pancreatic cancer patients. We have also measured the soluble mesothelin concentration in some highly metastatic colon and pancreas cancer patients' blood serum and compared them with the results obtained from healthy donors (figure 23). We observed a significant increase of mesothelin concentration in some patients compared to healthy donors.

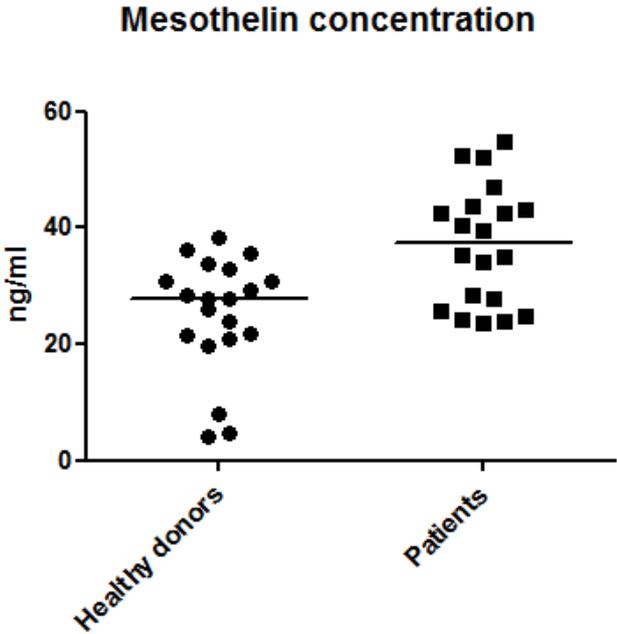


Figure 23. Mesothelin concentration in the serum of healthy donors and highly metastatic colon and pancreas cancer patients.

These preliminary data must be confirmed in a larger cohort with separating the colon and pancreas cancers. Mesothelin expression on colon cancer patients has not yet been clearly described (264,338).

Antibody based immunotherapies showed a great success in cancer treatment. Although hematological malignancies could already be successfully treated by immune based therapy, in solid tumors it needs further development of immunologic drugs. There are more than 50 antigens targeted by monoclonal antibodies or CAR T cells. Among them because of its selective expression on high range of cancer cells, mesothelin is a promising candidate for immune based therapy. In clinical development there is already some drug or immunotoxin conjugated or nude therapeutic antibodies or several CAR T cells which target mesothelin and show encouraging results in several phase I or II clinical trials (324,326).

Mesothelin is a membrane expressing protein, and its soluble, shedded, form also present in tumor micro-environment and in blood serum which creates obstacles for antibody based targeting of tumor cells. A great part of mesothelin targeting antibodies contains the SS1 single chain which was already described as a non-discriminating mAb recognizing both the membrane and the soluble form of mesothelin (259,339). This binding of SS1 to soluble mesothelin has been also demonstrated to correlate with decreased efficacy against cancer cells (299). The concentration of soluble mesothelin is increased differentially closest to cancer site. When the cancers are treated with non-discriminating antibodies, they are neutralized by soluble form before arriving to cancer cells.

For the reasons described above we sought to develop a membrane form discriminating antibody by using a novel method of immunization which became an object for patent application. By coupling to phage display this method of immunization allowed us to identify several numbers of antibodies with different VH-CDR3 sequences which selectively target membrane form of mesothelin. In our submitted article we showed with the example of one of these antibodies (1H7) that they do not recognize any other soluble form of

mesothelin present in physiological liquids and bind specifically mesothelin expressing cells. We have also demonstrated that this antibody do not target the stub region of mesothelin which remains on the cell surface after physiological cleavage.

Although the presence of mesothelin in tumor micro-environment can be an advantage for CAR T cell expansion in TME, but its presence in blood serum could be a barrier for mesothelin targeting CAR T cell therapies. CAR T cells activation by soluble antigens is not desirable because of cytokine-release syndrome (340).

Once CAR T cells are activated by binding its target, signaling pathway triggered by CD3zeta leads to cytokine expression. In many cases high concentration of released cytokines promote a toxicity called cytokine release syndrome characterized by nausea, fever, hypotension, vascular leakage and life-threatening multiple organ failure. This syndrome is highly associated with systemic macrophage activation. Secreted IFN γ and TNF by T cells activate macrophages which in turn release IL6. (340–343). In two clinical trials death of two patients have been reported because of the cytokine-release syndrome (344,345). *Shannon et al.* reported in 2014 that all the patients treated with CD19 expressing CAR T cells had have the cytokine-release syndrome. Severe cytokine-release syndrome, which developed in 27% of the patients, was associated with a higher disease burden before infusion and was effectively treated with the anti–interleukin-6 receptor antibody tocilizumab (214).

Second obstacle in front of the CAR T cell therapy is the absence of real tumor selective antigen. Nearly all of the targeted antigens by CAR T cells are expressed in healthy tissues at low levels. Although the low expression of the antigen in healthy cells is not a real problem for antibody therapy, it becomes plausible that low concentrations of antigens might induce severe side effects in the context of CAR therapy. A case report by *Richard A.M. et al.*

reported the death of a patient after respiratory distress, and accumulation of a dramatic pulmonary infiltrate following the administration of T cells transduced with a chimeric antigen receptor recognizing HER2 (346).

Therefore, securisation of CAR expression became constrained. T cells were co-transfected with CAR and inducible suicide genes (herpes simplex virus thymidine kinase, HSV-tk; inducible caspase 9, iCasp9). These suicide genes can be expressed after treatment with chemical compound (ganciclovir, AP1903) to induce lymphocyte apoptosis and limit the occurrence of severe toxicity.

Another barrier for CAR T cell treatment in solid tumors is tumor burden. Intravenously injected CAR T cells could not infiltrate successfully into tumor micro-environment. Even if they succeed to infiltrate, the presence of inhibitory molecules expressed by tumor cells impair the CAR activation. To avoid these limiting factors, injection of CAR T cells into the tumor or the co-administration of antibodies targeting lymphocyte homing inhibitory molecules have been tested in some trials. Koneru et al. reported a phase I study assessing the interest of MUC16 antigen specific CAR T cells genetically engineered to produce IL-12 after antigen recognition. This CAR T cells reprogrammed with a promoter including NFAT-response elements were tested in ovarian cancer. This was the first time where CAR T cells were injected intraperitoneally directly into the site of the tumor within the abdomen in humans. Furthermore, the ability of genetically modified cells to secrete IL-12 will potentially enhance CAR T cell persistence and modulate the tumor microenvironment (218).

We then decided to construct a mesothelin targeting CAR to show the beneficial effects of a membrane-form discriminating antibody in functional applications. We used a

gamma retroviral vector to construct a third generation CAR vector. We selected another membrane form discriminating clone 3C02. The CAR vector was successfully constructed and transduced to a packaging cell line (CD19). In this vector we used a reporter gene dCD19 to monitor the infection percentage of viral particles. Although we selected high dCD19 expressing PG13 cells for viral particle production, its supernatant could not transduce successfully T cells. Only 5 % of transduction efficacy was obtained. The monitoring of CD3 zeta chain in 95% dCD19 PG13 CAR-meso cells by western blotting has also showed the low expression which suggested that the CAR expression does not correlate with reporter gene expression. Despite the low expression of CD3 zeta we could preliminarily show that the CARmeso transduced T cells could bind, be activated and eliminate mesothelin expressing cancer cells.

For the next step the cytokine expression of activated CARmeso T cells will be determined. Then our experiments will pursuit with measuring the 'in vivo' efficacy in murine model.

To ameliorate and validate our results obtained with CAR-meso, we will clone the CAR transduced packaging cell line and screen the individual clones for CD3 zeta expression in western blotting. Transduction efficacy of the supernatant of each clone will also be tested. Then the clones which express high levels of CAR (CD3zeta) and have the best transduction efficacy will be selected for retroviral particle production.

We will also generate a lentiviral vector containing 3C02-CAR T cells. Lentiviral particles have the ability to transduce also non-proliferating cells with high efficacy. Comparing to retroviral particles, lentiviral particles could infect the cells with more copy numbers.

Our membrane form discriminating CAR will also be developed in association with gene therapy strategy to enhance IL-12 production(347–350). For that purpose, we will use an inducible promoter containing NFAT-response element to control IL-12 transcription. Moreover, our preclinical experiments will include the assessment of combination of CAR T cells adoptive transfer checkpoint inhibitor molecules such as anti-PD1 or anti-CTLA-4 neutralizing antibodies.

Another recent biotechnological approach to develop monoclonal antibodies is to produce nanobodies. Because of its similarity with human antibodies and the small size of its variable domain, cameloid antibodies (nanobodies) which consist only from heavy chain comprising variable domain (VHH) and constant fragment (CH2 and CH3) are also extensively produced by phage display and converted to monoclonal antibodies with high specificity and less immunogenicity in human (351). In 1993 *Hamers-Casterman et al.* investigated that the camel heavy-chain IgGs lack CH1, which in one IgG class might be structurally replaced by an extended hinge. These antibodies are composed of heavy-chain dimers and lack the light chains, but nevertheless have an extensive antigen-binding repertoire. Their single N-terminal domain (VHH, also referred to as Nanobody) binds antigen without requiring domain pairing (352,353). The structures of these antibodies have described as without having major backbone rearrangements in the VH framework compared to human and mouse VH domains. However, the architecture of the region of VH that interacts with a VL in a conventional Fv was different. Moreover, the CDR1 region, although in sequence homologous to human CDR1, deviated fundamentally from the canonical structure (354). Several advantages result from their single domain nature. Thus, VHH libraries generated from immunized camelids retain full functional diversity which allows to isolate high-affinity antigen-binding VHHs by directly screening a limited number of clones

from immune libraries without prior selection using display technologies (355,356). VHHs have been also demonstrated to recognize antigenic sites that are normally not recognized by conventional antibodies such as enzyme active sites and conserved cryptic epitopes (357–359). The small size of VHHs (15 Kda) results in a fast tissue penetration. This is advantageous for targeting of VHHs coupled to toxic substances to tumors (360). VHHs were also successfully linked to Fc portion of human antibodies to be used in therapy. An anti-VEGFR2 nanobody fused to Fc fragment of human IgG1 antibody have been shown ‘*in vitro*’ to bind to VEGFR2 and could induce the cell death by CDC and ADCC reaction (361). Here we suggest, because of its high stability in different physiological conditions and the simplicity of selection processes by phage display it could be interesting to develop a mesothelin targeting nanobody. This antibody could also be modified for preferred characteristics: for example, can be linked to a drug or a toxin to deliver them to tumor or could be fused to human Fc to be capable to induce ADCC or CDC.

Another application for anti-mesothelin antibodies could be to produce a bispecific antibody by conjugated them with immune system activator antibodies as anti-CD16 for activating NK cells, or by immune check-point inhibitor targeting antibodies for redirect the immune system against cancer cells or relieve the inhibitory effect of checkpoint molecules and targeting mesothelin expressing cells at the same time. Bispecific antibodies are engineered to enable the inhibition of two cell surface receptors, or the blocking of two ligands at the same time, or crosslinking the target receptor with T or NK cells (362). *Jimenez et al.* have generated a diabody, using the variable domain genes of two neutralizing antibodies, anti- VEGFR2 and anti-VEGFR3 which bound to both VEGFR2 and VEGFR3 in a dose-dependent manner, and blocked the interaction between VEGF/VEGFR2, VEGF-C/VEGFR2, and VEGF-C/VEGFR3 (363). The same group has also developed diabodies

which target simultaneously insulin growth factor (IGF) and EGF, or IGF and VEGF and showed their functionality *in vitro* and *in vivo* models (364,365). Another bispecific antibody, MM-111, which simultaneously targets HER2 and HER3 forms a trimeric complex, effectively inhibiting HER3 signaling and showing antitumor activity in preclinical models is in phase I clinical trial for HER2 expressing cancers (366). Other promising results have been obtained in phase I study to treat head and neck cancer by the bispecific antibodies targeting simultaneously HER2 or EGFR and the antibody directed to high affinity Fc receptor CD64 for activation and retargeting of NK cells (367,368). The development of tri-specific and quadro-specific antibodies has also been published (369,370).

Another perspective to validate the therapeutic functionality of our membrane-form discriminating antibodies can be to conjugate them with immunotoxins or other chemical drugs. This conjugation can be performed with genetic engineering the sc-Fv sequence of antibody to bacterial protein based toxins or by chemical linkage to non-protein based toxic drugs.

Finally, there are many further perspectives for future developments regarding the use of our anti-mesothelin antibodies.

- To conjugate our anti-mesothelin antibodies with drug delivering vesicles aiming to concentrate the toxic drug in the cancer micro-environment for more effective therapy and to decrease to the minimum the secondary effects of chemo-toxic drugs.

- Our antibodies could also be screened for the cross-reactivity with the mesothelin in other animals aiming to develop an animal model for further understanding of mesothelin expression, its role and anti-mesothelin treatment.

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ANNEXE 1. Trials using second or third generation CAR T cells in the treatment of malignant diseases and registered at <http://clinicaltrials.gov> as of May 2016 are listed

Target antigen	Disease	CAR	Gene transfer	Number of patients	Center	Identifier
BCMA	Myeloma	4-1BB-CD3ξ	NA		Abramson Cancer Center	NCT02546167
BCMA	Myeloma	CD28-CD3ξ			NCI	NCT02215967
CD19	FL	CD28-CD3ξ	RV	1	NCI	NCT00924326
CD19	DLBCL, FL	CD3ξ; CD28-CD3ξ	RV	6	BCM	
CD19	CLL	CD28-CD3ξ	RV	8	MSKCC	NCT00466531
CD19	CLL	CD28-CD3ξ	RV	4 (8 in total)	NCI	NCT00924326
CD19	CLL	CD28-CD3ξ	RV	4 (15 in total)	NCI	NCT00924326
CD19	CLL	CD28-CD3ξ	RV	4	BCM	NCT00840853
CD19	Leukemia	CD28-CD3ξ	RV	10	NCI	NCT01087294
CD19	CLL				MDACC	NCT01653717
CD19	CLL, SLL	4-1BB-CD3ξ	NA		Abramson Cancer Center	NCT01747486
CD19	ALL	4-1BB-CD3ξ	LV	2	UPenn	NCT01626495
CD19	ALL	4-1BB-CD3ξ	LV	30	UPenn	NCT01626495
CD19	ALL	CD28-CD3ξ	RV	2 enrolled, 1 treated	MSKCC	NCT01044069

Target antigen	Disease	CAR	Gene transfer	Number of patients	Center	Identifier
CD19	ALL	CD28-CD3ξ	RV	5	MSKCC	NCT01044069
CD19	ALL	CD28-CD3ξ	RV	16	MSKCC	NCT01044069
CD19	ALL	CD28-CD3ξ	RV	21 enrolled	NCI	NCT01593696
CD19	ALL	CD28-CD3ξ	RV	4	BCM	NCT00840853
CD19	ALL	4-1BB-CD3ξ	LV		Abramson Cancer Center	NCT01551043
CD19	ALL	CD28- CD137-CD3ξ	LV		Affiliated Hospital to Academy of Military Medical Sciences	NCT02186860
CD19	ALL pediatric	NA	LV		Seattle Children's Hospital	NCT01683279 4
CD19	ALL	CD28-CD3ξ	LV		COH	NCT02146924
CD19	ALL	CD28-CD3ξ	NA		MSKCC	NCT01860937
CD19	ALL	4-1BB-CD3ξ	LV		Abramson Cancer Center	NCT02030847
CD19	Leukemia	CD28-CD3ξ	RV		MSKCC	NCT01416974
CD19	Leukemia/lymphoma	4-1BB-CD3ξ	LV	30	Abramson Cancer Center	NCT01029366
CD19	Leukemia/lymphoma	CD28-CD3ξ versus CD3ξ	RV	14	BCM	NCT00586391
CD19	Leukemia/lymphoma	CD3ξ (EBV-CTLs), CD28-CD3ξ (T cells)	RV		BCM	NCT00709033

Target antigen	Disease	CAR	Gene transfer	Number of patients	Center	Identifier
CD19	Leukemia	4-1BB-CD3ξ	LV		Seattle Children's Hospital	NCT02028455
CD19	Leukemia/lymphoma	4-1BB-CD3ξ	RV		Chinese PLA General Hospital	NCT01864889
CD19	NHL	4-1BB-CD3ξ	NA		Abramson Cancer Center	NCT02030834
CD19	NHL	CD28-CD3ξ	RV		Jichi Medical University	NCT02134262
CD19	NHL	CD27-CD3ξ	LV		Peking University, University of Florida	NCT02247609
CD19	NHL	CD28-CD3ξ	LV		COH	NCT02051257
CD19	B-cell NHL	CD28-CD3ξ	LV		COH	NCT01815749
CD19	CLL, NHL, ALL	4-1BB-CD3ξ	LV		FHCRC	NCT01865617
CD19	B-cell NHL, ALL, or CLL	CD28-CD3ξ	RV		BCM	NCT02050347
CD19	MCL	4-1BB-CD3ξ	RV		Chinese PLA General Hospital	NCT02081937
CD19	Stratum 1: NHL stratum 2: CLL, PLL, SLL	CD28-CD3ξ	LV		COH	NCT02153580
CD19	B-cell NHL, ALL, CLL	CD28-CD3ξ, CD28-4-1BB-CD3ξ	RV		BCM	NCT01853631
CD19	HL	4-1BB-CD3ξ	RNA EP		Abramson Cancer Center	NCT02277522

Target antigen	Disease	CAR	Gene transfer	Number of patients	Center	Identifier
CD19	ALL, CLL, NHL	NA	NA		Southwest Hospital	NCT02349698
CD19	leukemia/lymphoma	CD28-CD3ξ	NA		MSKCC	NCT01430390
CD19	DLBCL, PMBCL, TFL	NA	NA		Kite Pharma	NCT02348216
CD19	Leukemia/lymphoma	CD28-4-1BB-CD3ξ	RV		Uppsala University	NCT02132624
CD19	NHL	CD28-CD3ξ	NA		MSKCC	NCT01840566
CD19	B-lymphoid malignancies post-HSCT	CD28-CD3ξ	DNA EP		MDACC	NCT00968760
CD19	B-lineage lymphoid malignancies post-UCBT	CD28-CD3ξ	DNA EP		MDACC	NCT01362452
CD19	Leukemia/lymphoma	NA	LV		COH	NCT01318317
CD19	Leukemia/lymphoma	NA	NA		FHCRC	NCT01475058
CD19	myeloma	4-1BB-CD3ξ	LV	10	Abramson Cancer Center	NCT02135406
CD19	Leukemia/lymphoma	NA	NA		Shanghai Tongji Hospital, Tongji University School of Medicine	NCT02537977
CD19	Leukemia/lymphoma	NA	NA		Shenzhen Second People's Hospital	NCT02456350

Target antigen	Disease	CAR	Gene transfer	Number of patients	Center	Identifier
CD19	Leukemia/lymphoma	NA	NA		Second Military Medical University	NCT02644655
CD19	Leukemia/lymphoma	NA	NA		Beijing Doing Biomedical Co.; First Hospital of Jilin University	NCT02546739
CD19	Lymphoma	CD28-CD3 ξ	RV		Xinqiao Hospital of Chongqing	NCT02652910
CD20	MCL, B-NHL	CD28-4-1BB-CD3 ξ	DNA EP	4 enrolled, 3 treated	FHCRC	NCT00621452
CD20	Lymphoma	4-1BB-Fc ϵ R1 γ	LV		Chinese PLA General Hospital	NCT01735604
CD22	ALL, FL, NHL, LCL	4-1BB-CD3 ξ	LV		NCI	NCT02315612
CD30	HL, NHL	CD28-CD3 ξ	NA		UNC Lineberger CCC	NCT01316146
CD30	Cutaneous T Cell lymphoma, transformed Mycosis fungoides	CD28 Δ -CD3 ξ	RV		University of Cologne	NCT01645293
CD30	HL, NHL	NA	NA		Chinese PLA General Hospital	NCT02259556
CD30	HL, NHL	CD28-CD3 ξ	NA		BCM	NCT01192464
CD30	Lymphoma	NA	LV		Peking University	NCT02274584

Target antigen	Disease	CAR	Gene transfer	Number of patients	Center	Identifier
CD33	AML	4-1BB-CD3ξ	LV	1	Chinese PLA General Hospital	NCT01864902
CD123	AML	CD28-CD3ξ	LV		COH	NCT02159495
CD133	Leukemia/lymphoma	CD3ξ; 4-1BB-CD3ξ	NA		Chinese PLA General Hospital	NCT02541370
CD138	Myeloma	4-1BB-CD3ξ	RV		Chinese PLA General Hospital	NCT01886976
LeY	AML	CD28-CD3ξ	RV	4	Peter MacCallum Cancer Center	
LeY	Myeloma, AML, myelodysplastic syndrome	CD28-CD3ξ	RV		Peter MacCallum Cancer Center	NCT01716364
NKG2D-L	Myeloma, AML, myelodysplastic syndrome	NA	NA		Celdara Medical, LLC	NCT02203825
Igk	Leukemia/lymphoma, myeloma	CD28-CD3ξ	RV		BCM	NCT00881920
ROR1	CLL, SLL	NA	NA		MDACC	NCT02194374
CEA	Carcinoma	CD28-CD3ξ	RV		Roger Williams MC	NCT01723306
CEA	Colorectal cancer	CD28-CD3ξ	RV		Roger Williams MC	NCT00673322
CEA	Breast cancer	CD28-CD3ξ	RV		Roger Williams MC	NCT00673829
CEA	Liver metastases	CD28-CD3ξ	RV		Roger Williams MC	NCT01373047

Target antigen	Disease	CAR	Gene transfer	Number of patients	Center	Identifier
CEA	Carcinoma	NA	NA		Southwest Hospital	NCT02349724
CEA	Carcinoma	NA	NA		Southwest Hospital	NCT02349724
cMet	Breast cancer	NA	RNA EP		Abramson Cancer Center	NCT01837602
ErbB2	Carcinoma	NA			Chinese PLA General Hospital	NCT01935843
ErbB2	Carcinoma	CD28-CD3ξ	RV		BCM	NCT00889954
ErbB2	Glioblastoma	CD28-CD3ξ	RV		BCM	NCT01109095
ErbB2	Sarcoma	CD28-CD3ξ	RV		BCM	NCT00902044
ErbB2	HNSCC	CD28-CD3ξ	RV		KCL	NCT01818323
ErbB2	Carcinoma	NA	NA		NCI	NCT00924287
ErbB2	Breast cancer	CD28-CD3ξ	RV		Fuda Cancer Hospital	NCT02547961
EGFRvII I	Glioblastoma	NA	LV		Abramson Cancer Center	NCT02209376
EGFRvII I	Glioma	CD28-4-1BB-CD3ξ	RV		NCI	NCT01454596
EGFR	Glioma	NA	NA		RenJi Hospital	NCT02331693
EGFR	Carcinoma	4-1BB-CD3ξ	LV		Chinese PLA General Hospital	NCT01869166
EphA2	Glioma	NA	NA		Fuda Cancer	NCT02575261

Target antigen	Disease	CAR	Gene transfer	Number of patients	Center	Identifier
					Hospital	
FAP	Mesothelioma	CD28-CD3ξ	RV		University Hospital Zurich	NCT01722149
FR-α	Ovarian cancer		NA		NCI	NCT00019136
GD2	Sarcoma, melanoma	OX40-CD3ξ	NA		NCI	NCT02107963
GD2	Neuroblastoma	OX40-CD3ξ	RV		BCM	NCT01822652
GD2	Neuroblastoma	NA	RV		Children's Mercy Hospital Kansas City	NCT01460901
GD2	Neuroblastoma	CD28-OX40- CD3ξ	RV		BCM	NCT02439788
GD2	Sarcoma	CD28-OX40- CD3ξ	NA		BCM	NCT01953900
IL-13Ra2	Glioma	4-1BB-CD3ξ	LV		COH	NCT02208362
L1-CAM	Neuroblastoma, ganglioneuroblastom a	4-1BB-CD3ξ; CD28-4- 1BB-CD3ξ	LV		Seattle Children's Hospital	NCT02311621
Mesothel in	Carcinoma	4-1BB-CD3ξ	LV		Abramson Cancer Center	NCT02159716
Mesothel in	Metastatic cancer	NA	RV		NCI	NCT01583686
Mesothel in	Metastatic pancreatic ductal adenocarcinoma (PDA)	4-1BB-CD3ξ	RNA EP		Abramson Cancer Center	NCT01897415

Target antigen	Disease	CAR	Gene transfer	Number of patients	Center	Identifier
Mesothelin	Malignant pleural mesothelioma	4-1BB-CD3 ξ	RNA EP	4	Abramson Cancer Center	NCT01355965
Mesothelin	Carcinoma	4-1BB-CD3 ξ	NA		Chinese PLA General Hospital	NCT02580747
Mesothelin	Pancreas carcinoma	4-1BB-CD3 ξ	LV		UPenn	NCT02465983
MUC1	Carcinoma	CD28-4-1BB-CD3 ξ (SM3scFv, IL-12; pSM3scFv)	LV	1	PersonGen Biomedicine	NCT02587689
MUC1	Carcinoma, glioma	NA	NA		PersonGen Biomedicine, Anhui General Hospital of Armed Police Forces	NCT02617134
PSMA	Prostate cancer	CD28-CD3 ξ	RV		MSKCC	NCT01140373
PSMA	Prostate cancer	NA	RV		Roger Williams MC	NCT00664196
PSMA	Prostate cancer	NA	RV		Roger Williams MC	NCT01929239
VEGFR-II	Metastatic cancer, melanoma, renal cancer	NA	RV		NCI	NCT01218867

Trials using second or third generation CAR T cells in the treatment of malignant diseases and registered at <http://clinicaltrials.gov> as of May 2016 are listed *Holzinger et al., 2016*. ALL acute lymphoblastic leukemia, BCM Baylor College of Medicine, BEN bendamustine, CLL chronic lymphocytic leukemia, COH City of Hope Medical Center, CR complete response, CTX cyclophosphamide, DLBCL diffuse large B cell lymphoma, EP electroporation, ETO etoposide, FHCRC Fred Hutchinson Cancer Research Center, FL follicular lymphoma, FLU fludarabine, HL Hodgkin's lymphoma, LV lentiviral, MC Medical Center, MCL mantle cell lymphoma, MDACC MD Anderson Cancer Center, MSKCC Memorial Sloan Kettering Cancer Center, NA not available, NCI National Cancer Institute, NHL non-Hodgkin lymphoma, NR non-responder, PEN pentostatin, PLL prolymphocytic leukemia, PMBCL primary mediastinal B cell lymphoma, PR partial response, RTX rituximab, RV retroviral, SD stable disease, SLL small lymphocytic lymphoma, TCM central memory T cell, TN/MEM naïve memory T cell, UCBT umbilical cord blood transplantation, UPenn University of Pennsylvania, wk week

