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# Hooking TRAIL-R2 to "educate" immune system to recognize cancer cells

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# Part I

## Abstract

Use of recombinant human tumor necrosis factor (TNF) related apoptosis inducing ligand (rhTRAIL) or TRAIL-receptors agonistic monoclonal antibodies (mAbs) promotes apoptosis in most cancer cells preserving normal cells. Furthermore, TRAIL-R2, one of the receptor that can trigger cell apoptotic machinery, is expressed at higher levels in tumor tissue as compared to normal tissues. Agonistic mAbs could mimic the tumor killing properties of rhTRAIL, with the advantage to have a prolonged half-life in vivo and to avoid binding of decoy TRAIL-receptors unable to transmit apoptotic signals. Although mAbs have proven to be highly effective, their use still unsolved the problem of TRAIL-resistance that some tumor cells are able to develop. To increase the anti-tumor activity of some mAbs, in last decades, bispecific antibodies (BsAbs) able to redirect cytotoxic activity of tumor resident/ circulating T cells against tumor cells in an MHC-independent fashion have been developed.

In this study, we isolated a BsAb able to bind the CD3, invariant component of the T-cell receptor, and TRAIL-R2. Among the different isolated BsAbs, one in particular presented good thermodynamic characteristics and showed a very good stability even after 2 years its purification. The BsAb was biochemically and functionally well characterized and the production method was optimized. We demonstrated that the BsAb could act with two mechanisms: retargeting T-cells to lyse tumor cells when acting as BsAb and activating apoptotic pathway by triggering TRAIL-R2 when acting as agonistic mAb. T-cell activation was confirmed by up-regulation of CD69 and CD25 and by production of inflammatory cytokines without off target toxicity. In vitro we demonstrated that, after BsAb-mediated retargeting, T cells were able to lyse tumors of different histotypes, like melanoma, breast and ovarian cancer while sparing normal cells.

# 1. State of the Art

## 1.1 TRAIL and its receptors

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), or Apo2L, is a 33-35 kDa type II transmembrane protein. TRAIL belong to TNF superfamily like CD95L (or Fas ligand) and TNF-α. It was discovered and characterised in 1995 [1] thanks to the presence in its sequence of a highly conserved motif characteristic for the TNF family members (28% amino acid sequence identity with Fas ligand and 23% identity with TNF- α). TRAIL is expressed as transmembrane protein (membrane TRAIL) by various activated cells of the immune system, like natural killer (NK), T- and B- cells, dendritic cells and macrophages [2]. Its extracellular region can be cleaved by metalloproteases to release a soluble molecule (sTRAIL) that could maintain its biological activity. Crystallography studies revealed that sTRAIL is present as a homotrimer stabilized by a cysteine residue, at position 230 of each sTRAIL subunit, which coordinate an internal zinc atom [3].

TRAIL receptor system is complex and comprises five different paralogous receptors (figure 1.1). DR4 or TRAIL-R1 and DR5 or TRAIL-R2 are intact functional transmembrane death receptors: they are characterized by an extracellular cysteine-rich domain and by a cytoplasmic death domain (DD) able, upon ligand stimulation, to trigger the assembly of the death-inducing signalling complex (DISC) and transduce the apoptotic signal [4].

By contrast the receptors DcR1 or TRAIL-R3 and DcR2 or TRAIL-R4 lack a functional intracellular DD (DD is not present in DcR1 while is truncated in DcR2 as in figure 1.1) and for this reason they are not able to initiate apoptotic events but act as decoy, or regulatory, receptors. The fifth member of the system is a decoy receptor called osteoprotegerin (OPG) whose main role is the regulation of osteoclastogenesis by competing with RANK (receptor activator of NF-KB) for

RANK ligand. OPG could also bind TRAIL with low affinity with a biological role, at the moment, not well defined [4].

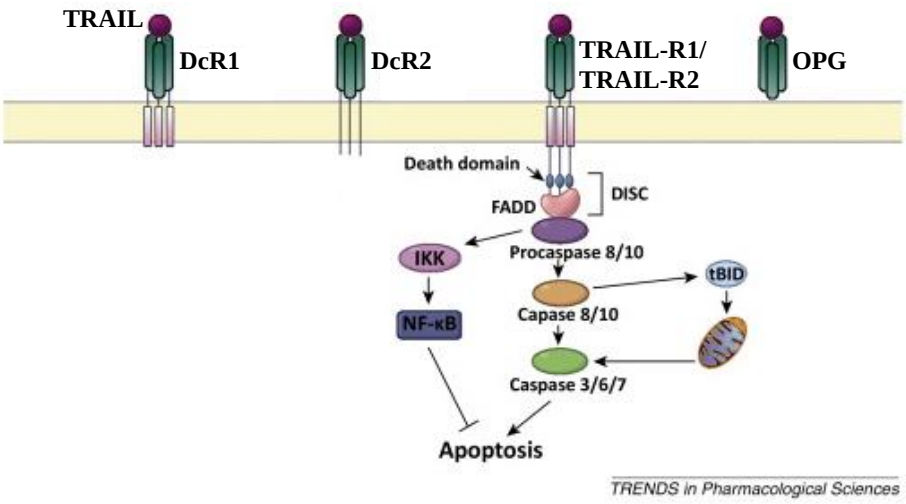


**Figure 1.1 TRAIL receptor system**
TRAIL-R1 and TRAIL-R2 have an intracellular death domain (DD) able to transduce the apoptotic signal after the binding of TRAIL. DcR1 (TRAIL-R3), DcR2 (TRAIL-R4) and OPG are not able to transmit the signal because they don't have a functional DD. [5]

In mice has been identified a homologous protein coding for TRAIL, with 65% of identity with the human TRAIL. This murine protein could interact with only one death inducing receptor (mTRAIL-R), homologous to human TRAIL-R1/R2, with two proteins homologue to decoy receptors and with a homologue to OPG [6]. Interestingly, human TRAIL could bind mTRAIL-R and cause apoptosis in murine cells. Nevertheless the agonistic activity of human TRAIL on murine cells is less potent than autologous TRAIL. Homology of human and monkey TRAIL ligand and receptors is almost complete with an identity around 90% in cynomologous monkey and around 100% in chimpanzee. Additionally, human TRAIL is capable of inducing apoptosis in the rhesus mammary tumor cell line CMMT110 and in the transformed baboon lymphoblastoid cell line CB1, further supporting its cross-

reactivity with nonhuman primate death receptors. Thus, monkeys may provide an optimal model for preclinical safety assessment of human TRAIL.

### 1.2 TRAIL-induced apoptosis

TRAIL-induced apoptosis is initiated upon binding of TRAIL to TRAIL-R1 or TRAIL-R2 on the surface of target cells and after the subsequent formation of DISC (figure 1.1 and figure 1.2). Binding of the ligand, in trimeric form, leads to trimerization of the receptors that are now able to recruit trough their DD the Fas-associated death domain (FADD) protein adaptor [7]. FADD contains two functional domains: the first takes contacts with TRAIL-receptor DD while the second, called death-effector domain (DED), could recruit procaspase-8 or -10. Once bound on DISC complex, procaspases -8 and -10 auto-catalytically cleave and become active initiating caspases. Activation of caspase-8 is, in type I cells, sufficient for subsequent cleavage and activation of the effector caspase-3. In type II cells, activation of caspase-8 at the DISC is not sufficient to activate effector caspases and amplification of the cascade trough mitochondrial (intrinsic) pathway has to occur to initiate apoptosis. In these cells caspase 8 induces cleavage of BH-3-only protein Bid. Truncated Bid translocates to the mitochondria where interacts with Bax and Bak which induce the formation of pores in the outer mitochondrial membrane resulting in the release of cytochrome-c (Cyt-c) and Smac/DIABLO. Free Cyt-c could bind apoptotic peptidase activating factor-1 (Apaf-1) and the complex, called apoptosome, cleaves and activates caspase-9. Smac/DIABLO blocks the activity of inhibitor of apoptosis proteins (IAP) allowing photolytic activity of caspase-9 which promotes caspase-3 activation (figure 1.2).

Caspase-3 is an effector caspase which executes the apoptotic process by cleaving several proteins including the enzyme poly (ADP-ribose) polymerase (PARP), cytokeratins, the plasma membrane cytoskeletal protein alpha fodrin and the nuclear mitotic apparatus (NuMA) protein [9].

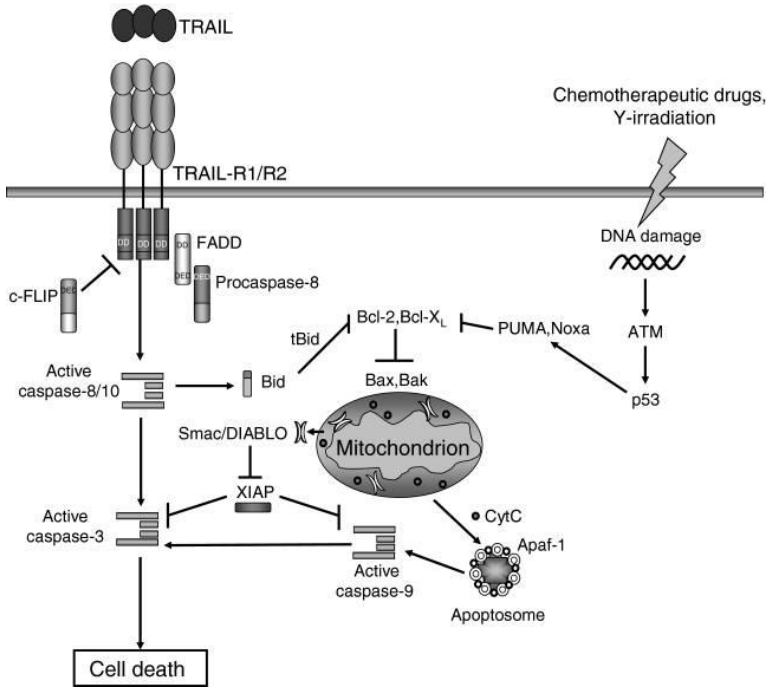


**Figure 1.2. Apoptotic intrinsic and extrinsic pathways [8]**

The principal negative regulator of TRAIL-mediated apoptotic pathway is cellular FLICE (caspase-8) -like inhibitory protein (c-FLIP). This protein has high sequence homology with caspase-8 but with catalytic subunit unable to activate its enzymatic activity. Three splice variants exist: c-FLIP$_L$, c-FLIP$_S$ and c-FLIP$_R$. Both c-FLIP$_L$ and c-FLIP$_S$ are recruited to FADD via the DED and cleaved into two peptides: p12, that is released and p43 that, remaining anchored to FADD, blocks the recruitment of caspase-8. Consequently, when there is a large amount of c-FLIP, the proteolytic cleavage of procaspase-8 is prevented and the apoptotic extrinsic pathway is inhibited [1]. In type II cells, important TRAIL-mediated apoptosis inhibitory molecules are Bcl-2 and Bcl-xL, which are able to prevent the release of Cyt-c and Smac/DIABLO from mitochondria, and IAPs (for example XIAP) that hinder the cleavage of initiating caspases like caspase 9 (figure 1.2).

### 1.3 TRAIL physiological role

The biological role of TRAIL is not fully understood, however it seems having a fundamental role in immune system. In fact, while the expression of TRAIL mRNA is found in wide variety of normal cell types, including fetal and adult tissues, the expression of high levels of functional protein seems to be restricted to immune cells [1;10]. Cells may overexpress TRAIL in response to insult or stress. For example human hepatocytes express at the same time TRAIL, TRAIL-R1 and R2 which seem to play a critical role in regulation of hepatic inflammation [11]. In addition, TRAIL is expressed at high level in neurons after ischemic damage and seems to have a role in cell death induction [12].

Immune cells, in particular NKs and cytotoxic T-cells, over-express TRAIL when they resulted activated. The expression of TRAIL, present on the surface or released like soluble molecule, furnish to immune cells a potent weapon to trigger apoptosis in cells expressing the death receptors like transformed cells and suggest that this molecule could have a role in the modulation of tumor immunosurveillance. NKs and T-cells activity is physiologically dependent on expression of major histocompatibility complex-1 (MHC-1) molecules on target cells. Tumor associated antigen (TAA) when exposed in a MHC-1 context could be recognized by T-cell receptor (TCR) of cytotoxic T-cells which are then activated, can proliferate and kill tumor cells [13]. Antigen-dependent activation of T cells induces expression of TRAIL enhancing their cytotoxic activity. NK cells on the contrary are activated by the lack of MHC-1 expression, a mechanism often used by tumor cells to escape T-cell recognition.

Indeed, malignant transformation may induce the down-regulation of MHC-1 expression and the up-regulation of CD70 or CD80. These two proteins when interact with CD27 and CD28, receptors present on T-cells and NKs surface, activate the signal cascade for immune cells activation. NK cytotoxic activity in tumor immunosurveillance seems to be mediated by TRAIL [14]. After activation

NK produce IFN-γ which up-regulates TRAIL on NK cells and sensitizes tumor cells to TRAIL activity [15]. The involvement of TRAIL in tumor immunosurveillance was confirmed performing experiments with mice models. In TRAIL knock-out mice a decreased tumor immunosurveillance was observed, specifically concerning initiation and metastatization processes together with a higher sensitivity to experimental autoimmune diseases. In an experimental skin carcinoma model it was demonstrated that in TRAIL-receptors null mice there was an increased rate of lymph node metastatization compared to TRAIL-Rs wt mice [16].

The binding of TRAIL to its agonist receptors seem to be specifically involved in the suppression of metastasis: after tumor cells detachment from primary site, there is an inhibition of the Erk pathway that causes the sensitization to TRAIL [17]. It was demonstrated that the impairment of TRAIL activity, by using neutralizing antibodies or in null mice, increased the development of experimental primary renal carcinomas and its metastatization capability [18].

Additionally, IFN-γ seems to play an important role in TRAIL mediated cytotoxicity also affecting macrophages activity. In fact IFN-γ causes macrophages activation that results in the release of molecules able of inducing up-regulation of TRAIL-R1 and TRAIL-R2 on tumor cells.

### 1.4 Differences between TRAIL-R1 and TRAIL-R2 death receptors

Human TRAIL-R1 and TRAIL-R2 death receptors are encoded by two genes located on the same chromosome: 8p. They are single-pass type-I membrane proteins and in human both have two splicing variants, the long and the short isoform. TRAIL-R2 isoforms differ for 29 aa located between extracellular cysteine rich domains (CRD) and transmembrane trait [19]. Short TRAIL-R1 isoform was identified in Ewing's sarcoma cell lines in which alternative splicing mechanism codes for a protein lacking of 158 aa in the TRAIL binding region [20].

Long isoforms are the most expressed on cells plasma membrane and for this reason are the most studied. The function of the short isoforms is unknown but it seems that they could be involved in the sensitization of the cell to the death ligand induced apoptosis. Comparing TRAIL-R1 and TRAIL-R2 there is a sequence similarity of about 46% but DD is highly conserved. Besides the same pro-apoptotic activity, structural differences between the two receptors affect their functionality. Experiments of receptor chimerization, in which transmembrane domain and extracellular regions of TRAIL-R1 or TRAIL-R2 were fused to portions of Fas, showed strongest caspase-8 and caspase-3 activation by chimaeras containing the TRAIL-R2-derived domain. No differences in ligand binding and internalization kinetics were observed for these chimaeras, thus providing no explanation for the differences in TRAIL signalling strength. Apparently these differences appear due to the variations found between TRAIL-R1 and TRAIL-R2 structures. In fact only TRAIL-R1 has, in the transmembrane domain, a S-palmitoylation site that seems to favour lipid raft-localization, leading to better responsiveness to the ligand. On the other hand only TRAIL-R2 has a GXXXG motif that could permit a more stabilization of the homotrimeric structure. Nevertheless further investigations would be required to elucidate the real effects of these structural differences on the regulatory function of these receptors [21].

### 1.5 TRAIL-R expression on tumors

The effect of TRAIL on tumors depends on the type of malignancy. Despite TRAIL receptors are upregulated in many tumors compared to corresponding normal tissues, the quantity of these receptors could vary among tumors even of the same histotype. In some tumors the upregulation of the two death receptors TRAIL-R1 and TRAIL-R2 influences the sensitization to TRAIL-based immunosurveillance of tumor. Investigations in normal colonic mucosa show that the two death receptors are expressed but their level is higher in adenomas and

carcinomas. In different cancer types, like ovarian [22], melanoma [23], bladder [24], glioblastoma [25] and pancreas [26] expression of TRAIL-R1/R2 correlates with better prognosis.

In other cases high death receptor expression, in particular TRAIL-R2 but occasionally also TRAIL-R1, correlates predominantly negatively on patients' prognosis. For example in human malignant breast cancer TRAIL-R1 expression is higher and can induce cell death [27] while high TRAIL-R2 expression correlated with higher tumor grade and shortened survival [28]. High TRAIL-R2 level also correlated with poor prognosis in renal carcinomas[29], head and neck squamous cell carcinomas [30] and non-small-cell lung cancer patients [31].

### 1.6 Scientific rationale for therapeutic use

The discovery of FasL, TNFα and TRAIL, natural cytokines members of the Tumor necrosis Factor superfamily, opened new possibilities for the development of new cancer therapeutics. Unlike many conventional antitumor therapeutics, these ligands are potentially useful because their capability of triggering apoptosis independently to the p53 gene, generally inactivated in a lot of tumors. The first two member of the superfamily, FasL and TNFα, were discovered about 20 years ago and were considered to be used as anti-cancer molecules. After initial excellent results in inducing apoptosis in tumor cells in vitro, their use *in vivo* preclinical models showed severe adverse effects: the use of TNFα provoked strong inflammatory response activating nuclear factor-kB in endothelial cells and macrophages [32], while the use of recombinant antiFas agonistic antibodies caused hepatocyte apoptosis resulting in severe liver toxicity [33].

TRAIL is the third discovered tumor pro-apoptotic ligand and, at variance from TNFα or FasL, *in vivo* studies excluded toxic side effects, its activity appeared specific for tumor cells, preserving normal cells [34]. Both TRAIL-R1 and TRAIL-R2 can trigger apoptosis and the majority of cancer cell lines and primary human

tumors were found to be positive for the two receptors by immunohistochemical staining. Preclinical efficacy studies in rodent xenograft models of colon cancer[35], breast cancer [36], glioma [37], prostate cancer [38], multiple myeloma [39] and lung cancer [40] demonstrate that TRAIL, administered as single agent or in combination with chemotherapy or radiotherapy, causes apoptosis in tumors, complete or partial tumor shrinkage and delayed tumor progression. Beyond the role of both death receptors in TRAIL therapy, several studies showed that TRAIL has a higher binding affinity to TRAILR2 as compared with TRAIL-R1 and that agonistic antibodies, which were developed to be specific to both receptors, have more potent activity when could bind TRAIL-R2 compared to TRAIL-R1.

### 1.7 Clinical trials with TRAIL

After the good results in preclinical models, different TRAIL receptor agonist compounds entered in clinic. These compounds included recombinant forms of TRAIL and agonistic antibodies directed against TRAIL-R1 or TRAIL-R2 [4]. Soluble recombinant human TRAIL (rhTRAIL) has the capability of target both receptors and, for this reason, to have a major spectrum of action. Moreover its small size allows perfusing the tumour much more efficiently than the larger antiTRAIL-R antibodies. A version of rhTRAIL that comprises the extracellular region of human TRAIL (amino acids 114–281), called Dulanermin, was the unique form of rhTRAIL that reached clinical trials. In these clinical trials Dulanermin showed some anti-tumour efficacy and only mild side effects were observed [41]. Despite promising initial clinical results, Dulanermin failed in phase II randomized studies (table 1.1). The lack of activity of wild-type rhTRAIL is due to its capability to bind all five TRAIL receptors: if the capability of binding both active receptors resulted in a broad effect, on the other hand rhTRAIL could be sequestered by decoy receptors diminishing apoptosis activation. RhTRAIL variants, which are able to bind only to TRAIL-R1 or TRAIL-R2 with very low

affinity for the other three receptors, have been proposed to reduce DcR-binding. TRAIL-R1- or TRAIL-R2-selective forms of recombinant TRAIL showed enhanced apoptotic activity in tumour cell lines compared to rhTRAIL WT [42-45]. This increased activity was the result of the high affinity binding of the selective rhTRAILs that allowed a more efficient receptor trimerization. Furthermore these molecules avoid the formation of heterotrimeric complexes consisting of TRAIL-R1, TRAIL-R2 and DcRs that were unable to transduce an efficient apoptotic signal [46].

Antibodies directed against TRAIL-R1 (mapatumuab) or TRAIL-R2 (drozitumab, conatumumab, lexatumumab and tigatuzumab) provide an alternative pro-apoptotic strategy to the use of selective rhTRAILs. Like selective rhTRAIL molecules, mAbs have a minor spectrum of action if compared to Dulanermin but on the other hand they have the advantage that could not be sequestered by decoy receptors and could not allow the formation of heterotrimeric receptor-complexes, whose apoptosis triggering is lower respect to homotrimeric complexes. Although their large size does not allow an efficient tumor perfusion, respect to selective rhTRAIL they could be administered in lower doses having a longer half-life (days in contrast to 1 hour of rhTRAIL). Like rhTRAIL, also with agonistic mAbs phase I clinical trials in subsets of patients were encouraging demonstrating safety and some antitumor activities with partial or complete responses, but randomized phase II clinical trials revealed no anti-cancer activity (table 1.2).

The cause of the insufficient antitumor activity in clinical trials has to be better evaluated. Reasonably, the first hypothesis is the insufficient agonistic activity of the TRAIL receptor targeting drugs. To improve the agonistic activity of rhTRAIL, several other recombinant forms have been developed in which tags were added at the amino terminus of TNF homology domain of TRAIL. Antibody crosslinked his-tagged and flag-tagged rhTRAIL resulted in more potent agonistic molecules but were also toxic to human hepatocytes in vitro. The addition of leucine or

isoleucine zipper to rhTRAIL allows the stabilization of the TRAIL trimeric structure and results in a more potent agonistic activity without off- target toxicity, as shown in preclinical experiments [47].

**TABLE 1.1. Results of Dulanermin clinical trials. Table revisited from [48]**

| Phase | n | Cancer | Combination | Safety | Efficacy |
|---|---|---|---|---|---|
| I | 71 | Advanced cancers | - | Safe | 2 PR |
| I | 23 | Colorectal | Chemo + Bevacizumab | Safe | 13 PR |
| I | 27 | Colorectal | Chemo + Bevacizumab | Safe | 6 PR |
| I | 30 | Colorectal | Chemo + Cetuximab | Safe | / |
| I | 24 | Lung | Chemo + Bevacizumab | Safe | 1 CR + 13 PR |
| I | 7 | Lymphoma | Rituximab | Safe | 2 CR + 1 PR |
| **II (RCT)** | 213 | Lung | Chemo + Bevacizumab | Safe | None |
| **II (RCT)** | 48 | Lymphoma | Rituximab | Safe | None |

Abbreviations :n, number or recruited patients; CR, complete response; PR, partial response; RCT, randomized clinical trials.

**TABLE 1.2/a. Results of agonistic antibodies in clinical trials. Table revisited from [48]**

| **TIGATUZUMAB (antiTRAIL-R2 mAb)** | | | | | |
|---|---|---|---|---|---|
| **Phase** | **n** | **Cancer** | **Combination** | **Safety** | **Efficacy** |
| I | 17 | Carcinoma or | - | Safe | None |
| II | 61 | Pancreatic | Chemo | Safe | 8 PR |
| II (RCT) | 97 | Lung | Chemo | Safe | None |
| **DROZITUMAB (antiTRAIL-R2 mAb)** | | | | | |
| **Phase** | **n** | **Cancer** | **Combination** | **Safety** | **Efficacy** |
| I | 9 | Colorectal | Chemo | Safe | 2 PR |
| I | 50 | Adv cancers | - | Safe | None |
| **LEXATUMUMAB (antiTRAIL-R2 mAb)** | | | | | |
| **Phase** | **n** | **Cancer** | **Combination** | **Safety** | **Efficacy** |
| **I/II** | 73 | Adv cancers | Chemo | Safe | 2 PR |

Abbreviations :n, number or recruited patients; CR, complete response; PR, partial response; RCT, randomized clinical trials.

**TABLE 1.2/b. Results of agonistic antibodies in clinical trials. Table revisited from [48]**

| MAPATUMUMAB (antiTRAIL-R1 mAb) | | | | | |
|---|---|---|---|---|---|
| **Phase** | **n** | **Cancer** | **Combination** | **Safety** | **Efficacy** |
| I | 49 | Adv cancers | - | Safe | None |
| I | 41 | Adv cancers | - | Safe | None |
| I | 49 | Adv cancers | Chemo | - | 12 PR |
| I | 27 | Adv cancers | Chemo | - | 5 PR |
| I/II | 40 | Lymphoma | - | Safe | 2 CR + 1 PR |
| II | 38 | Colorectal | - | Safe | None |
| II | 32 | Lung | - | Safe | None |
| II (RCT) | 104 | MM | Bortazomib | Safe | None |
| II (RCT) | 109 | Lung | Chemo | Safe | None |
| **CONATUMUMAB (antiTRAIL-R2 mAb)** | | | | | |
| **Phase** | **n** | **Cancer** | **Combination** | **Safety** | **Efficacy** |
| I | 37 | Adv cancers | - | Safe | 1 PR |
| I | 18 | Adv cancers | - | Safe | None |
| I | 6 | Sarcoma | Chemo | Safe | None |
| I | 9 | Adv cancers | Ganitumab | Safe | None |
| I | 12 | Lung | Chemo | Safe | 1 CR + 3 PR |
| I | 12 | Colorectal | Chemo | Safe | 5 PR |
| I | 13 | Pancreatic | Chemo | Safe | 4 PR |
| II (RCT) | 128 | Sarcoma | Chemo | Safe | None |
| II (RCT) | 172 | Lung | Chemo | Safe | None |
| II (RCT) | 83 | Pancreatic | Chemo | Safe | None |
| II (RCT) | 103 | Colorectal | Chemo | Safe | None |
| II (RCT) | 190 | Colorectal | Chemo + Bevacizumab | Safe | None |
| **LEXATUMUMAB (antiTRAIL-R2 mAb)** | | | | | |
| **Phase** | **n** | **Cancer** | **Combination** | **Safety** | **Efficacy** |
| **I** | 37 | Adv cancers | - | Safe | None |
| **I** | 31 | Adv cancers | - | Safe | None |
| **I** | 41 | Adv cancers | Chemo | Safe | Partial |
| **I** | 24 | Pediatric | - | Safe | None |

Abbreviations :n, number or recruited patients; CR, complete response; PR, partial response; RCT, randomized clinical trials.

### 1.8 Resistance to TRAIL

The failure of clinical trials could be explained also with a second hypothesis: the presence/development of an intrinsic TRAIL resistance within primary human cancers. Several studies demonstrated that tumour cells develop resistance to TRAIL-induced apoptosis: tumor cells acquire apoptosis-escape mechanisms that preserve them to TRAIL-mediated killing.

These mechanisms are not fully understood but the molecules that regulate TRAIL signaling in cell death are the same that are involved in resistance (figure 1.3). TRAIL resistance could be due to:

- expression of mutated death receptors genes, surface expression levels of TRAIL-R1 and TRAIL-R2 can decrease as a result of mutations or methylation of the receptor gene promoters. This mechanism seems to cause resistance in B-cell chronic lymphocytic and in acute myeloid leukemias, in human colon adenocarcinomas and in some type of head and neck and lung tumors [5];
- expression of decoy receptors or OPG: lacking the DD these receptors are not able to induce apoptosis and sequester TRAIL to functional active death receptors. For example in osteosarcoma cell line BTK-143 resistance is done to DcR2 and blocking this receptor restored the sensitivity to TRAIL [61]. DcR1 has been found to be overexpressed in TRAIL-resistant tumors of the gastrointestinal tract and has been suggested to compete with TRAIL-R1 for ligand binding, thus having a negative influence on apoptosis [49];
- overexpression of anti-apoptotic proteins like: c-FLIP, Bcl-2 family proteins or IAPs. c-FLIP, which acts in competition with caspase 8/10 to the same site of binding into DISC, was involved in TRAIL-resistance in various types of tumors [50] and was demonstrated that knock-down of c-FLIP restored TRAIL sensitivity in different cancer cell lines, including ovarian cancer cell lines [51]; Bcl-2 family proteins are key regulators of intrinsic apoptotic pathway, maintaining the integrity of the mitochondrial membrane, and, if highly

expressed, contribute to TRAIL resistance in different types of cancer [52]; myeloid cell leukemia-1 (Mcl-1) protein, another Bcl-2 family member, can inhibit BH-3 only proteins such as Bim, Bid, PUMA/NOXA and Bak and can thus cause TRAIL resistance in certain type II cells [53]; IAPs, which inhibit caspases provoking their degradation, are found to cause resistance in bladder cancer and in pancreatic adenocarcinomas[54];

- down-regulation or mutation of pro-apoptotic proteins like caspases and Bcl-2 family members Bax and Bak and Smac-Diablo. The dysregulation of caspase-8 is the principal mechanism of TRAIL-resistance: lack of transcription or expression of a mutant inactive form of this caspase or synthesis-down-regulation were found to be the causes of resistance respectively in primitive neuroectodermal tumor, colorectal carcinoma and leukemia cell lines [54].

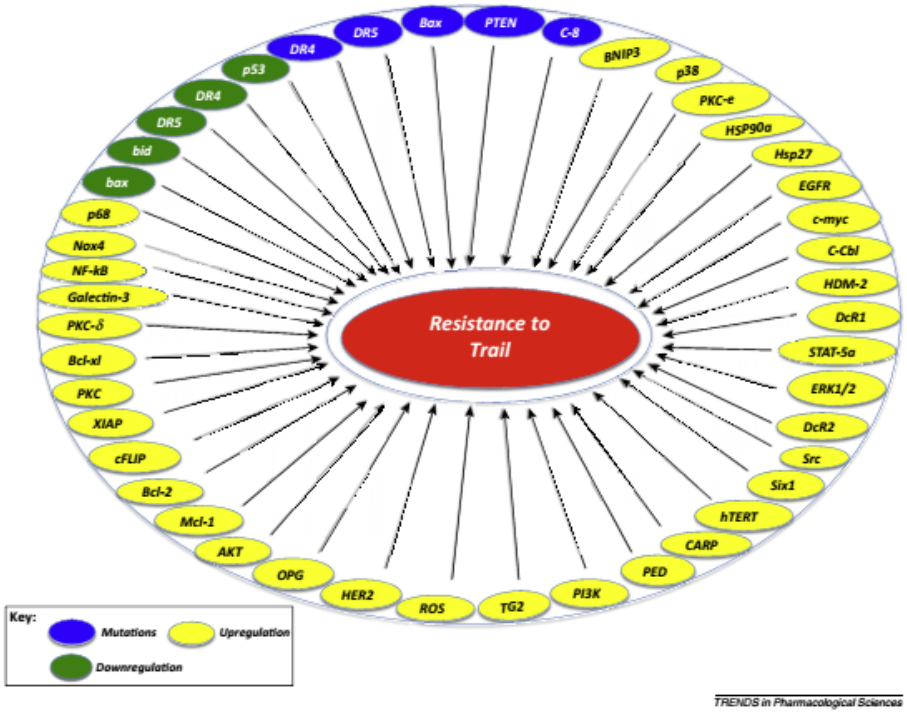


**Figure 1.3 Possible cellular mechanisms involved in TRAIL-resistance** [5]

Resistance to TRAIL-mediated apoptosis of human cancer cell lines can be caused also by a great number of different factors along the signaling pathway. For example activation of NF-κB was demonstrated to induce TRAIL-resistance in most cancer cells, like ductal pancreatic adenocarcinomas cells and HeLa cells, by overexpressing cell-survival genes like c-FLIP, IAPs and Bcl2-family protein Mcl-1 [5].

### 1.9 Sensitization of resistant tumors

All the different mechanisms of resistance to TRAIL underline the importance to understand why cells could be TRAIL-resistant in order to choose the right treatment that will effectively overcome resistance. To by-pass resistance TRAIL-R2 agonist compounds were used in association with drugs called “TRAIL sensitizers”. Several conventional chemotherapeutic agents are considered to be good “TRAIL-sensitizers” and showed good pre-clinical results but were disappointing in clinical trials [48]. Some chemotherapeutics are able to induce upregulation of TRAIL-R1 and TRAIL-R2. Although the upregulation of TRAIL-R1 and -R2 can contribute to TRAIL sensitization, it is not sufficient to explain the effect. For instance, Ganten et al. showed that upregulation of TRAIL-R1 and-R2 is not essential for the sensitization effect that they observed with 5-Fluourouracil (5-FU) to TRAIL-induced apoptosis [56]. The same conclusion was reached by Singh et al. and by Legadec et al. using Doxorubicin [57] or Tamoxifen [58]. TRAIL acts synergistically also when combined with IFN-α [59], etoposide [60], cisplatin [61], irinotecan [35] and Histone-Deacetylase (HDAC) or cyclooxygenase-2 (COX-2) inhibitors [62;63]. Although, the exact biochemical mechanism underlying sensitization remains unknown, these molecules act like TRAIL sensitizers by upregulating TRAIL-receptors, modulating the different family members, upregulating caspases or inhibiting IAP family members or c-FLIP. Also small molecule drugs, which were recently discovered for cancer therapy, have been

demonstrated to synergize with TRAIL. For example Bortezomib, a proteasome inhibitor approved by the FDA for the therapy of relapsed malignant melanoma and mantle cell lymphoma, has been shown to sensitize a variety of different tumour cell lines and primary tumors to TRAIL- induced apoptosis, without being toxic to normal cells [64]. Sensitization by proteasome inhibitors appeared to involve downregulation of the anti-apoptotic protein c-FLIP [65] or activation of proapoptotic caspases such as caspase-3 [66]. Smac mimetics took their name because they are able to mimic the structure of SMAC/DIABLO and thereby inhibit or degrade members of IAPs [67]. Of note is that SMAC mimetics alone caused very little toxicity, however, if combined with TRAIL its apoptosis-inducing potency can be dramatically increased [68;69]. Also the multi kinase inhibitor Sorafenib has been shown to sensitize to TRAIL-induced apoptosis and entered clinical trials in combination with rhTRAIL [70].

## 1.10 About Cancer

Cancer is a leading cause of death worldwide. In 2012, 14.1 million new cancer cases and 8.2 million cancer deaths were recorded but this numbers are expected to increase due to the increasing life expectation of the population of less developed countries [71]. The raising public awareness for this devastating disease bring, in the last 45 years, to an increase of investments in research and drug development that lead to extraordinary advances in the knowledge about the mechanisms of cancer development and progression. Cancers are described as complex tissues including cancer cells and tumor-associated stroma in which heterogeneous cell types reside. Tumors arise following genetic and molecular modifications of normal cells that result in hundreds of types and subtypes of cancer. Moreover also normal cells, like vascular cells, immune inflammatory cells and fibroblasts are recruited in the tumoral environment contributing to increase diseases heterogeneity. In particular it is paradoxical that the infiltrating immune cells may

in some cases induce inflammation in the tumor microenvironment that causes the production of growth, survival and proangiogenic factors, extracellular matrix-modifying enzymes and signals that cancer cells may use to survive and grow. The different types of tumors behave in a different way also to the point of view of the progression and the sensitivity/resistance to drugs. Complexity of cancer biology has been recently simplified by defining some principal hallmarks shared by all tumors: self-sufficiency in proliferative signals, evasion to growth suppressors, avoiding immune destruction, enabling limitless proliferation, induction of inflammation and angiogenesis, activating invasion and metastasis, genome instability and mutation, deregulating energy metabolism and resisting to apoptosis [72]. The definition of these hallmarks opened new horizons in the comprehension of cancer biology and in consequence in development of new anticancer drugs that should have been more suitable to the treatment of cancers. In fact despite remarkable progress in our understanding and in treatment of some forms of the disease, in the majority of cases, new treatment strategies, although with good results in preclinical studies, have often failed in subsequent clinical trials.

### 1.11 Conventional Cancer Therapy

Surgery, chemotherapy and radiation therapy have long been considered as the standard treatments of cancer. Surgery is the first attempt to eradicate tumors: the eradication of tumor in fact is the only option to defeat completely the disease. In recent years the development of laser technology has improved the precision of this technique. Unfortunately this treatment could be used only to benign cancers or confined tumors without metastases (10% of total cases of tumors). In all the other cases chemotherapy or radiation or a combination of the two treatment strategies have to be used. However they lack of a selective tumor toxicity causing very severe adverse effects towards healthy cells that have high rate of proliferation, for

example blood cells, pilous follicles, digestive and reproductive apparatuses mucosa [73].

The endocrine therapy, which is used for hormone-dependent tumors like prostate and breast cancers, is more selective but has like effect to slow or to stop tumor growth.

During last decades the increase in understanding cancer biology allowed the arising of a new more selective way to treat tumors: these new compounds were called targeted-cancer drugs. Agents such as monoclonal antibodies, small molecule inhibitors, antisense oligonucleotides and liposomes, have the capability to selectively hit specific modified factors that would not be present in normal cells like growth factors, signaling molecules, cell-cycle proteins, apoptosis and angiogenesis modulators [73]. Small molecule drugs are able to target particular mechanisms that allow cancer cells to grow in an uncontrolled manner. Although these drugs have anti-cancer activity, they can only kill the fraction of tumor bearing the molecular defect for which the drug has been designed.

Other therapies available are hyperthermia treatment that uses heat to kill cancer cells and photodynamic treatment that uses special drugs, called photosensitizing agents, to kill cancer cells [74;75]. Early attempts with these types of treatments showed discording results but newer molecules or newer tools allow a more precise delivery of heat or photosensitizing agent. A quite new attempt trying to “repair” errors that induce cells to grow in a non-controlled manner is represented by gene therapy. This approach considers treating cancer by replacing a missing or an altered gene with its healthy counterpart or by introducing “suicide genes”, normally vehiculated by viral particles or introduced by transposon technology, into tumor cells to cause their death [76]. All these developed anti-cancer drugs have improved the weapons to fight against tumors. Although that the way to discover the ideal anti-cancer therapy is long, one of the most promising fields in

cancer therapeutics is immunotherapy that furnishes systemic, tumor-specific and highly effective drugs.

## 1.12 Cancer immunotherapy

Immune system has the greatest potential for specific tumor eradication with no toxicity to normal tissue and with the possibility to develop immune memory that can prevent cancer recurrence. In the past two decades, due to the advances in mouse genetics, many studies have shown that in mice deficient in key immunologic molecules, the development of both chemically induced and spontaneous tumors is enhanced, demonstrating the ability of the immune system to recognize and reject tumors through a process called immunosurveillance [77-79]. The immune tumour specificity response resides in the recognition of tumour antigens by cells of the adaptive immune system, principally, $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells. However, most tumour antigens are ‘self-proteins’ to which the immune system has limited responsiveness, due to the development of tolerance, at thymus level, by clonal deletion or anergy. Immunotherapy is based on treatments that use component of the immune system to fight cancer. This can be done in different ways: principally by stimulating the own immune system to attack in a more efficient way cancer cells or giving to patient immune system components, such as man-made immune system cells or proteins. Tumor microenvironment is rich also in immunocompetent cells, which have a role in the natural process of immunosurveillance [80]. A central problem in tumor immunology is that an established tumor creates an environment of immune tolerance that promotes tumor growth, protects the tumor from immune attack and attenuates the efficacy of immunotherapy. Several mechanisms have been described by which tumors can suppress the immune system. Among these mechanisms the most studied are down-modulation of MHC class 1 molecule, secretion of cytokines, alterations in antigen-presenting cells, alterations in

costimulatory and co-inhibitory molecules and altered ratios of regulatory T cells (Treg) to effector T cells. Recently scientists demonstrated that Tregs-secreted cytokines and pro-survival molecules could help tumor to grow. Moreover, eliminating some tumor cells, immune cells could create the condition to the selection of the tumor variants with reduced immunogenicity [81]. These barriers of tolerance have to be overcome in a successful cancer therapy.

However the cohabitation of tumor and immune cells had opened to develop strategies that could take advantage of the immune cells weapons against tumors.

The starting points for immunotherapies against cancer was dated in 1890 when William Coley found that soft tissue sarcoma regressed in patients having acute bacterial infections. One of the first indications that metastatic human cancers could be eliminated using immunological manipulations came from studies of the administration of autologous lymphokine-activated killer (LAK) cells to patient with metastatic melanoma, colon cancer or renal-cell cancer. The great advances in the knowledge about molecular and cellular role of immune system against cancer encouraged to study new strategies to create new drugs to fight cancer. In this category of therapy we could find different classes of therapeutics that take advantage of different aspects of the immune system. Cancer immunotherapy approaches concentrate on killing tumor cells through effector cells of the immune system, which include B-cells, producing antibodies, $CD8^+$ cytotoxic lymphocytes, $CD4^+$ helper T cells, NK cells and NK-T cells. In the last 15 years, the most selective successful immunotherapy strategy for treating patients with hematological malignancies and solid tumors was antibody-based therapy.

### 1.13 Antibody structure and function

Antibodies (Ab), known also as immunoglobulins or as γ-globulins, are glycoproteins produced by differentiated B-lymphocytes named plasma cells. Abs could be expressed on B-cells plasma membrane or secreted like soluble molecules.

On the basis of differences in constant domains, antibody molecules can be divided into distinct classes, called isotypes: IgA, IgD, IgE, IgG, and IgM depending on which heavy chain they contain (α, δ, ε, γ, μ). IgG Abs consist of four disulfide bonds joined polypeptides, two identical heavy chains and two identical light chains joined to form a "Y" shaped molecule of about 150 kDa in which we could identify a steam and two identical arms that contain the antigen-binding sites (Figure 1.4). These proteins have as principal function that of recognize a particular aminoacidic (aa) sequence, called epitope, of a particular protein, the antigen. It was estimated that a mammal is able to produce more than 100 million Abs, each able to bind to a unique different epitope. This specificity of recognition is due to the presence, at the N-terminal of each chain, of variable region characterized by a high variability in aminoacidic sequence. Each variable region is composed of 4 low variable regions, called frameworks (FW1,FW2,FW3 and FW4), that support and coordinate 3 hypervariable regions, called complement determining regions (CDR1, CDR2, CDR3), whose sequences are responsible for antigen-binding. Besides the variable regions, Abs contain also 3 constant domains (4 if the Ab is an IgE or an IgM) in the heavy chain (the domains are numbered sequentially from N-terminal to C-terminal: CH1, CH2, CH3 and CH4) and 1 constant domain in the light chain (CL). Between CH1 and CH2 there is a hinge that confers flexibility to antibody arms. The stem region, formed by CH2 and CH3 (and CH4 if present), is called Fc (fragment crystallizable) and is responsible of the Ab biological activity. With a particular aa sequence present in the CH2 domain, Fc could recruit complement, leading to complement-mediated lysis of the targeted cell (CDC). In an analog way, each class of antibody contains a sequence in the CH3 domain that could be recognized by the class-specific Fc receptors (FcRα, δ, ε, γ, μ). FcRs are expressed differentially on immune cells like NK, dendritic cells, macrophages, mast cells and granulocytes. Depending to the recruited immune cell, the binding between Fc and FcR generates different responses against the antigen that will lead

to antibody-dependent cell-mediated cytotoxicity (ADCC), enhanced phagocytosis or in some cases allergy (mast cell degranulation).

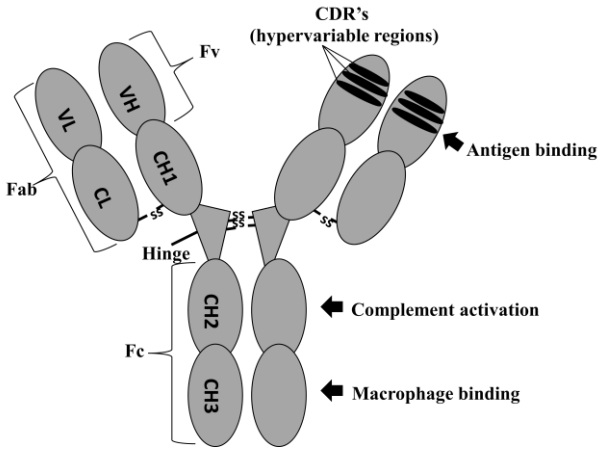


**Figure 1.4**. **Immunoglobulin G structure**

## 1.14 Monoclonal antibody and cancer therapy

Paul Ehrlich was the first who proposed an Ab therapy more than a century ago, but only in 1986 the first Ab, the murine antiCD3 Muromonab, had been approved by Food and Drug Administration (FDA) for human use. In 1976 the development of the hybridoma technology by Kohler and Milstein allowed to produce sufficient quantities of these reagents enabling clinical applicability of Ab therapy [82]. The Abs firstly used in human were of murine origin and, beyond some appreciable results, they encountered some immunogenicity problems because they were recognized as non-self in human inducing the formation of complexes after repeated administration, which resulted in mild allergic reactions and sometimes anaphylactic shock. The second problem is the inefficient stimulation of cytotoxicity because the murine Fc could not be recognized by human Fc receptors. Over the years with Polymerase Chain Reaction (PCR) discovery, Transgenic Mice generation and Phage Display Ab Library [83], hybridoma technology has been

replaced by recombinant DNA technology. With these techniques, it was possible to convert existing mouse mAbs or to de-novo generate chimeric, humanized or directly human mAbs with decreased immunogenicity and with an increased ability to activate the effector immune mechanisms [84]. Chimeric Abs are obtained by joining the antigen-binding variable domains of a mouse mAb to human constant domains. Humanized Abs are created by grafting the antigen-binding loops, (CDRs), from a mouse mAb into a human IgG.

The selection of the optimal cell surface antigen and targeting Ab is crucial to the success of a therapeutic program. An ideal antigen for mAb based-cancer immunotherapy is abundantly expressed on tumor cells, much less abundantly expressed on normal tissues and not ‘shed’ into the bloodstream, circumstance that could increase the sequestration of mAbs. Antigenic targets are usually tumour cell surface-expressed macromolecules, which are easily accessible from the blood and the extracellular fluid. Using currently available technological platforms, it is now possible to produce highly functional Abs against virtually any antigen or epitope. However, until recently, the number of clinically successful target antigens to which these technologies can be applied was surprisingly small.

In therapy the specificity of mAbs could be used to induce direct toxicity by ADCC or CDC, to prevent soluble grow factors from binding to cognate membrane receptors or to redirect against target antigens, toxic compounds, like radioactive isotopes, toxins or chemotherapeutic drugs [85].

Commonly, the mechanism of action of anti-cancer therapeutic mAbs is based on tumor destruction based on ADCC, ADCP (Ab dependent cellular phagocytosis) or CDC (figure 1.5). ADCC and ACDP are mechanisms of action by which effector cells are recruited by Fc binding to FcR expressed by NKs or macrophages respectively. Macrophages express all 3 FcR subtypes (FcγRIIIa/CD16, FcγRIIa/CD32 and FcγRI/CD64) and FcγRI is involved in ADCP activity, whereas NK cells, which are predominantly involved in ADCC activities, express

exclusively FcγRIIIa. These two mAb-mediated target cell killing are quite different: during ADCC NKs cells directly lyse tumor cells leading to antigen presentation and activation of adaptive immune components against cancer cells [84]; via ADCP macrophages destruct target cells by phagocytosis. CDC is the way based on the classical pathway of complement cascade that results in the formation of pores in mAb-targeted cell membrane, event that provokes cell death [86]. The contribution in the anti-cancer effects of Rituximab (antiCD20), Trastuzumab (antiHER2) and Cetuximab (antiEGFR) has been indicated in the clinical studies [86-88].

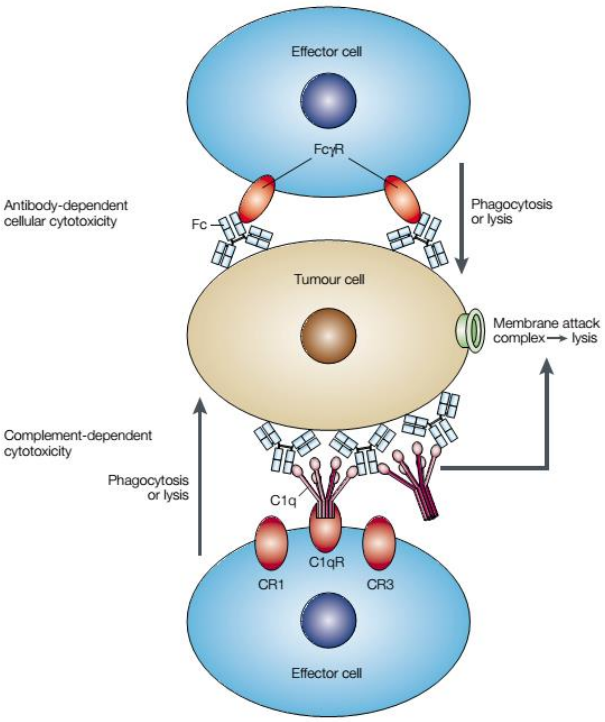


**Figure 1.5. Antibody effector functions [89]**

A second mAb mechanism of action consists of blocking physically the binding of the receptor to its ligand. It is the case of Cetuximab [90] and Panitumumab [91], which target cancer overexpressed EGFR, Pertuzumab [92] and Trastuzumab [93],

which target HER-2, and Bevacizumab [94], which targets VEGFA. Clinical activities of the these mAbs are promising, they provide high response and cure rates with increased survival advantages and also increased overall survival in patients with recurrent or metastatic disease [95]. In combination with standard chemotherapy, these agents reached a significant anti-tumor response in patients with breast cancer, metastatic colorectum cancers and in chemotherapy-refractory cancers [96-99].

The applications and efficacy of mAbs for anti-cancer therapy can be further improved by administrating them in combination with other anti-cancer therapies such as chemotherapy, radiotherapy, targeted therapy agents and cancer vaccines. Besides the applications of unconjugated (naked) mAbs, mAbs have been also conjugated to radioactive isotopes, toxins or chemotherapeutic drugs so that the toxicity of these agents is exclusively targeted to tumour cells. In cancer treatment, it is highly desirable to selectively target malignant cells sparing healthy tissues. However, cancer treatment usually remains a double-edged sword, therapeutic agents should be as aggressive as possible to kill the tumor cells, but it is precisely this aggressiveness that often causes severe side effects. It is for this reason that some promising chemotherapeutics cannot be delivered systemically. The specificity of monoclonal Abs to bind only particular targets extends the use of these proteins to vehiculate a toxic compound directly on tumor cells: the goal is to limit the toxicity of targeted compounds to tumour cells, leaving unaffected healthy cells.

To enhance the potency of antitumor mAbs, they could be direct armed with a radioactive atom (radioimmunotherapy), a chemotherapeutic drug (chemo-conjugated mAb) or a plant-derived or bacterial toxin (immunotoxins) [100]. Moreover mAbs could be used to functionalize the surface of liposomes and nanoparticles loaded with drugs/toxins for tumor specific delivery (figure 1.6).

Currently $^{90}$Y-ibritumomab tiuxetan (Zevalin®) and $^{131}$I-tositumomab (Bexxar®) are the unique FDA approved radioimmunotherapy agents. They are directed against the tumor associated antigen CD20 and used for the treatment of relapsed non-Hodgkin's lymphoma with great clinical results [101-103]. Other radioimmunotherapeutical drugs, to fight also solid tumors, are being in clinical trials demonstrating that the approach seems to be promising also for these types of cancers. Time will reveal whether this approach can be further developed and provide even better clinical outcomes [104].

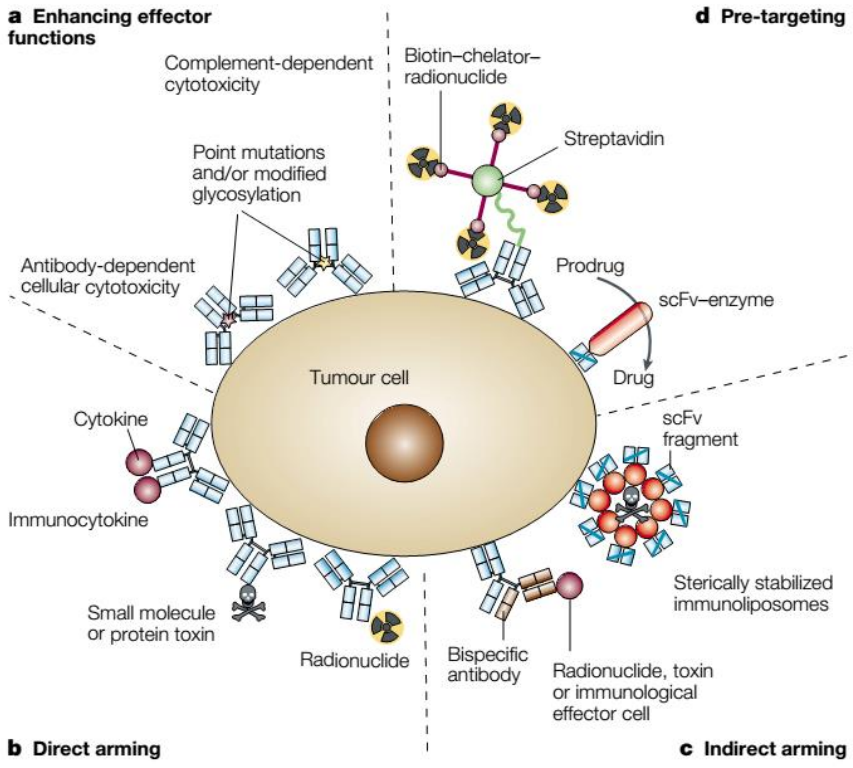


**Figure 1.6. Strategies for enhancing the potency of antitumor antibodies [89]**

Also Abs drug conjugates (ADC) development has demonstrate the potency of mAb to more efficiently vehiculate drugs to a tumor-associated target.

Brentuximab Vedotin (SGN-35 or Adcetris) [105] and Trastuzumab Emtansine (Kadcyla or T-DM1) have been approved by FDA after the successes obtained in clinical trials. These ADCs have been coupled respectively to mono-methylauristatin E (MMAE) or to mertansine, which are both powerful inhibitors of microtubule polymerization [105]. Spurred by these successes, drug discovery companies are ramping up development of a lot of ADCs. Much of the initial search for uses of ADCs was performed in hematological cancers, and five of eight ADCs in Phase II and III trials have been designed to treat blood cancers. Partly, this imbalance was driven by an early view that ADCs would penetrate poorly into solid tumors. However, the success of trastuzumab-DM1 in metastatic breast cancer has done away with the standpoint that ADCs are only useful against blood cancers.

The third strategy to employ mAb to vehiculate toxic substances to kill cancer cells is to generate immunotoxins. Immunotoxins are molecules that couple mAbs to plant or bacterial toxins. The first developed toxins for this purpose included gelonin, ricin, abrin, pokeweed antiviral protein, Pseudomonas exotoxin and Diphtheria toxin [106]. However, rapid clearance from blood and immunogenicity conduct to the generation of the second cohort immunotoxins such as BL22 and moxetumomab pasudotox [107]. Both immunotoxins are antiCD22-Pseudomonas exotoxins that are being tested in clinical trials for the treatment of B-cell malignancies and other hematological malignancies [108].

## 1.15 Harnessing the anti-tumour potential of T cells

T cells belong to the adaptive immune system and are further subdivided into $CD4^+$ and $CD8^+$ T cells, referring to co-receptors on their surfaces. $CD8^+$ cytotoxic T lymphocyte (CTL) precursors do not show lytic activity. CTLs are activated after the stimulation of the T cell receptor (TCR) by antigens charged on MHC molecules plus CD28 co-stimulation. After activation, lymphocytes are able to kill

malignant cells by the death receptor or the granule-mediated killing pathways. In the death-receptor mediated pathway TNF superfamily ligands, like TRAIL, are expressed in response to signal cascade induced by TCR activation and transported to T-cells surface. As described above, these ligands bind their receptors on tumor cells inducing the extrinsic apoptotic pathway. However the main mechanism of T-cell mediated killing is the granule mediated pathway. The recognition by T cell receptor of its cognate MHC-antigen complex on target cells allows the formation of an immunological synapse between the two cells. The synapse is stabilized by ICAM-1 and LFA-1 adhesion molecules allowing the formation of a tight intercellular junction. After TCR activation CTLs mobilize cytotoxic granules and secrete perforin and granzymes in the intercellular space. Perforins polymerize and initiate the formation of ring-like structures with a central pore in the target cell membrane, which allow granzymes to pass into the target cell [109;110]. Granzyme B is the most known member of a subclass of serine proteases and contributes to target cell destruction via different pathways (figure 1.7) [111]. Granzyme B could allow the release of the cytoplasmic endonuclease caspase-activated DNAse (CAD) from its inhibitor (ICAD), thus inducing chromosomal DNA fragmentation and chromatin degradation during apoptosis. It could act directly or by cleaving the executioner pro-caspase-3 that causes the activation of PARP that subsequently cleave ICAD [112]. Granzyme B could also truncate Bid, beginning the mitochondrial death pathway [113].

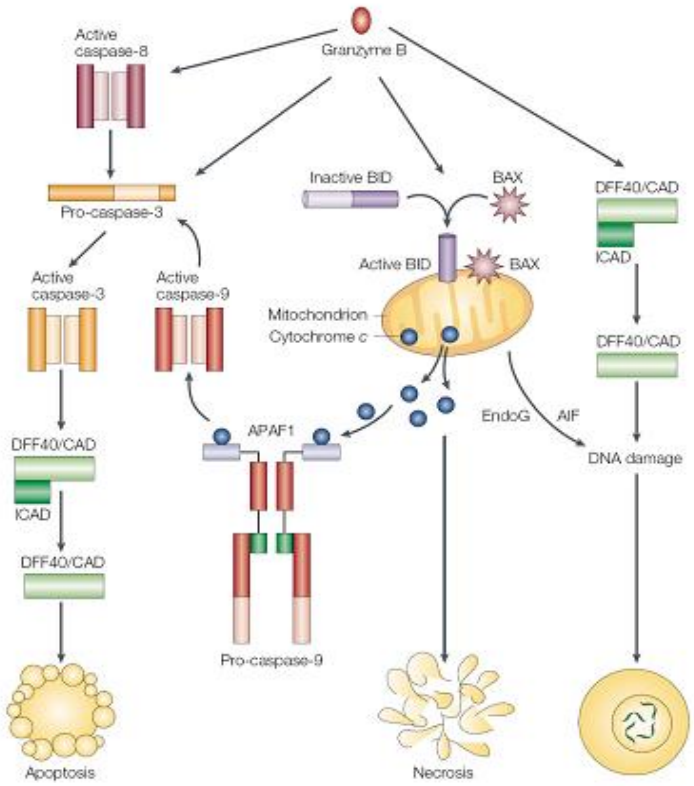


**Figure 1.7 Granzyme B-mediated apoptotic signals [114]**

CTLs are the most potent anti-tumor cells of the immune system. Some of the most exciting developments in cancer immunotherapy are those approaches that harness CTLs [114]. Although CTLs have the potential to recognize and destroy malignant cells, there are a number of inhibitory pathways (immune check-points) into the immune system that are crucial for the maintenance of self-tolerance in order to prevent autoimmunity. Furthermore, tumors are able to adopt certain immune-checkpoint pathways as a major mechanism of immune resistance, particularly against T cells that are specific for tumour antigens. Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is a key immune check-point that attenuates T cell activation. Ipilimumab is a monoclonal antibody directed against CTLA-4 and was the first agent able to demonstrate a survival benefit in patients with advanced

melanoma; Ipilimumab was approved by the FDA in March 2011 [115]. By blocking CTLA-4, Ipilimumab dampens the inhibitory signals that curtail the full power of CTLs to destroy cancer cells. Blockers of other immune-checkpoint proteins, such as programmed cell death protein-1 (PD-1) or its ligand PDL-1 are also showing promising anti-tumour effects in clinical trials.

### 1.16 Bispecific antibodies

A very effective approach for harnessing the anti-tumour potential of T cells is the use of bispecific antibodies (BsAbs). BsAbs are molecules that carry a different antigen binding capability on each arm. The possibility to obtain BsAbs with two different specificities was described more than 50 years ago and these reagents were first used in clinic in the early 1990s. The first generation of BsAbs was produced by chemical-linking of Abs with different specificities followed by quadroma technology (also called hybrid hybridomas) [116]. The use of these molecules was limited because of the difficulty to generate large, homogeneous batches of a well-defined and clinically useful product, due to the random combination of two mAbs in chemical cross-linking, or the random association of the two parental heavy (H) and light (L) chains that join spontaneously by Fc pairing forming also homodimers and H–L chain mismatches within one cell in the case of quadroma technique. Quadroma cells produce a mixture of ten different antibodies: only one molecule out of the mixture of potential products is the wanted BsAb and results very difficult to purify it. With advances in protein engineering and recombinant DNA technologies, homodimer formation and H-L mismatch problems were solved using knob into hole technology and chimeric mouse/rat quadroma cells. Besides the quadroma technique, other methods have been developed to generate different formats of bsAbs. New recombinant formats using antibody fragments were developed. At present there are more than 50 different ways to generate BsAbs [117] (figure 1.8). Recombinant BsAb formats include the

development of molecules more compact than IgG-based BsAb: bispecific tandem single chain ScFv antibodies (TaScFv) and diabodies (BsDb) [118;119]. BsDbs are produced from two different single chain variable fragments (scFv), comprising the heavy variable domain of one and the light variable domain of the other paternal mAb. In these scFvs the polypeptide linker connecting the variable domains is reduced to about five amino acid residues, thus forcing the crossover pairing of the two scFv polypeptide chains. Even though such BsDbs can be produced with high yield in bacteria, significant drawbacks of this approach are their reduced stability and the presence of inactive homodimers along with the functional heterodimers. In part these problems were overcome by introducing artificial cysteine residues that can be oxidized leading to stable disulfide bridges between the two scFvs in a diabody. A more promising format was developed by fusing the two antibody domains resulting in single-chain diabodies (scDb). ScDbs contain another peptidic linker of about 15 aa that connect the two polypeptide chains allowing the more efficient pairing between cognate variable domains [120]. TaScFvs consist of two different scFvs arranged in a unique chain, by fusing one to the C-terminus to the other, with the help of a short polypeptide linker. In this case, the two scFvs present in the TaScFv format separate folding entities.

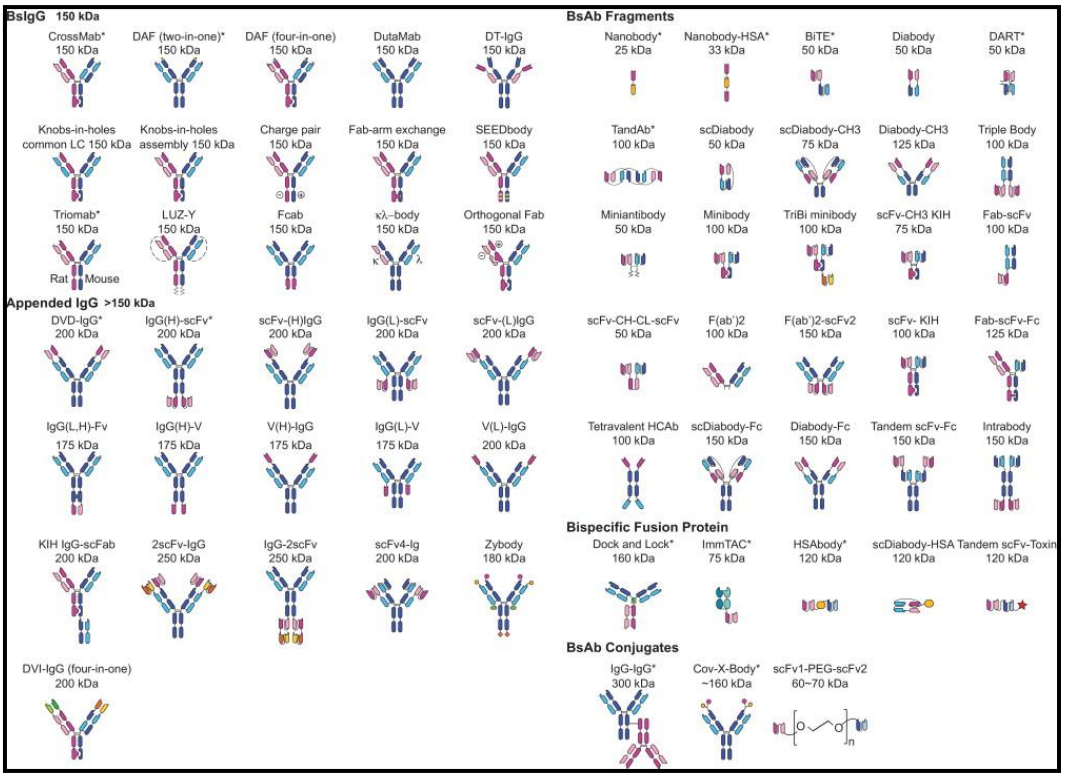


**Figure 1.8. BsAb formats that at the moment are been described in literature**
They could be subdivided into five major classes: IgG based BsAbs (**BsIgGs**) whose structure is similar to the normal IgG; **appended IgGs** in which to the IgG structure are linked other binding domains; **BsAb fragments** in which the arms are built using part of antibody like scFvs, Fab or in general variable domain joined by peptidic or chemical linkers or fragment of the constant portion of mAbs**; bispecific fusion proteins** that are composed of building blocks derived from antibody and from other proteins (for example albumin, toxins, TCR…) and **BsAb conjugates**.
**Legend**. Dark blue, dark green or dark pink: heavy chains of both specificities; light blue, light green and light pink: corresponding light chains. Thin lines: connecting and engineered disulfide bonds. BsAb formats that have advanced into clinical testing are highlighted (*). For a more detailed explanation of various formats please refer to the review from which this figure was taken [121].

## 1.17 Bispecific antibodies for targeted tumor therapy

MAb targeted immunotherapy cannot take advantage of the weapons of T-cells because they lack the Fc receptors. A class of bispecific antibodies was developed

to recruit tumor-resident and circulating $CD4^+$ and $CD8^+$ T cells for the destruction of tumour cells: they are equipped with one arm that could bind to an antigen on the tumour cell surface and another arm that could engage CD3. T-cell maturation, proliferation and activation are initiated upon binding to the invariant CD3 molecules associated to TCR. While the TCR sequence is variable from one lymphocyte to another, CD3 sequences are constant, a property that makes these proteins ideal as a target on T cells to redirect them to tumor cells with BsAbs. Between 1990 and 1997 different BsAbs were developed for T-cell retargeting: they were all first generation IgG-based molecules and had the production problems described above. Only four reagents reached the clinical trial phase: the antiCD19 × antiCD3, SHR-1, for treatment of non-Hodgkin’s lymphomas [122], the anti-epidermal GP2 × antiCD3, BIS-1, for treatment of renal and lung cancers [123], an anti-glioma × antiCD3 BsAb [124] and the anti-alpha folate receptor × antiCD3, OC/TR, for treatment of ovarian cancer that was produced in the lab where this thesis was performed [125]. The failure of these reagents was mainly due to the difficult in production and purification that increase enormously the costs inducing to the abandonment of these projects.

The growing knowledge in immune system mechanisms and the development of new approaches to avoid side effects have brought to the second generation class of BsAbs. Different formats of recombinant bispecific antibodies, described in the previous chapter, have been introduced and there are a number of studies, which demonstrated their efficiency in targeting malignancies in preclinical and clinical settings [126]. The first BsAb approved for clinical use was Catumaxomab (Removab). Catumaxomab (antiEpCAM × antiCD3) is a Triomab, an IgG-based BsAb produced using the quadroma technique: one arm is derived from mouse IgG2a and the other from rat IgG2b (TRION platform; TRION Pharma, Munich, Germany). In addition to the good results obtained in production and purification (reduction of H–L chain mismatch because of preferential intraspecies pairing),

triomabs have an Fc portion that can react with both human FcgRI and FcgRIII allowing the formation of a bridge between three cells: tumor target cell, T cell and accessory cell (macrophage, dendritic cell or NK cell). It was approved for the treatment of malignant ascites in patients with $EpCAM^+$ cancer [127].

In last decade small size recombinant antibodies have been produced: BsDbs, ScDbs and TaScFvs. The small size of these reagents (55 to 60 kDa molecular weight) allows them to bring T cells and tumor cells into close proximity when both cell populations are bound to the respective binding arm with the creation of an immunocytolitic synapsis. Several reports highlight potent anti-tumor response of BsDbs and scDbs in vitro and in preclinical models [128-131]. Although the promising preclinical results as therapeutic compounds, no BsDb or scDb entered into clinical trials so far [132].

The TaScFv format was the other major studied format of recombinant single-chain BsAb able to efficiently retarget T-cells. Several TaScFvs have shown their anti-tumoral efficacy against $CD33^+$ AML cells [133] or PSMA/PSCA positive prostate cancer cells [134]. Among BsAbs constructed in this format, Bispecific T-cell Engagers (BiTE) gained particular interest.

The BiTE Blinatumomab (named also Blincyto or MT-103) was the first BsAb approved by FDA. Blinatumomab is able to retarget T-cell to $CD19^+$ lymphoma and leukemia cells. This event leads to T-cells activation without the CD28- or CD4/CD8-mediated second stimulus that is generally required to activate T-cells. Blinatumomab is highly cytotoxic at picomolar concentration only when both arms are engaged [135]. Blinatumomab was approved by FDA for the treatment of Philadelphia chromosome-negative precursor B-cell acute lymphoblastic leukemia (B-cell ALL) and is the most advanced BiTE in clinical trials for the treatment of other leukemias and lymphomas [136;137]. Because of its small size BiTEs have a short serum half-life and in order to achieve the required concentration continuous infusion is required. Another BiTE is currently undergoing Phase I clinical studies:

the EpCAMxCD3 (MT110) is being tested for the treatment of solid tumors. Several new BiTEs are been developed using new isolated scFvs (for example antiCD33 and anti-melanoma associated chondroitin sulfate proteoglycan) or reformatting approved therapeutic antibodies, like Panitumumab or Cetuximab, as BiTE molecules [138].

Both formats seem to have advantages and disadvantages: scDbs are more resistant to proteases activity while domains of TaScFvs are more flexible. TaScFvs have a structure that could be more easily manipulated respect to scDbs. The more important feature to compare was if there would be a difference in term of efficacy. In a first study, the comparison between a ScDb and a TaScFv targeting the same TAA showed that TaScFvs are far more superior to scDbs [139]. However, the antibody components were not completely identical and a clear conclusion remained open. In the unique other study in which both antiPSCA BsAbs were prepared from the same antibody domains, the comparison of the two formats did not show obvious differences [134].

Bispecific antibodies as a way of re-directing T cell could make a significant contribution in the future to the immunotherapy of cancer.

## 1.18 Phage display

Phage display technology consists in the selection of peptides and proteins from combinatorial libraries displayed on the surface of filamentous phage [83]. This methodology is becoming an important tool in biotechnology for the generation of diagnostic and therapeutic mAbs [140] and for the study of natural immune responses. A crucial advantage of this technology is the linkage of displayed antibody phenotype with its encapsulated genotype which allows the evolution of the selected binders into optimized molecules and permits the rapid determination of the aminoacidic sequence of the specific binding peptide or protein molecule by DNA sequencing of the specific insert in the phage genome (Figure 1.9).

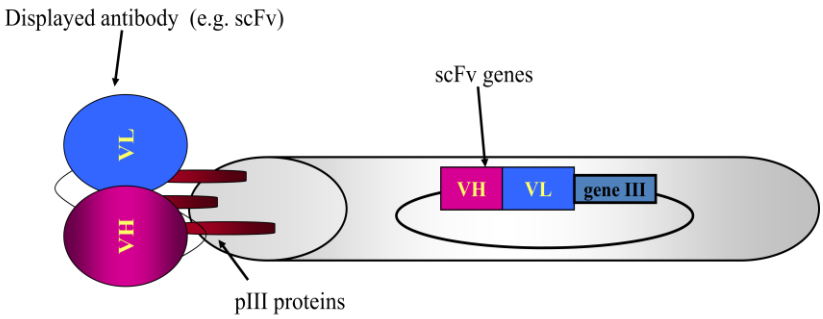


**Figure 1.9. ScFv expression on the surface of the filamentous phage**

Human antibody fragments against a variety of antigens could be isolated from diverse human antibody libraries. There are essentially three kinds of libraries: naïve, synthetic and immune. Naïve libraries are composed by antibody variable genes isolated from healthy donors or non-immunized animals. In synthetic libraries a combinatorial diversity is created by rearranging VH and VL gene segments *in vitro* and introducing artificial CDRs of varying loop lengths using PCR and randomized primers. Immune libraries are constructed using antibody V genes obtained from immunized animals, vaccinated patients or from patients that responded to a specific disease.

The phage antibody library could be selected against a specific antigen. The target antigen would be immobilized on a solid support and the phage library would be coincubated to allow binding of specific phages. Non-specific phages have no binding and could be washed away. After 3-5 cycles, specific phages enrichment occurred and phages were isolated and sequenced (figure 1.10).

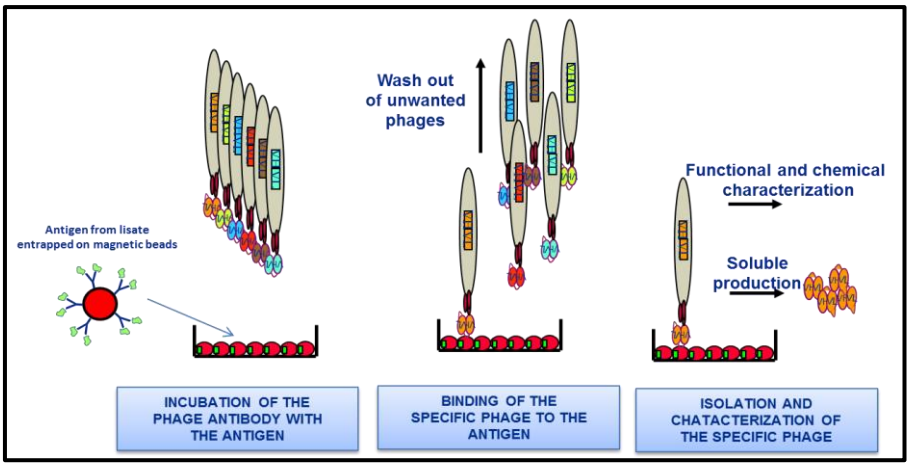


**Figure 1.10. Cartoon representing a cycle of panning (selection) of a phage display Ab library**

## Aim of the Project

Cancer is a leading cause of death worldwide. Despite remarkable progress in understanding and in treatment of some forms of the disease, in the majority of cases, new treatment strategies, although with good results in preclinical studies, have often failed in subsequent clinical trials.

The discovery of TRAIL, a natural cytokine member of the Tumor necrosis Factor superfamily, opened new possibilities for the development of new cancer therapeutics. In particular TRAIL, after binding TRAIL-R1/R2, is able to trigger apoptosis in tumor cells sparing normal cells. Recombinant form of TRAIL (rhTRAIL) showed good preclinical results but failed in clinical trials.

The lack of activity of rhTRAIL is due to its sequestration by decoy receptors (DcR) diminishing apoptosis activation. New rhTRAIL and agonistic antibodies, whose mimic TRAIL action, had the ability to bind exclusively TRAIL-R1 or TRAIL-R2 avoiding the DcR-sequestration. However tumor cells demonstrated new resistance mechanisms to this type of therapy.

In last decades scientists began studying methods to take advantage of T-cells potency in cancer therapy redirecting them on tumor independently from the TCR-defined specificity. Among different approaches, one of the most promising is the use of bispecific antibodies (BsAbs) that, by simultaneously recognizing an antigen on the cancer cells and an activating receptor on the surface of immune effector cells, offer an opportunity to redirect immune effector cells to kill cancer cells.

In this thesis it was proposed to evaluate the antitumor effects of targeting TRAIL-R2 by a novel BsAb, in scDb format, developed for recognizing this antigen and the CD3 epsilon chain of T-lymphocytes. The BsAb, binding TRAIL-R2, could trigger apoptosis in tumor cells. Its smaller size, compared with agonistic mAbs, has the advantage to allow a deeper penetration in the tumor. Moreover TRAIL-R2 should be a good target for Ab-based immunotherapy because it is overexpressed in tumor cells compared with normal cells. Using the BsAb, the TRAIL-resistance

could be bypassed using the second mechanism of action of the molecule. The BsAb, having a second arm against CD3, could redirect T-cells in proximity to tumor cells. In this way T-cells could be activated and lyse the tumor.

# 2. Methods

## 2.1 Cell lines

All the cell lines used in this thesis were listed in table 2.1. All cell lines were grown according to the culture media indicated in the table with 10% fetal calf serum and 2 mM L-glutamine, in a humidified atmosphere with 5% CO2 at 37°C.

**Table 2.1. Cell lines used.**

| Cell line | Histotype | Culture Medium | Source |
|---|---|---|---|
| M15 | Metastatic Melanoma | RPMI-1640 | Provided by Dr. Andrea Anichini [141] |
| M64 | Metastatic Melanoma | RPMI-1640 | Provided by Dr. Andrea Anichini [141] |
| M41 | Metastatic Melanoma | RPMI-1640 | Provided by Dr. Andrea Anichini [141] |
| A431 | Epidermoid epithelial carcinoma | RPMI-1640 | Laboratory cell bank, ATCC |
| HeLa | Cervix epithelial adenocarcinoma | DMEM | Laboratory cell bank, ATCC |
| INTOv11 | Epithelial ovarian carcinoma | RPMI-1640 | Cell line established in our laboratory |
| SKOV3 | Epithelial ovarian carcinoma | RPMI-1640 | Laboratory cell bank, ATCC |
| A2774 | Epithelial ovarian carcinoma | RPMI-1640 | Laboratory cell bank, ATCC |
| A2780 | Epithelial ovarian carcinoma | RPMI-1640 | Laboratory cell bank, ATCC |
| NL3507 | Epithelial ovarian carcinoma | RPMI-1640 | Laboratory cell bank, ATCC |
| MDA-MB-231 | Triple negative breast cancer | RPMI-1640 | Laboratory cell bank, ATCC |
| MDA-MB-468 | Triple negative breast cancer | DMEM | Laboratory cell bank, ATCC |
| MT-3 | Triple negative breast cancer | DMEM | Provided by Dr. Nadia Zaffaroni |
| HEK-293 | Normal embryonic kidney | DMEM | Laboratory cell bank, ATCC |
| Jurkat | Non Hodgkin Lymphoma | RPMI-1640 | Laboratory cell bank, ATCC |
| SU-DHL-4 | Non Hodgkin Lymphoma | RPMI-1640 | Laboratory cell bank, ATCC |

All media, sodium pyruvate, non-essential amminoacids, and L-glutamine were purchased from Hazleton Biologics, Inc. (Lenexa, US), and the fetal calf serum

from HyClone (Logan, US). Hybridoma producing the mAb the antiMyc tag mAb 9E10 (CRL-1729) was purchased from ATCC.

### 2.2 Bacterial strains

Escherichia coli strains TG1 {supEthi-1 ((lac-proAB) hsd (5[F' traD36+ proAB+ lacIq lacZ(M15)]} was used in antibody phage display technique and HB2151 {nalr thi-1 ara lac-proAB [F' proAB+ laciq lacZ(M15)]} was used for soluble production of the bispecific antibody.

### 2.3 Bispecific antibody phage library construction

ScDb was the format that was chosen to construct the phage display antibody library. A scDb format gene cassette ($VH_{TRAIL\text{-}R2}$-short Linker-$VL_{CD3}$-flexible Linker-$VH_{CD3}$-short Linker-$VL_{TRAIL\text{-}R2}$) was designed and synthetized by GeneArt (Life Technologies). The cassette contains two cloning sites flanked by restriction enzymes sites to clone the antiTRAIL-R2 variable domains: the first site is flanked by SfiI and ApalI for the insertion of the VH domain (Figure 2.1 – blue moiety) and the second site by NheI and NotI for the insertion of the VL domain (Figure 2.1 – red moiety). This cassette, which contains the antiCD3 epsilon-chain humanized UCTH1 variable domains at the centre of the sequence (Fig. 2.1- green moieties), was cloned into the phagemid vector pIT2 which is a derivative of pHEN1 [142]. Phagemids containing scFvs isolated after the second panning of naïve scFv library versus TRAIL-R2 protein (procedure described in Part III of this thesis) were collected using Quiafilter Midiprep (Qiagen) and used to amplify variable domains by PCR. In this amplification primers representative of the human VH-JH and VL-JL (λ and κ) domains were used. Restriction enzyme sequences for SfiI and ApalI or for NheI and NotI were added at the extremities of respectively VH and VL fragments.

PCR products were cut with correspondent restriction enzymes and cloned in the gene cassette to obtain the scDb phage library (figure 2.1).

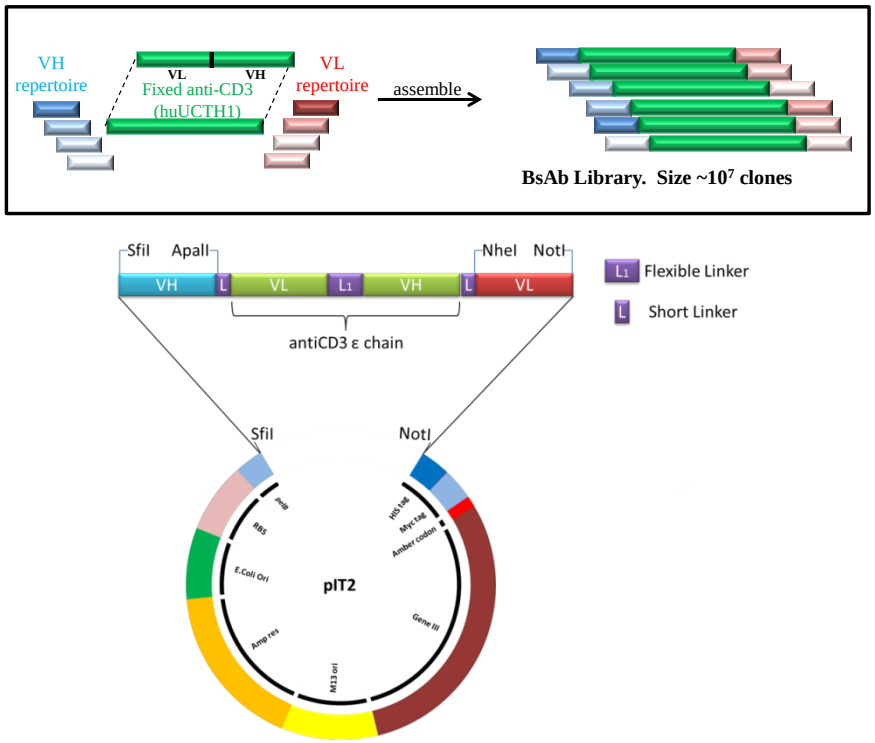


**Figure 2.1.** Cartoon representing the construction of a phage scDb antibody library as described in the text. Gene cassettes were engineered and cloned in piT2 vector.

## 2.4 Antibody Phage Display

A pre-made human ScFv phage library (proprietary) or the produced BsAb phage library were used and 3 rounds of selection versus TRAIL-R2. With single colonies derived after the enrichment, single phage ELISA was performed. All experiments were performed as described [140].

## 2.5 Bispecific antibody soluble expression

Soluble bispecific antibody was produced in HB2151 E.Coli and periplasmic preparations were purified using IMAC protocol with Nickel or L protein chromatography column as described [140].

### 2.6 Binding specificity of the recombinant antibody: FACS.

All the experiments procedures were performed as described [144]. BsAbs were detected using antiTag antibody followed by antiMouse IgG (H+L specific) Alexa 488 labeled. As positive control, mouse anti-human TRAIL-R2 (R&D Systems) and mouse TR66 mAb, derived from Hybridoma, were used. Fluorescence labeling was measured using FACSCalibur instrument (Becton Dickinson, Heidelberg, Germany) and data analysis was performed using FlowJo software (Tree Star Inc).

### 2.7 Dimerization and tetramerization of BsAb

To dimerize the BsAb, murine antiHis-tag mAb was incubated with a double molar concentration of BsAb for one hour in PBS at RT. Tetramerization was performed incubating 1 part of antiMouse mAb Fc-specific (SIGMA) with double concentration of antiHis-tag mAb (Pierce) and quadruple concentration of BsAb for 90 minutes in PBS at RT (figure 2.2).

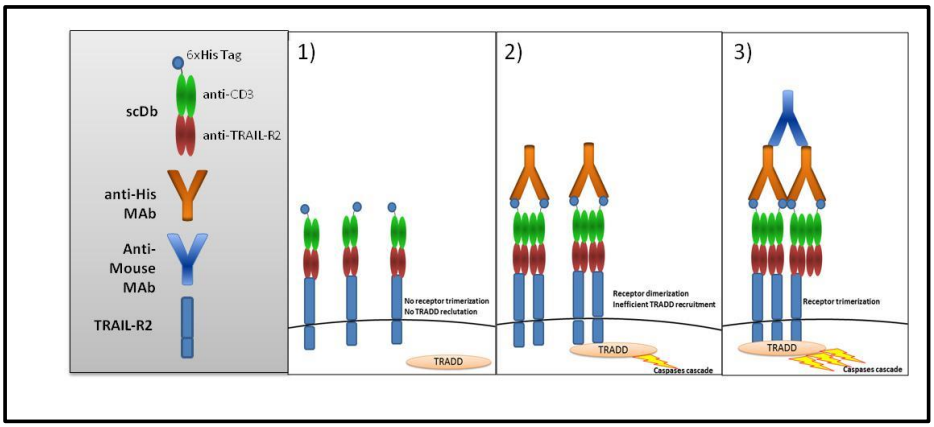


**Figure 2.2. ScDb multimerization**

Cartoon representing the way by which scDb has been multimerized: antiTag mAb could bind tag present on scDbs inducing dimerizion (2). The addition of antiMouse mAb to the antiTag/scDb complex induces tetramerization of BsAbs (3).

### 2.8 BIAcore - Surface Plasmon Resonance (SPR) analysis

SPR experiments were performed using Biacore 2000 (GE Healthcare). Standard EDC\NHS coupling was used to covalently immobilized recombinant TRAIL-R2 (R&D System) on CM5 (GE Healthcare) sensor chip. TRAIL-R2 immobilization was performed as described [151].

Binding Analysis Different concentrations of scDb or cross-linked scDb freshly diluted in HBS (12.5 nM, 50 nM and 200 nM) has been used to assay the binding and set up the system. The restoring of initial baseline was verified after each injection.

Kinetic analyses were performed at concentrations ranging from 400 to 25 nM of scDb. The data obtained were analyzed by the BIAevaluation software 3.2 (global fitting) assuming a 1:1 Langmuir-binding model.

Competition assay for the receptor between sTRAIL and the scDb was performed using 1 µM of sTRAIL to saturate all the receptor coated on the chip. Once saturation occurred, 400 nM scDb was injected on sTRAIL saturated TRAIL-R2.

**Abbreviations used**: NHS, N-hydroxysuccinimide; EDC, (N-ethyl-N-(3-dimethylaminopropyl) carbodiimide.

### 2.9 Biochemical characterization and integrity

The scDb size and the homogeneity were analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), western blotting and with mass spectrometry (SELDI-TOF).

*SDS PAGE-WESTERN BLOT*

Two samples containing 1 µg of purified scDb from two different batches of purification were used to test integrity of the molecule.

Sample preparation, SDS-PAGE, Coomassie staining and western blot were performed as described [140].

*SELDI-TOF*

Surface-enhanced laser desorption/ionization Time-of –flight mass spectrometry was performed following sample preparation, mass spectrometry acquisition and data analysis as described [143].

*SIZE EXCLUSION CHROMATOGRAPHY*

Potential dimerization was analyzed by size exclusion chromatography on a SUPEROSE 12 10/300 column (range separation: 300 Kd-10 Kd; GE Healthcare) as described [144].

## 2.10 Isolation of PBLs

Healthy donor buffy coats were gently provided by Dr Ravagnani (Head of INT Immuno-hematology and Transfusional Medicine unit). PBLs were isolated from buffy coat by Ficoll density gradient centrifugation standard protocol (Ficoll plus hystopaque, GE Healthcare). Isolated PBLs were cultured in RPMI1640 supplemented with 5% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 1 mM non-essential amminoacids, 0.1 mg/ml gentamycin, 1% of vitamin complex and cultured at 37°C with $CO_2$. For direct cytotoxicity assay, PBLs were activated using 150 IU of IL-2 (Chiron Corporation - Emeryville, US) for 4 days before use.

## 2.11 MTT viability assay

*Proliferation inhibition assay*

In the cytotoxicity titration experiment, to determine best dose and ratio, the scDb was used at a concentration of 1, 0.5 and 0.1 ug/ml while the E:T ratio started from 10:1 until 0.15:1 with F2 dilutions. In all the other assays, 0.5 µg/ml of scDb and E:T ratio of 5:1 were used.

Redirected T-cell proliferation inhibition was evaluated by MTT assay using PBLs and a panel of different TRAIL-$R2^+$ cell lines. 1.2 x $10^4$ cells were plated in each

well of a 96-well flat bottom plate with the appropriate medium and incubated ON to allow their attachment. After adding the scDb, the cells were incubated for 1 hour before adding PBLs. As negative controls, were used untreated cells, cells plus only scDb or plus only PBLs. After 48 or 96 hours, supernatant was removed and wells were washed three times with PBS to remove PBLs in suspension. In each well were added 100 µl of not metabolized medium containing 0.5 mg/ml of MTT salt. After 3 hours supernatant was discarded and 150 µl of MTT solvent (isopropanol + 4 mM HCl + 0.1% NP40) were used to dissolve formed formazan agitating the in an orbital shaker for 15 min. Absorbance at 590 nm (620 nm reference filter) was read using Biorad-550 microplate reader.

*Sensitivity to sTRAIL agonistic activity*

MTT assay was used also to assess sTRAIL sensitivity of all cell lines. Cells were incubated for 24 hours with 100 nM of sTRAIL.

*Agonistic activity assay*

Equimolar concentrations (0.5, 5, 50 and 100 µg/ml) of monomeric, dimeric or tetrameric BsAb were used to treat sTRAIL-sensitive M15 cells. Soluble TRAIL at the same concentrations was used as positive control. After 24 hour of incubation at 37°C with 5% $CO_2$, cell viability was analyzed by MTT assay.

### 2.12 Direct cytotoxicity assay

Redirected T-cell citotoxicity was assayed by the Calcein AM (Biovision Inc) release assay. $10^6$ target cells were resuspended in 1 ml of complete medium containing 1-15 µM of calcein-AM, incubated 30 minutes at 37°C and washed 3 times with fresh medium.$10^4$ cells were seeded in 96-well round bottom plates following the same treatment (three triplicates for each) used for proliferation inhibition assay. 6 replicate wells were used for the measurement of spontaneous

release and 6 for maximum release (target cells in medium containing 2% Triton X-100). After 4/16 hours of incubation with pre-activated PBLs with or without scDb, plates were centrifuged at 1500 RPM for 10 minutes and supernatant, containing released fluorescent calcein, was transferred in black walled 96-well plate. Fluorescence intensity was measured by Ultra multiplate reader (Tecan Group, Mannedorf/Zurich, Switzerland), with extinction/emission wavelengths of 485/535nm.

## 2.13 T CELL ACTIVATION ASSAY

Tumor TRAIL-$R2^+$ melanoma (M15, M41 and M64) and ovarian carcinoma (NL3507, A2774 and INTOv11) cells, TRAIL-$R2^+$ Hek-293 normal cells and TRAIl-$R2^-$ MDA-MB-468 cells were used as target cells and were grown with appropriate medium into 48-well plates (Corning) at a density of $3.5 \times 10^4$ cells for well. After 12 hours 0.5 µg/ml of scDb was added and incubated 1h at 37°C with 5% $CO_2$. Freshly isolated human PBLs were used as effector cells and added to the scDb treated/untreated target cells at an effector-to-target ratio of 5:1.

*CD69 and CD25 activation markers evaluation*

T-cell activation assay was performed for 16 h at 37 °C in 5% $CO_2$. T-cells contained in the supernatant were recovered, washed with PBS and stained with a PE conjugated mouse anti-human CD69 (BD Biosciences) and a FITC-conjugated mouse anti-human CD25 (Caltag Laboratories) for 30 min on ice. After washing three times with PBS+FCS 1%, the cells were analyzed by flow cytometry at FACSCalibur.

*Determination of cytokines release (Bioplex)*

T-cell activation assay was performed for 96 hours using M64, M41 and MDA-MB-468. Spontaneous release of PBLs was assessed incubating them with or without scDb. To determine amounts of secreted cytokines, supernatants were

collected daily for 4 days and were analyzed for IFN-γ and TNFα secretion using Bio-plex ProTM Human Cytokine standard 27-Plex, Group I (BIORAD), according to the manufacturer's protocol. The absorption of the samples was measured, and the obtained values were used to calculate the concentration of the cytokines in the samples, according to the values obtained for the standard series provided by the manufacturer.

### 2.14 In vivo experiment

All the procedures involving animals were conducted in conformity with institutional guidelines that comply with national and international laws and policies (D.L. 116/92 and subsequent implementing circulars).

The model that we decide to use is a modification of the established tumor model described before by Friedrich et al [145]. The scheme of treatment used is reported in figure 3.15A.

In our mouse model female NOD/SCID mice received injection of 2.5 x $10^6$ MT-3 cells orthotopically in one nipple. Approximatively 14 days later tumor reached a volume of about 150-200 $mm^3$ and could be considered established. Mice were divided into three groups: mice of the first group were inoculated with only saline; mice of the second and the third were inoculated into lateral tail vein with $10^7$ human non-activated PBLs. One day before PBLs engraftment, mice of all groups were irradiated with 2 Gy of $^{137}$Cs γ rays to eliminate murine circulating NKs. After 3 days mice of the third group received, daily for 18 days, 12.5 mg/kg of BsAb while the second group receive saline. Tumor volumes were monitored twice a week by using a digital caliber and tumor volume was calculated following the formula:

$$V = \frac{4}{3}\pi \text{ x } \frac{d^2}{2} \text{ x } \frac{D}{2}$$

where d is the minor diameter and D the major diameter.

A blood sample was collected at the sacrifice. Mice were monitored for 18 days and afterward mice were euthanized by anaesthetic overdose and cervical dislocation.

### 2.15 Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Bonferroni’s Multiple Comparison Test using PRISM 5 software.

# 3. Main Results

## 3.1 BsAb isolation from a BsAb antibody library

At the beginning of the project we start separately the selection of Ab fragment able to bind TRAIL-R2 from a naïve human scFv Ab phage library. After 3 panning cycles on TRAIL-R2 derived from positive cells lysates, 4 clones able to bind TRAIL-R2 were isolated. They were then cloned in a gene cassette which contains the antiCD3 scFv to generate the BsAb in scDb or TaScFv formats. This strategy failed due to the lack of stability of the constructed bispecific molecules (described in Part III). We decided to change strategy and to construct a library directly in scDb format (refer to figure 2.1). The pool of fragments obtained after 2 panning cycles against TRAIL-R2 was cloned in a vector containing variable domains of an antiCD3 Ab. As antiCD3 we chose the humanized UCTH1. In this way we had the possibility to select a humanized BsAb being composed of a fully human antiTRAIL-R2 scFv and a humanized antiCD3 scFv. The library obtained was heated transiently to induce protein unfolding and to promote aggregations of unstable clones [146]. After cooling, phage display antibody fragments that unfold reversibly were thereby enriched thus discharging molecules unable to refold. After three round of selection on naïve TRAIL-R2 there was an increase of number of positive colonies (figure 3.1A) and an increase in binding of polyclonal phages tested by ELISA on recombinant TRAIL-R2 (figure 3.1B). Single colonies, each containing a different BsAb-expressing clone, were tested in ELISA to isolate antiTRAIL-R2 specific clones. The assay was performed using the TRAIL-R2 expressing M15 melanoma cell line and negative MDA-MB-468 cell line. Six out of 90 tested clones specifically bound to M15 cells. These 6 clones were produced in soluble form and tested for binding ability by FACS analysis on relevant target cells. Only 3 clones (E7, F3 and F6) demonstrated good binding for both specificities (figure 3.1C). After sequencing it was evidentiated that two (E7 and F6) have identical sequence. Among the two clones, E7 maintained specificity and

stability over time whereas clone F3, after 1 week, lost the ability to recognize both antigens.

Analysis of E7 variable domains sequences demonstrated that they belong to VH3 (20*01 subgroup) and Vλ3 (subgroup 19*01) families.

The scDb E7/UCTH (figure 3.2A), cloned in pIT2 vector, was produced in soluble form in bacteria periplasm and it was purified with L-protein chromatography column. Method of scDb production was fine-tuned to reach a yield of BsAb in an average of about 5-6 mg/L (refer to Part III).

The size and the integrity of the purified reagent was first visualized on Coomassie blue-stained SDS-PAGE gel and detected by Western-blot with antiMyc Tag (9E10) antibody. The scDb migrated as a single band and, as expected, has a molecular size between the 50 kDa and 64 kDa. (figure 3.2B). The scDb exact molecular weight of 54.4 kDa was obtained after SELDI-TOF analysis (figure 3.2C).

To evaluate the potential formation of aggregates and fragments that could compromise the functionality of the reagent, E7/UCTH was analyzed by gel filtration on size-exclusion column. All performed tests indicated that the scDb was very stable. Stability of E7/UCTH was maintained over the time, more than 95% of the sample consisted of monomeric molecules also after 2 years from the production (figure 3.3).

### 3.2 BsAb binding specificity

The binding specificity of E7/UCTH against both TRAIL-R2 and CD3 antigens in native form was verified by FACS analysis on TRAIL-R2 expressing cells (M15; M41 and M64), on CD3 expressing cells (Jurkat) and on TRAIL-R2 and CD3 negative cells (MDA-MB-468). Commercial mAb specific for TRAL-R2 and hybridoma derived mAb specific for CD3 were used as positive binding controls. The BsAb showed a good and specific binding on both antigens only on positive

cells (figure 3.4). E7/UCTH binding ability and specificity was comparable to that of Abs used as positive controls. As expected, the monovalency of each arm does not allow the same binding strength of a bivalent antibody resulting in a lower fluorescent binding signal. Similar results were obtained when E7/UCTH was conjugated with fluorescein isothiocyanate or labeled with biotin followed by fluorochrome labeled avidin (data not shown) indicating that our reagent is suitable for a wide range of manipulations without losing its specific binding capability.

ScDb strength of binding was evaluated by BIAcore technique, based on Surface Plasmon Resonance for the detection of biomolecular interactions. BIAcore analyses, performed using as ligand the recombinant human TRAIL-R2 immobilized on CM5 chip, confirmed the ability of scDb to specifically bind the receptor. When the binding interactions between the ligand (TRAIL-R2) functionalized dextran layer of the sensor chip and the analyte (BsAb or sTRAIL) occurs, changes in the index of refraction at the surface of the sensor chip were detected and recorded as Resonance Units (RU). The output of BIAcore experiments is a sensogram, a plot of response versus time, showing the progress of the interaction between ligand and analyte (figure 3.5A). The first part of the curve indicates the speed of association and the second part of the curve shows the time of analyte dissociation from the ligand. The sensogram revealed that the BsAb had a fast association and a fast dissociation from the receptor. Based on experimental data, association and dissociation-curves were used to define binding kinetic and affinity constant (KD) which resulted to be of $1.48 \times 10^{-7}$ M (figure 3.5A).

Using BIAcore it is possible to evaluate the competition for the same binding site between two ligands. To evaluate the binding competition of the BsAb antiTRAIL-arm with sTRAIL, the recombinant receptor was immobilized on a chip and was saturated with 1 µM of soluble TRAIL. Soluble TRAIL bound the receptor with a very high strength: in fact it had a fast association and a very slow dissociation kinetic. The BsAb was unable to bind the receptor once sTRAIL was bound

demonstrating a strong competition for the same binding site (figure 3.5B). The fact that the BsAb could bind the same epitope of sTRAIL lets us to investigate if it could be agonist like sTRAIL inducing the death receptor-mediated apoptosis of targeted cells.

### 3.3 BsAb agonistic activity

Experiments performed with BIAcore revealing that the scDb and sTRAIL compete for the same binding site on TRAIL-R2, let us thinking that the antiTRAIL-R2 arm could have an agonistic activity like the death ligand. The scDb capability of inducing apoptosis via the activation of the death-receptor mediated pathway was examined using M15 TRAIL-sensitive cell line. As shown in figure 3.6B, the activity of monomeric scDb is very low (about 20% of cytotoxicity at the maximum dose tested) respect to the use of trimeric sTRAIL. Use of crosslinked scDb, dimerized using antiHis-tag antibody or tetramerized additioning also antiMouse mAb, showed improved cytotoxicity reaching a maximum of 65-80% of cell killing. Enhanced activity of tetramerized scDb are in line with the hypothesis that receptor trimerization is necessary for an efficient death receptor signaling [7]. These results were obtained only with a dose 20 fold higher of that used in sTRAIL treatment (100 µg/ml compared to 5 µg/ml of sTRAIL). This result could be explained by a different strength of binding of the two molecules for TRAIL-R2. In fact previous study [147] has calculated that the strength of binding of trimeric sTRAIL to TRAIL-R2 was $1.87 \times 10^{-9}$ about 100 times stronger if compared with that of monomeric BsAb evaluated with BIAcore. Soluble TRAIL that could bind 3 receptors at the same time has superior valency respect to the BsAb that could bind only one TRAIL-R2. For this reason, to compare the strength of binding of BsAb to sTRAIL a crosslink with antiTag antibody and with antiMouse was performed to multimerize the BsAb. As expected, the binding affinity of tetramerized molecule was superior respect to the dimeric and monomeric BsAb. However the tetrameric

molecule had a KD of about 9.52 x $10^{-9}$ revealing that it was ten times less affine to the receptor compared to the trimeric sTRAIL (figure 3.6A) In literature was described that a superior affinity influenced the triggering of the death receptors leading to an increased apoptotic activity [42;148]. At the same time we speculate that the loss in affinity of the scDb could have caused the lower cytotoxic response to the treatment.

### 3.4 BsAb-mediated tumor growth inhibition

Two different well characterized melanoma cell lines, M64 and M15, were initially used: the first cell line has low expression of TRAIL-R2 and results sTRAIL non-sensitive while M15 has higher expression of the receptor and is sensitive to sTRAIL (refer to figure 3.4 and figure 3.7). Cell lines were firstly characterized for their sensitivity to sTRAIL using 40% citotoxicity as cut-off of sensitivity (figure 3.7). The ability of the scDb to induce tumour growth inhibition was then investigated by redirecting non activated PBLs, isolated from healthy donor buffy coats, against tumor cells. To find the best treatment conditions three BsAb doses and 6 different E:T ratios were used. The best dose/response (minimum dose to obtain the maximum effect) ratio was observed using 0.5 µg/ml of scDb and an E:T ratio of 5:1 (figure 3.8). The test showed that the peak of cytotoxic activity using scDb to redirect PBLs was obtained when an E:T ratio of 10:1 was used. However at this E:T ratio cytotoxicity of PBLs alone was too high. To exclude a donor-dependent effect, the reproducibility of the experiment was evaluated using PBLs isolated from peripheral blood of 9 different healthy donors using the optimal scDb dose and E:T ratio. The data relative to M15, M64, M41 and TRAIL-R2 negative MDA-MB-468 are reported in figure 3.9. To avoid distortions due to different cytotoxic power of PBLs, CD69 high-expressing PBLs were considered just-activated and excluded. The scDb shows a good effect on all the used tumor cell lines. Treatment with scDb and PBLs from different healthy donors showed similar

results and the very low standard deviation calculated in each group indicated that the results are reproducible and not donor-dependent. No cytotoxicity was observed using TRAIL-R2 negative MDA-MB-468.

The activity of BsAb was also tested on other TRAIL-R2 positive cell lines; the membrane expression of the receptor was analyzed by FACS using BsAb plus antiMyc-Tag mAb plus antiMouse Alexa 488 mAb (figure 3.10A). We found that also HEK-293, a normal kidney immortalized cell line, presented a good level of TRAIL-R2 and for this reason it was used to evaluate toxicity on normal cells. The sensitivity to sTRAIL was evaluated on these cell lines (figure 3.10B). Results showed that HeLa, SKOV3, A2780 were resistant while INT-Ov-11, A2774, NL3507 and A431 were sensitive to sTRAIL treatment. As expected in normal HEK-293 no TRAIL-R2 mediated apoptosis was observed after treatment with the soluble ligand.

After retargeting of PBLs (E:T ratio of 5:1) with 0.5 µg/ml of scDb we observed that the scDb was capable of inducing target cell growth inhibition for all the cell lines used. No cytotoxicity was observed on treated HEK-293 despite the relatively high expression of TRAIL-R2 (figure 3.11).

### 3.5 Direct cytotoxicity evaluation

The measure of direct cytotoxicity of scDb retargeted PBLs we use a modification of the classical $^{51}$Cr-release assay to avoid the use of radioisotope. Among the different methods developed to bypass use of $^{51}$Cr, Calcein-acetoxymethil (calcein-AM) release assay demonstrated to be a good method [149]. Calcein-AM is liposoluble and could passively cross cell-membranes. Once calcein-AM entered in cytoplasm, it is converted in a lipid-insoluble molecule by cell esterases, that becomes fluorescent and remain trapped in cell until the cell membrane is damaged. With this method, cytotoxicity could be measured by quantification

fluorescent calcein released in the supernatant after target cell lysis by retargeted effector cells.

Pre-activated T-cells (E:T=5:1) were incubated with calcein labelled tumor cells with or without BsAb for 4 and 16 hours. Optimal calcein-AM concentration for tumor target cell number and density was determined by incubating cells to a concentration range from 1 to 15 μM, evaluating the optimal separation between maximal and spontaneous release (data not shown). The treatment with scDb was able to retarget T-cell to lyse melanoma cells M15, M64 and M41. After 4 hours cytotoxicity was about 50% for TRAIL-R2 high expression M15 and M41 and about 30% for low expression M64. After 16 hours the cytotoxicity reached 100% for all treated cell lines. No direct cytotoxicity was observed in MDA-MB-468, TRAIL-R2 negative cell line (figure 3.12).

### 3.6 T-cell activation

The ability of scDb to activate unstimulated PBLs from healthy donors after the co-incubation with TRAIL-R2 positive and negative tumor and normal cells was analyzed using T cells activation assay. After 16 hours of coculture with or without scDb the membrane expression of early activation marker CD69 and late activation marker CD25 was assessed by FACS analysis. Expression of the two activation markers on PBLs occurred in presence of all TRAIL-R2$^{+}$ tumor cells tested when treated with scDb (figure 3.13). While expression of early activation marker CD69 was evident with all TRAIL-R2 positive cell lines, independently from expression levels, expression of the late activation marker CD25 was induced only by cells with high TRAIL-R2 expression (see figure 3.4, figure 3.10 and figure 3.13). By contrast, the two markers were not up-regulated in the absence of scDb or when MDA-MB-468 TRAIL-R2 negative cells were treated, demonstrating that the scDb is able to activate PBLs in a target-dependent manner. The lack of overexpression of CD69 and CD25 was observed also when HEK-293 TRAIL-R2$^{+}$ normal cells

were used (figure 3.13). T-cell activation should occur after their BsAb-mediated redirection on cells bearing the antigen. If the antigen is not restricted to tumor, T-cells will be redirected also against normal cells and their activation will occur [136; 137]. Normal cells, which are resistant to direct T-cell mediated lysis, could be indirectly damaged by T-cell cytokines production. The lack of T-cell activation treating TRAIL-$R2^+$ HEK-293 cells is a promising result but further investigations are needed to understand why this event does not occur and if it would be confirmed treating other normal cells.

To understand if T cell activation leads to production of cytokines, level of TNFα and IFNγ was determined daily in supernatants by BIOPLEX assay starting 24 hours after scDb treatment in the presence of target cells. Concentration of IFNγ and TNFα released in the medium was evident for high TRAIL-R2 expressing M41 cells with a peak of production at 24 hours (figure 3.14). In M64, the level of cytokines production was low and, respect to M41, the peak of production of IFNγ occurs after 96 hours. These data agree with the difference in T-cells activation markers after incubation with M64. No cytokines production was observed using negative TRAIL-R2 cells.

### 3.7 *In vivo* experiments with an orthotopic triple negative breast cancer model.

The triple negative breast cancer cell line MT-3 was very efficiently lysed by BsAb-retargeted PBLs (refer to figure 3.11). We therefore used this model to test the effect of BsAb retargeting in the well-established orthotopic MT-3 xenograft. MT-3 cells were xenografted in mice nipples. When tumor reached a volume of about 150-200 $mm^3$, $10^7$ human non-activated PBLs, derived from healthy donor, were injected in a unique dose in lateral tail vein. Three days after PBLs engraftment, 2.5 mg/kg of BsAb were injected intraperitoneally daily (figure 3.15A). A significant delay of tumor growth compared to saline treated groups was observed in the scDb+PBLs treated group for 2 weeks (figure 3.15B). After 2

weeks, tumors of treated group restarted to grow. This was not surprising because, like described by Hoos et al [150], human T cells could enter in mice circulation and remain detectable for only two weeks. The lack of tumor eradication is probably due to human PBL depletion from mice circulation at the end of the experiment (mice were sacrificed at day 18). Indeed, no human PBLs were detected by FACS analysis at the end of the experiment in mice blood. We are aware that this is not the ideal model to test activity of BsAb considering the artificial transplant of human PBLs in a different organism. In fact transplanted PBLs have to circulate, enter and reside in tumor and animal organs without be eliminated. Nevertheless this experiment was useful to assess that BsAb could recircle and target the tumor and that the treatment would not be toxic on mice (no body weight loss in treated group respect to control). We think that a more appropriate model to test efficacy and toxicity could be an immunocompetent mouse in which BsAb could retarget autologous PBLs against autologous tumor expressing human TRAIL-R2.

# Figures and Tables

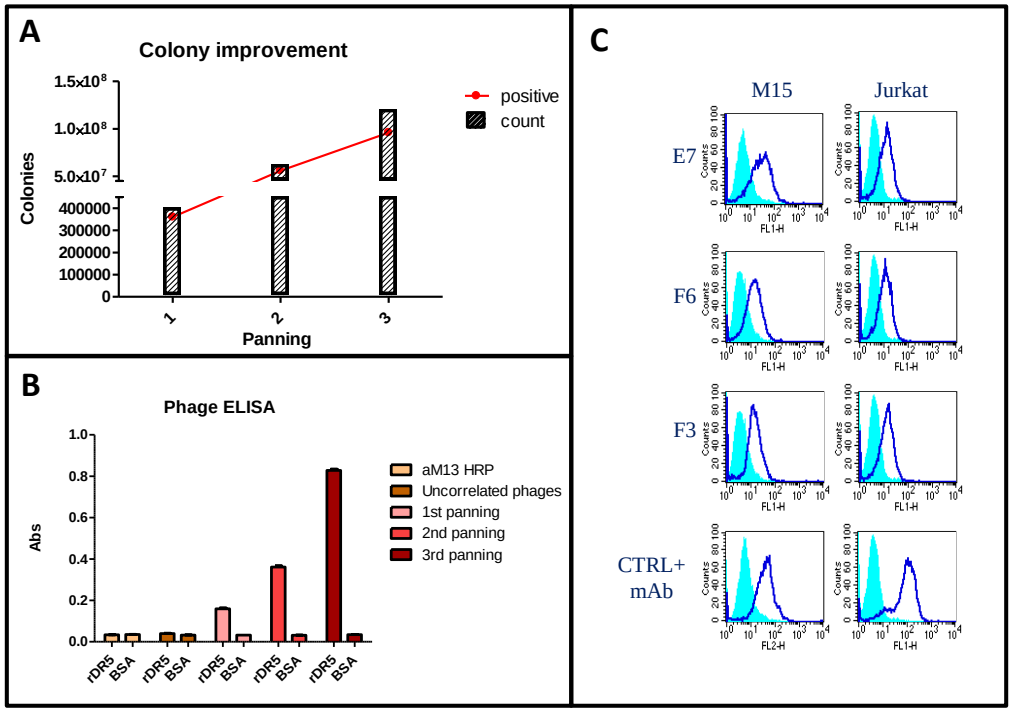


**Figure 3.1**

A) In graph is illustrated the number of colonies derived from phages infected bacteria obtained after each cycle of panning. 50 representative colonies were screened by PCR and colonies containing a BsAb gene cassette were considered positive.

B) ELISA analysis evaluating binding capability of recombinant TRAIL-R2 of phages isolated after each panning. BSA was used as uncorrelated protein and uncorrelated phages were used as negative control.

C) FACS analysis evaluating binding capability of the two specificities of the isolated scDbs on TRAIL-R2 positive M15 and CD3 positive Jurkat cell lines. As control, commercial mAb, recognizing TRAIL-R2, and hybridoma-derived TR66 mAb, recognizing CD3, were used.

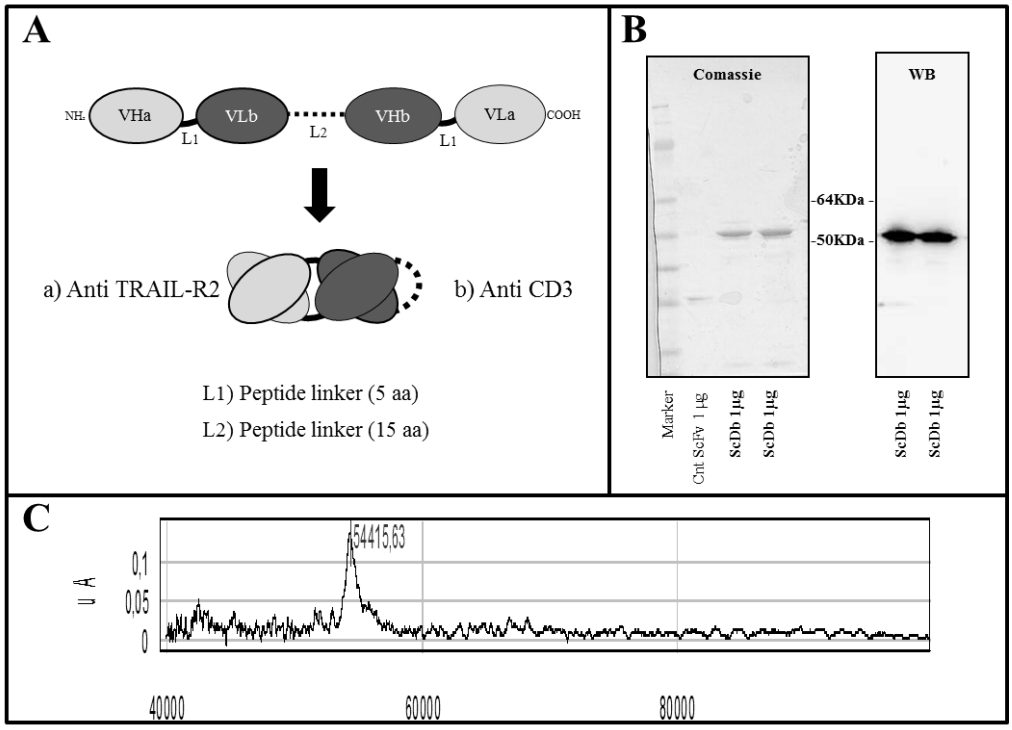


**Figure 3.2. Biochemical characterization of purified BsAb**

A) Cartoon represents the scDb construct which was engineered to create a bispecific antibody able to bind TRAIL-R2 and to retarget efficiently T-cells on tumor: antiTRAIL-R2 variable domains are at the extremities of the structure and are joined to antiCD3 scFv by two identical 5 amminoacids short linkers which avoid the formation of mismatched pairs.

B) Purified E7/UCTH1 was separated by SDS-PAGE and stained with Comassie blue or blotted on nitrocellulose membrane and detected with antiMyc tag and antiMouse HRP. The molecular weight is between 50 and 64 KDa.

C) SELDI-TOF analysis revealed that the mass of the scDb is about 54kDa.

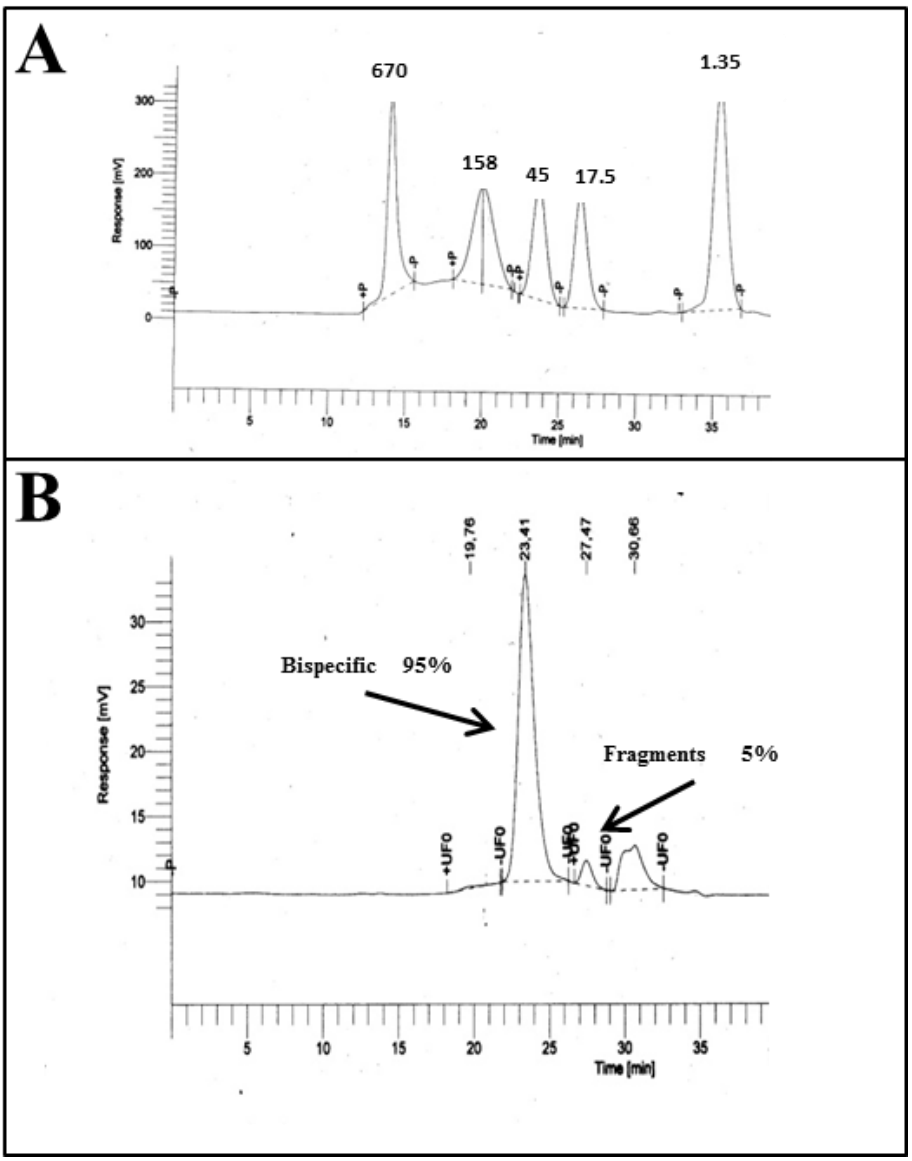


**Figure 3.3. Size exclusion chromatography (SEC)**

A) Instrument calibration with BIORAD standard. On each peak is indicated the molecular weight (KDa) of standard protein. In SEC chromatography the time of elution corresponds to mass of the protein.

B) SEC profile of scDb. The BsAb time of elution of 23.41 minutes was in agreement with the expected molecular weight.

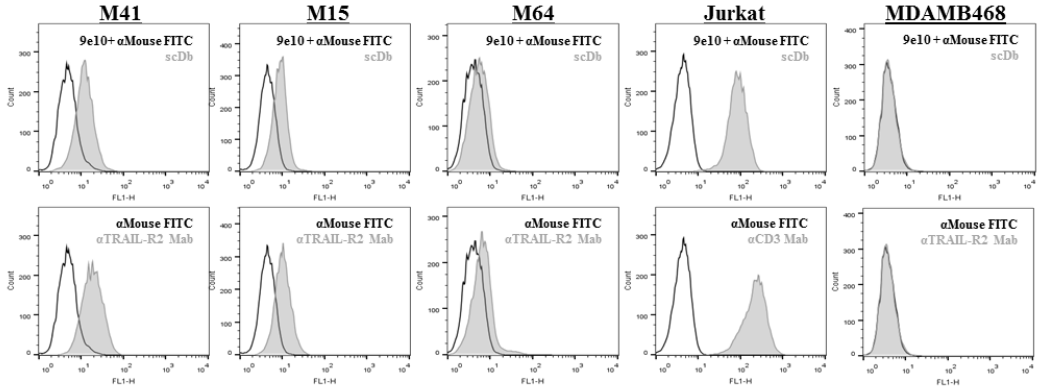


**Figure 3.4. Binding specificity of BsAb**

Flow cytometric analysis performed on a panel of TRAIL-R2$^{+}$ melanoma cells, CD3$^{+}$ Jurkat and TRAIL-R2$^{-}$ MDA-MB-468.

Top panels: binding of the BsAb revealed by antiMyc tag Ab (9E10) followed by alexa fluor 488 antiMouse Ab. Bottom panels: binding of positive controls antiTRAIL-R2 and antiCD3 mAbs on the same target cells, as revealed by secondary alexa fluor 488 conjugated antiMouse Ab. Empty peak: negative control; grey peak: tested Ab.

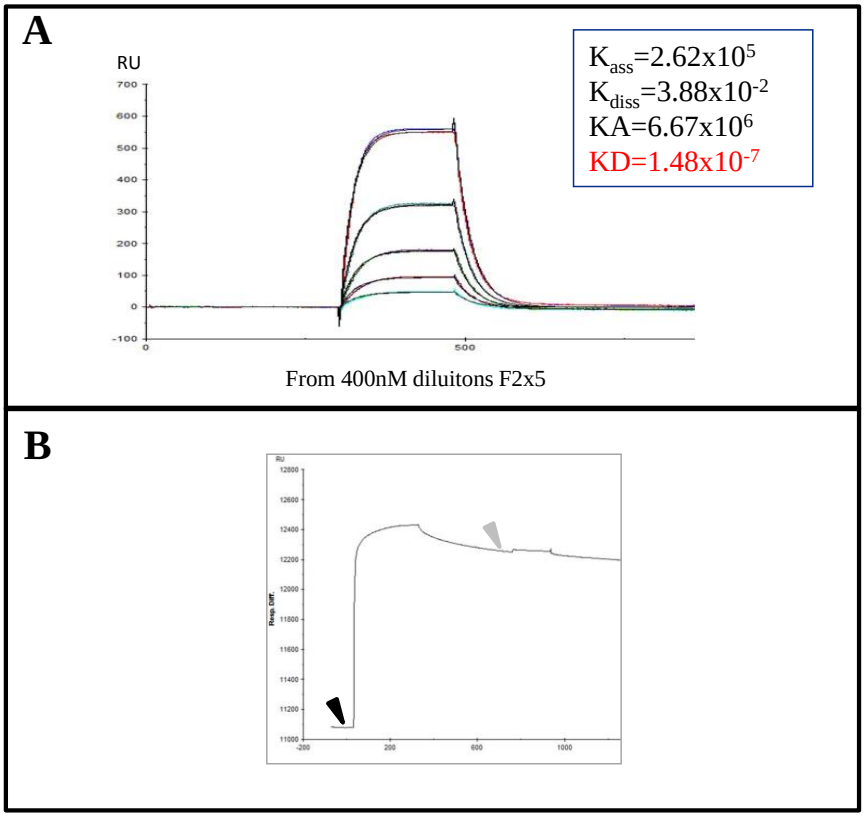


**Figure 3.5. BIAcore analysis**

**A)** Plasmon surface resonance sensogram demonstrated that the scDb had both a fast attach and a fast detach from TRAIL-R2 recombinant protein immobilized on a BIAcore CM5 chip. The dissociation constant at the equilibrium (KD) is 1,48x10$^{7}$ moles/L and was calculated using 5 different F2 dilutions, starting from 400 nM of scDb till 25 nM.

**B)** Sensogram from the BIAcore illustrating the competition study. 1 uM of sTRAIL was injected (black arrow); after saturation of all the TRAIL-R2 coated on the chip, 400 nM of scDb was injected (grey arrow). No binding of scDb on sTRAIL-saturated receptor was observed revealing that the two compounds are in competition for the same binding site.

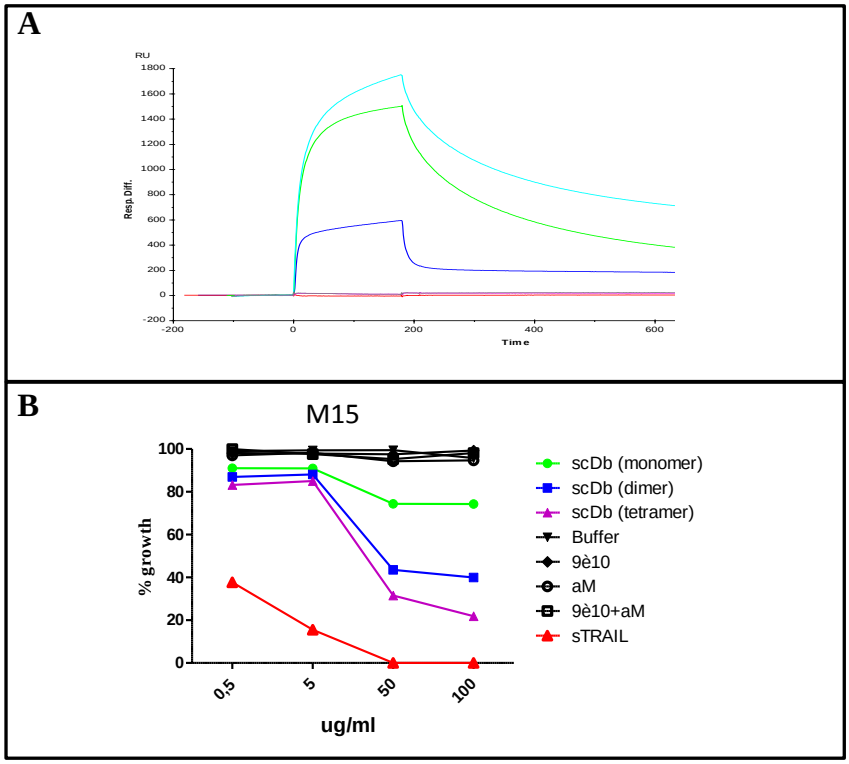


**Figure 3.6. Agonistic activity of BsAb antiTRAIL-R2 arm**

**A)** BIAcore sensogram illustrates binding of scDb in monomeric form (dark blue line) or multimerized with antiHis-tag antibody method. This method exploited the possibility of recognition of the His tag exerted by the specific antiTag monoclonal antibody allowing dimerization of the scDb (green line). If further incubated with Fc-specific antiMouse antibody, which could bind Fc of antiHis tag antibody, we could obtain an artificial tetrameric form of the scDb (light blue line).

**B)** M15 cells were treated with several doses of scDb or equal doses of scDb dimerized or tetramerized according to description in A. Cell toxicity was assessed after 24 hour of treatment using CellTiterGlo assay. sTRAIL, at equal scDb concentration, was used like positive control. Results were expressed as percentage of untreated cells.

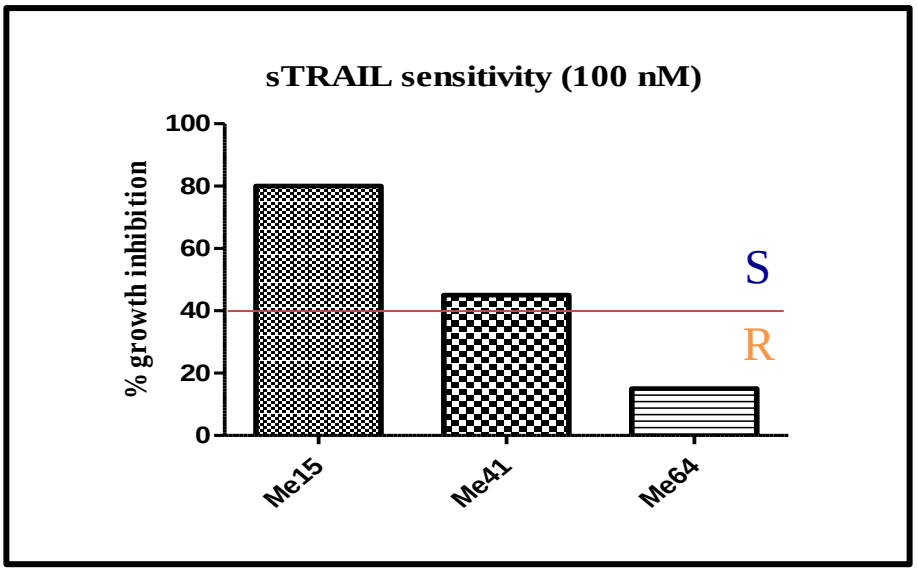


**Figure 3.7. sTRAIL sensitivity of all used cell lines.**
100 nM of sTRAIL were used for 24 hours of treatment. Cytotoxicity of 40% was considered the threshold between resistant (R) and sensitive (S) cell lines.

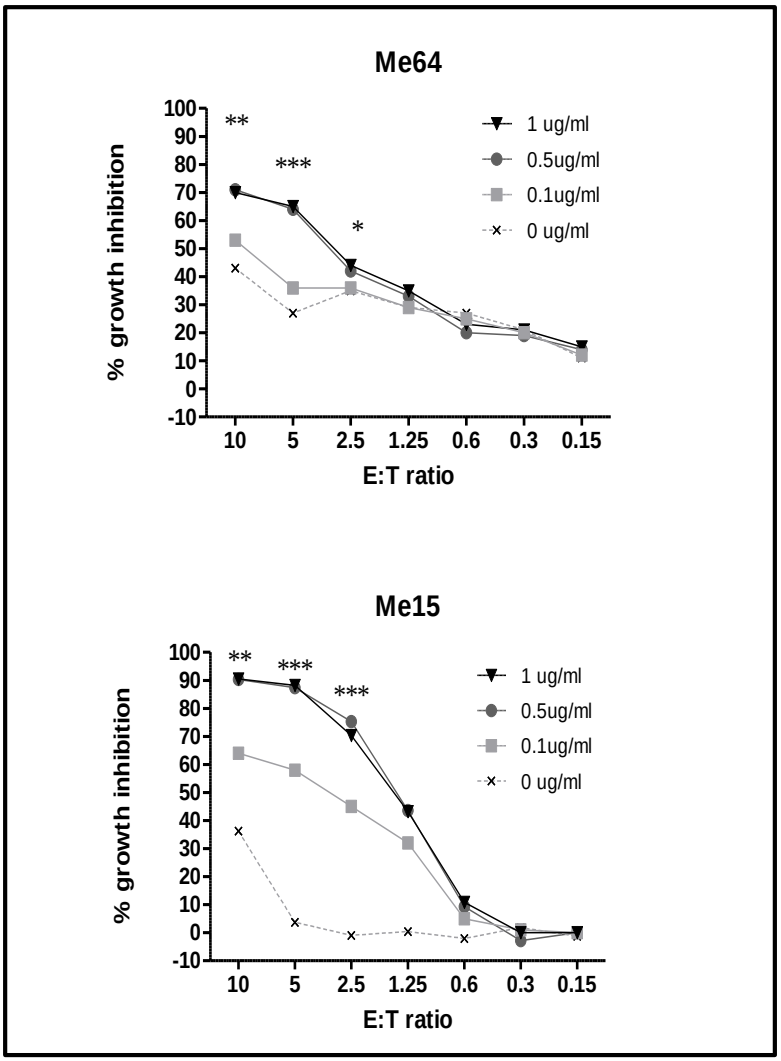


**Figure 3.8. Determination of best BsAb dose and E:T ratio**

Cytotoxicity titration graph of the bispecific scDb on M64 (low-expression of TRAIL-R2 and sTRAIL resistant) and M15 (high-expression of TRAIL-R2 and sTRAIL sensitive) melanoma cell lines. ScDb was used at 1, 0.5 and 0.1 µg/ml with E:T ratio starting from 10:1 until 0.15:1 with F2 dilutions. Experiments indicated that the best E:T ratio was 5:1 (absence of cytotoxicity on tumour cells without treatment) and the best scDb concentration was 0.5 µg/ml (activity plateau). Graph represents mean ± SD, n =3, * $p<0.05$, ** $p < 0.01$, *** $p < 0.001$

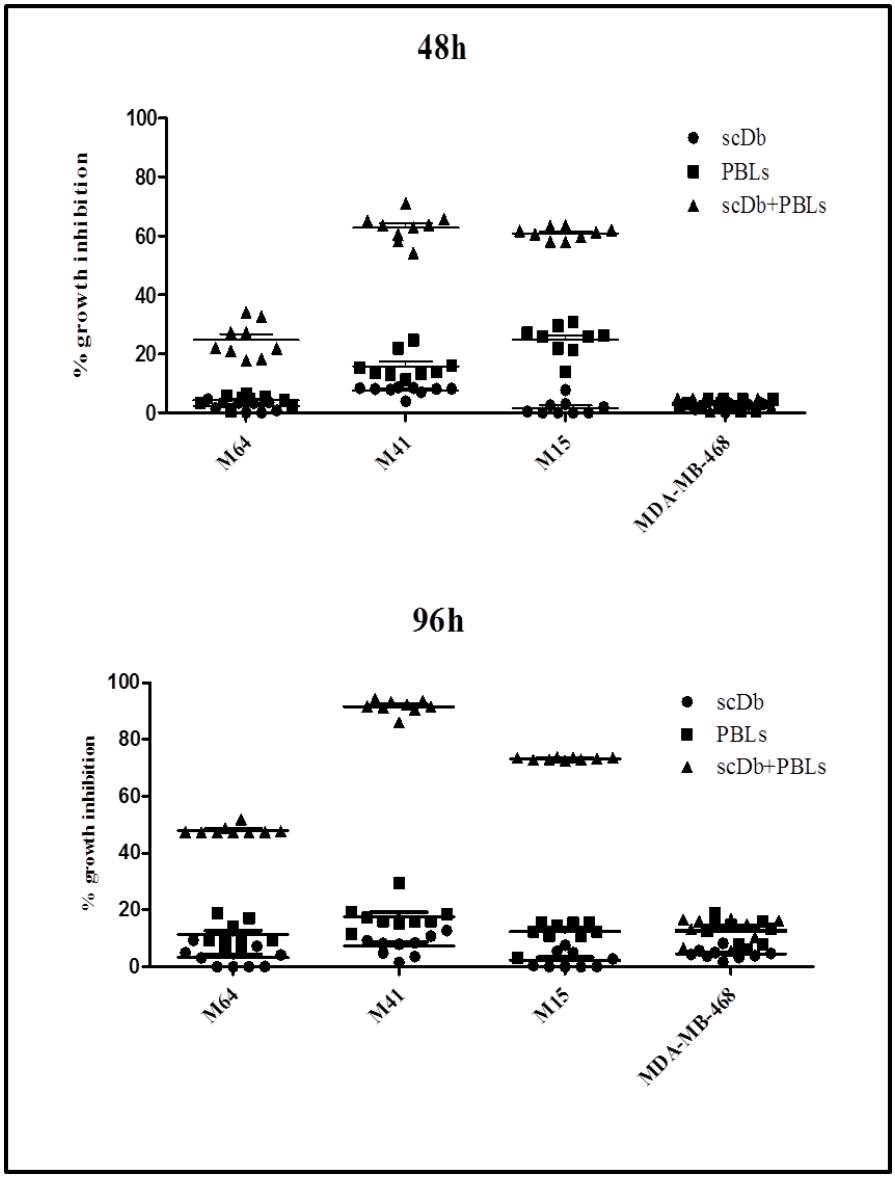


**Figure 3.9. Reproducibility of cytotoxicity results**

Graphs illustrated 9 different growth inhibition assays, performed to test results reproducibility. A different batch of PBLs (each derived from a different healthy donor) was used in each experiment. The treatment of M64, M41 and M13 with 0.5 ug/ml of scDb plus PBLs (E:T ratio of 5:1) gave similar results in all the experiments both after 48 or 96 hours of treatment. TRAIL-R2 negative MDA-MB-468 did not respond to treatment, as expected. Each point represents a single experiment. Bars represented the mean of all the nine experiments.

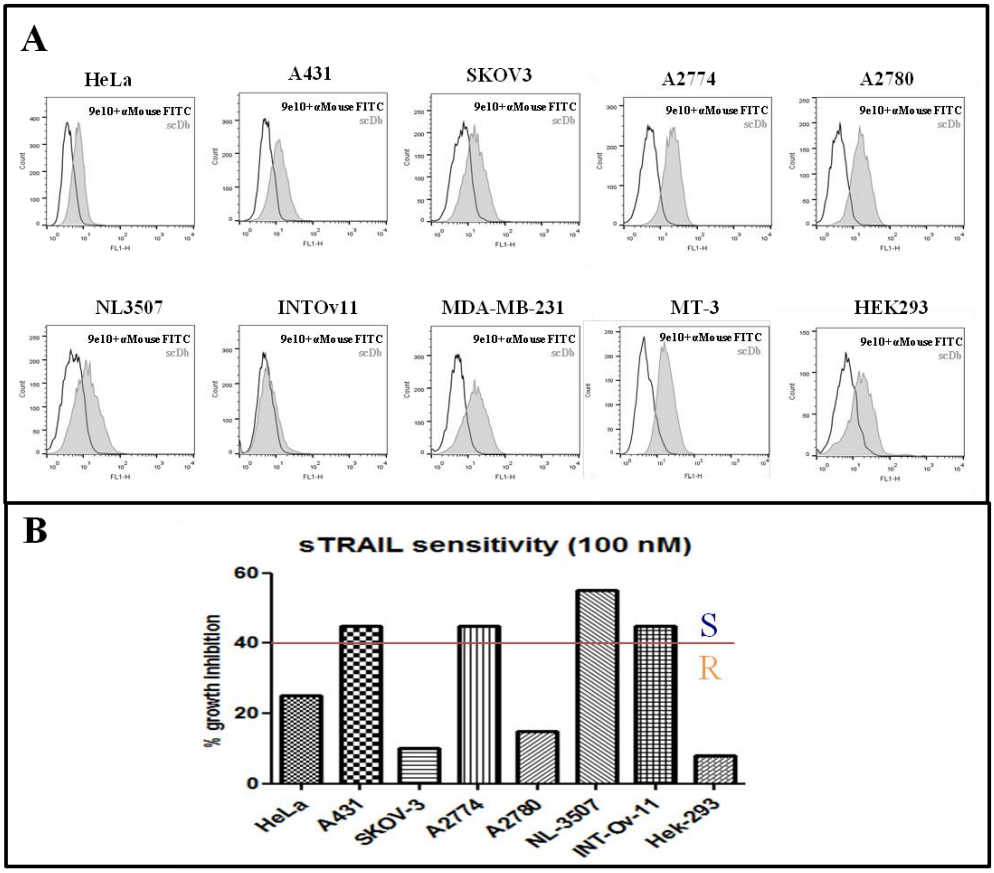


**Figure 3.10. Cell lines TRAIL-R2 expression and sTRAILsensitivity**

**A)** Flow cytometric analysis performed on a panel of TRAIL-R2$^{+}$ tumor cell lines. ScDb binding was revealed with antiMyc Tag and alexa fluor 488 conjugated antiMouse antibodies (grey peak). Empty peak indicated negative control. HeLa: cervix epithelial adenocarcinomas; A431: epidermoid epithelial carcinoma; SKOV3, A2774, A2780, NL3507 and INT-Ov-11: epithelial ovarian carcinomas; MDA-MB-231 and MT-3: triple negative breast cancer; HEK-293: normal embryonic kidney.

**B)** 100 nM of sTRAIL were used for 24 hours of treatment. Cytotoxicity of 40% was considered the threshold between resistant (R) and sensitive (S) cell lines

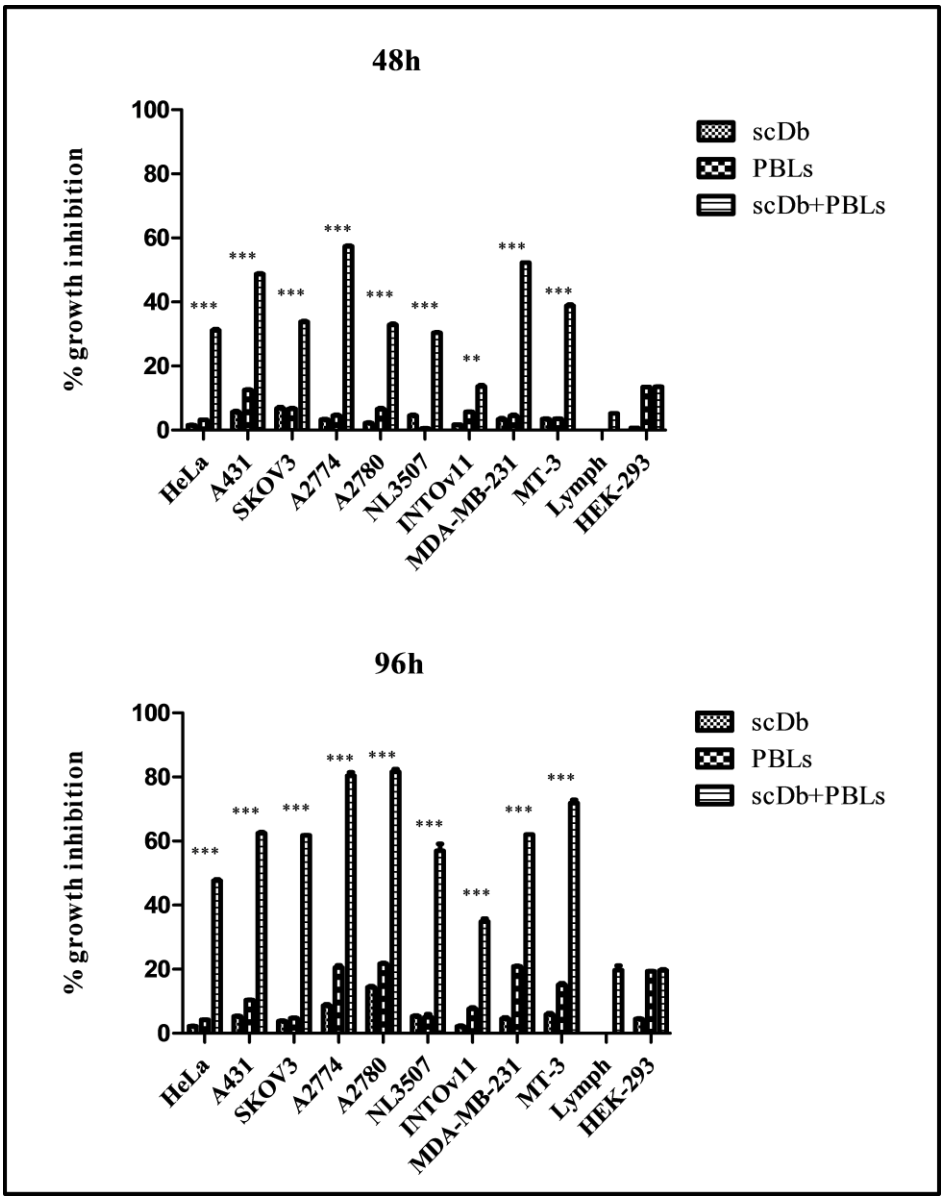


**Figure 3.11. Growth inhibition assay on a panel of TRAIL-R2 expressing cells**
ScDb-dependent PBLs -mediated growth inhibition was performed on several cancer cell lines using a concentration of scDb of 0.5 µg/ml and a E:T ratio of 5:1. Cells were exposed to treatment for 48 or 96 hours. The treatment induced growth inhibition on all tested tumor TRAIL-R2 positive cell lines; no toxicity was observed on normal TRAIL-R2-high expression HEK293 cells. Lymphocytes self-killing never exceed 20% after 96h. Graph represents mean ± SD, n =3, * $p<0.05$, ** $p < 0.01$, *** $p < 0.001$

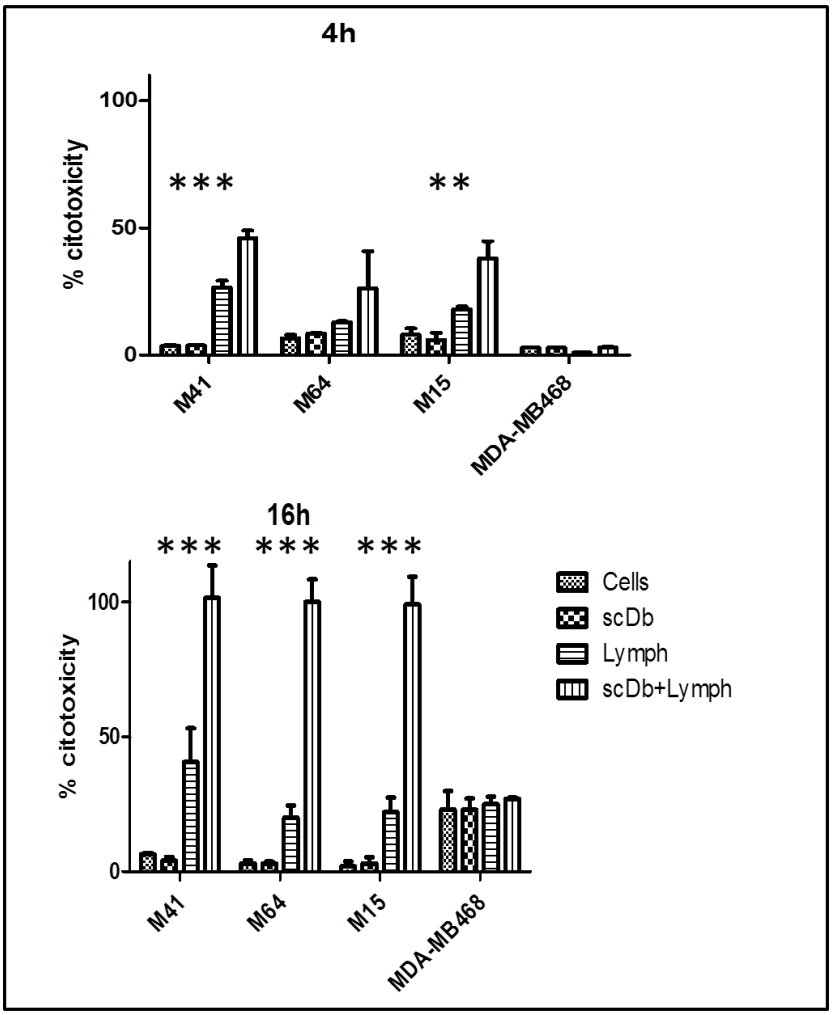


**Figure 3.12. Calcein-AM release assay**

Tumor cells were loaded with calcein-AM that, once entered in the cells, was esterified and became fluorescent. Cells were treated for 4 and 16 hours with PBLs ± scDb. Graphs show the percentage of cells lysis. After 4 hours about 45-50% of TRAIL-R2 high-expression M15 and M41 were lysed, while for low expression M64 level of cytotoxicity was not significant. After 16 hours 100% of the treated cells were lysed. No lysis was observed in TRAIL-R2$^-$ MDA-MB-468 cells. Graph represents mean ± SD, n =5, ** $p < 0.01$, *** $p < 0.001$

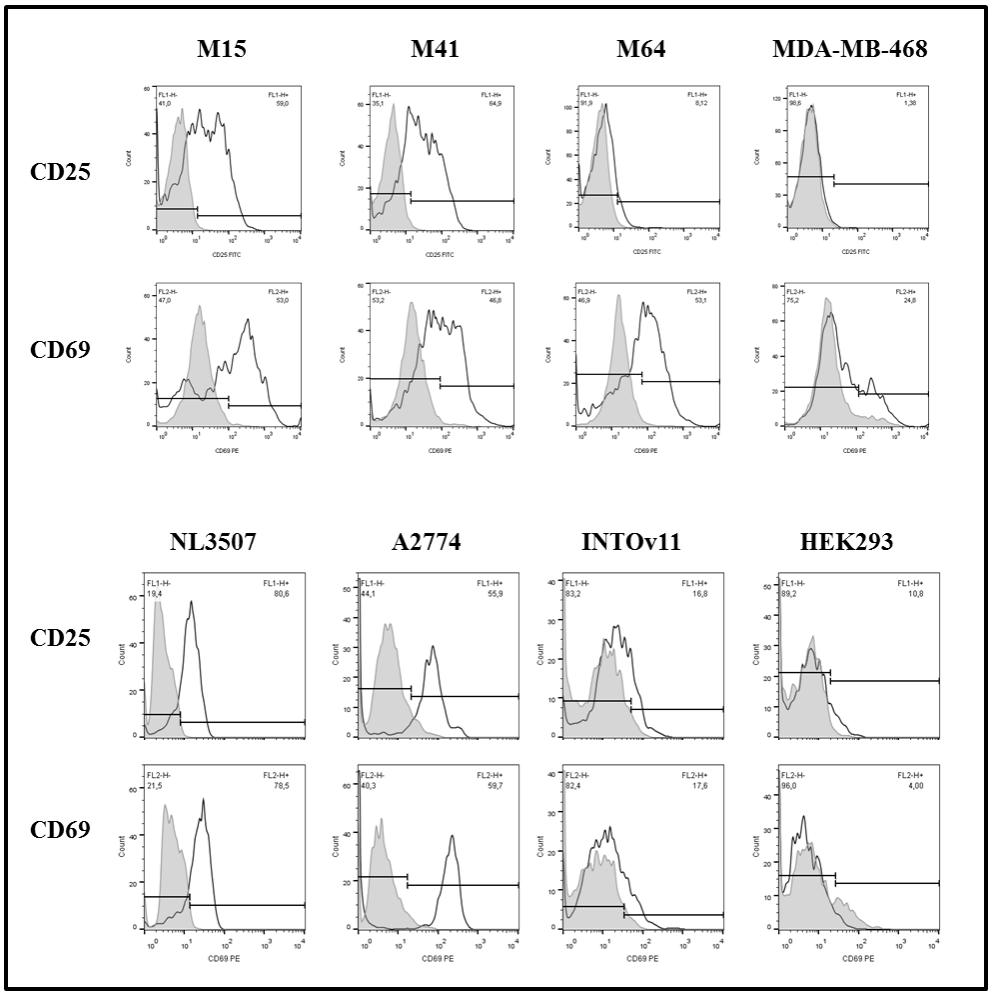


**Figure 3.13. ScDb-mediated T-cell activation analysis**

Freshly isolated T-cells were incubated, at E:T=5:1,with or without 0.5 ug/ml of scDb. 3 melanoma (M15, M41 and M64) and 3 ovarian carcinoma cell lines (NL-3507, A2774, INTOv11) which express TRAIL-R2 were used. Non-expressing TRAIL-R2 MDA-MB-468 and high-expression normal HEK293 were used. Cell surface expression of CD69 and CD25 was measured by flow cytometry analysis 16 h after starting the coculture. Grey peaks represent the quantity of markers expressed by T-cells when cocultured with tumor cells without BsAb while empty peaks illustrate marker expression when T-cells and tumor cells were incubated with scDb.

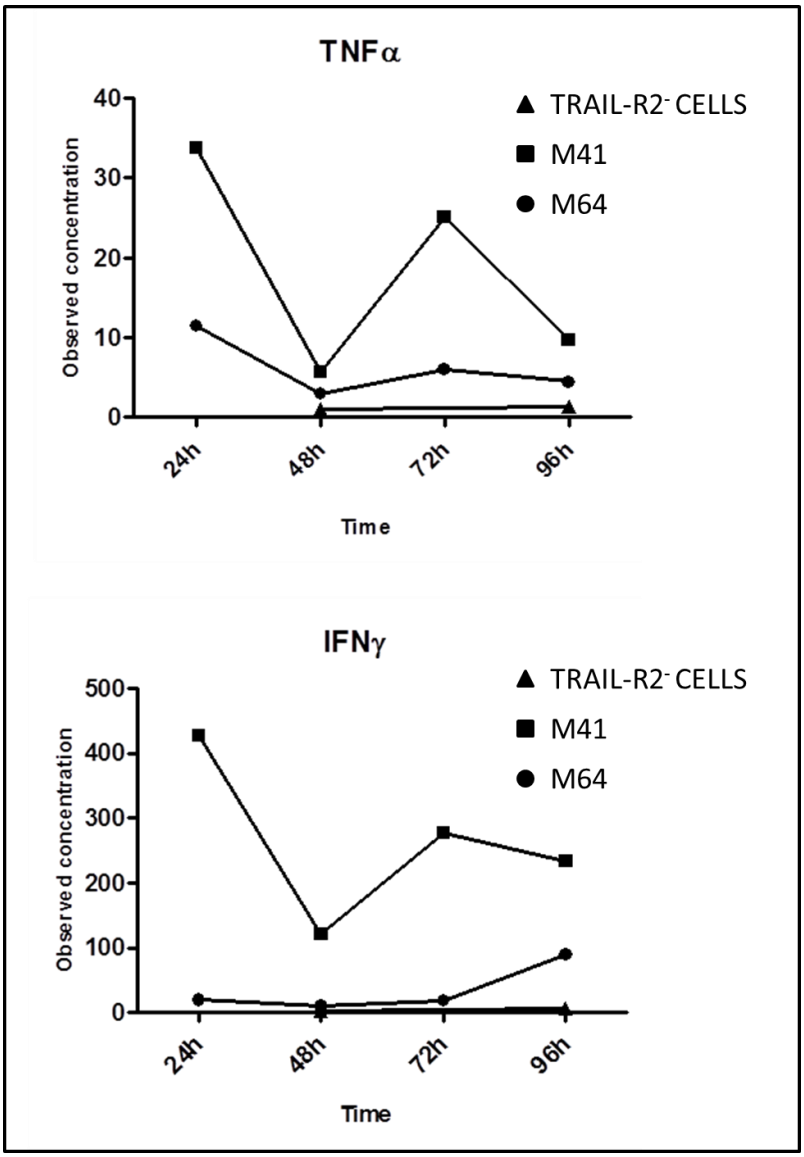


**Figure 3.14. Pro-inflammatory cytokines production by activated T-cells**
The medium of tumor cells, incubated with T-cells with or without scDb, was picked daily after 24 hours of treatment: the amount of TNFα and IFNγ was measured using Bioplex. The mean value of duplicate wells is represented in each graph. Each point represents level of cytokine produced after treatment with PBLs+scDb subtracted of cytokine production when PBLs were incubated with tumor cells or BsAb only.

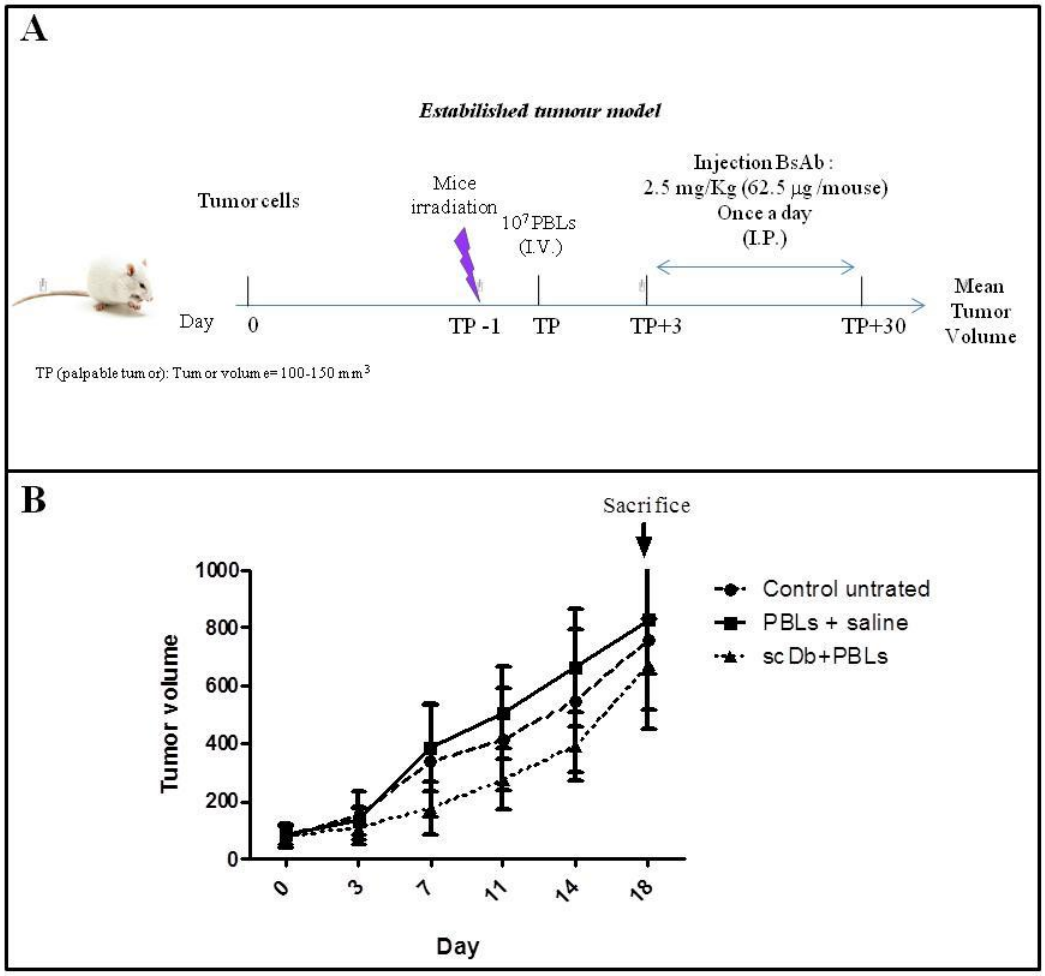


**Figure 3.15. Antitumor activity of E7/UCTH in mouse xenograft model**

A) Preclinical in vivo scheme of treatment using an established orthotopic model of triple negative breast cancer.

B) MT-3 xenografts were grown in the nipple of female NOD/SCID mice until they reached a volume of about 150 $mm^3$. Then mice were divided in 3 groups and two groups were injected into lateral tail vein with $10^7$ human non-activated PBLs derived from healthy donor. After 3 days the daily treatment with BsAb or with saline vehicle began (represented in the graph like Day 0). Tumor growth was determined by external caliper measurement and tumor volume was calculated using a standard hemiellipsoid formula. Mice were sacrificed at day 18. Each point represents mean tumor volume ($mm^3$) ± SD (n= 6 for each group).

## 4. Conclusions and Future Prospects

In vitro experiments demonstrated that ScDb antiTRAIL-R2 x antiCD3 is a good therapeutic compound to treat various tumors. If compared to sTRAIL the scDb has a lower agonistic activity due to its monomeric structure that does not allow an efficient trimerization of the death receptors. Nevertheless it could retarget efficiently T-cells, which become activated and lyse specifically tumor cells that express TRAIL-R2 on plasma membrane. This occurs also if tumor cells are resistant to agonistic TRAIL-mediated apoptosis as in the case of melanoma M64, ovarian carcinoma SKOV-3 and A2780 and cervix epithelial adenocarcinoma HeLa. We are confident that the treatment with this reagent would be safe: no T-cell activation was observed when normal cell lines were treated and subsequently no cytotoxicity was observed. However the mechanism by which T-cells do not activate, despite the high expression of TRAIL-R2, has to be elucidated and the panel of treated normal cells would be increased with other cells like hepatocytes whose are the more sensitive cells to TRAIL-treatment.

Also in vivo experiments were encouraging. A significant delay of growth of breast carcinoma xenograft was observed and we speculate that the lack of tumor eradication would be probably due to human PBL depletion in mice. The experiment could be repeated using infusion of PBLs every two weeks but we are aware that this is not the ideal model to test activity of BsAb considering the artificial transplant of human PBLs in a different organism. A more appropriate model to test efficacy and toxicity could be an immunocompetent transgenic mouse in which BsAb could retarget autologous T-cells against autologous tumor expressing human TRAIL-R2. For this reason we are constructing a scDb in with the anti- human CD3 arm is substituted by antibody variable domains which could recognize the mouse homologue protein. This experiment would represent a proof of concept of the activity of the BsAb and lay the foundation for preclinical studies on other animal species to assess safety and pharmacokinetic parameters.

A European patent application to protect the invention was filed on 1st July 2015 with the title: “Bispecific antibodies for use in cancer immunotherapy”.

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## Part III

## 4. Troubleshooting in BsAb construction

To construct the BsAb, at the beginning of the project, we decide to obtain first the two scFvs able to bind the two specificities (TRAIL-R2 or CD3 epsilon chain). Only in a second moment these antibody fragments were associated with appropriate linkers to generate the BsAb.

### 4.1 AntiTRAIL-R2 scFv isolation by phage display

To isolate scFv directed against TRAIL-R2 we used a pre-made human scFv phage display library. 3 rounds of selection were performed using TRAIL-R2 from SU-DHL-4 lysate. The titer of the eluted phages increased gradually after each cycle (figure 4.1A) and the binding of produced phages was tested in FACS and increased proportionally with the enrichment indicating that the selection was efficient (figure 4.1B). Randomly picked colonies, derived from bacteria infected with phages after the enrichment, were tested in single clone phage ELISA and 4 different clones (7, 8, 44 and 56), specific to naïve TRAIL-R2, were isolated, sequenced and characterized (figure 4.1C). The 4 clones were produced in soluble form and purified scFvs resulted capable of specifically binding TRAIL-R2$^{+}$ cells (figure 4.2A). Clone 7 and 8 resulted stable and were produced at good yield while clone 44 and 56 yield was very low and lost the ability to bind the receptor 3 days after production.

### 4.2 AntiCD3 scFv construction and production

To obtain the scFv against epsilon chain of human CD3, we converted two murine Hybridomas (OKT3 and TR66) in scFv form. The scFv of humanized antiCD3-mAb UCTH1, which sequence is described in literature, was generated by gene synthesis (GeneArt – Life technologies). All antiCD3 scFvs were cloned in pIT2

vector and produced in soluble form. FACS analysis using Jurkat cells showed that purified scFvs bound specifically CD3 (figure 4.2B).

### 4.3 BsAb construction

Isolated clone 7 and 8 and constructed antiCD3 scFvs were used to construct BsAbs in TaScFv and scDb formats. These formats are the most suitable for our purposes: in fact allowing the formation of a more compact reagent they permit a strictly approach of the T-cell with the tumoral one forming the immunocytolitic synapsis. Two gene cassettes were designed to create BsAb in TaScFv and scDb format. These cassettes vary for length of the peptidic linkers between the different moieties.

To choose the best pairing in TaScFv or scDb formats, constructs have been assembled with antiTRAIL-R2 clones 7 or 8 which have been paired with TR66, OKT3 and UCTH using appropriate formats linkers.

A total of 12 constructs have been developed and sequenced with positive results:

- Clone 7 + OKT3
- Clone 8 + OKT3
- Clone 7 + TR66
- Clone 8 + TR66
- Clone 7 + UCTH
- Clone 8 + UCTH

The different constructs, cloned in pIT2 vector, have been expressed in a prokaryotic system and in particular HB2151 E.Coli strain was used. Different conditions temperatures (30 and 37°C) and time of induction (3h and ON) were tested to optimize production of each construct. The presence of a histydine tail in

the vector makes possible the purification of soluble BsAbs with a nickel column by HPLC.

### 4.4 BsAbs biochemical characterization and binding ability

The evaluation of the 12 produced BsAbs by western blot showed the presence of the entire molecule for all the produced constructs (figure 4.3).

To test binding capability to the two antigens, purified BsAbs were tested by FACS on Jurkat, $CD3^+$ cells, and on Melanoma, TRAIL-$R2^+$ cells. Among the different BsAbs, only Clone 8 in pairing with TR66 TaScFv was able to bind both the two different target (figure 4.4A) and BsAbs with UCTH were able to recognize only the CD3 molecule. The rest of constructs did not recognize both antigens.

Unfortunately clone 8/TR66 TaScFv demonstrated a poor stability during time and after 48 hours it lost the ability to recognize both antigens (figure 4.4A).

This event was probably due to the presence of aggregates as demonstrated by Size Exclusion Chromatography (SEC) analysis (figure 4.4B).

In parallel we tested with SEC also all the other constructs: all during time aggregated and lost the ability to bind both antigen. Among different constructs UCTH of scDbs was the arm that maintained the ability of binding its target for longer time.

Data obtained showed that, although scFvs when tested by FACS are able singularly to bind their target antigen, when joined together to form a BsAb are no longer able to recognize the target. This could be attributed with a different conformation and stability that the scFvs have to adopt to stay in BsAb conformation. Moreover we observed that the constructed scDb molecules seemed to have slight more stability respect to TaScFv constructed with the same variable domains. For this reason we decide to restart the project constructing a phage library of BsAbs in scDb format. Among the other BsAbs, this format, with UCTH, was chosen because antiCD3 moiety conserved specificity for longer time and,

being humanized, has the advantage to produce a potentially less immunogenic reagent.

This approach revealed to be efficient like described in the principal part of this thesis.

**5. E7/UCTH scDB production and purification set-up**

To optimize the production of scDb E7/UCTH different conditions of temperature and period of induction were performed. It was shown that bacteria produced the protein in a more efficient way when they were induced ON at a temperature of 30°C (figure 5.1A).

To discover the method of purification that allows recovering the most quantity of protein contained in bacterial periplasm, different experiments were performed. The scDb was produced with a final polyhistidine tag and could be purified by immobilized metal-affinity chromatography (IMAC) column. Moreover, from analysis performed using VBASE2, an integrative database of germ-line variable genes from human and murine immunoglobulin loci (http://www.vbase2.org), we found that the UCTH light domain belongs to Vκ3 family and hence could be purified with affinity chromatography L-protein column. With this type of column two type of elution could be performed: acid elution with glycine (PH: 2.8) or basic elution with trimethylamine (PH: 8). The yield of recovery was about 1 mg/L for IMAC and 6.4 mg/L or 9.5 mg/L using L-protein, respectively with acid or basic elution. Best recovery was obtained using L-protein and basic elution but this method favored the formation of aggregates (figure 5.1B) and was not used. For Based on these results we decided to perform all the scDb purifications using L-protein with acid elution.

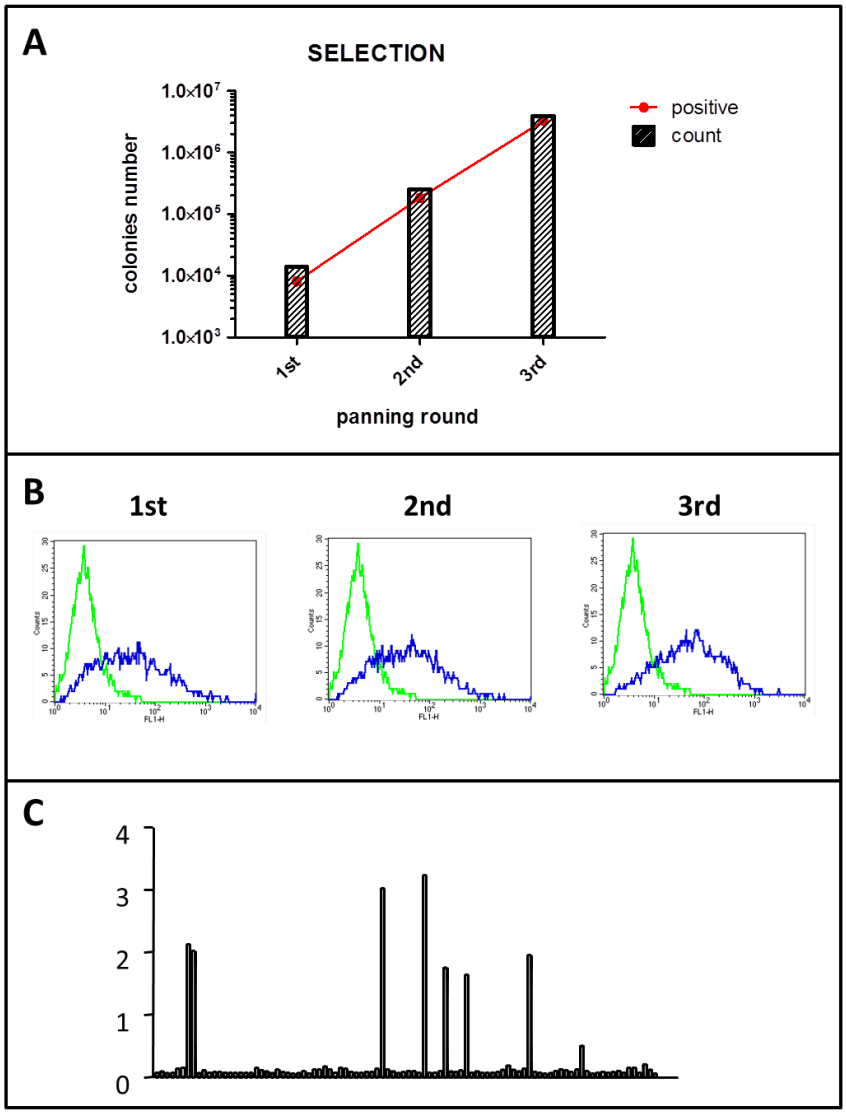


**Figure 4.1. AntiTRAIL-R2 scFvs selection**

**A)** A naïve antibody phage library was used for 3 rounds of selection using naïve TRAIL-R2. In graph is illustrated the number of colonies derived from phages infected bacteria obtained after each cycle of panning. 50 representative colonies were screened by PCR and colonies containing a BsAb gene cassette were considered positive. **B)** FACS analysis evaluating binding capability of M41 TRAIL-R2$^+$ cell line of phages isolated after each panning. AntiM13 mAb followed by antiMouse Ab Alexa Fluor 488 were used.

**C)** Single clone phage ELISA: after third cycle of panning, the binding capability of TRAIL-R2$^+$ cells of phages belonging to single colonies was tested.

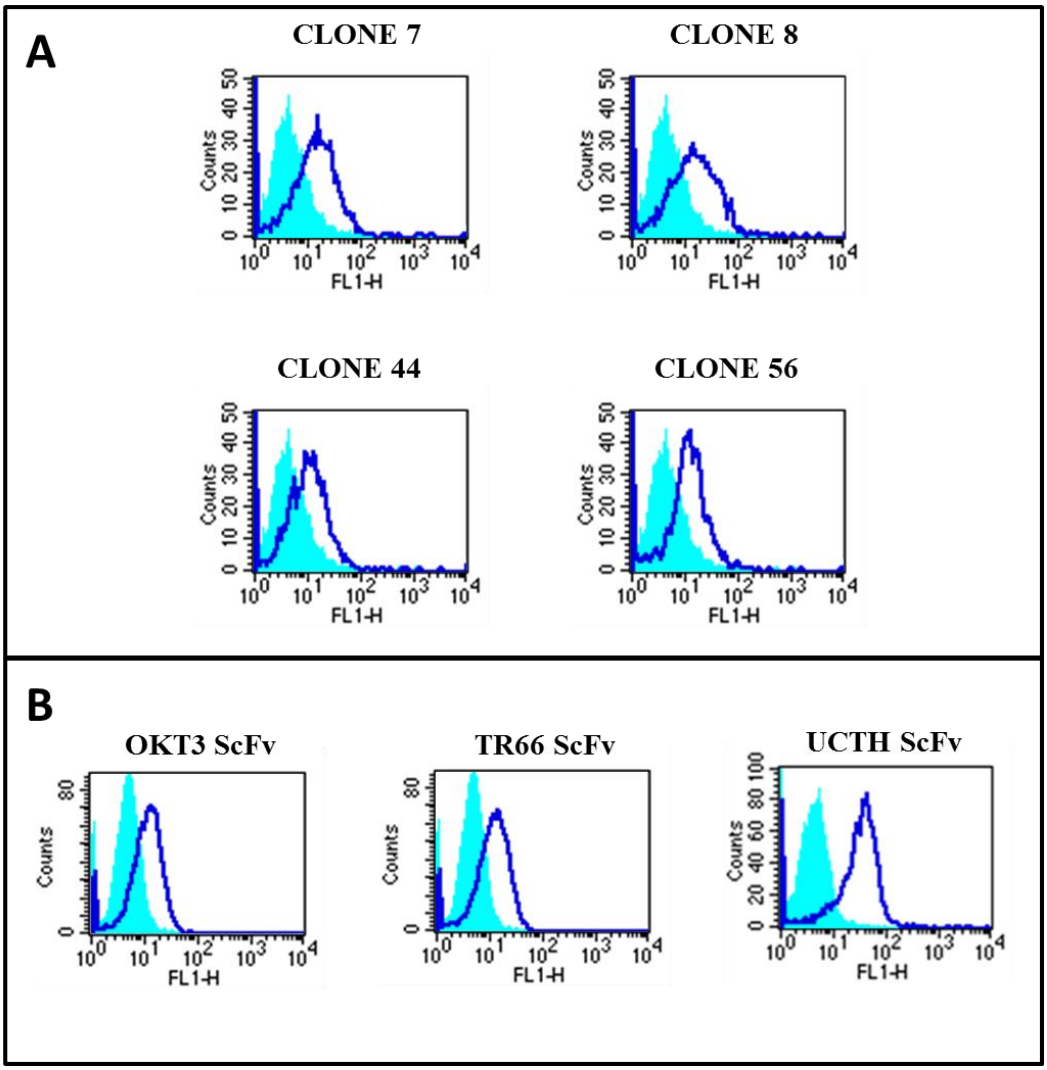


**Figure 4.2. FACS analysis** evaluating binding capability of M15 TRAIL-R2$^{+}$ cell line of isolated antiTRAIL-R2 scFvs (A) and of Jurkat cell line of antiCD3 scFvs (B). Binding was revealed using antiHis tag Ab followed by Alexa Fluor 488 conjugated antiMouse Ab.

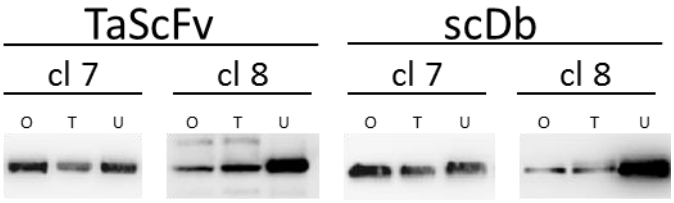


**Figure 4.3. SDS-PAGE and WB analysis of BsAb constructs.**
12 different BsAbs were constructed using all the different combination between the two stable scFvs antiTRAIL-R2 (cl 7 and cl 8) and the 3 scFvs antiCD3 (O:OKT3; T:TR66; U:UCTH). Produced TaScFvs and scDbs were run on SDS-PAGE. Western Blot, stained with antiHis-tag antibody and antiMouse HRP.

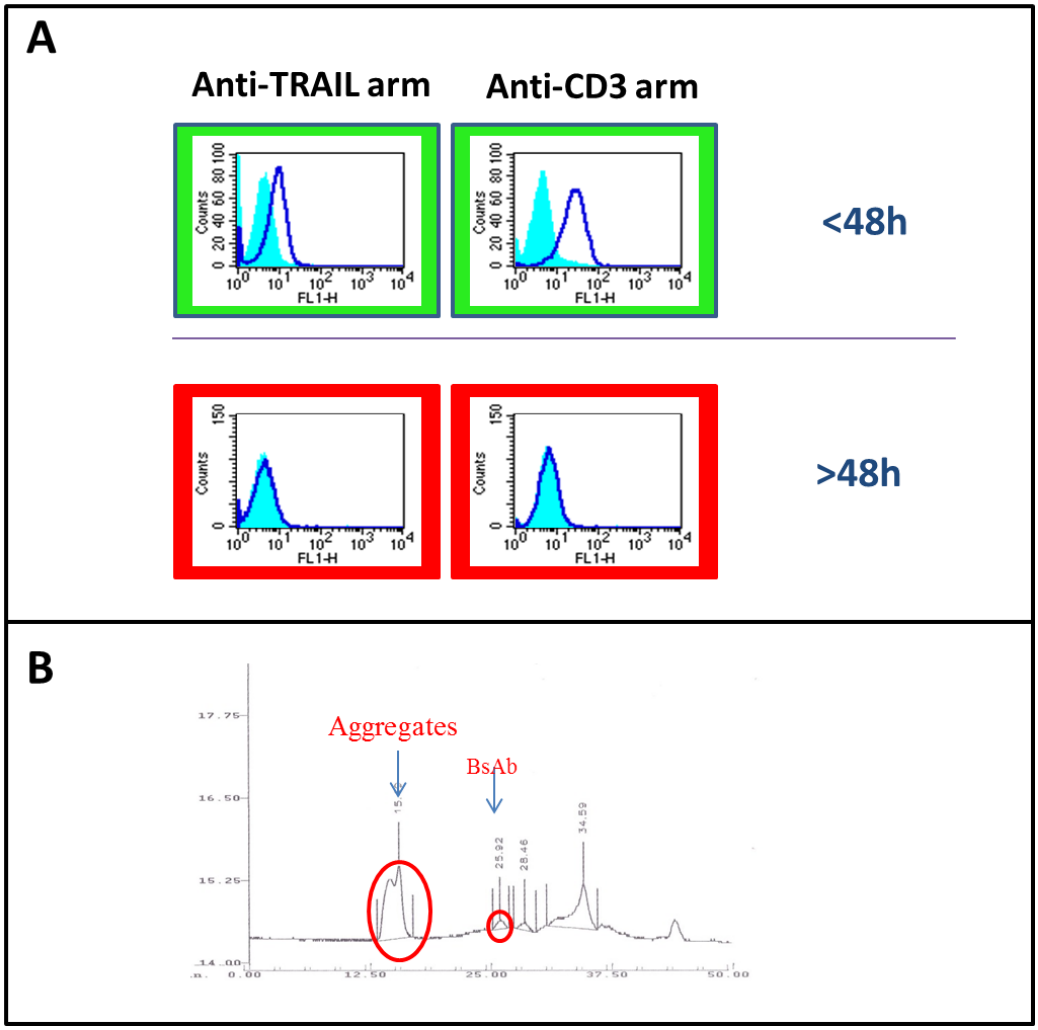


**Figure 4.4. Stability of TaScFv clone 8 + TR66**
**A)** FACS analysis evaluating binding capability of the two specificities of clone 8 + TR66 TaScFv on M15 TRAIL-R2$^{+}$ and Jurkat CD3$^{+}$ cell lines. Upon production, the assay was performed up to 48 hours **(upper panel)** and it was repeated after 48 hours **(lower panel)**.
**B)** SEC experiment evaluating scDb aggregation property after 48 hours.

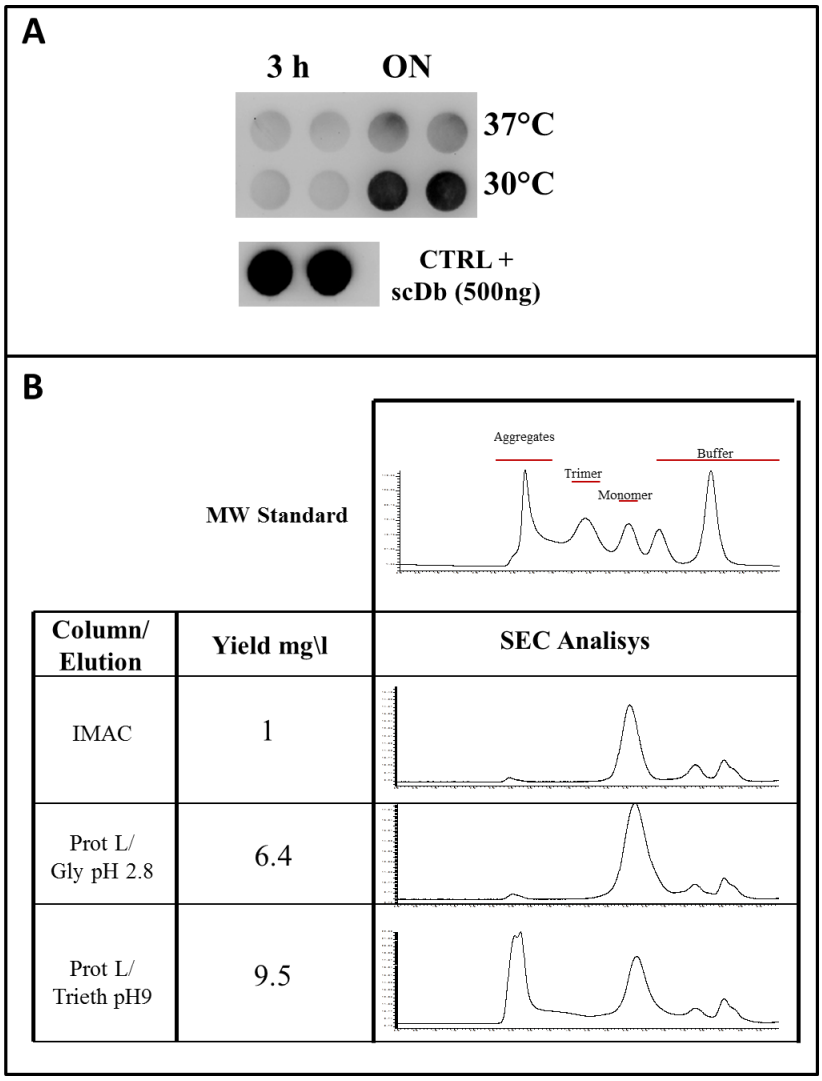


**Figure 5.1. E7/UCTH production and purification set-up**

**A)** Dot blot analysis indicating the quantity of scDb contained in periplasm of bacteria whose production was induced with different conditions. Different temperatures (30 or 37°C) and different times (3 hours or ON) were tested. As positive control 500 ng of purified scDb were used. Presence of scDb was revealed with antiHis tag Ab followed by antiMouse HRP.

**B)** Yields obtained and SEC profile of scDb purified with different methods. IMAC protocol with imidazole elution and L-protein column protocol with glycine (acid) or trimethylamine (basic) elution were performed. Instrument calibration with MW standard is reported in the first line.