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CICLO XXV

TITOLO DELLA TESI DI DOTTORATO

TARGETING CD38 ANTIGEN AS A THERAPEUTIC STRATEGY FOR HEMATOLOGICAL MALIGNANCIES

S.S.D. MED/04

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RIASSUNTO

Il successo di terapie convenzionali come la chemioterapia e la radioterapia per il trattamento delle neoplasie è stato limitato a causa di diversi fattori come la chemioresistenza ai farmaci e la tossicità periferica causata dalla mancanza di specificità di questi approcci. Per questo motivo l'interesse per le terapie selettive che prevedono l'uso di immunotossine, specialmente per il trattamento di tumori ematologici, è in aumento. Le immunotossine sono proteine chimeriche costituite da un ligando selettivo per la cellula bersaglio (dominio di origine anticorpale, citochina o fattore di crescita) che media il legame e l'internalizzazione della porzione tossica legata chimicamente o fusa geneticamente, generalmente rappresentata da una tossina di origine vegetale o batterica che agisce interferendo con la sintesi proteica.

In questo lavoro viene descritta la costruzione di nuove proteine di fusione ad uso terapeutico progettate per indurre apoptosi selettivamente in neoplasie umane dei linfociti B e la valutazione dell'effetto potenziante ottenuto attraverso l'associazione delle immunotossine con farmaci coinvolti in meccanismi metabolici intracellulari. Il dominio di legame delle nostre immunotossine è rappresentato da frammenti anticorpali a singola catena (scFv) diretti verso l'antigene CD38, una molecole di superficie espressa ad alti livelli dai linfociti B di un sottogruppo particolarmente aggressivo di Leucemia Linfatica Cronica (CLL) che evolve in una patologia dall'esito prognostico sfavorevole, nota come Sindome di Richter, e dalle plasmacellule tumorali immature nel Mieloma Multiplo (MM). L'scFv è fuso ad una porzione tossica che agisce inibendo il meccanismo della sintesi proteica negli organismi eucarioti e nel caso delle nostre immunotossine è rappresentato da una forma tronca della Esotossina A prodotta dal batterio *Pseudomonas aeruginosa* (PE40) o in alternativa dalla tossina di origine vegetale saporina.

Abbiamo inizialmente progettato una immunotossina con PE40 ed una con saporina contenenti un scFv derivato da un anticorpo monoclonale (mAb) sviluppato e caratterizzato nel nostro laboratorio. Tutti i costrutti ricombinanti sono stati prodotti nel sistema di espressione di origine batterica *Escherichia coli* e purificati da corpi di inclusione tramite IMAC. Tuttavia, l'scFv 1E8 non ha consentito di preservare l'efficienza di legame dell'anticorpo parentale. Inoltre, le immunotossine ricombinanti ottenute dalla fusione dell'scFv 1E8 con PE40 o saporina hanno mostrato una bassa affinità di legame

nei confronti delle cellule bersaglio esprimenti la molecola CD38 e, di conseguenza, è stata rilavata solo una trascurabile attività citotossica.

Con la progettazione della forma divalente dell'scFv 1E8, il nostro scopo è stato quello di aumentare l'affinità di legame dei costrutti. Nonostante i risultati sconfortanti del saggio di legame in citometria a flusso, la molecola DIV1E8-SAP ha dimostrato di inibire la sintesi proteica di cellule CD38-positive con una IC_{50} nell'ordine del sub-nanomolare.

Successivamente abbiamo progettato due immunotossine ricombinanti dirette verso l'antigene CD38, il cui dominio di legame era costituito da un scFv derivato da un mAb con una specificità epitopica diversa da quella del precedentemente descritto 1E8. Le immunotossine AT13/5-PE e AT13/5-SAP hanno dimostrato buone proprietà di legame con una elevata affinità e specificità per l'antigene CD38 espresso sulla superficie di cellule derivate da Linfoma di Burkitt e cellule di mieloma.

Abbiamo dimostrato l'abilità si queste immunotossine di inibire la sintesi proteica nelle linee cellulari studiate e ne abbiamo chiaramente dimostrato un effetto dose-risposta. Il blocco della sintesi proteica causato dalle immunotossine derivate da AT13/5 ha determinato infine l'innesco del processo di apoptosi e la morte cellulare. Attraverso saggi di apoptosi abbiamo dimostrato la capacità di AT13/5-PE e AT13/5-SAP di indurre apoptosi in cellule Daudi e RPMI8226.

Abbiamo perciò provato che l'associazione delle nostre immunotossine con molecole terapeutiche che agiscono su diversi bersagli dalla cascata di traduzione del segnale coinvolta nella crescita cellulare, nella sopravvivenza e nella proliferazione, potrebbe essere sinergica in alcune linee cellulari. In particolare abbiamo osservato che farmaci coinvolti nell'inibizione di Bcl-2, Bcl-xL e Bcl-w (noti come BH3-mimetics) possono aumentare la potenza delle nostre immunotossine.

Abbiamo infine dimostrato una prima prova di concetto riguardo l'efficacia delle immunotossine derivate da AT13/5 su linfociti B derivati da pazienti affetti da CLL, tuttavia questo studio necessita di essere implementato con una casistica più ampia.

ABSTRACT

The success of conventional chemotherapy and radiotherapy for the treatment of cancer has been limited due to several factors like chemoresistance to drugs and peripheral toxicity caused by the lack of specificity of these approaches. For this reason the interest in targeted therapies using immunotoxins (ITs) especially for the treatment of hematological malignancies is increasing. Immunotoxins are chimeric proteins with a cell-selective ligand (antibody-derived domain, cytokine or growth factor) which drives the binding and internalization of a chemically linked or genetically fused toxic portion, generally represented by a plant or bacterial toxin which acts by interfering with protein synthesis.

Here we report on the construction of novel therapeutic fusion proteins designed to induce target antigen-restricted apoptosis in human B-cell neoplasias and the evaluation of the potentiating effect obtained by the association of the ITs with drugs involved in intracellular metabolic pathways. The binding portion of our ITs is represented by a single-chain antibody fragment (scFv) directed against CD38 antigen, a surface molecule highly expressed by B lymphocytes of a particularly aggressive sub-group of Chronic Lymphocytic Leukemia (CLL) leading to the prognostically unfavorable Richter's Syndrome and by the neoplastic immature plasma cells in Multiple Myeloma (MM). The scFv is fused to a toxic portion which acts by inhibiting the mechanism of protein synthesis in eukaryotes and in our ITs is represented by a truncated version of the bacterial toxin *Pseudomonas aeruginosa* Exotoxin A (PE40) or alternatively by the plant toxin saporin.

We firstly designed a PE40- and a saporin-based IT comprising a scFv derived from a monoclonal antibody (mAb) developed and characterized in our laboratory. All the recombinant constructs were produced in the bacterial expression system *E. coli* and purified from inclusion bodies by IMAC. However, the scFv format (1E8) did not allow to preserve the binding efficiency of the parental monoclonal. Moreover, the recombinant ITs created by the fusion of 1E8 scFv with PE40 or saporin showed a low binding affinity to the CD38 target cells and, as a consequence, only negligible citotoxic activity was detected.

With the creation of the divalent form of the 1E8 scFv, our purpose was to increase the binding affinity of the constructs. Despite the discouraging results of the flow-cytometric

binding assay, DIV1E8-SAP demonstrated to inhibit protein synthesis of CD38-positive cells with an IC_{50} in the sub-nanomolar range.

Then we designed two anti-CD38 recombinant ITs whose binding portion was a scFv derived from a mAb with an epitope specificity different from that of the previously described 1E8. AT13/5-PE and AT13/5-SAP showed good binding properties with a high affinity and specificity for CD38 antigen expressed on the surface of Burkitt's lymphoma cells and myeloma cells.

We proved the ability of these ITs to inhibit protein synthesis in the cell lines studied and we clearly demonstrated a dose-response effect of the ITs. The arrest of protein synthesis caused by the AT13/5-derived ITs finally leads to the triggering of the apoptotic cascade and to cell death. By using apoptosis assays we demonstrated the capability of AT13/5-PE and AT13/5-SAP to induce apoptosis of Daudi and RPMI8226 cells.

Then we proved that the association of our ITs with therapeutic molecules acting on different targets of the signal transduction cascade involved in cell growth, survival and proliferation, could be synergistic in some cell lines. In particular we observed that drugs involved in the Bcl-2, Bcl-xL and Bcl-w inhibition (BH3-mimetics) can increase the potency of our ITs.

Finally we demonstrated a first proof of concept about the efficacy of AT13/5-derived ITs on B-lymphocytes derived from CLL patient, but this study needs to be implemented with a wider number of cases.

INDEX

ABBREVIATION USED:

Ab/Abs	Antibody/antibodies
Amp	Ampicillin
APS	Ammonium persulfate
Cam	Chloramphenicol
cpm	Counts per minute
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Gln	Glutamine
h	hour
H_2O	Water
Ig	Immunoglobulin
IMAC	Immobilized metal ion affinity chromatography
IPTG	isopropyl-beta-D-thiogalactopyranoside
IT /ITs	Immunotoxin(s)
mAb	Monoclonal antibody
MFI	Medium Fluorescence Intensity
NaOAc	Sodium acetate
NTA	Nitriloacetic acid
o.n.	overnight
OD	Optical density
PCR	Polymerase chain reaction
PE	*Pseudomonas aeruginosa* Exotoxin A
PE40	Truncated form of *Pseudomonas aeruginosa* Exotoxin A
PVDF	Polyvinylidene fluoride
rpm	Revolutions per minute
RT	Room temperature
scFv	Single-chain Fragment variable
SDS	Sodium dodecyl sulfate
$t_{½}$	Half-life
Temed	Tetramethylethylenediamine
Tet	Tetracycline
V_H	Heavy chain variable domain
V_L or V_k	Light chain variable domain

1. INTRODUCTION

1.1 CONVENTIONAL THERAPY OF CANCER

According to the World Health Organization (WHO), cancer is responsible for approximately 7.6 million (13%) of the 59 million deaths that occur each year. WHO estimates that if current cancer rates remain unchanged, new cases of cancer will increase from 12.7 million cases (2008) to 21.4 million cases (2030) [1]. Moreover, considering the more developed regions of the world it can be observed that cancer is responsible for about 25% of all deaths.

Among the over 400 types of cancer, a broad group is represented by hematological neoplasms affecting the blood, bone marrow and lymphoid system and characterized by an abnormal increase of immature white blood cells. As the disease progresses, leukemic cells move through the blood stream and invade other organs, such as the spleen, lymph nodes, liver, and central nervous system. As it can be observed in Figure 1.1, the incidence rate of leukemia in the industrialized countries is 11.4 cases per 100.000 person-years.

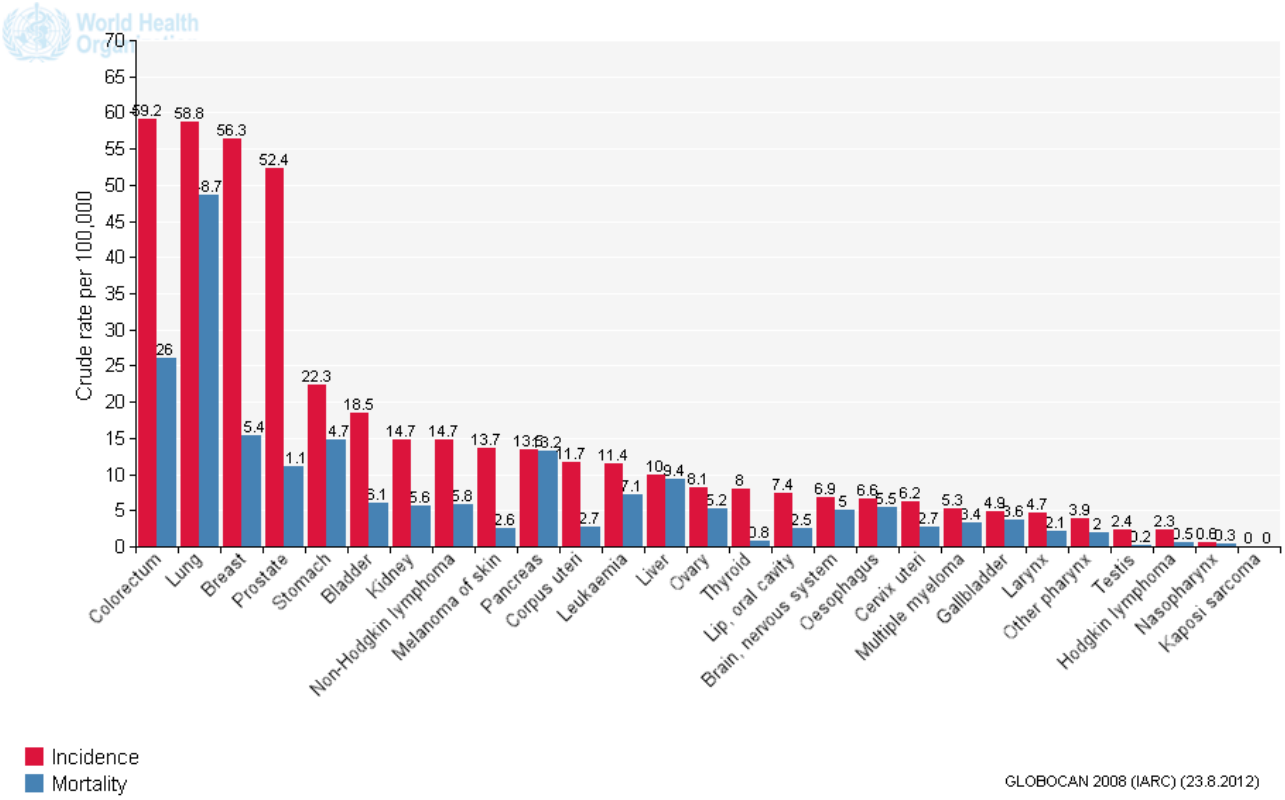


Figure 1.1 Incidence and mortality rate of cancer in the more developed regions of the world considered for both sexes of all ages (WHO, Globocan Project 2008).

The first line treatment for leukemia is represented by chemotherapy and radiation therapy, often used in combination.

Chemotherapy agents attack rapidly dividing cells and due to their inability to distinguish leukemia cells from other rapidly dividing but non-cancerous cells, they often cause toxicity towards healthy tissues with a rapid cell turnover [2] such as healthy red and white blood cells, blood-clotting platelets, hair follicles, and cells lining the gastrointestinal tract, thus creating unpleasant side effects. Furthermore the damage to white blood cells increases the immunodeficiency and the risk of infection.

Radiotherapy kills leukemia cells by exposing them to ionizing radiation that damages cell DNA but the treatment can lead to DNA mutations in the by-stander normal cells increasing the risk of the potential onset of a secondary radiation-associated cancer [3].

For the treatment of haematological malignancies, chemotherapy and radiotherapy are often supported by hematopoietic stem cells transplantation with the double purpose to replace disease-causing stem cells with healthy ones and to replenish a bone marrow which is damaged by the aggressiveness of the therapeutics mentioned above. Also stem cells therapy is not free of side effects and graft-versus-host disease is the major complication identified in the case of allogenic transplantation.

Along with the commonly known side effects of conventional treatments, frequent poor responses and relapses are observed especially in some indolent malignancies, due to their slow progress and inefficacy of the therapies. Besides, tumor cells can develop a resistance to chemotherapy drugs, hindering their mechanism of action or promoting their expulsion out of the cell before they can act [4].

As a consequence, the need for an improved efficacy in cancer therapies has been increasingly felt in the last few years, placing the focus of research on the development of new drugs that combine power of action and selective targeting of cancerous cells. An ever clearer understanding of the biochemical events that are implicated in the onset and progression of many malignancies has allowed to design tumor-specific therapies that directly target the molecules involved in the development of tumors or that selectively deliver the drug into cancerous cells. The opportunity of specifically affecting a tumor is provided by the presence of molecular targets being selectively expressed on the surface of cancerous cells: these are the tumor marker antigens [2].

1.2 ANTIBODY-BASED TARGETED THERAPIES

Antibody-based therapy for cancer has become established over the past 15 years and is now one of the most successful and important strategies for treating patients with hematological malignancies and solid tumors.

However, the history of true antibody therapy began about a century ago with the discovery by von Behring that resistance to infectious diseases like tetanus and diphteria could be transferred between animals through their sera, a strategy known as passive serotherapy [5]. Further developments of serotherapy came from Ehrlich, who forged the term “magic bullet” referring to immunoglobulins as the real responsible for immune protection, and from Köhler and Milstein, who developed in 1975 the hybridoma technology, a method for generation and large-scale production of monoclonal antibodies (mAbs) of murine origin [6]. However, the fundamental basis of antibody-based therapy of tumors dates back to the original observation of antigen expression by tumor cells through serological techniques in the 1960s [7]. The definition of cell surface antigens that are expressed by human cancers has revealed a broad array of targets that are overexpressed, mutated or selectively expressed compared to normal tissues. Therapeutics that target these antigens can function through mediating alterations in antigen or receptor function (such as agonist or antagonist functions), modulating the immune system or delivering a specific drug that is conjugated to an antibody [8].

Cancer immunotherapy can be considered from several perspectives, but one convenient way to categorize immunotherapy is to think of active or passive approaches:

- active immunotherapy involves the stimulation of the patient’s immune response against tumor cells (vaccination) through the administration of antigens (Ag) in various shapes (e.g. recombinant proteins, cDNAs inserted into plasmids or viral vectors, peptides) or by using tumor cells unable to replicate (apoptotic or necrotic) or by loaded immune cells;
- passive immunotherapy consists in the administration of immunological effectors (e.g. monoclonal antibodies alone or conjugated to drugs, toxins or cytokines, tumor-specific T lymphocytes).

Available clinically useful mAbs typically use a combination of direct and indirect mechanisms to perform their anti-tumor activity (Fig. 1.2). Indirect mechanisms involve the interaction of the Fc region of the mAb with components of the immune system which determine antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). On the contrary, through a direct approach, the binding of the antibody to a specific cell-surface antigen can lead to the neutralization of cytokines and angiogenic factors secreted by the tumor or to receptor blockade and interference with the signaling pathways of the cells including that involved in apoptosis induction. A more direct approach to kill the targeted cell entails the use of immunoconjugates which are often made by antibodies directly armed through their covalent linkage to toxic molecules, such as radionuclides or toxins (for example, small molecules or proteins). Alternatively, arming antibodies with cytokines is intended to create high intratumor concentrations of cytokines to stimulate the antitumor immune response (T cells, B cells or natural killer cells), while avoiding the toxicity associated with systemic cytokine delivery. Arming of antibodies can be also achieved by attaching engineered antibody fragments to the surface of liposomes loaded with drugs or toxins. Finally, pre-targeting strategies aim for the selective delivery of radionuclides to tumors or selective intratumor activation of prodrugs, thereby diminishing the systemic toxicities of these cytotoxic agents. Antibody-directed enzyme prodrug therapy (ADEPT) is a pre-targeting approach which specifically aims at causing bystander effects by targeting enzymes to the tumor cell and delivering a prodrug that is ideally converted to an active drug solely within the tumor [8].

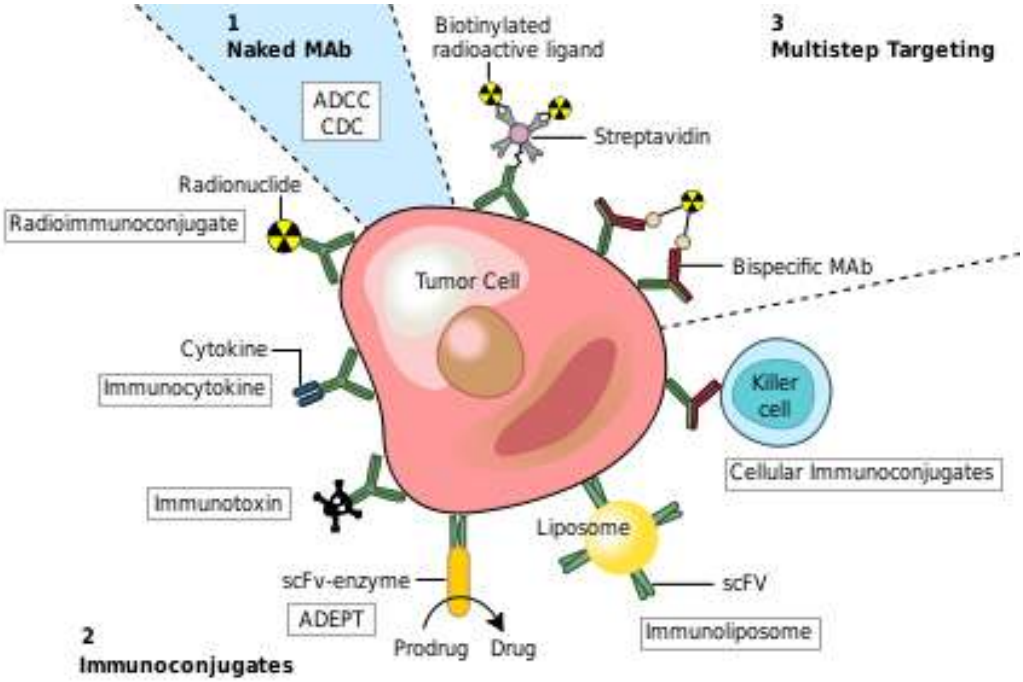


Figure 1.2 Antibody-based therapy of cancer. (1) Targeting "naked" monoclonal antibodies (not coupled to any effector molecule) to the tumor can result in the destruction of the cancerous cells by antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). **(2)** mAbs can also be used to target payloads (e.g., radioisotopes, drugs or toxins) to directly kill tumor cells or to activate prodrugs specifically within the tumor (antibody-directed enzyme prodrug therapy, ADEPT), or to target cytokines to stimulate the immune response against tumors. Similarly, mAbs and engineered antibody fragments can be armed with liposomes loaded with drugs or toxins. **(3)** The cytotoxicity of mAbs can be improved also through multistep targeting. Bispecific antibodies that bind to two different antigens can be pre-loaded with the cytotoxic machinery before administration (indirect arming) or alternatively pre-targeted to the tumor before delivery of the cytotoxic payload. In the same way, for radionuclide pre-targeting, an antibody–streptavidin conjugate is allowed to accrue within a tumor and is then used to capture a biotin–chelator–radionuclide complex. (Modified from Carter, P., 2001).

A number of antibodies have been approved for the treatment of either solid tumors and hematological malignancies, both as unconjugated antibodies and for the delivery of isotopes and drugs or toxins to cancer cells (Table 1.1).

Table 1.1 Monoclonal antibodies currently FDA approved in oncology

Generic name	Trade name	Antibody format	Target	FDA-approved indication	Mechanisms of action
Trastuzumab	**Herceptin**	**humanized IgG1**	HER2	Breast cancer Gastric or gastro-oesophagela junction carcinoma	Inhibition of HER2 signalling and ADCC
Bevacizumab	**Avastin**	**humanized IgG1**	VEGF	Colorectal cancer Non small-cell lung cancer Advanced breast cancer	Inhibition of VEGF signalling
Cetuximab	**Erbitux**	**chimeric IgG1**	EGFR	Colorectal cancer Head and neck cancers	Inhibition of EGFR signalling and ADCC
Panitumumab	**Vectibix**	**human IgG2**	EGFR	Colorectal cancer	Inhibition of EGFR signalling
Ipilimumab	**Yervoy**	**Human IgG1**	CTLA4	Metastatic melanoma	Inhibition of CTLA4 signalling
Rituximab	**Mabthera**	**chimeric IgG1**	CD20	Non-Hodgkin's lymphoma CLL	ADCC, direct induction of apoptosis and CDC
Alemtuzumab	**Campath**	**humanized IgG1**	CD52	CLL	Direct induction of apoptosis and CDC
Ofatumumab	**Arzerra**	**human IgG1**	CD20	CLL	ADCC and CDC
Gemtuzumab ozogamicin	**Mylotarg**	**humanized IgG4**	CD33	Acute myeloid leukaemia (withdrawn in June 2010)	Delivery of toxic payload, calicheamicin toxin
Brentuximab vedotin	**Adcetris**	**chimeric IgG1**	CD30	Hodgkin's lymphoma	Delivery of toxic payload, auristatin toxin
^{90}Y-labelled ibritumomab tiuxetan	**Zevalin**	**murine IgG1**	CD20	Non-Hodgkin's lymphoma	Delivery of the radioisotope ^{90}Y
^{131}I-labelled tositumomab	**Bexxar**	**murine IgG1**	CD20	Non-Hodgkin's lymphoma	Delivery of the radioisotope ^{131}I, ADCC and direct induction of apoptosis

HER2, human epidermal growth factor receptor 2; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; CTLA4, cytotoxic T lymphocyte-associated antigen 4; CLL, chronic lymphocytic leukemia.

1.3 TUMOR CELL ANTIGENS

The targets of cancer immunotherapy can be classified as:

a. Tumor Specific Antigens (TSAs), which are present only on tumor cells and are represented by new mutant proteins or aberrantly glycosilated versions of normal proteins;
b. Tumor Associated Antigens (TAAs), which are proteins being over-expressed on some tumor cells compared to healthy cells;
c. Oncofetal proteins, which are normally produced in the early stages of embryonic development and disappear by the time the immune system is fully developed.

The safety and efficacy of therapeutic mAbs and immunoconjugates in oncology vary depending on the nature of the target antigen. The ideal antigen for antibody-based therapy of leukemias should exhibit certain characteristics:

- its expression should be restricted to the surface of cancer cells. If the antigen is expressed on normal cells, the loss of these cells should not result in serious complications such as life-threatening cytopaenia or prolonged immunosuppression;
- it should not be expressed by early progenitors in bone marrow, thus allowing the reconstitution of B lymphocyte populations after the treatment;
- the target antigen should be expressed homogeneously and at high density on the leukemic cells to provide an adequate number of antibody binding sites. Studies suggest that tumor responses correlate with target density. The lower responsiveness of CD20-expressing CLL to rituximab compared with follicular B cell non-Hodgkin's Lymphoma appears to be due to the lower level of CD20 expressed in CLL [9];
- the target antigen should be accessible and its secretion should be minimal, as secreted antigens can bind the antibody in the circulation and could prevent sufficient antibody from binding to the tumor;
- if the desired mechanism of action is ADCC or CDC (as occurs for unmodified or "naked" antibodies), target antigens should not undergo internalization so as to maximize the availability of the Fc region to immune effector cells and

complement proteins. By contrast, good internalization is desirable for antibodies or proteins that deliver toxins into the cancer cell and for antibodies the action of which is primarily based on the downregulation of cell surface receptors.

The quest for surface molecules representing optimal targets for cancer immunotherapy is a major concern for solid tumors as well as hematologic malignancies. However, compared to solid tumors, blood-borne neoplasias have proven easier to treat with mAbs and their derivatives, because single circulating cells are more exposed to blood-infused drugs that can thus work efficiently at a lower dosage [10].

In lymphoproliferative diseases like leukemias and lymphomas most tumor antigens come into the category of proteins that constitute the *Cluster of Differentiation* (CD) of lymphocytes; they are mainly tumor-associated antigens (TAAs). The marker antigens that have turned out to be of particular interest as targets for immunotherapy of hematologic malignancies are CD20, CD22, CD19, CD38, CD52, CD30, CD33, CD25, CD80 and CD40 [11].

1.3.1 CD38

Human CD38 was originally designated as an activation marker during the quest to identify cell surface molecules involved in T cell recognition; indeed, this concept was validated by the observation of CD38 expression on thymocytes and T lymphocytes [12]. This definition was later proven inadequate when the molecule was shown to be neither lineage- nor activation-restricted and now CD38 expression is considered virtually ubiquitous with a widespread distribution either in lymphoid and nonlymphoid tissues (Table 1.2). However, the underlying mechanism of action of the molecule's expression in different cell lineage is still not entirely clear, and this is reflected in the sometimes contrasting reports in literature. A non negligible fact is that the expression of CD38 modifies significantly with age and, mostly for the hematological lineage, CD38 has been demonstrated to be expressed by immature hematopoietic cells, downregulated by mature cells and re-expressed at high levels by activated lymphocytes such as T cells, B cells, dendritic cells and natural killer (NK) cells [13].

Table 1.2. Distribution of CD38

Tissues	Cell population	Function
LYMPHOID		
blood	T cells (precursors, activated)	Activation and homing
	B cells (precursors, activated)	Inhibition of lymphopoiesis in BM
	Myeloid cells (monocytes, macrophages, dendritic cells)	Activation and homing
	NK cells	Redirection of lysis
	Erythrocytes	Unknown
	Platelets	Unknown
Cord blood	T and B lymphocytes, monocytes	Activation
Bone marrow	Precursors	Differentiation
	Plasma cells	Homing?
Thymus	Cortical thymocytes	Apoptosis
Lymph nodes	Germinal center B cells	Rescue from apoptosis
NON-LYMPHOID		
Brain	Purkinje cells, neurofibrillary tangles	Memory process?
	Cerebral cortex (rat)	Unknown
	Cultured astrocytes	Unknown
	Cerebellum	Unknown
Eye	Cornea	Unknown
	Retinal gangliar cells	Unknown
Prostate	Epithelial cells	Neoplastic transformation?
Gut	Intraepithelial lymphocytes	Activation
	Lamina propria lymphocytes	Cytokine secretion
	Small intestinal lymphatic vessels (rat)	Unknown
Pancreas	β cells	Insulin secretion
Muscle	Sarcolemma smooth and striated muscle	Contractility
	Myometrial smooth muscle cells (rat)	Contractility
Bone	Osteoclasts	Bone resorption
Kidney	Glomeruli	Unknown

1.3.1.1 CD38 STRUCTURE AND FUNCTION

Human CD38 is a pleiotropic type II surface glycoprotein made of 300 amino acid residues and with a molecular weight of 46 kDa comprising two to four *N*-linked oligosaccharide chains containing sialic acid residues [14].

The structure of CD38 proved difficult to establish and has been accomplished only recently thanks to a highly efficient yeast expression system which has been developed on purpose to enable structure-function studies and to facilitate purification of much of the cyclase for crystallography. This strategy has been used to obtain a construct with a missing transmembrane segment and mutated glycosylation sites [15]. The resulting extramembrane domain was fully active in terms of enzymatic functions and was crystallized as head-to-tail dimers [14].

The overall structure of the CD38 molecule is "L"-shaped and can be divided into two separate domains (Fig. 1.3). The N-terminal domain (residues 45-118 and 144-200) is formed by a bundle of α-helices (α1, α2, α3, α5, α6) and two short β-strands (β1. β3); and the C-terminal domain (residues 119-143 and 201-300) consists of a four-stranded parallel β-sheets (β2, β4, β5 and β6) surrounded by two long (α8 and α9) and two short α-helices (α4 and α7) [14]. These two distinct domains are connected by a hinge region composed of three peptide chains, and six disulfide bonds further stabilizes the relative conformations of the domains maintaining the monomeric and catalytically active structure of the molecule [16].

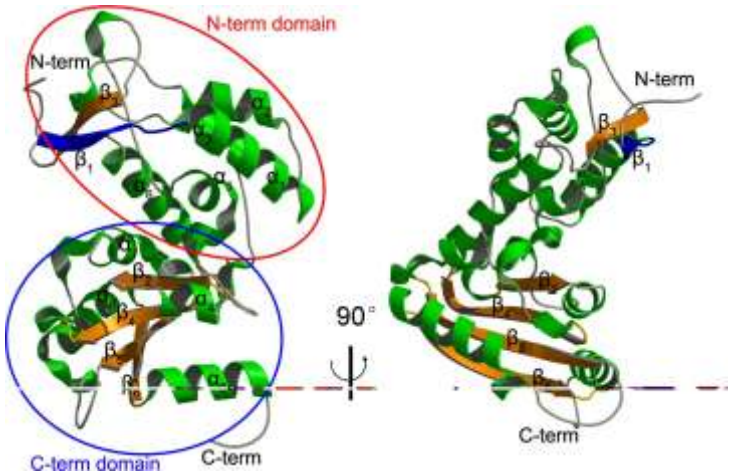


Figure 1.3 Two views of a ribbon representation of soluble human CD38 structure related by 90° rotation around a vertical axis.

CD38 was originally defined as an ectoenzyme (enzyme of the plasmatic membrane which catalyzes reaction taking place in the extracellular space), but during evolution it acquired the ability to mediate cell–cell interactions, acting as a receptor [17].

As an ectoenzyme, CD38 belongs to a complex family of the cell surface enzymes involved in the catabolism of extracellular nucleotides. With its ADP-ribosyl cyclase activity, CD38 generates cyclic ADP ribose (cADPR) and ADPR from NAD^+ and nicotinic acid adenine dinucleotide phosphate (NAADP) from $NADP^+$. These second messengers cooperate in the regulation and modulation of intracellular Ca^{2+} that plays a key role in several physiological processes, including cell proliferation, muscle contraction, stem cell regeneration and hormone secretion [18] (Fig. 1.4a).

The use of agonistic mAbs demonstrated that CD38 engagement is followed by signals that are apparently independent of CD38 enzymatic activities [19]. In fact, CD38 ligation determine tyrosine phosphorylation of a sequential number of intracellular signal transducers such as ZAP-70 and the proto-oncogene c-cbl (which drive life and death messages to the cells) or the phospholipase C-γ responsible for Ca^{2+} mobilization [20].

Study of CD38 as a receptor also demonstrated that the cross-linking with CD31 (also known as platelet endothelial cell adhesion molecule-1, PECAM-1), as a non-substrate ligand for CD38, activates a signaling which leads to proliferation of lymphocyte populations and to inhibition of apoptosis [21] (Fig. 1.4b).

Finally, studies by A. Funaro demonstrated that CD38 undergoes internalization following ligation with agonistic (IB4) or nonagonistic (IB6) specific mAbs [22]. Internalization, along with shedding, represents a mechanism of down-regulation of CD38 and this is independent on the amount of CD38 molecules constitutively expressed by different cells. Furthermore, this mechanism never involves the entire amount of surface molecule; on the contrary, the internalized fraction represents an almost constant percentage (30-40%) of the total amount of surface CD38 molecules, suggesting the existence of two pools of molecules, one of which undergoes internalization after Ab binding.

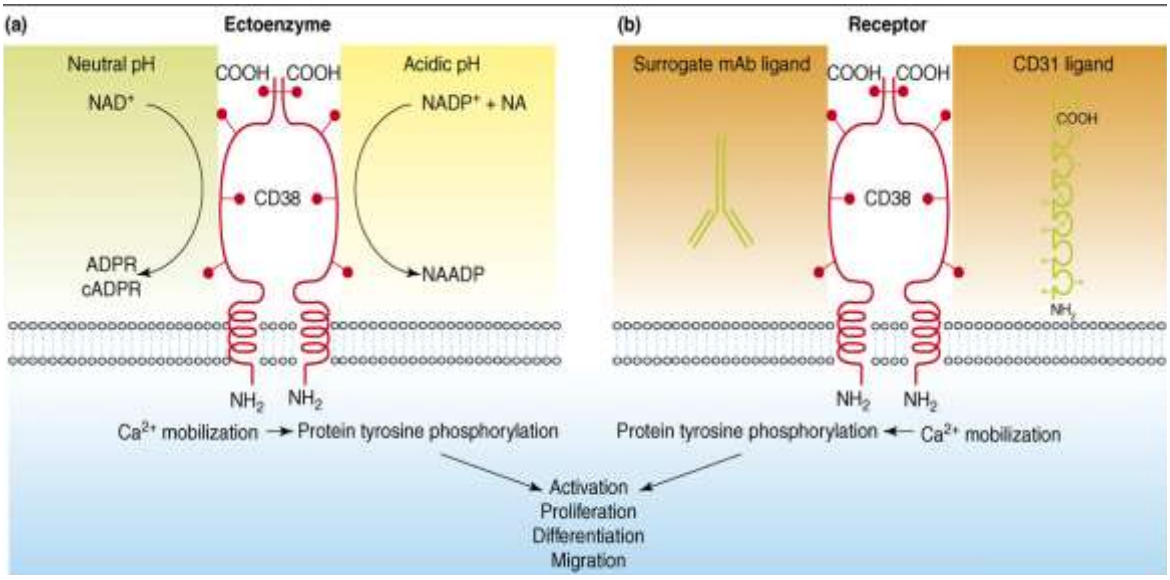


Figure 1.4 Schematic representation of the pleiotropism attributed to human CD38. The molecule works as an ectoenzyme **(a)**, transforming NAD+ and NADP+ into cADPR, ADPR and NAADP. The balance between the reactions is influenced by extracellular pH. The enzymatic products are powerful Ca^{2+}-mobilizing compounds inside the cell. CD38 also acts as a receptor **(b)** interacting with the non-substrate ligand CD31 or with surrogate agonistic mAbs. The resulting intracellular events include Ca^{2+} mobilization, cell activation, proliferation, differentiation and migration. Abbreviations: ADPR, adenosine diphosphate ribose; cADPR, cyclic adenosine diphosphate ribose; NA, nicotinic acid; NAADP, nicotinic acid adenine dinucleotide phosphate; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate. (Deaglio, S. *et al*., 2008)

1.3.1.2 CD38 AS A TARGET FOR IMMUNOTHERAPY

CD38 is expressed at high epitope density by a variety of lymphoid tumors, including most cases of myeloma [23], chronic lymphocytic leukaemia (CLL) [24], some cases of AIDS-associated lymphoma [25] and many cases of posttransplant lymphoproliferations [26]. The marked quantitative differences in cell surface expression between normal cells and their leukemic counterparts make CD38 an attractive target for immunotherapy (Fig. 1.5). Moreover, its specific internalization properties allow to consider CD38 a good candidate for the treatment of hematological malignancies with immunotoxins.

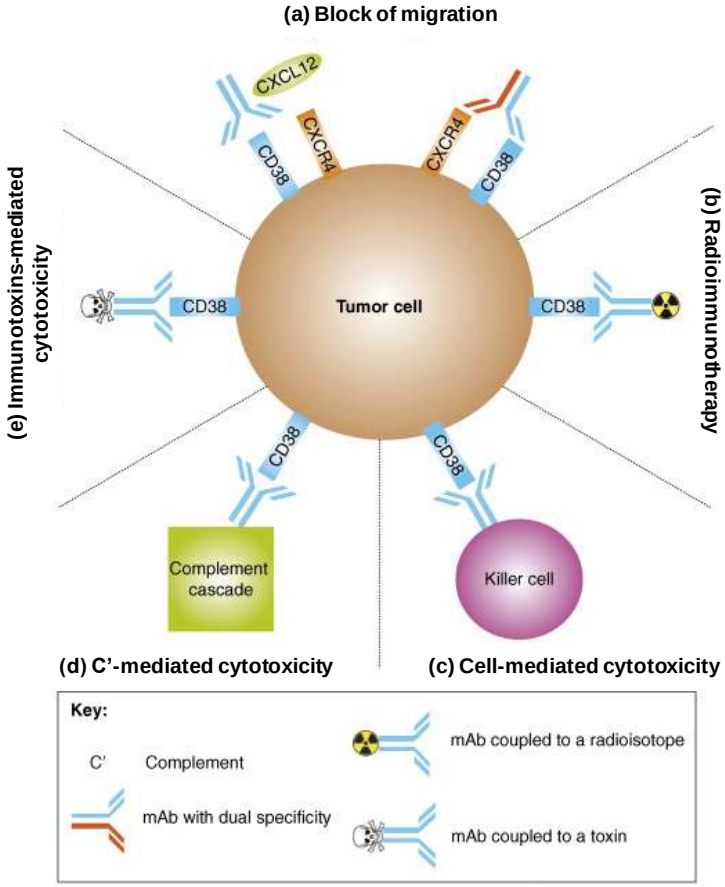


Figure 1.5 Potential applications of CD38 as a therapeutic target. The mechanisms of action of therapeutic anti-CD38 mAbs include: **(a)** the inhibition of a function, in this instance migration, considered critical for CLL progression. Inhibition might be the result of the action of a mAb binding a specific domain of CD38 and perturbing ligation of CXCL12 to its receptor. Alternatively, a divalent ligand of antibody origin might provide a simultaneous binding to CD38 and CXCR4, increasing ligand specificity. **(b)** The use of mAbs as carriers of radiopharmaceuticals delivering a lethal hit either by surface (γ emitters) or cytoplasmic (β emitters) irradiation. **(c)** The elicitation of cytotoxicity mediated *in vivo* by killer cells. **(d)** The elicitation of cytotoxicity mediated *in vivo* by complement. **(e)** The use of mAbs as carriers of toxins. (Modified from Deaglio, S. *et al.*, 2008).

1.3.1.2.1 Chronic Lymphocytic Leukemia (CLL)

B-cell chronic lymphocytic leukemia (B-CLL) is the abnormal progressive accumulation of functionally incompetent monoclonal B-lymphocytes in blood, bone marrow, lymph nodes and spleen [27]. It is the most common adult leukemia in Western countries, accounting for about 30% of total leukemias. Worldwide there are approximately 180.000 new cases every year. CLL is a pathology of the adult age which is rarely diagnosticated in individuals earlier than 40 years and its incidence rapidly increases with age after 55

years. The clinical course of CLL is extremely variable with survival ranging from 1 to more than 15 years.

The exact causes of CLL are unknown, with epidemiological studies finding no association with viral infection, chemical or radiation exposure [28]. Conventional therapies are ineffective and, although hematopoietic stem cell transplantation (HSCT) has led to complete remission for some patients [29], the development of new therapeutic strategies is critical to improve the clinical outcome.

Most of the circulating B-CLL cells are quiescent, resting in the G_0 phase of the cell cycle, and they can survive for a few months as opposed to a few days for normal B cells [30]. Thus, CLL can be considered a disease caused by a loss of appropriate apoptosis, rather than increased proliferation [31], even if no defects in the apoptotic pathways can be noticed suggesting that there are micro-environmental factors regulating death in B-CLL cells. As a consequence of B cells functional incompetence, patients become immunocompromised and easily exposed to recurrent infections which are often the cause of death. 10-15 % of patients develops autoimmune hemolytic anemia, while in 2-8% of the cases CLL tends to transform into an aggressive lymphoma called Richter's Syndrome which is associated with a rapid progression of the disease, chemotherapy resistance and a poor prognosis (which leads to death in 6 months) [32].

Several published works demonstrate that some diagnostic marker of CLL are associated with Richter transformation: overexpression of CD38 and ZAP-70 by B-CLL lymphocytes and absence of mutations on variable domains of their IgG gene are unfavorable characteristics which promote activation of B cells and that determine survival and proliferation in presence of cytokines, chemokines and other signals [33, 34].

Accumulated experience with CLL indicates that:

- CD38 is a marker selectively expressed by patients who are generally poorly responsive to conventional treatment;
- CD38 is a receptor for growth and survival signals mediated through interactions with favorable microenvironments and, finally;
- CD38 is a component of the multiple elements of the migratory machinery, which depends on chemokines and their receptors.

These overall observations suggest that CD38 is a potential therapeutic target for CLL [17].

1.3.1.2.2 MULTIPLE MYELOMA (MM)

Multiple myeloma (MM) is a malignant disorder of the B cell lineage, characterized by neoplastic monoclonal expansion of plasma cells able to spread within the bone marrow and produce osteolytic lesions resulting in destruction of adjacent bone tissue. MM accounts for approximately 1% of neoplastic disease and 13% of all hematological cancers, its incidence increases with age and the median age at diagnosis is about 70 years.

In recent years, the introduction of autologous hematopoietic stem cell transplantation (HSCT) together with the availability of novel drugs such as thalidomide, lenalidomide and bortezomib, especially when used in combination regimens, have dramatically improved initial response rates and prolonged overall survival [35]; nevertheless MM remains an incurable disease with a median overall survival of 4-7 years and new therapeutic options are needed for patients.

It has been demonstrated that myeloma cells themselves are not merely less sensitive to chemotherapy due to their dormancy but they are prone to develop multidrug resistance (MDR) due to the overexpression of the transporter P-glycoprotein (P-gp), the sigma receptors (σR_2) and to a defective apoptosis mechanism.

The strong expression of CD38 by myeloma cells has been exploited for the development of targeted therapies using both “naked” antibodies able to induce ADCC (as demonstrated by the works of G.T. Stevenson [36, 37]), and immunotoxins (e.g. IB4/saporin-S6, a mAb coupled to saporin-S6 which was created by the group of Bolognesi [38]). In spite of their promising results, these new approaches did not lead to clinical applications: the molecule’s widespread distribution in lymphoid, myeloid and epithelial cells as well as in specialized tissues and organs including the eyes (see Table 1.2), caused a general reluctance to use CD38 as a target in human therapy.

Nevertheless, further impetus for designing clinical models based on anti-CD38 molecules came from the observation by the K. Mehta group that the expression of CD38

is highly sensitive to exogenous and endogenous all-*trans* retinoic acid (ATRA) and derivatives and that the sensitivity is strongly magnified in tumors and leukemic cells [39]. These initial observations were reevaluated from therapeutic perspectives in acute promyelocytic leukemia (APL), as well as in other myeloid leukemias. ATRA is an *in vitro* inducer at nanomolar concentrations of the cell surface CD38 in myeloid leukemia blasts. The same reagent is a key component in clinical differentiation therapy adopted for APL cells, which are generally CD38- before treatment. The CD38 molecules expressed *de novo* at high epitope density may be used as therapeutic targets and the combination of ATRA with an anti-CD38 immunotoxin may result in a synergistic killing of leukemia cells [40].

1.4 IMMUNOTOXINS

Immunotoxins (ITs) are chimeric proteins composed of a targeting portion (usually an antibody fragment, a cytokine or a growth factor) linked to a toxin. Immunotoxins bind to surface antigens on a cancer cell, enter the cell by endocytosis, and kill it by enzimatically inhibiting protein synthesis. The most potent immunotoxins are made from bacterial and plant toxins.

First-generation ITs, obtained by chemically conjugating one whole toxin to a mAb, often showed no efficacy in animal models because they lacked specificity and they were toxic also for normal cells. Replacement of the cell-binding domain of the toxin with an antibody-derived domain led to the development of more specific compounds and much better tolerated by animals. However these second-generation ITs, as their precursors immunoconjugate, were unstable and not homogeneous in composition [41]. These difficulties were overcome exploiting recombinant DNA techniques and the principles of protein engineering to obtain third-generation immunotoxins, designed to contain only the elements required to recognized and kill the tumour cells. In the last 10-15 years several recombinant immunotoxins have been evaluated in clinical trials (Table 1.3).

Table 1.3 Clinically evaluated/under evaluation immunotoxins

Immunotoxin	Target antigen	Binding domain	Toxic domain	Diseases
LMB-2	CD25	scFv	PE38	NHL, Leukemias, Metastatic melanoma
RFB4(dsFv)-PE38 (BL22)	CD22	dsFv	PE38	NHL, CLL, HCL, ALL
Mutated RFB4(dsFv)-PE38 (HA22)	CD22	dsFv	PE38	NHL, CLL, HCL, ALL, PLL, SLL
OVB3-PE	Ovarian antigen	mAb	Full length PE	Ovarian cancer
ERB-38	erbB2/HER2	dsFv	PE38	Breast, esophageal cancers
SS1(dsFv)-PE38	Mesothelin	dsFv	PE38	Mesothelioma, ovarian and pancreatic cancers
LMB-1	Lewis Y	mAb	PE38	Adenocarcinomas
B3(Fv)-PE38 (LMB-7)	Lewis Y	scFv	PE38	Adenocarcinomas
B3(dsFv)-PE38 (LMB-9)	Lewis Y	dsFv	PE38	Adenocarcinomas
BR96(scFv)-PE40	Lewis Y	scFv	PE40	Adenocarcinomas
scFv(FRP5)-ETA	erbB2/HER2	scFv	PE40	Melanoma, breast, colon cancers
BL22	CD22	dsFv	PE38	HCL, CLL, NHL
NBI-3001	IL4R	IL4(38-37)	PE38KDEL	Glioma
IL13-PE38QQR	IL13R	IL13	PE38QQR	Glioblastomas
TP38	EGFR	TGFα	PE38	Glioblastoma
TP40	EGFR	TGFα	PE40	Bladder Cancer, CIS
DT_{388}-GMCSF	GMCSFR	GMCSF	DT_{388}	AML
DAB_{389}EGF	EGFR	EGF	DAB_{389}	Carcinoma
DAB_{389}IL2 (Ontak)	IL2R	IL2	DAB_{389}	CTCL, CLL, NHL

Abbreviations: CLL, chronic lymphocytic leukemia; HCL: hairy cell leukemia; NHL, non-Hodgkin's lymphoma; PLL, prolymphocytic leukemia; SLL, small lymphocytic leukemia; AML, acute myeloid leukemia; CTCL, cutaneous T-cell lymphoma.

1.4.1 THE BINDING DOMAIN

In the design of an IT a variety of binding domains can be used to selectively deliver the drug to the intended cell target; besides monoclonal antibodies and fragments thence derived, other small proteins are appropriate to fulfil this function, e.g. growth factors and cytokines. Such molecules impart specificity to the IT by virtue of the higher expression level of some receptors for growth factors and cytokines on tumor cells. Interleukin 2 (IL-2), IL-13, transforming growth factor α (TGFα) and granulocyte-macrophage colony stimulating factor (GMCSF), for instance, have been employed in the construction of immunotherapeutic agents to direct toxic molecules towards leukemia or lymphoma cells [42, 43].

However, due to their remarkable molecular versatility and to the possibility of being raised against virtually any target, antibodies are probably the best candidates as binding domains in ITs design [44].

1.4.1.1 ANTIBODIES

Antibodies are glycoproteins belonging to the immunoglobulin (Ig) superfamily; they are produced by B lymphocytes (B cells) in response to exposure to an antigen. They react specifically with that antigen *in vivo* or *in vitro* and are hence a part of the humoral adaptive immune response.

All antibody molecules share the same basic structural characteristics but display remarkable variability in the region that bind antigens. The basic structural unit of an antibody molecule consists of four polypeptide chains, two identical light chains (L) and two identical heavy chains (H). The four chains are linked covalently by disulfide bonds (Fig. 1.6). Each heavy chain has a molecular weight of 50-75 kDa and contains about 400 amino acids and the amino acid differences in its carboxy terminal portion identifies five isotypes (IgG, IgA, IgM, IgD and IgE). Light chains have a molecular weight of approximately 23 kDa, are composed of about 212 amino acids and are of two types, κ and λ, based on their structural (antigenic) differences.

Both heavy and light chains consist of amino terminal variable (V) regions that participate in antigen recognition and carboxy terminal constant (C) regions.

Variable regions are so named because most of the variability in amino acids sequence, that distinguish the antibodies made by different clones of B cells, is confined to three short stretches in the V regions of heavy and light chains (V_H and V_L). These hypervariable regions (each about 10 amino acid residues long) are called complementarity-determining regions (CDRs) and they are held in place by more conserved framework sequences [45]. The V_H is juxtaposed with the V_L to form an antigen-binding site.

The C region domains are separate from the antigen-binding site and do not participate in antigen recognition. The constant regions of the two heavy chains constitute the so-called Fragment Crystallizable (Fc) portion which, interacting with cells of the immune system, mediates effector functions.

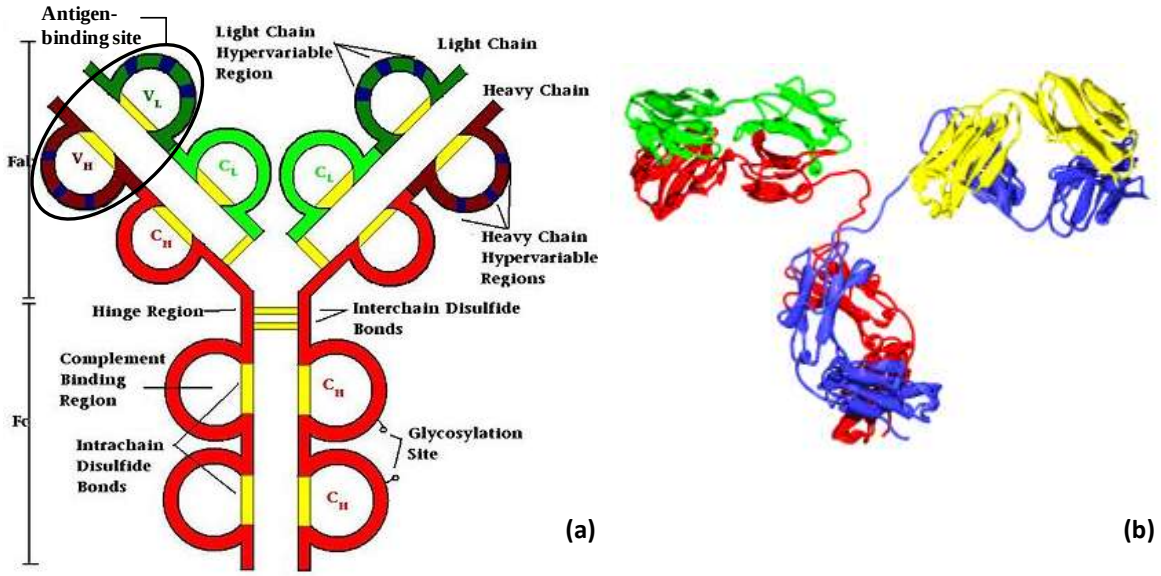


Figure 1.6 Structure of an antibody molecule. (a) Schematic diagram of a secreted IgG molecule. It is composed of four polypeptide chains, two light and two heavy chains, each of which is organized in domains of 110 amino acids containing a disulphide bridge that forms a *loop* of approximately 60 amino acids. The heavy chain of an IgG comprises three constant domain (C_H) and one variable domain (V_H), while the light chain is made by one single constant domain (C_L) and one variable domain (V_L). The antigen-binding sites are formed by the juxtaposition of the V_L and V_H domains. **(b)** Structure of a human IgG molecule as revealed by x-ray crystallography. In this ribbon diagram, the heavy chains are colored blue and red, and the light chains are colored green and yellow.

Early studies of antibody structure relied on antibodies purified from the serum of animals immunized with various antigens, yielding a polyclonal pool of reactive immunoglobulins that may respond to different epitopes of an antigen. Despite their high binding affinity, polyclonal antibodies are unsuitable for therapeutic use, due to their

heterogeneous composition. The most important breakthrough in the field of antibody-based therapies was the introduction, by Georges Köhler and Cesar Milstein in 1975, of the hybridoma technology for producing monoclonal antibodies. Hybridoma technology is based on the somatic fusion of myeloma cells and B lymphocytes from the spleen of an immunized mouse. The resulting chimeras are immortalized cells capable to secrete antibodies indefinitely (Fig. 1.7). Since each fusion yields mAbs with a unique and specific idiotype (i.e. antigen-binding site), the hybridoma clone producing the mAb with highest affinity can be selected and propagated, thus providing bulk amounts of a homogeneous immunoglobulin with the desired specificity [6].

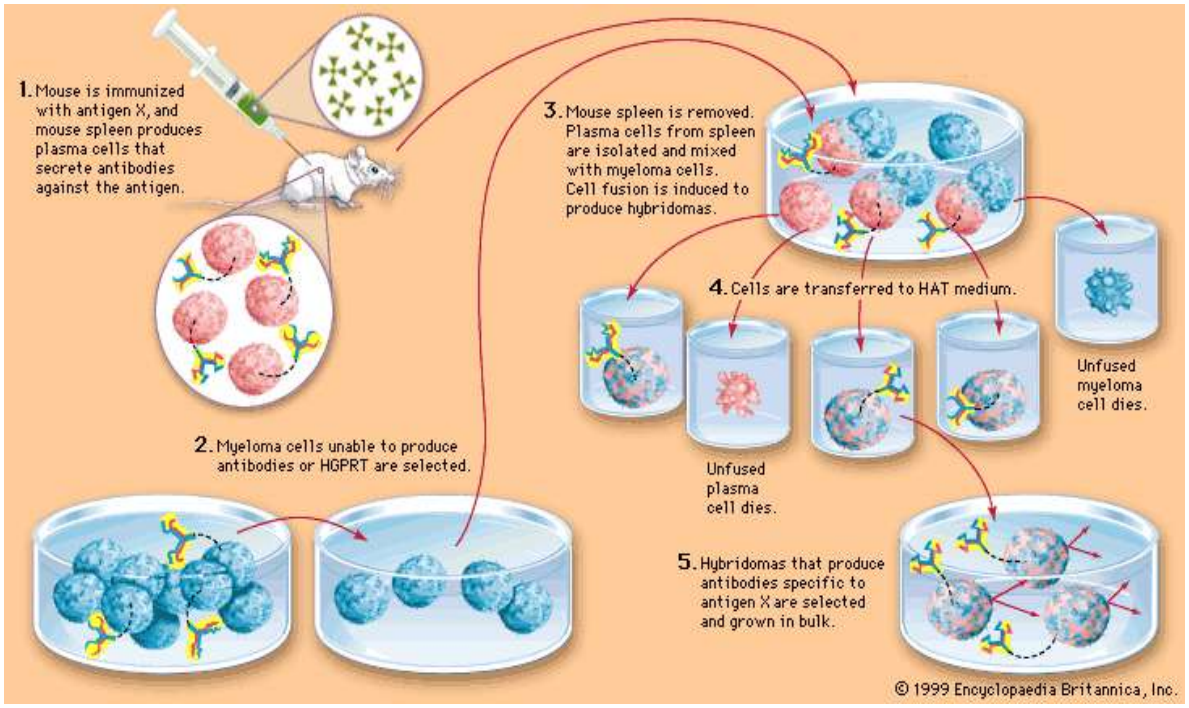


Figure 1.7 The hybridoma technology for producing monoclonal antibodies.
Abbreviations: HGPRT, hypoxanthine-guanine phosphoribosyltransferase; HAT medium, hypoxanthine-aminopterin-thymidine medium.

The ability of IgGs to bind two antigens greatly increases their functional affinity and confers high retention times (also called avidity) on many cell-surface receptors and polyvalent antigens. The Fc domain recruits cytotoxic effector functions through complement and/or through interactions with γFc receptors (Fc receptors for gamma globulins) and can provide long serum half-lives (>10 days) through interaction with the neonatal Fc receptor (FcRn).[46].

Monoclonal antibodies can be covalently coupled to toxins or toxin derivatives by chemical means. Generally, coupling reactions involve at least one accessible SH group. The connection of the antibody to the toxin utilizes two types of chemical bonds for conjugation. One class of immunotoxins contains a disulfide linker, so that the antibody and toxin separate upon reduction within the target cell. The other class of chemical linkers contains thioether bonds that cannot be cleaved by reduction. Different linkers for conjugation of proteins have been developed, varying in length, stability, flexibility and chemical reactivity: which linker class (cleavable or not) and which conjugation chemistry to be used depends first on the nature of the toxin and an experimental evaluation is usually required remembering that differences in linkers can greatly affect the activity of immunotoxins [47, 48].

1.4.1.2 Antibody fragments

To address some of the limitations of large IgG molecules, an ever increasing importance has been acknowledged to the study and development of antibody fragments (Fig. 1.8), which are smaller molecules with a potentially better tissue penetration in case of solid tumors. Antibody fragments still maintain an unaltered binding capability and specificity to the antigen while not having non-selective activity due to non specific binding of the antibody Fc portion.

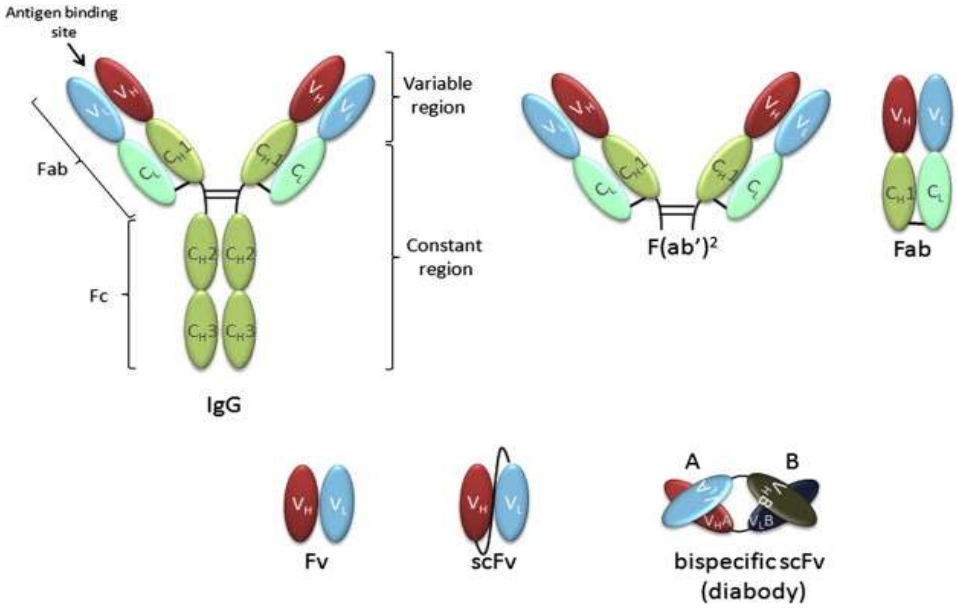


Figure 1.8 Schematic representation of different possible antibody configurations. The domain-based structure of immunoglobulins could be manipulated to yield a wide repertoire of antibody formats.

Initially, such fragments could be prepared by proteolytic digestion of whole immunoglobulins. Treatment of IgG with proteases like papain generates three separate fragments: the Fc region and two identical Fab (fragment, antigen binding) regions, comprising the complete light chain (V_L and C_L) associated with the variable domain and the first constant domain of the heavy chain. When pepsin (instead of papain) is used to cleave IgG, the released fragment is called F(ab')2 and is composed of a pair of Fab' units connected by two disulfide bonds. Fabs and F(ab')2 retain the ability to bind antigen because each contains paired V_L and V_H domains (Fig. 1.8) and at the same time their employment in ITs design brought about reduced levels of non-specific binding as compared to whole mAbs. However, since antibodies show variable susceptibility to proteolytic treatment, the final product can be highly heterogeneous, which accounts for the poor success claimed for immunotherapeutics manufactured by this kind of procedures [49]. The development of recombinant DNA technologies offered the chance to synthesize Fabs and even smaller fragments in heterologous expression systems.

The smallest unit of an immunoglobulin molecule with function in antigen-binding activities is called Fv fragment and it is represented by the heterodimer formed by the variable domains of heavy and light chains (Fig. 1.8). The genes encoding the V_H and V_L domains are usually cloned from hybridoma mRNA by reverse transcription, cDNA synthesis and subsequent PCR amplification using degenerate primers that are complementary to the conserved sequences at the 5' end of genes for the V_H and V_L domains and to the 3' ends of the J_H and J_L regions (located at the 3' ends of V_H and V_L) [50]. The PCR amplified variable regions can then be cloned into an appropriate plasmid vector for expression in a heterologous host, especially *Escherichia coli* [51]. In a recombinant Fv fragment V_H and V_L domains are associated through non-covalent interactions which may not be strong enough to assure the dimer stability; in fact the dissociation constant of the V_H-V_L complex, ranging between 10^{-5} and 10^{-8} M, is often not sufficient to keep the two domains together under slightly destabilizing conditions or when the protein concentration is low [49]. To improve their stability different strategy has been developed creating scFvs (single-chain Fragments variable) and dsFvs (disulphide-stabilized antibody fragments).

ScFvs are small antibody fragments in which the V_H and V_L antigen binding domains are held together and stabilized by a flexible peptide linker that connects the C-terminus of

the V_H (or V_L) with the N-terminus of the other domain (Fig. 1.8). Various linkers were designed to provide flexibility and enhance solubility, with the most widely used linker varying from 10 to 25 amino acids in length and typically including hydrophilic amino acids; the most common linker is the decapentapeptide $(Gly_4Ser)_3$. ScFvs often show a good binding capability, comparable to that of the mAb they derive from; while in some cases a considerable loss of affinity can be observed, probably associated with a high tendency to aggregate and form unstable multimers. The formation of dimers and trimers is primarily determined by linker length and it is favored by shorter linkers (0–12 amino acids) [52], while the V_H-V_L orientation can affect expression efficiency, stability, and antigen binding activity [53].

In dsFvs the connection between V_H and V_L chains is mediated by a disulfide bond which is the result of the insertion of one cysteine residue into the framework region of each of the two domain [54]. In order to avoid any hindrance to the antigen-binding, the cysteines involved in the covalent bridge should be located at a distance from the CDRs of their respective variable domains and they should be close enough to each other so as to allow the establishment of the bond.

A higher stability has been often observed for ITs containing a dsFv as their binding domain, as compared to those derived from a scFv. The interchain disulphide in a dsFv prevents the dissociation of V_H and V_L by providing a firm link between the two moieties; it also limits aggregation issues which would require the setting up of re-folding procedures and result in a decreased yield after purification [55]. In spite of the advantageous features of a dsFv format, the construction of an antibody fragment of this kind requires an accurate preliminary study of molecular modelling and an in-depth structural characterization of the antibody. Furthermore, fragments that are joined by both a peptide linker and a disulfide bond have been described and are known as sc-dsFvs [56]. In general, monovalent Ab fragments (i.e. scFv, dsFv and Fv) have a low functional affinity and a short in vivo half-life, due to their small size and valence, properties which are detrimental to some therapeutic applications. However, because recombinant antibody fragments are easily and cost effectively expressed and are handily subjected to genetic engineering to increase affinity and modify specificity, they remain attractive therapeutic candidates.

1.4.2 THE TOXIC DOMAIN

Several types of therapeutic agents have been used in the design of anticancer ITs and constructs containing cytotoxic drugs, cytokines, toxins or radionuclides have been evaluated in preclinical and clinical studies.

Standard chemotherapeutic drugs belonging to the antifolates, vinca alkaloids or anthracyclines have been chemically linked to mAbs, but these immunoconjugates proved to be inefficient in the clinical situation due to the moderate cytotoxic potential of these drugs and to the limited achievement of therapeutic levels within the cells [2].

Toxins constitute another class of highly cytotoxic agents that have been conjugated to mAbs and tested for antitumor therapy efficacy. Toxins are poisonous substances (usually protein) produced by living cells or organisms. In contrast to the low-molecular-mass chemical molecules adducted for chemotherapy, toxins used for anticancer therapy are generally enzymes that exert their cytotoxic activity inside the cell; in most cases one single molecule in the appropriate intracellular compartment is sufficient to kill the cell. To be used therapeutically, toxins mostly have to be modified to remove their binding sites for targets expressed in normal tissue. In addition, toxins often have to be deglycosylated to avoid rapid clearance by liver cells expressing mannose receptors.

The toxins that are best analyzed and most commonly used for making immunotoxins are the protein synthesis inhibiting toxins: ricin, diphtheria toxin (DT) and *Pseudomonas* exotoxin (PE). All these proteins have been crystallized, their structures determined and specific functions assigned to different structural subunits or domains of which they are composed. Also, the genes for these toxins have been cloned and expressed as recombinant proteins in *E. coli* [57]. Combining the understanding of their structure and function with molecular cloning techniques has made it possible to generate genetically altered toxin derivatives with improved properties for use as immunotoxins.

1.4.2.1 PLANT TOXINS

Plants synthesize and accumulate in seeds and leaves a broad range of secondary metabolites, including alkaloids and terpenoids, that are toxic to herbivores and pathogens, and so are believed to act as defense compounds [58].

Plant toxins belong to the ribosome inactivating proteins (RIPs), a class of potent inhibitors of protein synthesis that act by catalytically depurinating, thanks to their RNA N-glycosidase activity, an adenine residue (A4324 in rats) present in a conserved stem-loop region in 23/26/28S large ribosomal RNAs. The removal of this adenine, preventing association of the ribosome with the elongation factor 2 (eEF-2), causes an irreversible arrest of protein synthesis and consequently cell death occurs.

RIPs from plants have been classified into three main categories: type 1 are composed of a single polypeptide chain of approximately 30 KDa, type 2 are heterodimers consisting of an A chain, functionally equivalent to the type 1 polypeptide, linked to a B subunit, endowed with lectin-binding properties, while type 3 are synthesized as inactive precursors (ProRIPs) that require proteolytic processing events to form an active RIP and are not in use for therapeutic purposes.

Type 1 RIPs, like saporin (from the seeds of the soapwort *Saponaria officinalis*), PAP (pokeweed antiviral protein, from the plant *Phytolacca americana*) and gelonin (from the seeds of *Gelonium multiflorum*), are characterized by a high basicity (pI > 9.5) and can be glycosilated. Some of them have a N-terminal sequence that directs them to the endoplasmic reticulum. The routing of type 1 RIPs to reach their target into the cytosol is actually unclear but it has been demonstrated that the binding of saporin to the cell surface is at least in part mediated by the α2-macroglobulin receptor (α2MR; also termed *low density lipoprotein-receptor-related-protein*, LPR), indicating a general mechanism of interaction of plant RIPs with the α2MR system [59]. After being endocytosed, the toxin reaches the endo-lysosomal compartment from where it is delivered to the cytosol following as yet unidentified pathways.

As previously described, type 2 RIPs are holotoxins containing an A chain, which is the enzymatically active one (N-glycosidase), linked, through a disulphide bond, to a B chain that mediates binding to the terminal galactose or **N**-acetylgalactosamine residues

present on the surface of most mammalian cells and that promotes the translocation of the A chain into the cytoplasm.

Among type 2 RIPs, ricin, obtained from the seeds of the castor oil plant *Ricinus communis*, has been the one most widely used in preclinical and clinical studies [60]. After entering mammalian cells by endocytosis, ricin is transported to the early endosomes and undergoes retrograde transport via the Golgi complex to the endoplasmic reticulum (ER) where the catalytic moiety exploits the ER-associated degradation (ERAD) pathway, normally used for the disposal of misfolded or unassembled polypeptides, to reach and depurinate cytosolic ribosomes. This retrograde route of transport for ricin represents a highly effective strategy to deliver its A domain into the cytosol, which is a prerequisite for exerting toxicity [61].

Predictably, ITs containing native ricin and other type 2 RIPs lack specificity since they bind not only to target cells, but virtually to any other cell via the B chain. This problem has been successfully circumvented by altering or deleting the binding domain through two different approaches:

1) by separating the functionally active A chain from the B chain and deglycosylating the A chain (dgA) (this latter procedure prevents liver toxicity due to glycosylated residues on the A chain which recognize parenchymal and non parenchymal cells in the liver, causing hepatotoxicity and poor biodistribution)[62]. Alternatively, the A chain can be made as a recombinant protein in bacteria, but this last approach did not lead to obtain functional immuntoxins when A chain was conjugated to an antibody [63];
2) by attaching affinity ligands (i.e. galactose, lactose, or glycopeptides) to the sugar-binding sites of the B chain (blocked ricin) [64].

Due to their catalytic mechanism of action, these toxins are extremely potent; it has been estimated that a few molecules of ricin in the cytoplasm are enough to kill a cell. Moreover, since their mechanism of cell killing is different from those of standard chemotherapeutic agents, it is reasonable to expect that they could exert an efficient antitumor activity against chemoresistant and/or resting neoplastic cells without cumulative bone marrow toxicity.

1.4.2.1.1 SAPORIN

The plant toxin saporin, compared to other RIPs, shows various peculiar features in terms of remarkable stability and activity on a wide variety of substrates, that have made it an interesting protein to be employed in the design of immunotoxins [65, 66].

The term saporin collectively identifies a family of RIP isoforms that accumulate in different tissues of the soapwort *Saponaria officinalis*. Mixtures of closely related isoforms and several cDNA and genomic clones have been isolated; among them, SO6 saporin (or saporin-6) represents the major HPLC peak of purified seed protein and constitutes about 7% of the total proteins. Seed protein sequencing revealed heterogeneity at two positions, with either an aspartic or a glutamic acid in position 48, and either lysine or arginine present in position 91, indicating that the SO6 peak contains a set of correlated isoforms. In fact, HPLC analysis confirmed the presence of at least three different components in SO6 preparations while recombinant expression of single seed-like isoforms demonstrated the same RIP activity, except for a leaf-derived isoform [67].

While some characteristics of the saporin proteins, such as key catalytic residues and overall three-dimensional fold, are shared with RTA and the other known crystallized RIPs, other biochemical features clearly differ among type I plant RIPs and RTA. For example, the sequence identity is low and, in particular, only 22% of residues are conserved between RTA and saporin SO6. On the contrary, a high degree of sequence identity (about 80%) is found between saporin SO6 and dianthin from *Dianthus caryophyllus*, both of which are synthesized by plants belonging to the same subfamily of the Caryiophyllaceae family. Despite that, all the crystallized RIPs have been shown to share a common “RIP fold”, as can be estimated by the superimposition of the 3D structures of several type I RIPs and RTA, which is characterized by the presence of two major domains: an N-terminal domain, which is mainly β-stranded, and a C-terminal domain that is predominantly α–helical [68].

Saporin cytotoxicity varies in a wide range, with concentrations inhibiting protein synthesis by 50% (IC_{50}) changing from nanomolar to micromolar, depending on the cell lines investigated and on the expression of the α2-macroglobulin receptor/low-density

lipoprotein receptor-related protein (LRP1) which has been proved to bind saporin *in vitro* and mediate its internalization in human monocytes and in fibroblasts [67].

As compared to ricin-based ITs, saporin has been exploited relatively little as the toxin of choice for clinical uses, so far. A small clinical study with an anti-CD30 monoclonal conjugated to the seed extracted saporin (BERH2-SAP) for the treatment of Hodgkin's lymphoma, proved very encouraging [69] without reporting serious drug-related toxicities. However, antibodies against both domains were raised in the treated patients. ITs based on saporin have been also used by Flavell and coworkers for their clinical trials in adult and pediatric patients with hematological malignancies: their pre-clinical studies showed that an anti-CD19 immunotoxin, named BU12-saporin, and an anti-CD38 immunotoxin, OKT10-saporin [70], displayed selective antitumor activity both *in vitro* and *in vivo* against malignant target hematological cells.

1.4.2.2 BACTERIAL TOXINS

Pseudomonas aeruginosa Exotoxin A (PE) and *Corynebacterium diphtheria* toxin (DT) are the most widely exploited bacterial toxins for the immunotherapy of cancer. Both PE and DT enzymatically modify eEF-2 in the cytosol by catalyzing the adenosine diphosphate (ADP) ribosylation of residue His699 of eEF-2 which is post-translationally modified to a diphthimide residue [71, 72]. This modification irreversibly inactivates eEF-2 causing the arrest of cellular protein synthesis.

Both toxins are produced as single polypeptide chains and they share a similar structure made by three principal portions: a binding domain which mediates the interaction with the cell surface, a catalytic domain responsible for the ADP-ribosylating enzymatic activity and a translocation domain which facilitates the transfer of the catalytic domain into the cytosol. In each case, the toxin is proteolytically cleaved within the translocation domain, and a disulfide bond holds the two fragments together until it is reduced [44]. Despite their similar structure and mechanism of action, PE and DT differ greatly in their amino acid sequences; in fact, the enzymatic domain of PE is near the carboxyl terminus while that of DT is near the amino terminus. Conversely, the binding domain of PE is near its amino terminus and that of DT is near its carboxyl terminus [73].

1.4.2.2.1 *PSEUDOMONAS* EXOTOXIN A: STRUCTURE AND FUNCTION

Full-length *Pseudomonas* exotoxin A (PE) is a 66 kDa single-chain protein secreted by the Gram-negative, opportunistic and pathogenic bacterium *Pseudomonas aeruginosa*. PE belongs to a family of enzymes termed mono-ADP-ribosyltransferases, and more specifically is a NAD^+-diphthamide ADP-ribosyltransferase. An analysis of the 5' and 3' flanking regions indicated that PE is translated from a monocystronic message into a 638 amino acids precursor with a highly hydrophobic leader peptide of 25 amino acids, which is removed during the secretion process releasing the final 613 amino acids PE protein.

Analysis of the crystal structure of PE shows that it is composed of three major domains whose functions were assigned based on mutational analysis (Fig. 1.9). Domain Ia is located at the amino terminal portion of PE (residues 1-252) and it mediates binding to the eukaryotic cellular receptor which has been identified to be the large subunit of the α2MR (LRP1). Domain II (residues 253-364) is composed of 6 consecutive α helices and is required for the translocation of the toxin across cellular membranes: the translocation domain is responsible for enabling the carboxyl terminal ADP-ribosylating activity in domain III to reach the cytosol of target cells. Domain Ib is a small portion (residues 365-404) localized between domain Ia and III. The function of this domain has not been elucidated and may be required for the secretion of the toxin by the bacterium. Nevertheless, its last 4 residues (aa 400-404) together with domain III (aa 405-613) form the catalytic subunit of the protein with ADP-ribosyltransferase activity, which leads to an inhibition of protein synthesis and finally to cell death [74].

Two important amino acid motifs inside the PE molecule have been characterized by mutation analysis and are considered essential for its cytotoxicity. The first motif (aa 274-280, RHRQPRG) lies inside domain II of PE and it is exposed on the exterior surface of the protein, where it is accessible for the cleavage by the ubiquitous eukaryotic protease furin [75]. The second motif is the pentapeptide REDLK (aa 609-613) at the C-terminus of PE, which acts as an endoplasmic reticulum retention sequence [76].

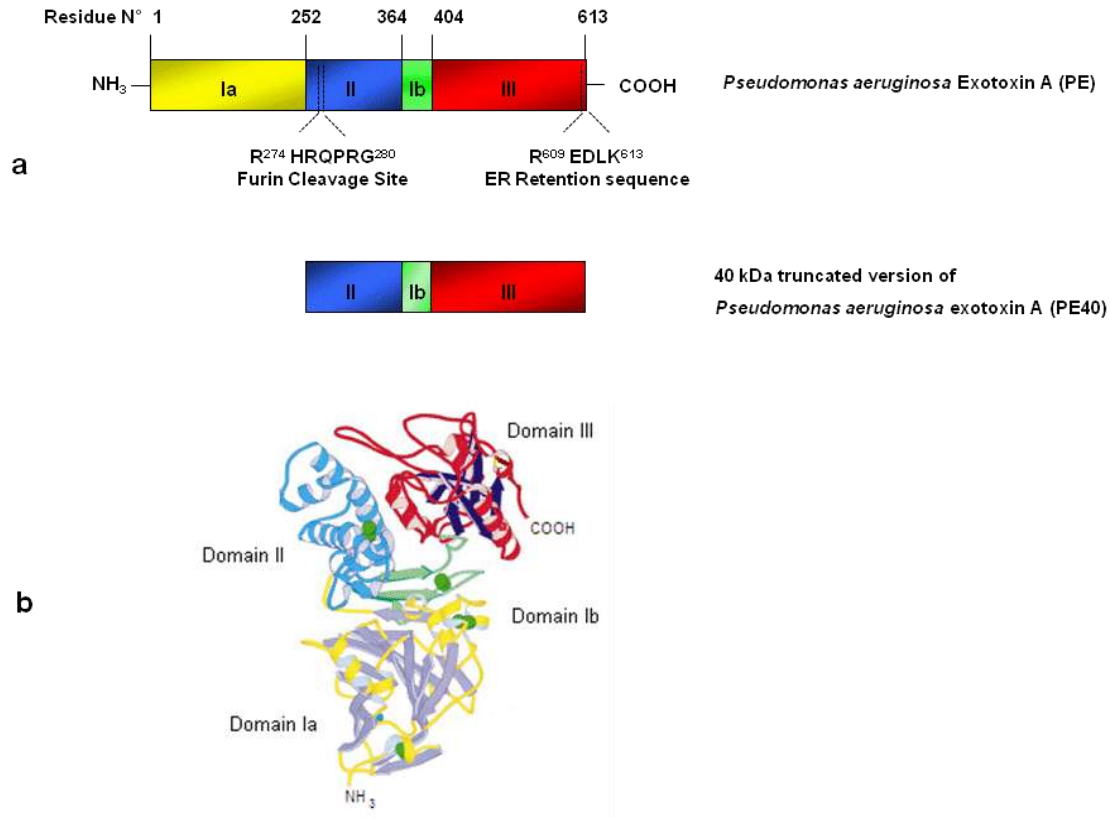


Figure 1.9 *Pseudomonas* exotoxin A (PE). (a) Schematic representation of the structural and functional domains of PE and PE40. Domain Ia (aa 1–252) represents the receptor binding domain. Domain II (aa 253–364) is required for the translocation of the toxin across cellular membranes. The catalytic subunit of PE with ADP-ribosyl transferase activity (aa 400–613) is located inside the structural domains Ib (aa 365–404) and III (aa 405–613). The furin cleavage site (aa 274–280) inside domain II and the ER retention sequence (aa 609–613) at the C-terminus represent further essential motifs for the cytotoxicity of PE. **(b)** 3D structure of the PE domains: domain Ia, purple β-sheets, yellow α-helices and coils; domain Ib, green β-sheets and coils; domain II, light blue α-helices and coils; domain III, red α-helices and coils, blue β-sheets. Cyan spheres represent Na^+ ions, yellow spheres represent Cl^- ions, disulfide positions are indicated as green spheres. (Modified from Wolf, P., 2008)

1.4.2.2.2 *Pseudomonas* Exotoxin A: Cytotoxic pathways

The first essential step in the cytotoxic pathway of PE is the cleavage of the C-terminal lysine residue (aa 613), presumably operated by plasma carboxy-peptidases. PE then binds via cell binding domain Ia to CD91 (the previously described α2MR/LPR) [77], which is expressed on the surface of several cell types, and at especially high levels in fibroblasts and hepatocytes, which appear 200-300 times more sensitive to PE as compared to other cells like those of lymphoid lineage.

Once PE has bound to CD91, it uses 2 retrograde pathways from the cell surface to the early endosomes (EE) [78]. The majority of the PE molecules are internalized into the cell

in a nonlipid-dependent manner via clathrin-coated pits. But in some cases, as demonstrated in HeLa cells, the association of a proportion of CD91-bound PE with a receptor in detergent-resistant microdomains (DRM), facilitates the uptake in caveosomes and the transport to the EE in vesicles containing the EE marker Rab5.

PE dissociates from CD91 in the acidic endosome environment, undergoes a conformational change, and is cleaved by the protease furin between residues R279 and G280 [79]. This results in an N-terminal fragment of 28 kDa and a C-terminal fragment of 37 kDa, the latter being composed of a portion of domain II, domain Ib and all of the enzymatic active domain III [80]. Both fragments are still connected by the disulfide bond between C265 and C287, encompassing the furin cleavage site. It is speculated that under the mildly acidic conditions in the endosomes, there is an unfolding event, possibly by the binding of chaperone proteins. This leads to a surface exposure of the disulfide bond with subsequent reduction, perhaps by protein disulfide isomerases (PDI), which is followed by a release of the C-terminal 37-kDa fragment [81].

The enzymatic active 37-kDa fragment travels to the trans-Golgi network (TGN) via late endosomes and a Rab9-dependent route [82]. There, it can bind in a pH-dependent manner to the KDEL receptor via its C-terminal KDEL-like sequence REDL (aa 609–612) and is transported to the endoplasmic reticulum (ER) [76]. The KDEL receptor cycles between the TGN and the ER via Golgi cisternae with the help of the tyrosine kinase Src and is responsible for recycling proteins bearing the C-terminal amino acid code KDEL to the ER [83]. Alternatively, the 37-kD fragment of PE, which bound to DRM at the cell surface, can directly reach the TGN from EE in a pathway independent of the small GTPase Rab9 and presumably reaches the endoplasmic reticulum (ER) in a lipid-dependent sorting pathway, controlled by Rab6 [78]. Sequences in the translocation domain II induce the dislocation of the 37-kDa fragment from the ER to the cytosol [80]. There is evidence that the Sec61p translocon, a protein used in eukaryotic cells for the dislocation of unfolded or misfolded proteins in the ER-associated protein degradation pathway (ERAD), is involved in this step [84].

Proteolytic cleavage and translocation into the cytoplasmic compartment are the rate-limiting steps in the intoxication process: Ogata and colleagues demonstrated that as little as 8-10% of the molecules interacting with a target cell are actually cleaved. This low

efficiency in proteolytic processing may be due to the short time during which PE and furin co-localize within the same compartment [80].

Once the enzymatic subunit of PE has reached the cytosol, it catalyzes the ADP ribosylation of its target protein, the eukaryotic elongation factor-2 (eEF-2), a single-chain polypeptide with a molecular mass of approximately 100 kDa, which is a member of the GTPase superfamily. The ADP ribosylation inactivates eEF-2 resulting in the inhibition of protein synthesis, which ultimately leads to cell death. The mechanisms of cell killing by PE were analyzed in several studies also demonstrating an involvement of caspases in PE-induced apoptosis [44].

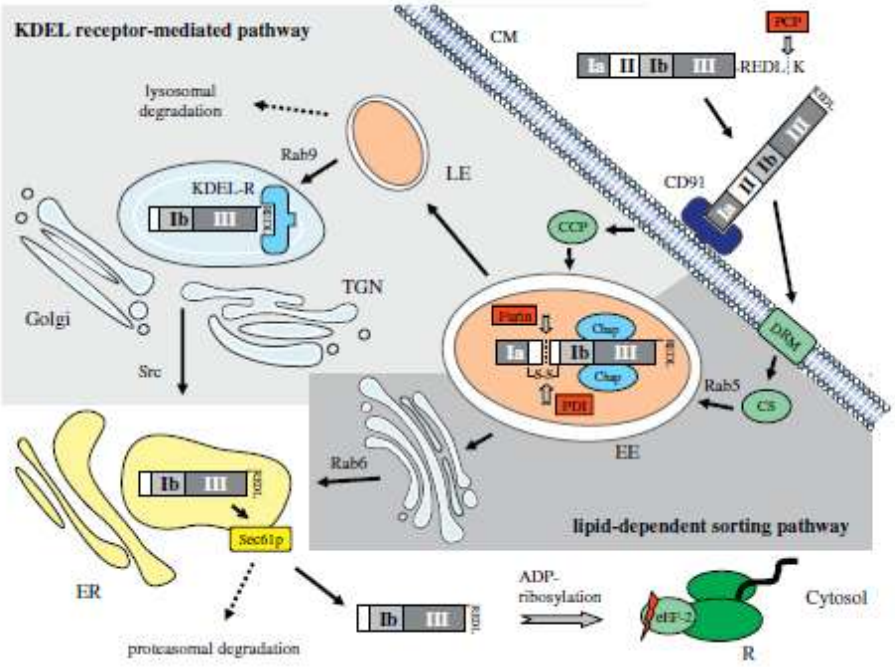


Figure 1.10 Cytotoxic pathways of *Pseudomonas* exotoxin A (PE). After cleavage of the C-terminal lysine (K) by plasma carboxypeptidases (PCP), PE binds to the CD91 receptor on the cell membrane (CM) and can then exploit different pathways to reach the endoplasmic reticulum (ER). On the one side, PE is internalized via clathrin-coated pits (CCP) into the cell. This is followed by furin cleavage in the early endosomes (EE) in cooperation with protein disulfide isomerase (PDI) and chaperons (Chap). Then the enzymatic active PE fragment travels via late endosomes (LE) in a Rab9-dependent manner to the trans-Golgi network (TGN). After binding to the KDEL receptor (KDEL-R), PE is transported to the ER under control of the tyrosine kinase Src. On the other side, CD91-bound PE can associate with detergent-resistant microdomains (DRM) and is transported via caveosomes (CS) to the EE in a Rab5-dependent manner. After cleavage in the EE, the toxic PE fragment directly travels to the ER via a lipid-dependent sorting pathway under the control of Rab6. PE fragments in the ER are secreted via the translocon Sec61p into the cytosol, where they inhibit the protein synthesis by ADP-ribosylating the eukaryotic elongation factor-2 (eEF-2) at the ribosomes (R). This finally leads to apoptosis of the host cell. (Modified from Wolf, P., 2008)

1.4.2.2.3 PE DERIVATIVES

The nonspecific toxicity of immunotoxins is often caused by binding of the toxic portion to normal cells. When using native toxins this is mediated, in the case of PE, by the binding domain Ia. Inactivation of this binding function was the major goal in developing PE derived immunotoxins and over the past several years a variety of modified PE molecules with altered cell-binding domains have been genetically engineered and then tested as immunotoxins. One of those PE derivatives is PE glu57, in which the lysine at position 57 in domain Ia is replaced by glutamate [85]. Another is PEglu57,246,247,249, in which the cell-binding domain is not only inactivated by the glu57 mutation, but is also unfolded due to the three further mutations [86]. Immunotoxins made from these PE derivatives show diminished nonspecific toxicity and therefore a wider therapeutic window is observed against cultured cells and in animal experiments. A further reduction of nonspecific toxicity can be reached by removing the entire domain Ia and replacing it with a tumor-specific ligand. The first truncated form of PE to be produced is known as PE40 (residues 253-613) to reflect its molecular weight of 40 kDa. Additionally, a large portion of PE domain Ib (residues 365-380) can be deleted: this modified form is called PE38. Both these truncated forms still retain full toxic and translocation activity and when coupled or recombinantly fused to antibodies or other targeting moieties make very active immunotoxins [87].

Several other modified versions of PE were created; some examples are: LysPE40 and LysPE38, which have an exposed lysine residue next to its amino terminus to facilitate conjugation to mAbs [88], while in PE40KDEL and PE38KDEL the original “REDLK” endoplasmic reticulum retention sequence at the carboxyl terminus of PE is replaced by a KDEL which is a preferred sequence [89]. These last immunotoxins show increased cytotoxic activity, but nonspecific toxicity is often increased by approximately the same factor.

1.4.3 IMMUNOGENICITY

Although ITs aroused interest in the scientific community, clinical trials underlined several limits to their use. One of the first disadvantages is the immunogenicity due to the carrier as much as to the toxic portion.

1.4.3.1 IMMUNOGENICITY OF THE BINDING DOMAIN

The binding domain, if derived from murine antibody, could be responsible for the activation of immune response (Human Anti-Murine Antibody, HAMA) with the subsequent half life reduction of the chimeric protein and the decrease of its antitumor effect [90]. This problem has been partially solved by the use of engineered chimeric, humanized, or human antibodies (Fig. 1.11).

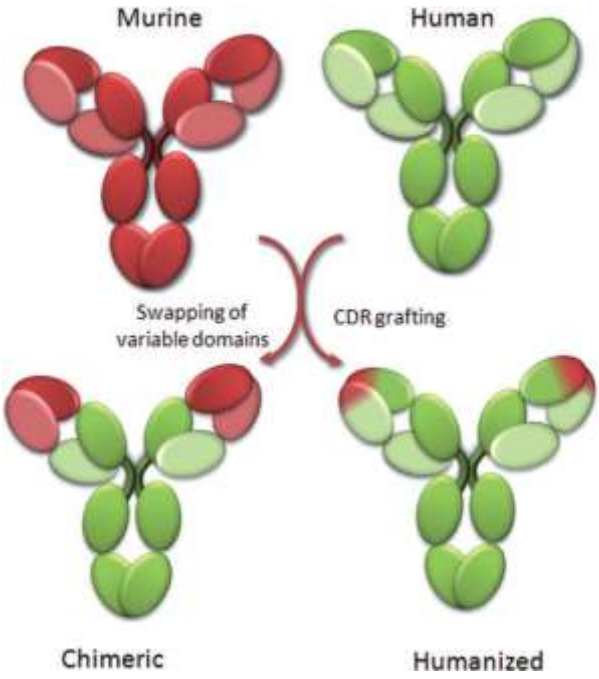


Figure 1.11 Chimeric and humanized antibodies. Murine sequences are depicted in red and human sequences in green, using light colours for light chain and dark colours for heavy chains.

A major application of antibody engineering was the possibility to create chimeric antibodies. As the binding activity of IgG molecules is generated by the variable domains of the heavy and light chains, it was possible to create chimeras by fusing murine variable domains with human constant domains [91] leading to the development of a new

generation of therapeutic candidates [92]. These chimeric antibodies are 70% human and possess a fully human Fc portion, which makes them considerably less immunogenic in humans and allows them to interact with human effector cells and the complement cascade.

With the development of antibody engineering techniques, it became possible to decrease even further the murine part of mAbs by replacing the hypervariable loops of a fully human antibody with the hypervariable loops of the murine antibody of interest, using an approach called complementarity-determining region grafting [93]. These antibodies, called “humanized”, are 85–90% human and are even less immunogenic than chimeric antibodies. However, complementarity-determining region grafting is more technically demanding than a mere fusion, and directed mutagenesis approaches are often needed to restore the affinity present in the murine parental antibody. Most of the approved mAbs in current use are either chimeric or humanized (Table 1.1).

Another major improvement came with the development of *in vitro* selection methods, the most successful being phage display. With the ever increasing power of antibody engineering, it became possible to clone entire repertoires of antibody fragment genes, from immunized or non-immunized animals, including humans. A powerful selection method was therefore needed to select from this large number of potential ligands, those able to bind the antigen of choice.

The first technique, and still by far the most common one was inspired by the work of George Smith [94]. Like all *in vitro* selection methods, this technique relies on the ability to establish a physical link between a protein and the gene encoding this protein, in this case between a protein fused to a filamentous phage capsid protein (p3 or p8) displayed at the surface of phage M13 and its corresponding gene contained in the encapsidated DNA. If the molecule is immunopurified by binding to the antigen of interest, its gene is immediately available, allowing sequencing and further multiplication of the specific clone. Because of these *in vitro* selection methods, it is now possible to rapidly and efficiently select fully human antibody fragments against virtually any antigen by using “universal”, large, non-immunized libraries [95].

Moreover, the same approach can be used to maximize the affinity of a valuable antibody by creating a secondary library consisting of mutants of the first candidate and

performing stringent *in vitro* selection against the antigen of choice. Phage display and more recently ribosome display have been used to obtain ligands with sub-picomolar affinities for the relevant antigen, outperforming the affinities of most conventional mAbs [96].

During the same decade, a complementary approach was developed to create fully human antibodies. Transgenic "humanized" mice were created by replacing the entire mouse IgG repertoire with a human repertoire [97]. Upon immunization, these humanized mice produce human IgGs and conventional hybridoma techniques can be used to clone human antibodies with the required properties. This approach has the advantage of yielding *in vivo* matured antibodies, circumventing the need for additional affinity maturation. However, humanized mice cannot be used effectively when the immunogen is toxic or when the targeted antigen shares a high degree of homology with its murine ortholog.

1.4.3.2 IMMUNOGENICITY OF THE TOXIC PORTION

Human immune response has been also observed against the toxic portion of immunotoxins: indeed, the presence of a bacterial or plant toxin can trigger the formation of neutralizing antibodies, hindering their efficacy. Especially PE and DT are very immunogenic [98] and cannot be humanized with standard techniques. DT-derived immunotoxins are in addition particularly affected because most people in developed countries have been vaccinated against DT and many adults have neutralizing antibodies to DT. In patients with B- or T-cell malignancies, the formation of neutralizing antibodies is less frequent because of their immunosuppressed state; in contrast, in patients with solid tumors, antibody responses are frequently detected as early as a few days after the first treatment regimen, preventing re-administration of the ITs [99]. Many efforts have been made to decrease immunogenicity of the toxin moiety; one possibility explored is masking of the therapeutic molecules by chemically modifying the immunotoxin with high molecular weight polyethylenglycol (PEG) [100], dextran, or other nonimmunogenic polymers. An impressive result was obtained recently by Onda and coworkers, who identified the major immunogenic B-cell epitopes in the truncated form of *Pseudomonas*

aeruginosa exotoxin A (PE38) [101]. A total of eight amino acids containing large bulky hydrophilic side chains have been replaced with smaller polar residues within these epitopes, resulting in a new toxin endowed with much less immunogenicity than the parental one, without any loss of cytotoxic activity also when recombinantly fused to an anti CD22 variable fragment [102]. Finally, a current successful approach to obtain less immunogenic or nonimmunogenic immunotoxins is the generation of a new class of recombinant molecules in which the cytotoxic moiety is an endogenous protein of human origin like proapoptotic protein (e.g TNF, TRAIL or granzyme B) or RNase [103].

A further complication observed in ITs administration in the past was due to nonspecific binding of the toxin domain to vascular endothelial cells, leading to the so-called "vascular leak syndrome" (VLS) [104], which is characterized by the damage of vascular endothelial cells, extravasation of fluids and proteins, interstitial edema, and organ failure. Although the mechanisms underlying this side effect are not completely understood, proteins such as RTA and some type I RIPs contain a consensus amino acid sequence which seems to induce vascular damage to human endothelial cells *in vitro* by binding to integrin receptors [105]. Indeed, in the case of RTA, molecular modeling suggested that these motifs were partially exposed on the surface of the molecule [105] and a similar motif is shared by viral disintegrins, which disrupt the function of integrin receptors [106]. Also PE-based immunotoxins have been shown to promote VLS in rats [107]. In the perspective of eliminating VLS during therapeutical use of ricin-based ITs, Vitetta and coworkers produced a series of RTA mutants, and identified the Asn 97 to Ala mutation, in a region flanking the VLS-responsible motif in the three-dimensional structure, as displaying significant less VLS in mice [108].

1.4.4 Expression systems

Diverse prokaryotic and eukaryotic expression systems have been developed for the production of recombinant proteins. These have included bacteria, yeast, filamentous fungus, eukaryotic alga, insect cell, plant, mammalian cell and transgenic animal systems. While in many instances heterologous proteins can be expressed in several different

systems, there is sometimes less flexibility in terms of choice of expression system due to structural requirements on the part of the specific protein to be produced.

The expression of heterologous proteins in eukaryotic organisms benefits by the presence of specialized compartments for the folding and assembly and of an efficient machinery for post-translational modifications. For this reason, eukaryotic expression systems are required for producing molecule, such as certain therapeutic IgGs, which requires appropriate glycosylation [109]. However, eukaryotic systems are not suitable for the expression of most ITs made by toxic domains which target the eukaryotic elongation factor of protein synthesis or a eukaryotic ribosomal subunit, thus resulting in a toxic effect on the host cell itself. Nevertheless some studies had shown that certain hosts are remarkably resistant to a number of toxins normally used in the design of ITs, while other expression systems (i.e. *Saccharomyces cerevisiae* and *Pichia pastoris*) are optimized for the production of these same toxins, allowing to recover fairly good amounts of the derived ITs [110].

Antibody fragments (mostly scFvs) and ITs have a much simpler structure and do not require glycosylation. Thus, bacterial expression has been the method of choice for the expression of these molecules. Further reasons for this choice lie in the cost-effectiveness of bacteria, their well-characterized genetics, and the availability of many different bacterial expression systems. Among the hosts available for recombinant expression, *Escherichia coli* is in an exceptional position. This stems from the many decades of intense research on its genetics as well as the broad scope of biotechnological tools available for genetic engineering of this organism. As a host for recombinant expression, *E. coli* is especially valued because of its rapid growth rate, capacity for continuous fermentation, low media costs and achievable high expression levels. One consequence of this popularity is that about 80% of all proteins used to solve three-dimensional structures submitted to the protein data bank (PDB) in 2003 were prepared in *E. coli* [111] and during 2003 and 2006, nine out of 31 approved therapeutic proteins were produced in *E. coli* [112], among them important growth factors, insulins and interferons [113].

The major drawbacks of using *E. coli* for recombinant protein production are its lack of secretion systems for efficient release of proteins to the growth medium, limited ability to facilitate extensive disulfide-bond formation and other post-translational modifications, inefficient cleavage of the amino terminal methionine which can result in

lowered protein stability and increased immunogenicity, and occasional poor folding due to lack of specific molecular chaperones [114].

Nevertheless, although refolding procedures require customization for each individual protein, the production of ITs in bacteria as inclusion bodies, followed by solubilization and *in vitro* renaturation, is still the most common route towards obtaining sufficient amounts of protein to be used for *in vitro*, lab-scale evaluation or for clinical trials [44].

1.5 COMBINATION THERAPIES

Despite their potency, immunotoxins produce complete remission infrequently when administered as single agents and relapses often occur because of the anti-apoptotic strategies frequently employed by tumor cells to overcome chemotherapy-mediated death [115, 116].

Programmed cell death (apoptosis) is now widely recognized as an evolutionarily conserved, genetically controlled process for killing damaged, infected, superfluous or potentially dangerous cells that is essential for the normal development and function of multicellular organisms. Defects in the control of apoptosis causing either the survival of unwanted cells or inappropriate killing of vital cells underlie a multitude of disorders, including autoimmunity, degenerative diseases and cancers [116]. Indeed, defects in apoptosis are now considered to be a hallmark of most, if not all, cancers.

Many cancer cells develop resistance to apoptosis by mechanisms involving members of the B-cell lymphoma-2 (Bcl-2) protein family, which are critical regulators of apoptosis consisting of anti- and pro-apoptotic proteins that determine the balance between survival and programmed cells death [117]. Structural and functional characteristics divide proteins of Bcl-2 family into three subgroups:

1) the anti-apoptotic family members Bcl-2, Bcl-xL , Bcl-W, A1 and Mcl-1 (myeloid cell leukemia-1), which promote cell survival by counteracting the apoptotic effector proteins, thus preventing their activation and mitochondrial outer membrane permeabilization with the consequent release of cytochrome c and caspase activation,

2) Bax and Bak proteins, which are the essential activators of the effector phase of apoptosis,
3) pro-apoptotic BH3 proteins, such as Bad, Bim (Bcl-2 interacting mediator of cell death), Bid (Bcl-2-interacting domain), Noxa and Puma (p53 upregulated mediator of apoptosis), which enable activation of Bax and Bak either by neutralizing anti-apoptotic Bcl-2 family proteins or by direct action.

Observations in human tumors and studies with genetically modified (transgenic or knock-out) mice have shown that tumourigenesis can be driven by gain-of-function mutations in cell death antagonists (e.g., Bcl-2 overexpression) or loss-of-function mutations in cell death activators (e.g., loss of Bim) [117].

Activation of the pro-apoptotic proteins Bax and Bak depends on the action of BH3-only proteins, which are up-regulated in response to stress or cell injury. BH3-only proteins can activate Bax/Bak directly or they can trigger apoptosis via the neutralization of the pro-survival proteins Bcl-2, Bcl-xL and Mcl-1 (Fig. 1.12).

Therefore, BH3 mimetics were developed as potential stand-alone antitumor agents or as sensitizers for chemotherapy, thus eliminating resistance to apoptosis. ABT-737, one such agent, was shown to have strong binding affinity for Bcl-2 and Bcl-xL but little or none for Mcl-1. Because inhibition of protein synthesis triggered by immunotoxins frequently results in the loss of Mcl-1, obtaining inhibition of Bcl-2 and Bcl-xL with ABT-737 seemed to be a good strategy to potentially sensitized tumor cells to immunotoxins action [115].

Combinatorial therapy using ABT-737 (and its clinical analog ABT-263) and immunotoxins has been demonstrated highly effective against molecular subgroups of multiple myeloma [118], adult T-cell leukemia/lymphoma (ATLL) [119] and many other types of tumor.

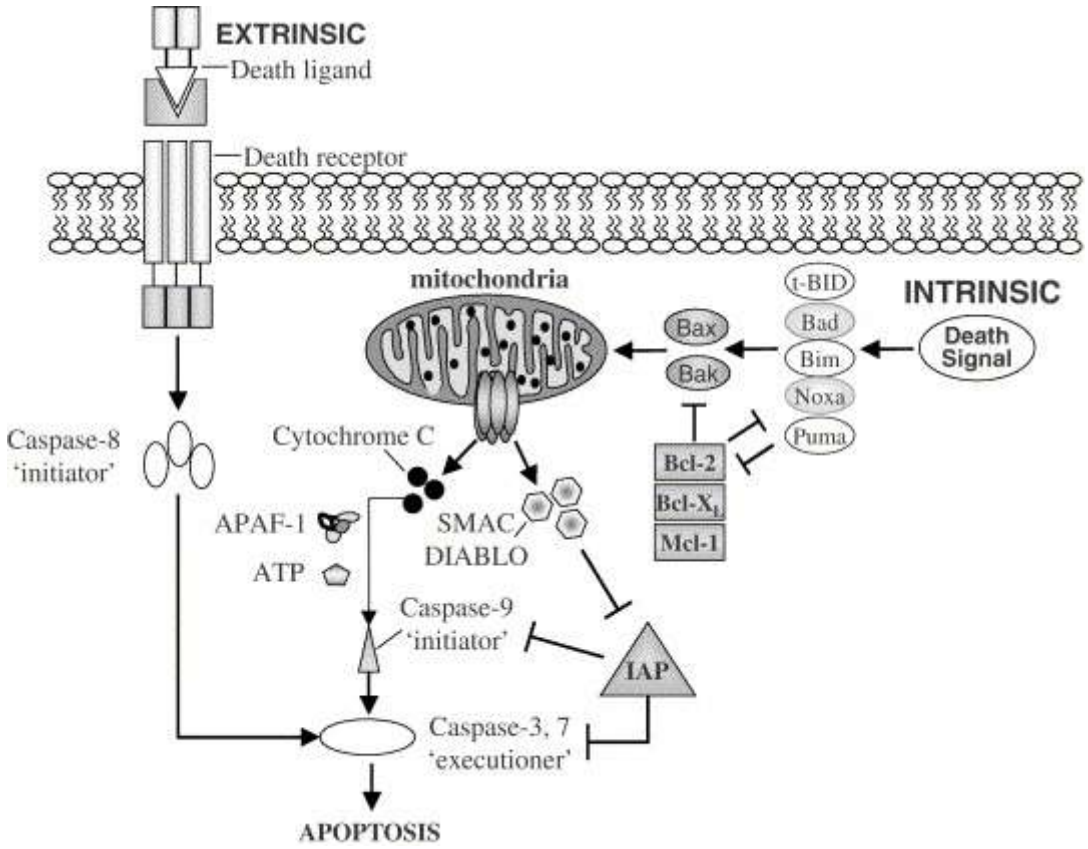


Figure 1.12 The extrinsic and intrinsic pathways of apoptosis. The extrinsic pathway is a receptor-dependent mechanism in which the activation of the death receptors leads to the formation of death-inducible signaling complex (DISC), activation of caspase-8 followed by a cascade of caspases that will execute cell death. The intrinsic pathway of apoptosis is activated in response to various stimuli including stress, DNA damage, and ultraviolet radiation (UV). The signal activates the pro-apoptotic Bcl-2 proteins Bax and Bak which complex to form pores in the outer mitochondrial membrane resulting in the release of cytochrome-c. This leads to the formation of the apoptosome, which is comprised of cytochrome-c, APAF-1 and caspase-9. The apoptosome cleaves caspase-3 leading to cell death. Anti-apoptotic proteins Bcl-2, Bcl-xL and Mcl-1 block the action of Bax and Bak, while BH3 mimetics like t-BID (truncated BID), Bad, Bim, Noxa and Puma act by activating the pro-apoptotic factors Bax and Bak and by inhibiting Bcl-2, Bcl-xL and Mcl-1.

Another important pathway involved in the control of programmed cell death is that mediated by Pim (Proviral Integration Moloney virus) kinases. The mammalian Pim family is composed by the highly homologous proteins Pim-1, -2 and -3 which are serine/threonine kinases with partially overlapping functions and expression patterns [120].

In nontransformed cells, the activity of these kinases is tightly controlled by cytokines and growth factor availability, whereas their sustained activation can lead to apoptotic resistance and uncontrolled cell proliferation promoting the processes of lymphomagenesis and malignant transformation [121]; in fact overexpression of one or

more Pim family members has been observed in multiple types of cancer including lymphomas and leukemias and frequently correlates with poor prognoses. Whereas elevated levels of PIM1 and PIM2 were mostly found in hematologic malignancies and prostate cancer, increased PIM3 expression was observed in different solid tumors (mostly in melanoma, pancreatic and gastric tumors) [122].

Pim kinases regulation occurs at the level of transcription, translation, and proteosomal degradation. In lymphocytes, Pim genes transcription is mainly mediated by the JAK/STAT signal transduction pathway: upon cytokine engagement of its receptor, JAK phosphorylates and activates STAT proteins which then translocate to the nucleus and serve as transcription factors for the Pim genes. In addition to transcriptional control, regulation of Pim mRNA stability is also a determinant of Pim activity [121].

Once translated, Pim kinases function by phosphorylating multiple downstream targets important for promoting tumor cell survival and proliferation including c-Myc, the pro-apoptotic protein Bad [123], members of the suppressor of cytokine signaling (SOCS) family [124] and the translational repressor eIF-4E binding protein 1 (4E-BP1) [125] (Fig. 1.13).

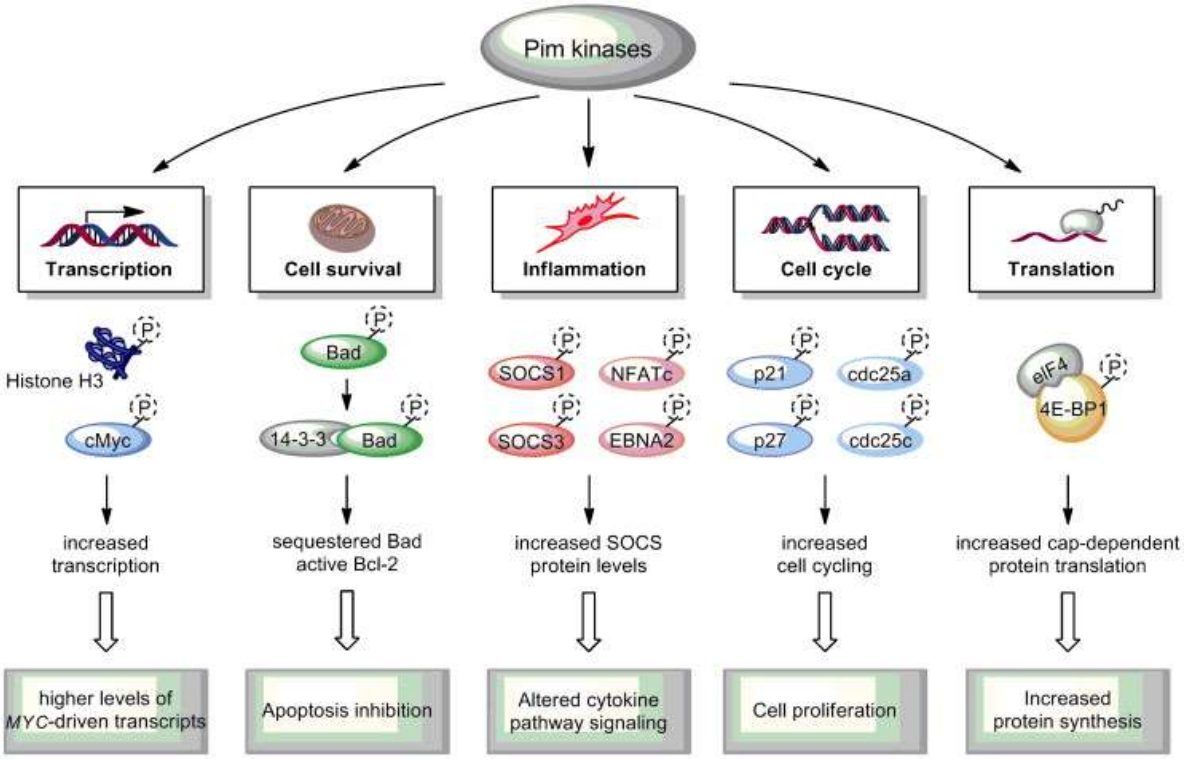


Figure 1.13 Potential downstream substrates of overexpressed PIM1 and correlated biological effects in hematologic malignancies. (Chen, L.S. *et al*. 2010)

Pim kinases represent validated drug targets in many hematologic cancers and select solid tumors and a number of small molecule PIM kinases inhibitors have emerged as therapeutics for hematological malignancies. Based on the known crystal structure of Pim-1, high-throughput screening, and lead optimization techniques, has been possible to develop compounds specifically binding to the ATP binding site of Pim-1 and competing with ATP binding. One of the most potent inhibitors of all 3 Pim kinases is SGI-1776, an imidazo[1,2-b] pyridazine compound which has been demonstrated efficient in preclinical models of human acute myeloid leukemia and CLL and prostate cancer, either as a single agent or in combination with other chemotherapeutic agents [126].

AIM OF THE RESEARCH

The main objectives of the study here presented are:

- construction and production in *E. coli* of a scFv binding to the B-cell surface antigen CD38, through the molecular cloning of the variable region of heavy and light chains (V_H and V_L, respectively);
- generation and bacterial expression of recombinant immunotoxins by genetic fusion of the scFv to a truncated version of *Pseudomonas aeruginosa* exotoxin A (PE40) or to the plant toxin saporin;
- characterization of the binding properties of the recombinant ITs on cells that express the target antigen on their surface;
- evaluation of the selective toxicity on target B cells of the ITs obtained;
- study of potentiating effects of drugs inhibiting intracellular pathways in association with recombinant ITs.

2. MATERIALS AND METHODS

2.1 MICROBIOLOGY TECHNIQUES

2.1.1 *ESCHERICHIA COLI* STRAINS

- **DH5α:** F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK^-, mK^+) *pho*A *sup*E44 λ *thi*-1 *gyr*A96 *rel*A1;
- **XL1-Blue**: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F^1 *proAB* $lacI^qZ\Delta M15$ Tn*10* (Tet^r)];
- **BL21 (DE3) pLysS**: F^- *ompT hsdS*(r_B^- m_B^-) dcm^+ *galλ*(DE3) *rne131* (pLysS Cam^r).

Bacterial stocks are kept at -80 °C in LB medium containing 20% glycerol.

2.1.2 *E. COLI* GROWTH MEDIA

LB-*medium*

Tryptone	10 g/l
Yeast Extract	5 g/l
NaCl	10 g/l

Adjust pH to 7.5 with 1M NaOH and sterilize at 121 °C for 20 minutes.

Agar medium for plates is prepared by addition of 15 g/l Agar before sterilization by autoclave.

LB broth is used for transformation, growth, maintenance and protein expression with DH5α, XL1-Blue and BL21(DE3)pLysS *E. coli* strains.

2.1.3 PLASMID VECTORS

For the molecular cloning of DNA sequences vector pET20b(+) (Novagen) was used.

pET20b(+)

pET20b(+) is an expression vector of 3716 bp carrying a multi-cloning site between T7 promoter and T7 terminator sequences.

In order to induce the expression of the protein coded by the sequence inserted in the polylinker, it is necessary to use *E. coli* strains carrying a copy of the gene for T7 RNA polymerase. These are lysogenic hosts of the DE3 bacteriophage, which has in its genome

the sequence coding for T7 RNA polymerase, under control of the promoter *lacUV5*. The *lacUV5* promoter is induced by the addition of IPTG and drives the transcription of the T7 RNA polymerase, which, in its turn, transcribes the sequence of interest. In this way the basal expression, i.e. the expression of the recombinant protein before induction, is very limited. The strain of *E. coli* employed for the expression also bears the plasmid pLysS, which determines a further repression of the basal expression level through the transcription of T7 lysozyme, a natural inhibitor of T7 RNA polymerase.

Vector pET20b(+), by virtue of the pelB signal peptide, promotes the periplasmic sorting of the protein coded by the inserted sequence, also providing a poly-histidine carboxy-terminal tag. pET20b(+) carries an ampicillin resistance and the pBR322 origin of replication.

In this plasmid the final scFv-coding sequence was inserted and subsequently fused to the fragment coding for PE40 or saporin, so as to obtain the constructs for the expression of the immunotoxins of interest.

2.1.4 Preparation of $CaCl_2$-competent *E. coli* cells

The strains of *Escherichia coli* utilized for the molecular cloning and for protein expression are made competent to transformation by plasmid DNA following treatment with $CaCl_2$.

A glycerol stock of DH5α, XL1-Blue or BL21 (DE3) pLysS cells (kept at -80 °C) is streaked on a LB-agar plate containing 25 µg/ml Tetracycline (for XL1-Blue), 34 µg/ml Chloramphenicol (for BL21 (DE3) pLysS) or no antibiotics (for DH5α) and incubated o.n. at 37 °C.

The day after a single colony is picked and used to start a 10 ml LB culture supplemented with the same antibiotics at the same concentrations used in the plates and grown o.n at 37 °C with shaking.

Few (1-2) ml of the overnight culture is then used to start a 50 ml LB culture with appropriate antibiotics. This culture is grown to OD_{600} = 0.3-0.4 at 25°C with shaking.

The bacterial culture is then kept on ice for 30 minutes, after which it is centrifuged at 4000 x *g*, 10 minutes at 4 °C. Always keeping the cells at ice temperature, they are recovered and resuspended in 25 ml of sterile, ice-cold 50 mM $CaCl_2$. After a one hour

incubation on ice the suspension is centrifuged at 3000 x *g*, 5 minutes at 4°C. The supernatant is removed and the bacterial pellet is resuspended in 3 ml of a sterile 50 mM $CaCl_2$, 20% glycerol solution. Cells are finally dispensed in aliquots, frozen in dry ice and kept at -80 °C.

2.1.5 HEAT-SHOCK MEDIATED TRANSFORMATION OF *ESCHERICHIA COLI*

A 100 µl aliquot of $CaCl_2$-competent cells are thawed on ice and mixed into a sterile tube with 5-10 ng of plasmid DNA; in case of transformation with a ligation, 5 µl of the reaction are used. The mixture is gently flicked and kept on ice for 20 minutes. Afterwards a heat shock is carried out by dipping the tube for one minute in a 42°C water bath, followed by a 2 minute incubation on ice. 1 ml of LB broth (without antibiotics) is then added into the tube which is then incubated 1 hour at 37°C with shaking. The desired amount of transformation is then plated (after centrifugation to concentrate cells if necessary) on LB-agar plates supplemented with the appropriate selective agent (100 µg/ml Amp + 25 µg/ml Tet or 100 µg/ml Amp + 34 µg/ml Cam). Plates are incubated inverted at 37 °C and grown o.n.

2.2 HUMAN CELL LINES

2.2.1 CELL LINES AND GROWTH MEDIA

- Ramos: human line of B lymphocytes derived from Burkitt's lymphoma (CD38-positive);
- Daudi: human line of B lymphocytes derived from Burkitt's lymphoma (CD38-positive);
- 3T3/7A: mouse fibroblasts tranfected with CD38 molecule (CD38-positive);
- RPMI8226: human line of Multiple Myeloma (CD38-positive);
- U266: human line of Multiple Myeloma (CD38-negative);
- Anti-CD38 hybridoma 1E82H11: the hybridoma clone was derived from spleen lymphocytes of a mouse previously immunized with intraperitoneal injection of a membrane preparation from 3T3 cells transfected with human CD38 antigen (kindly

provided by Prof. Ippoliti and co-workers from the University of L'Aquila); the myeloma cell line used in the fusion was NS-1.

All cell lines are grown in flasks at 37 °C, 5% CO_2, using the following medium:

RPMI 1640 medium (with 40 mg/l folic acid, 2 g/l $NaHCO_3$) (BiochromAG) supplemented with 10% Fetal Calf Serum (FCS), 2 mM L-Glutamine and antibiotics (100 U/ml penicilline and 100 µg/ml streptomycine-sulphate). Hybridoma clone 1E82H11 is grown in Hybridomed DIF 1000 Serum free medium (BiochromAG).

All supplements are added into the medium after sterilization through 0.22 µm filters.

Each cell line is cultured as a suspension and is constantly kept at an exponential growth phase by frequent medium changes. Cell stocks can be stored in liquid nitrogen tank at a concentration of 1 or 2 x 10^6 cells/ml RPMI medium with 20% FCS and 10% DMSO.

2.2.2 B-LYMPHOCYTES FROM PBMCS

B-lymphocytes were obtained by negative-selection (using *EasySep Human B Cell Enrichment Ki*t, StemCell Technologies) from a PBMC sampe of a CLL patient. Cells were mantained in IMDM medium (Life Technologies) supplemented with 10% FCS, 10 nM IL-4, 20 U/ml IL-2 and 50 ng/ml CD40L.

2.3 MOLECULAR BIOLOGY

2.3.1 RNA EXTRACTION FROM ANTI-CD38 HYBRIDOMA CELLS

3 x 10^6 cells of the anti-CD38 hybridoma are collected by centrifugation (5 minutes, 150 x *g*, 5°C) and likewise washed in physiologic solution. RNA extraction is obtained using *SV Total RNA Isolation System* (Promega) and finally RNA is eluted with 50 µl RNAse-free H_2O. Total RNA is quantified by spectrophotometry: 1 unit at OD_{260} corresponds to an RNA concentration of 40 µg/ml.

2.3.2 CDNA SYNTHESIS

Retrotranscription to cDNA is obtained using M-MLV retrotranscriptase (*Moloney Murine Leukemia Virus Reverse Transcriptase* - Invitrogen), following the manufacturer's

instructions: 4 µg of total RNA are mixed with 1 µl of *random primers* (Invitrogen), 1 µl of 10 mM dNTPs *mix* (Applied Biosystem) and 8 µl RNAse-free H_2O; this mixture is incubated at 65°C for 5 minutes and then kept on ice. The following must then be added: 4 µl of 5x *First Strand Buffer* (Invitrogen), 2 µl of 0.1 M DTT (Invitrogen), 1 µl of *RNase-OUT* (Invitrogen); the reaction is incubated 2 minutes at 37°C, after which 1 µl of M-MLV is added. Using a thermocycler, the reaction is then incubated 10 minutes at 25°C, followed by 50 minutes at 37°C and finally 15 minutes at 70 °C.

The quality of the cDNA is assessed through a PCR test with a couple of primers designed for the amplification of murine β-actine (see paragraph 2.3.3.1).

2.3.3 PCR AMPLIFICATION OF SPECIFIC DNA FRAGMENTS

The amplification of DNA fragments by polymerase-chain-reaction (PCR) was customized in order to fit the conditions required by specific templates and enzymes used in the reactions. All reactions were carried out using a GeneAmp PCR system 9700 thermocycler (Applied Biosystems).

2.3.3.1 AMPLIFICATION OF THE SEQUENCE CODING FOR MOUSE B-ACTIN

The outcome of this PCR provides an indication regarding the quality of the cDNA as a template, before proceeding with the amplification of the fragments of interest.

Cycling programme:

94°C	5	min	
94°C	45	sec	
59°C	45	sec	35 cycles
72°C	45	sec	
72°C	7	min	
4°C		end	

1 µl of cDNA is used as a template in a 50 µl reaction including a couple of primers (m-β-actin fw and m-β-actin rev) at a final concentration of 0.4 µM each, dNTPs (Applied Biosystems) at a concentration of 0.2 mM each, 2 units of Taq DNA polymerase (Fermentas), 5 µl of 10x buffer (Fermentas) and 4 µl of 25 mM $MgCl_2$.

2.3.3.2 AMPLIFICATION OF THE SEQUENCES CODING FOR VARIABLE DOMAINS OF HEAVY AND LIGHT CHAINS

In order to select the appropriate couple of primers for the amplification of fragments coding for the variable regions of heavy and light chains (V_H and V_k, respectively) of the anti-CD38 hybridoma clone, a panel of 25 forward and 4 reverse primers are considered for each variable domain (25 V_H forward primers and 4 J_H reverse primers; 25 V_k forward primers and 4 J_k reverse primers). Forward primers were designed based on highly conserved sequences at the 5'-end of DNA fragments for V_H and V_L domains from several families of murine immunoglobulins; reverse primers were instead inferred from the J regions located at the 3'-end of V_H and V_L DNA regions.

Each forward primer is tested in a PCR reaction that includes a mix of the four reverse primers. Once the best forward primer has been thus selected, it is used in four individual PCR reactions, each with a single reverse primer. The forward/reverse primers pair identified as the most appropriate for amplification are then re-designed as modified versions with suitable restriction enzymes.

The sequence coding for the V_k region of our anti-CD38 monoclonal antibody was PCR-amplified using the primers 5' *XhoI* $(G4S)_3$ 316 V_k and 3' J_k 4940 *Not*I, which introduce XhoI and NotI restriction sites, respectively, and also the sequence of hthe peptide linker $(G_4S)_3$ at the 5'; the V_H fragment was amplified with primers 5' *NcoI* 340 V_H and 3' J_H 353 *Xho*I, introducing NcoI and XhoI sites.

1 µl of cDNA is used as a template in each 50 µl PCR reaction, along with 0.4 µM of each primer, 0.2 mM of each dNTP (Applied Biosystems), 1 µl of PfuUltra II Fusion HS DNA polymerase (Stratagene) and 5 µl 10x buffer, providing a final Mg^{2+} concentration of 2 mM.

Cycling programme:

94°C	3	min	
94°C	1	min	
59°C	1	min	35 cycles
72°C	1	min	
72°C	10	min	
4°C		end	

2.3.3.3 AMPLIFICATION OF THE SEQUENCE CODING FOR THE TRUNCATED VERSION OF *PSEUDOMONAS AERUGINOSA* EXOTOXIN A (PE40)

The sequence of PE40 was amplified from a pre-existing plasmid construct. A PCR reaction in which the *Not*I-PE forward primer and the PE-*Not*I reverse primer were used, yielded the amplificate for the construction of an IT with a carboxy-terminal hexahistidine tag.

The reaction was prepared in a total volume of 50 µl, with 10 ng of plasmid template, 0.4 µM of each primer, 0.2 mM of each dNTP (Applied Biosystems), 1 µl of PfuUltra II Fusion HS DNA polymerase (Stratagene), 5% DMSO and 5 µl of 10x buffer. DMSO is a co-solvent that is often used in case of low yield with "GC-rich" templates (as in the case of PE40).

Cycling programme:

95°C	3	min	
95°C	30	sec	
55°C	30	sec	30 cycles
72°C	20	sec	
72°C	5	min	
4°C		end	

2.3.3.4 AMPLIFICATION OF THE SEQUENCE CODING FOR THE SAPORIN

The sequence of saporin was amplified from a pre-existing plasmid construct. A PCR reaction in which the *Not*I-SAP forward primer and the SAP-*Not*I reverse primer were used, yielded the amplificate for the construction of an IT with a carboxy-terminal hexahistidine tag.

The reaction was prepared in a total volume of 50 µl, with 10 ng of plasmid template, 0.4 µM of each primer, 0.2 mM of each dNTP (Applied Biosystems), 1 µl of PfuUltra II Fusion HS DNA polymerase (Stratagene) and 5 µl of 10x buffer. DMSO in this case is not required.

Cycling programme:

94°C	3	min	
94°C	1	min	
59°C	1	min	30 cycles
72°C	1	min	
72°C	10	min	
4°C		end	

2.3.4 DNA DIGESTION WITH RESTRICTION ENZYMES

Enzymatic digestion of plasmid DNA was performed according to the indications provided by manufacturers. Typically, in each reaction 5-10 units of enzyme are used to cut 1 μg of DNA. Reactions are usually incubated for 2 hours at the recommended temperature. If two or more enzymes need to be used, the DNA is ethanol-precipitated after the first digestion and then resuspended in deionized water before the second digestion.

Table 2.1 Restriction enzymes used.

	Manufacturer	Stock concentration
NcoI	Fermentas	10 U/μl
NotI	Takara	10 U/μl
XhoI	Neb	20 U/μl

2.3.5 PLASMID CONSTRUCTS

2.3.5.1 CLONING STRATEGY

The sequence of operations required to obtain the recombinant constructs for further expression is here described:

Step 1: scFv 1E8

The pET20b(+) vector containing the V_H and V_L of a pre-existing anti-CD22 scFv was digested with *NcoI* and *XhoI* and ligated with the PCR fragment coding for the anti-CD38 V_H domain (cut with the same enzymes), which thus replaced the V_H domain of the CD22 scFv. This intermediate construct was digested with enzymes *XhoI* and *NotI* (which removed the CD22-derived V_k fragment) and ligated with the V_k fragment from our anti-CD38 mAb obtained by PCR and digestion with the same enzymes (Fig. 2.1).

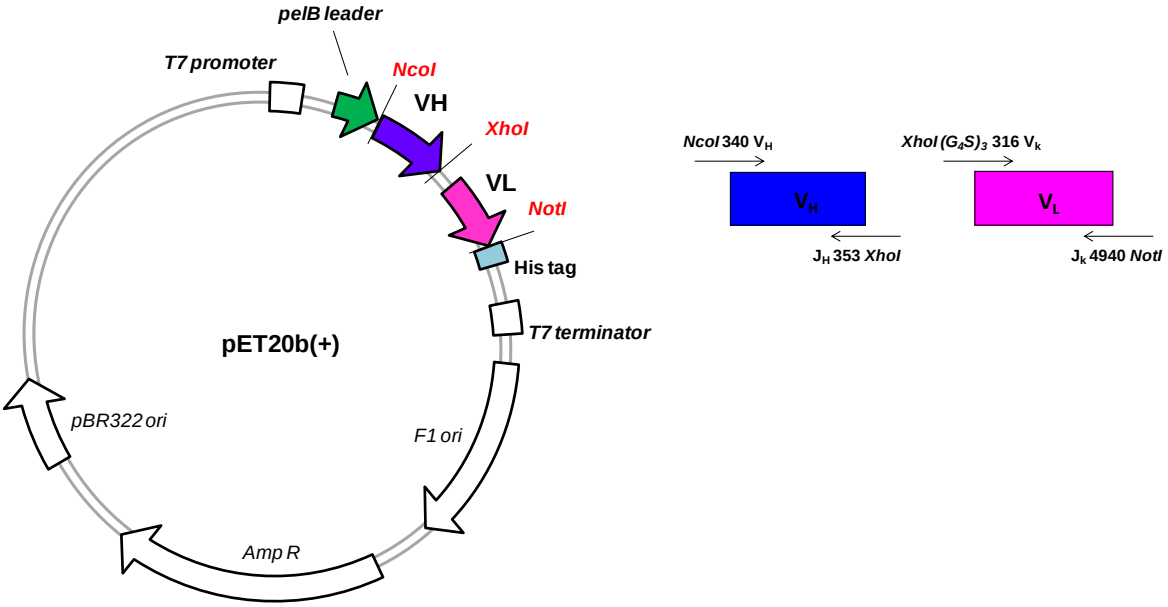


Figure 2.1 Construct for anti-CD38 scFv obtained as described in step 1.

Step 2: DIV1E8

The entire sequence obtained in step 1 was amplified by two distinct PCRs:

- PCR1 with oligonucleotides *NcoI* 340 V_H and V_k 1E8 *SacI*,
- PCR2 with *SfiI* V_H 1E8 and J_k 4940 *Not*I (see paragraph 2.3.10).

PCR1 was cut with *NcoI* and *SacI* and inserted into a pET20b(+) vector, carrying the sequence of a pre-existing divalent scFv, which was cut with the same enzymes to obtain the excision of the first scFv.

This intermediate construct was digested with enzymes *SfiI* and *NotI* (which removed the second scFv of the parental construct) and ligated with fragment obtained by PCR2 and digestion with the same enzymes (Fig. 2.2).

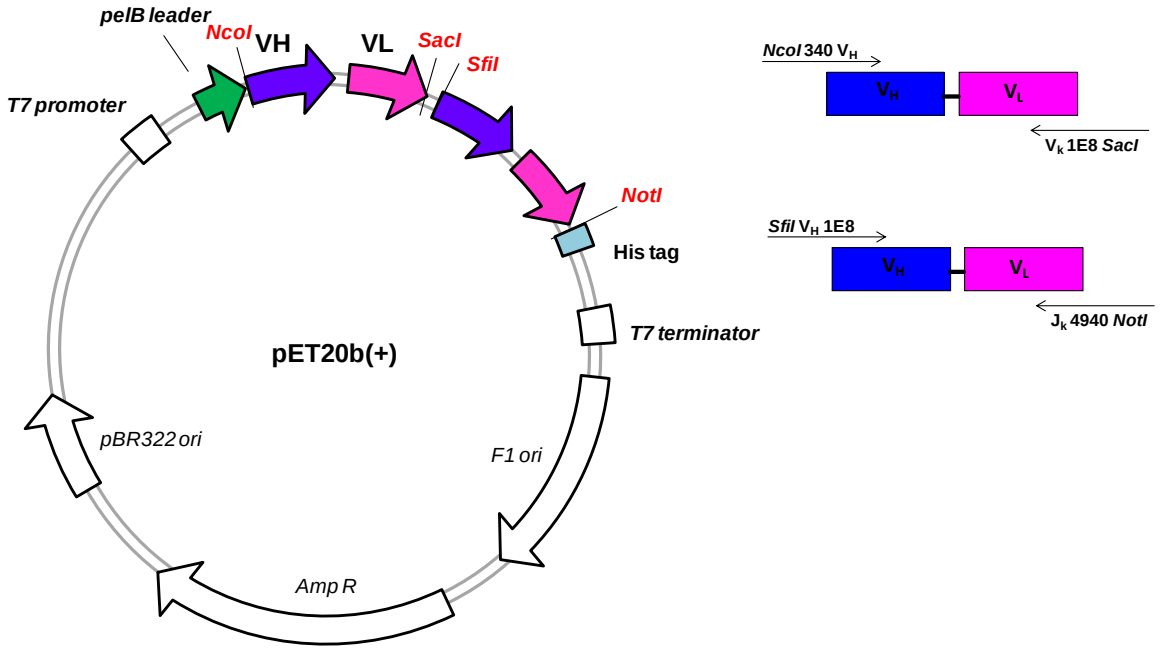


Figure 2.2 Construct for divalent anti-CD38 scFv obtained as described in step 2.

Step 3: AT13/5 scFv

The sequence of AT13/5 scFv, supplied in pDrive vector, was excised by *NcoI*/*NotI* digestion and ligated into pET20b(+) previously cut with the same enzymes.

Step 4: immunotoxins with PE40 or saporin

The plasmid constructs for the expression of anti-CD38 recombinant immunotoxins were obtained by insertion of the PCR fragments coding for PE40 or saporin into the constructs obtained in step 1, 2 and 3, previuosly digested with *NotI*. The correct orientation of the inserts was confirmed by restriction analysis (Fig. 2.3).

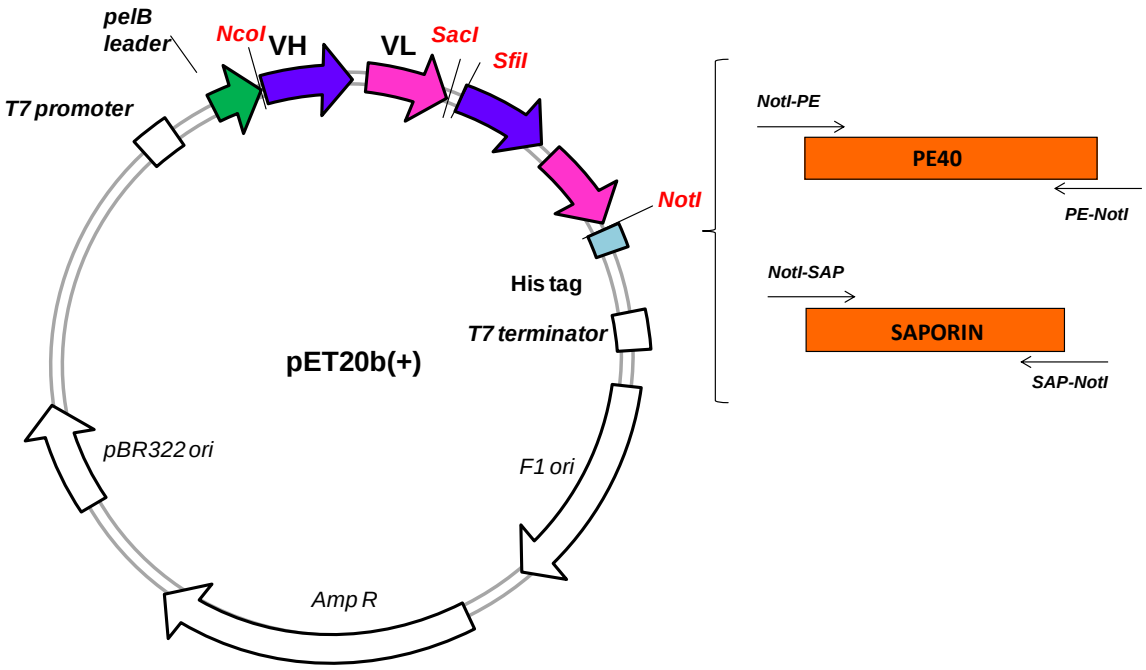


Figure 2.3 Cloning strategy for anti-CD38 immunotoxins as described in step 4.

2.3.5.2 Ligation

After digestion with the appropriate restriction enzymes, vectors and inserts for the preparation of all constructs were purified after agarose gel slices obtained by agarose gel electrophoresis using the *QIAquick Gel Extraction* kit (QIAGEN), and eventually ligated with T4 DNA ligase (Invitrogen). A 20 µl reaction is prepared in a clean tube containing 100 ng of vector, a five-fold molar excess of insert fragment, the provided ligation buffer and 2.5 units of enzyme. The ligation is incubated 1.5 hours at RT, after which 5 µl are used for the transformation of $CaCl_2$-competent *E. coli* cells (strain DH5α or XL1B).

2.3.5.3 COLONY-PCR SCREENING

For a rapid screening of *E. coli* colonies obtained after transformation with the ligation reaction, a PCR is performed directly on bacterial cells.

Using a sterile tip, cells from a single colony are picked from the LB-agar plate and dissolved in 10 µl sterile, deionized water in a PCR-tube; the same tip is dipped in 50-100 µl of LB broth with appropriate antibiotics in a 1.5 ml eppendorf tube, so that the positive bacterial clones can be recovered at the end of the screening. In each PCR tube is then added 15 µl of a concentrated reaction mix with: 2.5 µl of 10x reaction buffer (Fermentas), dNTPs mix (0.2 mM of each dNTP, Applied Biosystems), forward and reverse primers (10 pmoles each), 1 unit of Taq DNA polymerase (Fermentas). If necessary, also 5% DMSO is included.

Cycling programme:	95°C	4	min	
	95°C	30	sec	
	55°C	1	min	35 cycles
	72°C	1	min	
	72°C	7	min	
	4°C		end	

The PCR reactions are analysed by agarose-gel electrophoresis and staining with ethidium bromide. Amplificates of the expected size signify recombinant clones, that can be recovered from the small LB cultures and propagated for extraction of plasmid DNA.

2.3.6 PLASMID DNA EXTRACTION FROM *E. COLI* CULTURES

Single colonies picked from LB-agar plates (or from small LB cultures for colony-PCR screening) are inoculated in 5 mL for small-scale preparations (miniprep), or in 50 mL for medium-scale preparations (midiprep) of LB broth additioned with the appropriate antibiotics (100 µg/ml Amp); the culture is grown o.n. at 37 °C with shaking (250 rpm) and cells are recovered by centrifugation (5 minutes, 10000 rpm at RT in minifuge for minipreps; 10 minutes, 4000 x*g* at RT for midipreps) and the bacterial pellet is processed using the *Wizard Plus SV Minipreps DNA Purification System* (Promega) for minipreps and

the *PureLink HiPure Plasmid filter Purification kit* (Invitrogen) for midipreps, according to instructions provided by the manufacturers. Purity and concentration of plasmid DNA are assessed by agarose gel electrophoresis and ethidium bromide staining. Yields for pET20b(+)- derived plasmids range from 2 to 4 µg for minipreps and 10 to 100 µg for midipreps.

2.3.7 DNA SEQUENCING

The correctness of all plasmid constructs is confirmed by sequencing (BMR-Genomics, Padova, Italy). Analysis of the DNA sequences was performed using *Vector NTI Advance 10* software (Invitrogen).

2.3.8 OLIGONUCLEOTIDES USED

Oligonucleotide	Sequence
m-β-actin fw	caccctgtgctgctcaccgaggcc
m-β-actin rev	ccacacagatgacttgcgctcagg
NcoI 340 V_H	ataccatggccgatgtgcaactggtggagtctggg
J_H 353 *Xho*I	aataactcgaggatgcagagacagtgaccagag
XhoI $(G4S)_3$ 316 V_k	atactcgagtggtggaggcggttcaggcggaggtggctctgg cggtggcggatcggacattgtgatgacccagactcc
J_k 4940 *Not*I	attctgcggccgcctttgatttccagcttggtgcc
SfiI V_H 1E8	tataggccatcatggccgatgtgcaactggt
V_k 1E8 *SacI*	tagagctccctttgatttccagctt
*Not*I-PE	tatagcggccgcttccggaggtcccgag
PE-*Not*I	tatagcggccgccttcaggtcctcgcgcgg
NotI-SAP	tatagcggccgcttccggaggtgtcacatcaatc
SAP-*NotI*	tatagcggccgcctttggtttgcccaa

All oligonucleotides were synthesized by MWG Biotech.

2.4 PROTEIN EXPRESSION IN BACTERIA

2.4.1 EXPRESSION OF SCFV AND IMMUNOTOXIN IN *ESCHERICHIA COLI* BL21(DE3) PLYSS

A single colony of *E. coli* BL21 (DE3) pLysS transformed with pET20b(+)-based constructs coding for the scFv or immunotoxins was inoculated in 20 ml of Lb broth, 100 µg/ml Amp, 34 µg/ml Cam and grown o.n. at 37 °C with shaking at 250 rpm. The o.n. culture was used to start a bigger 1 l culture in LB broth, 100 µg/ml Amp which was grown at 37 °C with shaking to an OD_{600} between 0.7 and 0.8. Before induction, 0.5 ml of culture were transferred into an eppendorf tube and the cell pellet was recovered after centrifugation for 5 minutes at 11000 rpm in minifuge and stored at -20 °C for later SDS-PAGE analysis (non induced sample).

Expression of the recombinant protein was induced by addition of 1 mM IPTG into the culture and incubation was continued for 3 hours at 30 °C with shaking. 0.5 ml of culture (induced sample) were transferred into an eppendorf tube and treated as with the non induced sample. The bulk of the induced culture was centrifuged 15 minutes at 8000 x*g*, 4 °C and the bacterial pellet was frozen in liquid nitrogen and stored at -80 °C before protein extraction (see par. 2.5.1).

2.5 PROTEIN PROCESSING AND ANALYSIS

2.5.1 EXTRACTION OF PROTEINS FROM *E. COLI* BL21(DE3) PLYSS INCLUSION BODIES

Bacterial pellets obtained from 1 l induced cultures and stored at - 80 °C (see par. 2.4.1) were resuspended by sonication (3 bursts of 30 seconds each, with incubation of 30 seconds on ice after each burst) in 100 ml of lysis buffer (50 mM Na_2HPO_4 pH 7.5, 0.5 M NaCl, 1% Triton X-100, 1 mM PMSF).

After resuspension, the following were added: 10 mM $MgCl_2$, 20 units/ml DNAse I (Roche), 0.1 mg/ml lysozime. This was followed by an incubation of 20 minutes at 4°C.

The insoluble material was sedimented by centrifugation for 20 minutes at 13000 x*g*, at 4 °C. Inclusion bodies were then dissolved by sonication in 3 ml lysis buffer, added dropwise

into a beaker containing 47 ml of a denaturating buffer (50 mM Na_2HPO_4 pH 7.5, 0.5 M NaCl, 8 M urea) kept under magnetic stirring and then incubated for 1 hour at RT. The solubilized inclusion bodies were centrifuged 30 minutes at 20000 x*g*, 4 °C to separate persisting aggregates and the supernatant was then recovered for further purification of the recombinant proteins or store at 4°C.

2.5.2 PURIFICATION OF RECOMBINANT PROTEINS BY AFFINITY CHROMATOGRAPHY

Constructs for both scFv and ITs have a C-terminal hexahistidine tag, which was exploited for purification by *Immobilized metal-ion affinity chromatography* (IMAC). We used the *Ni Sepharose 6 Fast Flow* (GE Healthcare) that takes advantage of the coordination occurring between the Ni^{2+} ions immobilized on the resin beads and the imidazole rings of histidines.

The resin must first be equilibrated in the buffer in which inclusion bodies are resuspended and which is used for the chromatography. Two ml of *Ni Sepharose* slurry were mixed by inversion with 8 ml of denaturing buffer (see par. 2.5.1) containing 20 mM imidazole and then centrifuged 5 minutes at 2000 rpm to recovering the equilibrated resin. The sample (denatured inclusion bodies, see par. 2.5.1) was filtered by 0.45 µm syringe filter to remove possible aggregates and the supernatant was mixed with the sedimented resin into a beaker and incubated o.n. at 4°C. The day after, the sample was loaded onto a column (sealed at both ends) and incubated few mintes to allow the resin to settle. The column was then uncapped and the flow-through was collected. Three washing steps followed with 10 ml of Wash buffer with 20 mM imidazole, to remove unwanted bacterial proteins that could bind the resin aspecifically. Elution of His_6-tagged proteins was finally obtained in several steps, each time adding 1 ml of Elution buffer and collecting the eluate in eppendorf tubes.

Wash buffer:

NaH_2PO_4	50 mM	pH 7.5
NaCl	0.5 M	
Urea	8 M	
Imidazole	20 mM	

Elution buffer:

NaH_2PO_4	50 mM	pH 7.5
NaCl	0.5 M	
Urea	8 M	
Imidazole	0.5 mM	

Absorbance at 280 nm of the eluted samples gave an indication of the amount of purified protein: the samples containing a sufficient amount of protein (OD_{280} > 0.1), were pooled and dialyzed in the refolding process.

2.5.3 Refolding of proteins from inclusion bodies

Refolding of urea-denatured proteins purified by IMAC was attained by multi-step dialysis that gradually decreased the concentration of denaturant, therefore promoting protein refolding.
Tubular membranes with a 14 kDa cutoff (Carl Roth) were used.

Each dialysis steps were performed in a volume of 500 ml, for 12 hours at 4 °C according to the following scheme:

- Refolding buffer with 4 M urea
- Refolding buffer with 2 M urea
- Refolding buffer with 1 M urea
- Refolding buffer with 0.5 M urea
- Refolding buffer with 0.25 M urea
- Refolding buffer with no urea
- Refolding buffer without L-Arginine
- PBS

In case some precipitate formed during the dialysis procedure, it was removed by centrifugation at 7000 x*g*, 10 minutes at 4 °C. Supernatant was recovered and dialysis continued with the next step.

Refolding Buffer:

NaH_2PO_4	50 mM pH 8
NaCl	0.5 M
L-Arginine	0.4 M

10x PBS buffer:

NaCl	80 g/l
KCl	2 g/l
Na_2HPO_4 ($2H_2O$)	17.9 g/l
KH_2PO_4	2 g/l

Adjust pH to 7.2, sterilize 20 minutes at 121 °C and store at RT. Dilute to 1x in deionized water.

2.5.4 Purification of mAb from hybridoma culture medium

1E82H11 mAb was purified by affinity chromatography using the *Protein G Sepharose 6 Fast Flow* (GE Healthcare) which exploits the binding of protein G to the Fc region of murine IgG.

Two ml of *Protein G Sepharose* slurry were mixed with 8 ml of 20 mM sodium phosphate buffer, pH 7 and loaded into a column for resin equilibration. After sedimentation of the resin, the buffer was discarded as flow-through. Ten ml of hybridoma culture supernatant were centrifuged (1000 rpm, 10 minutes, 4 °C) to remove possible cell debris and aggregates and the supernatant was loaded onto the column. The column was sealed at both ends and incubated o.n. at 4°C on a rotating wheel. The column was then kept in a vertical position and, after the resin settled, it was uncapped and the flow-through was collected. Three washing steps followed with 10 ml of 20 mM sodium phosphate buffer, pH 7 to remove unwanted proteins that could bind the resin aspecifically. Elution of IgGs was finally obtained

adding 900 µl of 0.1 M glycine-HCl, pH 3 and collecting the eluate in eppendorf tubes where 100 µl of neutralizing buffer (1 M Tris-HCl, pH 9) were previously added.
Absorbance at 280 nm of the eluted samples gave an indication of the amount of purified protein: the samples containing a sufficient amount of protein were pooled and dialyzed in 2 l PBS buffer, o.n. at 4 °C.

2.5.5 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic separation of proteins from crude extracts, supernatants or purified samples was performed according to standard laboratory procedures, using mini-gels with 5% acrylamide stacking and 12% separating slabs. Electrophoresis was conducted in Tris-Glycine buffer (25 mM Tris, 190 mM Glycine, 0,1% SDS), using the *Mini-Protean III Cell* apparatus (BioRad) under reducing conditions (samples were denatured by boiling for 5 minutes in Sample Loading buffer (4X) - 0.32 M TrisHCl pH 6.8, 6% SDS, 50% glycerol, 2% β-mercaptoethanol, 0.006 g Bromophenol blue). A tension of 100 V was applied until the blue line (given by the bromophenol in Sample Loading Buffer) came out of the running gel. A protein size standard (*PageRuler Prestained Protein Ladder Plus*, Fermentas) was also loaded for molecular weight determination of the analyzed proteins.

2.5.6 Immunoblotting

2.5.6.1 Transfer of proteins on PVDF membrane

Proteins separated by SDS-PAGE were blotted on polyvinylidene fluoride (PVDF) membranes (*Immobilon–P*, Millipore) following the manufacturer's indications. The polyacrilamide gel and PVDF membrane were assembled as a sandwich in a *Mini Trans-Blot Electrophoretic Transfer Cell* (BioRad) according to the manufacturer's instructions. For proteins to be transferred, a tension of 100 was applied for 1 hour.

2.5.6.2 IMMUNODETECTION

After protein transfer, the PVDF membrane was incubated o.n. at 4 °C in blocking solution (5% w/v powder milk in 0.01% Tween-20, PBS) under stirring. After blocking, the membrane was first incubated with a primary antibody recognizing a specific epitope of the protein analysed (the hexahistidine tag of the recombinant proteins, GE Healthcare). This was followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma) that interacts with the murine Fc portion of the primary. For the detection of mAbs from hybridoma clones only the secondary anti-mouse IgG-HRP was used. Both antibodies were diluted in blocking solution and the membrane was rinsed twice for 5 minutes in 0.01% Tween-20, PBS and twice for 5 minutes in PBS after each incubation. Bands corresponding to the immunoselected polypeptides were finally detected by a chemiluminescent reaction using the *ECL Western Blotting Substrate* (Pierce), according to the manufacturer's instructions, and visualized by development of a photographic plate (*Hyperfilm MP High performance autoradiography film* - Amersham Biosciences).

2.5.7 PROTEIN QUANTIFICATION

2.5.7.1 SPECTROPHOTOMETRIC QUANTIFICATION

Absorbance at 280 nm (Lambda 35 UV/Vis Spectrometer, Perkin Elmer) provides the quantification of purified proteins (mAbs, scFv or ITs). As determined using the software *Vector NTI Advance 10,* 1 absorbance unit corresponds to:

mAb 1E82H11	0.77 mg/ml
scFv 1E8	0.46 mg/ml
1E8-PE	0.63 mg/ml
1E8-SAP	0.69 mg/ml
DIV1E8	0.46 mg/ml
DIV1E8-PE	0.58 mg/ml
DIV1E8-SAP	0.59 mg/ml
AT13/5-PE	0.71 mg/ml
AT13/5-SAP	0.81 mg/ml IT

2.5.7.2 COOMASSIE STAINING

A further means of protein quantification is provided by Coomassie staining after elctrophoresis of purified proteins. SDS-PAGE gels are stirred for 30 minutes at RT in Coomassie solution, followed by decoloration in Destaining solution until bands are clearly visible. Amounts of proteins in discrete bands are assessed by comparison with known quantities of bovine serum albumin (BSA, Sigma).

Coomassie solution	
Coomassie Brilliant Blue R250	0.25% w/v
Methanol	45% v/v
Acetic acid glacial	10% v/v
in deionized H_2O	

Destaining solution	
Methanol	45% v/v
Acetic acid glacial	10% v/v
in deionized H_2O	

2.6 ANALYSIS OF BINDING IN FLOW-CYTOMETRY

2.6.1 COMPARISON BETWEEN BINDING EFFICIENCIES OF HYBRIDOMAS

3T3/7A and Ramos cells were grown in T75 flasks to exponential phase and harvested by centrifugation at 150 x*g*, 5 minutes, 4°C in 15 ml tubes. After counting with a hemacytometer, cells were resuspended in a binding buffer (0.5% w/v BSA in PBS) and incubated 20 minutes on ice. Next, 2 x 10^5 cells were dispensed in each flow-cytometry tube and centrifuged as before. Cells were resuspended in serial dilutions of hybridoma supernatants and incubated on ice for 1 hour. Two washing steps follow, each with 2 ml of blocking buffer. For the detection of bound mAbs, cells were stained with an anti-mouse IgG-FITC antibody (goat polyclonal from Beckman Coulter, 1:100 diluted).

Incubation was carried out for 30 minutes on ice in 100 µl of binding buffer, followed by two washing steps with 2 ml binding buffer each. Background fluorescence was assessed by staining cells with anti-mouse-FITC.

At the end of the staining, cells in each tube were resuspended in 0.5 ml binding buffer and the mean fluorescence intensity (MFI) of each sample was determined using BD Facs Canto (BD Bioscience).

2.6.2 Competition assay for specific binding of new mAbs to CD38 on cells

2 x 10^5 Ramos cells were sedimented in single flow-cytometry tubes as described above (par. 2.6.1). Each tube was stained with 200 ng of biotinylated OKT10 mAb mixed with 100 µl of hybridoma supernatant containing an excess of IgG. After a 30 minutes incubation period on ice, two washes with 2 ml of binding buffer each were performed. A second staining with Streptavidin-FITC was used carried out for 30 minutes on ice, followed by two more washes, after which samples were resuspended in 0.5 ml binding buffer and analysed with BD Facs Canto (BD Bioscience).

The decrease of OKT10-biotin signal, evaluated by comparison with the maximal MFI obtained by incubation without a displacing mAb, was determined using BD Facs Canto (BD Bioscience).

2.6.3 Curves of binding to the CD38 antigen on cells

2 x 10^5 Daudi cells were sedimented in single flow-cytometry tubes as described above (par. 2.6.1). Cells were resuspended in 100 µl of binding buffer containing increasing amounts of mAb or scFv and incubated on ice for 1 hour. The staining with the recombinant ITs was performed with a fixed concentration of protein, without determining a curve. Two washing steps follow, each with 2 ml of blocking buffer. For the detection of bound scFvand ITs, cells were stained first with an anti-His_6 secondary antibody (mouse mAb from GEHealthcare, 1:200 diluted in binding buffer) and then with an anti-mouse IgG-FITC tertiary antibody (goat polyclonal from Beckman Coulter, 1:100 diluted). On the contrary, after staining with our anti-CD38 mAb or the commercial OKT10

mAb cells were directly incubated with anti-mouse IgG-FITC antibody. Incubations were for 30 minutes on ice, followed by two washing steps with 2 ml binding buffer each. Background fluorescence was assessed by staining cells with anti-mouse-FITC (for mAbs) or anti-His_6 followed by anti-mouse-FITC (for scFv and ITs). At the end of the staining, cells in each tube were resuspended in 0.5 ml binding buffer and MFI of each sample was determined as described above.

2.7 BIOLOGICAL ASSAYS

2.7.1 CYTOTOXICITY ASSESSMENT BY LEUCINE INCORPORATION

The effect on uptake of ^{14}C-leucine into cells was taken as a measure of the protein synthesis inhibition caused by treatment with a toxin or immunotoxins. Cells were resuspended in leucine-free RPMI-1640 medium supplemented with 2% FCS and $3x10^4$ cells in a volume of 90 µl were seeded in each well of round-bottom 96-well plates (Greiner bio-one). The molecules to be tested were dialysed in PBS, filter-sterilized through Spin-X tubes (Costar) and diluted in the same medium used for cell resuspension. Ten µl of differently diluted ITs were finally added in each well. The plate was incubated for 48 hours at 37 °C, 5% CO_2. Ten µl of ^{14}C-leucine from a 1-1.2x10^5 cpm/ml stock in leucine-free RPMI-1640 were added 16 hours before the end time, after which the content of each well was transferred to filter paper using a cell-harvester (Wesbart), and radioactivity was measured using a beta counter (Wallac 1409, Pharmacia).

Results obtained for experimental cultures are expressed as a percentage of the amount of ^{14}C-leucine incorporation observed in untreated control cultures maintained under identical conditions.

2.7.2 CELL PROLIFERATION ASSAY WITH XTT

The cytotoxic activity of the ITs was evaluated using a colorimetric cell proliferation assay based on the conversion of XTT (sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt) to orange-colored formazan compounds by cellular dehydrogenases. Thirty thousand cells, seeded in 96-well plate

and maintained in phenol red-free RPMI-1640 medium were treated with serial dilutions of IT for 72 hours. After this incubation period, 50 µl of 1 mg/ml XTT solution were added to each well and incubated for 3 hours. The absorbance was read at 490 and the 690 nm using a plate reader.

2.7.3 Apoptosis assay

CD38+ and CD38- cells were seeded in 24-well plates at a final concentration of $3x10^5$ and treated with 1 µg/ml of the different ITs, while B lymphocytes were seeded in 48-well plates at a final concentration of 2 x 10^6 cells. Cells were incubated for 48 hours at 37 °C, 5% CO_2, after which cells were harvested an sedimented in single flow-cytometry tubes and centrifuged at 150 x*g*, 5 minutes, 4°C. After wasting the supernatants, cells were stained with Annexin V-FITC/propidium iodide (PI) following the manufacturer's instructions for an incubation period of 15 minutes at RT. Cells were then washed with 2 ml of PBS and resuspended in a 0.5 final volume for further flow-cytometric analysis.

3. RESULTS

3.1 CHARACTERIZATION OF NEW ANTI-CD38 HYBRIDOMA CLONES

A standard procedure of mice immunization and hybridoma fusion was exploited for the generation of novel anti-CD38 clones. Briefly, Balb/c mice were first boosted with intraperitoneal (i.p.) injection of a membrane preparation from 3T3 cells expressing the human CD38 antigen (kindly provided by Prof. Ippoliti R. and co-workers, Università dell'Aquila). I.p. injection was repeated after seven days and three, four, five days later bacterially expressed CD38 ectodomain (from Prof. Ippoliti R. and co-workers) was intravenously (i.v.) injected. The sera from all the immunized animals were then tested and compared by immunoblotting and ELISA on the bacterially expressed CD38 (data not shown). The mouse showing the best anti-CD38 reactivity was used to carry out PEG-mediated fusion of spleen lymphocytes and the myeloma line Ag8. Fusions were dispensed in 48-well plates and the CD38-reactivity of supernatants was assayed by flow-cytometry, allowing to select positive wells for subsequent limiting-dilution cloning in 96-well plates. Different dilutions of supernatants from monoclonal wells were likewise tested by flow-cytometry on CD38+ 3T3 cells to assess the ability of the different clones to bind the target antigen. As shown in figure 3.1, four of the investigated clones, underlined with red color, were demonstrated not to bind CD38, while all the other ten clones showed a positive staining of CD38+ 3T3 cells. In particular it can be observed that two clones, highlighted with green color, exhibited high binding affinity. However, this first type of investigation did not take into account the quantification of the IgG contained into the supernatants of the different clones. Moreover, some clones may not secrete functional IgG and this may account for the apparent absence of binding.

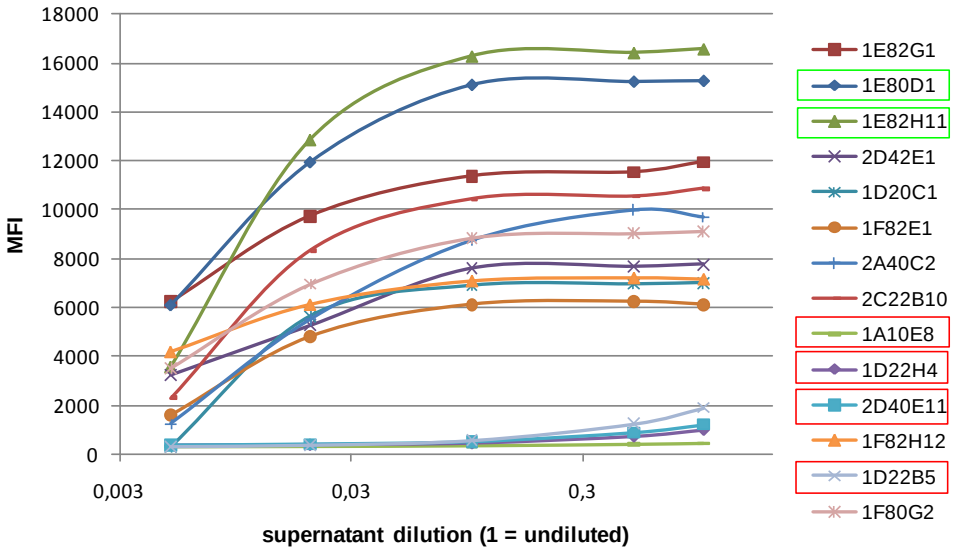


Figure 3.1 Flow-cytometric comparison between the binding efficiencies of different hybridoma clones on 3T3/7A cells (CD38+) stained with serial dilution of culture supernatants. Red rectangles highlight negative hybridoma clones, while green rectangles underline the clones showing the best binding properties.

To verify the effective secretion of IgG by hybridoma clones and to quantify their amount in the supernatants previously tested by flow-cytometry, an immunoblotting assay was performed. One and ten µl of each hybridoma supernatant were loaded onto a polyacrylamide gel and the electrophoresis was performed under reducing conditions; 250 ng of purified 4KB128 anti-CD22 mAb were loaded as positive control and as comparison parameter for the quantification. Secreted IgG were detected by staining with anti-mouse IgG-HRP, which in this case allowed to visualize the mAbs heavy chains at the molecular weight of 55 kDa and the light chains at 28 kDa.

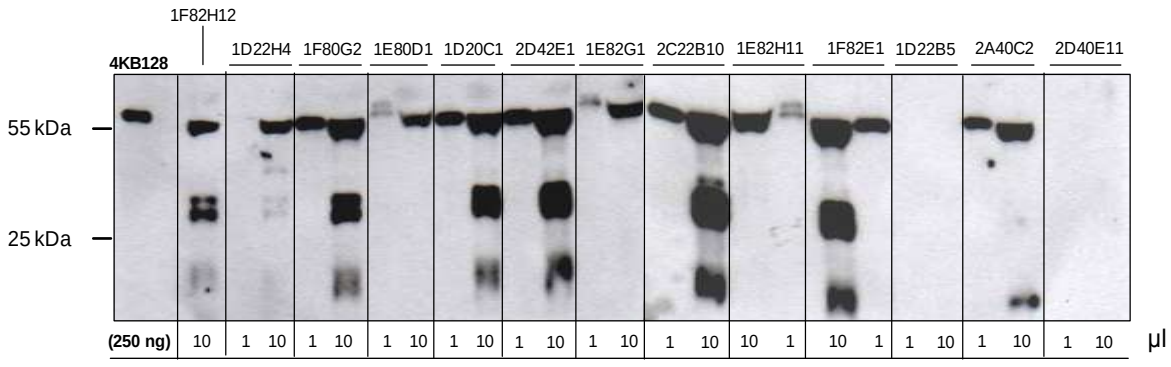


Figure 3.2 Immunoblotting analysis of supernatants from different anti-CD38 hybridoma clones. One and ten µl of cell culture were loaded and separated by SDS-PAGE under reducing conditions and then blotted on PVDF membrane that was hybridized with anti-mouse IgG-HRP (Sigma).

The immunoblotting confirmed our first hypothesis about the possibility to have isolated non-secreting hybridomas: in fact, two of the supernatants which had not shown a positive staining of the cells, did not contain a detectable amount of antibodies (Fig. 3.2), while the clone 1D22H4 probably secreted a mAb with low affinity for CD38 antigen.

Moreover, the quantification obtained by immunoblotting assay allowed us to correlate the antibody concentrations in the supernatants with the affinity for the molecular target, showing that the hybridoma supernatants of clones 1E82H11, 1E80D1, and 1E82G1, which showed the best binding curves on CD38+ 3T3 cells, were the same which showed a lower antibody concentration, therefore they could be considered to contain antibodies with a higher binding affinity.

Eight of the best performing supernatants were finally tested on Ramos, a B-cell line of Burkitt's lymphoma expressing high levels of CD38. The data shown in figure 3.3 confirmed the best binding properties of the mAbs secreted by clones 1E82H11 and 1E82G1.

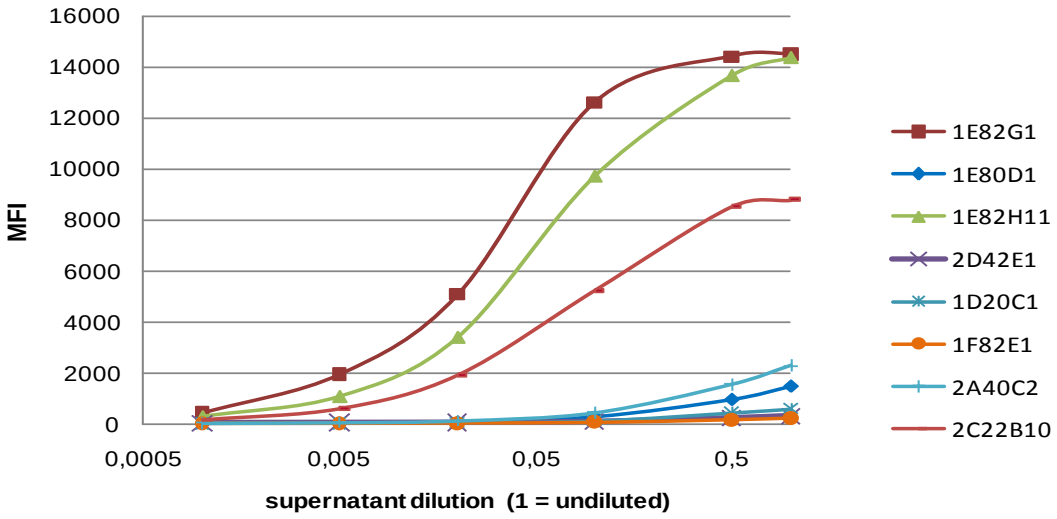


Figure 3.3 Staining of Ramos with supernatants dilutions of the best performing anti-CD38 clones.

Finally we wanted to compare the specific binding of our hybridoma supernatants with that of the well-characterized and commercially available mAb OKT10 which has been used in many studies on CD38 functions and internalization properties and has been also conjugated to toxic molecules to verify its therapeutic potential [70]. A competition assay was performed to assess the ability of the mAbs of the selected hybridomas to displace

OKT10 mAb. As shown in figure 3.4, only 1E82G1, 1E82H11 and 2C22B10, were able to displace OKT10 mAb chemically conjugated to biotin and identified in flow-cytometry by streptavidin-FITC staining; in fact the three clones presented a flow-cytometric pattern similar to that observable for OKT10 in figure 3.4a. This data suggests that the mAbs described above and OKT10 recognize the same epitope on the CD38 molecule.

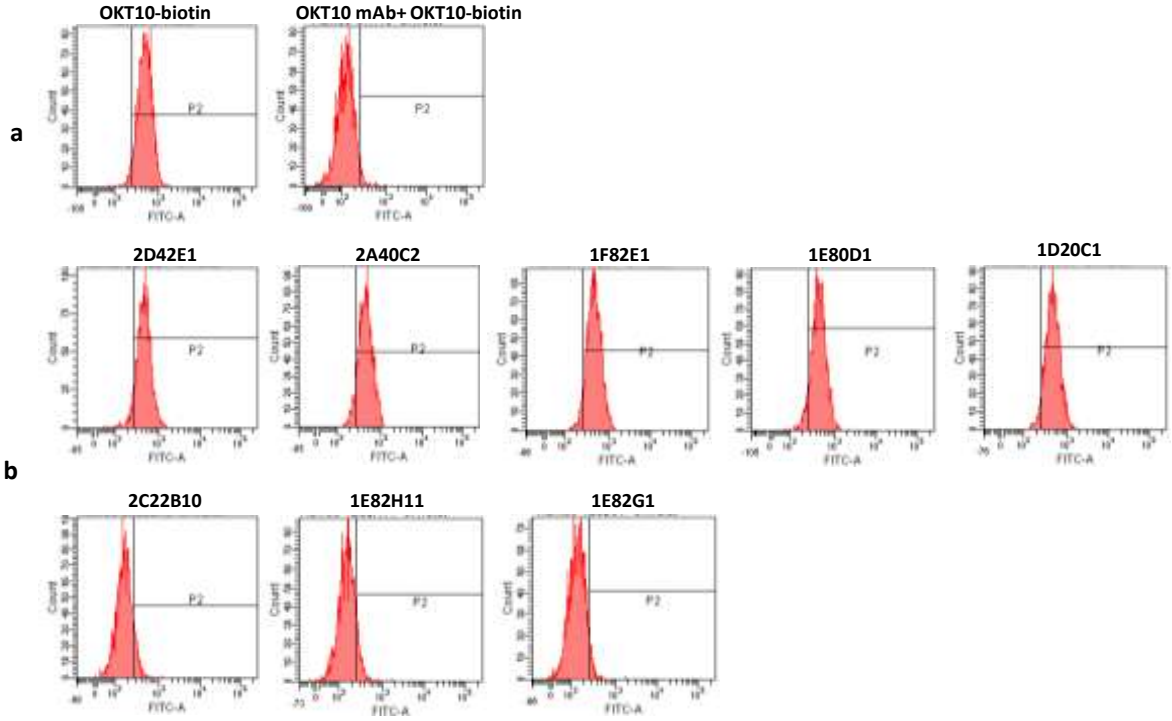


Figure 3.4 Competitive binding assay on Ramos cells. A fixed concentration (200 ng) of OKT10-biotin mAb, pre-mixed with 2 µg of non-biotinylated OKT10 mAb **(a)** or alternatively with 100 µl of supernatants from anti-CD38 hybridomas **(b)**, was used to stain Ramos cells. Binding of OKT10-biotin was detected by staining with streptavidin-FITC and subsequent flow-cytometric analysis. A positive staining means that OKT10-biotin has not been displaced by the the IgGs present in the supernatant of hybridomas, while a negative staining denote competition for the same binding site.

Summarizing the data obtained by the characterization of the supernatants from the hybridoma clones that we have developed, we finally chose 1E82H11 as the best performing in terms of binding properties and competition with OKT10.

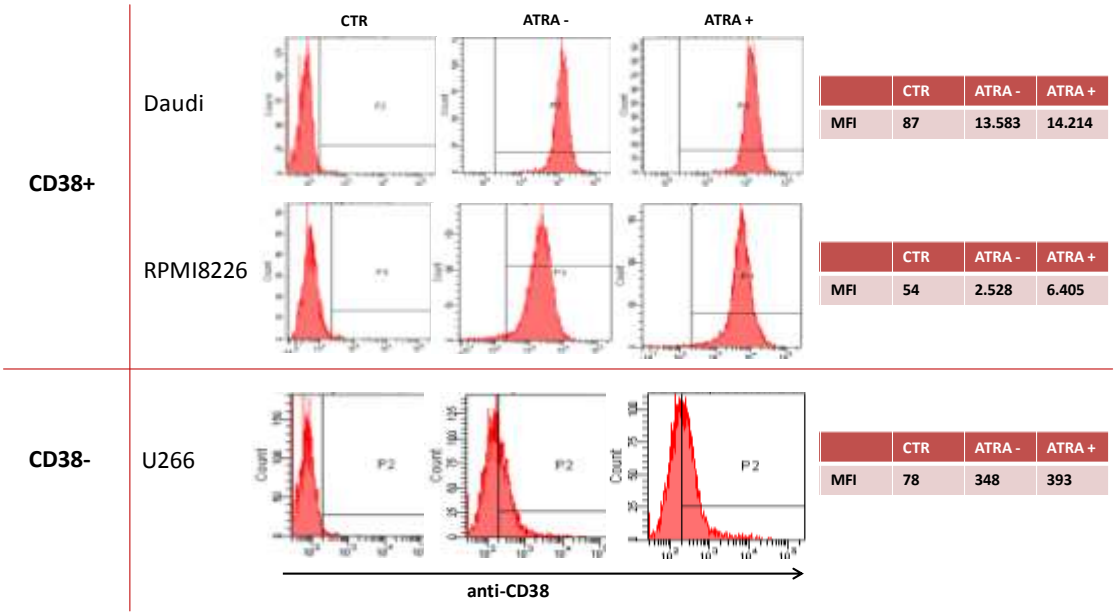
3.2 CHARACTERIZATION OF THE MONOCLONAL ANTIBODY 1E82H11

Hybridoma cells of the selected clone 1E82H11 were grown in a bioreactor (Sartorius) and the supernatant enriched of IgG was collected weekly. The mAbs were purified from the supernatants by affinity chromatography exploiting the binding of the Fc region of the IgGs to the G protein immobilized on a Sepharose matrix and allowing to collect 1 mg of purified antibody for each ml of culture supernatant.

Firstly the binding and specificity properties of the purified anti-CD38 mAb 1E82H11 to the native cellular antigen were verified by flow-cytometry on CD38+ cell lines (the lymphoid cell line Daudi and the multiple myeloma line RPMI8226) and the CD38- cell line of myeloid origin U266. As shown in figure 3.5, when stained with 5 µg of the 1E82H11 mAb, Daudi and RPMI8226 caused an evident rightward shift in the MFI (Mean Fluorescence Intensity) peak, as compared to the negative control, unlike U266 that do not express the antigen and therefore showed a negligible MFI shift. The MFI values suggest a high binding affinity and moreover a suitable specificity of 1E82H11 mAb for the target antigen since no binding could be observed on CD38- cells.

We observed a higher CD38 expression level on Daudi than RPMI8226, but also an increased expression when myeloma cells were exposed for 16 hours to 10 nM all-*trans* retinoic acid (ATRA), a conventional drug used in the therapy of Acute Promyelocytic Leukemia (PML) and described as a potent and selective inducer of CD38 expression in myeloid leukemia cells (see paragraph 1.3.1.2.2). On the contrary ATRA did not show any significant effect on CD38 expression of U266 cells; in fact, no increase in MFI value was observed with respect of cells not treated with ATRA. (Fig. 3.5).

Due to the higher levels of target antigen expressed, Daudi was selected as the line of choice for further experiments of characterization of the mAb and the other anti-CD38 constructs.



	CTR	ATRA -	ATRA +
MFI	87	13.583	14.214

	CTR	ATRA -	ATRA +
MFI	54	2.528	6.405

	CTR	ATRA -	ATRA +
MFI	78	348	393

Figure 3.5 Specificity of 1E82H11 mAb for CD38 antigen. The binding activity of 1E82H11 was analyzed by flow-cytometry using CD38 positive Daudi and RPMI8226 cells, and CD38 negative U266 cells (with or without pre-treatment with ATRA) incubated with 5 µg of mAb. The bound antibodies were detected by staining with anti-mouse IgG-FITC.

We plotted the data gathered by staining Daudi with increasing concentrations of the mAb obtaining a sigmoid curve which reached the plateau after incubation of the cells with over 10^{-8} M mAb (Fig. 3.6). In the same graph the binding affinity of the new mAb 1E82H11 was compared with that of OKT10 mAb, which was used as a positive control for our characterization tests.

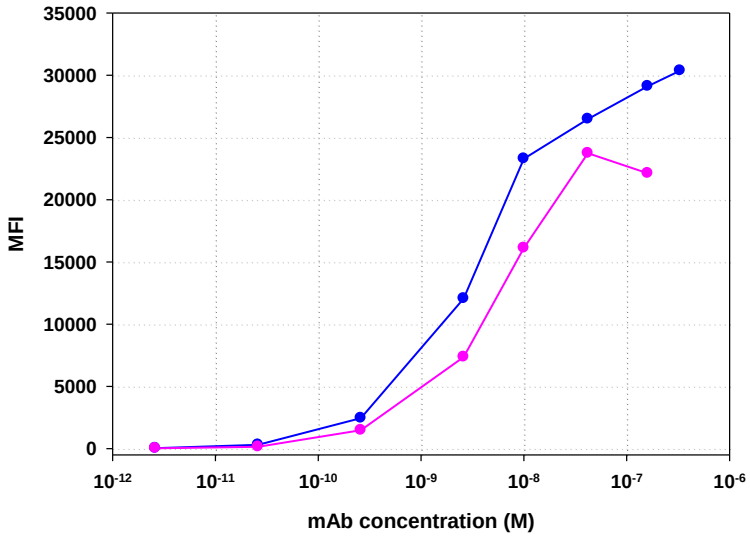


Figure 3.6 Binding curves of mAbs 1E82H11 and OKT10 on Daudi cells. Serial dilutions of the new 1E82H11 mAb (blue circles ●) and the commercially available OKT10 mAb (pink circles ●) were used to stain Daudi cells obtaining two almost overlapping sigmoid curves.

3.3 CLONING, EXPRESSION AND CHARACTERIZATION OF THE 1E8 SCFV AND DERIVED ITS

3.3.1 AMPLIFICATION OF THE V_H AND V_L DOMAINS OF THE ANTI-CD38 MAB

mRNA was extracted from the hybridoma cells of the clone 1E82H11 and retrotranscribed to cDNA as described in paragraph 2.3. Before proceeding with the amplification of the fragments of interest, the quality and integrity of the cDNA were assessed through a PCR test with a couple of primers designated for the amplification of murine β-actine (data not shown). The cDNA was then used as a template for the PCR screening of an array of mouse-specific primers designed to pair the flanking regions of V_H and V_L genes from a variety of IgG subfamilies. Twentyfive PCR reactions were first prepared to determine the best forward primer for V_L and V_H, respectively; next, each of the selected forward primers was tested with single reverse primers (Fig. 3.7).

As shown in figure 3.7a, all the forward primers for the amplification of the V_H provided one identical band which might represent a non-specific fragment since the molecular weight was too low to embody the sequence of an IgG variable region. Nevertheless, in lanes 14, 15 and 20 a second band of about 350 bp was present which might represent the V_H gene; thus, two of the forward primers corresponding to the most abundant amplificates were picked for further analysis. Subsequently, the PCR screening of the best forward primer for the amplification of V_L (Figure 3.7c) showed the presence of many DNA fragments of different size, but we could practically divide the most similar in size to the V_L gene into two groups and the forward primer corresponding to one representative of each group was chosen for the reverse primer selection. To identify the most convenient reverse primers for the cloning of both variable domains it was therefore necessary to prepare eight reactions for each of the variable domain (Fig. 3.7b and d).

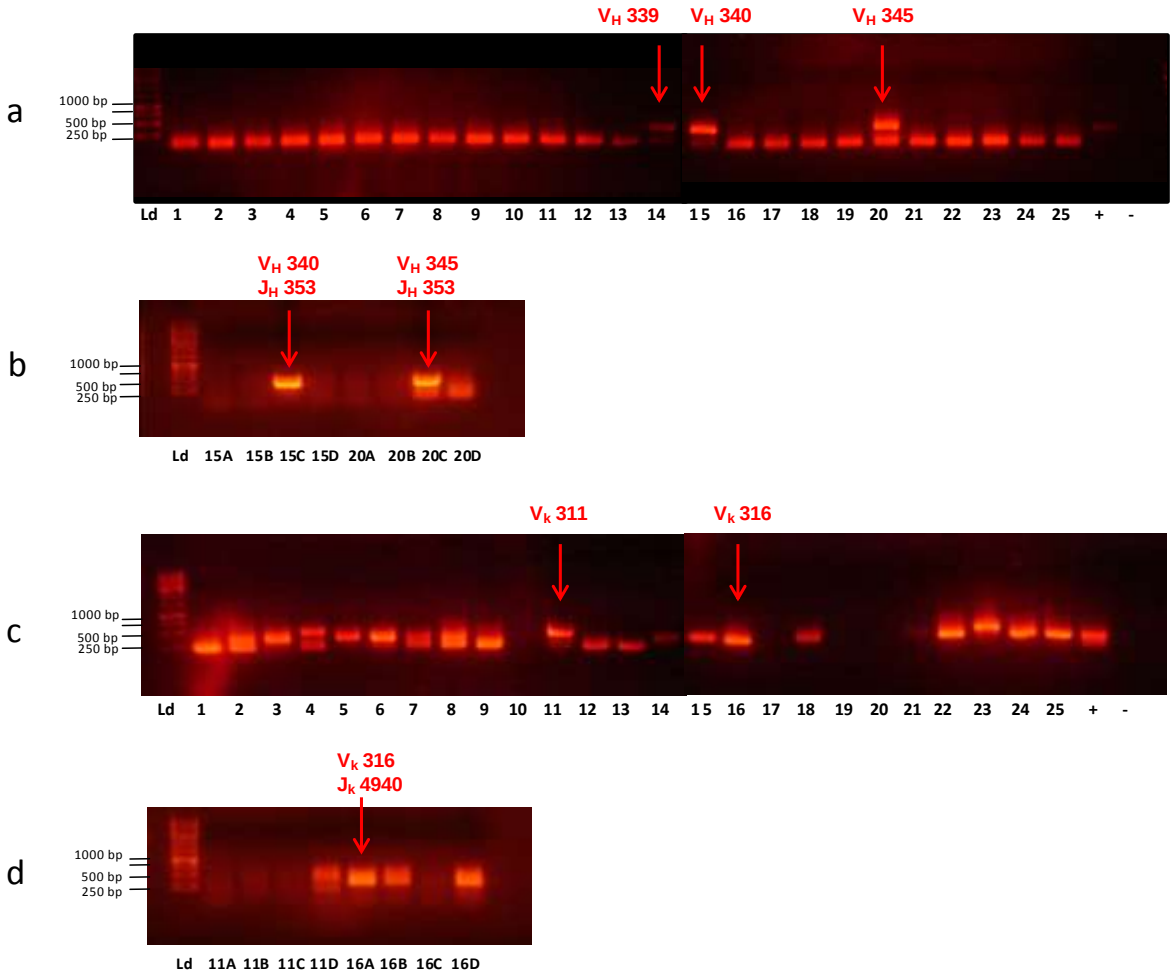


Figure 3.7 Screening of primers for the amplification of DNA sequences coding for the variable domains of the anti-CD38 mAb. PCR amplificates were analysed by agarose gel electrophoresis. **(a)** V_H amplification tested with different forward primers (lanes 1-25). **(b)** V_H amplification using two of the best forward primers with each of four reverse primers (lanes 15A-D and 20A-D). Forward and reverse primers for V_k were analogously selected as shown in **(c)** and **(d)**. The positive control (+) was a PCR reaction in which the template is a cDNA from a different hybridoma for which primer pairs had already been determined; negative control (-) was a PCR reaction with no template and the same primer pair as in the positive control. Arrows indicate amplificates from primers selected for further characterization. Ld = DNA Ladder.

The PCR product generated by the putative primer pairs for the isolation of the V_L gene (V_k 316/J_k 4940) and the two possible amplificates of V_H (obtained using V_H 340/J_H 353 or V_H 345/J_H 353 primer pairs) were sequenced and compared with sequences present in the Genbank database. This analysis showed that the alleged V_L PCR fragment shared major similarities with sequences from the Genbank database that code for murine antibody light chain variable domains. Similarly, the amplificates derived from the V_H 340/J_H 353 and V_H 345/J_H 353 primers pair, in addition to sharing the same nucleotidic sequence, proved to

have a highly superimposable sequence to those of previously characterized variable domains from murine antibodies heavy chains, so that both primer pairs were suitable for the V_H cloning.

3.3.2 EXPRESSION, PURIFICATION AND CHARACTERIZATION OF THE ANTIBODY FRAGMENT 1E8

The variable domains nucleotidic sequences of the anti-CD38 mAb were ligated, with a $(Gly_4S)_3$ linker between the V_H and the V_L, into the plasmid vector pET20b(+) which allowed to obtain the expression of the scFv with a six-hystidine tag at the C-terminus of the protein (Fig. 3.8a). The resulting construct, named 1E8 scFv, was transformed into the BL21(λDE3) pLysS *E. coli* strain, which is particularly suited for the heterologous expression of proteins, given the deletion of genes coding for some proteolytic enzymes that are often responsible for the degradation of the induced protein during the extraction and purification steps. Addition of IPTG in the culture medium triggers the overexpression of T7 RNA polymerase, which in turn superinduces the transcription of the scFv gene being placed under the control of the T7 promoter in pET20b(+).

Only 5 colonies of the transformed bacterial host grew in the selective medium and they were screened, following a small-scale induction, to assess their ability to produce the recombinant antibody fragment. The immunoblotting analysis of untreated bacterial pellets from the five clones (Fig. 3.8b) showed in all induced samples (excepting clone 3) a protein of 30 kDa, the size expected for our single-chain antibody. Clone 5 apparently gave the highest yield of induced scFv, so it was selected for a further large-scale expression.

Inclusion bodies from one liter of an induced culture of BL21(λDE3) pLysS expressing the construct for the His_6-tagged scFv (Fig. 3.8a) were solubilized in urea-containing buffer and the purification by affinity chromatography was performed under denaturing conditions, as described in Materials and Methods (paragraph 2.5.2), allowing the denatured and linearized proteins to bind the nickel ions anchored to the Sepharose matrix. Even though most of the proteins did not bind efficiently to the resin, as it is witnessed by the presence of scFv in the column flow-through and wash (Fig. 3.8c, lane 5, 6 and 7), the amount of the eluted protein, quantified in 4 mg/l, could be considered sufficient for our purposes.

Proteins were subsequently renatured by multi-step dialysis in a "Refolding buffer", however, this strategy did not allow the full recovery of the denatured proteins, which were largely lost as a precipitate due to the high hydrophobic composition of the scFv and to the incorrect refolding as the denaturing agent was gradually removed. As apparent from the gel in Fig. 3.8c, about 80% of the protein extracted from inclusion bodies and purified by IMAC exploiting the affinity of six-hystidine tag for nickel ions was lost during the refolding process (compare lanes 8 and 9).

Although a higher efficiency of renaturation would be highly desirable, this would require the methodical study and setting up of a denaturation/refolding approach promoting the oxidation of the expressed protein, while avoiding unwanted aggregation. For the purpose of preliminary, lab-scale characterization, the devised strategy was deemed appropriate, also considering the final scFv yield.

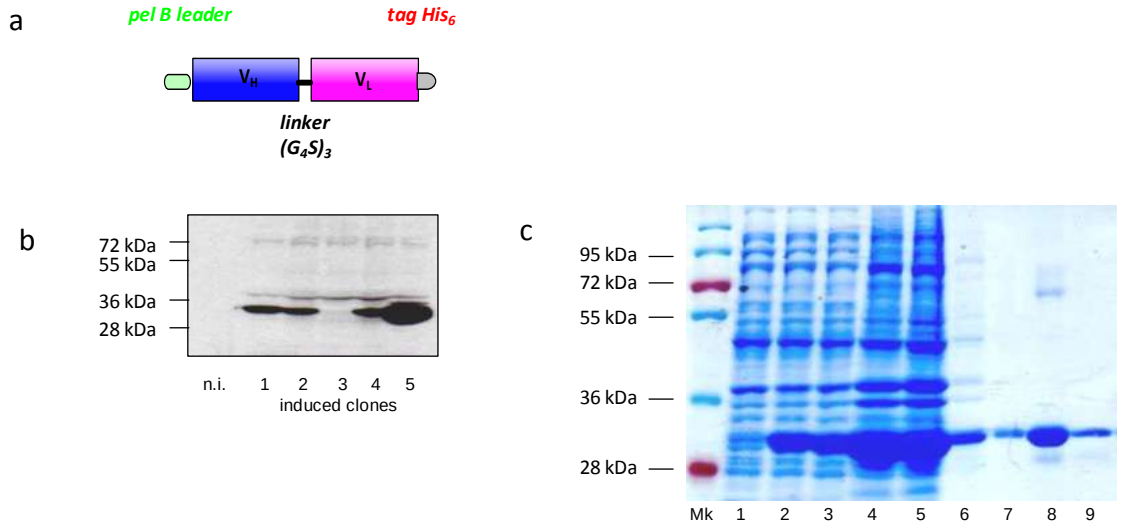


Figure 3.8 Expression and purification of 1E8 scFv. (a) Construct used for the expression of the anti-CD38 scFv. **(b)** Immunoblotting on cells from five independent BL21(λDE3) pLysS *E. coli* clones transformed with the scFv construct represented in (a). Each sample corresponds to bacterial pellets from 50 µl of culture. n.i. = sample before induction; induced clones are sample taken after o.n. induction with 1 mM IPTG at 30 °C. Samples were separated by SDS-PAGE under reducing conditions and then blotted on PVDF membrane that was hybridized with mouse anti-His_6 antibody (GE Healthcare). **(c)** SDS-PAGE and Coomassie staining of samples taken at different steps of the extraction and purification of anti-CD38 scFv from inclusion bodies. Abbreviations: Mk, protein size standards. Lanes: 1, bacterial pellet from 50 µl of non-induced culture; 2 and 3, bacterial pellet from 50 µl of induced culture; 4, 20 µl of urea-denatured inclusion bodies from one liter of induced bacterial culture; 5, flow-through sample after column loading (20 µl from 50 ml loaded); 6 and 7, 20 µl samples from 10 ml washes with 20 mM imidazole concentration; 8, eluted protein (10 µl); 9, protein after refolding (10 µl).

A preliminary flow-cytometric experiment was performed to estimate the binding properties of the scFv on Daudi cells (Fig. 3.9). Although 1E8 scFv did not preserve the binding affinity of the parental mAb, as it is demonstrated by the much lower MFI as compared with that of 1E82H11 mAb in figure 3.6, it was however able to bind the target antigen expressed by the cells and this represents a promising results with the perspective of creating the derived ITs.

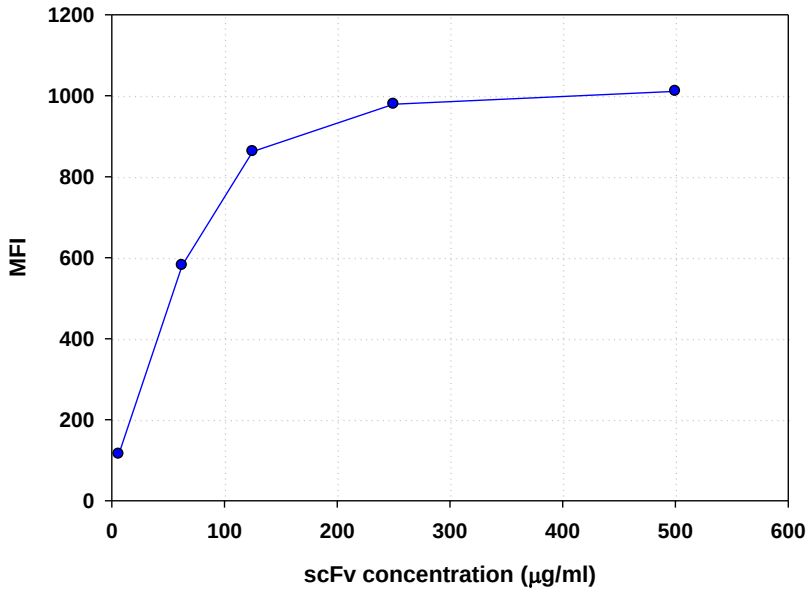


Figure 3.9 Curve of scFv binding to CD38-positive Daudi cells. The MFI is plotted against each scFv concentration tested.

3.3.3 EXPRESSION, PURIFICATION AND CHARACTERIZATION OF THE 1E8-DERIVED ITS

The nucleotidic sequence coding for the PE40 truncated version of *Pseudomonas* exotoxin A or alternatively for the plant toxin saporin was fused to the 3'-end of the scFv, generating two chimeric immunotoxins in the pET20b(+) vector which were called respectively 1E8-PE and 1E8-SAP (Fig. 3.10a). The C-terminal His_6 tag was exploited for purification and analytical purposes.

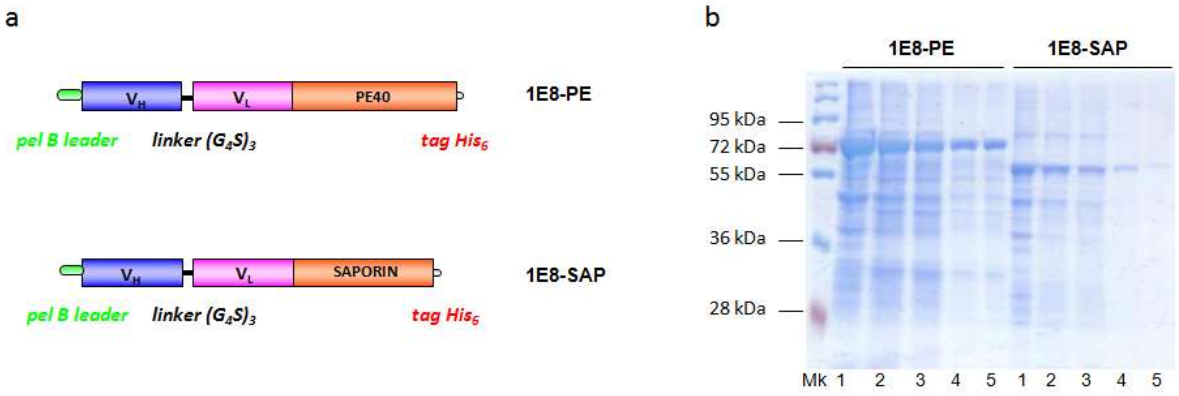


Figure 3.10 (a) The construct for expression of the scFv was modified by insertion of the coding sequences of PE40 or saporin. **(b)** Analysis by SDS-PAGE and Coomassie staining of samples taken at different steps of the purification/refolding procedure. Abbreviations: Mk, protein size standards. Lanes: 1, 20 µl of urea-denatured inclusion bodies from one liter of induced bacterial culture; 2, flow-through sample after column loading (20 µl from 50 ml loaded); 3, 20 µl samples from 10 ml washes with 20 mM imidazole concentration; 4, eluted protein (10 µl); 5, protein after refolding (10 µl).

A large-scale culture of BL21(λDE3) pLysS *E. coli* transformed with the vector containing the ITs constructs was induced and processed for extraction of inclusion bodies and purification of the proteins of interest, thus yielding a PE-derived IT of approximately 70 kDa and a saporin-derived protein of 60 kDa, as visualized by Coomassie staining (Fig. 3.10b), consistent with the expected size for a fusion between the scFv (30 kDa) and PE40 (40 kDa) or saporin (30 kDa).

The first consideration we could make observing the SDS-PAGE in figure 3.10b is the sensibly lower level of synthesis of the recombinant IT as compared to the scFv. This result is consistent with previously reported cases of heterologous proteins with a similar high molecular weight and that include toxic portions into their sequence. Moreover, the amount of expressed protein is higher in the case of 1E8-PE with respect to 1E8-SAP (lanes 1).

We could also observe that purification by IMAC technology caused a minor loss of IT in the flow-through with respect to the scFv alone; however, the amount recovered in the eluted fractions was not higher, especially for 1E8-SAP (lanes 4). Moreover, a negligible loss of the IT due to protein aggregation and precipitation during the renaturation steps of 1E8-PE was observed, and approximately 80% of the recombinant protein purified by IMAC could be recovered in soluble form after refolding of the eluted fractions (compare lanes 4 and 5 in Fig. 3.10b). On the contrary, high tendency to form insoluble aggregates

during the refolding process was observed for 1E8-SAP and more than 60% was lost before completing the renaturation.

The purified 1E8-PE immunotoxin (after dialysis of the eluted protein and filter-sterilization) had a concentration of 0.1 mg/ml, as assessed by comparison with BSA and confirmed by spectrophotometric quantification, amounting to 1.6 mg of recombinant protein from one liter of *E. coli* culture. Under the same conditions 1E8-SAP final yield was about 0.5 mg from one liter of culture, with a concentration of 0.02 mg/ml.

While higher amounts and yields (often requiring the implementation of fermentation facilities) are usually necessary to conduct *in vivo* experiments, our strategy of expression and purification is apt to provide sufficient material for a preliminary *in vitro* study of the ITs functional properties.

Unlike the scFv, both 1E8-derived ITs did not show an appreciable binding affinity to CD38 molecule expressed by Daudi cells (Fig. 3.11); only 1E8-PE IT showed a modest rightward shift in its MFI at the tested concentration. We can hypothesize that the binding properties described for the scFv could be at least partially hidden by a possible steric interference of both the toxic domains with the binding portion.

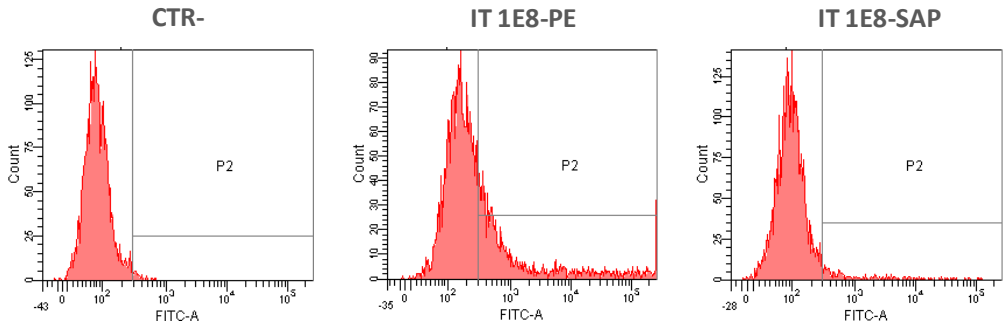


Figure 3.11 Flow-cytometric analysis of 1E8-PE and 1E8-SAP immunotoxins binding on Daudi cells. The boxes show the fluorescence profiles obtained by staining Daudi with 5 µg of each anti-CD38 recombinant immunotoxin followed by secondary staining with mouse anti-His_6 antibody and finally with anti-mouse IgG-FITC. Data are compared with the negative control (CTR-) obtained by staining cells with the secondary and tertiary antibodies alone. FITC-A, Fluorescein Isothiocyanate-Area.

Nevertheless, we tried to investigate the potential cytotoxic effects of these ITs, testing their ability to inhibit protein synthesis and comparing their properties with that of OKT10:SAP, an immunoconjugate molecule with a known and verified toxic potential [70], which was kindly provided by D. J. Flavell. We checked the effect of increasing

concentrations of ITs on the incorporation of ^{14}C radiolabeled leucine (^{14}C-Leu) by target cells observing no changes in leucine incorporation at the concentrations used (Fig. 3.12) and no detectable concentrations inhibiting 50% of protein synthesis (IC_{50}).

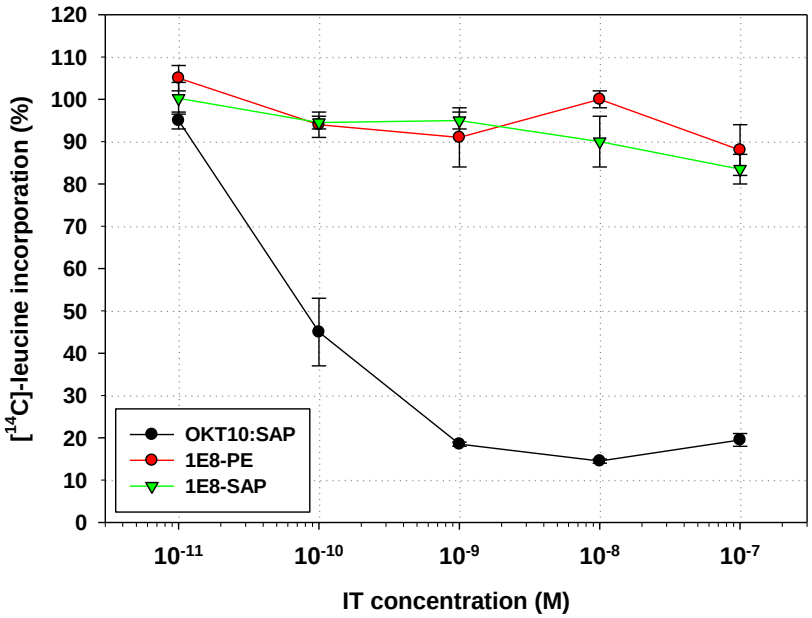


Figure 3.12 Cytotoxicity assay with 1E8-PE and 1E8-SAP immunotoxins on Daudi cells.
Cells were exposed for 72 h to increasing amounts of the recombinant ITs 1E8-PE and 1E8-SAP and inhibition of protein synthesis was measured by incorporation of ^{14}C-Leucine. OKT10:SAP immunoconjugate was used as a positive control. Data are expressed as percentage of control sample (untreated cells). Each value is the average of two independent experiments.

These negative results encouraged us to proceed our work by trying to restore the double valence of the mAb creating a divalent antibody fragment and its relative ITs.

3.3.4 EXPRESSION, PURIFICATION AND CHARACTERIZATION OF THE DIVALENT 1E8 ANTIBODY FRAGMENT AND THE DERIVED ITS

The divalent antibody fragment (DIV1E8) was obtained by cloning two scFv with a $(Gly_4S)_3$ linker between them into the expression vector pET20b(+) (Fig. 3.13a). This strategy was used to increase the binding affinity to the cell target creating a double binding site and also to promote the dimerization of the receptor and the subsequent internalization of the toxic molecule bound. Subsequently, the nucleotidic sequence coding for PE40 or alternatively for saporin was fused to the 3'-end of the divalent scFv as described in paragraph 2.3.5.1 of Material and Methods, generating two chimeric

immunotoxins in the pET20b(+) vector which were called respectively DIV1E8-PE and DIV1E8-SAP (Fig. 3.13a).

The expression and purification methods described for the scFv were applied to obtain the divalent antibody fragment alone (60 kDa) and the ITs DIV1E8-PE (100 kDa) and DIV1E8-SAP (90 kDa), but we observed, especially for the divalent ITs (Fig. 3.13c, lane 1), that the levels of synthesis of these constructs were sensibly lower than that of the scFv; however, almost all the purified protein could be recovered after the renaturation process (Fig. 3.13c lanes 4 and 5) as no aggregation and precipitation phenomena could be observed. In any case, the final yield of the two ITs was low (about 500 µg for one liter of bacterial culture) and the concentration was sufficient only to perform the cytotoxicity assays.

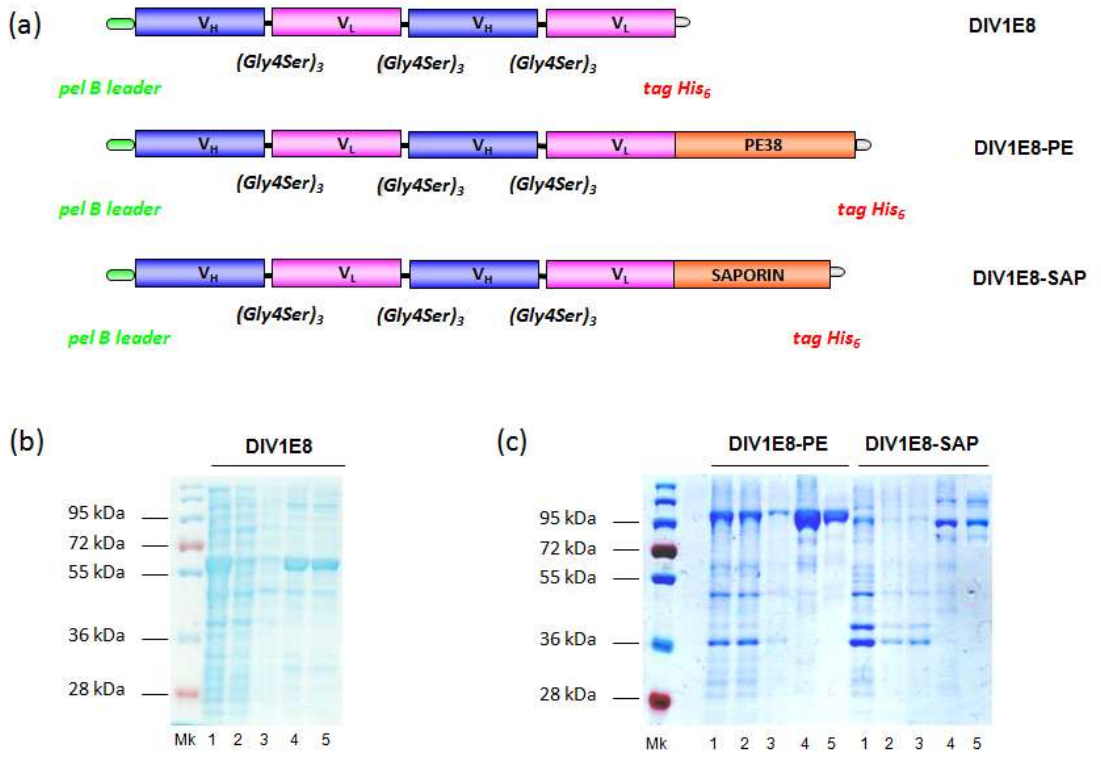


Figure 3.13 (a) The construct for expression of the scFv was modified by insertion of the sequence of a second identical scFv and subsequently the coding sequences of PE40 or saporin. **(b)** and **(c)** Analysis by SDS-PAGE and Coomassie staining of samples taken at different steps of the purification/refolding procedure. Abbreviations: Mk, protein size standards. Lanes: 1, 20 µl of urea-denatured inclusion bodies from one liter of induced bacterial culture; 2, flow-through samples after each column loading (20 µl from 50 ml loaded); 3, 20 µl samples from 10 ml washes with 20 mM imidazole concentration; 4, eluted protein (10 µl); 5, protein after refolding (10 µl).

All the divalent constructs were tested for their binding activity by a flow-cytometric assay. The MFI value of the DIV1E8 was approximately two-fold greater than that obtained with the scFv at the same concentration (Fig. 3.14), but the binding affinity was not preserved by the respective ITs, confirming our hypothesis about the alleged steric hindrance of the toxic domain probably preventing the binding of the divalent antibody fragment.

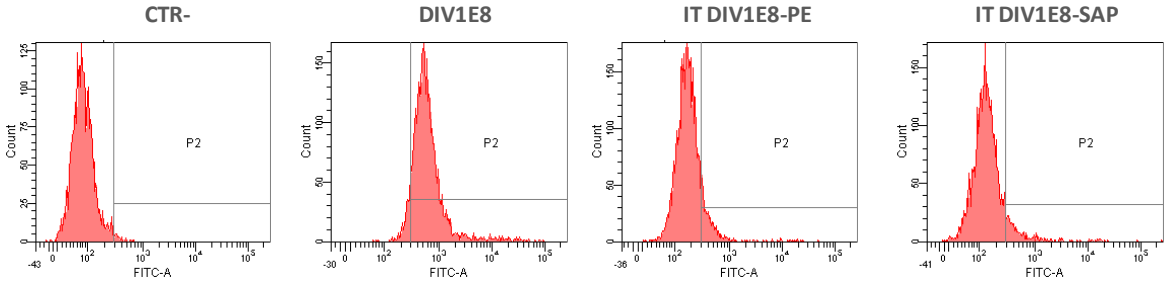


Figure 3.14 Flow-cytometric analysis of DIV1E8 antibody fragment and DIV1E8-PE and DIV1E8-SAP immunotoxins binding on Daudi cells. The boxes show the fluorescence profiles obtained by staining Daudi with 5 µg of each anti-CD38 divalent molecule followed by secondary staining with mouse anti-His_6 antibody and finally with anti-mouse IgG-FITC. Data are compared with the negative control (CTR-) obtained by staining cells with the secondary and tertiary antibodies alone. FITC-A, Fluorescein Isothiocyanate-Area.

We then performed a cytotoxicity assay by using the divalent ITs. Their cytotoxic activity was compared with that of OKT10:SAP immunoconjugate. In figure 3.15 is reported that both the recombinant ITs have an effect on protein synthesis. Within the range of concentrations used, only the divalent immunotoxin using saporin as a toxic portion was capable to inhibit the protein synthesis with an IC_{50} of $4.5x10^{-10}$ M, while in the case of the immunoconjugate OKT10:SAP the IC_{50} was $8.1x10^{-11}$ M. On the contrary, the DIV1E8-PE immunotoxin did not show a detectable IC_{50}, although it showed a tendency to result in greater protein synthesis inhibition with increasing concentrations.

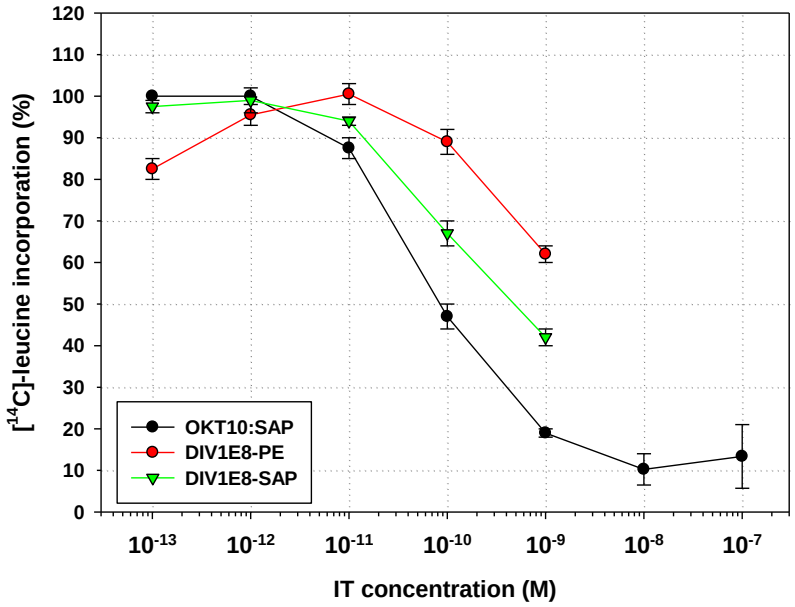


Figure 3.15 Cytotoxicity assay with DIV1E8-PE and DIV1E8-SAP immunotoxins on Daudi cells.
Cells were exposed for 72 h to increasing amounts of the recombinant ITs DIV1E8-PE and DIV1E8-SAP and inhibition of protein synthesis was measured by incorporation of ^{14}C-Leucine. OKT10:SAP immunoconjugate was used as a positive control. Data are expressed as percentage of control sample (untreated cells). Each value is the average of two independent experiments

3.4 AT13/5-DERIVED CONSTRUCTS

In our quest for functional recombinant ITs, and considering the deceiving results obtained with 1E8 mAb, we resorted to investigating the properties of a different anti-CD38 mAb and evaluated its suitability as the targeting element of new recombinant ITs.

The anti-CD38 hybridoma AT13/5 was produced by standard hybridoma technology by Ellis J.H. [127], who further characterized the specificity of the AT13/5 mAb through a series of flow-cytometric experiment. In addition to demonstrating the binding affinity of AT13/5 for CD38-transfected CHO cells and human PBL, he also investigated the relationship between the AT13/5 epitope and those of other anti-CD38 mAbs. He proved that IB4, but not OKT10, could inhibit the binding of FITC-conjugated AT13/5 to CD38-positive cells, thereby demonstrating that the new developed mAb, even if bound to the same molecule as OKT10 mAb, probably recognized a different epitope. Accordingly, the mAb 1E82H11 we have previously characterized (see paragraph 3.1 and 3.2) and AT13/5 would bind different epitopes on CD38 molecule.

3.4.1 EXPRESSION, PURIFICATION AND CHARACTERIZATION OF THE AT13/5-DERIVED ITS

The sequence of AT13/5 scFv was kindly provided by Dr. W. Helfrich ((University of Groningen, The Netherlands); it was subcloned into the vector pET20b(+) and the sequence coding for PE40 or saporin was cloned at the 3'-end of AT13/5 scFv obtaining the recombinant immunotoxins AT13/5-PE and AT13/5-SAP (Fig. 3.16).

The expression and purification methods were those previously described for the 1E8-derived constructs. The AT13/5-PE purification steps are shown in figure 3.16b: we can observe that AT13/5-PE is a protein of about 72 kDa expressed at high levels by the bacterial host (lane 1) and it owns such chemical characteristics that allowed to recover more than 90% of the purified protein after the renaturation process without a heavy loss of precipitated material (compare lane 3 and 4). Despite the inefficiency of the protein binding to the resin, which caused a strong loss of protein in the flow-through (lane 2), the final yield of protein was approximately 10 mg from one liter of bacterial culture.

AT13/5-SAP (60 kDa), on the contrary, as we had seen for other ITs containing saporin as the toxic portion, was poorly expressed by *E. coli* and, as a consequence, only few milligrams of recombinant immunotoxin could be purified by IMAC. Besides, a further loss of protein was observed in the renaturation steps allowing to obtain only 500 µg of IT for one liter of culture.

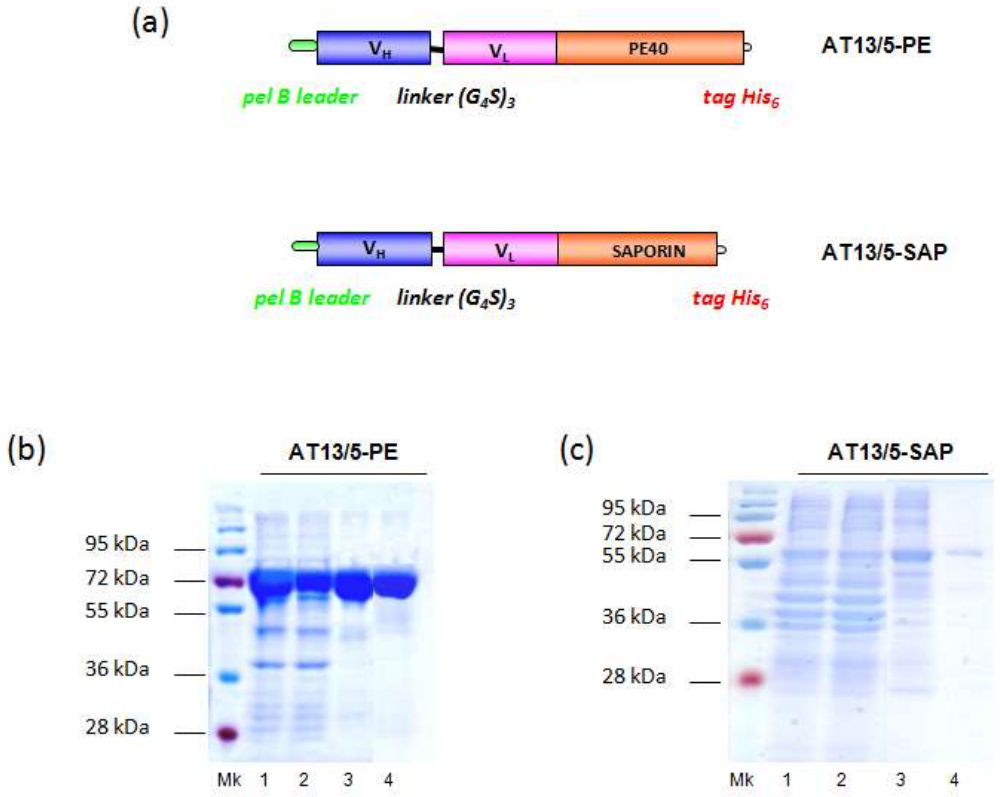
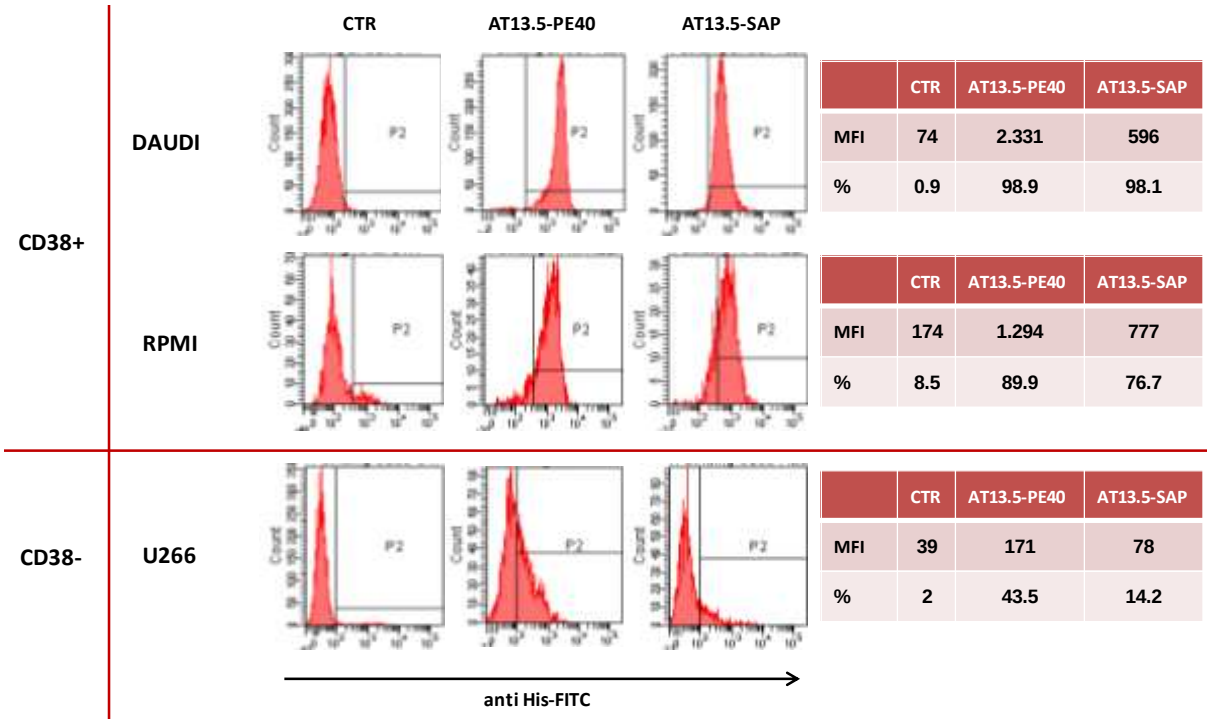


Figure 3.16 (a) The construct for expression of the scFv AT13/5 was modified by insertion of the coding sequences of PE40 or saporin. **(b)** and **(c)** Analysis by SDS-PAGE and Coomassie staining of samples taken at different steps of the purification/refolding procedure of AT13/5-PE **(b)** and AT13/5-SAP **(c)**. Abbreviations: Mk, protein size standards. Lanes: 1, 20 µl of urea-denatured inclusion bodies from one liter of induced bacterial culture; 2, flow-through samples after each column loading (20 µl from 50 ml loaded); 3, eluted protein (10 µl); 4, protein after refolding (10 µl).

Subsequently we checked the ability of the new recombinant ITs to bind CD38 antigen on the surface of positive and negative cells and we observed that, although the absolute MFI of the mAb 1E82H11 (which was comparable to that of the well characterized OKT10 mAb) was higher than that of the recombinant constructs as it would be expected, the recombinant ITs showed a good binding affinity for CD38 antigen constitutively expressed

by Daudi and RPMI8226 and overexpressed by RPMI8226 treated with ATRA (Fig. 3.17). It can be observed that the absolute MFI is higher for AT13/5-PE but this is necessarily due to the 5-fold higher concentration used to stain cells with respect to AT13/5-SAP.

Moreover, we verified the specificity of AT13/5-derived ITs binding for CD38 antigen showing that no positive staining was detectable on CD38-negative U266 cells.



	CTR	AT13.5-PE40	AT13.5-SAP
MFI	74	2.331	596
%	0.9	98.9	98.1

	CTR	AT13.5-PE40	AT13.5-SAP
MFI	174	1.294	777
%	8.5	89.9	76.7

	CTR	AT13.5-PE40	AT13.5-SAP
MFI	39	171	78
%	2	43.5	14.2

Figure 3.17. Characterization of recombinant immunotoxins AT13/5-PE and AT13/5-SAP expressed in *E. coli*. The binding activity of the recombinant ITs was analyzed by flow-cytometry using Daudi, RPMI8226 and U266 cells incubated with 100 µg/ml AT13.5-PE40 and 18 µg/ml AT13.5-SAP.

3.4.2 Cytotoxicity of the AT13/5-derived immunotoxins

The cytotoxic potential of AT13/5-PE and AT13/5-SAP was investigated considering different aspects of the phenomena induced in the cell physiology.

Firstly we evaluated the inhibition of protein synthesis by determining the effect of increasing concentrations of the recombinant ITs on the incorporation of ^{14}C-leucine by target and non-target cells. For comparison, also the immunoconjugate OKT10:SAP was tested. In figure 3.18a we can observe that AT13/5-PE, with a IC_{50} of 2.4 x 10^{-9}, and AT13/5-SAP (IC_{50} 8.6 x 10^{-9}), were highly effective on Daudi, although they were estimated to be 100-fold less potent than the immunoconjugate OKT10:SAP (IC_{50} 5.1 x 10^{-}

[11]). On the contrary, the effect on protein synthesis exerted by the ITs alone against RPMI8226 cells, as shown in figure 3.18b, was modest and did not reach a measurable IC_{50}. However, the simultaneous presence of ATRA during incubation with the ITs, promoting the overexpression of the target antigen (as previously demonstrated in figure 3.5), determined a strong increase in the toxic effect (Fig. 3.18c).

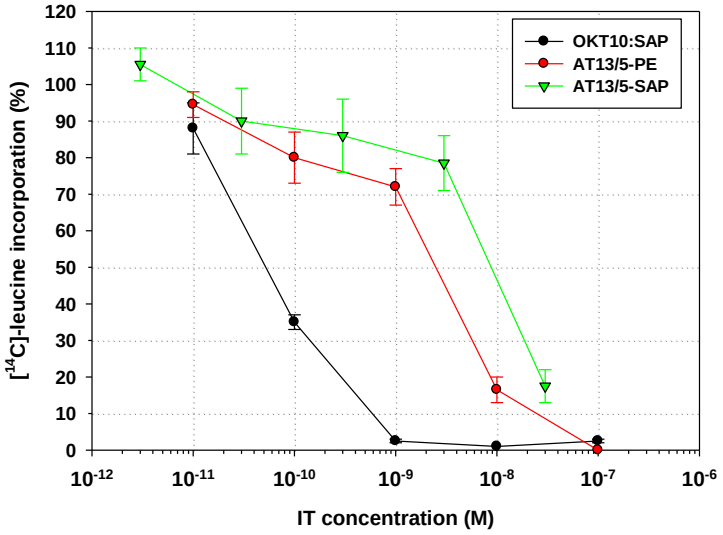
To demonstrate that the toxic activity of AT13/5-PE and AT13/5-SAP was selectively mediated by the binding portion of the ITs, specific for CD38 antigen, the same cytotoxic assay was performed on the CD38-negative U266 cell line, showing that, in the range of concentration considered, the recombinant ITs, just like the immunconjugate, did not reach the IC_{50}, even in presence of ATRA.

The IC_{50} values of the ITs are summarized in Table 3.1 and have been determined by the correspondent cytotoxicity curves in figure 3.18.

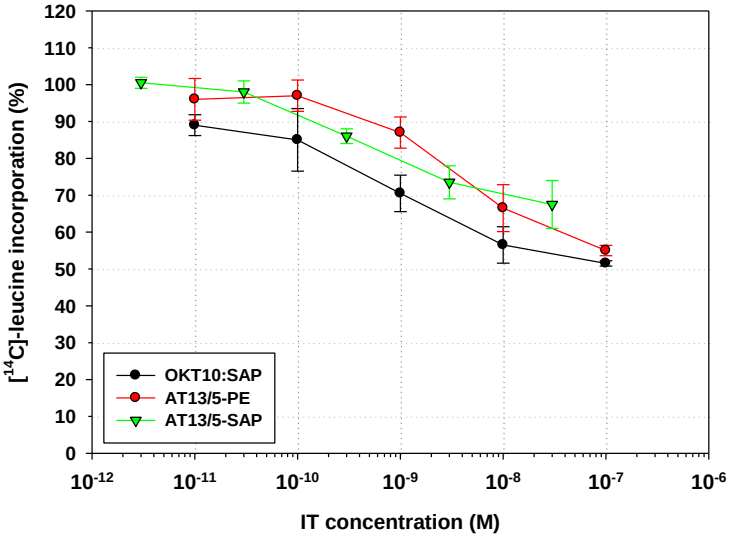
	IC50		
	OKT10:SAP	**AT13.5-PE40**	**AT13.5-SAP**
DAUDI	**$5.1 \cdot 10^{-11}$ M**	**$2.5 \cdot 10^{-9}$ M**	**$8.5 \cdot 10^{-9}$ M**
RPMI8226	**–**	**–**	**–**
RPMI8226 + 10 nM ATRA	**$2.5 \cdot 10^{-9}$ M**	**$3.6 \cdot 10^{-8}$ M**	**$1 \cdot 10^{-8}$**
U266	**–**	**–**	**–**
U266 + 10 nM ATRA	**–**	**–**	**–**

Table 3.1 IC_{50} values relative to ITs OKT10:SAP, AT13/5-PE and AT13/5-SAP on Daudi, RPMI8226 and U266.

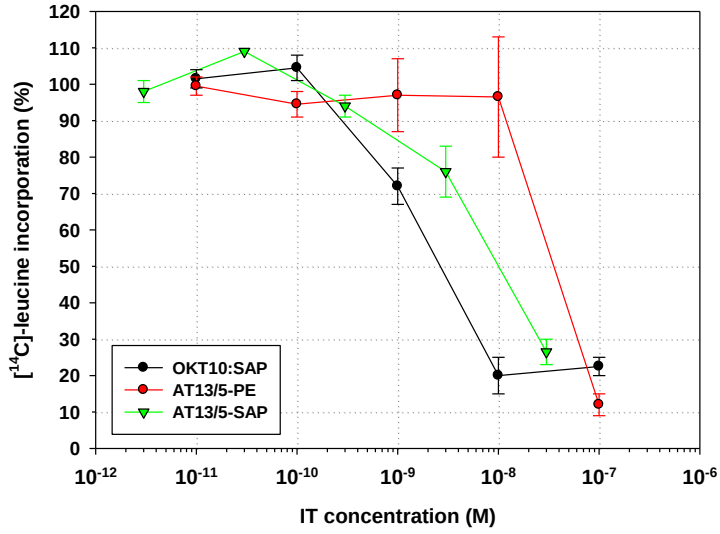
a) Daudi



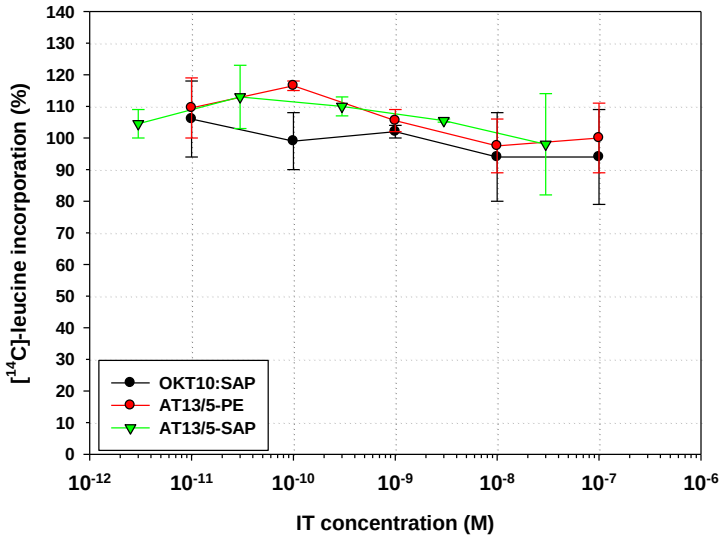
b) RPMI8226



c) RPMI8226
+ 10 nM ATRA



d) U266



e) U266
+ 10 nM ATRA

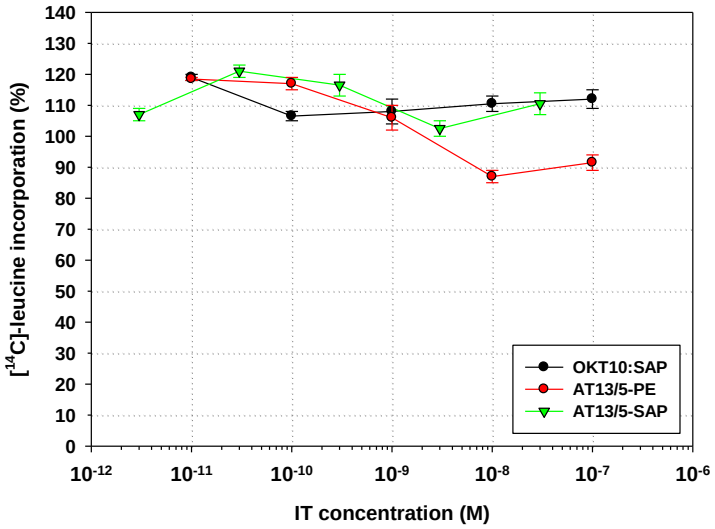


Figure 3.18 Cytotoxicity assay with AT13/5-PE and AT13/5-SAP ITs on Daudi, RPMI8226 and U266 cells treated or not with ATRA. Cells were exposed for 72 h to increasing amounts of the recombinant ITs AT13/5-PE and AT13/5-SAP (in presence or absence of 10 nM ATRA) and inhibition of protein synthesis was measured by incorporation of ^{14}C-Leucine. OKT10:SAP immunoconjugate was used as a positive control. Data are expressed as percentage of control sample (untreated cells). Each value is the average of two independent experiments.

As the final result of protein synthesis arrest is the inhibition of cell growth and finally death, we proceeded our analysis by evaluating the toxic effect of the recombinant ITs on cell proliferation. The XTT assay we used measures cell viability based on the activity of mitochondria enzymes in live cells that reduce XTT reagent (a tetrazolium derivative) to a highly water-soluble orange colored product. As the mitochondria enzymes are

inactivated shortly after cell death, the amount of water-soluble compound generated from XTT is proportional to the number of living cells in the sample and can be quantified by measuring absorbance at wavelength of 475 nm.

The curves in figure 3.19 clearly show that the ITs can inhibit the proliferation of cells expressing the CD38 antigen, while they have no detectable toxic activity on CD38-negative U266 cells within the range of concentrations used.

Although the immunoconjugate OKT10:SAP had a more potent action as compared to the recombinant ITs, we could observe that AT13/5-PE IC_{50} was lower than 10 nM for both Daudi and ATRA-treated RPMI8226, while AT13/5-SAP was capable to reduce by half the proliferation of CD38-expressing cell lines only at the higher concentration we could obtain by the purification process (3×10^{-8} M).

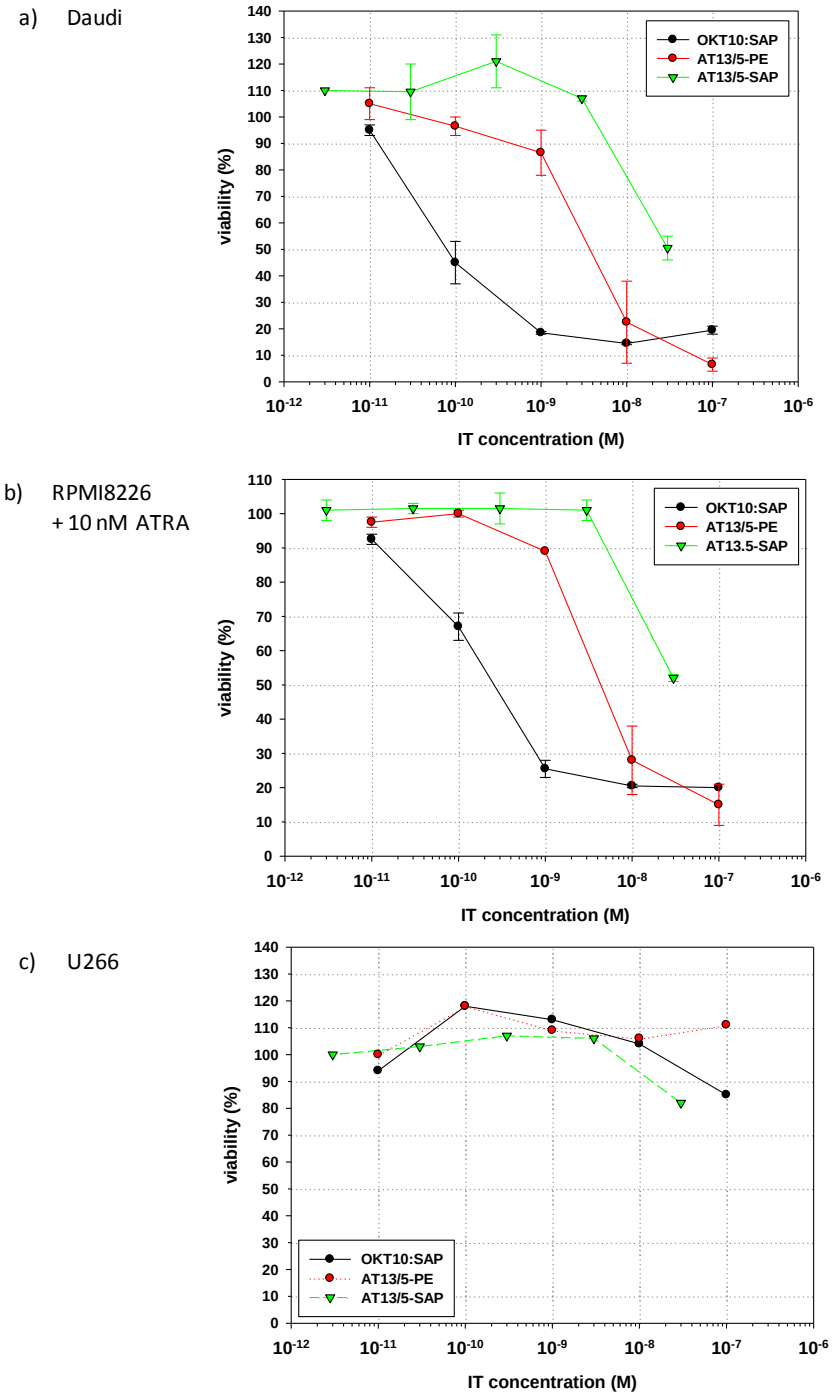


Figure 3.19 Effect of treatment with ITs on proliferation of Daudi **(A)**, ATRA-treated RPMI8226 **(B)**, and U266 **(C)** cells. Data are expressed as percentage of control sample (untreated cells). Each value is the average of two independent experiments.

The final parameter we wanted to evaluate for the complete characterization of our ITs was the ability to promote apoptosis of treated cells. This type of study was made by the flow-cytometric analysis of cells after a double staining with Annexin-V-FITC and Propidium iodide (PI). Annexin-V is a Ca^{2+}-dependent phospholipid-binding protein with

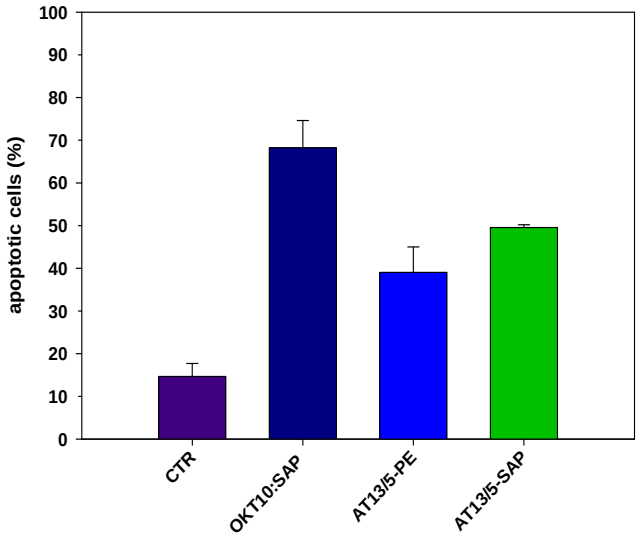
high affinity for phosphatidylserine which stains apoptotic as well as necrotic cells, while PI stains DNA of leaky necrotic cells only.

Initially we had to set up the best parameters to perform the experiments and we chose a time-point of 48 hours to better visualize the induction of apoptosis and a standard concentration of 1 μg/ml of each IT to compare the effect of the different molecules with each other and with the control non-treated cells. We also decided to pre-treat RPMI8226 cells with ATRA with the purpose of maximizing CD38 expression and obtaining the best results in terms of cell killing.

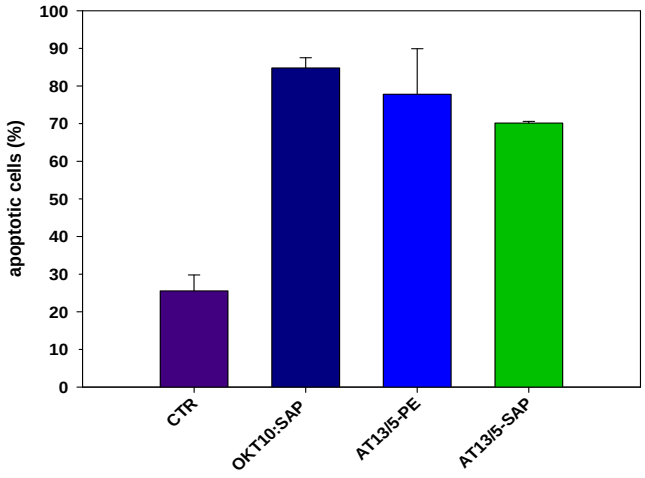
In figure 3.20a we can observe that a strong induction of apoptosis was determined by all anti-CD38 ITs, which were able to promote the killing of about 40% to 70% of treated Daudi cells. A similar, but stronger effect was observed in the case of RPMI8226 which seemed to be more prone to undergo cell death: in fact, in this case the level of apoptosis varied from 70% to 85% of treated cells. Moreover, the apoptosis induced by the recombinant ITs was almost comparable to the effect promoted by OKT10:SAP.

Once again we demonstrated that U266 viability/apoptosis was not influenced by the treatment with anti-CD38 ITs; in fact, as we can see in figure 3.20c, the percentage of apoptosis of treated cells was comparable to that of the control and this was not due to a form of apoptosis resistance because we can observe that ricin toxin at the concentration of 1 μg/ml was able to induce apoptosis of more than 70% of U266 cells.

a) Daudi



b) RPMI8226
+ 10 nM ATRA



c) U266

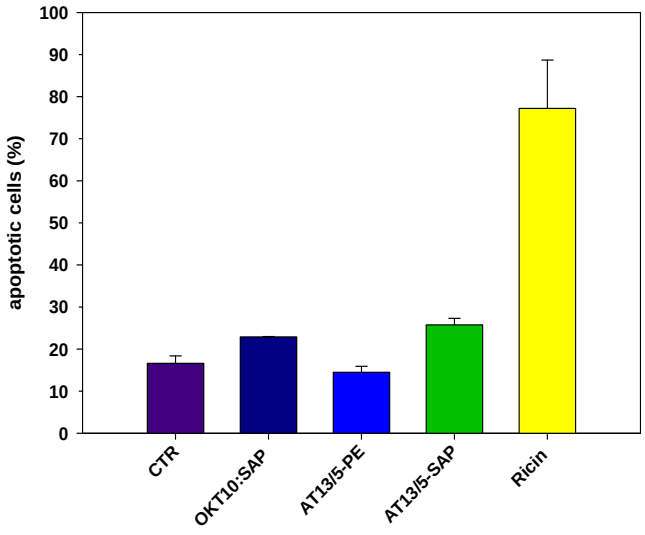


Figure 3.20 Induction of apoptosis by 1 µg/ml of anti-CD38 ITs on Daudi **(a)**, ATRA-treated RPMI8226 **(b)** and U266 **(c)** cells after an incubation period of 48 hours. Data derived from Annexin-V-FITC/PI staining.

3.4.3 COMBINATION TREATMENTS WITH AT13/5-DERIVED IMMUNOTOXINS

Subsequently we verified, by XTT proliferation assay, whether a synergistic effect could be observable by associating the recombinant ITs with commercial drugs which sensitize cells to apoptosis.

Firstly we chose ABT-737, a Bcl-2 Homology 3 (BH3)-mimetic that induces apoptosis by inhibiting pro-survival Bcl-2 proteins family, whose increased expression in cancer has been associated with chemotherapy resistance.

The second pathway that we decided to inhibit was that mediated by the proto-oncogenic Pim kinase family, a group of three constitutively active serine/threonine kinases which function by phosphorylating multiple downstream targets important for promoting tumor cell survival, proliferation and apoptosis inhibition.

A dose-response experiment was preliminarly set up to verify the sensitivity of Daudi and RPMI8226 to ABT-737 and to Pim inhibitors SGI1776 and SMI 4a (data not shown). Ten µM was chosen as the sub-toxic concentration of ABT-737 and SMI 4a while the same concentration of SGI 1776 led to the killing of 88-94% of treated cells, therefore 1 µM was the concentration chosen for the subsequent tests.

After treating Daudi and RPMI8226 with a fixed concentration of the Pim inhibitor and serial dilutions of the ITs we did not observe significant differences in the IC_{50} values with respect to the cells incubated with the ITs alone. We could therefore conclude that Pim inhibitors did not exert a synergistic effect in the presence of our ITs (Table 3.2 and 3.3).

A similar result was observed by co-incubating Daudi with ABT-737 and different concentrations of ITs (Fig. 3.21a). On the contrary the simultaneous presence of ABT-737 during incubation of RPMI8226 with ITs substantially decreased the IC_{50} of OKT10:SAP from $2.7x10^{-10}$ to $1.7x\ 10^{-11}$ M and that of AT13.5-PE40 from $3.5x10^{-9}$ to $2.8x\ 10^{-10}$ M. Moreover, while in standard conditions a concentration of AT13.5-SAP of $3x10^{-8}$ M was responsible for the killing of 50% of RPMI8226 cells, in presence of ABT-737 the death level was over 90% (Fig. 3.22a). These data suggest a synergistic effect of ABT-737 co-administered with ITs on RPMI8226 cells.

The differential ABT-737 sensitivity of different tumor cell lines has been widely discussed by researchers and seems to be related to the levels of expression of BCL-2

family protein: in fact ABT-737, being more effective in displacing BH3-only protein from Bcl-2 rather than from Bcl-xL and Bcl-w, is more active in tumors overexpressing Bcl-2.

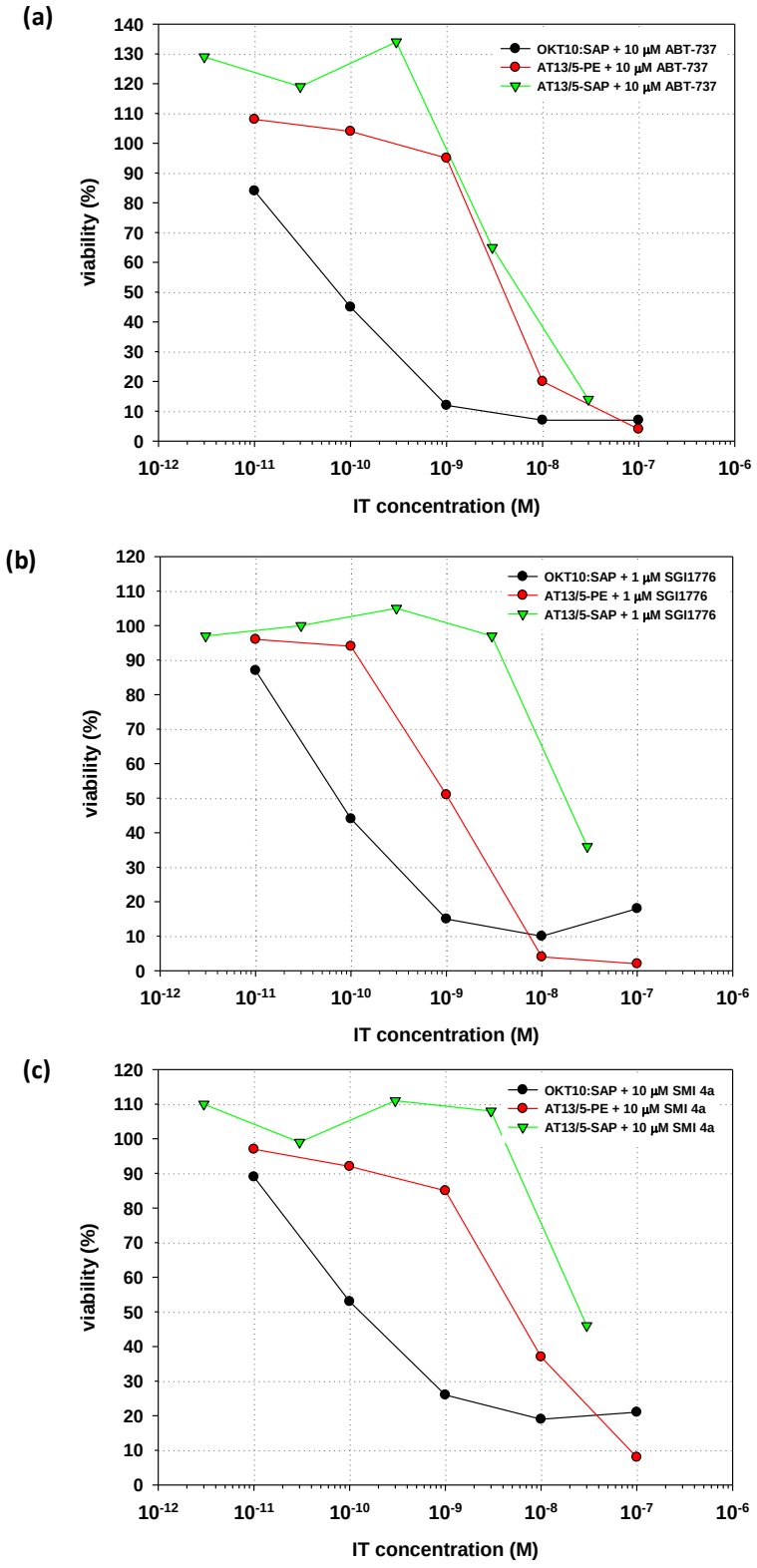


Figure 3.21 Effect of recombinant ITs on cell viability of Daudi co-incubated with 10 µM ABT-737 **(a)**, 1 µM SGI1776 **(b)**, or 10 µM SMI 4a **(c)**.

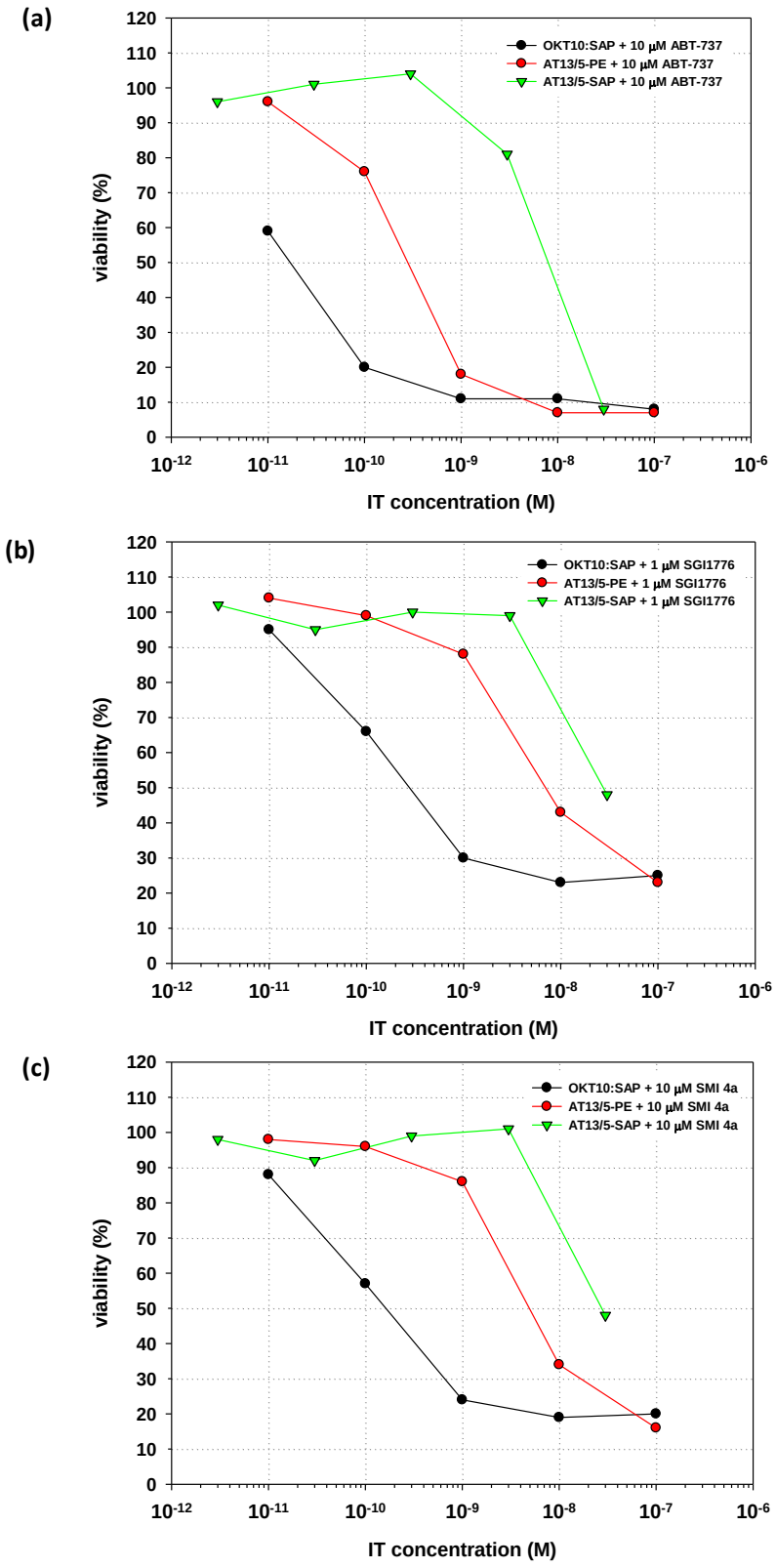


Figure 3.22 Effect of recombinant ITs on cell viability of ATRA-treated RPMI8226 co-incubated with 10 µM ABT-737 **(a)**, 1 µM SGI1776 **(b)**, or 10 µM SMI 4a **(c)**.

DAUDI	IC50		
	OKT10:SAP	AT13.5-PE40	AT13.5-SAP
untreated	$6.1 \cdot 10^{-11}$ M	$6 \cdot 10^{-9}$ M	$2.6 \cdot 10^{-8}$ M
10 µM ABT-737	$7.6 \cdot 10^{-11}$ M	$3.9 \cdot 10^{-9}$ M	$5.9 \cdot 10^{-9}$ M
1 µM SGI1776	$7.3 \cdot 10^{-11}$ M	$1 \cdot 10^{-9}$ M	$1.7 \cdot 10^{-8}$ M
10 µM SMI 4a	$1.2 \cdot 10^{-10}$ M	$5.2 \cdot 10^{-9}$ M	$2.5 \cdot 10^{-8}$ M

Table 3.2 IC_{50} values relative to ITs OKT10:SAP, AT13/5-PE and AT13/5-SAP on Daudi treated with BH3-mimetics or Pim inhibitors.

RPMI8226	IC50		
	OKT10:SAP	AT13.5-PE40	AT13.5-SAP
untreated	$2.7 \cdot 10^{-10}$ M	$3.5 \cdot 10^{-9}$ M	$3 \cdot 10^{-8}$ M
10 µM ABT-737	$1.7 \cdot 10^{-11}$ M	$2.8 \cdot 10^{-10}$ M	$7.9 \cdot 10^{-9}$ M
1 µM SGI1776	$2.7 \cdot 10^{-10}$ M	$6.8 \cdot 10^{-9}$ M	$2.7 \cdot 10^{-8}$ M
10 µM SMI 4a	$1.6 \cdot 10^{-10}$ M	$4.8 \cdot 10^{-9}$ M	$2.7 \cdot 10^{-8}$ M

Table 3.3 IC_{50} values relative to ITs OKT10:SAP, AT13/5-PE and AT13/5-SAP on ATRA-treated RPMI8226 treated with BH3-mimetics or Pim inhibitors.

3.4.4 Effect of the AT13/5-derived immunotoxins on B-CLL

We investigated the potential clinical application of our ITs by evaluating their specific cytotoxic activity on B-lymphocytes derived from B-CLL patients. This part of the study is now at an early stage of development for two main reasons: the first is the difficulty to find appropriate quantity of CD38-positive samples available for research purposes and suitable to perform reproducible experiments on samples from the same patient; the second is the need to optimize the culture conditions of these type of cells which are not immortalized and therefore show high level of apoptosis after 24 hours of incubation without any stimuli.

We firstly selected a PBMCs (Peripheral Blood Mononuclear Cell) sample showing high levels of CD38 expression and we purified B-lymphocytes by negative selection. The

subsequent flow-cytometric analysis of the purified cells demonstrated that the sample contained over 90% of CD38-positive cells (Fig. 3.23).

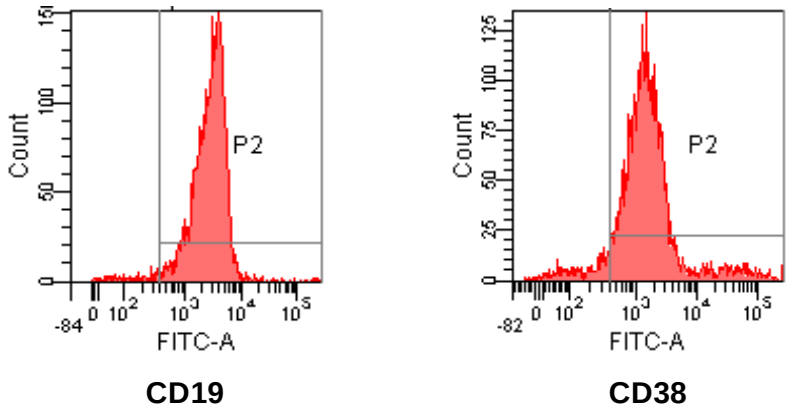


Figure 3.23 Flow-cytometric analysis of B-cell purity (CD19+ cells) and CD38 expression by B-lymphocytes after purification by negative selection from a PBMC sample from a CLL patient.

The culture conditions were optimized by the progressive addition of cytokines (IL-4, IL-2 and CD40-ligand) and the change of the culture medium with an enriched in nutrients one (IMDM), as shown in figure 3.24. Despite these solutions, as we can observe in Figure 3.25, untreated cells spontaneously underwent apoptosis at a level of 30% within 24 hours. However this time was sufficient to highlight the induction of apoptosis on B-cells of the first patient we treated with recombinant ITs.

This first apoptosis experiment, although yielding a moderately success, represents only a starting point for the future clinical development of anti-CD38 recombinant ITs.

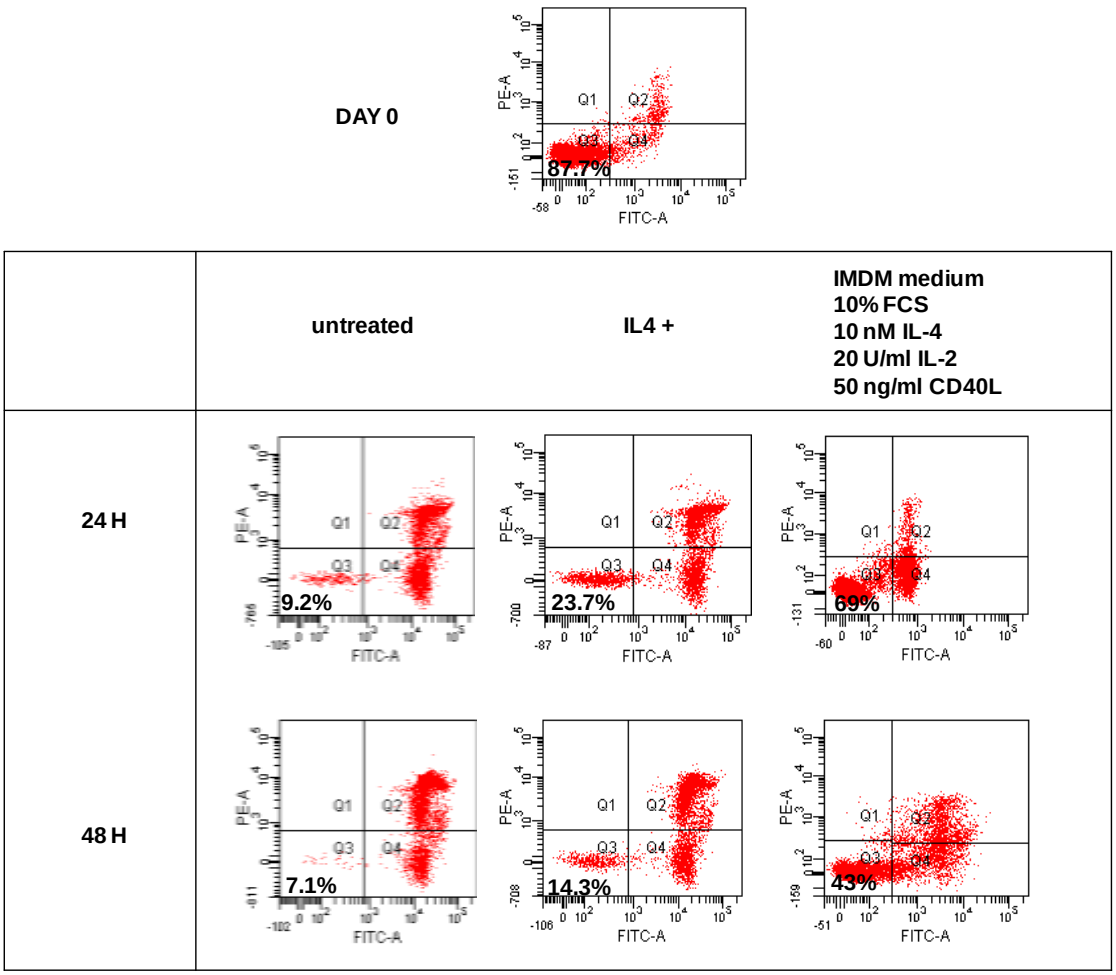


Figure 3.24 Stepwise optimization of culture condition of B-lymphocytes to diminish the level of spontaneous apoptosis. Annexin-V-FITC/PI staining was performed after 24 and 48 hours of incubation of cells with different cytokines

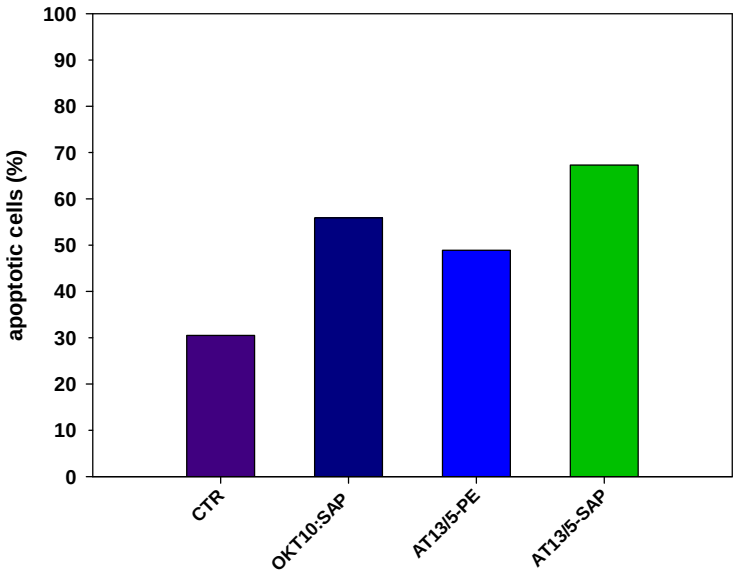


Figure 3.25 Induction of apoptosis by 1 µg/ml of anti-CD38 ITs on B-lymphocytes derived from a CLL patient after an incubation period of 24 hours. Data derived from Annexin-V-FITC/PI staining.

4. DISCUSSION

Rapid progress in understanding molecular mechanisms of cancer development and increased insights into the nature of tumor antigens made a large impact on the design and evaluation of novel therapeutic strategies focused on the specific targeting of tumor cells, thus obtaining an increase in efficacy with a concomitant reduction of side effects. ITs represent a very efficacious targeted immunotherapy approach for treating cancer patients, particularly when used in association with other therapeutic modalities. The best success of ITs has been observed in the field of hematological malignancies; in fact, cell from hematological tumors, being located intravascularly or perivascularly in well-perfused lymph nodes, are more exposed to permeation by ITs and therefore more accessible than cells of solid tumor masses.

The present thesis describes the construction and development of different recombinant ITs targeting the CD38 antigen, whose expression is mainly linked to the lymphoid lineage and whose overexpression has been demonstrated in hematological malignancies such as B-CLL and Multiple Myeloma [33]. CD38 is also known to undergo internalization upon binding of ligands to its extracellular portion [22], which is fundamental for the uptake of an antibody drug. We also explored an important concept about the effect on efficacy and selectivity of the association of our ITs with drugs involved in survival and apoptosis pathways of the cell.

Despite the interest in developing immunotherapeutic drugs targeting CD38 antigen, which led to the production of both anti-CD38 chimeric monoclonal antibodies and ITs, none of such compounds has been yet approved for clinical therapy.

In this work we pursued the construction of a panel of recombinant ITs which can be divided into different sub-groups depending on the nature of the binding domain as well as the toxic domain.

Considering the binding domain, we created ITs composed of single chain antibody fragments (scFv) directed against CD38 antigen and derived from two murine hybridomas secreting monoclonal antibodies with different epitope specificity. Hybridoma 1E82H11 was produced and characterized in our laboratory and the purified mAb from this clone showed good reactivity towards the native antigen expressed by lymphoma and myeloma cell lines. Moreover, 1E82H11 mAb binding efficiency was almost comparable to that of OKT10 mAb, a known mAb that we used as a reference standard. On the contrary AT13/5

hybridoma was developed by J. H. Ellis [127] and the sequence of the derived scFv was supplied to us by Dr. W. Helfrich (University of Groningen, The Netherlands). The construction of the scFvs by the traditional strategy of genetic fusion of the sequences coding for the variable domains implies that a future therapeutic utilization of such antibody fragments will likely require a "humanization" step by further genetic manipulation through a variety of approaches, in order to reduce their immunogenic potential and avoid the occurrence of HAMA [128]. With this perspective, CDR-grafting has already been employed by J.H. Ellis for the humanization of the entire AT13/5 IgG1.

The first choice for the toxic portion to be fused to our scFvs was a derivative of *Pseudomonas aeruginosa* exotoxin A (PE), which is a preferred molecule for the construction of ITs because its high toxic potential is well documented and its cytotoxic pathways are well understood [129]. Moreover, PE40, a mutant form of PE in which the largest portion of the cell-binding domain has been deleted and therefore, showing diminished nonspecific toxicity when administered to animals, has been widely used in the development of ITs and is now part of many recombinant molecules tested in clinical trials. Nevertheless, PE is known to be immunogenic, leading to the formation of neutralizing antibodies; it is therefore likely that a de-immunization strategy will be needed before exploring its *in vivo* efficacy. This can be accomplished, as shown by M. Onda and coworkers [101], through the replacement of the amino acids within the epitopes determining the reactivity of the immune system.

The first approach used to obtain a potent cytotoxic effect and possibly avoid a future immune response, was the employment of saporin as the toxic portion; indeed, saporin is reported to be a potent toxin and one of the less immunogenic among the toxins of plant origin.

Particular attention has been paid to setting up a suitable strategy for the expression and purification of the antibody fragments and the derived ITs. The main concern was manageability and final yield. For these reasons we opted for a prokaryotic host like *E. coli*, which is easily grown in shake flasks and allows the rapid induction and high-level accumulation of heterologous proteins. In order to maximize the amount of protein we decided to select the BL21(DE3)pLysS strain, which, being deficient in the expression of

several endogenous proteases, represents one of the most widely used prokaryotic systems for heterologous production of proteins.

Furthermore, all the recombinant constructs which have been inserted into the plasmid vector for the expression on the bacterial host present a N-terminal signal peptide for the sorting to the periplasm; this was done to ease the recovery of the heterologous proteins in soluble form. In spite of the presence of this signal sequence, the induced proteins were mainly accumulated as inclusion bodies which required the dissolution of the insoluble aggregates using a denaturing agent, such as urea, and a further renaturation process which is generally reported to be the most critical step affecting the yield of biologically active antibody fragments and ITs, as loss due to aggregation and precipitation can be substantial [130]. In fact, in spite of the application of an appropriate refolding procedure (i.e. the gradual removal of the denaturing agent and the use of reduced and oxidized glutathione and arginine that are known to limit the occurrence of aberrant protein folding and precipitation [131]), a sizeable loss of protein (especially for the scFv) due to formation of insoluble aggregates was observed. Fusions with PE40 proved less prone to aggregation during the renaturation procedure, while saporin-based ITs showed high levels of aggregation and precipitation. These different behaviour was probably conferred by the chemical and structural features of the toxin molecules, by their amino acid composition and their hydrophilic/hydrophobic profile. To avoid most of the disadvantages linked to the use of *E. coli*, especially the problem of protein aggregation following refolding, the use of eukaryotic systems and particularly of the yeast *Pichia pastoris*, has been proposed as the best-suited expression platform of saporin-based therapeutic molecules, allowing the recovery of proteins in soluble form from the culture medium [132].

Finally, our scFv and IT constructs contain a C-terminal hexahistidine tag to allow the purification by affinity chromatography. It should be noted, however, that an epitope tag represents an artificial element, unrelated to the rest of the polypeptide and with no pharmacological activity: a protein to be used as a therapeutic agent in humans should be ideally free from any such non-essential portion. Moreover, as it was described by J. L. Hessler et al., ITs with PE should have a free terminus because removal of the lysine residue of the C-terminal REDLK sequence is essential for the binding of PE to the KDEL receptor and therefore for the cellular intoxication of PE [133]. Thus, the presence of a

peptide tag could decrease PE activity. We have however maintained it in our PE-based ITs because in the present work we were not aiming at the best optimization obtainable but rather at proving the suitability of our ITs in the context studied. Optimization and further refinements will be addressed when the best candidate for use *in vivo* will be selected.

We began the analysis on the binding affinity of our recombinant constructs by a first characterization of the parental mAb. Flow-cytometric assays showed that the binding of our 1E82H11 mAb is restricted to CD38-positive cells (i.e. Daudi and RPMI8226) and that it targets the same epitope recognized by OKT10 mAb, by which it is displaced in a competitive staining.

The conversion of the mAb into the scFv format did not allow to preserve the binding efficiency of the parental monoclonal and not even that of the scFv derived from OKT10 mAb (data not shown). When generating a scFv from the immunoglobulin variable region genes isolated from a hybridoma cell line, a decrease in the apparent affinity of the resulting scFv as compared to the parental mAb has often been observed [134]. With many scFv molecules, the lower affinity results from the decrease in valence (number of binding sites) that occurs when the format is switched from the larger bivalent mAb to the smaller monovalent scFv. This is particularly true when multiple copies of the target epitope are present on a single antigen molecule as was the case with the CC49 MAb [135]. Decrease in affinity can also result from structural alterations between the IgG and scFv formats. In particular, the peptide spacer that joins the V_H and V_L chains can potentially interfere with the normal alignment of the two chains. Finally, the loss of scFv conformation due to the need of solubilizing proteins accumulated into inclusion bodies, and the further refolding procedure can affect molecules stability as it can be demonstrated by the high tendency to form aggregates showed by our 1E8 scFv.

We attempted to reconstitute the double valence of the parental IgG by genetically fusing a second scFv at the C-terminal of the first one, obtaining a divalent molecule with only a two-fold greater binding affinity with respect to the scFv and still far from the affinity showed by the mAb. These results suggested that a different approach for the design of multivalent antibody molecules could be considered: for example it could be possible to obtain diabody and triabody by shortening the peptide linker connecting the

V_H and V_L of a single scFv molecule from 15 amino acids to 5 amino acids or 0–3 amino acids respectively [136].

Genetic fusion of monovalent or divalent scFvs to the toxic portions resulted in a further loss of binding affinity probably ascribable to the steric hindrance of the toxic domain preventing the binding of the antibody fragment or possibly by the interference of the toxin sequence with the correct folding of the binding portion.

The substitution of the 1E8 scFv (derived from a mAb with the same specificity of OKT10) with that derived from AT13/5 mAb (a simil-IB4 mAb) implies the changing of the molecular epitope towards which the ITs were targeted. As reported by C. M. Ausiello *et al.* [137], competition binding analysis identified two families of mAbs, namely IB4, IB6 and AT2 on one side and OKT10, SUN-4B7 and AT1 on the other. Each mAb family binds epitopes that are completely or partially common. However, the functional activities of the CD38 molecule cannot be simply attributed to the epitope engaged: for instance, IB4 and OKT10 mAbs, which bind different epitopes, perform as agonistic mAbs in inducing PBMC proliferation and interferon (IFN)-γ secretion. On the contrary, IB4 is the only mAb able to induce significant intracellular Ca^{2+} fluxes [137]. Despite the considerations on the correlation between structural and functional properties of CD38 epitopes, we could observe that AT13/5-derived ITs showed good affinity and specificity for CD38 antigen expressed on the surface of Burkitt's lymphoma cells and myeloma cells proving that the steric organization of the scFv and the toxic domains in this case can favor the binding to a different epitope on CD38 molecule.

The toxic molecules that we used for the construction of our ITs have been described to induce inhibition of protein synthesis through the inactivation of the elongation factor eEF-2 after the internalization and the intracellular routing. This is the reason that justifies our investigation on the levels of protein synthesis inhibition (PSI) selectively induced by our ITs on CD38-positive cells.

With the creation of the divalent 1E8-derived ITs we demonstrated that, despite the discouraging binding properties of these constructs, they showed an acceptable cytotoxicity against Daudi cells. This observation could be explained with the small amount of toxic molecules needed to determine PSI. Moreover, the dimerization of the CD38 receptor upon binding to the divalent ITs could account for a better rate of

internalization of the divalent ITs with respect to the monovalent counterparts. However, similar results in terms of PSI were obtained using AT13/5-derived ITs, showing that the presence of a double binding site, as in the case of divalent ITs, is not essential for the cytotoxic activity if the binding affinity of the monovalent scFv is high, as demonstrated by AT13/5-derived ITs.

It is widely recognized that the arrest of protein synthesis is the main cause of cell death induced by ITs [138] and the cell proliferation assays that we performed on target cells with our anti-CD38 ITs confirmed the results obtained by the PSI assay. In fact, we observed that ITs concentrations able to induce arrest of protein synthesis also showed a high ability to inhibit cell proliferation. However, PE and PE-containing ITs, as well as some plant toxins, have been proved to induce programmed cell death by the activation of caspase-3-like protease [139]. Activated caspases can cleave structural proteins and enzymes necessary for the survival of both proliferating and resting cells; in addition, caspases activate the endonuclease responsible for the inter-nucleosomal cleavage of genomic DNA and cleave so-called “death substrates”, such as poly(ADP)-ribose polymerase (PARP), both hallmarks of apoptotic death [140]. Further molecular changes induced during apoptosis include randomization of the distribution of phosphatidyl serine between the inner and outer leaflets of the plasma membrane. The ability of our ITs to induce apoptosis was investigated by detecting some of these changes, such as surface-exposure of phosphatidyl serine with annexin V and detection of cells with subdiploid DNA content by staining with DNA intercalating dyes. We observed high levels of apoptosis induction both by PE- and saporin-based ITs. It should be noted that, although Daudi cells are reported to express CD38 at a higher density on their surface, RPMI8226 cells seem to be more prone to undergo apoptosis (Fig. 3.20). A possible explanation could be the different sensitivity of the cell lines derived from different types of tumors. Moreover, some cell lines, under certain culture conditions, could trigger mechanisms of resistance to apoptosis by the over-expression of multiple survival signals.

All the cytotoxicity experiments showed that the IC_{50} of the immunoconjugate OKT10:SAP was approximately 10- to 100-fold lower as compared to those of the recombinant constructs. The higher toxicity showed by OKT10:SAP could be explained by several factors: firstly by the double valence of the entire mAb compared to the scFvs,

which accounts for a best binding performance of the mAb, secondly by the possibility of an incorrect folding due to the renaturation process, which is a problem affecting the recombinant ITs but not the immunoconjugate molecules. Finally, the hexahistidine tag placed at the carboxy-terminal end of PE40 and saporin may interfere with the intracellular routing and consequently reduce ITs potency. Indeed, the addition of 6-11 amino acids at the C-terminus of PE40 has been demonstrated to be sufficient to bring about a tangible loss of enzymatic functionality [141]. While cytotoxicity results can be considered encouraging, it is quite probable that a significant increase in the ITs potency could be obtained by removing the hexahistidine tag from our constructs. This would however require the setting up of a different, tag-independent procedure for the purification of the resulting polypeptides.

It can be noticed that the specificity of our recombinant ITs was evaluated in PSI assays as well as in apoptosis and viability assays which confirmed that the potent effect of the new molecules is irrefutably mediated by the binding domain which selectively binds CD38-expressing cells.

We finally explored an important concept related to the association of recombinant ITs with drugs involved in the inhibition of essential intracellular pathways. The aim of this study was to assess if an increased cytotoxic and selective effect could be achieved by the association regimen. Combination therapy has become a mainstay of cancer chemotherapy because it consents to potentiate such compounds which show a modest activity when administered as single agents and allows to virtually affect different cellular targets . The association treatment here reported offers several advantages:

- it exploits drugs that have been already approved for clinical trials and whose pharmacology has been previously studied, so that the translation of the combination approach to the clinical development should be facilitated;
- it profits by the combination of chemical drugs which demonstrated to be functional in anti-cancer therapy;
- it allows to obtain a mutual potentiating effect of both ITs and chemical therapeutics.

The choice of a BH3-mimetic to be used in our combination therapy derived from two main observations:

1. the inhibition of protein synthesis mediated by the ITs frequently results in the loss of Mcl-1, a short-lived pro-survival Bcl-2 protein, and this may contribute to the potency of these protein toxins [142];
2. other pro-survival proteins such as Bcl-2 and Bcl-xL are longer lived and when cancer cells depend on one of these, toxin-mediated killing may be more difficult to achieve. Further, Bcl-2 and Bcl-xL are frequently associated with resistance to chemotherapy.

Binding of ABT-737 to either Bcl-2 or Bcl-xL neutralizes their pro-survival activity, allowing Bax or Bak to initiate the intrinsic arm of the apoptotic pathway. Here we showed that the BH3-only mimetic ABT-737 can increase ITs efficiency on RPMI8226 cells resulting in enhanced killing by as much as 10-fold. This activity was achieved using a concentration of 10 μM of ABT-737 that was non toxic when added alone. The same concentration did not determine a similar potentiating effect on Daudi cells. A possible explanation of this result can be inferred by the work of R. W. Rooswinkel and coworkers who demonstrated that Bcl-2, Bcl-xL and Bcl-w are not targeted with equal efficiency by ABT-737 in the cellular context [143]. As a consequence, ABT-737 was not equally effective in displacing BH3-only proteins or Bax from Bcl-2, as compared with Bcl-xL or Bcl-w, offering an explanation for the differential ABT-737 sensitivity of tumor cells overexpressing these proteins. Thus, in our experiments, the resistance of Daudi to ABT-737-induced apoptosis could be probably due to the variable pattern of pro-apoptotic molecules they express. This is an interesting concept that deserves to be explored in greater detail in future works.

A similar explanation can be given to justify the inefficiency of Pim-kinases inhibitors in potentiating the effect of recombinant ITs, which may be ascribable to the low expression of one or more Pim-kinase isoforms or to the fact that the growth and proliferation mechanisms induced by Pim-kinases could be poorly involved in the metabolic pathways of the cell lines considered.

As it was predictable, the association treatment of ABT-737 and ITs did not show any potentiating effect on U266 cells indicating that Bcl-2 mechanism of action is independent of that promoted by the ITs.

The work presented in this thesis is focalized on the functional characterization of recombinant ITs through the use of cancer cell lines. It is well-known that cell lines are not always true representatives of the parent tumors from which they are derived, in fact they might show altered properties due to prolonged time in culture and hence a heightened response to anticancer drugs; whereas tumors *in vivo* are influenced by microenvironment and can develop resistance to apoptosis by induction of several mechanisms. Here we reported only a preliminary *ex vivo* experiment on human cells derived from a CLL patient, but our intent is to extend the study to a wide panel of CD38-positive CLL and MM patients. The final goal of this work will be the *in vivo* evaluation of the therapeutic effect of anti-CD38 ITs in animal models.

A further development of the study of anti-CD38 ITs will be directed to overcome the problems related to immunogenicity of these heterologous proteins. The toxic domain PE40, which has revealed efficient in cell killing, could be engineered by the mutagenesis of the immunogenic epitopes or could be substituted by endogenous protein of human origin like proapoptotic protein (e.g TNF or TRAIL) or RNase, while humanization of the binding domain would be necessary.

Finally, to evaluate the possibility of enhancing the anti-tumor effects of our ITs, association with other therapeutics like pro-apoptotic agents and radiotherapy sensitizers could be explored.

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