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Depletion of T_{reg} cells for potentiation of cancer treatment with HPMA copolymer-bound cytostatic drug conjugates

Deplece T_{reg} buněk pro potenciaci nádorové léčby konjugáty léčiv vázaných na HPMA kopolymer

Diploma thesis

Supervisor: RNDr. Marek Kovář, PhD.

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Prohlášení

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Praha, 6.5.2013

Podpis

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Abstract

Tumor diseases are severe problem worldwide with increasing number of patients suffering from various types of malignancies. Many of approved therapeutics cause serious side toxicities. Therefore, there are intensive efforts to improve cancer treatment protocols.

The aim of this study was to deplete regulatory T (T_{reg}) cells without affecting other immunocompetent cells playing a positive role in tumor eradication. T_{reg} cells were reported to hamper anti-tumor immunity and promote tumor growth and survival. Thus, their selective elimination could lead to induction of anti-tumor responses and tumor rejection if combined with chemotherapy with selected *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-bound drug conjugates.

Original approach was to deplete of T_{reg} cells without the use of anti-CD25 mAb that has been widely exploited for T_{reg} cell elimination; however, its long-term persistence in circulation together with inhibitory effect on activated effector cells ($CD25^{+}$) are its main disadvantages. Thus, T_{reg} cells were sensitized to cell cycle-specific cytostatic drugs via application of IL-2/anti-IL-2 JES6.1 mAb immunocomplexes that induce vigorous selective proliferation of this cell population. Subsequent application of cell cycle-specific cytostatics showed steep decrease of T_{reg} cell counts but the resulting level of T_{reg} cell numbers was similar to the steady-state level in naïve mice. Therefore, alternative protocol for T_{reg} cell elimination was employed. The approach is based on application of biotinylated anti-CD25 mAb for T_{reg} cell depletion and its subsequent elimination from circulation by HPMA copolymer-bound avidin. It appears to be promising, as it negates disadvantages of anti-CD25 mAb treatment.

Next, maximal tolerated dose of selected HPMA copolymer-bound drug conjugates was determined. These conjugates showed also potent anti-tumor activity indicating they could be used in further experiments combining T_{reg} cell depletion with chemotherapy.

Keywords: cancer, regulatory T cells, IL-2, immunocomplexes, cytostatic drugs, avidin, biotin, anti-CD25 mAb, HPMA copolymer-bound drugs, doxorubicin

Abstrakt

Nádorová onemocnění představují závažný celosvětový problém, přičemž incidence jejich výskytu stále narůstá. Jelikož podávání řady běžně užívaných protinádorových terapeutik způsobuje vážné vedlejší účinky, je věnována velká pozornost tvorbě nových šetrnějších léčebných postupů.

Cílem této práce bylo odstranění regulačních T (T_{reg}) buněk z organismu aniž by došlo k ovlivnění dalších imunokompetentních buněk, které hrají významnou roli v eradikaci nádoru. Je známo, že T_{reg} buňky tlumí protinádorové imunitní reakce a podporují růst nádoru. Jejich eliminace by tudíž v kombinaci s chemoterapií vybranými konjugáty léčiv založených na *N*-(2-hydroxypropyl)methakrylamidu (HPMA) mohla vést k indukci protinádorové imunitní odpovědi a rejekci nádoru.

Původní snaha spočívala v odstranění T_{reg} buněk z organismu bez použití běžně užívané depleční anti-CD25 mAb. Daná protilátka je totiž charakteristická svým dlouhým setrváváním v oběhu a schopností inhibovat aktivované efektorové buňky ($CD25^{+}$). Pomocí IL-2/anti-IL-2 JES6.1 mAb imunokomplexů, které selektivně indukují robustní proliferaci T_{reg} buněk, byly tyto buňky senzitizovány k fázově specifickým cytostatikům. Aplikace vybraných cytostatik dokázala snížit množství proliferujících T_{reg} buněk pouze na hladinu srovnatelnou s bazální hladinou pozorovanou u naivních myší. Jako alternativní cesta byla proto zvolena aplikace biotinylované anti-CD25 mAb a její následné odstranění z oběhu pomocí avidinu vázaného na HPMA kopolymer.

Dále byla určena maximální tolerovaná dávka vybraných konjugátů léčiv vázaných na HPMA kopolymer. Testovaná léčiva vykazovala mimo jiné i silnou protinádorovou aktivitu, díky čemuž mohou být následně využita pro experimenty kombinující depleci T_{reg} buněk s chemoterapií.

Klíčová slova: nádor, regulační T buňky, IL-2, imunokomplexy, cytostatika, avidin, biotin, anti-CD25 mAb, léčiva vázaná na HPMA kopolymer, doxorubicin

List of Abbreviations

APC ..... Antigen presenting cell
ATRA ..... All *trans*-retinoic acid
B7-1, B7-2 ..... CD80, CD86
BCG ..... Bacillus Calmette-Guerin
CD ..... Cluster of differentiation
CTLA-4 ..... Cytotoxic T-lymphocyte antigen 4
DC ..... Dendritic cell
DOX ..... Doxorubicin
DR3 ..... TNF receptor superfamily member 25, TNFRSF25
DTX ..... Docetaxel
EAE ..... Experimental autoimmune encephalomyelitis
EPR ..... Enhanced Permeability and Retention
FDA ..... Food and Drug Administration
Foxp3 ..... Forkhead box p3, Scurfin
GARP ..... Glycoprotein A repetitions predominant
GITR ..... Glucocorticoid-induced TNFR-related protein
GM-CSF ..... Granulocyte-macrophage colony stimulating factor
HIV ..... Human immunodeficiency virus
HMW ..... High molecular weight
HPMA ..... *N*-(2-hydroxypropyl)methacrylamide
HPV ..... Human papiloma virus
IDO Indoleamin-2,3-dioxygenase
IFN ..... Interferon
IL ..... Interleukin
IL-2R ..... Interleukin-2 receptor
iNKT ..... Invariant NKT
iT_{reg} ..... Induced regulatory T
LAG-3 ..... Lymphocyte activation antigen 3
LMW ..... Low molecular weight

mAb ..... Monoclonal antibody

MDR ..... Multidrug resistance

MHC ..... Major histocompatibility complex

MP..... Memory phenotype

MTD..... Maximum tolerated dose

NK..... Natural killer

NKT ..... Natural killer T

nT_{reg}..... Natural regulatory T

OVA..... Ovalbumin

PAP ..... Prostate acid phosphatase

PEG..... Polyethylene glycol

r ..... Recombinant

rh ..... Recombinant human

rHSA ..... Recombinant human serum albumin

RT ..... Room temperature

s..... Soluble

TAMs..... Tumor-associated macrophages

TANs..... Tumor-associated neutrophils

TCR..... T cell receptor

TGF-β..... Tumor growth factor-β

T_h..... T helper

TLR..... Toll-like receptors

TNF..... Tumor necrosis factor

TNFR ..... Tumor necrosis factor receptor

T_{reg}..... Regulatory T

Contents

1 Introduction

One of the most common causes of death worldwide are malignant diseases with rapidly increasing incidence worldwide. Clinical treatment of cancer exploits beneficial effects of surgery, chemotherapy and radiotherapy together with immunotherapy. However, these approaches can be accompanied with serious side toxicities inducing damage to normal tissues. Thus, development of other approaches diminishing toxicities and improving above mentioned methods has been in progress. Mostly, there are efforts to boost immune system to react against tumor tissues using various immunostimulants, passive vaccination with tumor-specific antibodies or adoptive transfer of tumor-specific effector T cells.

1.1 Tumor immunity

Immune system has the ability to regulate reactions of organism either to self or non-self antigens and prevent pathological and aberrant responses. Moreover, it can eliminate malignantly transformed tumor cells upon recognition, inhibit their uncontrolled proliferation and metastatic potential. The first one to suggest the existence of so called cancer immune surveillance was MacFarlane Burnet in 1957 [1]. Subsequently, on the basis of accumulated data from investigation of different murine and human tumors it was proposed that certain immune cell subsets or produced factors indeed promote anti-tumor reactions [2].

Tumor cells are identified by effector cells via recognition of tumor antigens, some of which are also exploited in the field of cancer vaccination and antibody immunotherapy, on the surface of tumor cells. Tumor antigens were divided into two classes, either tumor-specific antigens expressed only on tumor cells [3], or tumor-associated antigens expressed both on tumor or normal cells alike with aberrant or dysregulated expression on tumors [4]. Following tumor recognition, cell and humoral anti-tumor immune responses leading to elimination of tumor cells are employed. Several studies reported immune cell infiltrates in tumor sites and draining lymph nodes in the tumor vicinity, mainly composed of T cells, natural killer (NK) cells, and macrophages. In some types of tumors it is a sign of good prognosis. However, few tumor cells can escape deletion and enter the equilibrium with the immune system. During this phase tumor cells that acquired resistance to immune reactions can be generated. Such cells may completely escape the immune surveillance, rapidly

proliferate and cause severe damage to the organism [2]. These three phases, i.e. elimination, equilibrium, escape, present parts of the process known as cancer immunoediting (Figure 1.1).

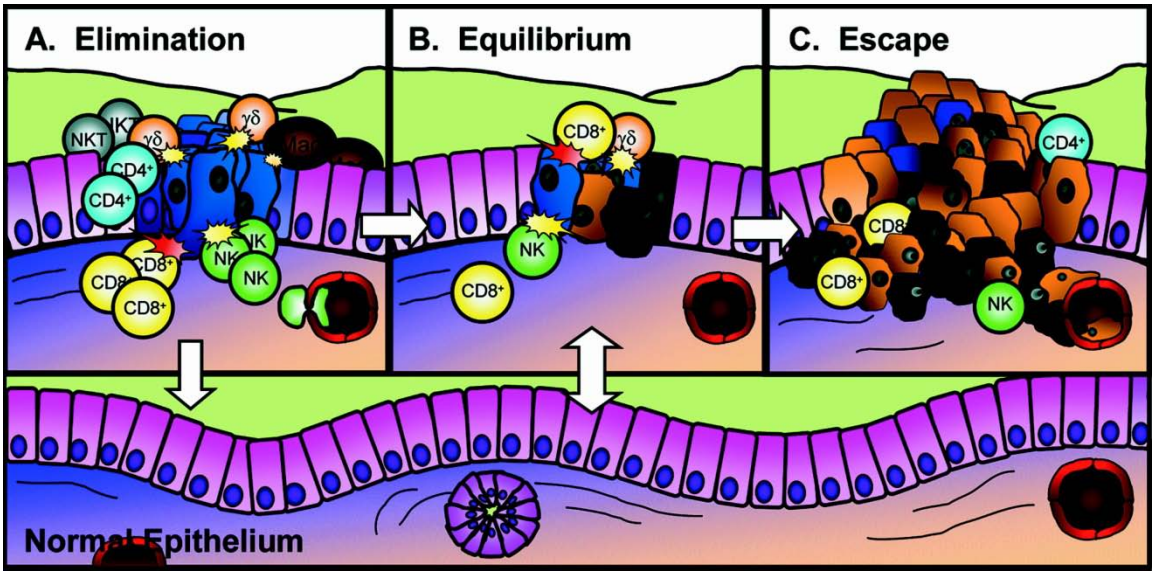


Figure 1.1: Three phases of cancer immunoediting. Adopted from Dunn *et al.* [2].

Unfortunately, immune system often fails to protect against growth of malignancies mostly due to suppressive effects of regulatory T (T_{reg}) cells. These cells protect tumors since they originate, after all, from normal cells of self origin thus being only weakly immunogenic, except for those induced by oncogenic viruses or carcinogenic agents. Moreover, tumors themselves have developed a number of mechanisms enabling them to escape from immune responses such as expression of pro-apoptotic ligands by tumor cells, down-regulated or absent expression of major histocompatibility complex (MHC) class molecules on the tumor cell surface or production of immunosuppressive factors (e.g. tumor growth factor-β, TGF-β) [5]. Apart from T_{reg} cells, tumors also exploit the anti-tumor activity of tumor-associated macrophages (TAMs) and neutrophils (TANs) [6] or myeloid-derived suppressor cells (MDSC) [5].

Malignant transformation of normal cells can be induced by various stimuli. Several carcinogenic chemicals (e.g. alkylating agents) [7] have been described, as well as the role of some viruses in carcinogenesis have been implied (e.g. Human Papilloma Virus (HPV) and its involvement in induction of cervical cancer development [8]). Nevertheless, the question of cancer development and its causes has been a matter of intensive research.

1.2 Cancer therapy

1.2.1 Radiotherapy

One of the approaches of cancer treatment is the use of ionizing radiation for elimination of tumor cells. Even though it has been successful in therapy of a number of tumors (e.g. early stages of breast carcinoma), it has serious disadvantages such as affecting and damaging normal tissues or high risk of secondary cancer development. Radiotherapy acts through direct or indirect damage of DNA that is mediated via photon or charged particles. Usually, it is combined with surgical removal of tumor tissue and chemotherapy [9].

The disadvantages of radiotherapy were reduced by targeted radiation beams [9] or modification of radioactive isotopes with various agents. Structures containing tumor-specific mAb covalently coupled to radioactive atom have been used for cancer radioimmunotherapy. They can exert site-specific elimination of tumor cells exploiting mAb's specificity and radioactivity of attached atoms. ^{90}Y-ibtritumomab tiuxetan (also with ^{111}In; Zevalin) and ^{131}I-tositumomab (Bexxar) targeted to the surface molecule of B cells and B-cell derived lymphomas, CD20 [10], belong among such structures. They show significant anti-tumor activity without the occurrence of side toxicities associated with conventional radiotherapy. Both agents possess potent anti-tumor reactivity against B-cell non-Hodgkin lymphoma and refractory B-cell lymphoma and were approved by Food and Drug Administration (FDA) for clinical use. Covalent attachment of tumor-specific mAb together with toxin and radioactive isotope forms so called radioimmunotoxins used in tumor eradication and in bone marrow transplantation [11]. Conjugate of phytotoxin ricin and ^{125}I both linked to anti-CD5 mAb was showed to specifically react against $CD5^+$ human leukemia cells *in vitro* and in nude mice *in vivo* [12].

1.2.2 Chemotherapy

A large number of low molecular weight (LMW) drugs of various origin and functions have been used in eradication or attenuation of tumor diseases for decades [13]. Commonly used anti-cancer drugs in chemotherapy are anthracycline antibiotics (e.g. doxorubicin), taxanes (e.g. docetaxel), vinca alkaloids (e.g. vinblastine), alkylating agents (e.g. cyclophosphamide), antimetabolites (e.g. methotrexate) or topoisomerase I and II inhibitors (e.g. camptothecin or etoposide, respectively). The main mechanism of

action of these drugs is destruction of rapidly dividing cells. Unfortunately, this includes not only tumor cells but several normal cell subsets such as bone marrow cells or hair follicles as well. Moreover, most of these agents are highly immunosuppressive and abrogate induction of anti-tumor immune responses. Therefore, development of therapeutics with lowered toxicities or modification of drugs already used in clinical practice have been in progress in order to improve cancer treatment and diminish side effects, including immunosuppression.

In order to overcome above mentioned disadvantages, structures containing tumor-specific mAb with attached cytostatic drug or toxin destroying tumor cells were developed and are also known as immunotoxins. Following FDA approval, immunotoxin of mAb against CD30 (some lymphomas) with anti-tubulin mitosis-blocking agent vedotin bound via degradable bridge have been used for treatment of Hodgkin's lymphoma and anaplastic large cell lymphoma [14-15]. Several other immunotoxins are under investigation in clinical trials.

Another promising way of cancer treatment improvement is development of high molecular weight (HMW) systems for drug delivery, e.g. liposomes [16], self-assembling micelles [17], dendrimers [18], nanoparticles [19] or water soluble polymers [20]. These systems ensure that attached (or entrapped) drugs do not damage normal tissues and are selectively accumulated in solid tumors via Enhanced Permeability and Retention (EPR) effect. This effect was proved to work in most types of solid tumors. Abnormal vasculature of tumor tissue characterized by discontinuous epithelium enables extravasation of macromolecules from circulation into the tumor site and due to the insufficient lymphatic drainage, they are accumulated in tumor tissue unlike small molecules which quickly diffuse from the tissue back to the circulation via capillaries (Figure 1.2) [21]. One of the most intensively studied delivery system is based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) (see chapter 1.3). HPMA copolymer-bound drug conjugates are characterized by significantly lowered toxicity and prolonged circulation half-life in comparison to free drugs. Even though some of these conjugates showed potent anti-tumor activity *in vitro* and *in vivo* on a number of tumor models, none of them have been approved for cancer treatment so far.

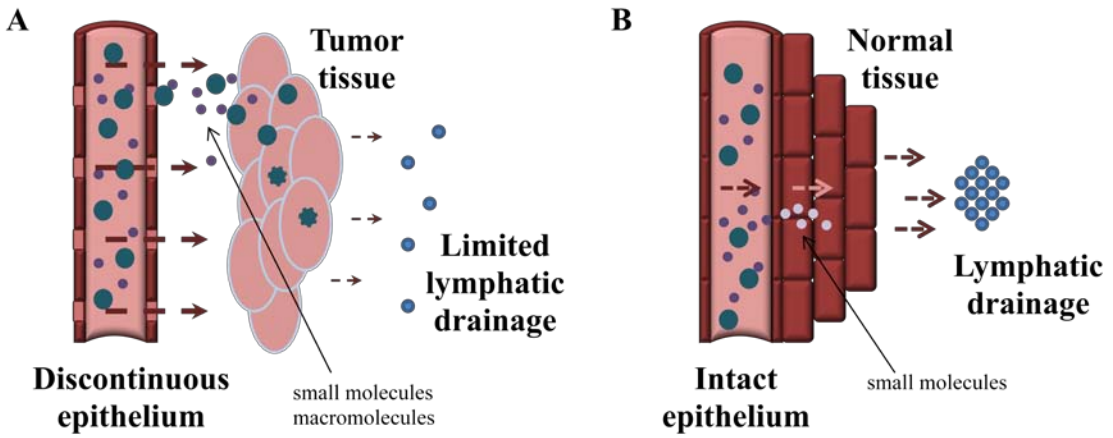


Figure 1.2: Scheme of Enhanced Permeability and Retention (EPR) effect in tumor tissue (A) in comparison to normal tissue (B).

1.2.3 Immunotherapy

There have been vigorous efforts to induce immune responses against tumors in tumor-bearing patients. Several specific and non-specific agents have been used for immunotherapy and some of them showed such promising anti-tumor activity to enter clinical trials or even being approved by FDA for cancer treatment. Non-specific immunostimulatory agents include interleukin-2 (IL-2), interferon α (IFN-α) [22-23] or adjuvant-like agents (bacillus Calmette-Guerin). IL-2 was approved by FDA to be used in high doses for treatment of metastatic renal cell carcinoma and malignant melanoma. However, this therapy is limited by severe side toxicities [24-25]. Clinical trials focused on IL-2 treatment of certain autoimmunities have been conducted [26] together with the studies concerning its modifications in order to improve its clinical potential (see chapter 1.4.3). The intravesical bacillus Calmette-Guerin (BCG) therapy has been successfully used for treatment of high-risk and non-muscle invasive bladder cancer since its FDA approval even though it is accompanied by severe side toxicities resulting from application of the live pathogen. The research focusing on improvement of this approach is currently in progress, investigating the possibilities of modification of BCG or application of non-live vaccines derived from BCG [27].

Other approach includes development of cancer vaccination. The first therapeutic anti-tumor vaccine used for treatment of metastatic castration-resistant prostate cancer was approved by FDA in 2010 [28]. Vaccine is directed against tumor-specific antigen (prostate acid phosphatase, PAP) of prostate cancer using patient's dendritic cells activated with fusion protein of PAP and granulocyte-macrophage colony stimulating

factor (GM-CSF). This induces generation of PAP-specific effector T cells upon injection back to the patient.

Potent tool of cancer treatment is adoptive cell therapy based on a transfer of autologous tumor infiltrating immune cells [29]. Such immune cells are present in tumor site in an anergic or tolerogenic state due to tumor microenvironment. After isolation from tumor, they are cultured and activated *ex vivo* and subsequently returned back into the patient together with adjuvants and/or growth factors. This approach has been successfully used for treatment of metastatic melanoma. Moreover, genetic modification of autologous lymphocytes with retroviral vector encoding tumor antigen-specific T cell receptors (TCR) has been reported and these cells have been tested for treatment of patients suffering from common epithelial cancers. Apart from that, genetically modified effector T cells expressing chimeric antigen receptor for B-cell antigen CD19 were reported to be reactive against B cells and potent for treatment of chronic lymphocytic leukemia [30].

Nevertheless, probably the most explored field of cancer immunotherapy is application of mAbs to defined tumor-specific or tumor-associated antigens. Among those belong rituximab (Mabthera; targets CD20 on B cell lymphoma) [31] or trastuzumab (Herceptin; targets HER2 on certain breast cancers) [32]. Several antibodies, such as anti-CD25 mAb or agonistic anti-GITR mAb, have been used for elimination of T_{reg} cell population in order to overcome T_{reg} cell-mediated suppression of anti-tumor immune responses. Besides monospecific mAbs, bispecific mAbs were developed. They form a bridge between tumor cells and effector T cells, which enables specific T cell-mediated elimination of tumor cells. Phase I/II clinical trials of blinatumomab specific for CD19 (expressed on B-cell lymphoma) and CD3 (expressed on T cells) showed promising anti-tumor immunity against non-Hodgkin's lymphoma and acute lymphoblastic leukemia [33].

1.3 HPMA copolymer-based conjugates

1.3.1 Biological properties and structure of polymer-bound drug conjugates

Construction of polymer-bound drug conjugates was a result of efforts to overcome disadvantages of classical chemotherapy with LMW drugs such as severe side toxicity and multidrug resistance of cancers [34-35]. Polymer carriers have been used to transport selected drug(s) or biologically active agent(s) in their inactive form to the target tumor site or tumor cell compartment where they are released from the polymer backbone and activated without damaging normal tissues.

The basic structure of a polymer-bound drug conjugate contains water soluble polymer carrier bearing desired anti-cancer agent bound via biodegradable linker [34-35]. Hydrophilic character of the carrier confers mostly hydrophobic drugs with water solubility and better availability. Moreover, due to the high molecular weight, polymer-bound drug conjugates are able to passively accumulate in the solid tumor tissue via Enhanced Permeability and Retention (EPR) effect [21], while the addition of targeting moiety (e.g. tumor antigen-specific antibodies) to the conjugate results in its specific and active accumulation in the tumor site in addition to EPR effect [36]. Furthermore, polymer-bound drug conjugates are characterized by their long persistence in the circulation, thus increasing the half-life of attached drug and lowering its side toxicity [34]. Another advantage is a change of the route of drug cell entry; free drugs enter cell directly via plasma membrane, whereas polymer-bound drug conjugates enter through endocytosis. That leads to partial bypass of multidrug cell resistance (MDR) based on pump-mediated drug efflux.

1.3.2 HPMA copolymer-bound drug conjugates

Since 1970s, when *N*-(2-hydroxypropyl)methacrylamide (HPMA) homopolymer was designed and synthesized for the first time [37], the intensive research concerning polymer carriers based on HPMA (Figure 1.3) has been in progress.

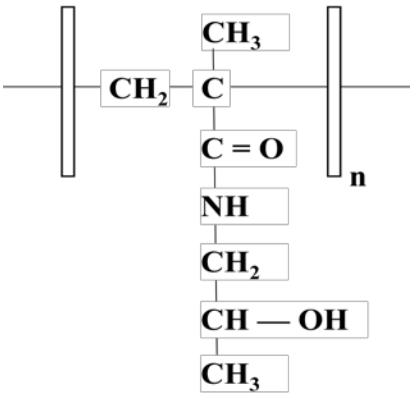


Figure 1.3: HPMA monomer.

The main reason is biocompatibility and immunocompatibility of HPMA homo- and copolymer making it almost ideal drug carrier together with its ability to carry multiple pendant groups due to several functional groups present on the polymer backbone [35, 38-40]. It was reported the HPMA homo- and copolymers exert no side toxicity, do not bind plasma proteins and do not form deposits in the organism. However, they are not biodegradable and are excreted via renal filtration if smaller than approximately 45 kDa (defined renal excretion limit) [35, 39-40]. Concerning the immunogenicity, HPMA homopolymers were proved not to trigger any immune responses, whereas HPMA copolymers containing oligopeptide sequences were observed to be weakly immunogenic with immunogenicity positively correlated with molecular weight of the copolymer [41-43]. Attachment of various drugs to the polymer backbone led to significantly reduced toxicity [35, 43-44]. Moreover, proteins bound to the HPMA copolymers induced about 250-fold lower antibody titers than their native forms [35, 39]. A number of various substances have been bound to HPMA copolymer, either alone or in combination [34, 45]. Among those, the most studied is probably anthracycline antibiotic doxorubicin (DOX).

There are two basic ways of drug release from HPMA-based polymer carrier depending on the bond through which they are attached to the polymer. The first type of conjugates bears amide bond which is sensitive to enzymatic cleavage, with tetrapeptide GlyPheLeuGly (GFLG) being the most widely used enzymatically degradable linker [46-47]. The other type of conjugates utilizes hydrazon bond or *cis*-aconityl acid residue and is therefore susceptible to pH-controlled hydrolysis. Such conjugates are stable in the bloodstream (pH 7,4) and hydrolyzed in acidic environment of endosomes and lysosomes or in acidic microenvironment of tumor tissue [48-50].

In order to increase the ability to accumulate in tumor site, HMW HPMA copolymer-bound drug conjugates were developed. They were designed in a way ensuring their biodegradability into short linear polymer chains so they could be excreted via kidney [51-52]. HPMA copolymers containing enzymatically degradable oligopeptide cross-links connecting short linear polymer chains into the branched structure were firstly synthesized. However, the synthesis was difficult to reproduce and generated carriers had high polydispersity. Subsequently, conjugates of polymers with dendrimer core [52] or graft polymers with semitelechelic HPMA homopolymer side chains bound to the multivalent HPMA copolymer backbone [51] were designed. Both types of conjugates carried DOX bound through pH-sensitive hydrazone bond to the polymer backbone. Apart from the above mentioned structures, HPMA copolymer-bound drug conjugates forming supramolecular micellar nanoparticles 13-37 nm in diameter in aqueous solutions were also developed [53].

1.3.2.1 HPMA copolymer-bound doxorubicin conjugates

The first HPMA copolymer-bound DOX conjugate DOX^{AM}-PHPMA (FCE 28068, also known as PK1) was designed in the middle of 1980s as a conjugate of linear HPMA copolymer backbone bearing DOX bound via amide bond to the GFLG side linker [47]. It belongs to non-targeted HPMA copolymer-bound drug conjugates and it is characterized by prolonged serum half-life and increased accumulation in solid tumors [54]. Preclinical experiments showed its potent anti-tumor activity *in vitro* and *in vivo* against variety of tumor models derived from mouse, rat or human cell lines [55]. These results led to the assumption it could be useful therapeutic agent in cancer treatment. Phase I/II clinical trials [54, 56] showed 4 to 5-fold reduced side toxicity of DOX and increased biological activity in selected tumors (e.g. chemotherapy resistant breast carcinoma). Other non-targeted HPMA copolymer-bound DOX conjugates include conjugates containing DOX bound via pH-sensitive bond to the polymer. They were described to have potent anti-tumor effects on tumor cell lines *in vitro* [50, 57] as well as on established tumors *in vivo* [57-58].

HPMA copolymer-bound DOX conjugates could be further modified with attachment of targeting moiety. Among various agents used for site-specific delivery of conjugates belong antibodies, lectins, carbohydrates, asialoglycoproteins or hormones [36]. Nevertheless, the most exploited targeting agents are antibodies. The random binding of antibody primary amino groups to the polymer chains, i.e. “classic structure”

(Figure 1.4), proved to be only partially efficient due to several disadvantages; mainly high polydispersity and possible decrease of antibody binding activity. Contrary, conjugates with “star structure” (Figure 1.4) were reported to have higher anti-tumor potential and lower polydispersity than the above mentioned structures both *in vivo* and *in vitro* [59-60]. They have antibody as a central molecule surrounded by semitelechelic HPMA copolymer chains bearing DOX bound through pH-sensitive hydrazone bond or enzymatically degradable amide bond.

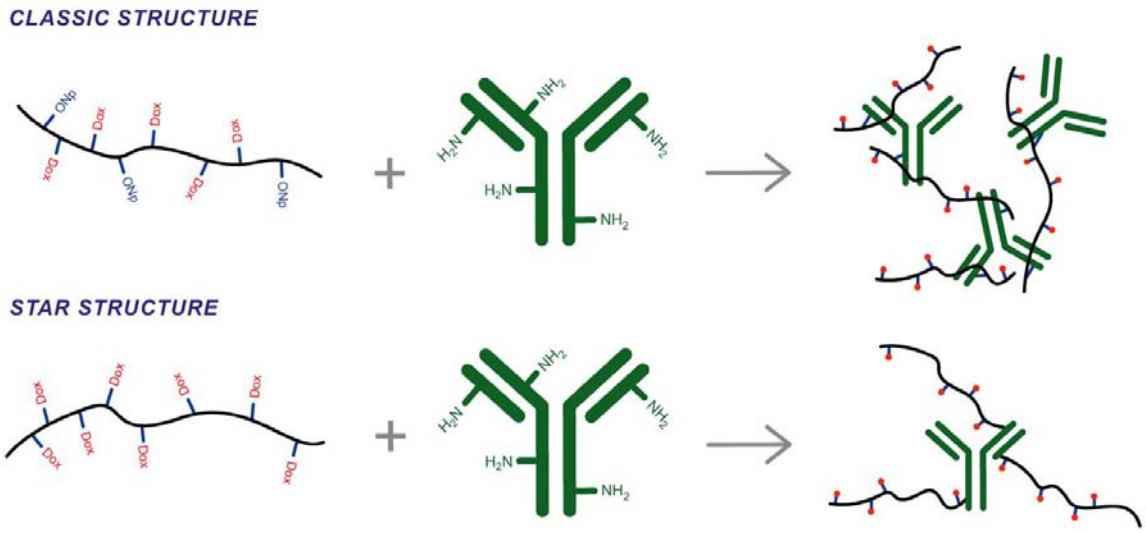


Figure 1.4: Schematic picture of classic and star structure of antibody-targeted HPMA copolymer-bound DOX conjugates.

Potential of several HPMA copolymer-bound DOX conjugates in cancer therapy, either targeted or non-targeted, was investigated in several clinical trials. Preclinical data showed very promising anti-tumor activity of several conjugates, some of them even inducing complete tumor regression and establishment of long-lasting tumor resistance [61]. Apart from PK1 mentioned above, DOX^{AM}-PHPMA-galactosamine (FCE 28069, also known as PK2) targeted to asialoglycoprotein receptor entered phase I/II clinical trials but further testing was abandoned due to the comparable accumulation of conjugate in tumor site and normal liver tissue alike [62]. Pilot clinical study focusing on therapeutic effects of HPMA copolymer bearing DOX or epirubicine together with autologous or human polyclonal immunoglobulin was performed in Prague [63]. Tested patients were suffering from metastatic generalized cancer and underwent all available cancer therapy. Conjugate of epirubicine bound to HPMA copolymer targeted with autologous IgG was tested on metastatic angiosarcoma bearing

patient and showed anti-tumor activity and no side toxicity. Conjugate of DOX bound to HPMA copolymer either targeted with autologous IgG or human immunoglobulin (DOX-HPMA-HuIg) was tested on five patients suffering from metastatic breast cancer. Positive response to the treatment, disease stabilization and overall survival from 6 to 18 months after the initial application of the conjugate was observed in three tested patients. DOX-HPMA-HuIg was proved to be stable, non-toxic, and to have anti-tumor and immunoprotective character. Nevertheless, there are no ongoing clinical trials investigating this conjugate and its potential for cancer treatment.

Other HPMA copolymer-bound drug conjugates that entered clinical trials showed varying anti-tumor activity but up to this date, there is no trial concerning HPMA copolymer-bound DOX conjugates in progress.

1.4 Interleukin-2

Cytokines compose vast family of glycoproteins and peptides that is involved in regulation of immune responses and maintenance of immune homeostasis via intercellular signaling. They can act as soluble or membrane bound factors produced by variety of cells of lymphoid and non-lymphoid origin [64]. One of the most prominent and studied cytokine is interleukin-2 (IL-2) as it is the first described interleukin in terms of gene and protein structure, together with its receptor identification [65]. Since it plays an essential role in T cell development, proliferation and survival it is involved in regulation of immune reactions as well. Functions and biological effects of IL-2 have been exploited in several clinic approaches including treatment of cancer, autoimmunities and chronic viral infections [24-26, 66].

1.4.1 Receptor

IL-2 exerts its activity through binding to IL-2 receptor complex (IL-2R) [67] that is composed either from IL-2β (CD122) and common cytokine receptor γ-chain (CD132) subunits and form a dimeric receptor binding IL-2 with intermediate affinity ($Kd=10^{-9}$ M), or from IL-2α (CD25), CD122 and CD132 subunits forming trimeric high-affinity ($Kd=10^{-11}$ M) receptor complex. Moreover, CD25 can bind IL-2 without the need for the other subunits, although with low affinity ($Kd=10^{-8}$ M).

The specificity of IL-2R for IL-2 is conferred by CD25 that is present only in IL-2R [67]. Other IL-2R subunits are rather promiscuous as they can be found in other receptor complexes as well, CD122 being a part of IL-15 receptor and CD132 being a subunit in receptors of a whole cytokine family that, beside IL-2, includes also IL-4, IL-7, IL-9, IL-15 and IL-21.

Dimeric receptor is mainly present on $CD122^{high}$ populations such as memory phenotype $CD8^+$ T cells and NK cells, in lower amounts on naïve $CD8^+$ T cells and memory $CD4^+$ T cells and at extremely low levels on naïve $CD4^+$ T cells. In order to be able to respond to physiologic concentrations of IL-2 it needs to be expressed in high levels [68]. Nevertheless, it is not sufficient enough to bind steady state low levels of IL-2, whereas high concentrations or exogenously administered IL-2 triggers IL-2 signaling even through the dimeric IL-2R and induce proliferation [69].

Trimeric receptor is up-regulated on the surface of activated T cells and B cells and constitutively expressed on T_{reg} cells [68]. Its high affinity for IL-2 is mediated by CD25 which is co-expressed with CD122 and CD132 upon TCR-stimulation. Wang *et al.* [70] crystallized the structure of quaternary complex of IL-2 and trimeric receptor and showed four binding sites (IL-2/CD25, IL-2/CD122, IL-2/CD132, CD122/CD132) mediating its formation (Figure 1.5). Moreover, it seems there is no contact between CD25 and CD122 or CD132, and the induction of structural changes in IL-2 upon CD25 binding, which stabilizes it and makes it accessible for CD122/CD132 complex, was reported as well. The IL-2/IL-2R quaternary complex is rapidly internalized and its components degraded with exception of CD25 that is recycled back onto the cell surface [70].

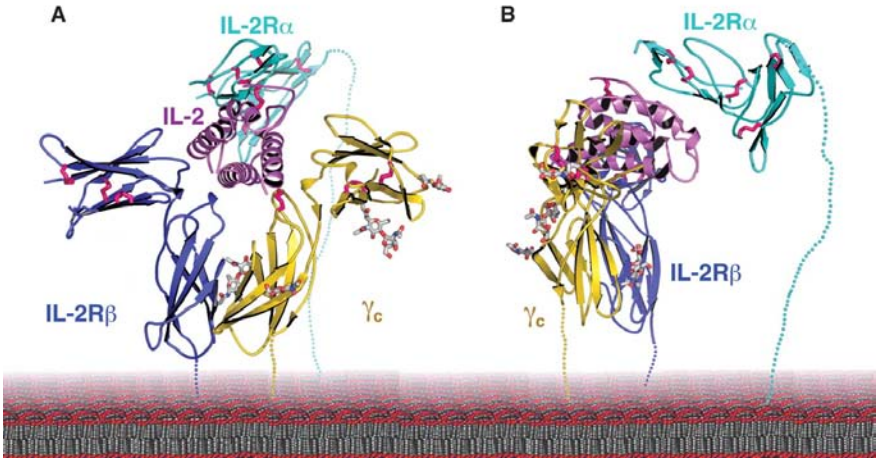


Figure 1.5: Ribbon diagram of quaternary complex of IL-2/IL-2R. The complex is shown in two views related by a 90° rotation about vertical axis. IL-2 is shown in purple, IL-2Rα in cyan, IL-2Rβ in blue and IL-2Rγ_c in gold. Adopted from Wang *et al.* [70] with minor changes made.

1.4.2 Biological functions

IL-2 was defined as a crucial pro-survival factor of T cells playing key role in their development, proliferation upon activation, differentiation into cytotoxic T cells and homeostasis of T cell memory populations [67, 71]. It was described to be secreted by effector $CD4^+$ T cells upon TCR stimulation as well as by other cell subset, though in a lesser extent, including $CD8^+$ T cells, NK cells, NKT cells and some of the activated dendritic cells (DCs) or mast cells [72-74]. Its production was not recorded in T_{reg} cells, since it is repressed by Foxp3 transcription factor, thus they are completely dependent on IL-2 excreted from other cells [75] contrary to above mentioned cell subsets which can use IL-2 in autocrine manner. Some studies reported IL-2 could be captured by CD25 on the surface of DCs and presented in *trans* to T cells *in vitro* [76]. It could be also associated with endothelial and smooth muscle cells of arteries [77] or even bound to the extracellular matrix heparan sulphate that regulates its availability to cells [78].

Besides the importance for effector T cell functions and homeostasis and thus promotion of immune system activation, IL-2 act as an essential molecule for T_{reg} cell development, survival and T_{reg} cell-mediated suppression of immune reactions (see below). Moreover, it appears IL-2 triggers NK cell proliferation and cytolytic activity as well as influence B cell immunoglobulin production [79-80]. Therefore, IL-2 plays a dual role in the immune system regulation.

1.4.2.1 IL-2 and effector T cells

As mentioned above, T cell subpopulations are dependent on IL-2/IL-2R pathway in many ways. Upon TCR/peptide-MHC and costimulation (CD28/CD80 or CD86) engagement, cells start to produce IL-2. Activated T cells then utilize generated IL-2 in autocrine/paracrine manner and $CD4^+$ or $CD8^+$ T cells subsequently start to differentiate into effector or memory cells [81-83] depending on the duration and intensity of the IL-2 signal. Strong and sustained signaling results in generation of effector clones [82, 84], whereas low-level signal leads to differentiation into memory phenotype in case of $CD8^+$ T cell [84], and into memory phenotype or follicular T helper cells in case of $CD4^+$ T cells [82].

1.4.2.2 IL-2 and immunological tolerance

There are many studies reporting severe autoimmunities leading to premature lethality of mice with disrupted IL-2 signaling either being a result of the IL-2 absence or dysfunction, or disruption of receptor function [73, 85-87]. The main cause of this observation is probably due to the fact that IL-2 is crucial for induction of Foxp3 expression in immature thymic T_{reg} cells [88], for T_{reg} cell proliferation and maintenance of metabolic fitness and peripheral T_{reg} cell homeostasis [73, 89].

Several studies reported that IL-2 signaling influences reciprocal balance of T_{reg} and T_h17 population via interference with IL-6 signaling important for T_h17 development and promotion of the induction of T_{reg} cell phenotype [81]. On the other hand, generated T_{reg} cells in turn consume IL-2 and deplete it from the environment which facilitates T_h17 cell differentiation [90]. This led to suggestion that IL-2 application could prevent IL-17-mediated inflammation. Together with recently described phenomenon of IL-2-mediated inhibition of germinal center follicular helper T cell development [80, 91], these data show IL-2 mediates control of inflammatory as well as humoral immune reactions.

1.4.2.3 IL-2 in immunotherapy

IL-2 and its immunostimulatory functions, mainly the promotion of $CD8^+$ T cell-mediated responses, were exploited in the research of therapy of several autoimmune or immunopathologic diseases. Even though it was approved by FDA for treatment of malignant melanoma and metastatic renal cell carcinoma with complete response in minor fraction of patients [24-25], its clinical application bears a number of disadvantages. Since it has an extremely short half-life in circulation [92], the dosage have to be rather high with maximum tolerated dose of intravenously administered IL-2 determined to be 10 mg/m^2 in a bolus or 1 mg/m^2 in a 24h continuous infusion [79]. High-dose therapy, however, results in development of severe side toxicities, such as vascular leak syndrome associated with pulmonary edema, liver cell damage or renal failure. The toxicity was thought to be mainly caused by activation of NK cell via IL-2 binding to dimeric IL-2R on their surface [79] but recently it was shown it is probably the consequence of IL-2 interaction with CD25 on the surface of endothelial cells [93]. Nevertheless, IL-2 administration, apart from tumor-specific effector T cells, induces robust proliferation of T_{reg} cells that could impede the IL-2 treatment. Thus it was

suggested to combine this therapy with the selective T_{reg} cell-expansion inhibition that could promote the effectiveness of IL-2 application [94].

Besides high-dose IL-2 treatment in case of tumor therapy, it was investigated whether the low-dose IL-2 treatment could lead to improvement of patients suffering from of HCV-induced vasculitis characterized by reduced T_{reg} cell counts. Saadoun *et al.* [26] reported in their clinical trial study such approach is promising as they observed recovery of T_{reg} cells and no induction of effector T cell activation or side toxicity, followed by the improvement of autoimmune conditions.

Potential use of IL-2 in treatment in chronic viral infections was investigated e.g. in human immunodeficiency virus (HIV) infected patients. However, IL-2 effectivity for HIV therapy was not proved to be sufficient enough and research concerning this matter was stopped by FDA since number of disadvantages (side toxicities, prize) exceeded potential advantages (increase of T cell population counts) of this approach [66].

1.4.3 Modifications

There are a number of studies focusing on modification of IL-2 in order to overcome the disadvantages of IL-2 treatment, mainly side toxicity associated with high-dose therapy together with the “non-specificity” of IL-2 stimulatory effects, and improve IL-2 biological activity and stability to make it more useful for immunotherapy. Mostly, IL-2 was conjugated with HMW molecules conferring it with possible prolonged maintenance in circulation.

1.4.3.1 IL-2 attachment onto polymer carriers

Strategies concerning conjugation of IL-2 and hydrophilic polymer carriers showed promising results in mice. In case of polyethylene glycol (PEG)-IL-2 conjugate, its intravenous administration into murine system proved it has significantly prolonged half-life and superior anti-tumor activity than free IL-2 [95]. However, clinical study of its beneficial effects in humans showed no significant difference between anti-tumor activity of PEG-IL-2 and free IL-2 and no improvement of tumor patients’ conditions was recorded [96].

Another polymer used as a carrier of IL-2 is based on HPMA. At first, it seemed to be rather potent since it had superior biological activity and significantly prolonged circulation half-life in comparison to free IL-2 *in vivo.* The investigation of its effects on

cell subsets showed it induced robust expansion of recently activated $CD8^+$ T and NK cells as well as T_{reg} cells [97]. However, its anti-tumor activity tested on murine tumor models was negligible and it was claimed to be not suitable for cancer treatment. Nevertheless, it was suggested to be useful as a potent adjuvant in vaccination protocols.

1.4.3.2 Fusion protein of IL-2 and tumor-specific mAbs (immunocytokines)

The potential of complexes covalently linking IL-2 and C'-terminus of heavy chain of selected tumor-specific mAbs, structures also known as immunocytokines, were investigated by several research groups [98]. Preclinical data indicated their strong potential for cancer therapy, since they showed potent tumor antigen-binding activity as well as activation of tumor-specific effector T and NK cells in murine tumor models. Indeed, phase I/II clinical trials of certain IL-2 immunocytokines showed promising improvement of cancer treatment, e.g immunocytokine L19-IL-2 (Darleukin) has significant anti-tumor activity against metastatic melanoma and metastatic renal cell cancer [99].

1.4.3.3 IL-2 modification by serum proteins

Another approach exploring the potential of IL-2 modification is based on fusion of IL-2 and serum proteins. Fusion protein of recombinant human serum albumin (rHSA) and recombinant human IL-2 (rhIL-2), also known as albuleukin, showed significantly prolonged circulation half-life (6-8h) and potent anti-tumor activity [100]. Unfortunately, phase I/II clinical studies proved it is not suitable for cancer treatment.

Vaccination by tumor cells genetically engineered to produce chimeric protein of IL-2 and IgG (structure also known as immunoligand) showed greater potential of such cells to induce local anti-tumor reactions leading to tumor rejection [101] than injection of tumor cells transfected with IL-2 gene alone. IL-2 is bound to the N'-terminus of IgG constant regions and this chimera is able to exert IL-2 stimulatory effects on cell populations as well as immunoglobulin effector function, i.e. Fc-triggered complement-mediated cell lysis [102].

1.4.3.4 IL-2/anti-IL-2 mAb immunocomplexes

The first reports focusing on and describing a formation of cytokine/anti-cytokine mAb immunocoplexes was published in 1990s [103]. Such complexes are less potent

than free cytokines *in vitro*, but *in vivo* they possess significantly higher biological activity, probably due to prolonged circulation half-life, in comparison to free cytokines. These complexes were reported to be formed by several cytokines such as IL-2, IL-3, IL-4, IL-5, IL-6 or IL-7 [69, 103-105].

Studies of IL-2/anti-IL-2 mAb immunocomplexes (IL-2 immunocomplexes) showed their significant potential in immunotherapy of various diseases, predominantly cancer treatment [92], induction of bone marrow cells expansion after bone marrow transplantation [69], prevention of graft rejection and attenuation of autoimmune disorders [106], since their application is accompanied by much lower toxicities than those seen in treatment with free IL-2 and their biological effects are far superior [69, 93]. The half-life of these complexes in mice was determined to be ~3h [92], while half-life of IL-2 in mice is approximately 3.7± 0.8min thus enabling lowering the dosage required for effective treatment [107]. The seminal work of Boyman *et al.* [69] describes formation of IL-2 immunocomplexes upon co-injection of recombinant IL-2 (rIL-2) with particular anti-IL-2 mAb and their selective specificity depending on used mAb clone. They defined two basic types of IL-2 immunocomplexes represented by IL-2/anti-IL-2 S4B6 mAb (IL-2/S4B6) and IL-2/ anti-IL-2 JES6.1A12 mAb (IL-2/JES6.1) immunocomplexes specific for stimulation of distinct cell subsets in comparison to free IL-2 which induce expansion of cells in non-specific manner. It was suggested the different specificity of these immunocomplexes is probably due to binding of S4B6 and JES6.1 mAbs to different epitopes on IL-2 molecule, therefore modulating its ability to bind to either dimeric ($CD122^{hi}$ cells) or trimeric ($CD25^{hi}$ cells) receptor on the target cells.

Complexes of IL-2 with anti-IL-2 S4B6 mAb and related mAbs (human IL-2 specific MAB602 mAb, mouse IL-2 specific JES6.5H4 mAb) were initially reported to be highly stimulatory for $CD122^{hi}$ memory phenotype $CD8^+$ T and NK cells [69]. Later on, research data showed their potential to induce moderate proliferation of naïve activated $CD8^+$ T cells that are able to form functional memory $CD8^+$ T cell population, moderate proliferation of T_{reg} cells [92] and, furthermore, proliferation of NKT cells together with γδ T cells (Figure 1.6).

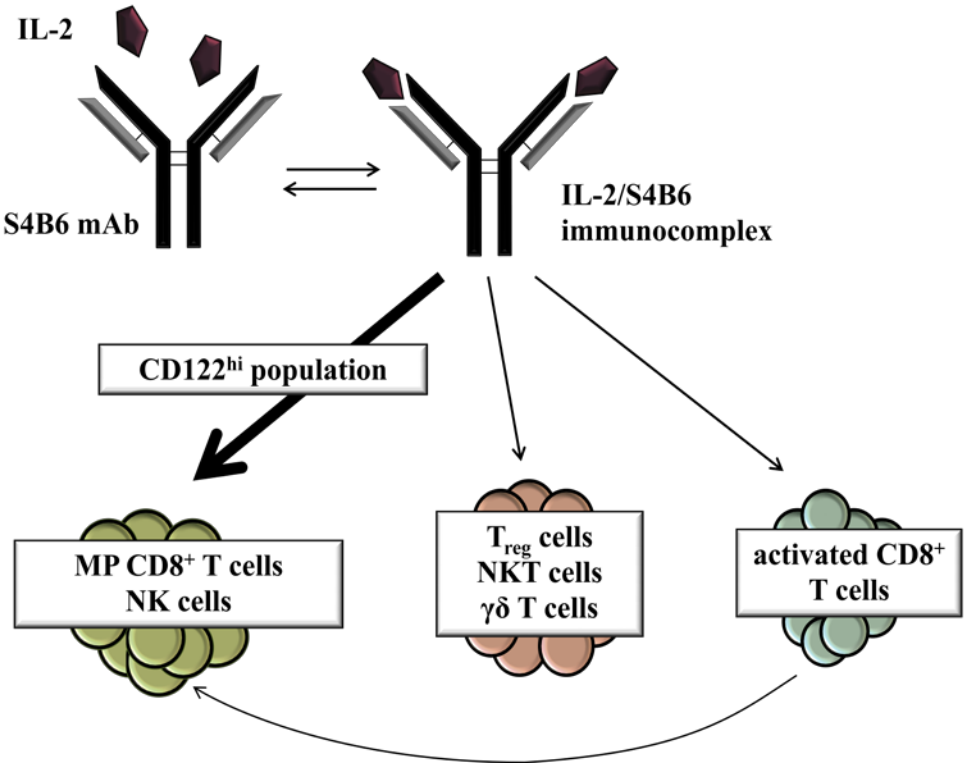


Figure 1.6: IL-2/anti-IL-2 S4B6 mAb immunocomplexes and their selective biological activity. MP, memory phenotype.

Higher cytolytic activity of IL-2/S4B6 immunocomplexes-stimulated NK cells could be further enhanced via addition of IL-12 [108]. Moreover, the administration of free S4B6 mAb results in the decrease of T_{reg} cells accompanied with the increase of memory phenotype $CD8^+$ T cells, presumably due to depletion of IL-2 by S4B6 mAb, thus imparing T_{reg} cell homeostasis, leading to the formation of memory phenotype $CD8^+$ T cell-specific IL-2/S4B6 complexes [69]. These data led to the investigation whether IL-2/S4B6 immunocomplexes could be useful in tumor treatment and therapy of chronic viral infections. Experiments on murine tumor models (e.g. metastatic melanoma (B16F10), or B-cell leukemia (BCL1)) showed their considerable anti-tumor activity if given prophylacticaly early after tumor cell inoculation [92], while their application in treatment of murine models of acute and chronic bacterial and viral infections proved their protective abilities [109-110]. The combination of IL-2/S4B6 immunocomplexes, alone or co-administered with IL-12, and HPMA copolymer-bound DOX conjugate showed potent synergy of this two anti-tumor agents and led to prolonged survival or complete cure of mice with established tumors [108]. Thus, IL-2/S4B6 immunocomplexes were suggested potentially useful for clinical treatment of above mentioned diseases.

Recently, Tomala *et al.* [111] designed and produced a protein chimera of IL-2 linked via oligopeptide spacer to the light chain of S4B6 mAb, thus the problem with possible dissociation of IL-2/S4B6 immunocomplexes or excess of either molecule was overcome (Figure 1.7). Its biological activity is similar to that of IL-2/S4B6 immunocomplexes *in vitro*, and probably even higher *in vivo*.

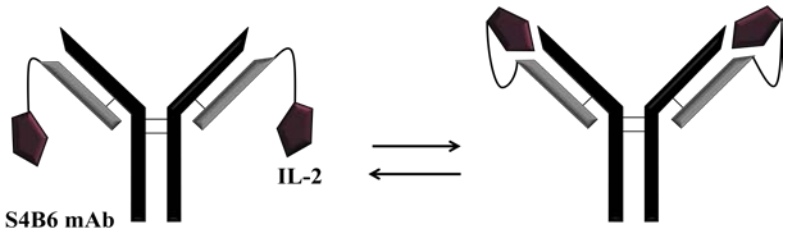


Figure 1.7: Schematic picture of protein chimera of IL-2 and anti-IL-2 S4B6 mAb.

On the contrary, complexes of IL-2 with anti-IL-2 JES6.1 mAb or related mAbs (human IL-2 specific 5344 mAb) are highly stimulatory for T_{reg} cells and for recently activated $CD8^+$ T cells (Figure 1.8) [69, 92]. Exploiting their ability to induce robust expansion of T_{reg} cells, Kovar *et al*. [112] provided the first direct prove that T_{reg} cells promote tumor growth and disease progression using a murine model of B-cell leukemia (BCL1). Nevertheless, there are many studies dealing with IL-2/JES6.1 immunocomplexes potential for induction of immune tolerance to grafts or establishment of state of tolerance in patients suffering from autoimmune disorders. Webster *et al.* [106] reported IL-2/JES6.1 immunocomplexes are able to successfully promote indefinite tolerance to allografts of pancreatic islets without the further need for immunosuppressive treatment. IL-2/JES6.1 immunocomplexes are also able to prevent the onset of experimental autoimmune encephalomyelitis (EAE) if given prophylactically or even suppress the ongoing disease if applied in combination with rapamycin. Furthermore, prevention of type I diabetes development in non-obese diabetic mice [113], suppression of allergic airway disease [114] or attenuation of Myasthenia gravis progression [115] were reported by other groups. Interestingly, the research team of Marek Kovar observed IL-2/JES6.1 immunocomplexes can exert potent anti-tumor activity probably via stimulation of expansion of recently activated $CD8^+$ T cells (unpublished data).

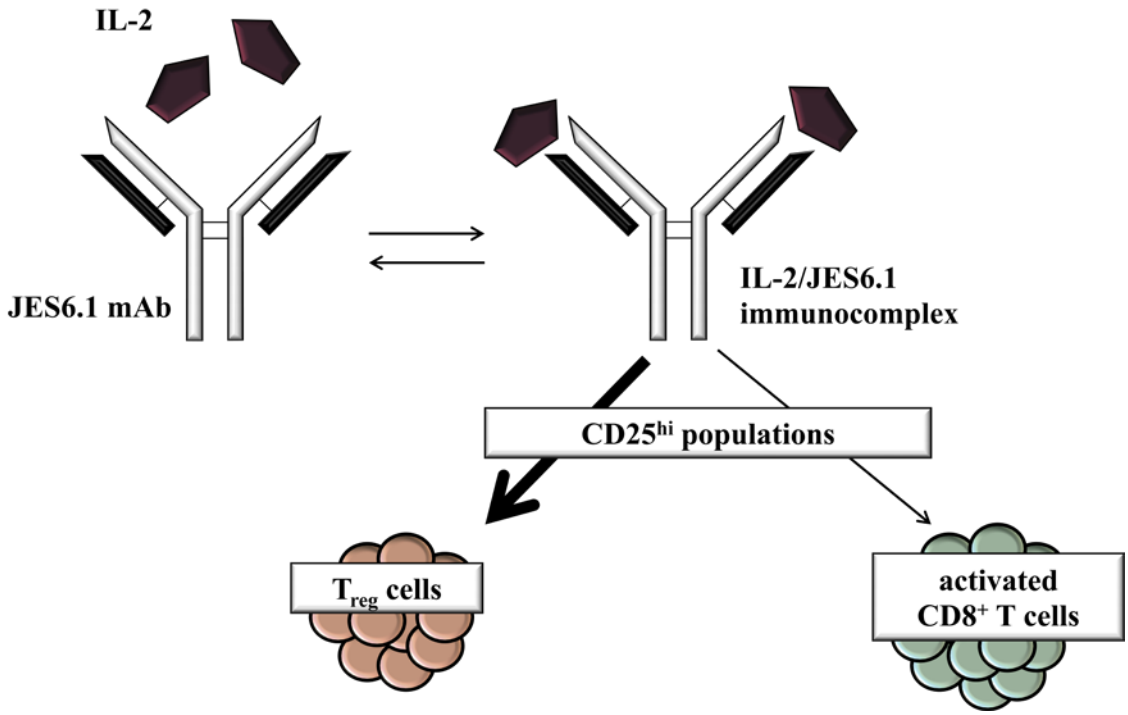


Figure 1.8: IL-2/anti-IL-2 JES6.1 mAb immunocomplexes and their selective biological activity.

In conclusion, IL-2 immunocomplexes present a potent tool for improvement of IL-2 based treatment of immunopathologies.

1.5 Regulatory T cells

The immune system represents complex machinery whose main function is to protect the organism against pathogens by distinguishing self and non-self antigens. It comprises of various different cell populations developing and differentiating mainly in primary and secondary lymphatic organs. The special role among immune cells has the population of regulatory T (T_{reg}) cells which maintains the immunological self-tolerance in periphery by suppression of autoreactive effector T cells and inhibition of various types of immune responses [116-117]. Besides preventing autoimmunities [118-119], T_{reg} cells have also a role in maintaining tolerance to gut microbial flora [120], in inhibition of graft rejection [118, 121-122], abnormal reactions to pathogens and non-pathogenic xenogeneic proteins (e.g. allergens, food antigens) [118, 123-125], or in impairment of anti-tumor responses [112].

1.5.1 T_{reg} cell characteristics and molecular pattern

Since the first reports of suppressive T cells in late 1960s, this subject has been rather controversial. Only after several years of research it was considered these suppressive cells form a functionally distinct population that is highly specialized for regulation of immune responses [118]. They are produced mostly in thymus (natural, nT_{reg} cells) and in lesser extent in periphery (induced, iT_{reg} cells) (see chapter 1.5.3). Their development, function and survival is highly dependent on various set of molecules especially cytokines and costimulators.

T_{reg} cells was defined as $CD4^{+}CD25^{high}Foxp3^{+}$ T cell population [117-118, 126-127]. CD4 molecule is one of the T helper cell markers being a co-receptor of T cell receptor (TCR) [64]. As for the CD25 molecule, it has a crucial role in T_{reg} cell function, survival and generation since it is the α chain of trimeric high-affinity IL-2 receptor complex that is able to bind IL-2 even at very low concentrations present in the body at steady state levels [70]. It was the first suggested surface marker of T_{reg} cells [118] as it is constitutively expressed on most of this cell subset, however, it occurs also on activated T and B cells and some other cell populations. The transcription factor Foxp3 (Forkhead box p3) also known as Scurfin is essential for the development and suppressive functions of T_{reg} cells and acts as master regulator [117, 126-127] that either activates or represses its target genes [128]. Originally, it was assumed Foxp3 expression defines immature thymocytes to undergo differentiation into T_{reg} cells, however studies investigating this matter indicate the commitment to T_{reg} cell lineage is before the induction of Foxp3 expression [129]. Accumulating data also show Foxp3 is not strictly T_{reg} cell-specific marker as it could be transiently expressed in other cell subsets, e.g. some activated T cells [130]. Foxp3 is essential for acquisition of suppressive phenotype and amplification and stabilization of molecular pattern of T_{reg} cell lineage [129] together with other environmental signals such as TCR, IL-2 and TGF-β signaling (see below). Dysfunction, attenuation or mutation of Foxp3 or its gene leads to the defective development of T_{reg} cells [117, 131] resulting in severe immunopathology characterized by multiorgan autoimmune and inflammatory disorder reported both in mice and humans alike [117, 132]. The expression of Foxp3 itself is regulated by number of transcription factors, as well as TCR signaling, cytokines and epigenetic mechanisms [133] - particularly methylation of Foxp3 locus which was reported to be crucial for stable maintenance of Foxp3 expression [134] and thus for stability of T_{reg} cell lineage.

Apart from above mentioned molecules there are other surface and intracellular molecules associated with T_{reg} cell phenotype. Recently, several studies suggested transcription factor Helios to be another marker of thymus-derived nT_{reg} cells. It is strongly expressed in nT_{reg} cells of mice and human alike, while its expression by iT_{regs} remains to be fully elucidated [135-136]. It was described that Helios binds *Foxp3* promoter [136] which led to the suggestion it might be involved in regulation of Foxp3 expression and thus suppressive activity of T_{reg} cells.

Costimulatory molecules and several other molecules expressed on the T_{reg} cell surface belong among essential molecules involved in T_{reg} cell development, function and survival. However, their expression is not restricted only to the T_{reg} cell subset as they can be found also on the surface of other cell populations such as activated T and B cells, exhausted effector T cells, double-positive and double-negative thymocytes, activated monocytes, macrophages and DCs. Currently there are no strictly T_{reg}-cell specific molecular markers and their determination has been under intensive investigation.

1.5.1.1 Costimulatory molecules

The CD28 immunoglobulin family members expressed on the surface of T_{reg} cells play essential role in the regulation of this cell lineage, especially CD28 and CTLA-4. In 2000, Salomon *et al.* [137] were the first to report CD28 signals are critical for homeostasis of T_{reg} cells, their thymic generation, survival and maintenance in periphery, as well as for a control of the onset and progression of autoimmune disorders. Later on, it was reported CD28 costimulation promotes induction of Foxp3 expression, up-regulation of GITR and CTLA-4, the initial signals for T_{reg} cell generation, and production of IL-2 by other T cells required by T_{reg} cells for survival [138]. CD28 exerts its functions via binding of its ligands CD80 or CD86 (B7-1 and B7-2, respectively) [139]. Even though it has an essential role in generation of thymus-derived T_{reg} cells, recent study showed CD28 signaling plays a dual role in T_{reg} cell development as it negatively regulates the generation of iT_{reg} cells in periphery [140]. Contrary, cytotoxic T-lymphocyte antigen 4 (CTLA-4, CD152) constitutively expressed on T_{reg} cells is an inhibitory molecule [141]. Although it is a structural homologue of CD28 [142] it exerts 20-fold higher avidity for its respective ligands (CD80, CD86) than CD28 [143] leading to the abrogation of CD28 signaling pathway, down-regulation

of CD80 and CD86 costimulatory molecules on antigen presenting cells (APCs) and B cells [144-145], hampering of DC maturation [145], and subsequent suppression of immune responses, e.g. inhibition of effector T cell activation, down-regulation of antibody-mediated responses [144] or prevention of the xenogeneic transplant rejection [146], thus protecting against autoimmune diseases [141, 144] and graft-versus-host disease onset [146], as well as hampering of anti-tumor responses [144].

Glucocorticoid-induced TNFR-related protein (GITR) belongs among the set of costimulatory molecules constitutively expressed on T_{reg} cells [147] as well as other cell subsets upon activation [148]. It has a role in suppression of T_{reg} cell functions upon binding of its respective ligand GITRL expressed on macrophages, DCs and B cells [147, 149]. GITR acts as a costimulator for TCR signaling and promotes TCR-triggered activation and proliferation of effector T cell [148]. The activation of GITR by agonistic anti-GITR mAbs can lead to the onset of severe autoimmune disorders [148] as well as initiation of anti-tumor reponses [150], whereas neutralization of GITRL results in reduction or prevention of autoimmune reactions [151].

1.5.1.2 Activation markers

Molecules that identify "activated" T_{reg} cells include lymphocyte activation antigen 3 (LAG-3) [152] and glycoprotein A repetitions predominant (GARP) [153]. LAG-3 is a transmembrane protein related to CD4 molecule that interacts with MHC class II molecules expressed on APCs and blocks CD4/MHC class II engagement [154] leading to down-regulation of costimulatory molecules and inhibition of DC and subsequent effector T cell activation [155]. T_{reg} cells expressing LAG-3 were described to mediate the suppressive functions via cell-cell interaction and via production of interleukin-10 (IL-10) and TGF-β immunosuppressive cytokines. Furthermore, it was reported LAG-3^+ T_{reg} cell subset is significantly expanded at tumor sites in cancer patients where it abrogates the anti-tumor immune responses [156]. Nevertheless, it plays an important role in mediation of activated T_{reg} cell suppressive functions and maintenance of immune tolerance.

Expression of GARP is strongly correlated with the expression of Foxp3 and CD25 on thymus-derived nT_{reg} cells [153] while iT_{reg} cells and T_h cell subsets show no expression of GARP even after the activation and it is thus considered to be a marker of activated nT_{reg} cells. On the other hand, transduction of $CD4^+CD25^-$ T cells with GARP

induced expression of Foxp3 and acquirement of suppressor activity [157]. Moreover, GARP was also proposed to be a putative T_{reg} cell receptor for TGF-β [158].

1.5.1.3 Other molecules

T_{reg} cells are characterized by expression of several other molecules such as low levels of IL-7 receptor α chain (CD127) [159] that is also down-regulated upon activation of T cells; enhanced expression of CD39, CD73 and CD44 [118, 159] that strongly correlates with Foxp3 expression; expression of CD45RO and MHC class II molecules [160], or high expression of DR3 (TNF receptor superfamily member 25, TNFRSF25) while its expression on effector T cells is quite low [159]. Potent immunosuppressive molecule expressed on the surface of T_{reg} cells or released in its soluble form from B cells and DCs is Ig-like type I transmembrane protein CD83 (or sCD83 for soluble form), however, its respective ligand has yet to be identified. The ability of T_{reg} cells to induce apoptosis in target cells is due to expression of high levels of FasL [161] on T_{reg} cell surface that interacts with Fas on target cells. Except for above mentioned molecules, Toll-like receptors (TLR) were reported to be present on the surface of T_{reg} cells (TLR-4, -5, -7 and -8) [162]. They confer the ability to control immune reactions to gut commensal microbial flora and non-pathogenic microbes. CD40L (CD154) also belongs among T_{reg} cell surface molecules involved in the maintenance of developing thymic T_{reg} cell homeostasis [163] and has a role in T_{reg} cell-mediated regulation of immune system via engagement of CD40 on APCs.

Human and murine T_{reg} cells express wide range of selectins (CD62L) [118], galectins, integrins and chemokine receptors [159]. Regulation of T_{reg} cell migratory activity between lymphoid and non-lymphoid tissues is mediated via chemokine receptors expressed on T_{reg} cells (e.g. CXCR3, CCR4, CCR6, CCR7 or CCR9), whereas the accumulation and retention of T_{reg} cells in target sites is maintained by α_E integrin subunit (CD103).

1.5.2 Biological functions and effector mechanisms

T_{reg} cells constitute specialized population that maintains immune tolerance by abrogation of aberrant immune responses to self and non-self antigens via suppression of effector T cells and APCs activation and functions. Functions and activity of T_{reg} cells are dependent on TCR stimulation, costimulation and high levels of IL-2 (see below). T_{reg} cell TCR ligands are peptide-MHC complexes where the peptides are

derived from normal self-antigens, tumor antigens, non-self antigens and allogeneic transplantation antigens. Upon TCR and/or IL-2 stimulation, T_{reg} cells down-regulate autoimmune reactions [118], confer protection to tumor cells [112] and allografts [118, 121] as well as allogeneic fetus [122]. They also prevent aberrant reactions to xenogeneic proteins (allergens) [118, 123-125] or to commensal microbes in gut [120].

Regulation of immune reactions is mainly mediated through cell-cell contact via several above described surface molecules, e.g. CTLA-4, LAG-3 or GITR. However, some T_{reg} cell subsets rely on production of soluble immunosuppressive molecules, especially cytokines. IL-10 has been reported to belong among anti-inflammatory cytokines with potent immunoregulatory functions [164]. It plays an important role in maintenance of immune homeostasis as its dysfunction leads into development of autoimmune disease. Its effect is mediated via inhibition of pro-inflammatory cytokines production, suppression of effector T cell proliferation or hampering of DC activation by down-regulation of costimulatory and MHC class II molecules on their surface. IL-10 was reported to be produced by almost all leukocytes: macrophages, monocytes, activated T cells and B cells. Production of IL-10 by T_{reg} cells is elusive since there are a number of contradictory studies [131, 165]. It is probable, that IL-10 is produced only by certain T_{reg} cell subsets.

In contrast to IL-10, tumor growth factor-β (TGF-β) is commonly considered to be one of the T_{reg} cells effector molecules. It is involved in down-regulation of autoreactive effector T cells [166] and anti-tumor immune responses [167] as it was shown that its deficiency or deficiency of its receptors results in fatal autoinflammatory scurfy-like disorder [166, 168]. It was suggested that latent TGF-β can bound to GARP on T_{reg} cell surface and either confer cell-cell mediated suppression or it can be released in its active form and act as a soluble molecule [158].

Recently discovered interleukin-35 (IL-35) was described to be specifically produced by murine T_{reg} cells [169-170] and it presents an additional effector molecule involved in T_{reg} cell-mediated suppression. Studies focusing on the expression of IL-35 by human T_{reg} cells seem to be rather contradictory [171-172]. Nevertheless, its absence or dysfunction in mice leads to impaired T_{reg} cell function, while the overexpression results in high suppressive activity of T_{reg} cells [169] and leads to IL-35-dependent conversion of naïve T cells into iT_r35 suppressive cells characterized by no expression of Foxp3, TGF-β and IL-10 [173]. Thus, IL-35 not only regulates the immune reactions, it also affects T_{reg} cell homeostasis.

Depletion of pro-survival molecules or factors by T_{reg} cells (e.g. IL-2 or cysteine elimination) [159], down-regulation of costimulatory molecules, or induction of indoleamin-2,3-dioxygenase (IDO) production by APCs leading to triggering of T_{reg} cell generation from conventional T cells, belong among other mechanisms of effector cell inhibition. Moreover, T_{reg} cells can directly induce apoptosis via engagement of Fas/FasL pathway, by secretion of granzyme A and B and perforin.

1.5.2.1 T_{reg} cells in tumor immunity

As mentioned above, T_{reg} cells are involved in abrogation of anti-tumor immune responses. Several studies showed their increase in tumor site, while their modulation or depletion led to tumor regression and possible induction of tumor-specific effector cell reactions [174]. The first direct evidence that T_{reg} cells present another tumor mechanism for immune system evasion was made in 2008 [112]. To impair T_{reg} cell protection of cancer and promote anti-tumor immunity, a number of approaches have been developed and entered clinical trials [174]. Among these belong application of antibodies specific for T_{reg} cell surface molecules such as anti-GITR agonistic mAb (clone DTA1) that reduces T_{reg} cell-mediated suppression and confers immunity to various mouse tumor models upon re-challenge, or anti-CTLA-4 mAb that induces tumor rejection and tumor immunity with no obvious effect on T_{reg} cell population in terms of number and function. Furthermore, application of anti-CTLA-4 mAb led in some tumor bearing patients to the onset of autoimmune diseases [174]. The application of anti-CD25 mAb (clone PC61.5) is widely used for T_{reg} cell depletion in various experimental schedules. Recently, it was reported that the main cell population playing the role of major effector of αCD25 mAb mediated T_{reg} cell depletion *in vivo* is Fcγ$RIII^+$ phagocytes. A single i.p. injection of αCD25 mAb is able to significantly reduce T_{reg} cell numbers with the difference between peripheral blood (~70% reduction of T_{reg} cells), spleen (40% reduction of T_{reg} cells) and lymph nodes (47% reduction of T_{reg} cells) [175]. It was reported that anti-CD25 mAb (clone PC61.5) promotes tumor regression in several mouse tumor models [174], however, a number of groups showed that it negatively influences not only T_{reg} cell functions, but also other activated $CD25^+$ effector cells important for development of anti-tumor responses [176-180].

Other approaches include protein chimera of IL-2 linked to the diphteria toxin via degradable spacer (denileukin diftitox) used for treatment of $CD25^+$ cutaneous T-cell

leukemia and lymphoma, or application of chemotherapeutics (e.g. cyclophosphamide) [174].

1.5.3 Development of T_{reg} cells

Originally, it was presumed T_{reg} cell differentiation and development mainly takes place in thymus where natural T_{reg} (nT_{reg}) cells are generated and instructed to maintain the immunological tolerance to self-antigens encountered in periphery thus preventing the onset of autoimmune disorders [118]. However, there have been increasing number of reports describing the generation of T_{reg} cells also in periphery (induced T_{reg} cells, iT_{reg}) that confer the tolerance to non-pathogenic xenogeneic proteins preventing allergies or inflammatory reactions to microbial flora of intestine [120, 123, 181]. These two subsets probably use non-overlapping TCR repertoires and their functions appear to be non-synonymous [181].

Many reports showed indispensability of several costimulatory molecules in T_{reg} cell development, such as CD28 signaling involved in thymic T_{reg} cell generation [137-138] or CD40/CD40L pathway maintaining homeostasis of thymic T_{reg} cells [163]. Apart from costimulation, role of cytokines is also essential. One of the most important cytokine for T_{reg} cell development, survival, proliferation and stability, is IL-2 whose role in the T_{reg} cell homeostasis was showed mainly on models based on the deficiency of IL-2 or impairment of its high-affinity receptor [73, 85-87]. In 2005 Setoguchi *et al*. [73] showed IL-2 is vital for the maintenance and activation of peripheral T_{reg} cells and its source are other T cells which suggest there is a feedback loop controlling these two cell subsets. However, the exact source of IL-2 until now is still not clearly identified, since apart from T cell it is possible IL-2 is produced also by other cells including DCs [72]. Moreover, IL-2 was described as one of the cytokine inducing the Foxp3 expression in the thymic T_{reg} cell precursors [88].

TGF-β was reported to be crucial for the homeostasis and maintenance of already generated T_{reg} cells [166, 168] as well as for the conversion of peripheral naïve $CD4^+CD25^-$ T cells into T_{reg} cells via the induction of Foxp3 expression [134]. Data concerning the importance of TGF-β in thymic T_{reg} cell development are contradictive since some reports show its dysfunction does not influence the number of thymic nT_{reg} cells [134, 168], while the other reports describe its indispensability for thymic T_{reg} cell generation [182-183], e.g. via protection of thymic T_{reg} cell precursors from cell death

induced by strong TCR engagement with self-antigen [183]. This matter needs to be further investigated.

1.5.3.1 Generation of nT_{reg} cells in thymus

Thymus is primary lymphoid organ specialized for the development of immunocompetent T cells. During the generation and differentiation, thymocytes undergo several selection steps that ensure deletion of defective or autoreactive thymocytes from T cell population pool before entering periphery. The elimination of immature self-reactive cells is termed negative selection although only the most aggressive autoreactive cells are deleted by this mechanism of central tolerance. Some of the autoreactive clones are able to escape to periphery where they are usually suppressed by the mechanisms of peripheral tolerance or undergo conversion to iT_{reg} cell subset [184]. However, recent study of Enouz *et al*. [185] indicates some of autoreactive cells that escaped into periphery are not anergized or converted, but can be activated by certain infections and can induce the development of autoimmune disorders.

Compared to the generation of conventional T cells, development of T_{reg} cells in thymus is delayed in ontogeny [119, 186]. The very first T_{reg} cells are detectable in periphery past the third day after the birth. Several studies proposed TCR specificity of developing thymocytes for rare tissue-specific self-antigens expressed by thymic cells and tissues in autoimmune regulator (AIRE)-dependent or independent manner as a main factor determining differentiation into T_{reg} cell lineage [184]. Such TCRs lie in the avidity window between positive and negative selection. The recognition of rare self-antigen could lead to the triggering of T_{reg} cell developmental program contrary to high-affinity recognition of ubiquitously expressed self-antigens that maily results in deletion of thymocytes. Studies focusing on thymic generation of T_{reg} cells placed its localization into thymus medula where immature $CD4^+CD8^-$ single positive T cells [186-187] start to differentiate towards T_{reg} cell phenotype in the costimulatory and cytokine dependent manner. Double positive $CD4^+CD8^+$ cells expressing Foxp3 were reported to be rare [187]. In 2008, Lio and Hsieh [88] proposed the "two step" model of thymic T_{reg} cell differentiation. The "first" TCR-dependent step includes generation of $CD25^+Foxp3^-$ T_{reg} cell precursors induced by CD28 signaling, while the "second" TCR-independent step involves cytokine-mediated (IL-2, IL-7, IL-15) induction of Foxp3 expression. This model has to be further discussed and investigated as there is controversy around the

putative $CD25^{+}Foxp3^{-}$ precursor which does not have to be involved in differentiation of all nT_{reg} cells.

1.5.3.2 Development of iT_{reg} cells in periphery

Several reports suggest majority of T_{reg} cells are of thymic origin, as the TCR repertoires of $CD4^{+}Foxp3^{+}$ thymocytes and mature peripheral T_{reg} cells show overlaps, and only a fraction of T_{reg} cell population pool arise from peripheral conversion of naïve $CD4^{+}CD25^{-}$ T cells into iT_{reg} cells triggered in response to foreign antigens. Similarly to thymic development, generation of iT_{reg} cells is governed by wide range of molecules. It is triggered by TCR signaling in the presence of IL-2, TGF-β and all *trans*-retinoic acid (ATRA), that induce expression of Foxp3 and acquisition of suppressive phenotype. Also, it is negatively correlated with the maturation state of T cells [184]. Among regulators of iT_{reg} cell generation belong cytokines or transcription factors of effector T cells such as T_h1 (IFN-γ or IL-12, and T-bet, respectively) or T_h2 (IL-4 and GATA-3, respectively) cell subsets, that were reported to effectively inhibit the conversion to iT_{reg} cell phenotype. The main function of iT_{reg} cells is to maintain the immunological tolerance to gut commensal microbial flora and non-pathogenic foreign antigens, although it plays a role in hampering the anti-tumor immunity and reaction to some microbial pathogens as well.

1.5.3.3 Stability and putative plasticity of T_{reg} cells

Recent studies came with the controversial data indicating T_{reg} cells might not be as stable cell line as was supposed to. Possible plasticity of T_{reg} cells was reported by several research groups [133] describing ability of T_{reg} cells to convert to effector cells of T_h cell subsets specialized for inhibition of different effector T_h cell types under the certain circumstances in response to environmental signals. Such cells was termed "hybrid" T cells since their maintain the Foxp3 expression while acquiring T_h cell phenotype, e.g. T_{reg} cells expressing Foxp3 together with T_h1 transcription factor T-bet and IFN-γ that could regulate T_h1 inflammatory responses. Thus, it seems T_{reg} cells can adapt to the environment in order to confer regulation of immune responses. Moreover, several groups described T_{reg} cells can even loose Foxp3 expression and become "exFoxp3" T cells of T_h cell phenotype characterized by effector functions and production of effector cytokines. Nevertheless, some of these cells can retain the ability to re-express Foxp3 in response to certain signals.

Previously mentioned data are rather controversial and it has been discussed whether they are relevant or not since there have been reported a number of contradictive studies showing remarkable stability of T_{reg} cell line in various conditions. Taken together, these findings led to the formulation of the "heterogeneity" model hypothesis [188] suggesting the majority of peripheral $Foxp3^+$ T cells is irreversibly committed to T_{reg} cell lineage with a small fraction of uncommitted $Foxp3^+$ T cell population present in $CD25^-$ T cell subset that has the potential to convert to "exFoxp3" T cells. Whether this model is correct or not remains to be further investigated.

2 Aims

The aims of this study are:

1) Investigation of the possibility to deplete regulatory T cells (T_{reg} cells) from the organism via induction of T_{reg} cell proliferation by administration of IL-2/anti-IL-2 mAb immunocomplexes and subsequent application of cell cycle-specific cytostatic drug(s).
    - Determination of IL-2/anti-IL-2 immunocomplexes optimal dosage for induction of T_{reg} cell expansion sufficient enough for their sensitization to cell cycle-specific cytostatic drugs.
    - Determination of optimal time frame for cell cycle-specific cytostatic drugs administration.
    - Determination of cell cycle-specific cytostatic drugs dosage.
2) Investigation of the possibility to deplete T_{reg} cells from the organism via application of biotinylated anti-CD4 mAb or anti-CD25 mAb and employment of avidin-biotin system for their subsequent elimination from organism.
    - Determination of anti-CD4 and anti-CD25 mAb dosage and optimal time for their elimination from organism.
    - Comparison of biological activity of biotinylated and native mAbs.
    - Determination of biotinylated mAbs persistence in organism.
    - Comparison of avidin and *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-bound avidin ability to eliminate biotinylated mAbs from organism.
    - Determination of HPMA copolymer-bound avidin dosage for sufficient biotinylated mAbs elimination from organism.
3) Determination of maximum tolerated dose and anti-tumor activity of selected HPMA copolymer-bound drug conjugates which would be used in further studies focused on combination of T_{reg} cell depletion and chemotherapy in cancer treatment.

3 Material

3.1 Mice

C57BL/6 (B6) mice and BALB/c mice were obtained from breeding colony of the Institute of Physiology of ASCR, v.v.i. All mice were kept in the animal facility of Institute of Microbiology of ASCR, v.v.i, and were used at the age between 9 and 15 weeks. All experiments were carried out in conventional conditions and were approved by the Animal Welfare Committee of the Institute of Microbiology of ASCR, v.v.i.

3.2 Cell lines

EL4 lymphoma (ATCC) cell line is a murine model of T-cell lymphoma derived from ascetic fluid of B6 mouse lymphoma. Cells of this line are described to be resistant to cortisol and dexamethasone and sensitive to phytohaemaglutinin.

3.3 Solutions

Following solutions were used for experiments and analyses:

Phosphate buffered saline (PBS):	deionized H_2O
	0.9% NaCl
	1.4% $Na_2HPO_4.12H_2O$
	0.12% $Na_2HPO_4.2H_2O$
	pH 7.2-7.4 (4M NaOH)
Flow cytometry buffer:	PBS
	2% fetal calf serum (FCS)
	2 mM EDTA
Blocking buffer:	PBS
	1% gelatine
Dilution buffer:	PBS
	0.5% gelatine

3% PEG 6000
0.1% Tween 20

Rinsing buffer: PBS
0.1% Tween 20

3.4 HPMA copolymer-bound drug conjugates

Following HPMA copolymer-bound drug conjugates designed and synthesized at Institute of Macromolecular Chemistry of AS CR, v.v.i., were used for elimination of biotinylated anti-CD25 mAb from mouse system:

Conjugate 1: HPMA-based polymer-avidin conjugate (branched structure, see Figure 3.1A)
Mw = 550000 g/mol; Mw/Mn = 1.5
67 wt% of egg white avidin (bound via amide bond)
Precursor: linear poly(HPMA-*co*-MA-Acap-TT)
Mw = 79000 g/ml

Conjugate 2: HPMA-based polymer-avidin conjugate (bridge structure, see Figure 3.1B)
Mw = 550000 g/mol; Mw/Mn = 1.95
35 wt% of egg white avidin (bound via amide bond)
Precursor: linear poly(HPMA-*co*-MA-Acap-TT)
Mw = 84000 g/ml

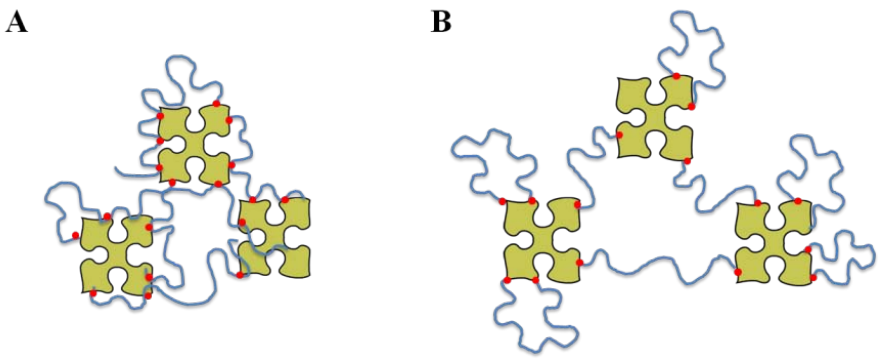


Figure 3.1: Schematic structure of HPMA-based polymer-avidin conjugate. A) Branched Structure. HPMA copolymer carrier with 10 reactive groups along its backbone that is able to cross-link several avidin molecules and form robust net structure. **B)** Bridge structure. HPMA copolymer carrier with reactive groups only on the ends of its backbone that is able to bind one or two avidin molecules at maximum and the resulting structure is more relaxed.

Characteristics of biological functions (i.e. maximum tolerated dose and anti-tumor activity) were determined for following HPMA copolymer-bound drug conjugates designed and synthesized at Institute of Macromolecular Chemistry of AS CR, v.v.i.:

Conjugate 3: linear poly(HPMA-*co*-MA-Acap-NHN=doxorubicin)
Mw = 32500 g/mol; Mw/Mn = 1.86
9.8 wt% of doxorubicin (bound via hydrazone bond)
Precursor: linear poly(HPMA-*co*-MA-Acap-$NHNH_2$)
Mw = 27 000

Conjugate 4: linear poly(HPMA-*co*-MA-Acap-NHN=LEV-docetaxel)
Mw = 34200 g/mol; Mw/Mn = 1.62
6.9 wt% of docetaxel (bound via hydrazone bond)
Precursor: linear poly(HPMA-*co*-MA-Acap-$NHNH_2$)
Mw = 27000

Conjugate 5: star-like HPMA-based polymer-doxorubicin conjugate
Mw = 250000 g/mol; In = 2.21
9.9 wt% of doxorubicin (bound via hydrazone bond)
Precursor: star-like polymer carrier with hydrazide groups
Mw = 225 000

Conjugate 6: star-like HPMA-based polymer-docetaxel conjugate

Mw = 236000 g/mol; In = 1.91

7.7 wt% of docetaxel (bound via hydrazone bond)

Precursor: star-like polymer carrier with hydrazide groups

Mw = 225000

4 Methods

4.1 Regulatory T cell proliferation and preparation of immunocomplexes

Immunocomplexes of IL-2/anti-IL-2 JES6.1A12 mAb (henceforth IL-2/JES6.1) and IL-2/anti-IL-2 S4B6 mA (henceforth IL-2/S4B6) at selected doses and time schedules were used for induction of regulatory T (T_{reg}) cells proliferation. Combination of IL-2/JES6.1 and IL-2/S4B6 immunocomplexes for determination of possible synergistic effects was applied as well.

Immunocomplexes were prepared *in vitro* by mixing of rmIL-2 (Peprotech) and selected anti-IL-2 mAb at molar ratio 2:1. After 15min incubation at room temperature (RT), the immunocomplexes were diluted in PBS to desired concentration and injected i.p. into mice.

4.1.1 The bromodeoxyuridine incorporation assay

FITC BrdU Flow Kit (BD Biosciences) was used for monitoring of T_{reg} cell proliferation and subsequent determination of the optimal dose of IL-2/JES6.1 mAb immunocomplexes and the optimal time for administration of cell cycle-specific drugs.

Simultaneously with i.p. administration of immunocomplexes, bromodeoxyuridine (BrdU) was i.p. injected into mice (0.5 mg/dose). Apart from that, BrdU was also added into the drinking water (0.8 mg/ml). Mice were euthanized at desired time and their splenocytes were isolated. Selected surface markers, transcription factor Foxp3 and BrdU incorporated into DNA were labeled. Splenocytes were analyzed by flow cytometry (see chapter 4.4).

4.2 Elimination of proliferating T_{reg} cells with cell cycle-specific drugs

Mice were i.p. injected with one or two doses of IL-2/JES6.1 immunocomplexes (3.2 μg/dose of IL-2 equivalent) at selected time points. The injection was followed with i.p. application of selected cell cycle-specific cytostatic drug either alone or in combination at two or three daily doses. Some groups were i.p. injected with free anti-IL-2 S4B6 mAb (75 μg/dose) on day 2, between days 2 and 3 or on day 3. All mice were i.p. injected with BrdU (0.5 mg/dose) either on day 0 (2h prior the experiment) or

on day 0 and day 3. Average weight of mice was approximately 25 g. T_{reg} cell counts were checked by flow cytometry upon isolation of splenocytes from experimental mice. Selected cytostatic drugs were methotrexate (MTX), hydroxyurea (HU), actinomycine D (AcD) and aminopterin (AMT), all purchased from Sigma-Aldrich.

4.3 Monoclonal antibodies

Following monoclonal anti-mouse antibodies were used for *in vivo* experiments: anti-CD25 (henceforth αCD25) mAb, anti-CD4 (henceforth αCD4) mAb (ExBio), anti-IL-2 mAb JES6.1-A12 (eBioscience) and anti-IL-2 mAb S4B6 (Bio-port).

Following monoclonal anti-mouse antibody conjugates were used for cell surface staining: CD3-eF450, CD4-FITC (clone RM4.4), CD8-PerCP-Cy55, CD25-APC (clone PC61.5), CD25-PE (clone PC61.5) and CD25-eF660 (clone 7D4), all purchased from eBioscience; CD25-APC (clone 3C7) and CD25-PE (clone 3C7) purchased from Biolegend; and CD4-PerCP purchased from BD Biosciences.

Following monoclonal anti-mouse antibody conjugates were used for intracellular staining: Foxp3-PE (clone FJK-16, clone NRRF-30) and Foxp3-A700 purchased from eBioscience; and BrdU-FITC purchased from BD Biosciences.

Purified anti-rat IgG1 (clone MRG1-58) (Biolegend) was used for ELISA.

4.4 Flow Cytometry

Cells isolated from spleens of experimental mice were analyzed on LSR II flow cytometer (BD Biosciences) and data were evaluated in FlowJo software (Tree Star).

4.4.1 Preparation of cell suspensions

Experimental mice were euthanized by cervical dislocation, spleens were harvested and homogenized by gentleMACS Dissociator (Miltenyi Biotech) in flow cytometry buffer (PBS, 2% FCS, 2 nM EDTA). Cell suspensions were filtered (70μm cell strainer; BD Biosciences) and after red blood cell lysis with ACK lysing buffer (GIBCO) resuspended in flow cytometry buffer and filtered again (30μm cell strainer; BD Biosciences). Resultant cell suspensions were resuspended in flow cytometry buffer and stained for selected surface and intracellular markers.

4.4.2 Cell surface molecules staining

Single cell suspensions were prepared from spleens as described above. Afterwards, they were blocked by 20% healthy mouse serum for 30min on ice and stained with mAbs for 30min on ice in dark. Cells were then washed twice in flow cytometry buffer, fixed in Cytofix/Cytoperm Fixation and Permeabilization (henceforth Fix/Perm) solution (BD Biosciences) for 1h on ice, followed by staining of intracellular markers.

4.4.3 Intracellular molecules staining

Cells were prepared and stained for surface markers as described in 4.4.1 and 4.4.2. Foxp3 staining buffer set (BD Biosciences) was used for Foxp3 staining. After fixation in Fix/Perm solution, cells were washed twice in Perm/Wash buffer and stained with anti-Foxp3 mAb for 30min on ice in dark. Subsequently, cells were washed twice in Perm/Wash buffer, transferred to flow cytometry buffer and analyzed.

Intracellular staining for BrDU was performed using FITC BrdU Flow Kit (BD Biosciences). Following fixation in Fix/Perm solution for 30min on ice, cells were incubated with Cytofix/Cytoperm Plus buffer for 10min on ice, with Fix/Perm solution for 5min on ice and with DNAse for 1h in 37°C, respectively. Then, cells were stained with anti-BrdU mAb together with anti-Foxp3 mAb for 30min on ice. Cells were washed twice in Perm/Wash buffer after each step. Finally, cells were transferred to flow cytometry buffer and analyzed.

4.5 Depletion of T_{reg} cells via αCD4 or αCD25 mAb administration

Populations of $CD4^+$ and $CD25^+$ cells were depleted by i.p. injection of selected doses of either αCD4 mAb (clone GK1.5) or αCD25 mAb (clone PC61.5). Number of T_{reg} cells was checked by flow cytometry.

4.6 Elimination of αCD25 mAb via avidin-biotin system

4.6.1 Biotinylation of αCD25 mAb

Depleting αCD25 mAb was biotinylated by EZ-LINK™ Sulfo-NHS-LC-Biotin (Pierce) according to the standard protocol. Briefly, EZ-LINK™ Sulfo-NHS-LC-Biotin

was equilibrated to RT and diluted in deionized H_2O (1 mg/ml). Defined amount of resultant solution (75 µl) was added to αCD25 mAb diluted in PBS (2 mg/ml) and incubated for 30min at RT. Solution was then dialyzed against PBS overnight in 4°C. Biotinylated αCD25 mAb filtered (0.22 µm filter, Millipore), transferred to a microtube and stored at 4°C.

4.6.2 HABA assay

The amount of biotin molecules per one molecule of biotinylated αCD25 (henceforth αCD25-BIO) mAb was determined by Biotin Quantitation Kit (Pierce) according to the standard protocol. Briefly, HABA/avidin Premix was equilibrated to RT and mixed with deionized H_2O (100 µl). Defined amount of solution (20 µl) was pipetted into the microplate well containing PBS (160 µl) and the absorbance was measured (λ = 500 nm; A_{500} HABA/avidin). Defined amount of either biotinylated sample (20 µl) or biotinylated HRP (positive control) was added to the solution and the absorbance was measured (λ = 500 nm; A_{500} HABA/avidin/biotin). The calculation of moles of biotin per mole of protein was made using the HABA Calculator available on manufacturer's website [189].

Solution of biotinylated HRP was prepared by mixing of biotinylated HRP in deionized H_2O (1 mg/ml) and incubating 5min in RT.

4.6.3 Elimination of biotinylated αCD25 mAb from organism

Mice were i.p. injected with αCD25-BIO mAb and in selected time point with HPMA-avidin conjugate or avidin. Blood was collected at desired time intervals from carotid artery and the concentration of αCD25-BIO mAb in plasma was determined by ELISA.

4.6.4 Determination of αCD25 mAb plasma concentrations by ELISA

Blood from experimental mice was collected to heparinized microtubes and centrifuged (20min, 12 000 rpmi) to separate plasma from the other blood compounds and erythrocytes. Plasma was transferred into microtubes and stored at -20°C.

COSTAR 96-well clear flat-bottom plates were plated with 50 µl of anti-rat IgG1 mAb (4µg/ml) and incubated overnight in 4°C. After rinsing, wells were incubated in blocking buffer for 2h in RT on shaking machine. Collected plasma samples were plated in desired dilution (30x-10000x) together with blank (PBS) and plasma of naïve

mice enriched with titrated αCD25-BIO mAb as a standard. Each sample was plated in triplicate, standard in doublet. After 2h incubation in RT on shaking machine, Extravidin-Peroxidase conjugate (Sigma) diluted 1:5000 in dilution buffer was added for 1h (RT, shaking machine). Finally, wells were developed using 3,3´,5,5´-tetramethylbenzidine (Sigma) for 1min in RT in dark and stopped by 2M H_2SO_4. Wells were washed in rinsing buffer after each step. Absorbance (λ = 450 nm) was measured on Tecan Infinite M200 Pro ELISA reader and Magellan software.

Data obtained from Tecan Infinite M200 Pro ELISA reader and Magellan software were analyzed using Prism software (GraphPad Software).

4.7 Characterization of HPMA copolymer-bound drug conjugates

Non-degradable low molecular weight (LMW) linear and high molecular weight (HMW) star-like HPMA copolymer-bound drug conjugates bearing either doxorubicin (Conjugate 3 and Conjugate 5, respectively) or docetaxel (Conjugate 4 and Conjugate 6, respectively) bound via hydrazone bond were tested for the maximum tolerated dose (MTD) and their possible anti-tumor activity.

4.7.1 Determination of maximum tolerated dose

Mice were i.v. injected with titrated doses of selected HPMA copolymer-bound drug conjugates, their body weight was recorded and general condition was observed. MTD of each conjugate was defined as a dose that does not cause mortality and any signs of side toxicity such as ruffled fur, cachexy, neurotoxicity or reduction of weight higher than 15% of body weight.

4.7.2 Determination of anti-tumor activity

Mice were s.c. inoculated with $1x10^5$ EL4 lymphoma cells on day 0. Titrated doses of selected HPMA copolymer-bound drug conjugates were i.v. injected on desired time points. Tumor growth was measured and survival of experimental mice was observed.

5 Results

5.1 Sensitization of regulatory T cells to cell cycle-specific cytostatic drugs

Cell cycle-specific cytostatic drugs are characterized by their ability to eliminate rapidly proliferating cells via interference with cell division processes [190]. Thus, it was proposed that the induction of vigorous proliferation of regulatory T (T_{reg}) cells mediated by IL-2/anti-IL-2 mAb immunocomplexes could lead to their sensitization to such drugs.

5.1.1 Determination of IL-2/anti-IL-2 mAb immunocomplexes dosage

Firstly, the optimal dosage of IL-2/anti-IL-2 mAb immunocomplexes which would potently induce T_{reg} cell proliferation was determined. Titrated dose of either IL-2/JES6.1 or IL-2/S4B6 immunocomplexes (0.32, 1, 3.2 and 10 μg of IL-2 equivalent) was i.p. injected into C57BL/6 (B6) mice on day 0 together with prior i.p. administration of bromodeoxyuridine (BrdU), i.e. agent used for detection of proliferating (DNA synthesizing) cells. Furthermore, BrdU was also added into drinking water of experimental mice. Used schedule for BrdU administration was applied in all experiments in chapters 5.1 and 5.2. Mice were euthanized 48h after administration of IL-2 immunocomplexes, their spleens were harvested and splenocytes isolated, labeled for selected markers and analyzed by flow cytometry.

Significant increase of T_{reg} cell counts was induced by both types of IL-2 immunocomplexes. However, it is obvious IL-2/JES6.1 immunocomplexes are more specific for T_{reg} cell population then IL-2/S4B6 immunocomplexes, which considerably stimulate $CD4^+CD25^-$ T cells as well (Figure 5.1). Thus, only IL-2/JES6.1 immunocomplexes were chosen to be used in further experiments. The expansion of T_{reg} cells was dose-dependent up to the dose of 3.2 μg of IL-2 equivalent. Higher dose does not show significant increase in T_{reg} cell numbers. In conclusion, the optimal dose of IL-2/JES6.1 immunocomplexes was determined to be 3.2 μg of IL-2 equivalent.

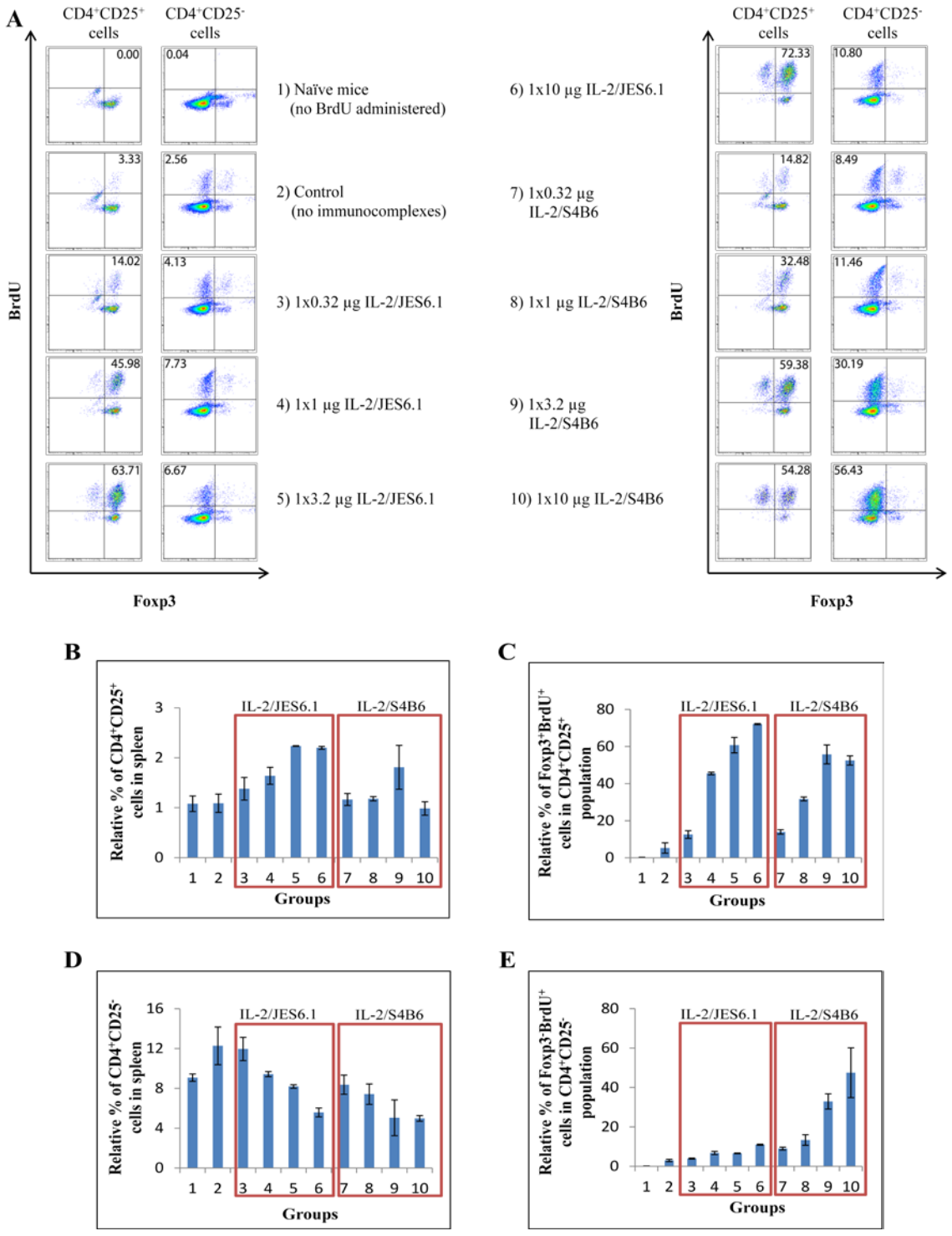


Figure 5.1: Titration of optimal dosage of IL-2/anti-IL-2 mAb immunocomplexes. B6 mice were i.p. injected with 0.5 mg BrdU and subsequently with titrated doses of IL-2/JES6.1 or IL-2/S4B6 immunocomplexes (0.32, 1, 3.2 and 10 µg of IL-2 equivalent). They were euthanized 48h afterwards and their splenocytes were harvested for flow cytometry analysis. Apart from i.p. administration of BrdU, mice were given BrdU *per os* in drinking water (0.8 mg/ml). Groups 1 and 2 represent controls; naïve mice (group 1) and mice i.p. injected with BrdU but not with immunocomplexes (group 2). Groups 3-6 show proliferation of T_{reg} cells after administration of IL-2/JES6.1 immunocomplexes. Groups 7-10 show proliferation of T_{reg} cells after administration of IL-2/S4B6 immunocomplexes. **A)** Flow cytometry analysis. **B)** Relative percent of $CD4^+CD25^+$ T cells in spleen. **C)** Relative content of $Foxp3^+BrdU^+$ T cells in the population shown in B. **D)** Relative content of $CD4^+CD25^-$ cells in spleen. **E)** Relative percent of $Foxp3^-BrdU^+$ T cells in the population shown in D. Presented data are mean±SD of 2 mice per each condition. Dot plots show 1 representative mouse.

5.1.2 Combination of IL-2/JES6.1 and IL-2/S4B6 immunocomplexes

In order to investigate whether IL-2/JES6.1 and IL-2/S4B6 immunocomplexes act in synergy, combination of IL-2/JES6.1 and IL-2/S4B6 immunocomplexes was i.p. injected into B6 mice at the dose of 1.6 μg of IL-2 equivalent for IL-2/JES6.1 as well as for IL-2/S4B6 immunocomplexes, thus resulting dose of IL-2 equivalent was 3.2 μg. Mice were euthanized 48h after the administration of immunocomplexes, their spleens were harvested and splenocytes isolated, labeled for selected markers and analyzed by flow cytometry. As in 5.1.1, BrdU was used as a marker of cell proliferation. Data show no synergistic effects of these two IL-2 immunocomplexes, as their combination did not induce significantly higher increase of T_{reg} cell proliferation than either immunocomplexes alone (Figure 5.2).

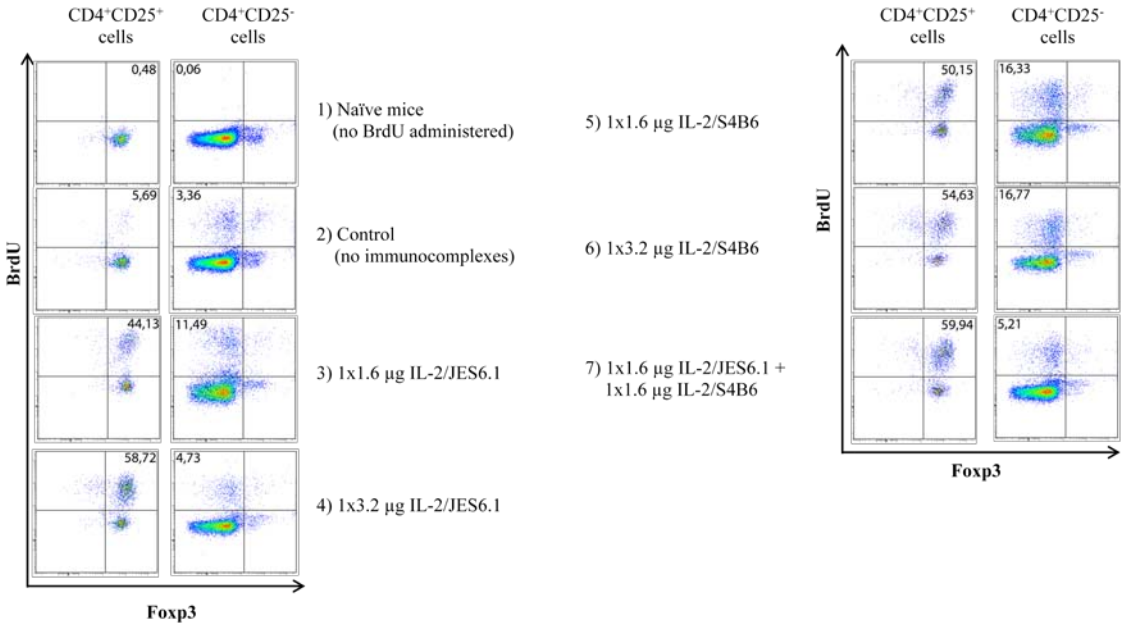


Figure 5.2: Effect of IL-2/JES6.1 and IL-2/S4B6 immunocomplexes co-administration on T_{reg} cell proliferation. B6 mice were i.p. injected with 0.5 mg BrdU and subsequently with IL-2/JES6.1 (1.6 μg or 3.2 μg of IL-2 equivalent), IL-2/S4B6 immunocomplexes (1.6 μg or 3.2 μg of IL-2 equivalent) or combination of both (3.2 μg of IL-2 equivalent in total). Mice were euthanized 48h afterwards and their splenocytes were harvested for flow cytometry analysis. Apart from i.p. administration of BrdU, mice were given BrdU *per os* in drinking water (0.8 mg/ml). Groups 1 and 2 represent controls; naïve mice (group 1) and mice i.p. injected with BrdU but not with immunocomplexes (group 2). Dot plots show 1 representative mouse.

5.1.3 Determination of optimal time for cell cycle-specific drugs administration

The optimal time frame for administration of selected cell cycle-specific cytostatic drugs either alone or in combination was determined in the second step. B6 mice were i.p. injected with IL-2/JES6.1 immunocomplexes either in one (on day 0) or two (on days 0 and 1) doses. Each dose was corresponding to 3.2 µg of IL-2 equivalent. At selected time points (24h, 48h, and 72h after the first dose), mice were euthanized and their splenocytes were analyzed by flow cytometry. There was no significant increase of T_{reg} cell numbers observed until 24h after the immunocomplexes administration. However, robust expansion of this cell population was recorded between 24h and 48h. Approximately 80% of proliferating cells inside the T_{reg} cell population was observed 72h after a single injection of IL-2/JES6.1 immunocomplexes. Two doses of immunocomplexes led to similar results as the single dose, i.e. single dose of IL-2/JE6.1 immunocomplexes induced proliferation of comparable numbers of T_{reg} cells after 48h as a double dose. Similar results were found also in 72h interval (Figure 5.3). Therefore, the optimal time for cell cycle-specific cytostatic drugs administration was determined to be between 24h and 48h after IL-2/JES6.1 immunocomplexes administration.

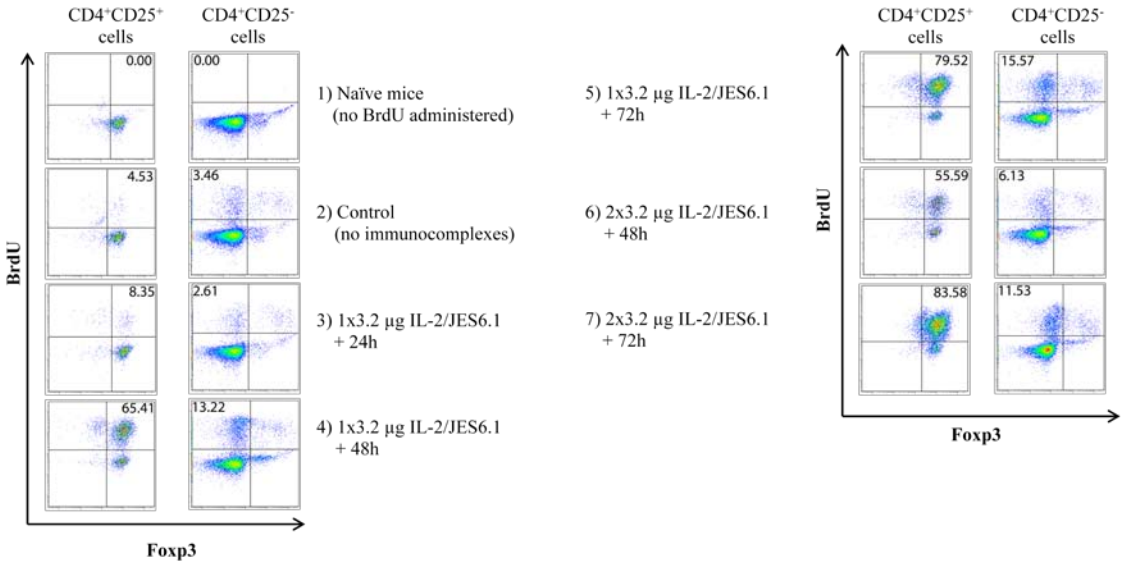


Figure 5.3: Kinetics of IL-2/JES6.1 immunocomplexes-induced T_{reg} cell proliferation. B6 mice were i.p. injected with 0.5 mg BrdU and subsequently with IL-2/JES6.1 immunocomplexes at dose corresponding to 3.2 µg of IL-2 equivalent; either one dose (groups 3-5) or two doses separated by 24 h (groups 6 and 7). Apart from i.p. administration of BrdU, mice were given BrdU *per os* in drinking water (0.8 mg/ml). Mice were euthanized 24h, 48h or 72h after the administration of the first dose of immunocomplexes, their splenocytes were harvested and analyzed by flow cytometry. Groups 1 and 2 represent controls; naïve mice (group 1) and mice injected with BrdU but not with immunocomplexes (group 2). Dot plots show 1 representative mouse.

5.2 Depletion of proliferating T_{reg} cells via application of cell cycle-specific drugs

Selected cell cycle-specific cytostatic drugs either alone or in combination were used for elimination of proliferating T_{reg} cells. At first, the mixture of methotrexate (MTX), hydroxyurea (HU) and actinomycin D (AcD) was used in various schedules. Dosage of these drugs was chosen according to the literature [191-194] and personal experience with application of cytostatic drugs in experimental therapy of mice tumor models. Furthermore, free S4B6 mAb was applied in several groups in order to neutralize IL-2/JES6.1 immunocomplexes and thus stop the strong IL-2 signal providing a pro-survival environment for T_{reg} cells.

Mice were i.p. injected with IL-2/JES6.1 immunocomplexes at dose of 3.2 μg of IL-2 equivalent, in one or two doses separated by 24h. Mice were injected either with cytostatic drug mix (8 mg/kg MTX, 80 mg/kg HU and 0.8 mg/kg AcD in two doses separated by 12h) or free S4B6 mAb or both at selected time points. Mice were euthanized 72h after the first dose of IL-2/JES6.1 immunocomplexes and their splenocytes were analyzed by flow cytometry (Figure 5.4). The most considerable reduction of T_{reg} cells was seen in groups treated with two doses of IL-2/JES6.1 together with cytostatic drug mix. Unfortunately, the amount of administered cytostatics was too high and severe cytotoxicity and cachexy of treated mice were observed.

Next experiment was made under different conditions (data not shown). The dosage of applied cytostatics in cytostatic drug mix were significantly lowered and administered in 2 or 3 daily doses in one or two consecutive days (0.5 mg/kg/dose MTX, 5 mg/kg/dose HU and 0.05 mg/kg/dose AcD). Moreover, groups injected only with single selected cytostatic drug (0.5 mg/kg/dose AcD or 0.25 mg/kg/dose of aminopterin, AMT) were added. There was no significant reduction in T_{reg} cell counts in groups that received cytostatic drug mix indicating selected dosage was too low to deplete proliferating T_{reg} cells. Group that received AMT alone showed severe side toxicity and group injected with AcD alone died by day 5. The other groups appeared healthy. In conclusion, the applied amounts of cytostatics were either too high and led to serious side toxicity or too low to cause significant decrease of T_{reg} cell population below the homeostatic threshold. Thus, although the original idea of T_{reg} cell depletion via IL-2 immunocomplexes-mediated induction of their proliferation and subsequent administration of cell cycle-specific cytostatic drugs appears attractive, it does not

provide sufficiently strong results to be further used for T_{reg} cell depletion during cancer treatment experiments.

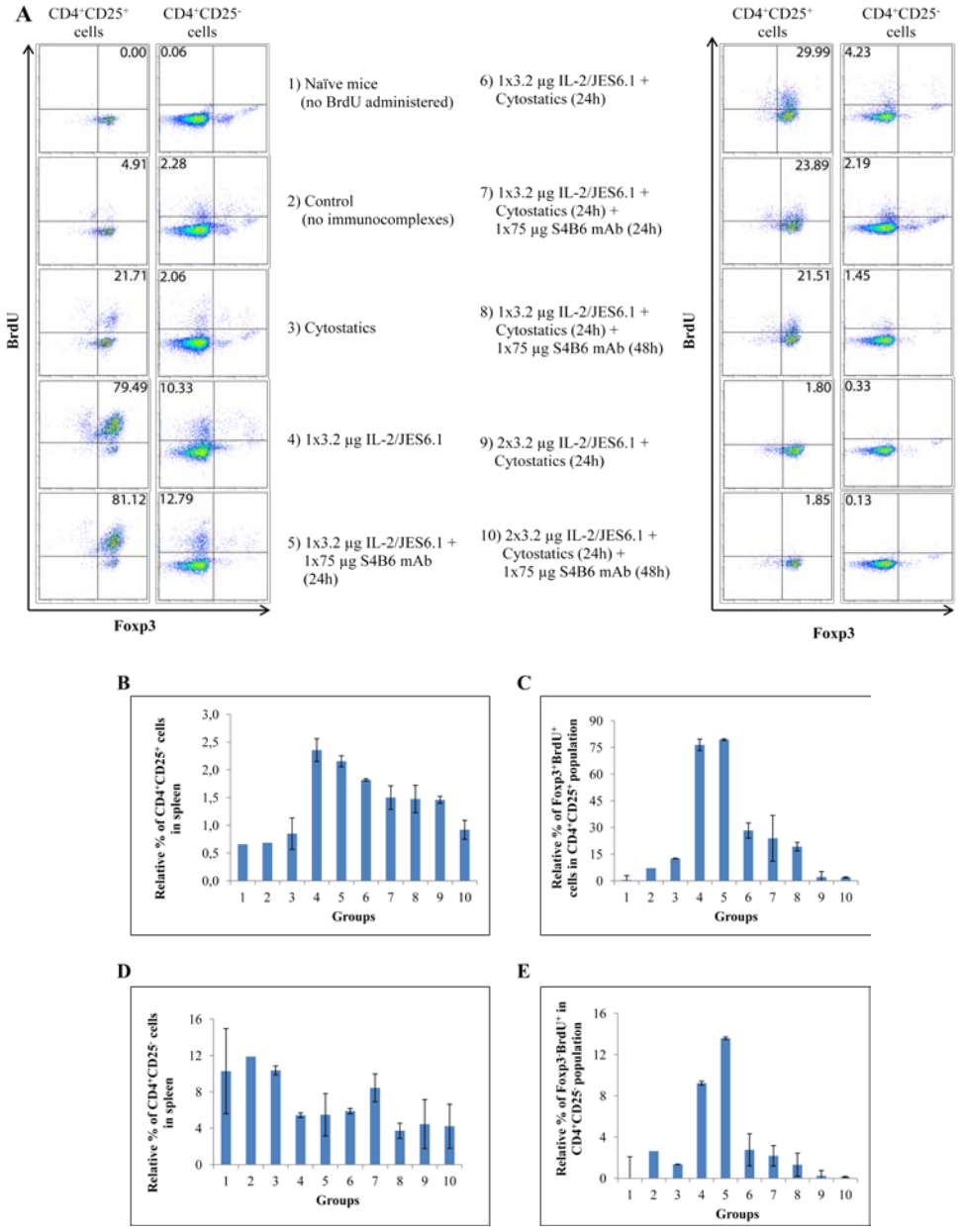


Figure 5.4: Application of selected cell cycle-specific cytostatic drugs for elimination of proliferating T_{reg} cells. B6 mice were i.p. injected with 0.5 mg BrdU and IL-2/JES6.1 immunocomplexes at dose corresponding to 3.2 µg of IL-2 equivalent – either 1 dose (groups 5-8) or 2 doses separated by 24 h (groups 9 and 10). Mice were i.p. injected either with cytostatic drugs mix (8 mg/kg MTX, 80 mg/kg HU, 0.8 mg/kg AcD in two doses separated by 12h) or S4B6 mAb (75 µg/dose) or both 24h after the first dose of immunocomplexes. Groups 8 and 10 received S4B6 mAb 48h after the first dose of immunocomplexes. Mice were euthanized 72h after the first dose of immunocomplexes, their splenocytes were harvested and analyzed by flow cytometry. **A)** Flow cytometry analysis. **B)** Relative content of $CD4^+CD25^+$ T cells in spleen. **C)** Relative content of $Foxp3^+BrdU^+$ T cells in the population shown in B. **D)** Relative content of $CD4^+CD25^-$ cells in spleen. **E)** Relative content of $Foxp3^-BrdU^+$ T cells in the population shown in D. Naïve mice, mice injected with BrdU but not with immunocomplexes, mice injected with BrdU and cytostatic drug mix but not immunocomplexes, mice injected with BrdU and immunocomplexes, and mice injected with BrdU, immunocomplexes and S4B6 mAb were used as controls. Apart from i.p. administration of BrdU, mice were given BrdU *per os* in drinking water (0.8 mg/ml). Presented data are mean±SD of 2 mice per each condition. Dot plots show 1 representative mouse.

5.3 Depletion of T_{reg} cells via application of αCD4 depleting mAb

Since the original approach to deplete T_{reg} cells via their sensitization to cell cycle-specific cytostatic drug-mediated elimination appears to be only partially effective and accompanied with serious side toxicities if given in higher doses, the effects of αCD4 mAb (clone GK1.5) application on T_{reg} cell numbers were investigated. This mAb specifically depletes $CD4^+$ T cells, therefore it should eliminate T_{reg} cells as well.

BALB/c mice were i.p. injected with 100 μg of αCD4 mAb and euthanized in selected time points. Their spleens were harvested and splenocytes were analyzed by flow cytometry (Figure 5.5). $CD4^+$ T cells were significantly decreased after administration of αCD4 mAb. Gradual decrease of $CD4^+$ T cell population can be seen in the Figure 5.5A while in the Figure 5.5B, there is obvious relative increase of T_{reg} cells within $CD4^+$ T cell population on days 2 and 3, and a mild decrease on day 4 compared to control.

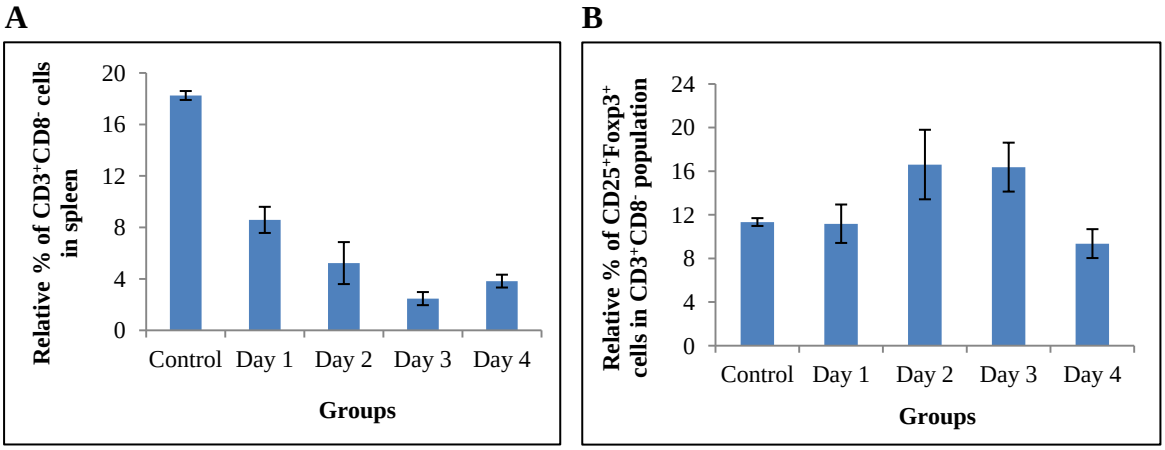


Figure 5.5: Kinetics of $CD4^+$ T cell and T_{reg} cell depletion by αCD4 mAb. BALB/c mice were i.p. injected with 100 μg of αCD4 mAb on day 0 and sacrificed on days 1, 2, 3 and 4 after the mAb administration. Their splenocytes were were isolated and analysed by flow cytometry. Naïve mice were used as controls. **A**) Relative content of $CD3^+CD8^-$ cells (i.e. $CD4^+$ T cells) in spleen in selected time points. **B**) Relative content of $CD25^+Foxp3^+$ cells (i.e. T_{reg} cells) in population shown in A. Presented data are mean±SD of 3 mice per each condition.

To determine the effects of different doses of αCD4 mAb, B6 mice were i.p. injected with three different doses of αCD4 mAb (50, 100 and 150 μg), euthanized on day 4 after the αCD4 mAb administration and their splenocytes were analyzed by flow cytometry (Figure 5.6). Similar observation as in the first experiment was made, i.e. T_{reg} cells appear to be less sensitive to αCD4 mAb-mediated depletion in comparison to the other $CD4^+$ T cells. Furthermore, increasing relative number of T_{reg} cells in $CD4^+$ T cell population can be seen with increasing dose of αCD4 mAb. These data indicate that T_{reg}

cells are less sensitive to GK1.5 mAb-mediated depletion compared to other $CD4^+$ T cells in dose-dependent manner.

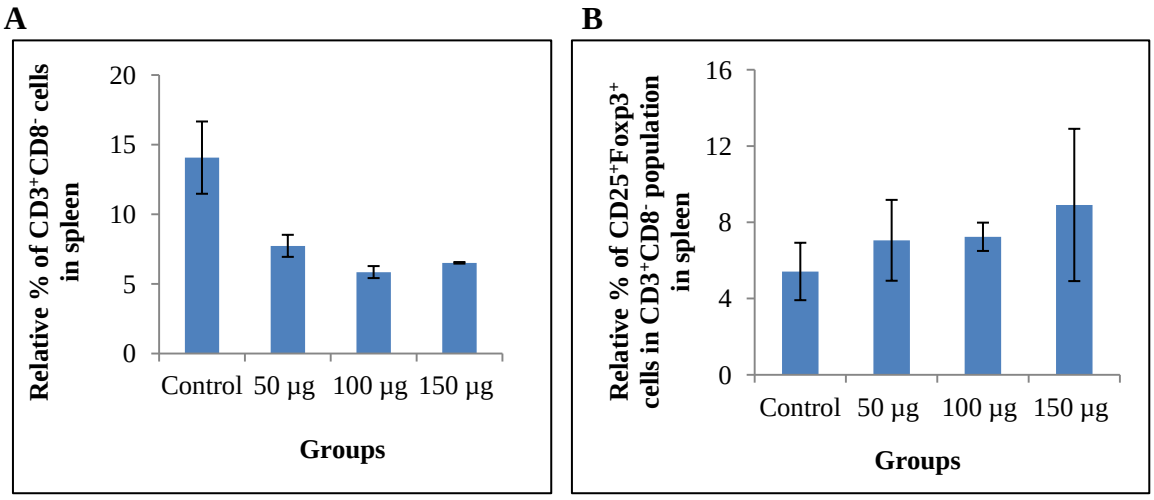


Figure 5.6: Effects of αCD4 mAb application on $CD4^+$ T cell and T_{reg} cell populations. B6 mice were i.p. injected with titrated doses of αCD4 mAb (50, 100, 150 µg) on day 0 and sacrificed 4 days after the mAb administration. Their splenocytes were isolated and analyzed by flow cytometry. Naïve mice were used as controls. **A**) Relative content of $CD3^+CD8^-$ cell population (i.e. $CD4^+$ T cells) in spleen. **B**) Relative content of $CD25^+Foxp3^+$ cells (i.e. T_{reg} cells) in population shown in A. Presented data are mean±SD of 3 mice per each condition. The experiment was done twice with similar results.

5.4 Depletion of T_{reg} cells via application of αCD25 depleting mAb

Employment of αCD4 mAb showed to be only partially suitable for T_{reg} cell depletion, thus the use of αCD25 mAb was reconsidered. An approach overcoming the main problem of αCD25 mAb application, i.e. long persistence of αCD25 mAb in the organism leading to the elimination of potentially activated tumor-specific effector cells [177-180], was developed. It is based on exceptionally strong avidin-biotin interaction. αCD25 mAb is biotinylated and administered into mice and eliminated by HPMA copolymer-bound avidin conjugate after significant lowering of T_{reg} cell counts is achieved. Such conjugate, which contains several avidin molecules bound to one HPMA copolymer carrier and thus it better cross-links and eliminates biotinylated mAbs, was used. The amount of biotin bound to one molecule of αCD25 mAb was determined by HABA assay and it ranges from 5 to 10 molecules of biotin per αCD25 mAb molecule.

5.4.1 Titration of αCD25 mAb dose

The optimal dosage of αCD25 mAb was determined together with estimation of optimal time for its elimination from organism. B6 mice were i.p. injected with two different doses of αCD25 mAb (100 μg and 250 μg), euthanized on selected time points and their splenocytes were analyzed by flow cytometry (Figure 5.7).

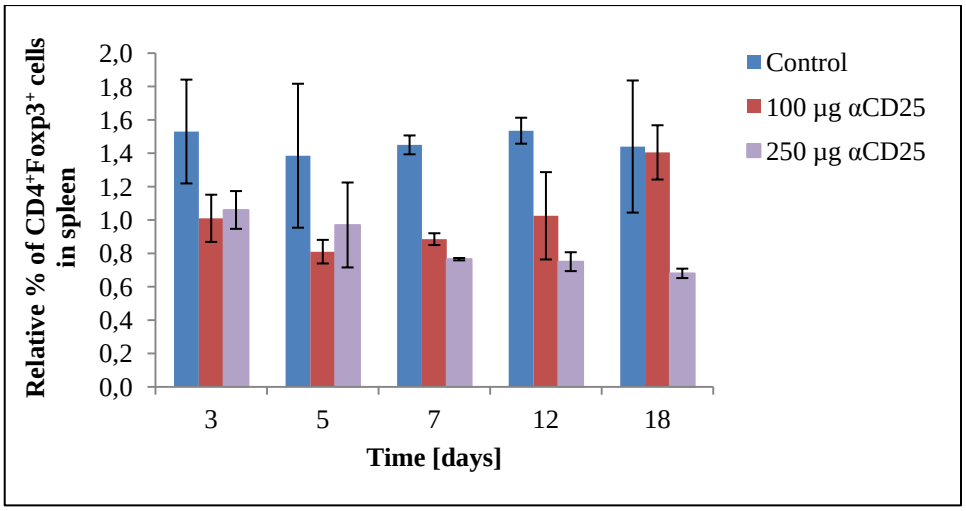


Figure 5.7: Kinetics of αCD25 mAb effect on T_{reg} cell counts. B6 mice were i.p. injected with 100 μg or 250 μg of αCD25 mAb on day 0 and sacrificed 3, 5, 7, 12 and 18 days after αCD25 mAb administration. Their splenocytes were isolated and analyzed by flow cytometry. Naïve mice were used as controls. Presented data are mean±SD of 2 mice per each condition.

The lowest number of T_{reg} cells was seen on day 5 in case of 100 μg of αCD25 mAb, whereas 250 μg of αCD25 mAb caused continuous decrease of T_{reg} cell number till day 18. 100 μg of αCD25 mAb was decided to be used for further experiments. The optimal time for αCD25 mAb elimination from organism was determined to be day 5 after the mAb administration, since the elimination of T_{reg} cells is planned to be combined with chemotherapy and stimulation of anti-tumor responses by IL-2/S4B6 immunocomplexes which would be severely impaired in the longer presence of αCD25-mAb as mentioned above.

5.4.2 Comparison of native αCD25 mAb and biotinylated αCD25 mAb *in vivo*

The biological activity of native and biotinylated αCD25 (henceforth αCD25-BIO) mAb was compared after the determination of optimal dosage of αCD25 mAb and the optimal time for its elimination from organism. Mice were i.p. injected with 100 μg of αCD25 mAb or 100 μg of αCD25-BIO mAb, euthanized 5 days afterwards, and their spleens were harvested and analyzed by flow cytometry (Figure 5.8). Data indicate that

the activity of both agents is similar. Therefore, αCD25-BIO mAb was used in further experiments.

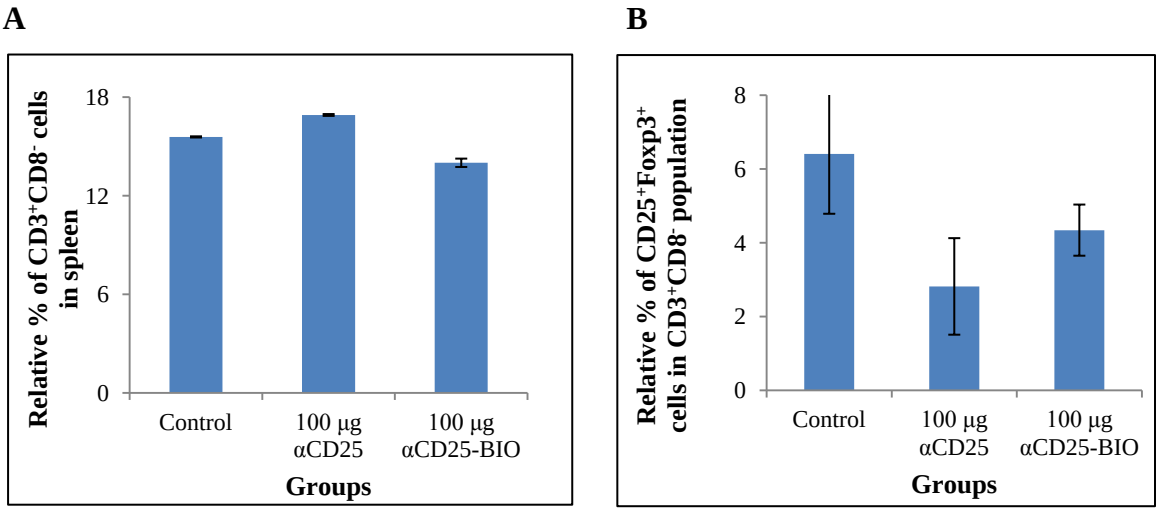


Figure 5.8: Comparison of biological activity of native and biotinylated αCD25 mAb. B6 mice were i.p. injected with 100 µg of αCD25 mAb or αCD25-BIO mAb on day 0 and sacrificed 5 days after mAb administration. Their splenocytes were isolated and analyzed by flow cytometry. Naïve mice were used as controls. **A**) Relative content of $CD3^+CD8^-$ cells (i.e. $CD4^+$ T cells) in spleen. **B**) Relative content of $CD25^+Foxp3^+$ cells (i.e. T_{reg} cells) in population shown in A. Presented data are mean±SD of 2 mice per each condition. The experiment was done twice with similar results.

5.4.3 Persistence of αCD25-BIO mAb in organism

Mice were injected with two different doses of αCD25-BIO mAb for investigation of αCD25 mAb persistence in organism. Blood was collected at selected time points, and the concentration of αCD25-BIO mAb in their plasma was measured by ELISA (detection limit for αCD25-BIO mAb was 0.3 µg/ml). αCD25-BIO mAb was detectable in blood up to 12 and 18 days after i.p. administration of 100 µg of αCD25-BIO mAb and 250 µg of αCD25-BIO mAb, respectively (Figure 5.9).

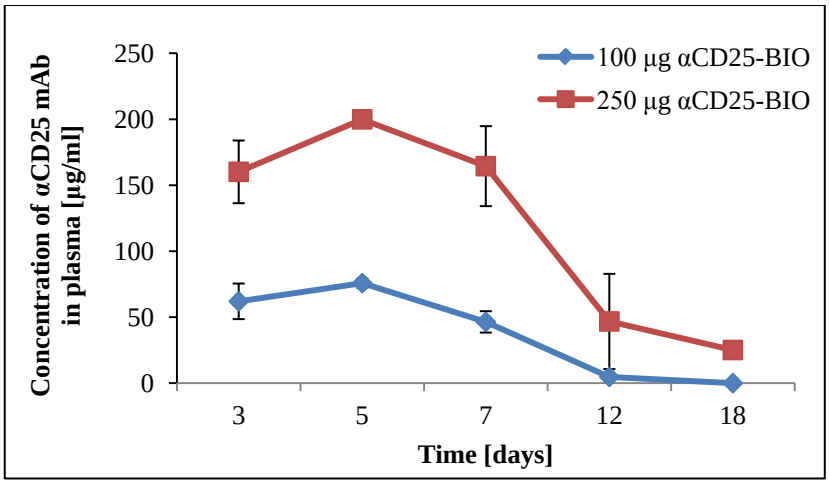


Figure 5.9: Kinetics of αCD25-BIO mAb concentration in plasma after i.p. injection. B6 mice were i.p. injected with 100 μg or 250 μg of αCD25-BIO mAb on day 0. Blood was collected from carotid artery on days 3, 5, 7, 12 and 18, and concentration of αCD25-BIO mAb in the plasma was measured by ELISA. Naïve mice were used as a negative control. Presented data are mean±SD of 2 mice per each condition. The experiment was done twice with similar results.

5.4.4 Elimination of αCD25-BIO mAb from organism by the use of HPMA copolymer-bound avidin conjugate

Mice were i.p. injected with 100 μg of αCD25-BIO mAb and with HPMA copolymer-bound avidin conjugate (100 μg of avidin equivalent; HPMA-avidin conjugate) or PBS (control) 24h afterwards for determination whether HPMA-avidin conjugate can affect pharmacokinetics of αCD25-BIO mAb. Mice were euthanized at selected time points, their blood was collected and plasma was analyzed by ELISA (Figure 5.10). Significant decrease of αCD25-BIO mAb was observed between 4h and 8h after HPMA-avidin conjugate administration, thus the employed system appears to be suitable for this application and was used in further experiments.

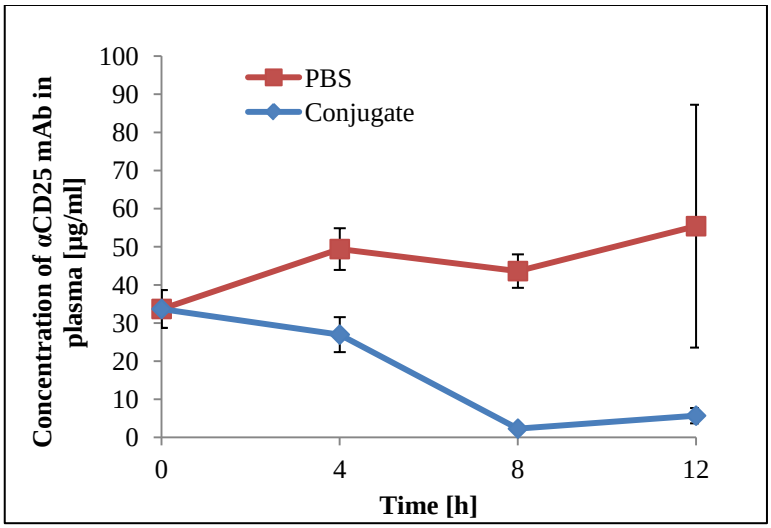


Figure 5.10: Kinetics of αCD25-BIO mAb elimination from circulation via application of HPMA-avidin conjugate (Conjugate). B6 mice were i.p. injected with 100 μg of αCD25-BIO mAb on day 0. Mice were i.p. injected either with Conjugate at dose of 100 μg of avidin equivalent or PBS (control) 24h later. Mice were euthanized 0, 4, 8 and 12h after the Conjugate or PBS administration, their blood was collected from carotid artery and the concentration of αCD25-BIO mAb in their plasma was measured by ELISA. Mice injected with 100 μg of αCD25-BIO mAb and euthanized 24h later were used as a positive control. Naïve mice were used as a negative control. Presented data are mean±SD of 2 mice per each condition. The experiment was done twice with similar results.

5.4.5 Comparison of avidin and HPMA-avidin conjugate and determination of their optimal dose

Mice were i.p. injected with 100 μg of αCD25-BIO mAb and 24h later with titrated doses of HPMA-avidin conjugate, avidin or PBS (control) in order to investigate whether there is a difference in extent of αCD25-BIO mAb elimination between HPMA-avidin conjugate and avidin, and to determine the optimal dosage of both agents. Mice were euthanized 4h after the administration of HPMA-avidin conjugate, avidin or PBS, their blood was collected and plasma analyzed by ELISA (Figure 5.11). Doses up to 20 μg of avidin or avidin equivalent are insufficient for αCD25-BIO mAb elimination. Dose of 64 μg of avidin or avidin equivalent caused only mild decrease of αCD25-BIO mAb. Only HPMA-avidin conjugate at dose of 200 μg of avidin equivalent was able to reduce the amount of αCD25-BIO mAb in plasma to non-detectable concentrations. Therefore, it seems that HPMA-avidin conjugate is more potent than avidin in elimination of αCD25-BIO mAb from organism.

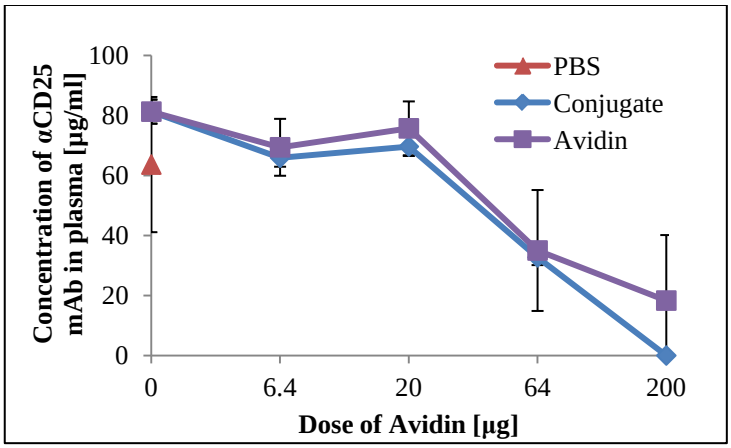


Figure 5.11: Titration of HPMA-avidin conjugate (Conjugate) dosage and comparison of its activity with avidin. B6 mice were i.p. injected with 100 µg of αCD25-BIO mAb on day 0 and 24h later either with Conjugate (6.4, 20, 64 and 200 µg of avidin equivalent), avidin (6.4, 20, 64 and 200 µg), or PBS (control). Mice were euthanized 4h after the administration of Conjugate, avidin or PBS, their blood was collected from carotid artery and the concentration of αCD25-BIO mAb in their plasma was measured by ELISA. Mice injected with 100 µg of αCD25-BIO mAb and euthanized 24h later were used as a positive control. Naïve mice were used as a negative control. Presented data are mean±SD of 2 mice per each condition. The experiment was done twice with similar results.

5.4.6 Kinetics of αCD25-BIO mAb elimination

Mice were i.p. injected with 100 µg of αCD25-BIO mAb followed 24h later with HPMA-avidin conjugate at dose of 250 µg of avidin equivalent, 250 µg of avidin or PBS (control). Mice were euthanized at selected time points, their blood was collected from carotid artery and plasma was analyzed by ELISA (Figure 5.12).

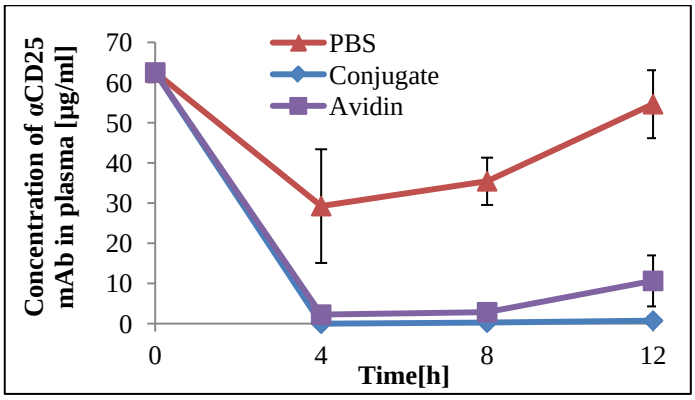


Figure 5.12: Kinetics of αCD25-BIO mAb elimination via application of avidin or HPMA-avidin conjugate (Conjugate). B6 mice were i.p. injected with 100 µg of αCD25-BIO mAb and 24h later with either Conjugate at dose of 250 µg of avidin equivalent, 250 µg avidin, or PBS. Mice were euthanized 0, 4, 8 and 12h afterwards. Their blood was collected from carotid artery and the concentration of αCD25-BIO mAb in their plasma was measured by ELISA. Mice injected with 100 µg of αCD25-BIO mAb and euthanized 24h later were used as a positive control. Naïve mice were used as a negative control. Presented data are mean±SD of 2 mice per each condition. The experiment was done twice with similar results.

The majority of αCD25-BIO mAb is eliminated during first 4 hours after mAb administration (Figure 5.12). HPMA-avidin conjugate is capable to reduce the concentration to non-detectable levels in comparison to avidin.

In conclusion, the proposed approach is assumed to be effective in terms of T_{reg} cell depletion followed by αCD25 mAb elimination from organism and can be thus employed in experiments focusing on its usage in tumor therapy.

5.5 Maximum tolerated dose and therapeutic activity of HPMA copolymer-bound drug conjugates

The development and establishment of suitable method for T_{reg} cell elimination without hampering tumor-specific immune reactions was followed by testing of maximum tolerated dose (MTD, see chapter 4.7.1) and anti-tumor activity of selected polymer-bound drug conjugates that would be used as chemotherapeutics after T_{reg} cell depletion.

MTD of selected non-degradable low molecular weight (LMW) linear HPMA copolymer-bound drug conjugates bearing either doxorubicin (DOX; Conjugate 3) or docetaxel (DTX; Conjugate 4) bound via hydrazone bond to polymer carrier was determined. Selected non-degradable high molecular weight (HMW) star-like HPMA copolymer-bound drug conjugates bearing either DOX (Conjugate 5) or DTX (Conjugate 6) bound via hydrazone bond to polymer carrier was tested for their MTD as well. Anti-tumor activity of these conjugates was investigated on murine model of EL4 T-cell lymphoma growing on syngeneic B6 mice.

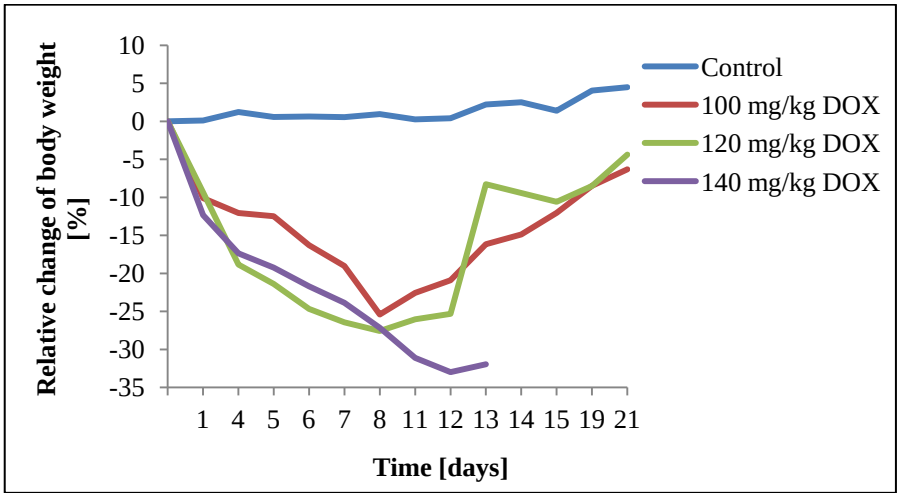
5.5.1 MTD of LMW HPMA copolymer-bound drug conjugates

Mice were i.v. injected with titrated doses of Conjugate 3 (100, 120 or 140 mg/kg of DOX equivalent) or Conjugate 4 (100, 125 or 150 mg/kg of DTX equivalent). Their weight was recorded and general condition was observed.

Resulting data indicate that MTD of Conjugate 3 is lower than the lowest applied dose, i.e. 100 mg/kg of DOX equivalent (Figure 5.13), as all mice in this group showed significant loss of body weight and mild signs of side toxicity (e.g. ruffled fur). Group injected with 120 mg/kg of DOX equivalent showed severe side toxicity which led to

premature death of 2 out of 3 of mice. Group injected with 140 mg/kg of DOX equivalent died within 13 days after the administration of Conjugate 3.

A



B

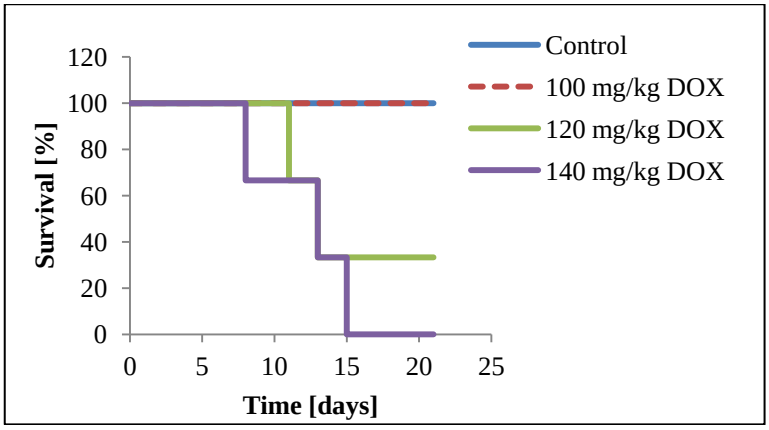


Figure 5.13: Determination of MTD of Conjugate 3. B6 mice were i.v. injected with Conjugate 3 at doses of 100, 120 or 140 mg/kg of DOX equivalents. Weight of the mice was recorded (**A**) and survival was monitored (**B**) until day 21 after the administration of Conjugate 3. Naïve mice i.v. injected with PBS were used as controls. Presented data are mean±SD of 3 mice per each condition.

On the other hand, MTD of Conjugate 4 appears to be considerably higher than the highest applied dose, i.e. 150 mg/kg of DTX equivalent (Figure 5.14). No signs of side toxicity were seen and only very small decrease in body weight was observed in all groups.

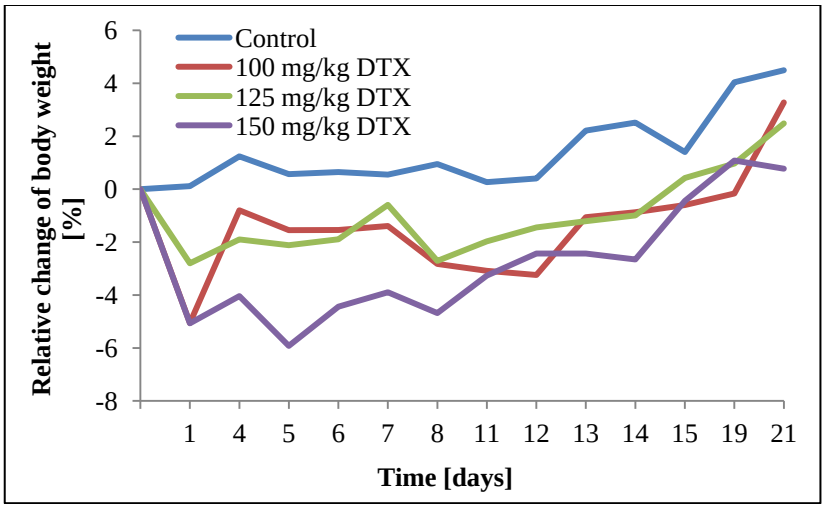


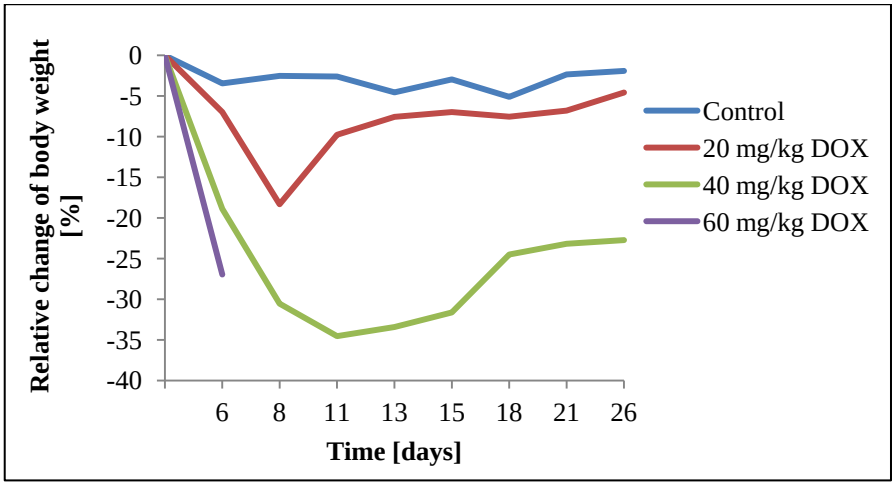
Figure 5.14: Determination of MTD of Conjugate 4. B6 mice were i.v. injected with Conjugate 4 at doses of 100, 125 or 150 mg/kg of DTX equivalent. Weight of the mice was recorded (**A**) and their survival was monitored (**B**) until day 21 after the administration of Conjugate 4. Naïve mice i.v. injected with PBS were used as controls. Presented data are mean±SD of 3 mice per each condition.

5.5.2 MTD of HMW HPMA copolymer-bound drug conjugates

Mice were i.v. injected with titrated doses of Conjugate 5 or Conjugate 6. Their weight was recorded and general condition was observed.

MTD of Conjugate 5 seems to lie between 20 and 40 mg/kg of DOX equivalent, since the dose of 20 mg/kg of DOX equivalent led to decrease of body weight (slightly higher than 15%) but otherwise mice appeared healthy, whereas the dose of 40 mg/kg of DOX equivalent led to severe side toxicity and premature death of 2 out of 3 of mice (Figure 5.15). Mice given 60 mg/kg of DOX equivalent died within 8 days.

A



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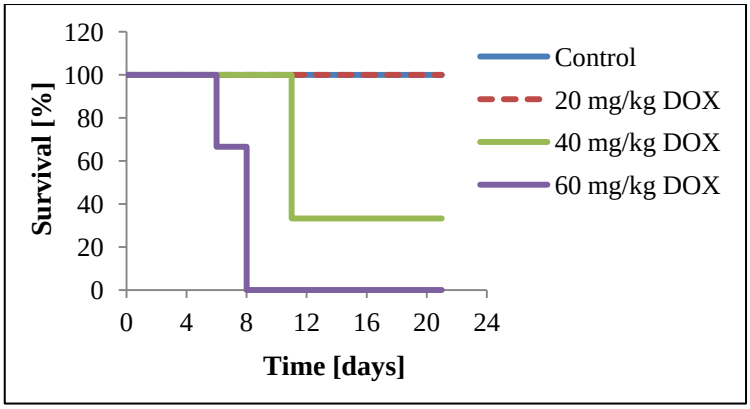

Figure 5.15: Determination of MTD of Conjugate 5 – I. BALB/c mice were i.v. injected with Conjugate 5 at doses of 20, 40 or 60 mg/kg of DOX equivalent. Weight of the mice was recorded (**A**) and their survival was monitored (**B**) until day 21 after the Conjugate 5 administration. Naïve mice i.v. injected with PBS were used as controls. Presented data are mean±SD of 3 mice per each condition.

Next, mice were i.v. injected with Conjugate 5 at doses of 25, 30 or 35 mg/kg of DOX equivalent, their weight was recorded (Figure 5.16) and general condition was observed. No mortality was recorded in any group. Nevertheless, average weight of the groups that received 30 or 35 mg/kg of DOX equivalent was reduced for more than 15% and mice showed moderate signs of side toxicity (e.g. diarrhea, ruffled fur). Group that received 25 mg/kg of DOX equivalent appeared healthy with no signs of toxicity. Thus, the MTD of Conjugate 5 is around 20-25 mg/kg of DOX equivalent.

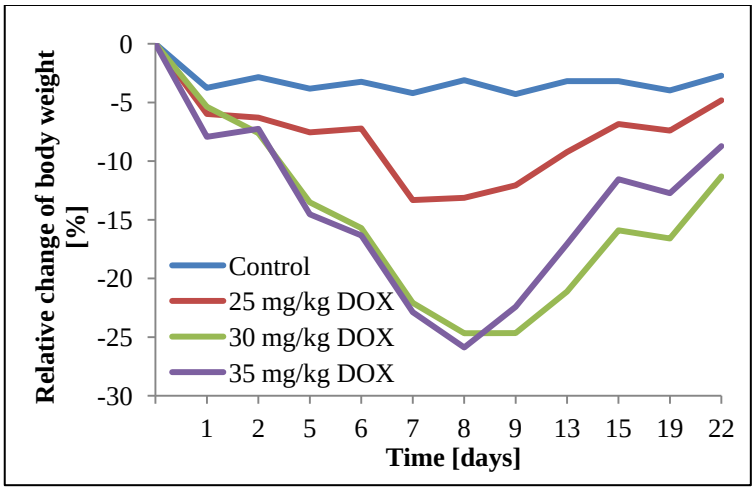

Figure 5.16: Determination of MTD of Conjugate 5 – II. BALB/c mice were i.v. injected with Conjugate 5 at doses of 25, 30 or 35 mg/kg of DOX equivalent. Weight of the mice was recorded until day 22 after the Conjugate 5 administration. Naïve mice i.v. injected with PBS were used as controls. Presented data are mean±SD of 3 mice per each condition.

To estimate the MTD of Conjugate 6, mice were i.v. injected with 20, 40 or 60 mg/kg of DTX equivalent. However, neither dose caused side toxicity or led to significant reduction of body weight (Figure 5.17). Therefore, mice were injected with higher doses of Conjugate 6 (80 and 100 mg/kg of DTX equivalent). It appears that MTD of Conjugate 6 is approximately around 90 mg/kg of DTX equivalent (Figure 5.18), since the dose of 80 mg/kg of DTX equivalent did not cause any health problems and body weight reduction, however, the dose of 100 mg/kg caused significant decrease of body weight accompanied by cachexy and neurotoxicity and led to premature death of 2 out 3 mice.

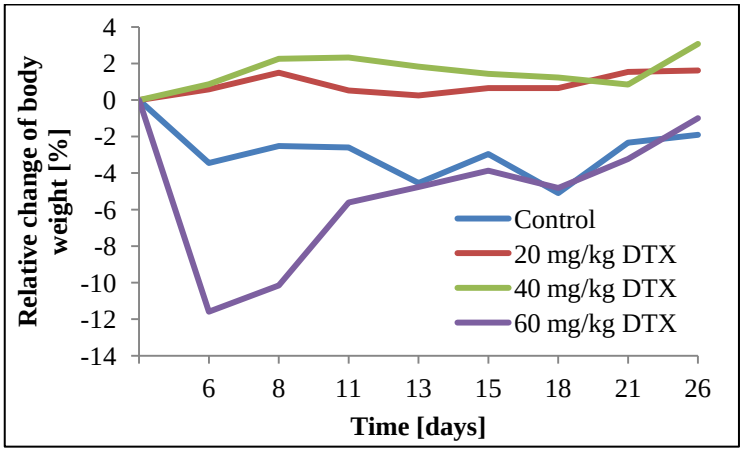

Figure 5.17: Determination of MTD of Conjugate 6 – I. BALB/c mice were i.v. injected with Conjugate 6 at doses of 20, 40 or 60 mg/kg of DTX equivalent. Weight of the mice was recorded until day 21 after the Conjugate 6 administration. Naïve mice i.v. injected with PBS were used as controls. Presented data are mean±SD of 3 mice per each condition.

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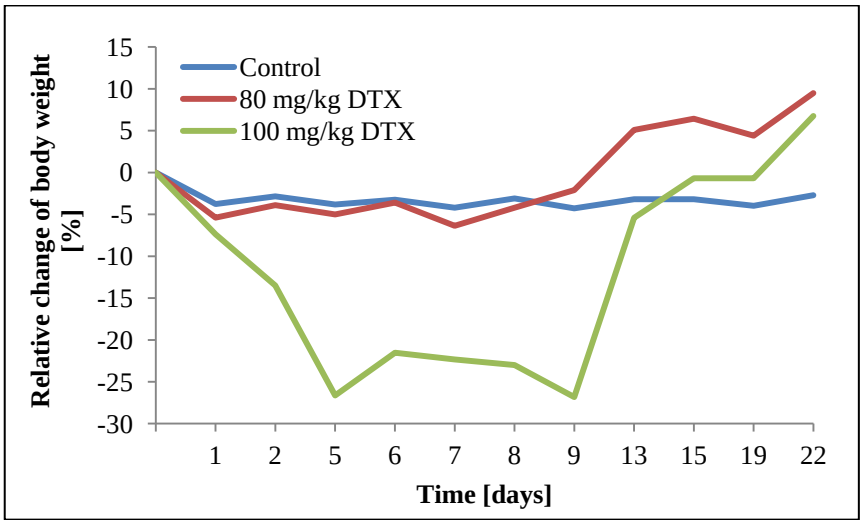

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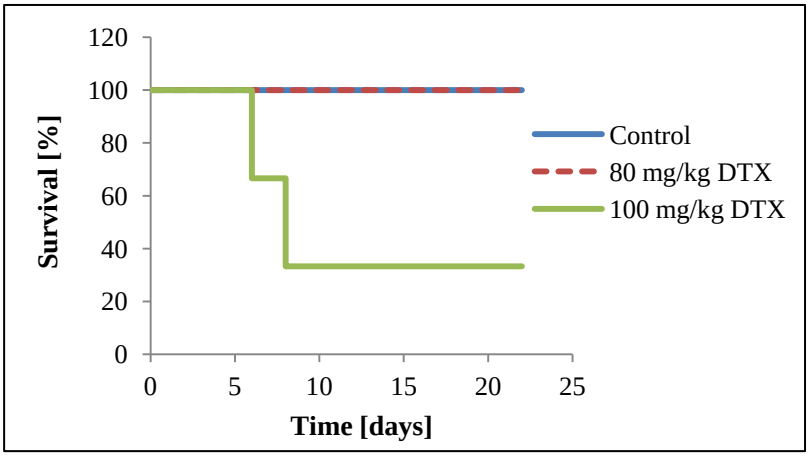

Figure 5.18: Determination of MTD of Conjugate 6 - II. BALB/c mice were i.v. injected with Conjugate 6 at doses of 80 or 100 mg/kg of DTX equivalent. Weight of the mice was recorded (**A**) and their survival was monitored (**B**) until day 22 after the Conjugate 6 administration. Naïve mice i.v. injected with PBS were used as controls. Presented data are mean±SD of 3 mice per each condition.

5.5.3 Anti-tumor activity of LMW HPMA copolymer-bound drug conjugates

The anti-tumor activity of all Conjugates was tested in EL4 T-cell lymphoma tumor model growing in syngeneic B6 mice. Mice with established tumors were i.v. injected with two doses of Conjugate 3 (2x10 mg/kg DOX equivalent) on days 8 and 12 after the tumor cell inoculation. Significant inhibition of tumor growth compared to control mice (untreated mice s.c. injected with EL4 lymphoma cells) was observed (Figure 5.19) with 5 out 8 mice surviving with no apparent signs of disease up to day 92.

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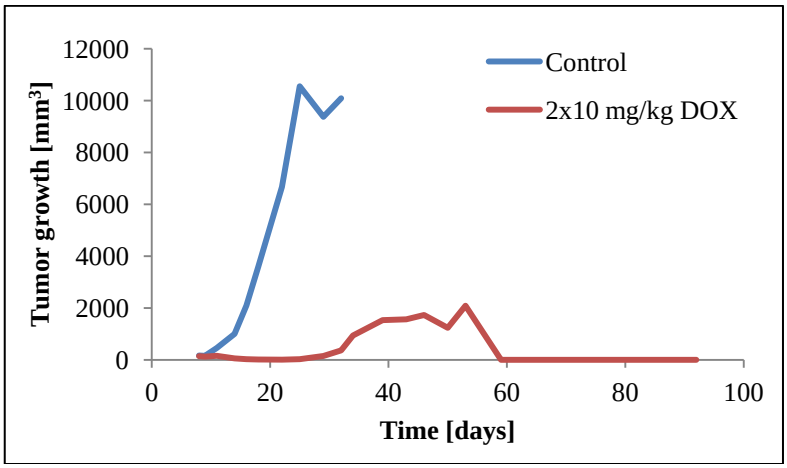

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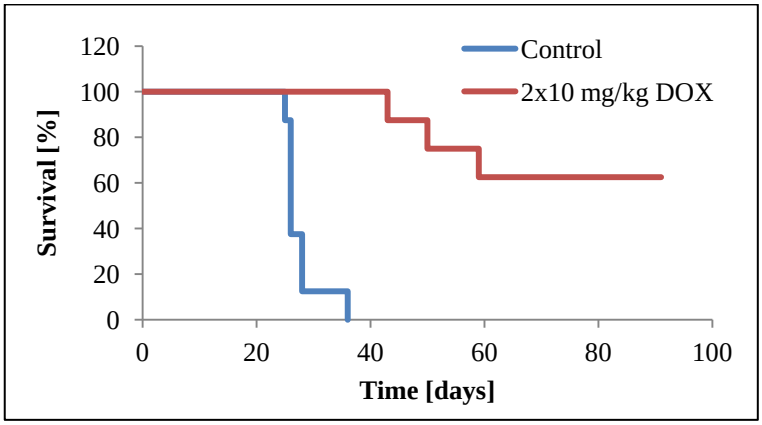

Figure 5.19: Anti-tumor activityct of Conjugate 3 in mouse model of EL4 T-cell lymphoma. B6 mice were s.c. injected with $1x10^5$ of EL4 lymphoma cells on day 0 and i.v. injected with Conjugate 3 at 10 mg/kg/dose of DOX equivalent on days 8 and 12 after tumor cell inoculation. Tumor size was measured (**A**) and survival was monitored (**B**) until day 92. Untreated mice s.c. injected with EL4 lymphoma cells were used as controls. Presented data are mean±SD of 8 mice per each condition.

Similar schedule as described above was used in case of Conjugate 4. Mice were i.v. injected with two doses of Conjugate 4 (2x20 mg/kg DTX equivalent) on days 8 and 12 after the tumor cell inoculation. Conjugate 4 was also capable to significantly inhibit the growth of the tumor, however, only 2 out 8 mice survived with no signs of disease up to day 92 (Figure 5.20).

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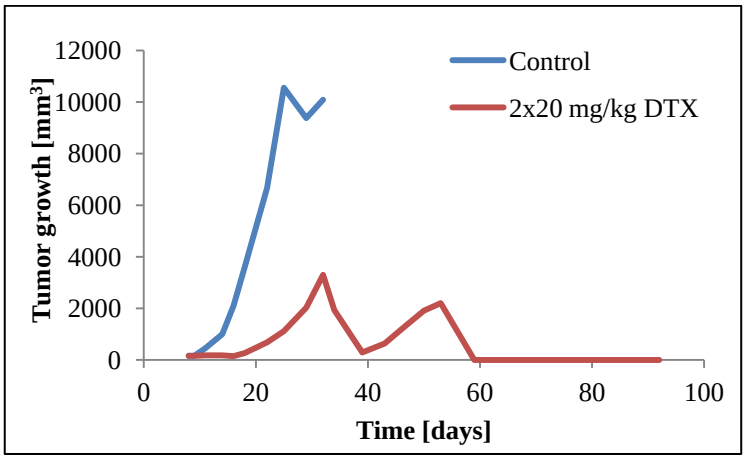

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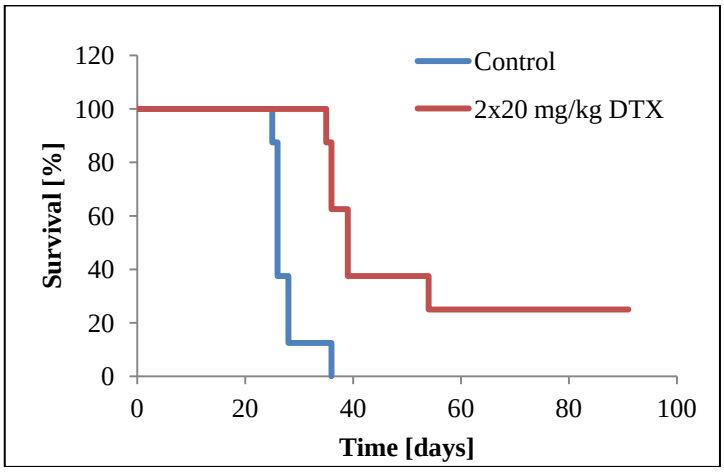

Figure 5.20: Anti-tumor activity of Conjugate 4 in mouse model of EL4 T-cell lymphoma. B6 mice were s.c. injected with $1x10^5$ of EL4 lymphoma cells on day 0 and i.v. injected with Conjugate 4 at 20 mg/kg/dose of DTX equivalent on days 8 and 12 after tumor cell inoculation. Tumor size was measured (**A**) and survival was monitored (**B**) until day 92. Untreated mice s.c. injected with EL4 cells were used as controls. Presented data are mean±SD of 8 mice per each condition.

Data show that both tested conjugates are able to retard tumor growth and even completely cure some of the experimental mice to the state with no signs of disease. Therefore, they were considered to be used for further experiments concerning testing of combination of T_{reg} cell depletion with chemotherapy in treatment of tumor diseases. Higher therapeutic activity of the conjugate bearing DOX probably reflects the fact that the used tumor model is lymphoma. Hematological malignancies are known to be relatively sensitive to anthracyclines (i.e. DOX) but taxanes (i.e. DTX) are practically not used to treat this kind of malignancies since they show only limited effect.

5.5.4 Anti-tumor activity of HMW HPMA copolymer-bound drug conjugates

Titrated doses of Conjugate 5 (5, 7.5 or 10 mg/kg of DOX equivalent) were i.v. injected into mice on day 8 after EL4 tumor cells inoculation. Similarly to LMW HPMA copolymer-bound drug conjugates, the application of Conjugate 5 caused significant inhibition of tumor growth compared to control mice (Figure 5.21). Moreover, several mice in each group were completely cured with no signs of the disease up to day 63 – 3 out 7 mice in group injected with 5 mg/kg DOX equivalent, 5 out 7 mice in group injected with 7.5 mg/kg DOX equivalent and 6 out 8 mice in group injected with 10 mg/kg DOX equivalent.

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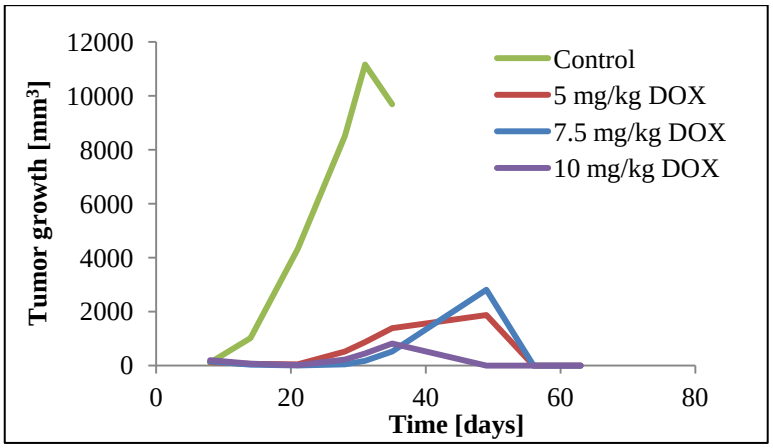

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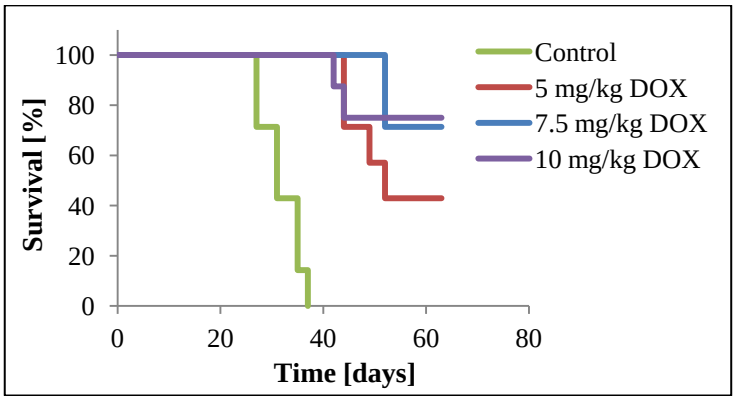

Figure 5.21: Anti-tumor activity of Conjugate 5 in mouse model of EL4 T-cell lymphoma. B6 mice were s.c. injected with $1x10^5$ of EL4 lymphoma cells on day 0 and i.v. injected with Conjugate 5 at doses of 5, 7.5 or 10 mg/kg of DOX equivalent on day 8 after tumor cell inoculation. Tumor size was measured (**A**) and survival was monitored (**B**) until day 63. Untreated mice s.c. injected with EL4 cells were used as controls. Presented data are mean±SD of 7 mice per each condition, except for group injected with 10 mg/kg of DOX equivalent which contained 8 mice.

Mice were i.v. injected with two doses of Conjugate 6 (2x20 mg/kg of DTX equivalent) on days 8 and 12 after tumor cells inoculation to estimate the anti-tumor

activity of Conjugate 6 (Figure 5.22). Significant suppression of tumor growth and survival of 4 out of 8 experimental mice was observed. Of note, this is much better therapeutic activity than the one found for Conjugate 4 (i.e. LMW conjugate with DTX) showing that HMW conjugates possess higher therapeutic activity at comparable doses. The question that needs to be answered is the ratio of MTDs of these conjugate.

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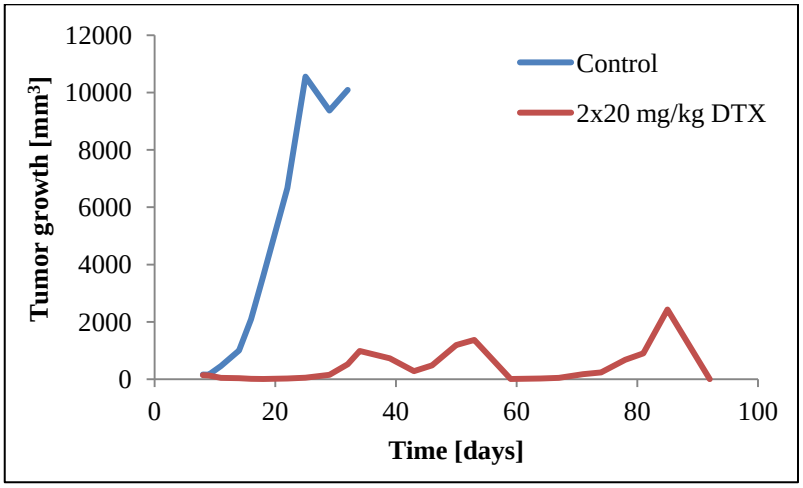

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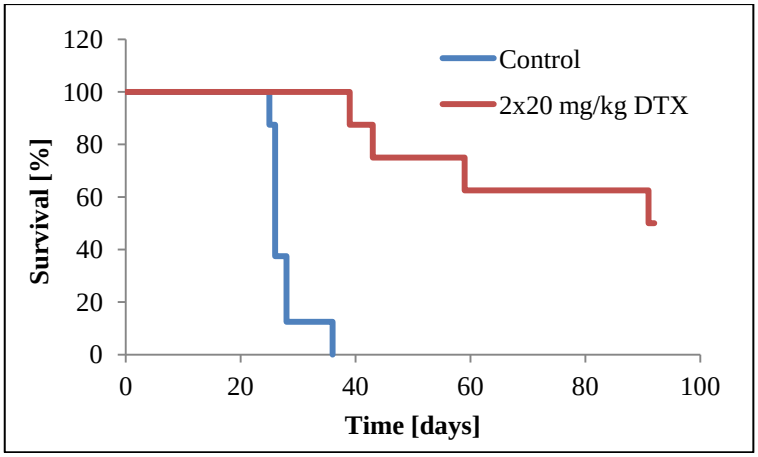

Figure 5.22: Anti-tumor activity of Conjugate 6 in mouse model of EL4 T-cell lymphoma. B6 mice were s.c. injected with $1x10^5$ of EL4 lymphoma cells on day 0 and i.v. injected with two doses of Conjugate 6 (20 mg/kg/dose of DOX equivalent) on days 8 and 12 after tumor cell inoculation. Tumor size was measured (**A**) and survival was monitored (**B**) until day 92. Untreated mice s.c. injected with EL4 cells were used as controls. Presented data are mean±SD of 8 mice per each condition.

In conclusion, both conjugates bearing either DOX or DTX show considerable anti-tumor activity and could be used in further experiments focusing on combined immunotherapy and chemotherapy for cancer treatment.

6 Discussion

Clinical treatment of cancer diseases employs various therapeutics, most of which are accompanied with serious side effects. There is an intensive research concerning improvement of cancer treatment protocols in terms of modification of therapeutics or development of novel anti-cancer drugs that would be more specific for tumor cells and thus would not cause damage to normal tissue.

Since tumors originate from normal cells and are often protected from immune system by T_{reg} cells, the effort to overcome regulatory T (T_{reg}) cell-mediated inhibition of anti-tumor immune reactions has been addressed as well. Several mAbs were employed for elimination of this cell subset, e.g. anti-GITR mAb or particularly anti-CD25 mAb (henceforth αCD25 mAb) [174]. However, this approach shows serious disadvantages. Antibody-targeted molecules are present not only on the surface of T_{reg} cells, but also on other immune cell subsets, especially activated T cells. Moreover, circulation half-life of IgG molecule is rather long, ranging from 21 to 28 days in humans [64], thus it could interfere with development of anti-tumor responses conducted by activated effector immune cells. The impairment of effector cell functions upon application of αCD25 mAb was reported in several studies demonstrating negative effect of αCD25 mAb on activated T cells [177, 179-180], B cells, NK cells [177] and iNKT cells [178].

Therefore, another approach how to eliminate T_{reg} cells from organism was proposed and its relevance was investigated in this thesis. It is based on reports describing that IL-2/anti-IL-2 mAb immunocomplexes possess selective stimulatory activity for various immunocompetent cell subsets depending on anti-IL-2 mAb clone used. The ability of IL-2/anti-IL-2 JES6.1A12 mAb (IL-2/JES6.1) immunocomplexes to trigger vigorous yet very selective proliferation of T_{reg} cells was exploited for sensitization of T_{reg} cells to cell cycle-specific cytostatic drugs. Employment of the other described IL-2/anti-IL-2 mAb immunocomplexes, i.e. IL-2/anti-IL-2 S4B6 mAb immunocomplexes, was abandoned after confirmation of their stimulatory activity not only for T_{reg} cells, but also for non-T_{reg} cell subsets whose proliferation even exceeded the level of proliferating T_{reg} cells.

The application of selected cell cycle-specific cytostatic drugs after induction of IL-2/JES6.1 immunocomplexes mediated T_{reg} cell proliferation showed that this approach is not suitable for depletion of T_{reg} cells. The drugs' dosage was either too

high and led to severe side toxicity or too low to reduce T_{reg} cell numbers under the level found in control mice. No significant reduction below this level was achieved.

Since the original approach was not efficient enough, another way how to deplete T_{reg} cells was considered. The potential of αCD4 depleting mAb (clone GK1.5) was investigated as it should eliminate whole $CD4^+$ T cell population including T_{reg} cells. Nevertheless, it was observed that T_{reg} cells are less sensitive to αCD4 mAb mediated depletion when compared to the rest of $CD4^+$ T cells and this observation was also made by other research group [195]. The mechanism of this phenomenon remains elusive.

The above discussed data led to reconsideration of αCD25 mAb (clone PC61.5) usage for T_{reg} cell depletion, however, the system for αCD25 mAb efficient elimination from organism had to be developed. It is based on avidin-biotin system. Avidin has extremely high affinity for biotin and the bond between these two molecules was defined to be the strongest known non-covalent interaction ($K_d = 10^{-15}$ M) with 1 molecule of avidin able to bind 4 molecules of biotin. These characteristics were exploited in this thesis where αCD25 mAb was biotinylated (henceforth αCD25-BIO mAb), i.p. administered to mouse organism for T_{reg} depletion and eliminated by application of HPMA copolymer-based conjugate bearing avidin (henceforth HPMA-avidin conjugate). It was proved that the biotinylation does not interfere with biological activity of αCD25 mAb *in vivo* as the comparison of biotinylated and native αCD25 mAb showed no significant difference in T_{reg} cell depletion. The persistence of αCD25-BIO mAb in mice plasma was also investigated. Levels of αCD25-BIO mAb remain in the circulation up to 12 and 18 days (detection limit of the assay was 0.3 μg/ml) when 100 μg and 250 μg of αCD25-BIO mAb are i.p. administered, respectively. Both doses caused considerable reduction of T_{reg} cell population with the peak decrease on day 5 (100 μg of αCD25-BIO mAb) or day 18 (250 μg of αCD25-BIO mAb) and with noticeable decrease of T_{reg} cell numbers already on day 3 after mAb administration. These results are more or less in accordance with the literature as studies employing αCD25 mAb reports that the peak depletion lies between 8-11 days after mAb administration and it stays stable for several weeks depending on the dose [175-176, 196]. Even though 250 μg of αCD25-BIO mAb appeared to be more effective, it was decided to use 100 μg of αCD25-BIO mAb in further experiments. This decision was made due to the fact that application of αCD25 mAb will be exploited in cancer therapy, thus it is necessary to eliminate the antibody from the organism relatively soon after its administration, i.e. about 5 days after the αCD25-BIO mAb injection. Both doses

showed comparable reduction of T_{reg} cell population on this day, therefore the lower dose was chosen to better suit further plans in research concept.

Conjugate of avidin bound to the HPMA copolymer via amide bond was designed and synthesized for αCD25-BIO mAb elimination from organism. It was proposed HPMA-avidin conjugate could be more suitable for αCD25-BIO mAb elimination from the body than avidin as it has higher molecular weight (550 000 g/mol; one molecule of the conjugate contains several avidins) and thus prolonged half-life in circulation, together with higher capability to cross-link αCD25-BIO mAb molecules. Moreover, avidin bound to the HPMA copolymer carrier has almost certainly significantly lowered immunogenicity [35, 43-44]. Two types of HPMA-avidin conjugates differing in structure were tested (see Figure 3.1). Both showed same biological effects *in vivo*. Application of HPMA-avidin conjugate led to reduction of concentration of αCD25-BIO mAb in circulation in dose-dependent manner (the higher dose of conjugate, the faster and more efficient αCD25-BIO mAb elimination from organism) with 100 μg of avidin equivalent being the minimal amount needed for effective elimination (lowering the concentration of αCD25-BIO mAb in circulation to non-detectable levels) of αCD25-BIO mAb from organism. Moreover, in comparison to avidin, HPMA-avidin conjugate proved to be more potent. Such difference between avidin and HPMA-avidin conjugate may be due to the polyvalency of HPMA-avidin conjugate, i.e. the ability to bind many biotin molecules and thus form large cross-linked multiprotein complexes.

Therefore, this system proved to be effective for T_{reg} cell depletion and αCD25-BIO mAb elimination from organism. Furthermore, it is rather general system and could be used for elimination of any biotinylated protein from organism. Whereas it also overcomes the main disadvantage of αCD25 mAb application, i.e. hampering development of anti-tumor responses, will be further tested.

Depletion of T_{reg} cells from organism is mostly not efficient enough to trigger anti-tumor immune reactions that would be capable to eradicate already established tumors. However, it was proposed to combine its anti-tumor activity with chemotherapy with HPMA copolymer-bound drug conjugates as these conjugates were reported not to, or only to low extent, suppress cell populations responsible for anti-tumor immunity [108]. Several studies already investigated the effects of αCD25 mAb (clone PC61.5) mediated T_{reg} cell depletion in combination with cancer treatment modalities. In 2005, Van Meirvenne *et al.* [196] reported that T_{reg} cell elimination *in vivo* before

immunization with tumor-antigen pulsed dendritic cells (DCs) enhances recently activated and memory phenotype antigen-specific $CD8^+$ T cell responses and inhibits growth of MO4 tumor model (mouse melanoma line B16F10 transfected with full-length OVA) *in vivo*. Study of Ma *et al.* [197] shows that partial T_{reg} cell depletion could be employed before radiation therapy. Irradiation causes increase of T_{reg} cell counts and prior use of αCD25 depleting mAb could diminish this effect and lead to improvement of such therapy. The authors conclude this combined treatment is safe and well tolerated in mice. Furthermore, it was reported that T_{reg} cell depletion in combination with chemotherapy (pemetrexed) has synergistic anti-tumor effect on established tumor (murine malignant mesothelioma) *in vivo* [198]. Prolonged survival, decrease of tumor infiltrating T_{reg} cells, increase of IL-2 production, DC maturation and increase of $CD3^+CD8^+IFN\text{-}\gamma^+$ tumor-infiltrating T cells was recorded in mice treated with αCD25 mAb and pemetrexed combined therapy in comparison with mice treated with pemetrexed or αCD25 mAb only. However, as it was already mentioned, such combined treatment could be affected by αCD25 mAb acting on activated non-T_{reg} $CD25^+$ cells important for development of anti-tumor immunity. The approach for T_{reg} cell depletion proposed in this thesis could overcome this disadvantage and would be potentially useful for combined therapy of cancer. Several HPMA copolymer-bound doxorubicin (DOX) or docetaxel (DTX) conjugates were tested in this study for their biological activity in terms of maximum tolerated dose (MTD) and anti-tumor effect in order to determine their optimal dosage for efficient cancer therapy following T_{reg} cell depletion. The conjugates have either linear (low molecular weight, LMW, conjugates) or star-like (high molecular weight, HMW, conjugates) structure and the drug is bound to the polymer carrier via pH-sensitive hydrazon bond. HPMA copolymer-based conjugates carrying DOX bound via hydrazon bond were reported to be stable in physiological pH (circulation) but not in acidic environment, where they are hydrolyzed and the drug is quickly released from polymer carrier (endosomes, lysosomes, tumor tissue) [48-50].

MTD of free DOX or DTX in mice differs in literature depending on used mouse strain, route of administration or experimental set up. MTD of free DOX ranges between 5-10 mg/kg/dose (i.v.) [199-202], whereas MTD of free DTX is reported to be 25 mg/kg/dose (i.v.) [199], 3x20 mg/kg separated by 4 days (i.v.) [203] or even 40 mg/kg (3-hours i.v. infusion) [202]. Indeed, tested HPMA copolymer-bound drug conjugates have higher MTD then free drugs with linear conjugates having significantly

higher MTD in comparison to star-like conjugates. The initial dosage for determination of MTD of HMW HPMA copolymer-bound DOX conjugate was chosen in concordance with the report of Lee *et al.* [200] who investigated characteristics of similar polymer-bound DOX conjugate. Dosage of other tested HMW and LMW HPMA copolymer-bound conjugates was selected according to the previously gathered data and personal experience with application of HPMA copolymer-bound drug conjugates for past 20 years.

Anti-tumor activity of selected conjugates was tested on the mouse tumor model of EL4 T-cell lymphoma. All conjugates proved to be useful for cancer treatment and were thus claimed to be suitable for further experiments which would combine T_{reg} cell depletion and chemotherapy in cancer treatment.

These data show new promising approach for T_{reg} cell elimination and potentiation of anti-tumor immunity, as well as strong potential of developed HPMA copolymer-bound drug conjugates for cancer therapy. T_{reg} cell depletion and HPMA copolymer-bound drug conjugates treatment could be combined together and lead to the improvement of cancer treatment exploiting not only the beneficial effects of HPMA copolymer-bound drugs, but also depleting effects of αCD25 mAb on T_{reg} cell population resulting in unleashed development of anti-tumor immune responses.

7 Conclusions

The original aim to deplete T_{reg} cells via IL-2/anti-IL-2 mAb immunocomplexes-mediated induction of proliferation and their subsequent elimination by cell cycle-specific cytostatic drugs led to following results:

- Dose of IL-2/JES6.1 immunocomplexes efficient enough for significant induction of T_{reg} cell proliferation is 3.2 μg of IL-2 equivalent.
- IL-2/S4B6 immunocomplexes are not suitable for selective induction of T_{reg} cell proliferation as they trigger robust proliferation not only of T_{reg} cells but also non-T_{reg} cells as well.
- The combination of IL-2/JES6.1 and IL-2/S4B6 immunocomplexes did not increase higher induction of T_{reg} cell proliferation than IL-2/JES6.1 immunocomplexes alone.
- The optimal time frame for application of cell cycle-specific cytostatic drugs is between 24 and 48 hours after the application of IL-2/JES6.1 immunocomplexes.
- Cell cycle-specific cytostatic drugs used at doses associated with acceptable toxicity are not able to lower the number of proliferating T_{reg} cells below the level found in control mice.

In conclusion, the application of cell cycle-specific cytostatic drugs was proved to be not suitable for sufficient elimination of T_{reg} cells from organism upon induction of their proliferation by IL-2/JES6.1 immunocomplexes.

Since the above mentioned approach proved to be not efficient enought in T_{reg} cell elimination, the alternative way was investigated.

- Depletion of T_{reg} cells by application of αCD4 mAb clone GK1.5 showed T_{reg} cells are less sensitive to GK1.5 mAb-mediated depletion in comparison to other $CD4^+$ T cell subsets.
- Administration of αCD25 mAb (clone PC61.5) efficiently reduced T_{reg} cell numbers without any signs of side toxicity. Dose chosen for further experiments was 100 μg and the optimal time for αCD25 mAb elimination from organism was determined to be day 5 after the mAb administration.
- Biotinylated αCD25 (αCD25-BIO) mAb shows similar biological activity as native mAb *in vivo*. The number of biotins per αCD25 mAb ranges from 5 to 10.

- αCD25-BIO mAb is detectable in plasma for several weeks in dose-dependent manner (detection limit was 0.3 μg/ml).
- The HPMA-avidin conjugate is more potent then avidin in elimination of αCD25-BIO mAb from circulation.
- The minimal dose of HPMA-avidin conjugate for successful elimination from organism is 100 μg of avidin equivalent and more. Effectiveness of αCD25-BIO mAb elimination increase with higher doses.

The avidin-biotin system was proved to be suitable for overcoming the main disadvantage of using αCD25 mAb for T_{reg} cell depletion which is long persistence of αCD25 mAb in organism affecting subsequently activated effector cells and suppressing induction of anti-tumor immunity.

Experiments focusing on characterization of synthesized HPMA copolymer-bound DOX or DTX conjugates showed that:

- Maximum tolerated dose of linear LMW HPMA copolymer-bound DOX (Conjugate 3) is significantly lower than 100 mg/kg of DOX equivalent.
- Maximum tolerated dose of linear LMW HPMA copolymer-bound DTX (Conjugate 4) is significantly higher than 150 mg/kg of DTX equivalent.
- Maximum tolerated dose of star-like HMW HPMA copolymer-bound DOX (Conjugate 5) is between 20-25 mg/kg of DOX equivalent.
- Maximum tolerated dose of star-like HMW HPMA copolymer-bound DTX (Conjugate 6) is around 90 mg/kg of DTX equivalent.
- All tested conjugates possess potent anti-tumor activity, in mouse tumor model of EL4 T-cell lymphoma.

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