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ASSESSMENT OF THE MODULATION OF PHOTODYNAMIC EFFECT BY β-GLUCAN AND CHARACTERISTICS OF ANTI-CD7 MONOCLONAL ANTIBODY DURING TUMOR PROCESS

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FOTODINAMINIO POVEIKIO MODULIACIJOS β-GLIUKANU VERTINIMAS IR MONOKLONINIO ANTIKŪNO PRIEŠ CD7 SAVYBIŲ TYRIMAS NAVIKINIO PROCESO METU

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CONTENTS

ABBREVIATIONS

ADCC	– antibody dependent cell cytotoxicity
BRM	– biological response modifier
BSA	– bovine serum albumin
βGR	– β-glucan receptor
CD	– cluster of differentiation
CDC	– complement dependent cytotoxicity
CDR	– complementary determining region
COX	– cyclooxigenase
CR3	– complement receptor 3
CR3-DCC	– complement receptor 3 dependent cell cytotoxicity
Cy	– cyclophosphamide
DEN	– dielthylnitrosamine
DNA	– deoxyribonucleic acid
EGFR	– endothelial growth factor receptor
FACS	– fluorescence activated cell sorter
FcR	– Fc receptor
FITC	– fluorescein isothiocyanate
GM-CSF	– granulocyte-macrophages colony stimulating factor
HAMA	– human anti-mouse antibody
HARA	– human anti-rat antibody
H_2O_2	– hydrogen peroxide
IFNγ	– interferon γ
IgG	– Immunoglobulin G
IL	– interleukin
LacCer	– lactosylceramide
LLC	– Lewis lung carcinoma
LPS	– lipopolysaccharide
mAb	– monoclonal antibody
MAC	– membrane attack complex
MHC	– major histocompatibility complex
MNC	– mononuclear cell
NK	– natural killer cell
NO	– nitric oxide
NSCLC	– non-small cell lung cancer
PAMP	– pathogen-associated molecular pattern
PBS	– phosphate buffered saline
PCNA	– proliferating cell nuclear antigen

PDT – photodynamic therapy
PMNS – polymorphonuclear leukocyte
PRR – pattern recognition receptor
PSP – polysaccharopeptide
T-ALL – T cell acute lympholeukemia
TLR – Tool-like receptors
TNF – Tumor necrosis factor
TRAIL – TNF-related apoptosis-inducing ligand
sc – single chain
SCID – severe combined immunodeficiency
SCG – glucan from *Sparassis cripsa*
SIV – swine influenza virus
VEGF – vascular endothelial growth factor
V_H – heavy chain variable domain
V_L – light chain variable domain
WBC – white blood cell

INTRODUCTION

Cancer has become more and more prevalent over the last few years, reaching its highest ever levels per capita in Lithuania and in the world. Cancer is a group of diseases characterized by the uncontrolled growth and spreading of abnormal cells. This is related to dynamic changes in the genome. Typical treatments for cancer include surgery, radiation therapy and chemotherapy. Cancer cells are very sensitive to chemotherapeutic agents. However, tumor cells are not specifically targeted and chemotherapy also kills normal cells of the body, therefore it results in significant side effects. In the last years, the problem of resistance against chemotherapeutic agents has become more common, and further investigation revealed that the resistant tumor cells contained mutations that allowed them to regulate drug cytotoxicity [24]. In recent years, given an important role in the immune system of cancer patients, encouraging them to recognize and destroy malignant cells and cause tumor regression. Therefore, other methods of treatment based on the host's immune system functioning, such as biological therapy with monoclonal antibodies (immunotherapy) and photodynamic therapy becomes important for a complex treatment of tumors. Change of factors of the immune system during these treatments gives the opportunity to develop new strategies in tumor therapy.

Photodynamic therapy (PDT) is a minimally invasive therapeutic modality approved for the treatment of some vascular and cancer disease. In addition to producing necrosis and/or prompting apoptosis in the tumor, PDT also triggers the immune system of the host that results in damages of nutrient blood vessels to the tumor [39, 153, 38, 101]. Activation of complement cascade plays an important role in the PDT-induced response and treatment outcome [71, 73]. After the activation of complement system via alternative pathway cells are opsonised with iC3b frgment, which interacts with complement receptor 3 (CR3). The CR3 on human leucocytes do not trigger the killing of tumour cells coated with their ligand iC3b. But CR3 priming for cytotoxic function requires ligation of both, the I-domain and lectin-like domain of CR3 [111]. CR3-DCC is normally reserved for yeast and fungi that have β-glucan as an exposed component of their cell wall [42]. In contrast to microorganisms, tumor cells lack β-glucan as a surface component and can‘t trigger complement receptor 3-dependent cellular cytotoxicity and initiate tumor-killing activity.

The findings mentioned above gave rise to the hypothesis that β-glucan in combination with PDT will produce more effective killing of PDT-treated tumor cells.

With the development of hybridoma technology by Georges J.F. Kohler and Cesar Milstein, monoclonal antibodies (mAbs) have attracted renewed strong interest as therapeutics in clinical oncology. This strategy is more specific to cancer cells and their metabolism. In clinic, they achieve promising levels of cytotoxicity towards cancer cells and reduce unwanted side effects. Early clinical trials using rodent mAbs failed due to rapid formation of human anti-mouse antibody (HAMA) or human anti-rat antibody (HARA). These host responses dramatically altered the pharmacokinetic profile of the antibody, leading to rapid clearance of the mAb and preventing repeat dosing [107]. In addition to immunogenicity, murine antibodies have a short life in humans and are ineffective in effector functions, which are essential components of the mechanism of action of many mAbs [158, 128]. Immunogenicity raises serious problems in terms of acute side effects and influences drug pharmacokinetics and decreases drug efficacy. So there is a requirement developing less immunogenic mAbs. These have included chimerization, humanization, and the development of human antibodies from transgenic mice or phage display libraries [2, 75].

Today some of monoclonal antibodies are successfully used in clinical practice for B-cell lymphoma, breast and colorectal cancer treatment [115, 118, 2, 130, 137], all have impressive activity in tumor patients. However, patients suffering from T-cell leukaemias (T-ALL) and lymphomas still have limited treatment options. Prognosis of childhood acute T-ALL has improved with modern chemotherapy, but T-ALL patients with remission induction failure after induction chemotherapy or with relapse of T-ALL still have a very poor prognosis [104].

One of the prerequisites for successful immunotherapy of T-cell neoplasias is the selection of an appropriate target antigen, which ideally should be T cell specific and expressed on most T-cell lymphomas and leukaemias but absent on at least a portion of normal T lymphocytes [104]. The CD7 antigen meets these requirements. CD7 is a marker for very early stages of T-cell maturation and is already present on lineage-committed hematopoietic progenitors in the fetal liver and on pluripotent progenitors of T cells in the thymus, bone marrow, and cord blood [49, 125]. CD7 is further expressed on a majority of human thymocytes and a large subset (`~85%) of peripheral blood T cells and natural killer cells [1, 125, 120, 102]. The remaining subset of CD7-negative peripheral T cells maintains immune functions needed for the prevention of opportunistic infections and the engraftment of hematopoietic stem cells. Therefore, this subset may become relevant for therapeutic purposes, because it may serve to repopulate the T-cell compartment at least in part after a CD7-directed therapy [104, 1].

The use of recombinant DNA technology allows to reduce antigenicity of murine monoclonal antibodies. Chimeric antibodies were developed, in which the constant domains of the human IgG_1 molecule were combined with mouse heavy and light chain antibody variable regions (Fv fragment) [43, 130, 54]. Fv fragment is the smallest antibody fragment with the entire antigen-binding site. However, the absence of covalent bonds makes this fragment unstable and it is unable to trigger effector functions without Fc part [86, 43, 91].

The hybridoma monoclonal antibody TH-69, generated by Dr. Martin Gramatzki (University of Erlangen-Nurnberg, Erlangen, Germany), directed against human CD7, produced significant antitumor effects in athymic ("nude", $nu^{-/-}$) and SCID mice xenografted with human T-ALL cell lines (CEM or MOLT-16 cells) [14]. Also the high binding affinity for TH-69 contributes to the therapeutic efficacy. The human Fc portion is essential for the recruting of human effector immune cells to produce antitumor effect [14, 158]. Therefore, connection of Fv portion of murine anti-CD7 antibody (TH-69) with Fc portion of human IgG_1 can be helpful for such protein to obtain ideal feathers. However, each modification of the monoclonal antibody can cause the lost or decrease in the rate of protein expression and antigen-binding properties. Monoclonal antibody products are unique in their molecules. Because of post-translational modifications that often occur during the fermentation process, the final product is heterogeneous [150]. Therefore, careful characterization of monoclonal antibodies is required in order to assess their identity, purity, potency and safety [77, 91, 158].

Activation of the immune system during photodynamic therapy and improvement of the effector functions of mAbs – these are the ways to use and enhance the potential of the immune system to fight cancer.

1. THE AIM AND OBJECTIVES OF THE STUDY, SCIENTIFIC NOVELITY OF THE WORK

The aim of the study:

To determine the possibility to modulate tumor response to photodynamic therapy with β-glucans and characterize a chimeric single chain anti-CD7 Fc-fusion antibody *in vitro*.

The objectives of the study:

1. To evaluate the growth of Lewis lung carcinoma tumor, treated by photodynamic therapy or/and β-glucan.
2. To define survival of Lewis lung carcinoma tumor bearing mice, treated by photodynamic therapy or/and β-glucan.
3. To determine the expression level of proliferating cell nuclear antigen in the cells of Lewis lung carcinoma tumor, treated by photodynamic therapy or/and β-glucan.
4. To define the size of necrotic areas in Lewis lung carcinoma tissue after photodynamic therapy or/and treatment with β-glucan.
5. To determine which β-glucan – either β-glucan from barley, or from brown algae, or from baker's yeast – is most effective in combination with photodynamic therapy for Lewis lung carcinoma treatment.
6. To express and purify chimeric single chain anti-CD7 Fc-fusion antibody and to determine its binding specificity to CD7 antigen.
7. To determine the capacity of chimeric single chain anti-CD7 Fc-fusion antibody to mediate lysis of CD7-possitive tumor cells *in vitro*.

The scientific novelty of the work

There are various cancer treatment methods currently available. However, treatment outcomes are not completely succesful. Special attention is paid to research in order to improve the effectiveness of treatment, because the incidence of cancer is growing around the world.

A lot of studies were performed for enhancing PDT effect by combination with other treatment modalities. There was only one report before, where β-glucan was used in combination with PDT. However, the single dose of β-glucan used in the mentioned study. Original part of our work is that we used multiple dosage of β-glucan according to the recently characterized mechanisms of action for β-glucan and complement activation during PDT. Tumor response to the treatment was evaluated by monitoring the changes in tumor volume and survival of mice. The significant part of the study is immunohystochemical analysis, where changes in proliferating activity of tumor cells and necrosis in tumor tissue were measured to estimate the efficiency of the treatments. In addition to cell killing, PDT activates the cellular DNA damage repair system, which could be an important factor that modulates tumor sensitivity to this treatment. Therefore, the novel part of this study is that effect of β-glucans on the activity of DNA damage repair system was investigated in this study as well.

The effect of different structure β-glucans was investigated in our study in order to determine influence of the molecular weight and structure on its effectivity. To our knowledge, there was no any research done in Lithuania before on β-glucans, well known as non-specific stimulators of the immune system and a very powerful antagonists to both benign and malignant tumors.

Many studies are performed in the world in order to decrease immunogenicity and to improve pharmacokinetic properties of mAbs. To our knowledge and literature data, this is the first study, where Fv fragment of anti-human-CD7 molecule has been incorporated into human immunoglobulin framework. The work has been performed together with the colleges from Christian-Albrecht University (Kiel, Germany). Modern investigation methods were used to characterize the novel antibody. Any research on anticancer mAbs has not been performed in Lithuania before.

The results of this study confirmed the anticancer activity of β-glucans. It was shown that β-glucan in combination with PDT supress tumor growth and proliferation activity, and produce effective necrosis of tumor tissue. Therefore, PDT in combination with β-glucan might be the effective therapeutic strategy.

Our data indicate that fusion of scFv fragment with Fc part of human IgG_1 has improved cytotoxicity to tumor cells *in vitro*. That is why, chimeric single chain anti-CD7 Fc-fusion protein might be a promising construct for further modifications to improve Fcy receptor binding or to diminish interaction with Fcy inhibitory receptors [46, 77, 92].

2. LITERATURE REVIEW

2.1. Photodynamic therapy

Photodynamic therapy (PDT) is a treatment method that combines the administration of a light-sensitive drug and lesion-directed activation of the photosensitizer with visible light.

2.1.1. History of photodynamic therapy

Light has been used for treatment purpose for more than three thousand years. Ancient Egyptian, Indian and Chinese civilizations used light to treat some disease, as psoriasis, rickets and skin cancer [38, 114]. More than 100 years ago, German medical student Oscar Raab described that combination of light and certain chemicals could induce lethal effect on bacteria [114]. Herman Von Tappainer in 1904 discovered that oxygen was essential for the process and described this phenomenon as "photodynamic action"[114, 38]. In 1911 W. Hausmann performed the first studies of the biological effect of hematoporphyrin. In the 1960s, Richard Lipson and Samuel Schwartz initiated the modern era of PDT at the Mayo Clinic. In 1972, I. Diamond and colleagues postulated that the combination of the tumor-localizing and tumor-phototoxic properties of porphyrins might be used to kill cancer cells [114].

In Lithuania first investigations in the field of PDT were initiated in 1985. Researches in Vilnius University and Lithuanian Oncology Center are concentrated on *in vitro* and *in vivo* spectroscopy of biologically active pigments, spectroscopy of photosensitizers in model systems. PDT has been used in the Clinics of Lithuanian Oncology Center since 1989 [114].

To date, the U.S. Food and Drug Administration (FDA) has approved the photosensitizing agent porfimer sodium, or Photofrin, for use in PDT to treat or relieve the symptoms of esophageal cancer and non-small cell lung cancer [140]. Porfimer sodium is approved to relieve symptoms of esophageal cancer when the cancer obstructs the esophagus or when the cancer cannot be satisfactorily treated with laser therapy alone. Porfimer sodium is used to treat non-small cell lung cancer in patients for whom the usual treatments are not appropriate, and to relieve symptoms in patients with non-small cell lung cancer that obstructs the airways. In 2003, the FDA approved porfimer sodium for the treatment of precancerous lesions in patients with Barrett's esophagus (a condition that can lead to esophageal cancer) [141].

2.1.2. Mechanism of action

In the first step of PDT for cancer treatment, a photosensitizing agent is used by intravenous injection or topical application to the skin. If agent (photosensitizer) is injected intravenously, it is absorbed by cells all over the body, but stays in rapidly dividing cells longer than it does in normal cells. Approximately 24 to 72 hours after injection [97, 114], when most of the agent has left normal cells but remains in rapidly dividing cells, as cancer cells, the target tissue is exposed to light of a specific wavelength that activates the photosensitizer. The photosensitizer in the target tissue absorbs the light (photons) and is transformed from its ground state (singlet state) into a relatively long-lived excited state (triplet state) via a short-lived exited singlet state [114, 144, 37]. The exited triplet state can react in two ways. The first way involves direct reaction with a substrate, such as cell membrane or a molecule, and transferring of a hydrogen atom (electron) to form free radicals, which further react with molecular oxygen producing superoxide radical anion, hydrogen peroxide or hydroxyl radical. These free radical species are generally highly reactive and can cause irreparable biological damage. By the second way the triplet state sensitizer can transfer its energy directly to molecular oxygen, to form singlet oxygen, $^1O_2$,– this species is extremely reactive and can interact with a large number of biological substrates, inducing oxidative damage and cell death and is responsible for majority of lesions generated during PDT [114, 38, 121].

The wavelengths used in PDT are in the red or infrared range of electromagnetic waves. For a photobiological reaction to occur light must be absorbed by the photosensitizer. This is possible when the wavelength of light matches the electron absorption spectrum of the photosensitizer [61, 62]. For clinical use the activating light is usually between 600 and 900 nm. This is because endogenous dyes, mainly hemoglobin, strongly absorb light below 600 nm and longer wavelengths are energetically insufficient to produce $^1O_2$, which is the most important although not the only cytotoxic effector of PDT [121]. A critical parameter for consideration in discussing the efficacy of PDT is the depth of light penetration through tissues, which is dependent on several processes. Tissue penetration is also affected by the wavelength of the light. Longer wavelengths of visible light penetrate tissues better than shorter ones, and for most PDT application the lower limit is 580 nm largely because of strong absorption by hemoglobin [97, 114]. Typically, the depth of penetration is from 3 to 8 mm for light in the range from 630 to 800 nm. It was observed that tumors of up to 1 cm of depth can be effectively eradicated by PDT, an effect that can be explained

by the concomitant activation of local immune response [97]. Halogen, fluorescent, xenon lamps are used as a light source for PDT, but optimal illumination is obtained with laser light due to it features, as collimation, coherence and monochromacity [97].

Currently, over 30 different photosensitizers are used in preclinical studies [98]. The most extensively studied photosensitizers are porphyrins. From this group of photosensitizers, Photofrin is the most commonly used in the clinic today. But there are several limitations of this drug – it is not very selective for tumor tissue and causes long-lasting cutaneus photosensitivity, as it is absorbed by the skin [97, 114]. Currently new photosensitizers were developed, because there was a need of compound, which absorbs light at longer wavelength (that means better penetration of the light), has greater tumor specificity and less skin photosensitivity [98].

Researchers continue to study ways to improve the effectiveness of PDT and expand it to other cancers. Clinical trials are under way to evaluate the use of PDT for cancers of the brain, skin, prostate, cervix, and peritoneal cavity. Other research is focused on the development of photosensitizers that are more powerful [38], more specifically target cancer cells [114, 144, 37], and are activated by light that can penetrate tissue and treat deep or large tumors [144]. Researchers are also investigating ways to improve equipment [144, 114] and the delivery of the activating light [37].

2.1.3. PDT effects on tumors

In addition to directly killing cancer cells, PDT appears to shrink or destroy tumors in two other ways [39, 153, 114, 144]. The photosensitizer can damage blood vessels in the tumor, thereby preventing the cancer from receiving necessary nutrients. In addition, photooxidative lesions produced by PDT are recognized by the host as self alteration. It activates the immune system to attack the tumor cells and major effectors, inflammation and acute phase response, are mobilized. Activation of the complement cascade has an important role in the initiation and orchestration of the PDT-induced response and treatment outcome [71]. The blockage of the receptors of the anaphylatoxins C3a and C5a [71] or depletion of neutrophils [73], both significantly decrease tumour cure rates. In contrast, adjuvant treatment with complement –activating agents enhanced the therapeutic effect of PDT [25, 69]. Complement proteins, which act as opsonins, bind to PDT-treated cells that attract neutrophils, macrophages, dendritic and other immune cells displaying complement receptors. Complement system is activated via alternative pathway during the post-PDT treatment [68, 71]. After several cascade reactions it results in the covalent attachment of C3b to the cell

surface, where then it is rapidly degrade into the fragments iC3b and C3dg. Then these fragments bind to complement receptor 3 (CR3; CD11b/CD18) on the leucocytes.

2.2. β-glucans

β-glucan has been known to scientists as a plant constituent for decades. For over twenty years, it has been studied for the favorable biological effects on mammals. It has been common knowledge in the scientific community that β-glucan is the most known powerful immune stimulant and a very powerful antagonist to both benign and malignant tumors; it lowers cholesterol and triglyceride level [122, 131], normalizes blood sugar level, heals and rejuvenates the skin [34] and has various other benefits.

2.2.1. β-glucan sources and structure

β-glucans are naturally occurring polysaccharides. These glucose polymers are produced by a variety of plants, such as oat, barley, and seaweed. β-glucans are the constituents of the cell wall of certain pathogenic bacteria (*Pneumocystis carinii, Cryptococcus neoformans, Aspergillus fumigatus, Histoplasma capsulatum, Candida albicans*) and fungi (*Saccharomyces cerevisiae*). The main components of the fungal cell wall are polysaccharides and glycoproteins (Fig. 2.2.1). β-glucan has been purified from brewer's and backer's yeast [136], from oats and barley bran [15]. The healing and immunostimulating properties of mushrooms have been known for thousands of years. The extracts of these mushrooms were widely used for treatment purpose in East countries. The number of mushrooms on Earth is estimated to be 140 000, but approximately 10% are known (14 000 named species) [148]. These mushrooms contain biologically active polysaccharides in fruit bodies and cultured mycelium. These polysaccharides are of different chemical composition, with most belonging to the group of β-glucans and they have attracted the most attention [148]. β-glucan has been isolated from some mushrooms as shiitake (*Lentinus edodes*), maitake (*Grifola frondosa*) [87], schizophylan (*Schizophillum commune*), and SSG (*Sclerotinia sclerotiorum)* [20]. β-glucan extracts from *Lentinus edodes* and *Schizophillum commune* are used in traditional medicine for cancer treatment in Japan since 1980. β-glucans derived from different sources have some differences in their structure. Glucans are a heterogeneous group of glucose polymers, consisting of a backbone of β (1,3)-linked β-D-glucopyranosyl units with β(1,6)-linked side chains of varying distribution and length. Oat and barley β-glucans are primarily linear with large regions of

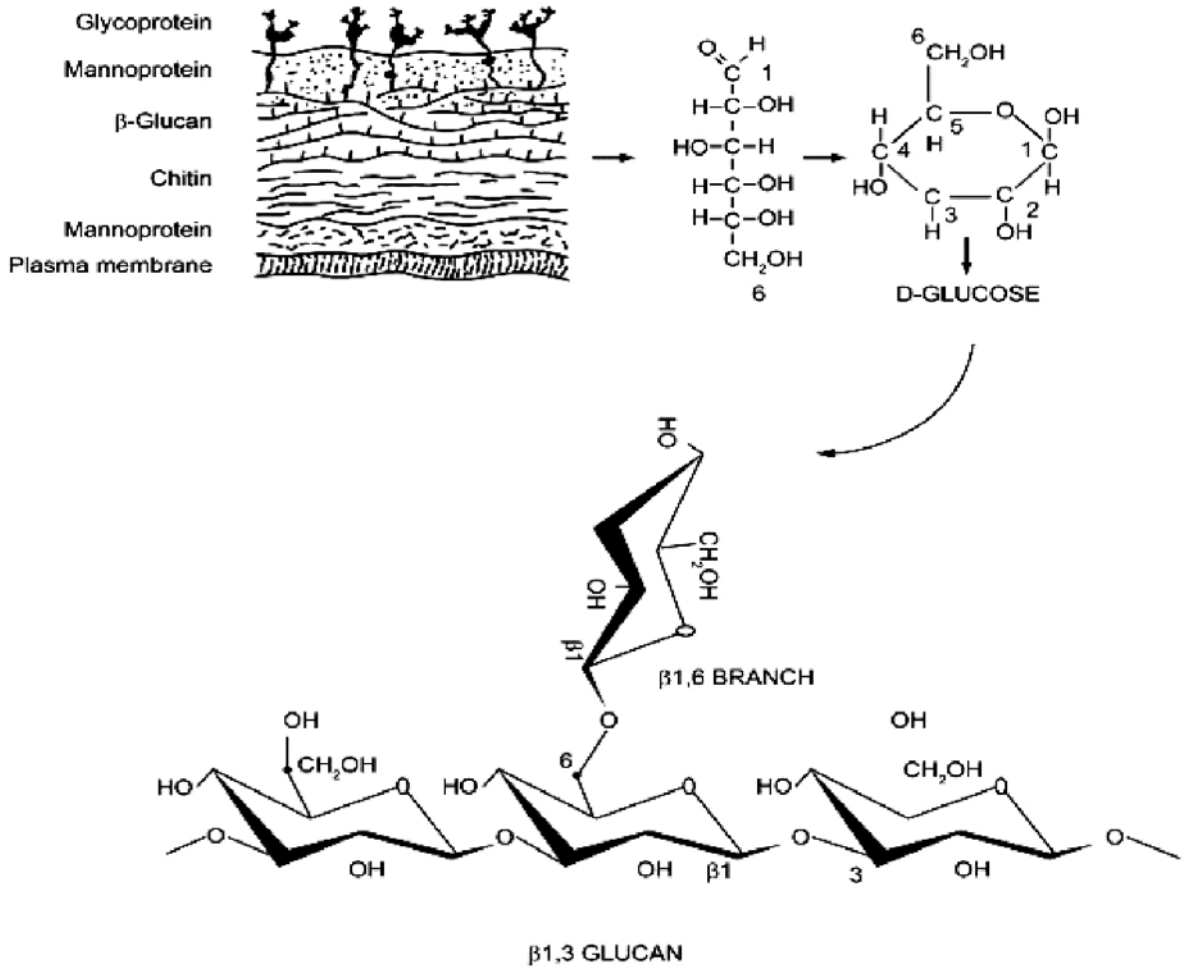


Fig. 2.2.1. *β-glucan is one of the key components of the fungal cell wall.* The basic subunit of the fungal β-glucan is β-D-glucose linked to one another by 1→3 glycosidic chain with 1→6 glycosidic branches. The length and branches of the β-glucan from various fungi are widely different. Source: Chan et al., J Hemat Oncol 2009.

β-(1,4) linkages separating shorter stretches of β-(1,3) structures. Mushrooms β-glucans have short β-(1,6)-linked branches coming of the β-(1,3) backbone. Yeast β-glucans have β-(1,6) branches that are further elaborated with additional β-(1,3) regions. These structural differences can have large implications for the activity of the β-glucan. For example, differences in the length of the polysaccharide chain, extent of branching, and the length of those branches can result in the difference between material extractable by hot water, as mushroom β-glucans, and insoluble cell wall component, as yeast β-glucan, and in different molecular weight. In general, *in vitro* studies have suggested that large molecular weight or particular β-glucans (such as zymosan) can directly activate leukocytes, stimulating their phagocytic, cytotoxic, and antimicrobial activities, including the production of reactive oxygen and nitrogen intermediates. Intermediate or low molecular weight

β-glucans (such as glucan phosphate) possess biological activity *in vivo*, but their cellular effects are less clear. Very short β-glucans (<5000–10 000 molecular weight; such as laminarin) are generally considered inactive [21, 56, 80]. Yeast β-glucan, because it is easily purified, and mushrooms β-glucans, because there are a lot of experiments performed in Japan, China, and Korea, are mostly investigated.

Therefore, β-glucan from different sources may affect tumor growth differently and that is why different structure β-glucans were used in this study.

2.2.2. β-glucan immunostimulating activity

Patients who suffer from systemic fungal infections including those caused by *Candida*, *Aspergillus*, and *Cryptococcus* species have been described to possess high levels of circulating β-glucans in their plasma. It is possible that they have modulating effects on the immune system by activating of macrophages, phagocytosis of the pathogen, and release of proinflammatory cytokines [119]. There was established, that β-glucan is a key molecular pattern recognized by neutrophils (or polymorphonuclear leukocytes (PMNs)) in response to *Candida albicans,* because antibody specific for β-glucan, a major component of yeast cell walls, blocks this response [78]. This mechanism – to recognize and respond to their conserved structural components, particularly β-glucans – has evolved in mammals as defense against fungal pathogen. Macrophages play a critical role in all phases of host defense that are both innate and adaptive immune responses in case of an infection. When pathogen crosses an epithelial barrier, it is affected by phagocytosis of macrophages and digested by lysosomal enzymes released from them. Lysosomal enzymes and phagocytic activity determine the macrophage function. The secretion of cytokines (IL-1, IL-6, IL-8, IL-12, TNF-α) and inflammatory mediators (nitric oxide, NO, and hydrogen peroxide, H_2O_2) are other effects of these cells. Therefore, activation of macrophage functions by β-glucans increases host immune defense. However, polysaccharides stimulate a dose-dependent increase in NO and TNF-α, but not in reactive oxygen intermediate production in peritoneum macrophages [62]. It is suggested that the ability of polysaccharides upon the up-regulation of these surface molecules involved in antigen-presenting processes may, by inference, activate T-cell-mediated immunity against malignant cells *in vivo*. Taken together, these results suggest that β-glucan acts as an effective immunomodulator and enhances the anti-tumoral activity of peritoneum macrophages. *In vitro* studies have

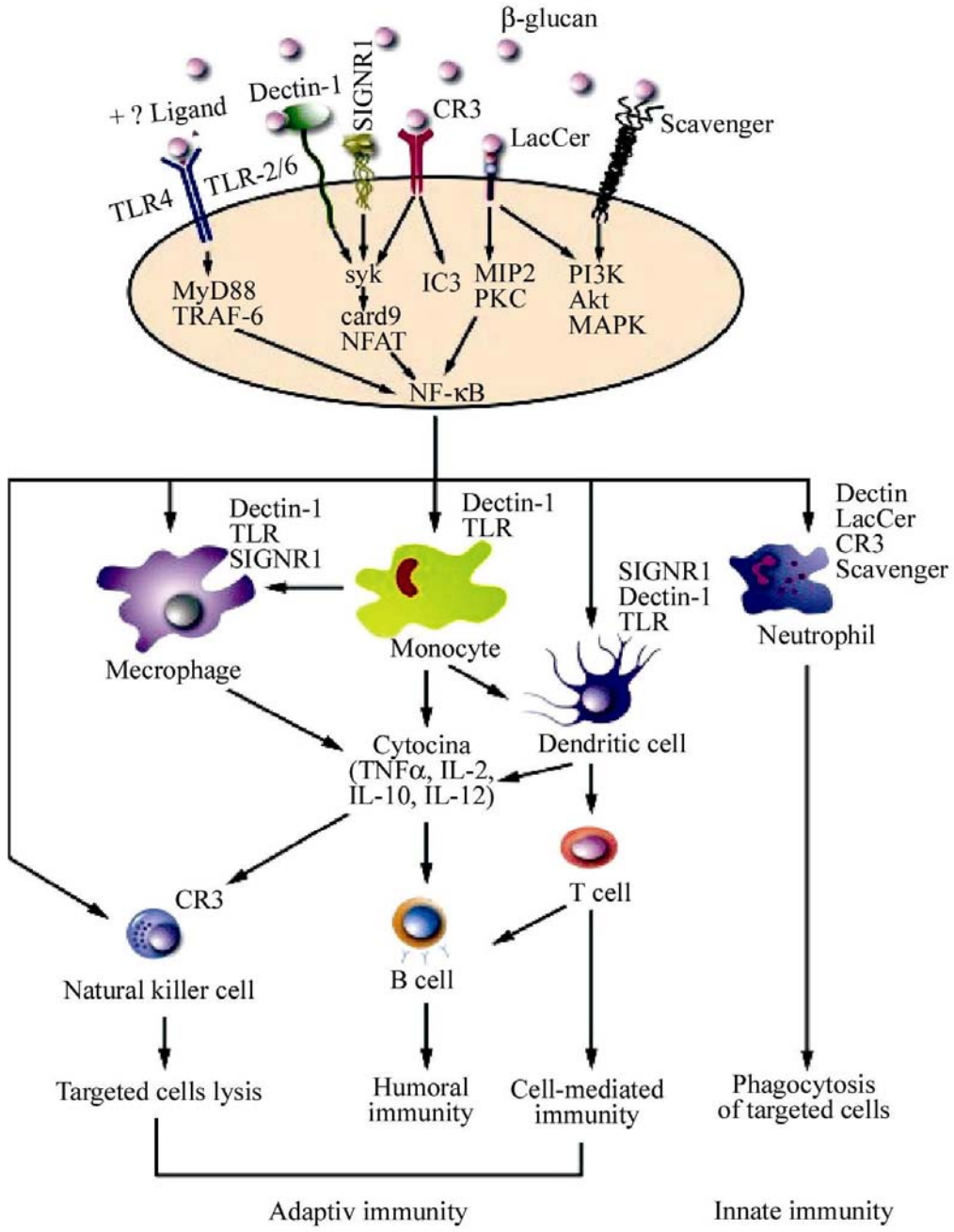


Fig. 2.2.2. *Immune activation induced by β-glucans.*

β-glucans can act on a variety of membrane receptors found on the immune cells. It may act singly or in combine with other ligands. Various signaling pathway are activated and their respective signaling molecules are shown. Corresponding surface receptors are listed. The immunomodulatory functions induced by β-glucans involve both innate and adaptive immune response. Source: Chan et al., J Hemat Oncol 2009.

demonstrated enhanced microbial killing by monocytes and neutrophils in healthy volunteers after β-glucan administration. Besides activation of macrophages, T cells, natural killer (NK) cells, β-glucan activates complement

by alternative activation pathway (Fig. 2.2.2). Pathogens that activate complement are first coated with the C3b fragment of C3, which is rapidly proteolysed into the iC3b fragment by serum factor I. These iC3b fragments serve to promote a high-avidity attachment of the iC3b-opsonized pathogens to the iC3b receptors (CR3, CD11b/CD18) of phagocytic cells and NK cells, stimulating phagocytosis and/or cytotoxic degranulation [113].

2.2.3. β-glucan receptors

The induction of cellular responses by mushroom and other β-glucans is likely to involve their specific interaction with one or more cell surface receptors. This has been the focus of intense research in recent years. Unfortunately, one quite commonly finds the results obtained with one or two β-glucans extrapolated to β-glucans in general, which are then referred to "β-glucan" [19]. Now it should be evident that such substance does not exist. Even β-glucans of similar structure, molecular weight, and solution conformation exhibit vastly differing biological activities *in vitro* and *in vivo*, and these differences are more pronounced when structurally less similar β-glucans are included in the discussion. To complicate matters even more, much of what is currently known about the molecular interaction of β-glucans and various cell types comes from studies conducted with zymosan. Zymosan is a particle obtained from yeast (*Saccharomyces cerevisiae*) consisting of a variety of different substances, including mannans, glucans, glucosamine, and glycoproteins and is, therefore, not ideally suited for the investigation of β-glucan-specific activities. If, however, a specific effect resulting from the binding of zymosan to a cell can be inhibited by a variety of β-glucans, it can be concluded that these β-glucans bind to the same receptor(s) as the β-glucan part of the zymosan particle. Glucans are thought to mediate their effects via interaction with membrane receptors on macrophages, neutrophils, and NK cells (Fig. 2.2.2). β-glucan receptors were firstly identified on the surface of monocytes by Czop and Austen in 1985 as opsonin-independent receptors for particulate activators of the alternative complement activation pathway [32]. Until now, four β-glucan receptors have been identified as candidates mediating these activities. It is namely complement receptor 3 (CR3; CD11b/CD18), lactosylceramide, selected scavenger receptors, and dectin-1 (βGR) (Fig. 2.2.2). CR3 (complement receptor 3) is a heterodimeric transmembrane glycoprotein, belonging to the β_2-integrin family, consisting of CD11b noncovalently associated with CD18. Distinct functional domains have been identified in the extracellular portion of the CD11b subunit of CR3: the I- or A-domain is essential for

binding and phagocytosis of iC3b-coated particles, and the lectin domain located C-terminal to the I-domain is responsible for the nonopsonic binding properties of CR3 [134]. The leukocyte $\alpha_M\beta_2$ integrin known also as Mac-1, complement receptor type 3 (CR3), and CD11b/CD18 functions both as an adhesion molecule facilitating diapedesis and as a C3R enabling phagocytosis or degranulation in response to factor I-cleaved C3b fragment of C3 (iC3b)- opsonized microorganisms. The same lectin domain within CD11b regulates both the cytotoxic and adhesion functions of Mac-1/CR3. CR3 is highly expressed on neutrophils, monocytes, and NK cells and less present on macrophages. Lactosylceramide (LacCer; CDw17) is a glycosphingolipid found in the plasma membranes of many cells and was identified as a β-glucan receptor from biochemical analyses of the interactions between β-glucan and isolated human leukocyte membrane components [160]. It has been suggested that the interaction of β-glucan with this receptor can induce macrophage inflammatory protein (MIP)-2 and the activation of NFκB and can enhance the neutrophil oxidative burst and antimicrobial functions, but the mechanisms behind these activities are unknown. Adaptive immunity uses somatically generated receptors that recognize antigenic patterns to which the host has been previously exposed. In contrast, innate immunity relies on genetically predetermined pattern recognition receptors (PRRs) that recognize carbohydrates, lipids, and proteins that are unique to microorganisms and are not produced by the host. These macromolecular structures, usually found in the cell wall, are referred to as pathogen-associated molecular patterns (PAMPs). Glucans may be fungal recognition molecules (PAMPs) for the innate-immune system of the host [109]. Dectin-1 (or β-glucan receptor, βGR) was described by Brown and Gordon by using a blocking monoclonal antibody against CR3 and anti-Dectin1 antibody [22]. βGR consists of a single C-type, lectin-like, carbohydrate recognition domain, a short stalk, and a cytoplasmic tail possessing an immunoreceptor tyrosine-based activation motif. It recognizes carbohydrates containing β-1,3 and/or β-1,6 glucan linkages. βGR is expressed on cells of the monocyte/ macrophages lineage, neutrophils. Dendritic cells and a subpopulation of T cells also express the βGR, but at lower levels [133]. Phagocytosis of nonopsonized microorganisms by macrophages initiates innate immune responses for host defense against infection. Cytosolic phospholipase A2 is activated during phagocytosis, releasing arachidonic acid for production of substances, which initiate acute inflammation. Dectin-1 receptor was identified as pattern recognition receptor that stimulates arachidonic acid release and cyclooxygenase 2 (COX2) expression in macrophages by pathogenic yeast and yeast cell walls. Pure particulate (1, 3)-β-D-glucan stimulated arachidonic acid release and COX2 expression were augmented in a Toll-like

receptor 2 (TLR2)-dependent manner by macrophage-activating lipopeptide-2 [127]. There were first results established concerning a significant role for dectin-1, in cooperation with TLR2, to activate a macrophage's proinflammatory response to a mycobacterial infection [155].

2.2.4. β-glucan increases resistance to infectious challenge

β-glucan itself can elicit broad anti-infective effects. *Staphylococcus aureus, Escherichia coli, Candida albicans, Pneumocystis carinii, Listeria monocytogenes, Leishmania donovani, Influenza virus* are the microorganisms, against which a protective effect of β-glucan has been established. The potential antiviral effect of *Saccharomyces cerevisiae* β-glucan was investigated on the pneumonia induced by swine influenza virus (SIV). The microscopic lung lesions induced by SIV infection were significantly more severe than those induced by infection in animals pre-administered β-glucan. Significantly more SIV nucleic acid was detected in the lungs of pigs experimentally infected with SIV only at 5, 7, and 10 days post-inoculation (dpi) compared with lungs from pigs pre-administered β-glucan and infected with SIV. The concentrations of interferon γ (IFN-γ) and nitric oxide in bronchoalveolar lavage fluid from pigs pre-administered β-glucan and infected with SIV were significantly higher than for any other group at 7 and 10 dpi for IFN-gamma, and at 5, 7, and 10 dpi for NO [59]. In the tther study the hypothesis, that systemic β-glucan treatment would result in enhanced migration of neutrophils into a site of inflammation and improve antimicrobial function, was tested in a model of acute inflammation in rats. Animals treated with β-glucan showed a 66±6% and 186±42% increase in wound cell number recovered 6 and 18 h post-wounding, respectively. Increased migration did not correlate with increased chemo attractant content of wound fluid, alterations in neutrophil-induced loss of endothelial barrier function, or changes in neutrophils adhesion to endothelial cells. Studies also showed a priming effect for chemotaxis and respiratory burst in circulating neutrophils isolated from β-glucan-treated animals [79]. There are some reports that acidic polysaccharide isolated from *Phellinus linteus* (PL) alleviated the septic shock induced by high dose lipopolysaccharide (LPS) injection in mice [63]. To examine the origin of this effect, cytokine production in serum and the expression of MHC II in B cells and macrophages in areas of inflammation was investigated. Pretreatment with PL 24 h before LPS administration resulted in a significant inhibition of up to 68% of circulating tumor necrosis factor-α, a moderate reduction of 45% of IL-12 and 23% of IL-1β, but no significant reduction in IL-6. The

decrease of IL-1β, IL-12 and TNF-α in sera and the down-modulation of MHC II during septic shock may contribute to the long survival of mice by PL. Administration of PL *in vivo* decreases IL-2, IFN-γ and TNF-α production in splenocytes and enhances spontaneous cell apoptosis in macrophages and lymphocytes stimulated with LPS *in vitro*. But β-glucans induce secretion of TNF-α in dose-dependent manner. High concentration (≥500 μg/mL) causes suppression of the TNF-α release. As previously described, β-glucan administration enhances microbial killing by monocytes and neutrophils. It may play a role in decreasing the infectious complication rate in patients undergoing major surgery. The safety and efficacy of β-glucan in surgical patients at high risk for postoperative infection were determined in a double-blind, placebo-controlled randomized phase I/II trial [7]. The trial was performed in 34 high-risk patients undergoing major abdominal or thoracic surgery. Patients who received β-glucan had significantly fewer infectious complications (3.4 infections per infected patient *vs.* 1.4 infections per infected patient), decreased intravenous antibiotic requirement (10.3 days *vs.* 0.4 days), and shorter intensive care unit length of stay (3.3 days *vs.* 0.1 days). A dose-response trend with regard to infection incidence among patients who received β-glucan at a dose of 0.1–2.0 mg/kg was observed in a phase II multicenter, double-blind, randomized, placebocontrolled study [8]. Perioperative administration of β-glucan also reduces serious postoperative infections or death by 39% after high-risk noncolorectal gastrointestinal operations [35]. Kournikakis et al. [72] have evaluated the anthrax-protective effect of β-glucan in an experimental animal model. β-glucan was administered by subcutaneous injection to Balb/c mice 2 days prior to anthrax challenge, or daily oral gavage for 7 days prior to challenge, or in drinking water for 10 days post-challenge with a lethal dose of *Bacillus anthracis* spores.

2.2.5. β-glucan anticarcinogenic activity

Carcinogenesis can be separated to different stages. The initiation phase involves exposure to a mutagen and often requires its subsequent metabolic transformation into a biologically active form. This exposure, even if resulting in permanent damage to DNA, is often insufficient by itself to cause cancer. At least in chemically induced tumors in experimental animals, a tumor promoter is often required to stimulate cell division and result in the formation of small, benign tumors. A similar promotion phase is thought to exist in naturally occurring cancers, but the actual events are still only poorly understood. Progression to malignancy occurs when the tight

controls that normally govern cell cycle progression break down, resulting in the uncontrolled proliferation of cancerous cells. It also involves the ability of these cells to invade surrounding tissue and to metastasize. The anticarcinogenic activity of two medicinal mushrooms, such *Ganoderma lucidum* and *Tricholoma lobayense,* extracts was tested against cell transformation induced by a defined *ras* oncogene [152]. Investigation was performed using R6/*ras* assay system.

Ras proteins play a pivotal role in regulating cell growth and the development of human cancer. This study is the first to demonstrate that the polysaccharide-enriched mushroom extracts can inhibit cell transformation induced by a defined oncogene through a novel non-cytocidal route. In this study, the *ras*-transformed cells in focus formation were effectively inhibited during the early stage of transformation, and were equally inhibited when the stably transformed cells were mixed with normal cells. It means that the inhibitory effect of mushroom extracts against *ras*-transformed cells requires the presence of normal cells. The demonstration of the inhibitory effect of mushroom extracts on *ras*-induced transformation in this study may have broad implications for cancer prevention. The protective influence against dielthylnitrosamine (200 mg/kg i.p.) genotoxicity, cytotoxicity, and carcinogenicity of aqueous extracts of *Agaricus blazei* mushroom was tested in rats [11, 12, 105]. Dielthylnitrosamine (DEN) is a potent genotoxic carcinogen that has been used as initiating agent in some two-stage (initiation-promotion) alternative protocols for hepatocarcinogenesis [Dragan et al., 1994]. It has been reported that after its metabolic biotransformation, DEN produces the promutagenic adducts O6-ethyldeoxyguanosine and O4- and O6-ethyldeoxythymidine that may initiate liver carcinogenesis [Dragan et al., 1994; Verna et al., 1996]. The findings were controversial. In one experiment [105] there was no protective effect on post-initiation stage of hepato-carcinogenesis. In others [11, 12] the previous treatment with *A. blazei* showed a hepatoprotective effect on both liver toxicity and hepato-carcinogenesis process induced by a moderately toxic dose of DEN. However, effect depends on both the dose of the chemopreventive agent and of the carcinogen used. The highest concentration of *A. blazei* extract (11.5 mg/mL) demonstrated procarcinogenic properties by reducing the elimination of damaged cells (there was less apoptosis/necrosis of liver cells after DEN injection in rats receiving the mushroom extract) leading to the formation of an increased number of preneoplastic lesions. It means that mechanism of action is not clear yet.

Natural killer (NK) cells are directly cytotoxic for tumor cells and play a primary role in regulating immune responses. As immunostimulating agent which acts through the activation of macrophages and NK cells cytotoxicity,

β-glucan can inhibit tumor growth in promotion stage too. There were monitored levels of NK cell cytotoxic activity in MM-46 carcinoma-bearing C3H/HeN [66] and C3H/HeJ [67] mice treated with D-fraction extracted from maitake mushrooms (*Grfola frondosa*). A result showed that D-Fraction markedly suppressed tumor growth, corresponding with increases in TNF-α and IFN-y released from spleen cells and significantly increases TNF-α expressed in NK cells. Furthermore, D-Fraction increased macrophage-derived interleukin (IL)-12, which activates NK cells.

Angiogenesis is crucial to tumor growth and metastasis, and interruption of this process is the purpose for therapeutic intervention of tumor proliferation. The Sarcoma-180 tumor-bearing mouse model was used to investigate the polysaccharopeptide, PSP, isolated from the edible mushroom *Coriolus versicolor* [50]. Quantitative analysis of microcorrosion casting of the tumor tissue showed more angiogenic features such as dense sinusoids and hot spots, in control (untreated) than in PSP-treated animals. Immunostaining of tumor tissues with antibody against the endothelial cell marker (Factor VIII) demonstrated a positive correlation in that both the vascular density and tumor weight were lower in mice treated with PSP. The total amount of new vessels production was reduced, the basic tumor type-specific vascular architecture was retained. However, the expression of vascular endothelial cell growth factor (VEGF) in these tumors was suppressed. So, anti-angiogenesis can be one of the pathways through which β-glucans mediate anticarcinogenic activity.

Double-blind placebo-controlled randomized study was conducted to evaluate the effects of 28-day administration of polysaccharide peptides (PSP), isolated from the fungus *Coriolus versicolor,* on patients, who had completed conventional treatment for advanced non-small cell lung cancer (NSCLC) [138]. Thirty-four patients, with no significant difference in their baseline demographic, clinical or tumor characteristics, or previous treatment regimes were recruited into each of the PSP and control arms. After 28-day treatment, there was a significant improvement in blood leukocyte and neutrophil counts, serum IgG and IgM, and percent of body fat among the PSP, but not the control, patients. Although the evaluable PSP patients did not improve in NSCLC-related symptoms, there were significantly less PSP patients withdrawn due to disease progression, than their control counterparts (5.9 and 23.5%, respectively). There was no reported adverse reaction attributable to the trial medications. PSP treatment appears to be associated with slower deterioration in patients with advanced NSCLC. In non-random case series, maitake mushroom (*Grifola frondosa*) MD-fraction was investigated to determine its effectiveness for 36 (22- to 57-year-old)

cancer patients in stages II-IV. MD-fraction contains β (1,3)-glucan with β (1,6)-branched chains. Cancer regression or significant symptom improvement was observed in 58.3% of liver cancer patients, 68.8% of breast cancer patients, and 62.5% of lung cancer patients. The trial found a less than 10–20% improvement for leukemia, stomach cancer, and brain cancer patients. Furthermore, when maitake was taken in addition to chemotherapy, immune-competent cell activities were enhanced 1.2–1.4 times, compared with chemotherapy alone. Animal studies have supported the use of maitake MD-fraction for cancer [65]. All these data suggest that polysaccharides, β-glucans, could influence on initiation phase of carcinogenesis, but mechanism of action is not clear. By activating NK cells function, interfering with tumor angiogenesis, β-glucans can inhibit promotion and progression of the tumors.

2.2.6. β-glucan as adjuvant to cancer chemo- and radiotherapy

The major side effect of most chemotherapeutic drugs is neutropenia. The administration of these anticancer drugs impairs blood forming function. These functions are important to maintain defense system of the patient. As a result, chemotherapy may accelerate risk of infections that decrease the quality of life for cancer patients. The effect of β-glucan (SCG), purified from edible mushroom *Sparassis crispa,* on cyclophosphamide (CY)-induced leukopenia was tested [47]. 200 mg/kg of CY was administered i.p. to mice. Immediately after this SCG was administered (125, 250, 500, 1000, 2000 μg/mouse) i.p. to CY-treated mice. The number of white blood cells (WBCs) was reduced significantly within 3 day after CY treatment. The peak of the WBC count appeared on day 7, only in SCG 2000 μg-dose group and CY group – on day 9. The effect of SCG was dose-dependent, but high concentration of SCG showed lower efficacy. IL-6 concentration in spleen cells was also increased. IL-6 is involved in B cells differentiation, T cells activation, induction of acute phase proteins and reduction of G0 – residence time of the hematopoietic cells. Therefore, by increasing IL-6 concentration SCG enhances hematopoietic response.

Radiotherapy often results in hematopoietic and immune depletion. Consequently, patients often experience anemia, lymphocytopenia, thrombocytopenia, and granulocytopenia. This leads to high risk of development of serious and lethal infections and increasing the mortality and morbidity of these patients. A water-soluble glucan from *Lentinus lepideus* was orally administered every day for 24 days to irradiated with 6 Gy mice [58]. The levels of IL-1β, IL-6, and GM-CSF were significantly increased in glucan-treated mice. In contrast, the level of TNF-α, whose level had been in-

creased after irradiation, was decreased over time. These results suggested that glucan could increase serum levels of radioprotective cytokines, while decreasing the level of radioinduced TNF-α, which is increased as a consequence of tissue injury and anemia due to radiation. Also the number of colony forming cells was already close to the level seen in nonirradiated mice at Day 8, and continued to show such levels during the 24-day period. All these data suggested that glucan is able to modulate the dysregulation of cytokine production in radiation damage. In other experiment it was demonstrated, that soluble yeast β-glucan could enhance the proliferation of hematopoietic cells, promote leukocyte recovery following sublethal irradiation, and increase the survival of lethally irradiated animals following allogeneic hematopoietic cells transplantation in a CR3-dependent manner.

Taken together, these observations suggest a novel role for complement, CR3, and β-glucan in the restoration of hematopiesis following by bone marrow injury [31].

2.2.7. CR3-DCC and β-glucan

Complement initiates few mechanisms that can be used for the destruction of antibody-coated pathogens. The same mechanisms, typically combination of them, are used by anticancer monoclonal antibodies (mAbs) (see chapter 2.3.1) in directing cytotoxic effect to a tumor cells [41, 2]. Most interact with components of the immune system through antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). ADCC occurs when antibodies bind to antigens on tumor cells and the antibody Fc domains engage Fc receptors (FcR) on the surface of immune effector cells [2]. Complement-dependent cytotoxicity (CDC) is another cell-killing method that can be directed by antibodies. The complement cascade ends in the formation of a membrane attack complex (MAC), which creates pores in the cell membrane that facilitate free passage of water and solutes into and out of the cell [156]. There is the third mechanism of cytotoxicity – CR3-dependent cellular cytotoxicity (CR3-DCC) [42]. But CR3 priming for cytotoxic function requires ligation of both, the I-domain and lectin-like domain of CR3 [111]. This mechanism is normally reserved for yeast and fungi and some microorganisms, which have β-glucan as an exposed component of their cell wall. Yeast cell wall β-glucan binds to a C-terminal lectin domain of CD11b, and also iCR3b binds to N-terminal I-domain binding site of CD11b. After this dual ligation efficient cytotoxic degranulation and phagocytosis are primed. In contrast to microorganisms, tumor cells, as well as other host cells, lack β-glucan as a surface component and cannot trigger CR3-DCC and initiate tumor-killing

activity [42]. This mechanism could be induced in the presence of β-glucans. However mAb therapy is not very effective, because in some patients tumors don't express a high level of tumor antigens or tumor cells are protected from complement-mediated injury by membrane regulators of the complement system, such as CD55 and CD59 [42], that are overexpressed on tumors [84]. Therefore, there is a need for agents that might increase the effectiveness of anticancer mAbs. ADCC can be enhanced by CR3-dependent mechanism (CR3-DCC) [111]. According this mechanism, CR3 binds to iC3b, thus enhancing FcR-mediated effector cell binding to opsonized cell.

The effect of mAb against ganglioside GD2 (experimental neuroblastoma model) [28], G250 (renal carcinoma model) [28] and CD20 (Rituximab) (CD20 lymphoma model) [88] together with β-glucan was investigated. Synergistic effect of mAk and β-glucan for tumor regression was demonstrated in all these experiments. The mechanism by which orally [51] and i.v. [52] administered β-glucans enhance the tumoricidal activity of antitumor mAb was demonstrated in murine tumor models. Oral and i.v. β-glucans function by a similar mechanism. Orally administered β-glucan goes through an intermediate step in which gastrointestinal macrophages process and deliver soluble β-glucan to the CR3 of granulocytes in bone marrow and tissue macrophages. Soluble i.v. administered β-glucan is delivered directly to the bone marrow and tissue macrophages. It was shown that β-glucan-mediated tumor regression requires antitumor Ab that activates complement and deposition of iCR3 on the tumor cells. This was demonstrated by failures of therapy in mice deficient in CR3 (CD11b–/–) or C3 (C3–/–) [51, 52]. The mechanism involved in the *in vivo* priming of CR3 by β-glucan and signaling pathway that activate effector cells was described in the recent years [81]. There was shown that *in vivo* intact β-glucan is first taken up by macrophages and cleaved in to a 25-kDa molecular weight active fragment that binds to CR3, and primes the effector cells for target killing through the activation of 3-Syk-Phosphatidylinositol 3-Kinase signaling pathway. Its mean, that this fragment, but not parent β-glucan, can bind and mediate CR3-DCC *in vitro.* However, the up taking of either soluble or particular β-glucan *in vivo* is CR3 independent. It depends on Dectin-1 receptor, whish is present on macrophages [26] (Fig. 2.2.1).

So, we postulate that CR3-DCC mechanism can be mediated by any agent, which initiated tumor cell opsonization by iC3b fragment, just presents of β-glucan is required.

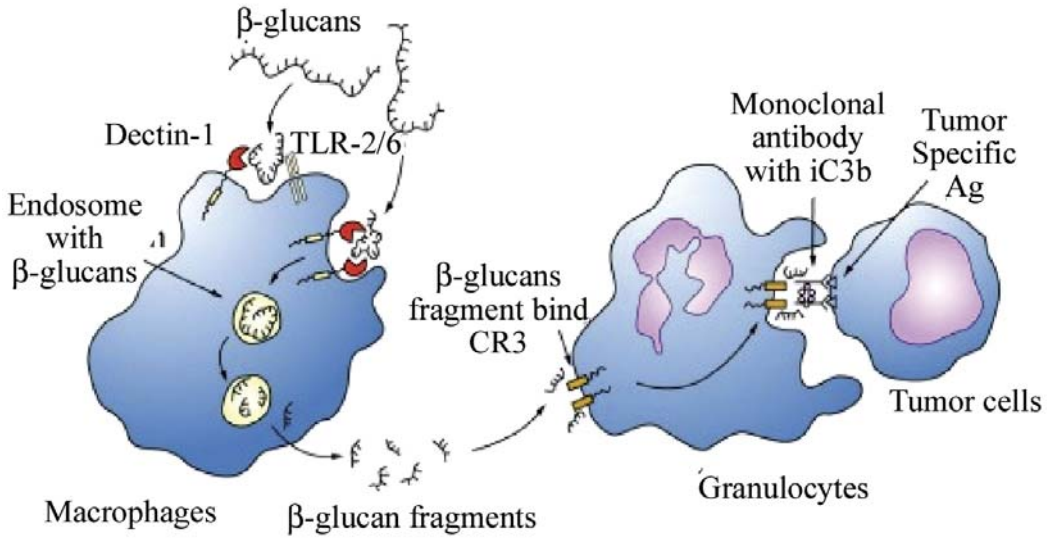


Fig. 2.2.1. *The uptake and subsequent actions of β-glucan on immune cells.* β-glucans are captured by the macrophages via the Dectin-1 receptor . The large β-glucan molecules are then internalized and fragmented into smaller sized β-glucan fragments within the macrophages. They are carried to the marrow and endothelial reticular system and subsequently released. These small β-glucan fragments are eventually taken up by the circulating granulocytes, monocytes or macrophages via the complement receptor 3 (CR3). The immune response will then be turned on, one of the actions is the phagocytosis of the monoclonal antibody tagged tumor cells. Source: Chan et al., J Hemat Oncol 2009.

2.3. Immunotherapy

The immune system works to defend the body against attacks by "foreign" or "non-self" invaders. This network is one of the body's main defenses against infection and disease. The immune system works against diseases, including cancer, in a variety of ways. However, the immune system does not always recognize cancer cells as "foreign." Also, cancer may develop when the immune system does not function adequately. Biological therapies are designed to repair, stimulate, or enhance the immune system's responses [161]. Some antibodies, cytokines, and other immune system substances can be produced in the laboratory for use in cancer treatment. These substances are often called biological response modifiers (BRMs)[161]. They alter the interaction between the body's immune defenses and cancer cells to boost, direct, or restore the body's ability to fight the disease. BRMs include interferons, interleukins, colony-stimulating factors, monoclonal antibodies, vaccines, gene therapy, and nonspecific immunomodulating agents.

Biological therapies may be used to stop, control, or suppress processes that permit cancer growth; make cancer cells more recognizable and susceptible to destruction by the immune system; boost the killing power of immune system cells, such as T cells, NK cells, and macrophages; enhance the body's ability to repair or replace normal cells damaged or destroyed by other forms of cancer treatment, such as chemotherapy or radiation; prevent cancer cells from spreading to other parts of the body [161]. Some BRMs are a standard part of treatment for certain types of cancer, while others are being studied in clinical trials. BRMs are being used alone or in combination with each other. They are also being used with other treatments, such as radiation therapy and chemotherapy.

2.3.1. Monoclonal antibodies

When Georges Kohler and Cesar Milstein invented hybridoma technology in 1975, the new era of cancer therapy has been started. A hybridoma is a hybrid cell produced by injecting a specific antigen into a mouse, collecting an antibody-producing cell from the mouse's spleen, and fusing it with a long-lived cancerous immune cell called a myeloma cell. The hybrid cell, which is thus produced, can be cloned to produce many identical

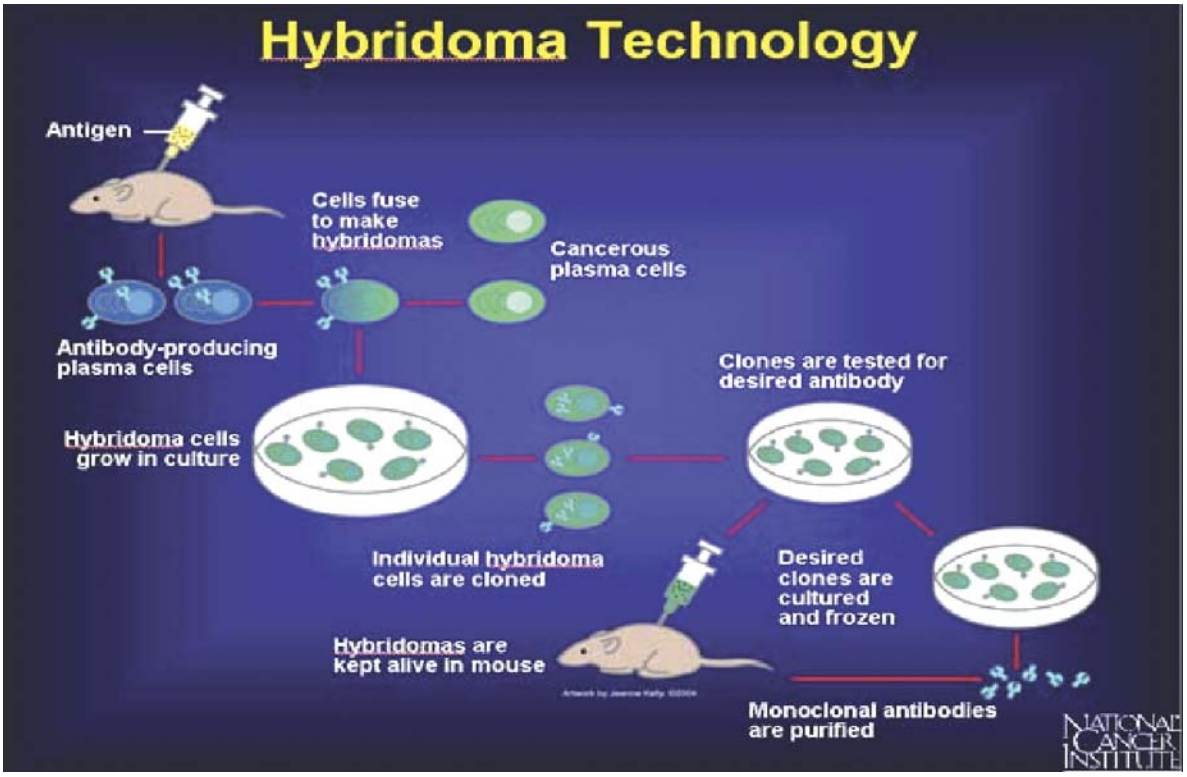


Fig. 2.3.1. *Principle of hybridoma technology***.**
Source http://www.nci.nih.gov/cancertopics

daughter clones. These daughter clones then secrete the immune cell product. Since these antibodies come from only one type of cell (the hybridoma cell) they are called monoclonal antibodies (mAbs). Hybridoma technology (Fig. 2.3.1) allows making the large quantities of the specific antibodies.

Immunotherapy using monoclonal antibodies is a novel cancer treatment strategy. It holds a great promise because of ability to target cancer cells specifically and minimize damage to normal tissues. It is an important advantage over the chemotherapy and radiotherapy.

MAbs react with specific antigens on the surface of certain types of cancer cells. Some of mAbs are already used in clinical oncology for treatment of malignant diseases. As described above, they typically use a combination of mechanisms in directing cytotoxic effect to a tumor cells. Most interact with components of the immune system through antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) [42, 2, 91]. Many of them alter signal transduction within the tumor cell or act to eliminate a critical cell-surface antigen. MAbs can also be used to target payloads, as radioisotopes, drugs or toxins, to directly kill tumor cells or to activate prodrugs specifically within the tumor (antibody-directed enzyme prodrug therapy, ADEPT) [2, 118, 46].

Due to their murine origins the first generation of mAbs evaluated in the clinics were limited by their immunogenicity and poor ability to recruit immune efector mechanisms [2, 96, 95]. Rodent mAbs are recognized by the human immune system as foreign, and it responses against them, producing human anti-mouse antibodies (HAMA). These not only cause the therapeutic antibodies to be quickly eliminated from the host, but also form immune complexes that cause damage to the kidneys. Many efforts have been directed to reduce the immunogenicity and improve effector functions of antibodies. Using genetic engineering it is possible to make mouse-human hybrid antibodies in an attempt to reduce the problem of HAMA. These have included chimerization, humanization, and the development of human antibodies from transgenic mice or phage display libraries [14]. *Chimeric antibodies* combine the variable regions (antigen-binding parts) of the mouse antibody with the constant regions (effector parts) of a human antibody. *Humanized antibodies*, or *CDR-grafted antibodies*, combine only the amino acids (complementary determed regions, CDRs) responsible for making the hypervariable regions (the antigen binding site) of a mouse (or rat) antibody with the rest of a human antibody molecule thus replacing its own hypervariable regions. Fully human antibodies have been derived from mice that have had their endogenous immunoglobulin genes inactivated and

human immunoglobulin genes placed into their genome. In addition, fully human variable domains have been isolated from phage display libraries and then converted into whole mAb [75] (Fig. 2.3.2).

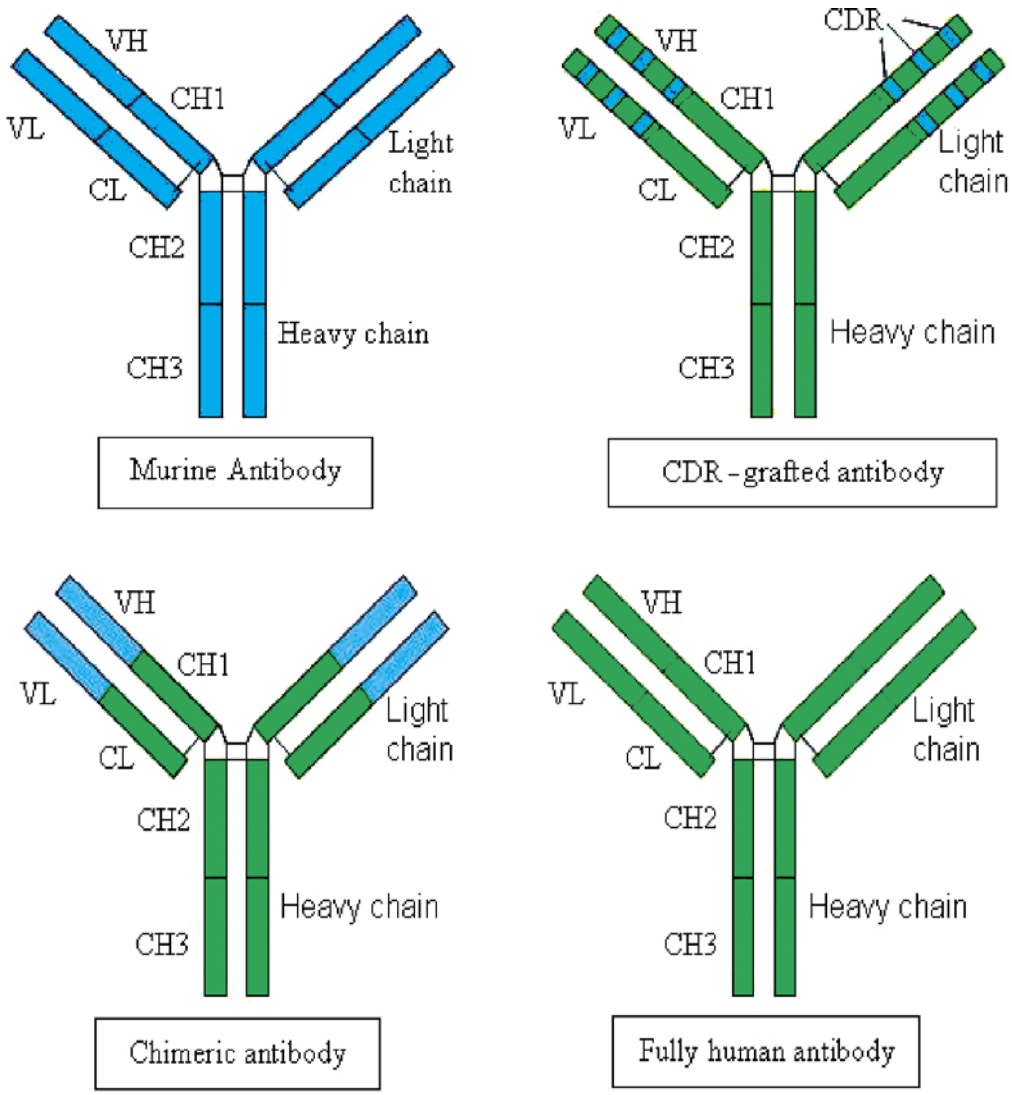


Fig. 2.3.2. *Schematic representation of modified monoclonal antibodies.*
Source: *Fusion Antibodies Ltd.*

Antitumor mAbs direct cytotoxic effect to cancer cell when interact with components of the immune system via ADCC or CDC [43, 45, 79]. ADCC occurs when antibody binds to antigen on tumor cell and antibody Fc domain to Fc receptor, which is present on the surface of immune effector cells [46, 2]. IgG isotype Fc region interact with Fcγ receptors [46, 96, 95]. Dependently on the receptor, effector cells can be activated or inhibited. There are three activating FcγR characterized– FcγRI, FcγRIIa and FcRIIIa. FcRIIb is inhibitory one [46]. Interaction with Fc receptors is important in clinical efficacy of mAb. Antitumor activity of mAb against human tumor

xenografts was diminished in the FcγR knockout mice and was enhanced when the inhibitory FcγRIIb was knocked-out [30].

During the CDC mechanism complement cascade is activated. The complement cascade ends with the formation of a membrane attack complex, which creates pores in the cell membrane that allows free passage of water and solutes into and out the cell [2, 42].

2.3.2. Recombinant antibody constructs in cancer therapy

Among the human antibody isotypes (IgG_{1-4}, IgA_{1-2}, IgM, IgD, IgE), IgG_1 is most widely used isotope today as the backbone for therapeutics mAbs. Schematical structure of human IgG_1 is shown in Fig. 2.3.3.

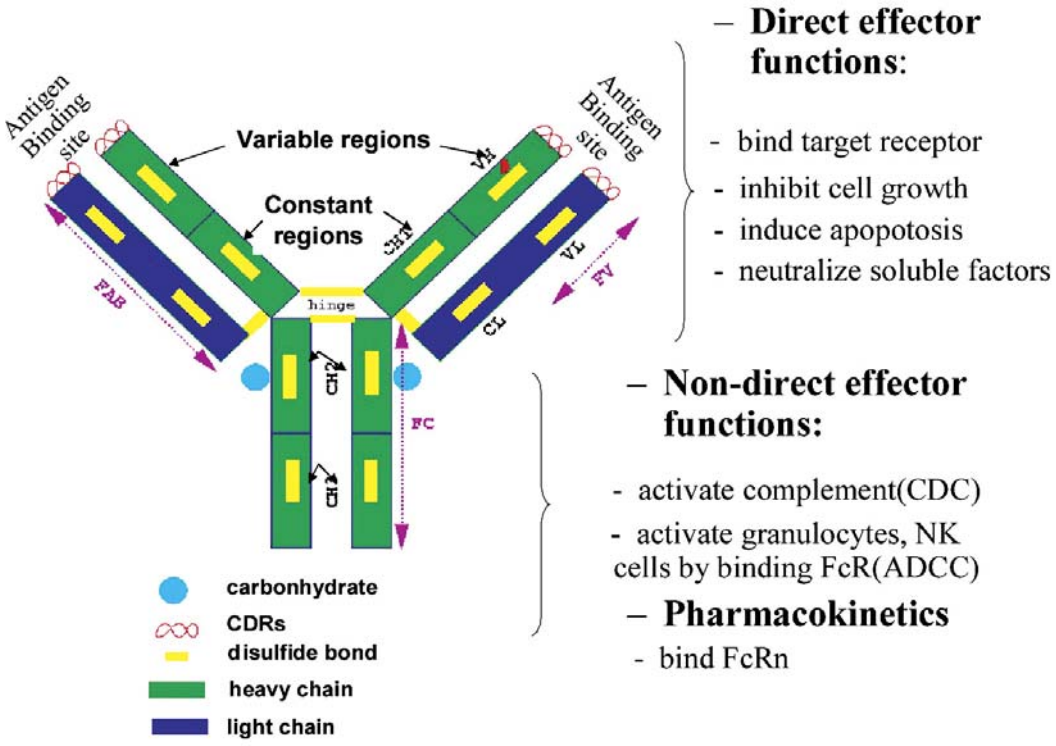


Fig. 2.3.3. *Schematic structure of human IgG_1.*
Functions of each part of immunoglobulin molecule are shown.

This isotype effectively mediates Fc domain-based functions, such as antibody-dependent cellular cytotoxicity and complement fixation [2]. Other isotypes, such as human IgG_2, have been used when the mAb was designed to act simply through its antigen-binding properties, because it has low complement activating capacity and poorly interact with most cellular Fc receptors [2, 46]. An example of such antibody is anti-epidermal growth factor receptor (EGFR) antibody. IgG_4 is consistently inactive in comple-

ment activation [46]. Although IgM is the most effective isotype for complement activation, it is not widely used in clinical oncology because IgM does not extravasate from vascular structures [2, 91]. IgG_3 isotype activates complement, but has a half life of only 7 days [46]. IgA activates human neutrophils more effectively than IgG antibodies and is effectively transported to mucosal surfaces of the gut, the airways and the urogenital tract, that gives the potential advantage to target tumors (lung, colon cancers) from the luminal surface, which is enriched in neutrophils [46].

In the past year, important advances have been made in the design, selection and production of new types of engineered antibodies. Lower toxicity for antibodies versus small molecules, the potential for increased efficacy by conjugation to radioisotopes and cellular toxins, or the ability to enhance immune cell function have made them very attractive in clinical use compered to conventional drug therapies [130].

There are several immunoglobulin design strategies currently available. One of them is when variable regions of light and heavy chain (V_L and V_H), for chimerized antibodies, or complementary determining regions, antibody peptide sequences which recognize the prospective antigen epitope), for humanized antibodies, are properly placed within the framework of a fully human immunoglobulin [130, 54, 75]. But still these antibodies are capable to stimulate the patient's immune system to generate anti-antibodies [130]. More recently for generation of fully human antibodies new technologies started to be used widely. It involves the use of bacteriophage libraries expressing human Ig variable region cDNAs, or transgenic mice containing portions of human chromosomes that express fully human Ig in response to antigenic challenge [130, 54, 75]. One important variation on bacteriophage Ig libraries is the expression of Fab fragments in bacteria as single chain variable region antibody fragments (scFv) [130]. ScFv can be used alone or can be fused to Fc domain and function as minibody, which better penetrates solid tumor's tissue [112]. ScFv can be served as targeting agents for radioisotopes and chemotherapeutics as well [2].

2.4. Anti-CD7 antibodies

Human CD7 is a 40 kDa single domain Ig molecule that is expressed on human T and NK cells, on cells in the early stages of T, B and myeloid cell differenciation [120]. It plays an essential role in T-cell interactions and also in T-cell/B-cell interactions during early lymphpoiesis [120, 13]. CD7 is expressed on malignant precursor T-cells as well, whereas normal bone marrow progenitor cells are CD7-negative [9]. In addition, it was identified that CD7 expression predicts poor disease free survival and post-remission

survival in patients with acute myeloid leukemia [27]. Since this antigen is an early prognostic marker detected at the chronic stage of chronic myeloid leukemia, changes in its gene methylation level could be an early indicator of disease progression [110].

The hybridoma monoclonal antibody TH-69, generated by Dr. Martin Gramatzki (University of Erlangen-Nurnberg, Erlangen, Germany), directed against human CD7, produced significant antitumor effects in athymic ("nude," *nu*$^{-/-}$) and SCID mice xenografted with human T-ALL cell lines (CEM or MOLT-16 cells) [14]. The complete lysis of 2 g T-cell ALL tumor in mice was achieved. SCID mice showed significantly prolonged survival although treated at advance stage by single ntibody dose only. Also the high binding affinity for TH-69 contributes to the therapeutic efficacy. Tumor penetration of antibody was excellent as well. The TH-69 Fab_2 fragment was tested as well, but it was completely ineffective. Complement binding was essential for tumor destruction, as high deposition of C3 and C1q was detected in tumors. The Fc portion of murine unmodified TH-69 antibody is essential for its effectiveness. Nevertheless, other Fc-dependent mechanism, as ADCC, was not involved.

Binding of antibody caused a rapid down-regulation ("modulation") of the CD7 antigen and this property makes the antigen well suited for targeting of immunotoxins [14, 104]. Therefore, the antibody was subcloned as single chain Fv fragment (scFv) and genetically linked to a truncated pseudomonas exotoxin A fragment [104],or ricin toxin A chain [Frankel et al., 1997], or poeweed antiviral protein [149]. However, using of mAb-toxin conjugates causes toxin- related side effects *in vivo*. Later on, scFvCD7 was linked to death-inducing tumor nrcrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) [18] and Fas ligand [17]. The modified agent bound to CD7- positive T-lymphoid cells and killed them by the induction of apoptosis.

Because of these promising results obtained with the unmodified antibody [14], the antibody has been used in pilot studies with patients with advanced T-cell tumors [104]. But human anti-mouse antibodies (HAMA) are produced and decrease the effects of the CD7 murine antibody. To reduce the antigenicity of murine antibody and the constract of a chimeric antibody were developed. This construct of humanised CD7 antibody has been created by Dr. Matthias Peipp (Christian-Albrecht University, Kiel, Germany) (Fig. 3.9.1). The construct of this chimeric scCD7 Fc fusion antibody has been expressed and functions of the purified protein were tested in this study.

Since the CD7 antigen is expressed on ALL and AML cells, anti CD7 antibody could be useful for the treatment of these malignancies.

3. MATERIALS AND METHODS

3.1. Animals and tumor model

C57BL/6 female mice (obtained from Immunology Institute, Vilnius, Lithuania) at 8–10 weeks of age and 19–22 g body weight were used. Mice were injected subcutaneously with 0.2 ml of 5-fold diluted LLC tumor mass suspension (2×10^6 cells) in the right groin. Ten days after implantation, tumors reached 400–600 mm^3 volume and the mice were then subjected to treatment. Tumor volume was determined by measuring the tumor diameter with vernier calipers and calculating the volume according to the following formula: tumor volume = $L \times W \times H \times \pi/6$ (L is length, W is width and H is height of the tumor).

All animal procedures were performed in accordance with the guidelines established by the Lithuanian Animal Care Committee, which approved the study (No. 0179).

3.2. Photosensitizer

Photofrin (porfimer sodium, a kind gift from Axan Pharma Inc., Mont-Saint-Hilaire, Quebec, Canada) was dissolved in 0.9% sodium chloride solution and used at a concentration of 10 mg/kg. It was injected intravenously into tumor-bearing mice, except for those in the control group, 24 h before the tumors were exposed to laser irradiation.

3.3. Laser irradiation

Tumors (400–600 mm^3) in PDT-treated groups were irradiated with light from a diode laser (Institute of Oncology, Vilnius University, Vilnius, Lithuania) with a 630±2 nm wavelength and a 160 mW/cm^2 fluence rate for 15 min, to achieve a dose of 200 J/cm^2. During laser treatment individual animals were anesthetized (Fig. 3.3.1).

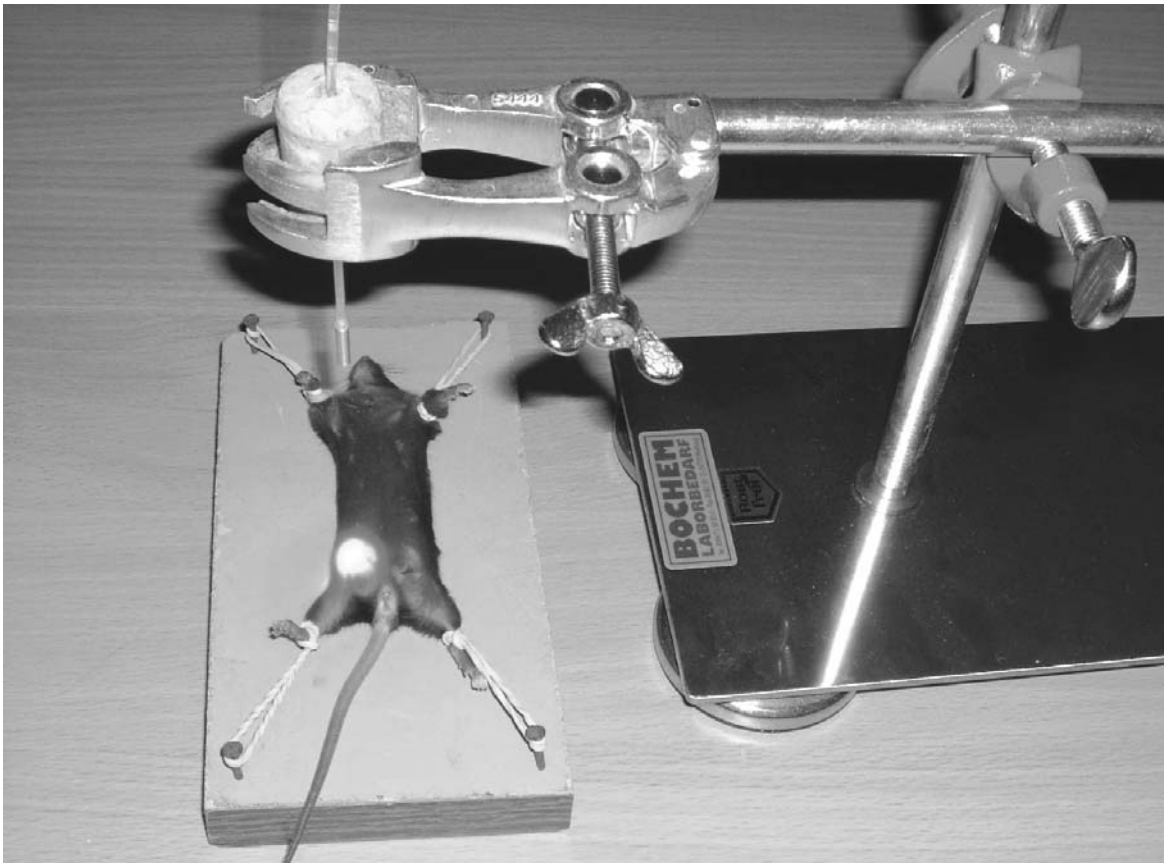


Fig. 3.3.1. *Laser irradiation of LLC tumor bearing mouse*

3.4. Glucans

Three types of β-glucans were used in the study:

β-glucan from baker's yeast (powder, Sigma-Aldrich, Steinheim, Germany) is (1→3),(1→6)-β-glucan with a high number of β-(1→6)-linked branches;

Laminarin, a storage polysaccharide of the marine brown algae *L. digitata* (powder, Sigma-Aldrich, Steinheim, Germany), is a linear β-(1→3)-linked glucan with occasional β-(1→6)-linked branches [β(1→3):β(1→6) ratio of 3:1];

β-glucan from barley (powder, Sigma-Aldrich, Steinheim, Germany) is a large molecular weight, unbranched (1→3),(1→4)-β-glucan.

All β-glucans were dissolved in phosphate-buffered saline (PBS) and administered orally at a dose of 400 μg/mouse (volume 0.2 ml) every day up to 5 days, starting on the same day as Photofrin administration (Fig. 3.6.1 b).

3.5. Immunohistochemical analysis

Every day, starting on the day of Photofrin injection (Fig. 3.6.2 c.), 3 mice from each group were sacrificed and LLC tumors were excised. Immediately following excision, the tumors were placed in formalin solution for 1 week. Then 3 sections from the each tumor were cut for immunohistochemical analysis.

Immunohistochemical reactions were carried out using a ChemMate EnVision (DAKO A/S, Glostrup, Denmark) technique. Tissue sections were incubated for 10 min in 0.3% hydrogen peroxide solution in absolute methanol to block endogenous peroxidase. After microwave retrieval of antigens in 10 mmol/L (pH 6.0) citrate buffer and washes in PBS, all sections were treated with 1% normal horse serum in PBS and then incubated for 30 min with mAbs PC10 against proliferating cell nuclear antigen (PCNA). After washes in PBS, dextran coupled with peroxidase and goat secondary antibody against rabbit and mouse immunoglobulins (ChemMate EnVision detection kit, DAKO A/S, Glostrup, Denmark) were applied. Between incubations the sections were washed 3 times in PBS. Chromogenic development was obtained using 3,3-diaminobensidine (DAB) and hydrogen peroxide (ChemMate EnVision detection kit; DAKO A/S, Glostrup, Denmark). Negative controls were performed, omitting primary antibodies. Finally, sections were counterstained with hematoxylin, cleared, mounted and examined at 400× magnification. Immunohisto-chemical expression was visualized by the product of the chromogenic reaction (oxidized DAB precipitate) staining the cells. Color of the oxidized DAB precipitate varies in intensity (from light brown to dark brown) and indicates positive expression. For evaluation of PCNA scores, 1000 tumor cells were counted from throughout the entirety of each section. The necrotic areas in all tissue samples were analyzed using the fields of vision method [132].

3.6. Experimental design

The experiment with each type of β-glucan was performed separately. Mice were coded and randomized into 4 groups during each experiment (n = 10 each group) (Table 3.6.1):

I group – Control group, mice did not receive any treatment;

II group – PDT group, Photofrin was injected intravenously 24 h prior to laser irradiation (Fig. 3.6.1 a);

III group – β-glucan group, β-glucan, either from barley, or from baker's yeast or laminarin, was administered orally at a dose of 400 μg/mouse daily

up to 5 days (Fig. 3.6.1 b);

IV group- PDT + β-glucan group, Photofrin was injected intravenously 24 h prior to laser irradiation and β-glucan, from either barley, baker's yeast, or marine brown algae, laminarin, was administered orally at a dose of 400 μg/mouse daily up to 5 days starting on the same day as Photofrin injection (Fig. 3.6.1 a, b).

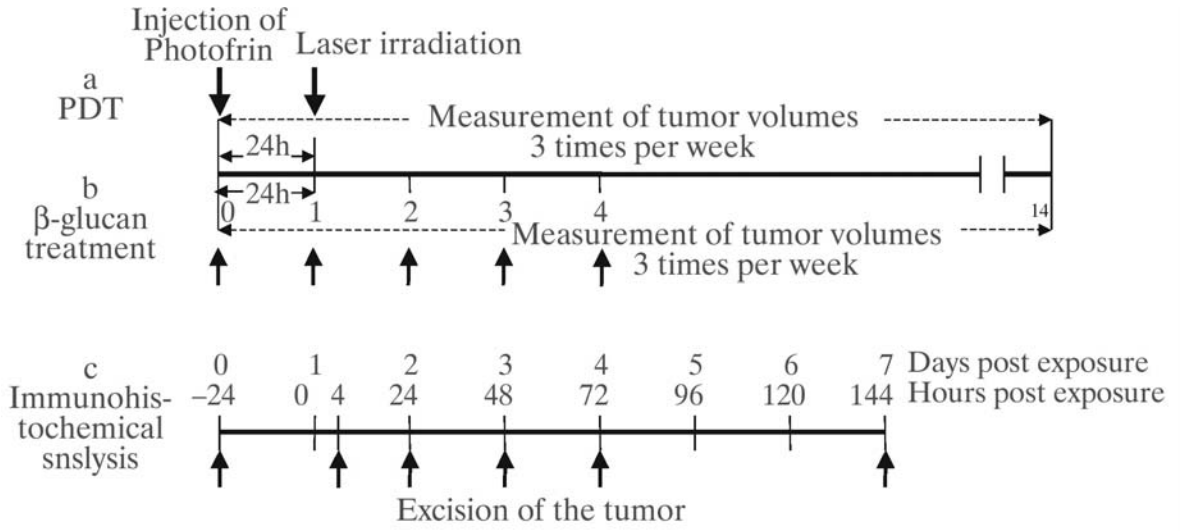


Fig. 3.6.1. *Experimental design.*
Shown are the schedules for PDT (a), β-glucan treatment (b) and immunohistochemical analysis (c).

Table 3.6.1. *Experimental groups.*

Experimental groups	**Experiment I**	**Experiment II**	**Experiment III**
I group Control	n=10	n=10	n=10
II Group PDT	n=10	n=10	n=10
III Group β-glucan	n=10 Laminarin, β-glucanfrom brown algae	n=10 β-glucan from barley	n=10 β-glucan from baker's yeast
IV Group PDT+β-glucan	n=10 PDT+ laminarin, β-glucanfrom brown algae	n=10 PDT+ β-glucan from barley	n=10 PDT+ β-glucan from baker's yeast

3.7. Blood donors

For effector cells isolation 100 ml of blood was taken from healthy volunteers after written consent. Experiments of this study were approved by Ethics Committee of the Christian-Albrecht University (Kiel, Germany), in accordance with the Declaration of Helsinki.

3.8. Cell lines

CEM (Human T cell lymphoblast-like cells) and ARH77 (Peripheral blood leukemia plasma cells) were obtained from American Tissue Culture Cell bank and cultured in RPMI 1640-Glutamax-I (Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum (FCS) and penicillin and streptomycin at 100 units/ml and 100 µg/ml, respectively.

Human 293T cells were cultured in DMEM-Glutamax-I medium (Invitrogen, Karlsruhe,Germany) supplemented with 10% fetal calf serum and penicillin and streptomycin at 100 units/ml and 100 µg/ml, respectively.

3.9. Bacterial expression and purification of scCD7 Fc-fusion fragment DNA

Here scFv fragment of murine anti-CD7 antibody (TH-69) was fused with Fc part of human IgG_1 . The CD7-scFv SfiI-cassette was cloned into SfiI cut pSEC-IgG1-Fc (Fig. 3.9.1). DNA of this protein was kindly provided by dr. M. Peipp.

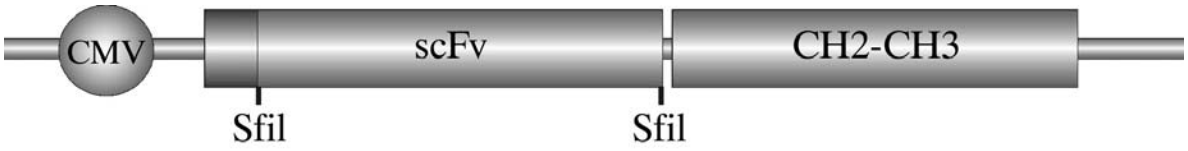


Fig. 3.9.1. *Construction scheme for chimeric single chain anti-CD7 Fc-fusion antibody molecule***.**

scFv- single chain Fv fragment of CD7 murine antibody; CH2-CH3 – constant regions of the human IgG_1 heavy chain; CMV – cytomegal virus promoter.
Author: Dr. Matthias Peipp, Christian-Albrecht University, Kiel, Germany.

Escherichia coli Mach1^{TM} –T1^{R} strain (Invitrogen, Karlsruhe, Germany) was used for the amplification of plasmids. Plasmids were purified using Plasmid DNA purification kit (Macherey-Nagel, Düren, Germany). DNA concentration was determined by optical spectrometry.

3.10. Expression of scCD7 Fc-fusion protein

Single chain anti-CD7 Fc-fusion protein was expressed in mammalian cells 293T. 293T cells were transiently transfected with the calcium phosphate method including the addition of chloroquine to the transfection mix to enhance transfection efficiency. $6x10^6$ 293T cells were seeded and grown per 100 mm dish at least for 24 h. Then medium of the cells was changed and 8 ml of fresh medium was added for a 100 mm plate. 20 µg (20 µl) DNA was prepared in 875 µl sterile water, 100 µl 2.5 M $CaCl_2$ and 5 µl 100 mM Cloroquine were added. Air was bubbled through 1 ml 2xHBD (Table 3.10.1) in 15 ml Falcon and the DNA/Ca/chloroquine solution was added dropwise. After short vortex 2 ml transfection solution was added dropwise to the cell plate under swirling. Cells were incubated for 10 hours. Then medium was changed. Cells were harvested 48 h after transfection, then medium was exchanged daily, and culture supernatant was collected each day for 6 days.

Table 3.10.1. *Composition of 2xHBS buffer*

2×HBS	**1 liter**
HEPES	11.9 g
NaCl	16.4 g
Na_2HPO_4	0.21 g

3.11. Two-step purification of the chimeric scCD7 Fc-fusion antibody

Purification of the recombinant protein was performed by two-step affinity chromatography. Before purification cell culture supernatant was centrifuged and filtered. In 10% of whole volume 200 mM sodium phosphate buffer (pH 7.0) was added to supernatant.

In the first step Protein A column (HiTrap Protein A HP 1 ml, GE Healthcare, Sweden) were according to the manufacturer's conditions. The following buffers were used: binding buffer – 20 mM sodium phosphate, pH 7.0; elution buffer – 0.1 M citric acid, pH 3–6. Collection tubes were prepared by adding 200 µl of 1 M Tris-HCl pH 9.0. Elution fractions were pooled and applied to affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) agarose column (HisTrap HP 1ml, GE Healthcare, Sweden) was used for the second purification step. The following buffers were used: binding buffer – 20 mM sodium phosphate, 0.5 M NaCl, 20–40 mM

imidazole, pH 7.4; elution buffer – 20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4.

Re-buffering of the eluted recombinant protein in PBS was carried out using a Slide-A-Lyzer dialyze cassette (Pierce, Rockford, Illinois, United States).

3.12. Detection of purity and concentration of the chimeric scCD7 Fc fusion antibody

Purity of purified protein was roughly estimated by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE), using 15% (w/v) SDS gels (Tab. 3.12.1), followed by staining with 0.25 % (w/v) Coomassie brilliant blue G250 (Tab. 3.12.2)

Table 3.12.1. *Buffers composition for Tris/Glycine SDS-PAGE*

Chemicals	**Separating gel 15% 15 ml**	**Stacking gel 3 ml**
H_2O	3.5 ml	2.1 ml
30% Acrylamide mix	7.5 ml	0.5 ml
1.5 M Tris (pH 8.8)	3.8 ml	_____
1.0 M Tris (pH 6.8)	_____	0.38 ml
10% SDS (Sodium dodecyl sulfate)	0.15 ml	0.03 ml
10% APS (Amonium persulfate)	0.15 ml	0.03 ml
TEMED (N,N,N',N'-Tetramethylethylenediamine)	0.006 ml	0.003 ml

Table 3.12.2. *Composition of Coomassie staining solution.*

Water	**60 ml**
Methanol	20 ml
CBBG-250 (5 × concentrate)	20 ml

3.13. Flow cytometric analysis

To monitor binding of scCD7 Fc-fusion molecules to CD7-positive and CD7-negaive cells, 5×10^5 CEM and ARH77 cells respectively were incubated with mentioned protein and murine anti-CD7 antibody (TH-69 protein, was kindly provided by dr. M. Gramatzki for this study) and cetuximab (Erbitux, Bristol-Myers Squibb Company, Princeton, USA), as negative controll, at various concentrations for 1 h on ice. Non-bound antibodies were removed by washing with PBS containing 1% BSA. Then cells were incubated with FITC-labeled goat anti-human IgG Fc secondary antibody diluted 1:500 in 1x PBS/1% BSA for 30 min in the dark on ice. Finally, the cells were washed twice with 1×PBS, and resuspended in 500 µl 1×PBS. Fluorescence activated cell sorter (FACS) analysis was performed using a FACS Calibur instrument and CellQuest software (Becton Dickinson, Heidelberg, Germany). Ten thousand events were collected for each sample and all analyses of whole cells were performed using appropriate scatter gates to exclude cellular debris and aggregates.

3.14. Isolation of mononuclear effector cells

Citrate-anticoagulated blood from healthy volunteers was layered over a discontinuous gradient consisting of 70% and 62% Percoll (Biochrom, Berlin, Germany). Human mononuclear cells (MNC) were used as effector cells. ADCC assay was performed with the blood of three donors. After centrifugation, MNCs were collected from the serum/Percoll interface, then washed. Viability of cells tested by Trypan blue exclusion was higher than 95%.

3.15. ADCC assay

Tumor cell lysis mediated by scCD7 Fc-fusion antibody or murine anti-CD7 antibody was analyzed in 51Cr-release assays (Fig. 3.15.1). Chromium-51 release as measured on the MicroBeta Trilux counter (Perkin Elmer Life Science, Massachusetts, USA). Viable CEM cell and MNC count were determined by Trypan blue staining. Target CEM (CD7-positive) cells were labeled with ^{51}Cr (1.2×10^6 cells: 100 µl ^{51}Cr) by the incubation at 37˚C in a CO_2 incubator for 3 hours. After incubation, the cells were washed 3 times and diluted with media.

CEM cells were seeded in U-bottom 96-well plates (0.2×10^5 cells/per well), and scCD7 Fc-fusion antibody, or murine anti-CD7 antibody, or Cetuximab (as control) was added at varying concentration.

Human MNCs were used as effector cells. ADCC assay was performed with the blood of three donors. MNCs were added to wells at effector to target (E:T) cell ratio 40:1. Target CEM cells were incubated with antibody and MNC or in the presence of antibody or MNC only for 4 h. Then the plates were centrifuged in order to separate the cell mass from the supernatant containing the released chromium and 25 µl of supernatant was transferred to a suitable 96-well microtitration plates. 150 µl of scintillation solution (SuperMix, Applied Biosystems) was added; the plates were sealed, shaken for 15 min and then transferred to MicroBeta counter.

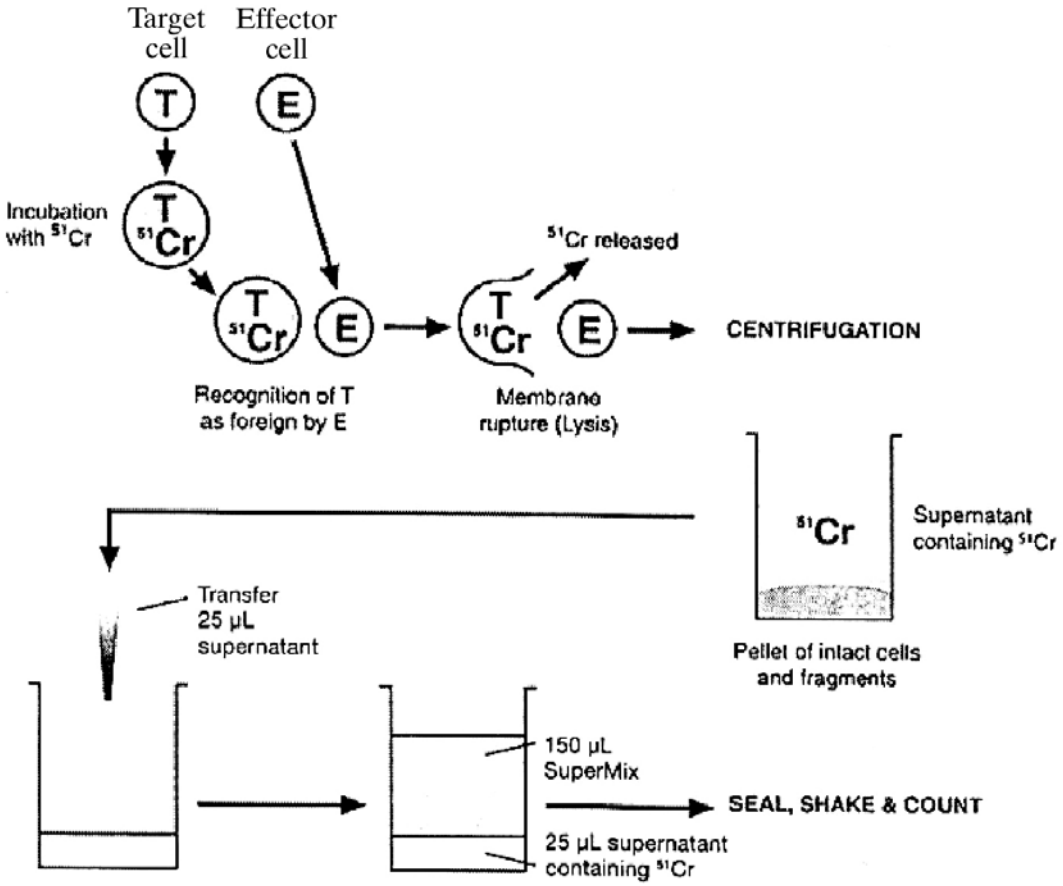


Fig. 3.15.1. *Principle of the chromium release assay.*
Source: producer's instruction www.perkinelmer.com/lifesciences

As a control, the total amount of chromium taken up by the cells must be quantified. This is accomplished by using a detergent (2% Triton X-100) to completely disrupt all cells in a known sample. The value obtained after the measuring was referred to as the maximum chromium release. As a second control, the activity of the intact labeled cells (without addition of detergent or effector cells) was measured. This value was referred to as the spontaneous release.

The results of the percentage lysis of the cells or specific cytotoxicity were calculated according the formula:

$$\frac{\text{Sample } ^{51}\text{Cr release value} - \text{spontaneous release value}}{\text{Maximum } ^{51}\text{Cr release value} - \text{spontaneous release value}} \times 100\%$$

3.16. Data analysis

GraphPad Prism 3.0 software (GraphPad Software, Inc. CA, USA) was used for the statistical analysis. Tumor response results were statistically analyzed using two-way ANOVA. Data are given as group means ± standard deviation (SD). Average tumors size and PCNA expression level over time between two groups was tested for significant difference using Fisher's *F* test. The medians of necrotic area between two groups were compared by Mann-Whitney test. The Kaplan-Meier method was used for survival analysis. The level of significance of the differences between the survival curves was assessed by Gehan's Wilcoxon test. Cytotoxicity assay data are given as means± standard deviation (SD). Statistical differences were considered significant when $P<0.05$.

4. RESULTS

4.1. Tumor growth dynamic

To evaluate the treatment effect in LLC-bearing mice, tumor volume was measured every second day (3 times per week) starting on the day treatment began till the end of the experiment. The pilot experiments revealed the absence of anti-tumor activity of laser irradiation without Photofrin or vice versa (data not shown).

Tumor volumes in all treated groups were significantly less ($P<0.05$) than those in the control group (Fig. 4.1.1 a, b, c). As shown in Fig. 4.1.1 a, LLC tumor volumes were lowest in the group in which mice were treated with PDT in combination with laminarin. Tumor volume differed signifycantly ($P<0.05$) compared with tumors in the control group, but the difference was not significant between tumor volume size of mice treated with PDT alone or by laminarin alone ($P>0.05$).

Mean tumor volume in all treated groups was significantly lower ($P<0.05$) than that in the control group (Fig. 4.1.1 b). Both treatment regiments, administration of PDT alone and β-glucan from barley alone, have shown a significant efficacy ($P<0.001$) in tumor growth suppression in LLC tumor-bearing mice as compared with untreated mice. However, tumor volume after the treatment with β-glucan from barley alone was less in comparison to treatment with PDT alone, but the difference was not significant ($P>0.05$). The most effective tumor growth suppression was achieved in mice treated with combination of PDT and β-glucan from barley, and the difference was significant ($P<0.05$) as compared with PDT alone.

The most pronounced effect was achieved with the treatment by PDT in combination with β-glucan from baker's yeast (Fig. 4.1.1 c). Tumor volumes in this group were significantly lower than in the control ($P < 0.01$) and the PDT ($P<0.05$) groups. β-glucan from baker's yeast showed better antitumor effect than laminarin.

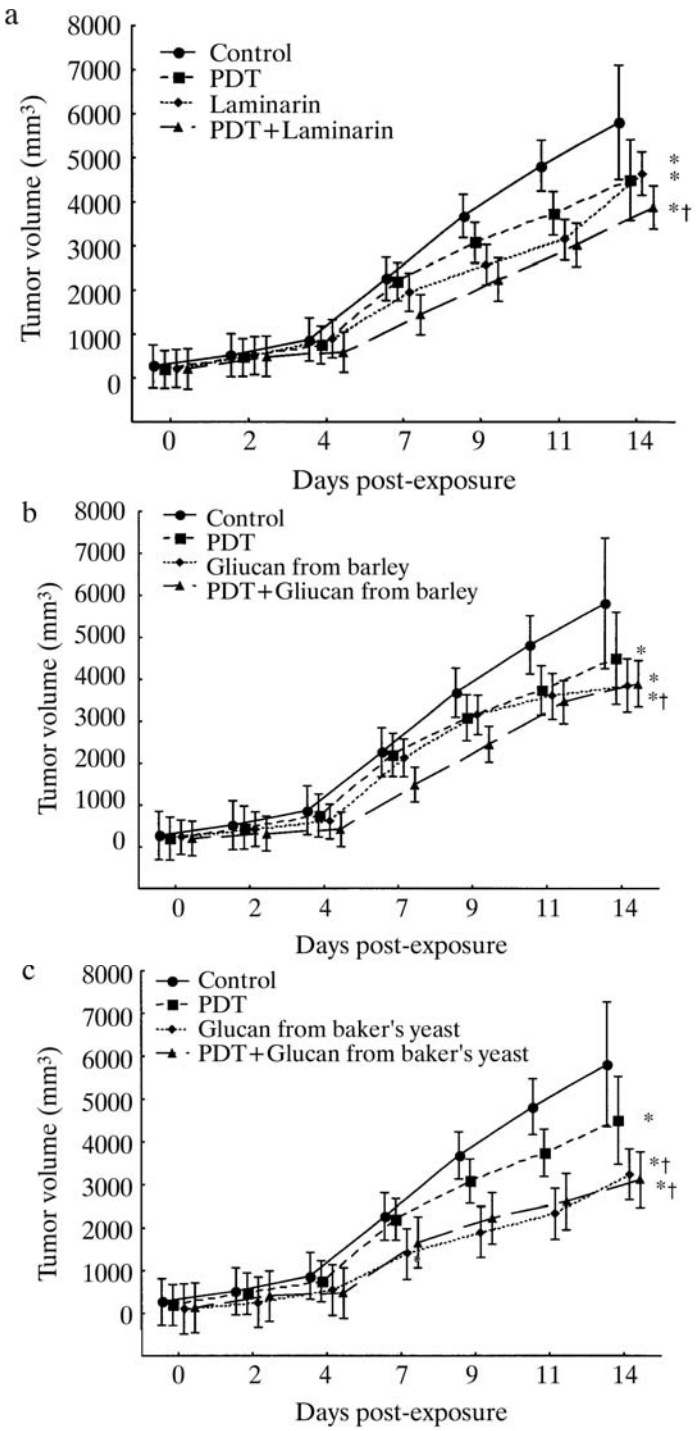
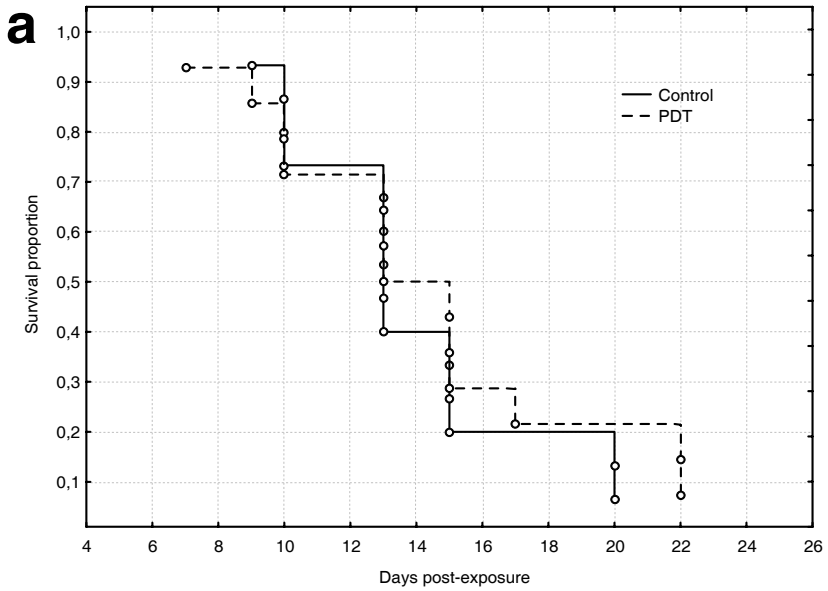


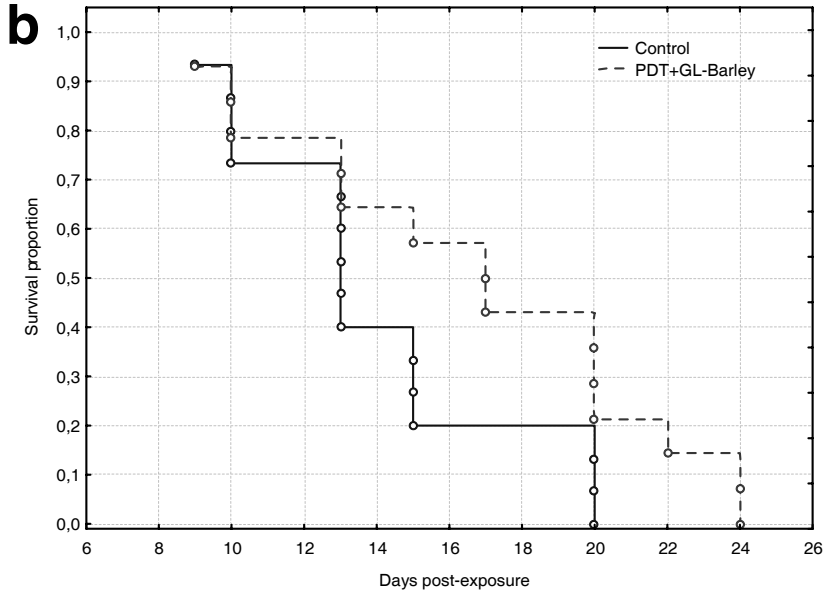
Fig. 4.1.1. *LLC tumor growth dynamics.*

The curves show changes in tumor volume in nontreated mice (Control); in mice treated by PDT (Photofrin intravenous injection followed by laser irradiation 24 h later); or treated with β-glucan either from brown algae, laminarin (**a**), from barley (**b**), or from baker's yeast (**c**), which was administered orally at a dose of 400 μg/mouse daily up to 5 days starting on the same day as Photofrin injection; or treated by combination of above mentioned method (PDT+ β-Glucan): Photofrin intravenous injection followed by laser irradiation 24 h later in combination with β-glucan, which was administered orally at a dose of 400 μg/mouse daily up to 5 days starting on the same day as Photofrin injection. Points represent means ±SD; * P<0.001 vs. control; † P < 0.001 vs. PDT; Fisher's test.

4.2. Survival of mice bearing LLC tumors treated by PDT or/and β-glucan

There was no difference registered in survival of the LLC tumor bearing mice treated with PDT alone compare to untreated mice (Fig. 4.2.1 a). The best survival has been achieved in the group of mice treated with combination of PDT and glucan regiment, but difference was not significant compare to control group (P=0.143) ant to PDT alone group (P=0.319) (Fig. 4.2.1 b, c).





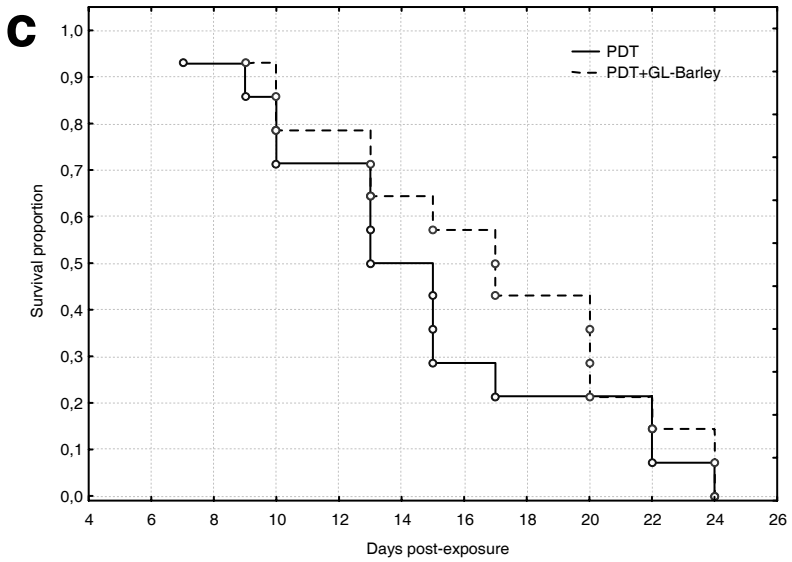
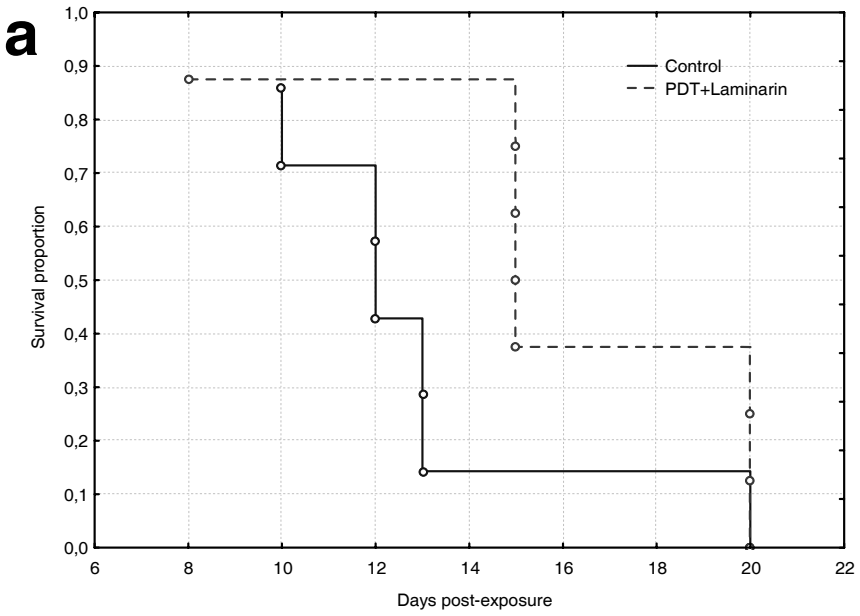
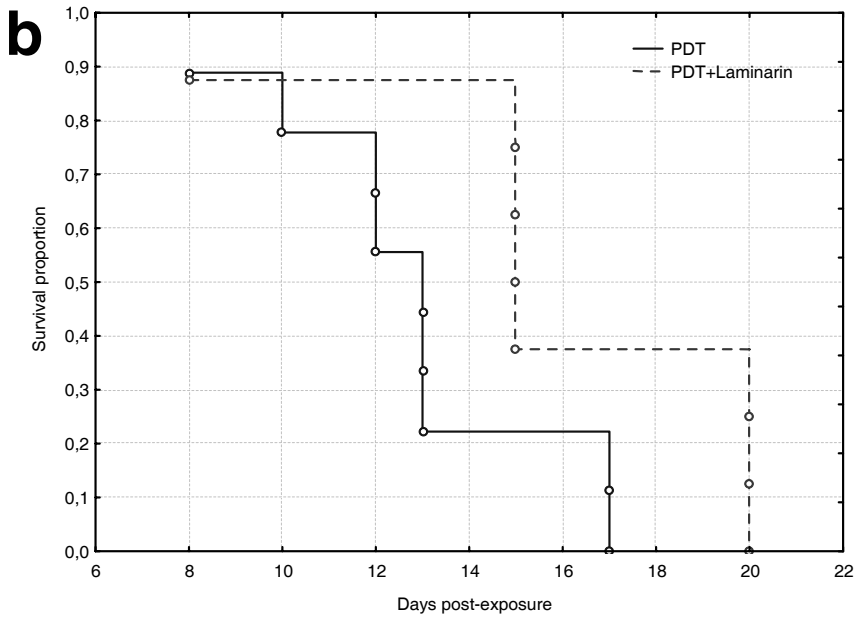


Fig. 4.2.1. *Survival curves of LLC tumor – bearing mice after PDT and/or β-glucan from barley treatment.*

a – PDT vs. control; **b** – PDT+GL-Barley vs. control; **c** – PDT vs. PDT+GL-Barley. Control – nontreated mice; PDT – Photofrin i.v. injection followed by laser irradiation 24h later; GL-Barley – β-glucan from barley was administered orally at dose 400μg/mouse/ daily up to 5 days starting on the same day as Photofrin injection; PDT+GL-Barley – Photofrin i.v. injection followed by laser irradiation 24h later in combination with β-glucan from barley, which was administered orally at dose 400 μg/mouse/daily up to 5 days starting on the same day as Photofrin injection.

As Fig. 4.2.2 shows there is no significant difference in survival between nontreated LLC tumor – bearing mice and treated with PDT and/or laminarin. Slight prolongation in the survival of mice, treated by PDT in combination with laminarin can be seen, but difference is not significant compare either with control group (P=0.08) or PDT group (P=0.06).


a
Control
PDT+Laminarin
Survival proportion
Days post-exposure
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
6
8
10
12
14
16
18
20
22


b
PDT
PDT+Laminarin
Survival proportion
Days post-exposure
1,0
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0,8
0,7
0,6
0,5
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0,3
0,2
0,1
0,0
6
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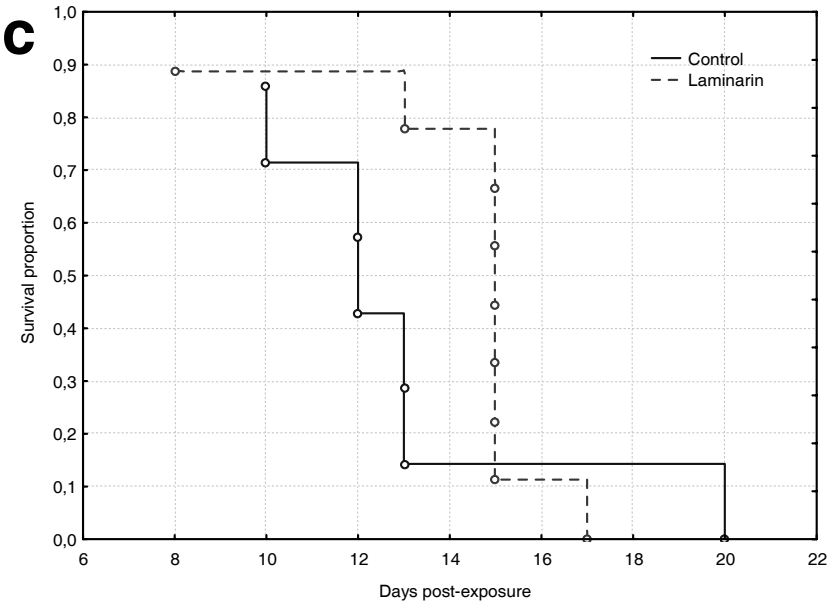
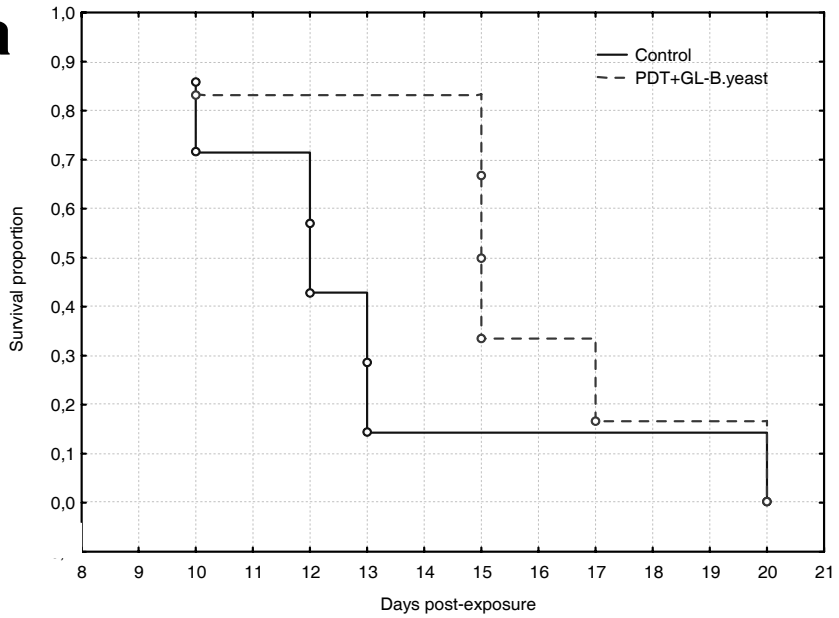


Fig. 4.2.2. *Survival curves of LLC tumor – bearing mice after PDT and/or laminarin treatment.*

a – PDT+Laminarin vs. control; **b** – PDT vs. PDT+Laminarin; **c** – control vs. laminarin. Control – nontreated mice; PDT – Photofrin i.v. injection followed by laser irradiation 24h later; Laminarin – β-glucan from brown algae – was administered orally at dose 400μg/mouse/daily up to 5 days starting on the same day as Photofrin injection; PDT+Laminarin – Photofrin i.v. injection followed laser illumination 24 h later in combination with laminarin, which was administered orally at dose 400 μg/mouse/daily up to 5 days starting on the same day as Photofrin injection.

Treatment with β-glucan from baker's yeast significantly prologs survival of LLC tumor – bearing mice compare with nontreated mice (P=0.03) (Fig. 4.2.3 c). Slight prolongation can be seen in the survival of mice which were treated with combination of PDT and β-glucan from baker's yeast, but difference is not significant compare with control group (P=0.14) (Fig. 4.2.3 a) and PDT group (P=0.15) (Fig. 4.2.3 b).

a


Control
PDT+GL-B.yeast
Survival proportion
Days post-exposure
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
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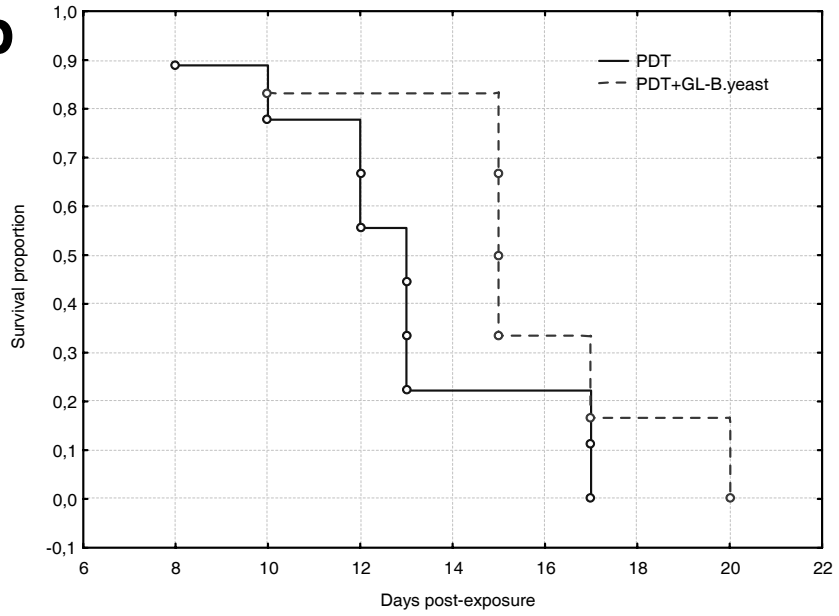
b


PDT
PDT+GL-B.yeast
Survival proportion
Days post-exposure
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0,8
0,7
0,6
0,5
0,4
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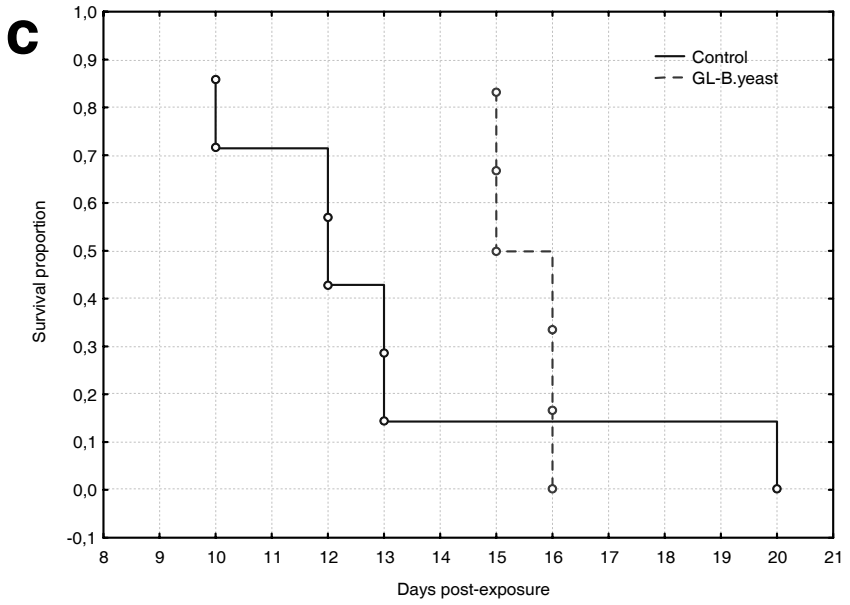

Fig. 4.2.3. *Survival curves of LLC tumor – bearing mice after PDT and/or β- glucan from baker's yeast treatment.*

a – PDT+GL-B.yeast vs. control; **b** – PDT vs. PDT+GL-B. yeast; **c** – control vs. glucan from baker's yeast. Control – nontreated mice; PDT – Photofrin i.v. injection followed by laser irradiation 24 h later; GL-B. yeast – β-glucan from baker's yeast was administered orally at dose 400 µg/mouse/daily up to 5 days starting on the same day as Photofrin injection; PDT+GL-B. yeast – Photofrin i.v. injection followed by laser irradiation 24 h later in combination with β-glucan from baker's yeast, which was administered orally at dose 400µg/mouse/daily up to 5 days starting on the same day as Photofrin injection.

4.3. PCNA expression and necrosis in tumor tissue

The results of immunohistochemical staining show that PCNA expression in LLC tumors increased significantly after PDT treatment (P<0.001) starting on the same day as laser irradiation (Fig. 4.3.1). Necrosis was already observed in LLC tumors treated with PDT on the same day after the laser irradiation (Fig. 4.3.2).

Both, combination treatment and treatment with β-glucan from barley significantly decreased PCNA expression in LLC tumors (P<0.001) compared with nontreated tumors (Fig. 4.3.1 b). Mostly PCNA expression decreased in tumors treated with PDT in combination with β-glucan from barley, but not significantly (P>0.05) compare with tumors treated with β-glucan from barley alone.

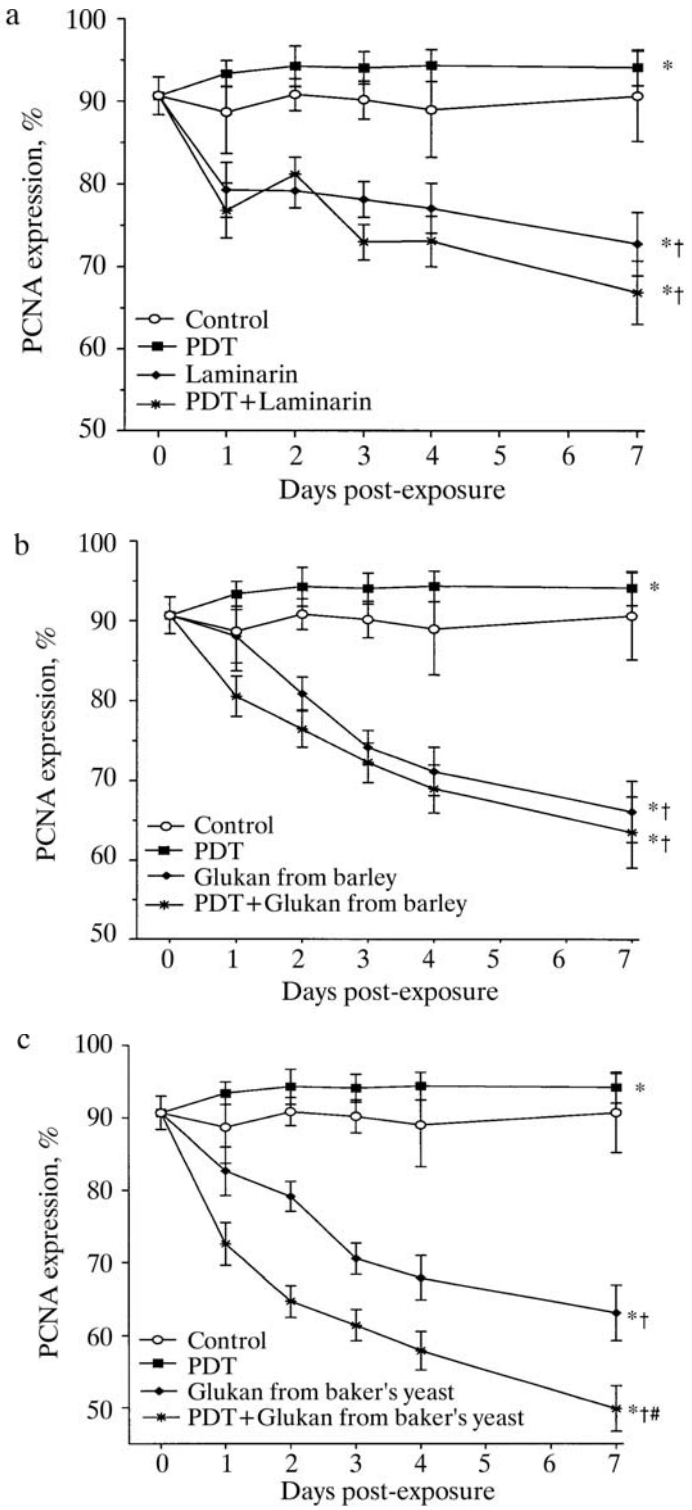

Fig. 4.3.1. *PCNA expression in LLC tumor.*

PCNA expression level detected by immunohystochemistry in the tumors of mice treated by PDT or β-glucan either from brown algae, laminarin (**a**), from barley (**b**), or from baker's yeast (**c**) alone, or in combination of these as described in Fig 3.6.1. Tumors of nontreated mice served as control. The points are means ± SD from 3 tumors (3 sections of each tumor). * $P<0.001$ vs. control; † $P<0.001$ vs. PDT; # $P<0.001$ vs. β-glucan alone; Fisher's test.

Table 4.3.1. *Significance of difference in PCNA expression in the sections of non- treated tumors and by PDT treated tumors.*

Days post-exposure	*P*
1	<0.01
2	<0.05
3	<0.01
4	<0.01
7	<0.05

The largest necrotic area was detected in tumors treated with PDT in combination with β-glucan from barley (Fig. 4.3.2 b). Despite decreased PCNA expression in tumors treated with β-glucan from barley alone, only single necrotic areas were detected in the tumors. But larger necrotic areas were seen in tumors treated with PDT compared with nontreated tumors and tumors treated with β-glucan from barley alone ($P<0.001$), even though the highest PCNA expression was detected in these tumors.

As shown in Fig. 4.3.1 a, PCNA expression significantly decreased in tumors, treated with laminarin alone or by PDT and laminarin ($P<0.01$) in comparison to nontreated tumors or those treated with PDT alone. No difference in PCNA expression was observed between PDT with the laminarin combination group and the laminarin alone group. A slight, but not significant ($P>0.05$) decrease in PCNA expression in the group with combined treatment was seen starting on day 4 post-treatment. However the area of necrosis in tumors treated with PDT in combination with laminarin is significantly ($P>0.001$) higher compared with PDT alone (Fig. 4.3.2 a). Only a few necrotic areas were detected in tumors treated with laminarin alone.

Both, combination treatment and treatment with β-glucan from baker's yeast decreased PCNA expression in LLC tumors significantly ($P<0.001$) compare with nontreated tumors (Fig. 4.3.1 c), but the lowest PCNA expression level was achieved with PDT combined with β-glucan from baker's yeast ($P<0.001$). The largest necrotic area was detected in tumors treated with PDT in combination with β-glucan from baker's yeast (Fig. 4.3.2 c). Despite decreased PCNA expression in tumors treated with β-glucan from baker's yeast alone, only single necrotic areas were detected in the tumors. In contrast, despite having the highest PCNA expression compared with other treatment groups and the control group, LLC tumors treated with PDT had larger necrotic areas compared with nontreated tumors and tumors treated with β-glucan from baker's yeast alone ($P<0.001$).

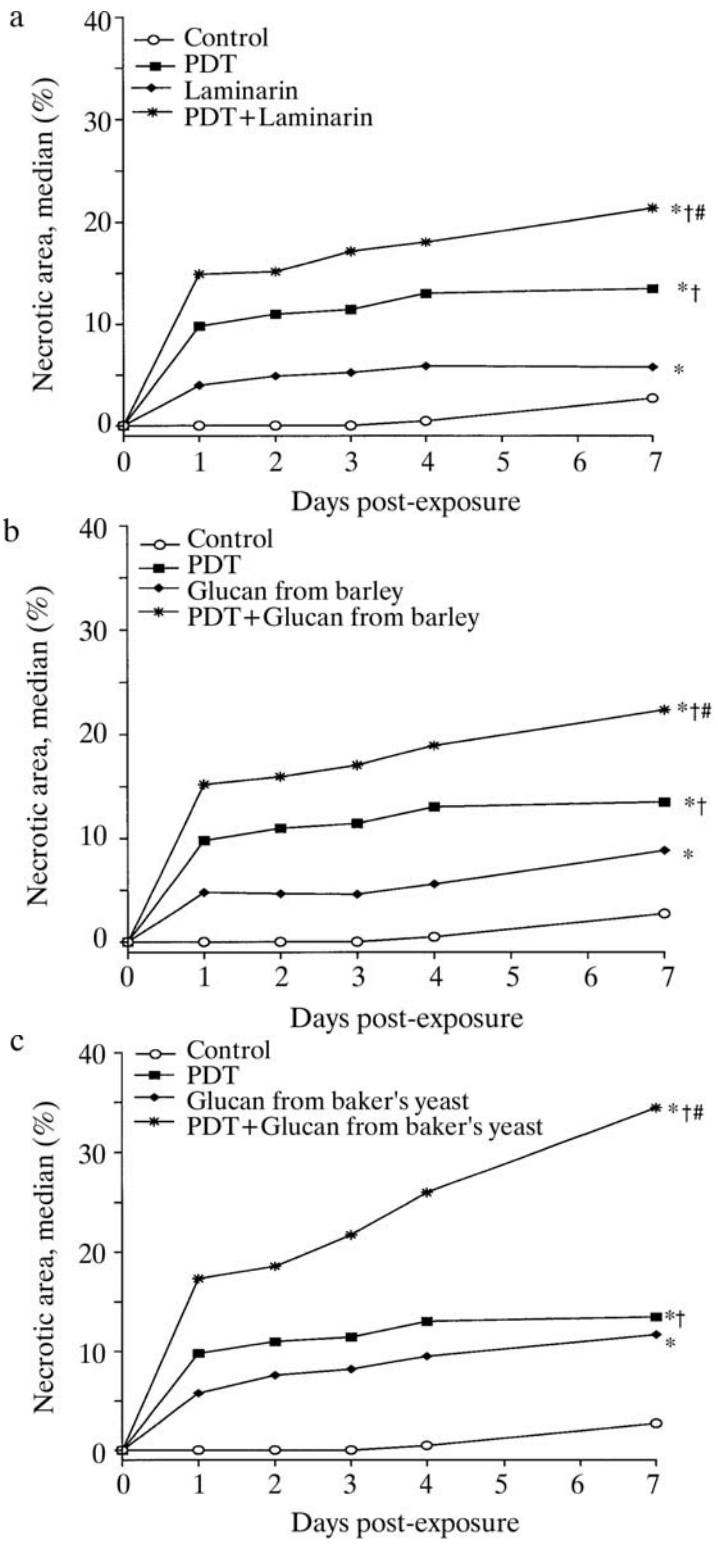

Fig. 4.3.2. *Necrotic areas in LLC tumor tissue.*

The curves show changes in necrotic areas in tumors treated by PDT or laminarin (**a**), β-glucan from barley (**b**), or from baker's yeast (**c**) alone, or in combination of these as described in Fig 3.6.1. Each point represents the median of necrotic area from 3 tumors, in 3 sections of each tumor. * $P<0.001$ vs. control; † $P<0.001$ vs. β-glucan alone; # $P<0.001$ vs. PDT; Mann-Whitney test.

The lowest PCNA expression (P<0.001) was detected in LLC tumors treated with PDT combined with β-glucan from baker's yeast compared with tumors treated with PDT in combination with either β-glucan from barley or laminarin (Fig. 4.3.3). No significant difference was observed between combinations of PDT with β-glucan from barley or with laminarin (P>0.05) (Table 4.3.2). A similar necrotic area was detected in tumors of mice treated with PDT and laminarin or β-glucan from barley. PDT in combination with β-glucan from baker's yeast produced significantly wider necrosis in tumor tissue.

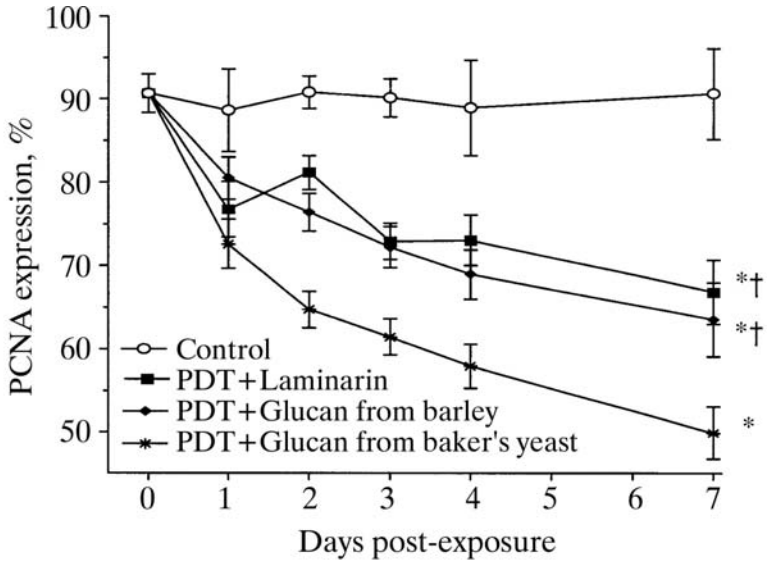

Fig. 4.3.3. *PCNA expression in LLC tumor treated by PDT and Laminarin, or β-glucan from barley, or β-glucan from baker's yeast.*

Control – nontreated mice; PDT+Glucan from barley– Photofrin i.v. injection followed by laser irradiation 24 h later in combination with β-glucan from barley, which was administered orally at dose 400μg/mouse/daily up to 5 days starting on the same day as Photofrin injection; PDT+Laminarin – Photofrin i.v. injection followed by laser irradiation 24h later in combination with laminarin, which was administered orally at dose 400μg/mouse/daily up to 5 days starting on the same day as Photofrin injection; PDT+Glucan from B. yeast – Photofrin i.v. injection followed by laser irradiation 24 h later in combination with β-glucan from baker's yeast, which was administered orally at dose 400 μg/mouse/daily up to 5 days starting on the same day as Photofrin injection. Tumors of nontreated mice served as control. The points are means ± SD from 3 tumors (3 sections of each tumor). * P<0.001 vs. control; † P<0.001 vs. PDT+Glucan from baker's yeast; Fisher's test.

Table 4.3.2. *Significance (P value) of differences in PCNA expression in LLC tumor treated with PDT in combination with β-glucan from barley, or laminarin, or β-glucan from baker's yeast.*

Days post-exposure	PDT+ β-glucan from barley *vs.* PDT+Laminarin	PDT+ β-glucan from barley vs. PDT+ β-Glucan from baker's yeast	PDT+Laminarin *vs.* PDT+ β-glucan from baker's yeast
1	<0.05	<0.001	<0.05
2	<0.001	<0.001	<0.001
3	>0.05	<0.001	<0.001
4	<0.05	<0.001	<0.001
7	>0.05	<0.001	<0.001

In LLC tumors treated with β-glucan from barley or laminarin or with β-glucan from baker's yeast alone, PCNA expression was also significantly lower (P<0.001) compared with nontreated tumors (Fig. 4.3.4, 4.3.5). As shown here, the lowest PCNA expression in tumors was achieved after treatment with β-glucan from baker's yeast starting on day 3 post-exposure to treatment, but the difference was not significant (P>0.05) compared with tumors treated with β-glucan from barley (Table 4.3.3).

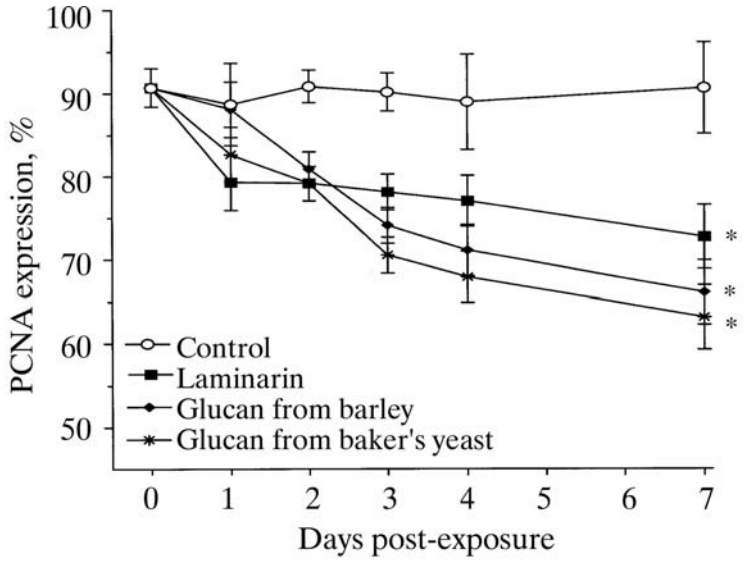

Fig. 4.3.4. *PCNA expression in LLC tumor treated by β-glucan from barley or Laminarin, or β-glucan from baker's yeast.*

Control – nontreated mice; Laminarin –laminarin, β-glucan from brown algae; Glucan from barley – β-glucan from barley; Glucan from B. yeast – β-glucan from baker's yeast. All β-glucans were administered orally to mice at dose 400 μg/mouse/ daily up to 5 days starting on the same day as Photofrin injection (Fig. 3.6.1 b). Tumors of nontreated mice served as control. The points are means ± SD from 3 tumors (3 sections of each tumor). * $P<0.001$ vs. control; *Fisher's* test.

Table. 4.3.3. *Significance (P value) of differences in PCNA expression in LLC tumor treated with β-glucan from barley, or laminarin, or β-glucan from baker's yeast.*

Days post-exposure	**β-glucan from barley *vs.* Laminarin**	**β-glucan from barley vs. β-glucan from baker's yeast**	**Laminarin *vs.* β-glucan from baker's yeast**
1	<0.01	<0.01	<0.05
2	>0.05	>0.05	>0.05
3	<0.05	<0.05	<0.001
4	<0.01	>0.05	<0.001
7	<0.01	>0.05	<0.001

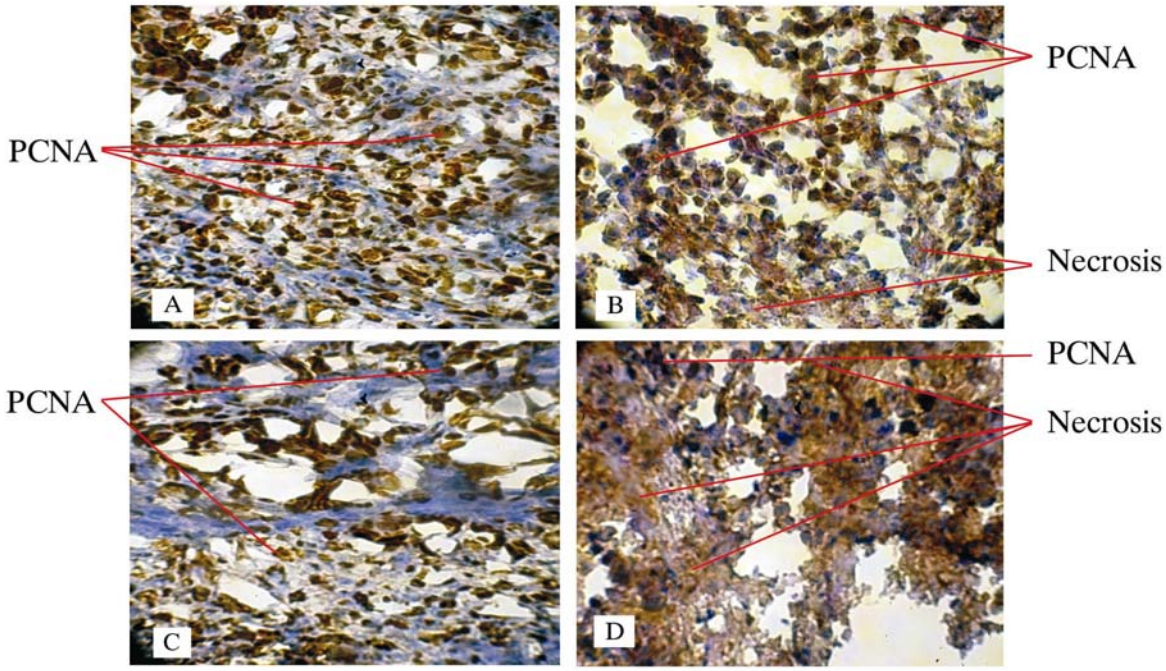

Fig. 4.3.5. *Immunohistochemical staining of LLC tumor tissue.*
A – nontreated tumor's tissue; B – PDT treated tumor's tissue; C – tissue of tumor treated by β-glucan only; D – tissue of tumor treated by PDT and β-glucan.

4.4. Detection of protein purity and concentration

Chimeric scCD7 Fc-fusion antibody was expressed in mammalian cells (293T) and purified from culture supernatant. For this purpose, 293T cells were transiently transfected with the calcium phosphate method including chloroquine additionally to the transfection mix to enhance transfection efficiency. Purification of the protein was made by two-step affinity chromatography. Purified protein was analyzed by SDS-PAGE and migrated at the expected size of 60-65 kDa. The material was more than 90% pure as estimated by visual inspection of coomassie blue-stained gel (Fig. 4.4.1).

About 200 µg of purified protein were obtained from 800 ml of culture supernatant. After the first step purification with protein A, the IgG heavy and light chain from bovine serum, and scCD7 Fc fusion protein are seen on SDS-PAGE gel (Fig. 4.4.1). After the second step purification one band appears on the gel.

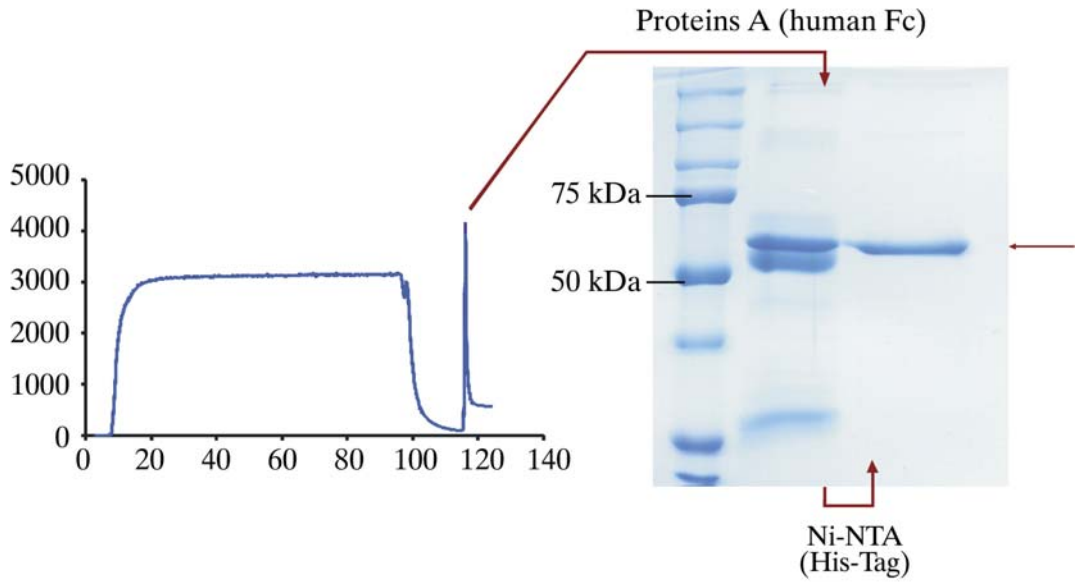

Fig. 4.4.1. *Two-Step-Purification of the chimeric scCD7 Fc-fusion antibody***.** Purification of the protein was performed by two-step affinity chromatography. In the first step Protein A columns were used; Ni-NTA agarose columns were used for the second purification step. Purity of purified protein was estimated by SDS-PAGE followed by coomassie staining (red arrow on the right).

Detection of scCD7 Fc-fusion antibody concentration was made on SDS-PAGE gel followed by coomassie staining. BSA was used as a standard at the different concentrations (Fig. 4.4.2). Concentration of scCD7 Fc-fusion antibody was considered as 0.1 µg/µl.

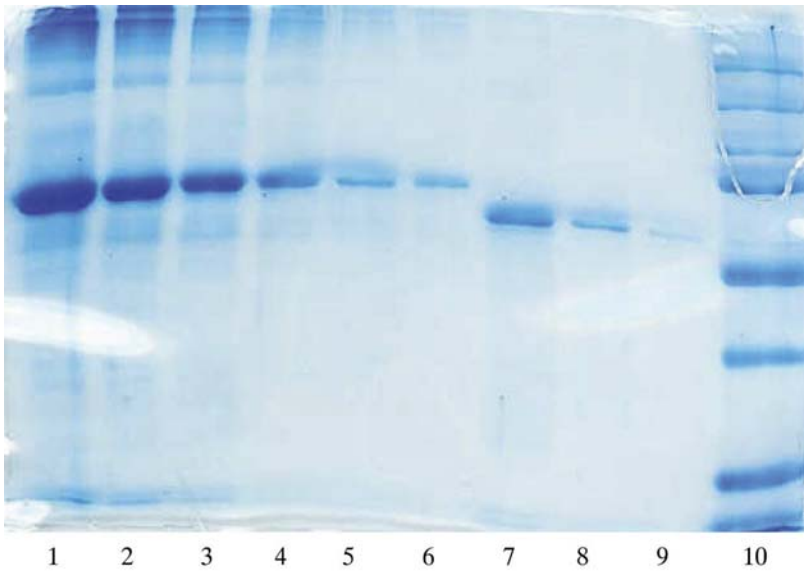

Fig. 4.4.2. *Detection of scCD7 Fc-fusion antibody concentration on SDS-PAGE gel followed by Coomassie staining.*

Lane 1 – 10 µg BSA; Lane 2 – 5 µg BSA; Lane 3 – 2.5 µg BSA; Lane 4 – 1.5 µg BSA; Lane 5 – 0.625 µg BSA; Lane 6 – 0.3125 µg BSA; Lane 7 – 10 µl of protein; Lane 8 – 5 µl of protein; Lane 9 – 2.5 µl of protein; Lane 10 – standard.

4.5. Binding specificity

Purified scCD7 Fc-fusion antibody was analyzed for binding by Fluorescence Activated Cell Sorting method (FACS). This recombinant protein retained specific binding for the CD7-positive T-cell line CEM, but failed to bind to the CD7-negative plasma-cell line ARH77 (Fig. 4.5.1).

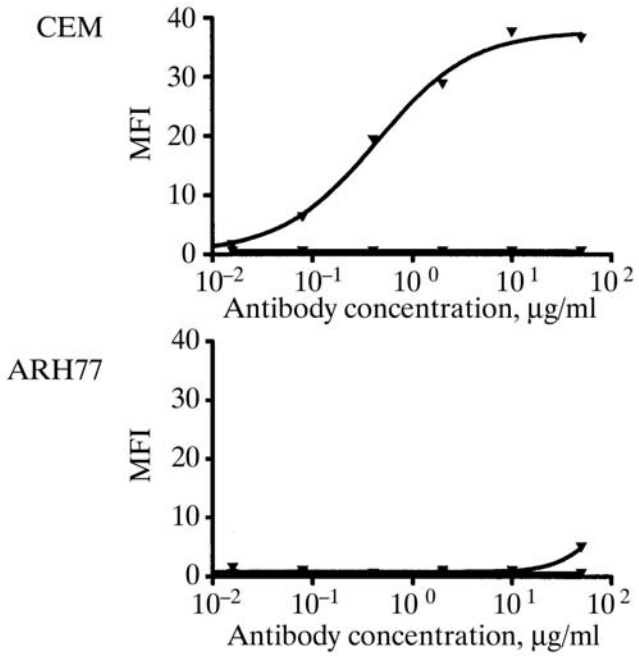

Fig. 4.5.1. *Specific binding of chimeric scCD7 Fc-fusion antibody to target cells.*

FACS analysis was performed using CD7-positive T-ALL line CEM and CD7-negative lymphoma line ARH-77 cells.

Thus, the process of fusion of Fv fragment of TH-69 hybridoma protein with human Fc part did not detectably alter the binding specificity to the antigen. The binding saturation has been reached at 10 µg/ml concentration. An additional control was performed with rituximab to exclude that any recombinant protein may have binding properties.

4.6. ADCC killing

To investigate the cytotoxic properties of the chimeric scCD7 Fc-fusion antibody and CD7 murine antibody were incubated at different concentration with ^{51}Cr labeled CEM target cells. Human MNCs from three donors as effector cells were used at E:T ration 40:1.

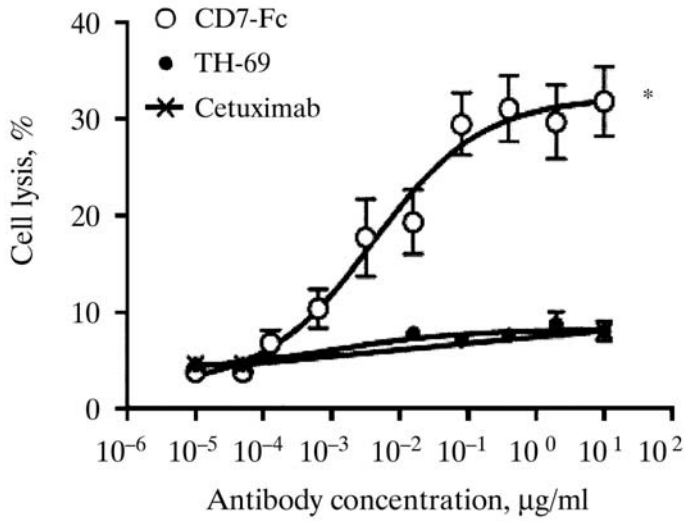

Fig. 4.6.1. *Tumor cell lysis mediated by scCD7 Fc-fusion antibody was analyzed in ^{51}Cr-release assays.*

CD7-positive CEM cells or CD7-negative ARH-77 cells were incubated with scCD7 Fc-fusion antibody (CD7-Fc), or murine anti-CD7antibody (TH-69), or cetuximab (anti-EGFR Ab) and human MNC as effector cells (E:T ratio 40:1). Each point represents mean ± SD. *P < 0.01, paired Wilcoxon rank sum test.

To exclude nonspecific lysis of the CEM cells cetuximab was used as negative control. Results showed that no killing of CD7-psitive cells was observed. Murine anti-CD7 antibody (TH-69) did not show any killing of CD7-positive cells. Chimeric scCD7 Fc-fusion antibody produced lysis of CD7-positive cells up to 35% (Fig. 4.6.1). Killing capacity increased with the increasing of Ab concentration and has reached maximal killing at concentration 10 μg/ml.

5. DISCUSSION

5.1. Response of LLC tumors to PDT treatment modulated by β-glucans

PDT induces cancer tissue damage, which initiate inflammatory and immune responses. The complement system plays a pivotal role in these responses and its activity contributes to the cure rate of tumors treated with PDT [71; 97; 116]. Tumor-localized treatment with zymosan, an alternative complement pathway activator, and TLR2 and TLR6 ligands, reduced the number of recurrent tumors after PDT [71]. However, the treatment with a classical complement pathway activator heat-aggregated y-globulin had no significant effect as a PDT adjuvant [71]. The combination of intratumoral dentritic cells and PDT against CT26 tumors in mice [116] or peritumoral and intravenous injection of NK cells, genetically altered to produce IL2, in combination with PDT in SCID mice [70] produced improvement in the outcome of PDT. Better effect of PDT on cancer has been achieved with co-administration of y-inulin, which is complement activator [69]. The kinetics of systemic and PDT-treated tumor-localized activation of the complement system have been described by Cecic et al. [25]. They showed that the level of complement 3, the key protein in the complement cascade, in PDT-treated tumors peaks at 3 h after phototherapy and remains highly activated until 24 h post-PDT, and the potential for complement activation via alternative pathway persists to 72 h, finally returning to the pretreatment level at 7 days post-PDT. During this period of time, PDT-treated tumor cells are opsonized by iC3b fragments and cytotoxic degranulation of the effector cells can be induced after the priming of CR3 by β-glucan. Therefore, this period of time is essential for the action of β-glucan. As previously reported, a minimum of 3 days after oral administration is needed for macrophages to degrade the large molecules of digested β-glucan into smaller active fragments [51, 26], which primes CR3 on the effector cells. However, another report [108] shows that a high concentration of β-glucan appears 3 h following oral administration. Therefore, β-glucans were administered 24 h before the laser irradiation in our study to. Orally and intravenously administered β-glucan functions by a similar mechanism [51]. Intravenous β-glucan is delivered directly to the CR3 on circulating granulocytes. Orally administered high molecular weight β-1,3 glucans, such as glucan from baker's yeast and barley [51], or small molecular weight β-1,3 glucans, such as laminarin [108], go through an intermediate step in which they are taken up by gastrointestinal macrophages and shuttled to reticuloendothelial tissue and bone marrow. Once there, macrophages degrade

large molecules of β-glucan into smaller biologically active fragments [81]. The active fragments are slowly released from the macrophages, bind to mature bone marrow neutrophil or NK cell CR3, and prime these cells for targeted killing through the 3-Syk-phosphatidylinositol 3-kinase signaling pathway [81].

Treatment with a combination of PDT and β-glucan in the current study produced significantly larger necrotic area in tumor tissue than PDT alone (Fig. 4.3.2) and this supports the findings of other researches, that CR3-dependent phagocytosis and cytotoxic degranulation requires dual ligation of both the lectin-like domain and the inserted (I) domain of the CD11b subunit of CR3 (CD11b/CD18, Mac-1) [142, 154, 111]. Without β-glucan, iC3b-opsonized tumor cells are resistant to killing, because they, like all human and mammalian cells, lack β-glucan in their membrane. Microbes can activate the lectin domain on CR3, leading to effective phagocytosis and cytotoxic degranulation [28, 42, 85]. Therefore, these processes can be induced by coadministration of β-glucan together with the agents, which cause opsonization of tumor cells by iC3b fragment. After photodynamic treatment, due to the activation of complement system, tumor cells are opsonized by iC3b fragment and CR3-DCC mechanism can be initiated.

The ability of β-glucan to enhance the activity of antitumor monoclonal antibodies (mAbs) also requires that they activate complement and deposit iC3b on tumor cells for recognition by CR3 on granulocytes and NK cells [28, 52, 40]. It has been demonstrated that β-glucan from both barley [28, 88] and yeast [157, 52, 51, 81, 82, 117] has synergistic effects when used with anticancer mAbs in various tumor models. The mechanism of action has been explained in several studies [51, 52, 84].

It has been reported that PDT results in DNA lesions such as single-strand breaks and DNA degradation as well as in chromosome aberrations [41, 99]. DNA damage induces several cellular responses including DNA repair and triggering of apoptotic pathways [93]. Lower expression of PCNA after the treatment with β-glucan reflects decrease in cellular prolixferation. However, it also indicates lower potential for DNA damage repair in these cells. The absence of the activation of PCNA expression in tumors treated with combination of PDT and β-glucan indicates both the low prolixferation and low DNA damage repair. This is consistent with an observation that combined treatment results in the best photodynamic effect. On the other hand, treatment with PDT alone results in a significantly higher PCNA level, which may be related to an activation of DNA damage repair that prevents necrosis induction in tumor tissue. Therefore, the treatment with β-glucan in combination with PDT could positively modulate the photodynamic effect by inhibition of the DNA damage repair system, which can

be activated by PDT alone.

Our data show that the most pronounced effect was achieved when PDT was combined with (1→3),(1→6)-β-glucan from baker's yeast. Therefore, it supports an idea, which was noted by Mueller et al. [89] and Adams et al. [3] that branching frequency may enhance the affinity of the polymer for the glucan receptor, and a polymer with greater molecular weight exhibits higher binding affinity. In addition, larger molecular weight β-glucans are absorbed more rapidly from the intestinal tract [108], which can cause higher concentration of the substance at the tumor site. According to our results β-glucan from brown algae, laminarin, has less effect on LLC tumor growth and suppresses PCNA expression less than β-glucan from barley or from baker's yeast. It can be due to the low molecular weigh of laminarin. Due to that it cleared from the blood plasma after 8 h already, whereas big molecular weight β-glucan from barley and insoluble β-glucan circulate more than 24h in blood plasma [108].

Current data show that all β-glucans, when used alone, also suppress LLC tumor growth (Fig. 4.1.1) and proliferation (Fig. 4.3.1, 4.3.4). The antitumor activity of β-glucans is known and has been reported in several studies with fungal (1→3),(1→6)-β-glucan, such as shizophylan, lentinam [100, 159], (1→3),(1→6)-β-glucan from yeast [61]. It can be explained that after implantation of tumor cells naturally elicited antitumor antibodies most probably occur, and they function similarly to exogenous antitumor mAbs by coating tumor cells with iC3b fragments [51]. So, CR3-DCC is initiated here, but is not very active. Moreover, β-glucan converts the nonprotective Th2-type response, which has been reported as dominant in the course of cancer, to a protective Th1-type response that favors cytotoxic T lymphocytes activity [10, 83]. Therefore, it can result in more effective tumor elimination and produce necrosis of tumor tissue when is given alone to treat LLC tumors.

The anti-angiogenetic activity of β-glucan can not be excluded as well. Polysaccharopeptide, which has β-glucan as the main polysaccharide component and is isolated from the edible mushroom *Coriolus versicolor*, was tested in the mouse sarcoma-S180 tumor model [50]. Immunostaining of tumor tissues with antibody against the endothelial cell marker (factor VIII) demonstrated a positive correlation in that both the vascular density and tumor weight were lower in mice treated with PSP. The total amount of new vessel production was reduced, and the basic tumor type-specific vascular architecture was retained. However, the expression of vascular endothelial cell growth factor in these tumors was suppressed. Angiogenesis is crucial to tumor growth and metastasis, and interruption of this process is one of the therapeutic interventions against tumor proliferation. This may explain why

PCNA expression decreased also in tumors, treated by β-glucans alone (Fig. 4.3.4). In the current study, it was observed that tumor tissue from mice treated with β-glucan alone or in combination with PDT was less infiltrated by erythrocytes compared with nontreated tissue or tissue treated with PDT alone. It can be postulated that this could be due to suppression of tumor angiogenesis. Thus, anti-angiogenesis can be one of the pathways, through which β-glucans mediate antitumor activity.

Data of this study suggest that the activation of DNA repair system by the PDT and the significant decrease in this activity when PDT is used in combination with β-glucans (Fig. 4.3.1). Tumor growth dynamics (Fig. 4.1.1), the expression levels of PCNA (Fig. 4.3.1) and evaluation of the necrotic areas in tumor tissue (Fig. 4.3.2) suggest that the activity of DNA damage repair system is an important factor, which modulates the efficacy of PDT. Unbranched, high molecular weight (1→3),(1→4)-β-glucan from barley, branched high molecular weight (1→3),(1→6)-β-glucan from baker's yeast and low molecular weight (1→3),(1→6)-β-glucan from brown algae, can all enhance the efficacy of photodynamic treatment. Coadministration of these β-glucans results in more effective necrosis of PDT-treated tumor cells (Fig. 4.3.2, 4.3.5), but the presence of the (1→3),(1→6)-β linkage is essential to achieve better effect (Fig. 4.3.3). In our study we confirmed the antitumor activity of β-glucan, but the mechanisms of this effect are not not yet clear and further investigations are needed. Our data support an idea that PDT in combination with β-glucan might be the effective therapeutic strategy.

5.2. In vitro characterization of chimeric scCD7 Fc-fusion antibody

Monoclonal antibodies have many features that make them attractive as therapeutic targeting agents. Immunotherapy using MAbs is quite safe and selective method for the treatment of cancer. At the beginning, monoclonal antibodies in clinical use were murine antibodies. In addition to immunogenicity, murine antibodies have a short life in humans and are ineffective in effector functions, which are essential components of the mechanism of action of many mAbs [150]. Immunogenicity raises serious problems in terms of acute side effects and influences drug pharmacokinetics and decreases drug efficacy. In addition, human Fc part of antibody is essential for the recruiting of human immune cells for ADCC and CDC. Chimeric antibodies were developed in which the constant domains of the human IgG molecule were combined with mouse heavy and light chain antibody

variable regions (Fv fragment) [130, 54]. Fv fragment is the smallest antibody fragment with the antigen-binding site. So, connection of scFv fragment of anti-CD7 antibody with Fc portion of human IgG_1 can be helpful for scFvCD7-Fc-fusion protein (Fig. 3.9.1) to obtain ideal feathers. However, this modification of the monoclonal antibody can cause the lost or decrease in the rate of protein expression and antigen-binding properties. That is why chimeric scCD7 Fc-fusion antibody requires careful *in vitro* characterization.

Chimeric scCD7 antibody was derived from mammalian cells in this study. The derivation of therapeutics products from live cells raises the possibility of potential contamination in the final preparation [150]. One major contaminant found in conventional serum-based culture medium is bovine Ig. Bovine Ig shares most of the physical and biological activities with its therapeutic Ig counterparts and has proven difficult to remove during down stream purification [130]. Antibody purification typically begins with a protein A column to concentrate the antibody from medium and to begin removal of medium contaminants [130]. After first step purification both chimeric scCD7 Fc-fusion antibody and bovine Ig (dissociated heavy and light chain) are seen in SDS-PADE electrophoresis (Fig 4.4.1). However, presence of six histidine molecules in the construct of scCD7 Fc-fusion antibody (NH_2-terminal hexa-histidine tag) allows further removing of contaminants. As our results show, after the second step purification on Ni-NTA agarose column only one band appears on the gel. The size of the protein is ~60 kDa as expected (Fig. 4.4.1).

Similar single chain CD20-directed molecule (TRU-015) was described [45]. Here anti CD20 scFv fragment was fused to human IgG_1 Fc domain. It significantly reduced tumor volumes and increased long-term complete tumor regression compared with rituximab (full molecule mAb) in xenograft mice tumors. Both, molecule size and binding affinity are factors that influence the diffusion rate of protein through tissues [2]. These differencies may contribute to the better activity of TRU-015 protein [45]. Also, alteration of human IgG_1 hinge region in TRU-015 reduces its ability to medite CDC, but not alters ADCC activity. This slower rate of complement activation compared with rituximab may reduce the occurance of infusion ractions.

The complexity of protein products might have an impact on product efficacy [150, 130, 75]. Therefore, fusion of scFv fragment with Fc fragment of human Ig also can alter functions of this chimeric Ab. Thus, the bioassay is used to monitor biological activity that may not be reflected in biochemical or physiochemical characterization [150]. The most common bioassays used for cancer therapies are cell-based assays that measure cell

survival, proliferation, or downstream signaling such as phosphorilation or cytokine release [150]. Depending on the mechanism of action of the antibody or fusion protein appropriate assay should be designed to capture the integrity of each structural component necessary for activity. Antibody specificity is determined by the *in vitro* binding affinity of the antibody to the prospective target. The affinity of scCD7 Fc-fusion protein was tested by FACS with CD7-positive and CD7-negative cell lines in this study (Fig. 4.5.1) and specific binding affinity to the antigen was confirmed.

In addition, cell culture-based activity assays that are appropriate for the antibody's indication and mechanism of action (antibody-dependent cytotoxicity, complement-dependent cytotoxicity, anti-angiogenesis activity) are evaluated [150]. The principal mechanism by which IgG antibodies engage the cellular immune system is via interaction of Fc domain with Fcy receptors [94, 29]. Fc part is responsible for activation and fixation of complement fragments on cancer cell and by this inducing of complement-dependent cytotoxicity. Another killing mechanism of cancer cells is antibody-dependent cytotoxicity, where binding of Fc receptor by mAb recruits leucocytes and NK cells for killing of cancer cells [2, 55]. As the main our idea was to use effector functions of Ab's Fc part, ADCC killing is crucial for chimeric scCD7 Fc-fusion antibody. The most commonly used is ADCC assay, in which the spilling out of natural (e.g. lactate dehydrogenase) or artificial (e.g. chromium) target cell ingredients is measured to monitor the extent of lysis [36]. NK cells are the best appreciated among anti-tumor effectors and they are unique that typically express the activating receptor FcyIIIa, but are not a subject to regulation by the inhibitory receptor FcyIIb [36]. However, Fc part of murine CD7 antibody does not recruit human immune effector cells. But Fc part of human IgG_1 recruits them producing effective killing of CD7-positive target cells, as results of our study show (Fig. 4.6.1).

The support for safe and effective use of chimeric antibodies comes from clinical data for anti-CD20 antibody Rituxan, used for B-cell non-Hodgkin's lymphoma treatment [43], and anti-TNF-α antibody Infliximab, used for the treatment of rheumatoid arthritis and Crohn's disease [130]. Therefore, scCD7 Fc-fusion antibody is promising construct for further modifications: glyco-engineering; engineering to improve Fcy receptor binding or to diminish interaction with Fcy inhibitory receptors [46]. In addition, pharmacokinetic studies to define the plasma half life and biodistribution studies in leukemia xenograft model will also be important to determine the clinical application of this novel targeted therapy in $CD7^+$ malignancies.

To summarise the findigs of this study, it can support the idea to recruite the immune system for cancer treatment. During PDT it can be done by involving of CR3-DCC mechanism, when effective cell necrosis can be produced, and by decreasing of DNA damage repair system in the presence of β-glucan. Chimerization of anti CD7 mAb gives opportunity to enhance ADCC mechanism by recruiting of human effector immune cells, where Fc part of human Ab is essential. .

CONCLUSIONS

1. The growth of Lewis lung carcinoma tumor is suppressed more when treated by photodynamic therapy in combination with β-glucan than tumors, treated by photodynamic therapy alone.
2. Co-administration of β-glucan does not prolong survival of Lewis lung carcinoma tumor bearing mice, treated by photodynamic therapy.
3. The expression level of proliferating cell nuclear antigen in Lewis lung carcinoma tumor, treated by photodynamic therapy, is decreased with co-administration of β-glucan. PCNA expression in LLC tumor, treated by β-glucans alone is lower than in LLC tumors treated by PDT alone.
4. Photodynamic therapy in combination with β-glucan is more effective to produce necrosis in Lewis lung carcinoma tumors, than photodynamic therapy alone.
5. β-glucan from baker's yeast is most effective to modulate Lewis lung carcinoma tumor response to photodynamic therapy.
6. Chimeric single chain anti-CD7 Fc-fusion antibody have been successfully expressed and purified and it retains binding specificity to CD7 antigen.
7. Chimeric single chain anti-CD7 Fc-fusion antibody mediates lysis of CD7-possitive tumor cells by antibody dependent cell cytotoxicity mechanism in the presence of human mononuclear cells.

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REFFERENCES

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