University of South Bohemia in České Budějovice

Faculty of Science

The use of artificial opsonization for cancer treatment

Bachelor thesis

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Abstract

This work is using the innate immunity to fight against cancer. Artificial opsonization of B16-F10 mice melanoma cancer cells was studied in vivo and in vitro. For this purpose, syngeneic mice IgG was isolated and the linkage to cancer cells was provided using bifunctional crosslinker SMCC. Survival analysis was evaluated for the in vivo experiment and spectrophotometric and fluorometric methods were used to investigate the extent of the linkage of protein on B16-F10 cells via SMCC. The extent was evaluated using regression lines of corresponding compounds. In vivo and in vitro results were compared.

Tato práce se zabývá možností využití přirozené imunity v boji proti rakovině. Umělá opsonizace buněk B16-F10 myšího melanomu byla zkoumána in vivo a in vitro. K tomuto účelu bylo izolováno IgG ze syngenních myší. Vazba na rakovinnou buňku byla zprostředkována pomocí bifunkčního crosslinkeru SMCC. Pro in vivo experiment byla vyhodnocena analýza přežití. Míra vazby proteinu na buňky B16-F10 pomocí SMCC byla in vitro vyhodnocena za použití spektrofotometrických a fluorimetrických metod. Míra vazby byla vyhodnocována pomocí regresních křivek odpovídajících látek. Výsledky in vivo a in vitro pokusů byly porovnány.

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1 Introduction

Cancer is statistically one of the main causes of death in these days. It endangers a wide range of population and the current medical treatment is often not sufficient. Therefore, it is a topic of high interest in pharmacy and cure development.

Melanoma present 2% of all new cases of cancer and 1% of deaths caused by cancer [1]. It is estimated to affect 132 000 new patients worldwide every year [2]. This type of cancer presents an especially big problem. Its incidence is increasing and patients with a later stadium of the disease have a poor prognosis. Moreover, melanoma are often resistant to oncological treatments and tend to show remission [3], [4].

The goal of this bachelor thesis was to study and experimentally apply artificial opsonization of cancer cells in order to reduce melanoma tumors. For this purpose, a bifunctional cross-linker SMCC was used to link immunoglobuline IgG from syngeneic mice to cancer cells [5]. Innate immunity was used in this way to promote the defense of the organism against cancer. In vivo and in vitro experiments monitoring functioning of linkage and its usability were performed.

Cancer cells B16-F10 were transplanted to induce melanoma tumor in mice. After progression of the tumor, the mice were treated with opsonizing agent. Several control groups were included in order to obtain relevant results. The mice were observed and the tumor sizes were measured periodically. Finally, a survival analysis was performed and conclusions were made.

The in vitro experiment was carried out with the mice cancer cells B16-F10 [6]. In order to determine the efficiency of the in vitro conjugation, both spectrophotometric and fluorometric methods were used. Absorbance and emission measurements were used to calculate the change in protein concentration caused by conjugation with cells. Proteins used in the experiment were hemoglobin, bovine serum albumin and phycoerytrin.

2 Cancers

Being statistically one of the main causes of death in these days, cancer presents a topic of high interest in pharmaceutical industry and cure development research.

The immune system is well equipped to fight back in case of an infection by exogenic organisms and it possesses sufficient defense in everyday life. Nevertheless, it can be unsuccessful when dealing with a tumor. As the immune system protects the organism against self damage, the tumor, as a part of the body, may be protected as well [7].

Origin of tumors comes from the deformation and/or the destruction of genes. Deformation itself can be caused by various sources - chemically, electromagnetically induced by UV radiation or even by viruses. The epidemiology of cancer is moreover influenced genetically and dietetically and its survival is usually influenced by age [8], [9].

Proliferation of cells with deformed DNA and the expression of deformed genes when unstopped may lead to the generation of a malignant tumor [10]. Nevertheless, this is the black scenario when DNA is not repaired and no further successful regulation from an organism is involved. There are, of course, mechanisms that usually contribute to the reparation.

As it is caused by uncontrollable progressive growth and division of very vital and differentiated cancer cells, it is difficult to reach the full restoration of a diseased organism [11]. When compared to normal cells, cancer cells are able to resist mechanisms of growth control and successfully avoid apoptosis [10].

2.1 Classification

Tumors, in general, can be described as benign or malignant. Benign tumors are bounded, non invasive and not forming methastasis. The danger behind benign tumors lies in the suppression of the space necessary for other tissues and the risk of transformation into a malignant form. Such tumors can often be removed surgically without danger of further occurrence. On the other hand, malignant tumors are forming methastasis in other kinds of tissues than the primary. Such cases are then extremely difficult to stop when disease is already well established [10].

Furthermore, tumors can be classified according to the tissue of their origin and according to their stage. The information about the stage is particularly useful when dealing with prognostics of the case. Yet the first surgeons involved in oncology knew that localized primary tumor has better prognosis than that being beyond this phase [12].

There are several staging systems used worldwide according to particular demands. The most often used is the TNM system that classifies cancer according to the extent and size of the primary tumor (denoting "T"), regional lymph node involvement ("N") and presence of distant methastasis ("M"). This system has been updated over years according to the knowledge and clinical needs. For categorization, the stages are divided into stage I, II, III and IV. Stage I corresponds to the cases with smaller or less invasive tumors and negative nodes. Stages II and III are assigned for cases with increasing tumor extent and lymph node involvement. Stage IV are cases with present distant methastasis. Additionally, there also exists a term for Stage 0, the carcinoma in situ with no metastatic potential [13].

2.2 Melanoma

Melanine is a pigment having a function of protecting the skin from UV light irradiation. Its function lies in absorbing the harmful light that could induce mutations in potential oncogenes and tumor supressor genes or could alter the expression of gene products [14]. Moreover, melanine can protect the organism from reactive oxygen species (ROS). Nevertheless, in-vitro studies state that melanin, when irradiated by UV light, may promote generation of ROS. Unradiated melanine is also able to cause DNA breakage and prevent its reparation and toxic intermediates generated during melanin synthesis can cause damage of the cell [15]. The absorbance spectrum of melanine can be seen in Fig. 1.

Melanosomes are organelles of cells, melanocytes, producing melanine. These can be found in skin, eye and inner ear [16]. The production of melanine is influenced hormonally. The uncontrollable progression of melanocytes due to damage of DNA upon UV light irradiation can lead to melanoma (Fig. 2).

There are several factors influencing melanoma incidence. There is an apparent relation with latitude and with the degree of skin pigmentation. Melanoma predominantly appears at fair-skinned people. Another factor is a level of exposure to UV light [17].

Melanoma can be, as cancer in general, classified using staging systems as TNM that takes into account three main factors - the size of the tumor, lymph nodes presence in the vicinity and metastasis formation. The other factor that is important and can bring information about the prognosis of melanoma is the mitotic rate. It describes the progression of the cells and contributes to differentiation between distinct phases [18]. The summary table of melanoma staging according to AJCC can be seen in Tab. 1.

In research, melanoma often serve as a model for understanding immunology of cancer in general as it is easily adaptable to cell cultures. Further reasons are that this type of cancer is very problematic as it is often resistant to chemotherapy and radiotherapy and therefore requires intensive research to find suitable treatments [16].

Mouse melanoma has been used as a model from the beginning of the 20th century in order to study the disease and treatment in general. Probably the most often used and the best known cell line is B16. Mice cell lines bring the advantage of being transplantable for in vivo experiments as well as being available for in vitro cultivation and application [6].

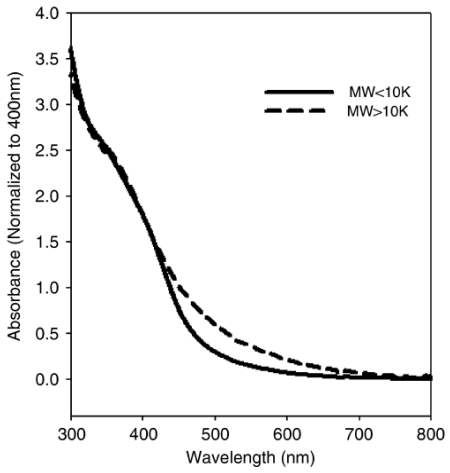

Fig. 1 Eumelanin absorbance spectra (two lines present eumalin of two different Mw) [19].

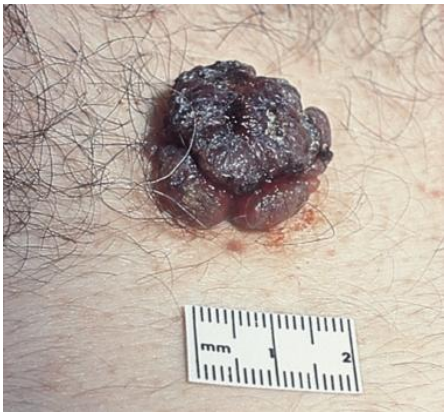

Fig. 2 Primary malignant melanoma in human. [20]

Tab. 1 Overview of cutaneus melanoma staging classification according to AJCC taken from the literature [1].

AJCC classification/staging for cutaneous melanoma [3]

AJCC staging	Characteristics	TNM classification	Characteristics	Clinical staging	Characteristics
I	Primary tumour thickness < 1.5 mm with no regional lymph node metastasis, no distant metastasis	T-primary tumour	PTX primary tumour cannot be assessed PT0 no evidence of primary tumour Ptis melanoma in situ PT1 tumour 0.75 mm or less and invades papillary dermis PT2 tumour more than 0.75 mm but no more than 1.5 mm and/or invades the papillary-reticular dermal interface	I	Localised disease
IIA	Primary tumour thickness 1.5–4.0 mm with no regional lymph node metastasis, no distant metastasis		PT3 tumour more than 1.5 mm but no more than 4.0 mm in thickness and/or invades the reticular dermis PT3a tumour more than 1.5 mm but no more than 3.0 mm thickness PT3b tumour more than 3.0 mm but no more than 4.0 mm thickness		
IIB	Primary tumour thickness > 4.0 mm with no regional lymph node metastasis, no distant metastasis		PT4 tumour more than 4.0 mm thickness and/or invades subcutaneous tissue PT4b satellite(s) within 2.0 cm of the primary tumour		
III	Any primary tumour with lymph node metastasis, no distant metastasis	N-regional lymph nodes	NX regional lymph nodes cannot be assessed N0 no regional lymph node metastasis N1 metastasis 3.0 cm or less in greatest dimension in any regional lymph node(s) N2 metastasis more than 3.0 cm in greatest dimension in any regional lymph node(s) N2a metastasis more than 3.0 cm in greatest dimension in any regional lymph node(s) N2b in-transit metastasis N2c both	II	Palpable regional lymph nodes
IV	Any primary tumour with lymph node metastasis, distant metastasis	M-distant metastasis	MX distant metastasis cannot be assessed M0 no distant metastasis M1 distant metastasis M1a metastasis in skin or subcutaneous tissue or lymph node(s) beyond the regional lymph nodes M1b visceral metastasis	III	Presence of distant metastases

AJCC, American Joint Committee on Classification.

2.3 Immunology and cancer

Deregulated, autonomous proliferation of cells is one of the main characteristics of both benign and malignant tumors [21]. The immune system has dispositions for regulation or elimination of such cells, but not all of them are always sufficient.

The immune system provides humoral and cellular response. As a complex, it develops during life. The immunity obtained with birth is called the innate immunity. The adaptive immunity is the adaptation due to experience during lifetime [22].

The innate immunity is providing recognition between self and non-self non-specifically. When recognized as a non-self, the object is destroyed by non-specific effector cells like macrophages, granulocytes, dendritic cells and NK cells [7].

The innate immunity is able to distinguish pathogen associated molecular patterns (PAMPs) using pathogen pattern receptors (PPR). PPR enable innate immunity to recognize a dangerous stimulus from a safe, non pathogenic one and eliminate in this way non necessary reactions and damage of own structures. Furthermore, PPR are able to provide synergy with specific immunity when cooperating with effector cells. The information on pathogens is presented on HLA providing the information to T cells of specific immunity.

Granulocytes form the biggest group of leukocytes. They are able to kill microorganisms and decompose them when behaving as effectors. Moreover, they present an important source of cytokines induced by bacterial products, e. g. LPS.

Macrophages are cells performing phagocytosis of own destroyed or infected cells being activated by T lymphocytes, NK cells and also able to recognize PAMPs using PPR. They are having effector functions as well as regulation functions when dealing with specific T cell immunity.

Dendritic cells are important as they provide an expansion of specific T cells activation followed by activation of B cells in secondary lymphatic organs. The main function of natural killer (NK) cells is the ability to cytotoxically destroy cells infected by a virus or cancer cells. Their importance also lies in the fact that they are a source of cytokines influencing hematopoiesis and those that are influencing T cells [21].

Complement is the part of the innate immunity that recognizes external microorganisms as well as pathogenic molecules in order to promote phagocytosis [23]. In addition, it clears unwanted debris like apoptic and necrotic cells and gives feedback to adaptive immunity. Complement itself consists of 35 proteins participating in three main activation pathways: classical, lectin and alternative. The classical pathway can be trigerred by interaction of C1q from the C1 complex, immune complexes and other molecules like DNA, misfolded proteins and CRP. The lectin pathway is activated when recognizing certain saccharides by MBL or ficolines. The alternative pathway is triggered via autoactivation or unstable factor C3. All three pathways are cascades of protein cleavages resulting in the formation of enzymatic complexes C3 and C5 convertases, target opsonization using C3b/iC3b in order to promote phagocytosis, release of inflammatory anaphylatoxins C5a and C3a to attract leukocytes and formation of MAC that provides perforation of the target membrane [23].

Interferons are an important part of both innate and specific immunity. The strongest stimulus for production of intereferons is viral infection. Interferons, when binding to the specific cell receptor, induce in the cell that was not infected yet a state of non-permission against viral agens. They also provide an anti-proliferative effect that is in praxis used for the treatment of hematological malignancies [21].

The adaptive immunity consists mainly of B and T lymphocytes and the production of antibodies. There are various kinds of B and T cells having a lot of important functions. Though, these are not of the main interest of this work.

In the matter of adaptive immune response to cancer, there are tumor associated antigens (TAA) that can be recognized specifically by T lymphocytes and trigger the response. Three main groups of TAA are known - specific for the particular tumor, the TAA specific for the group of tumors and TAA specific for the type of tissue from which the tumor cells are developed [7].

The escape of tumor can occur at several levels of tumor antigen presentation. Melanoma cells can lose the antigen. Defects in antigen procession may interfere with generation of immunogenic peptides in cytosol or loading of peptides into ER. The HLA allele can get downregulated or its allele can be absent causing MHC (Fig. 2) not being presented on the melanoma cell surface [14]. Moreover, tumors are able to create an immunosuppressive environment by the production of immunosuppressive cytokines.

TGF-β may be involved in this situation inducing suppression of inflammatory T-cells response [11].

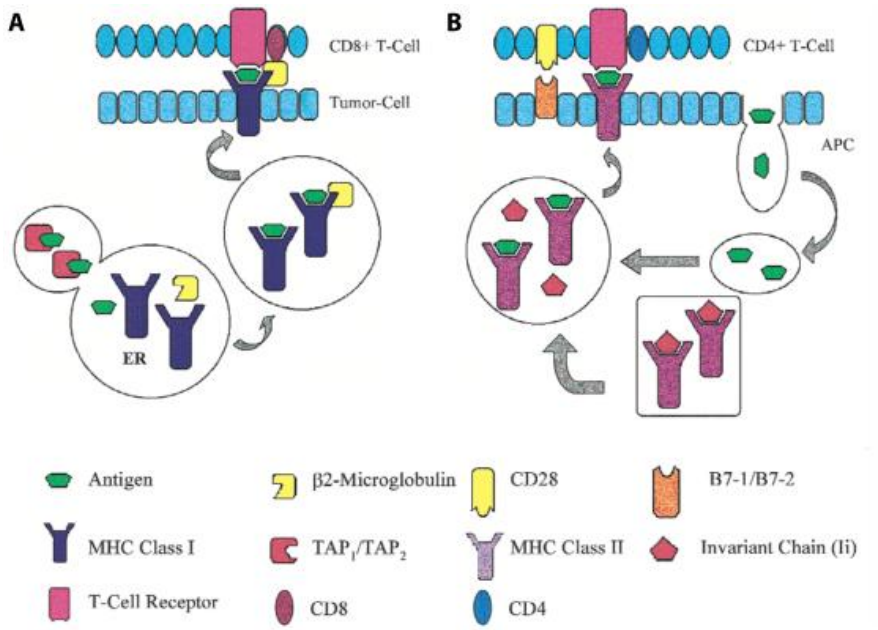

Fig. 2 Antigen presentation in MHC pathways. (A) MHC I. (B) MHC II [24].

3 Therapy of cancer

In order to reach full recovery, various methods and their combinations are used. It is necessary to mention that it is not always possible to reach full recovery and even improvement of the state can be considered a success..

The earliest written evidences about cancer can be found in Edwin Smith papyrus from approximately 3000 BC describing breast cancer. The later written artifacts include the treatment. Many cultures were using metals in form of their salts. Egyptians were using Arsenic paste until the 19th century. Hippocrates described several kinds of cancer and was recommending as a treatment either palliative care or, in case of deep tumors, excision. By the beginning of the 13th century, surgeries were prohibited by the pope. Nevertheless, Middle Ages physicians contributed to the knowledge of cancer and its classification [25]. The 19th century introduced microscopy as a useful tool for research of cancer and its diagnostics. The cells were known and microscopic analysis of the tissue sample made it possible to choose the suitable surgical treatment [26].

Surgical treatment of cancer is still very often used, usually combined with other types of therapies. Especially in earliest stages it is often the best option. The prognosis depends on the extent of tumor, respectively the stage of disease. It is often applied in combination with adjuvant therapies [17].

Another treatment that is used very often nowadays is chemotherapy. It is based on the reduction of growth of rapidly growing cells. As there is a different rate of the mitotic cycle of cancer cells (slower) and normal cells, normal cells are usually able to regenerate when treated with chemotherapeutics [27].

Radiotherapy is one of the most used therapies of cancer. It involves the destruction of DNA of cells - both cancer cells and normal body cells. Normal cells usually regenerate and cancer cells, ideally, do not. Though, the process of chemotherapy is more complex. Just as in the case of chemotherapy, radiotherapy has a lot of side effects, too. However, despite its side effects, it is used due to its efficiency [28].

3.1 Immunother apy

The idea behind immunotherapy in the second half of the last century was the treatment of a tumor by vaccination in the analogous way as it has been used in the cases of external pathogens. The theory suggested that the vaccine would prepare the immune system for recognition of a tumor in a similar way as it was used for dealing successfully with external pathogens [29].

There were already attempts to treat patients with a direct crude tumor extract vaccination in the beginning of the 20^{th} century [20]. The other strategies were using bacterial extracts in order to promote the immune system [7]. Nevertheless, the early attempts were often not very successful solving the problem and it is still a challenging issue nowadays.

Nowadays, various strategies are applied and tested. These include the usage of recombinant cytokines to promote tumor specific T cells action and protection. Other options are the activation of antigen presenting cells via CD40 providing further protection of dendritic and T cells from apoptosis caused by a tumor or the application of CpG oligonucleotides for the activation of antigen presenting cells via toll like receptors. Last but not least, there is a strategy that is blocking anti-CTLA 4 antibodies for removal of negative signal given to activated T cells allowing proliferation of tumor specific T cells induced by a vaccine [29]. Different types of vacciness and their advantages and disadvantages are summarized Tab. 2.

One of the problems occuring when applying vaccinations is that a lot of them proved to be efficient in vitro but not in vivo. The tumor cells have the ability to induce an immunosuppressive microenvironment serving as a possible explanation of vaccines failing in praxis [30].

Tab. 2 Types of vaccines in cancer immunotherapy [20]

Type of vaccine	Advantages	Disadvantages
Allogeneic cellular	Simple to prepare	Presents irrelevant 'allo' antigens
	Presents broad spectrum of potential antigens	Difficult to precisely characterise components
	Currently in phase II clinical trials	Requires adjuvant
Autologous cellular	Presents patient-specific unique antigens	Requires laborious individual vaccine production
	Presents numerous antigens	Requires adjuvant
Autologous heat shock protein	Presents patient-specific unique antigens	Requires laborious individual vaccine production
	Presents numerous antigens	Unproven immunogenicity
Purified protein or carbohydrate	Well-defined components	Production can be difficult
	Safety and immunogenicity established (carbohydrates) in mature clinical trials	Requires adjuvant
Peptide	Simple to prepare	Requires adjuvant
	Safety established in early trials	Only presents single epitope
		HLA-restricted
DNA	Simple to prepare	Little clinical data to date
	Numerous epitopes presented	
	Immunostimulatory sequences in vector	
Recombinant virus	Inherently immunogenic	Neutralising immunity to vector
	Presents numerous epitopes	

There are yet established vaccines used in cancer prevention that showed to be relatively efficient and have been taken into praxis. One of them is the vaccine against HPV that successfully completed clinical tests in 2005. It seems that the effect of a vaccine lies in inducing anti-HPV antibodies preventing a viral infection of cervical epithelium. The vaccine showed to be 100% effective against 2 main strains causing HPV and is now used for the prevention of cervical cancer [11].

Immunotherapy is not always sufficient to fully treat cancer. Nevertheless, it proved to be efficient for some patients. Intensive research is necessary in order to find the exact mechanisms and investigate the immunotherapeutic applicability of particular treatments at different phases of the disease. Despite all of that, there is already a number of immunotherapeutic methods applied in praxis using cytokines, monoclonal antibodies and active specific immunotherapy [7].

3.2 Therapy of melanoma

In the earliest stages, surgical treatment showed to be the best option when dealing with melanoma. The variable which shows best which method is the most suitable for the treatment and what is the prognosis, is tumor thickness. When diagnosed early, more than 90 % of melanomas can be treated just with the use of surgical impact [17].

As surgical treatment does not often posses the full treatment, adjuvant therapies might be necessary and are often applied. The usual procedure is to remove the tumor surgically and in order to prevent its remission, an additional treatment is applied. An often used adjuvant treatment is radiotherapy or chemotherapy. As targeted therapy and immunotherapy including interferon vaccination progressed in the last decades, these also gain their place between adjuvant methods for treatment cancer [1].

3.2.1 Immunotherapy of melanoma

As melanoma is resistant to cytotoxic chemotherapy leaving behind its viable stem cells, there is a need for an alternative treatment. Nowadays, there are studies dealing with the migration of circulating tumor cells of melanoma in order to observe the formation of methastasis [4]. Spontaneous remissions are not rare and indicate genetical heterogeneity and instability of the tumor [3] or an immunological mechanism. Therefore, immunotherapy of melanoma shows to be relevant as an adjuvant therapy to avoid remission in patients after surgical impact [20].

In praxis, cytokines interferon-α (IFN-α) and interleukin-2 (IL-2) have been used in immunotherapy of melanoma showing response of 15% in the case of metastatic melanoma [20]. Radioimmunotherapy, on the other hand, combines radioactive isotopes connected with antibodies [2].

Interferons are another well studied subject of a possible immunotherapy of melanoma. As they are proteins, which are able to inhibit virus replication, protein synthesis and tumor cell replication, interferons attracted the interest of research in field of immunotherapy of cancer several decades ago. The exact mechanism of their action is not known yet. The proposed ways are including stimulation of macrophages and NK cells activity, support of cell surface antigen expression of MHC and tumor antigens, suppressing the tumor cell growth and further effects [1]. The MHC I pathway is then critical for the tumor specific T-cells generation in order to exert corresponding epitopes [31]. Moreover, the latest researches also concentrate on innate immune system as the key component in development of the immune network regulating melanoma and tumor antigens [32].

In order to find sufficient and efficient ways of treatment, immunotherapy needs to deal with the melanoma cells immune escape mechanisms and support and restore the function of APCs and T cells. Furthermore, various methods of immunotherapy need to be studied and optimized in order to bring more benefits [24].

4 Heterobifunctional crosslinkers

Conjugates with immunoglobulines are used for the indirect detection of antibodies or antigens in various assays. The important condition in this case is the availability of particular functional groups such as primary amines and sulfhydryls when thinking of linker containing maleimid contact groups. This then determine the degree of binding and in the case of assays, its sensitivity [33].

Heterobifunctional cross-linkers can be used both for the preparation of conjugates of proteins with proteins and proteins with cells. Their advantage lies in the presence of two functional groups enabling one to control reaction sequences and stoichiometries. Succininimidyl-4-(N-maleinimidomethylcyclohexane)-1-carboxylate (SMCC) reacts stoichiometrically with a protein to bind to the amino and thiol groups rapidly and efficiently. Such a conjugation shows to have minimal effects on the structural integrity of the cell and of the protein as the conjugate is very stable [5].

The reacting groups on SMCC are N-hydroxysuccinimide (NHS ester) and maleimide. The NHS ester reacts with primary amines to form an amide bond at pH of 7-9. Subsequently, maleimide reacts with the sulfhydryl groups to form a stable thioether bond at pH of 6.5-7.5. The maleimide group is more stable than NHS ester but tends to hydrolyze when exposed to a pH higher than 7.5. Therefore, the conjugation is usually performed at pH 7.2-7.5. The cyclohexane ring in the structure decreases the rate of hydrolysis of SMCC-conjugate compared to similar reagents.

The advantage of SMCC is its stability and wide applicability for enzyme labeling of antibodies, creating bioconjugates and sulhydryl-reactive maleimide-activated carrier proteins for coupling haptens. The structure and the scheme of conjugation reactions can be seen on Fig. 3 and Fig. 4.

Sulfo-SMCC is a water soluble analogue of SMCC. SMCC is not soluble in water and has to be predissolved in an organic solvent. Subsequently, it can be diluted into aqueous solution and further reacted with proteins if present in low concentration [34].

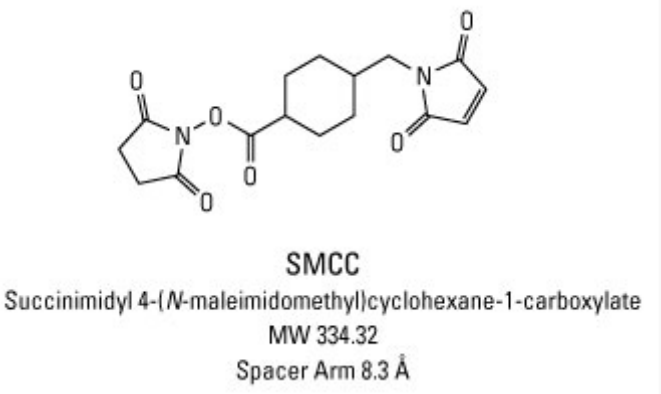

Fig. 3 Structure of SMCC [35].

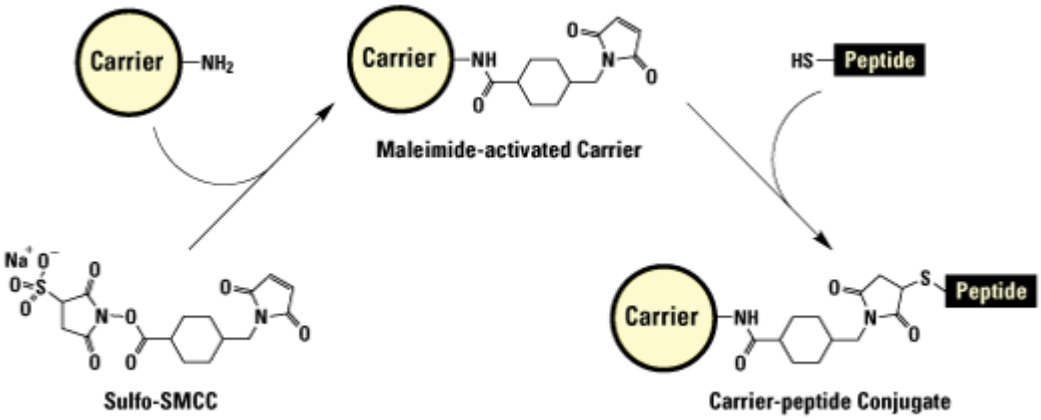

Fig. 4 Anchoring of SMCC on protein [35].

5 Experimental

5.1 Instrumentation

Centrifuge NF 400 R

Centrifuge Universal 32 R (Hettich Zentrifugen)

Spectrophotometric device ELx800^{TM} (BioTek)

Fluorimeter TBS-380 (Turner BIOSYSTEMS)

Fluorimeter Infinite M200 (Tecan)

5.2 Chemicals

SMCC (Pierce SCIENTIFIC)

RPMI 1640 (Sigma Aldrich)

HBS

PBS

DTT (Sigma Aldrich)

B - phycoerythrin (AnaSpec)

Trypan blue

DMSO

Bovine serum albumin (Sigma Aldrich)

Hemoglobin (Sigma Aldrich)

Ammonium sulphate

LPS z E. Coli 0111.B4 (Sigma Aldrich)

Bradford reagent

Trypsin (Sigma Aldrich)

5.3 Mice

The 4 weeks old female mice C57BL/6N were obtained from the Charles River Laboratories. The experiment started when their age reached 8 weeks. The mice were kept separately in boxes at 12/12 photoperiode and had nonlimited access to water and food.

5.4 Cell line

The cells of the mice melanoma B16-F10 were cultivated in RPMI 1640 medium with 10% FCS, antibiotics, glutamine and mercaptothenol. The cultivation was performed at 37 °C in an atmosphere saturated with water vapor containing 5% CO_2.

5.5 Methods

5.5.1 IgG preparation

Blood was obtained from the shoulder girdle of five three months old female mice C57BL/6N Charles River Laboratories and kept overnight at 4 °C. Subsequently, it was centrifuged (600 g, 10 min). 1.32 mL of serum were obtained.

The serum was left under stirring at 4 °C while saturated ammonium sulphate solution was addded dropwise. For 1.32 mL of the serum, 0.713 mL ammonium sulphate solution were used, reaching a final saturation of 35%. Afterwards, the mixture was stirred for one hour at 4 °C.

Subsequently, the mixture was centrifuged at 7000 RPM for 1 minute (r = 7.5 cm, corresponding to 4116 g). The precipitate was dried and redisolved in 200 μL of PBS. Another 400 μL of PBS were added and the mixture was dialyzed overnight using 1 L PBS of pH 7.3 and filter of Mw cut-off 12 - 14 000 in order to clear the mixture from the sulphate.

The solution of 920 μL was obtained. Using the Bradford determination of protein, the concentration was calculated. The solution contained 2.74 mg/mL.

5.5.2 In vivo experiment

When the mice reached an age of 8 weeks, 400 000 cells of B16F10 were transplanted subcutaneously into the back of each mouse. The twelfth day after the transplantation, the mice were randomised into groups (6 mice each) and the size of the tumors were measured. This day was considered day 0 of the therapy. The reduction

solution (or control solution) of 50 μL was injected into the mice and they were left for one hour. Subsequently, the corresponding mixture of 50 μL was injected into the mice according to the groups to which they belonged to. The injection mixtures are summarized in Tab. 3 and Tab. 4. SMCC was applied according to the literature procedure [5].

The next day, the same procedure including reduction was repeated and the tumor sizes were measured. The same was repeated the third day. During the following days, the mice were observed and the tumor size was measured every two days. The volume of the tumor was calculated using the formula V = π/6 AB2 where A is the length of the tumor and B its height.

Tab. 3 Injections.

Group	1st injection	2nd injection
A	Reduction solution	Solution A
B	Reduction solution	Solution B
C	Reduction solution	Solution C
D	Reduction solution	Solution D
E	Solution E	Solution E

Tab. 4 Preparation of injection solutions.

Solution	Preparation
A	1.7 mg SMCC dissolved in 100 μL DMSO, 5 mL PBS added to reach 1 mM solution. Immediately afterwards 16.4 μL 1mM SMCC added to 900 μL IgG v PBS pH 7.3 (16.4 nmol IgG), left 1 hour at room T, filled up to 2 mL with PBS.
B	1 mL of solution A mixed with 0.5 mg of LPS.
C	1 mL of PBS mixed with 0.5 mg of LPS.
D	PBS

E	PBS
Reduction solution	6.3 mL 0.05 M PBS mixed with 90.3 mg TCEP (Sigma Aldrich C4706) .

5.5.3 Cell preparation for in vitro experiments

The cells were kept in RPMI medium. Before usage, the medium was poured out and the cells were washed three times using HBS. In order to detach the cells from the walls of the flask, trypsin (0,02% trypsin and 0,02% EDTA in PBS) was applied. The cells with trypsin were shaken and left in a thermostat for a few minutes. When most of the cells were detached, they were washed using 50 mL RPMI in centrifuge (10 min,150 g, 4 °C) and subsequently using the same amount of HBS and the same conditions. The supernatant was poured out and 1 mL of DTT solution (10 mM in PBS) was added on the pelet. It was placed on ice and kept to reduce for one hour. DTT was used for reduction instead of TCEP due to the toxicity issues.

When reduced, the cells were washed using 50 mL HBS. The pellet was carefully resuspended in a small amount of liquid still present. 20 µL of the suspension were mixed with 20 µL of trypan blue and the cells were counted in a Bürker chamber. Subsequently, they were diluted to reach the desired concentration and pipetted onto an assay plate.

5.5.4 In vitro bond quantification using BSA

SMCC of 1.7 mg was dissolved in 100 µL of DMSO. Afterwards, the solution was diluted in 5 mL PBS in order to obtain 1 mM solution.

The conjugate was prepared using 5 mL of 1 mM BSA solution and 5 mL of 1 mM SMCC solution. The control solution containing only BSA in PBS was prepared analogously. Both solutions were prepared by dilution with PBS. The conjugate mixture was incubated for one hour at 37 °C.

The cells were pipetted onto a plate in concentrations of 100000 cells per well (24 well plate) in 500 µL. Afterwards, 500 µL of conjugate or control solution were pipetted onto the cells and left to incubate for 1 hour at 37 °C.

Subsequently, the mixtures on the plate were centrifuged (3 min, 150 g, 4 °C) and the supernatant was used for the measurements. The concentration of BSA was measured using the Bradford method.The Bradford reagent was added and the samples and the calibration line absorbance at 595 nm was measured. The concentration of free, noncomplexed BSA was determined using the BSA calibration line.

5.5.5 In vitro bond quantification using hemoglobin

SMCC of 3.4 mg was weighed and dissolved in 200 μL of DMSO. Afterwards it was diluted with 10 mL HBS in order to obtain 1 mM solution.

The conjugate was prepared mixing 100 μL of 1 mM hemoglobin solution, 100 μL of 1 mM SMCC and 9.8 mL of PBS. It was left to react for 1 hour at room temperature. The control solution was prepared in an analogous way containing 100 μL of 1 mM hemoglobin and 9.9 mL of PBS.

The cells were set onto a plate in concentrations of 20000, 100000 and 200000 per well in 500 μL. Subsequently, 500 μL of conjugate or control solution were added into each well. After one hour, the plate with the mixtures was centrifuged (3 min, 150 g, 4 °C) and 400 μL of supernatant were pipetted out and its absorbance was measured. The rest was left to conjugate for two more hours. The calibration was measured using dilution series of the hemoglobin solution.

5.5.6 In vitro bond quantification using B - phycoerythrin

SMCC of 0.9 mg was weighed and dissolved in 60 μL DMSO. Subsequently, 20 μL of the solution were diluted with 180 μL DMSO. 5 μL of that solution were diluted with 1995 μL of PBS. Finally, 5 μL of this solution were diluted in 495 μL PBS. B-PE with concentration of 0.11 mg mL^{-1} was diluted 3 μL in 997 μL PBS. The formed solution was diluted 60 μL with 1590 μL PBS.

B-PE of 450 μL was mixed with 20 μL of SMCC solution and left to incubate for one hour in a dark environment (37 °C). The cells were pipetted onto a black plate in concentration of 100000 cells per hole and less in 150 μL. 150 μL of conjugate were added

on the cells. The control group of B-PE on cells of corresponding concentration was prepared as well. The mixtures were incubated for one hour at 37 °C.

Subsequently, the emission was measured using an excitation wavelength of 545 nm. B-PE was used for calibration and the pure conjugate was used for comparison when evaluating the bonding.

5.5.7 Statistical analysis

Statistical analysis was performed using two-tailed Student's t-test. Mice survival was evaluated using Kaplan-Meier test (MedCalc).

6 Results

6.1 In vivo experiment

The results of survival analysis can be seen on Fig. 5. There were no significant differences between any groups.

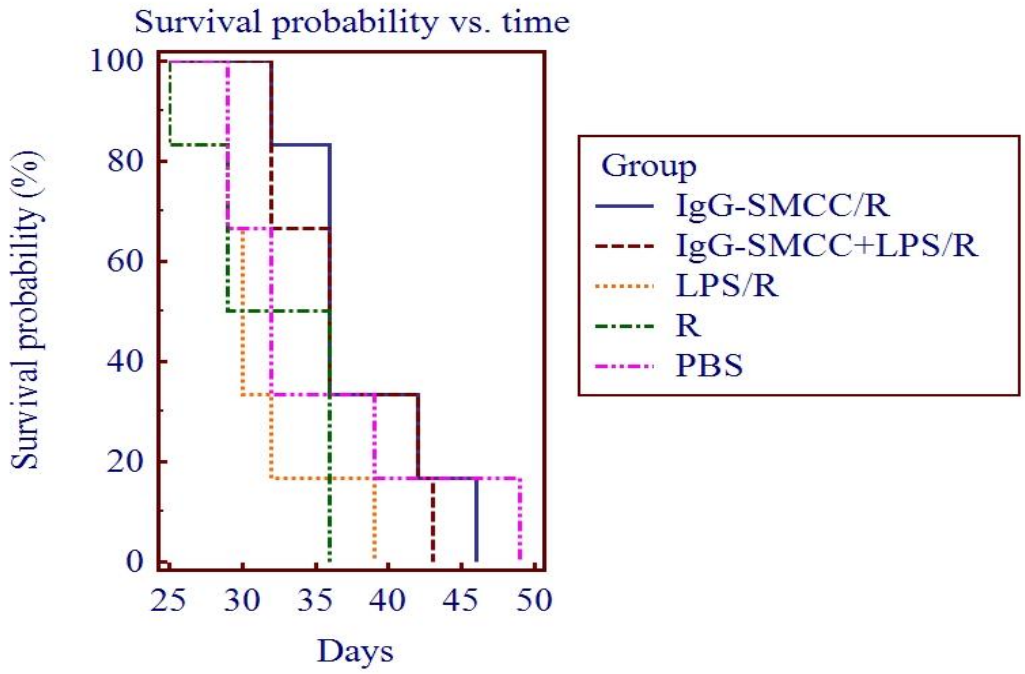

Fig. 5 Survival of mice artificially opsonized by IgG.

The statistically relevant reduction in tumor volume was observed in the case of treatment with the mixture of LPS and IgG-SMCC conjugate. There is sporadic statistically significant reduction of tumor volume observable also for the LPS group, but clearly the best effect can be seen for the combination of LPS with IgG-SMCC. This decrease of tumor growth was statistically significant not only in comparison with control, but also in comparison with IgG-SMCC. In case of 2 mice tumors temporarily (days 2 - 14 and 2 - 10) disappeared.

IgG-SMCC/R alone and reduction did not influence tumor growth. The plotted tumor volumes as a function of time for all groups can be seen in Fig. 6.

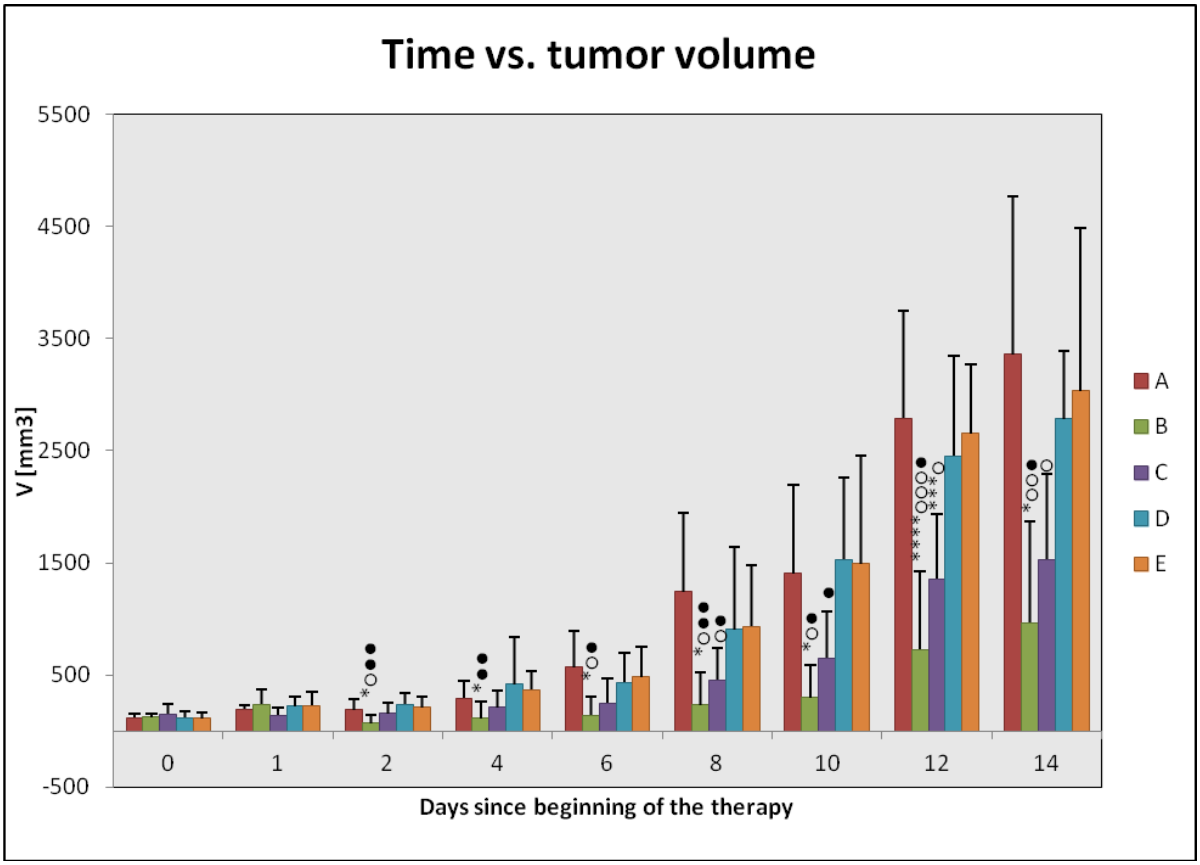

Fig. 6 A - IgG-SMCC/R, B - IgG-SMCC + LPS/R, C - LPS/R, D - reduction control group and E - PBS without reduction.

* P < 0.05 ** P < 0.01 *** P < 0.005 **** P < 0.001 (compared to PBS)

○ P < 0.05 ○○ P < 0.01 ○○○ P < 0.005 ○○○○ P < 0.001 (compared to IgG-SMCC)

● P < 0.05 ●● P < 0.01 ●●● P < 0.005 ●●●● P < 0.001 (compared to reduction)

6.2 In vitro bond quantification

Any of the in vitro methods did not prove significant bonding of BSA, hemoglobin and B-PE to cell via SMCC linkage. Even though the method was performed using varying conditions, trying to optimize the procedure to obtain relevant results, it seems that there were still many steps and too much of manipulation with the cells. which could cause destruction of cells or contribution of cellular substances. The same trends of fluorescence as a function of concentration of both conjugated and unconjugated B-PE indicates that no bond formation occurs. The resulting tables and calibration lines can be seen on Fig. 7 - Fig. 10 and Tab. 5 - Tab. 6.

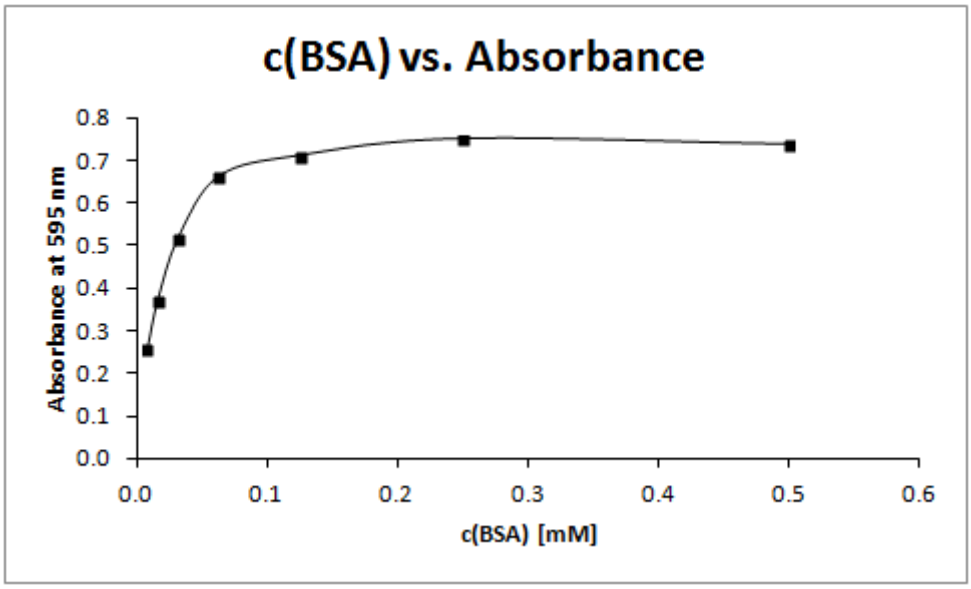

Fig. 7 Calibration curve for BSA, absorbance measured at 595 nm. The curve behaves nonlinearly at concentrations prescribed for the experiment.

Tab. 5 Absorbances at 595 nm for individual groups in BSA quantification. The expected decrease in absorbance due to bonding of BSA-SMCC to cells was not observed.

	Average absorbance per:			
	No cells	10 000 cells	50 000 cells	100 000 cells
BSA	0.738	0.841	0.778	0.817
BSA-SMCC	0.736	0.807	0.772	0.807

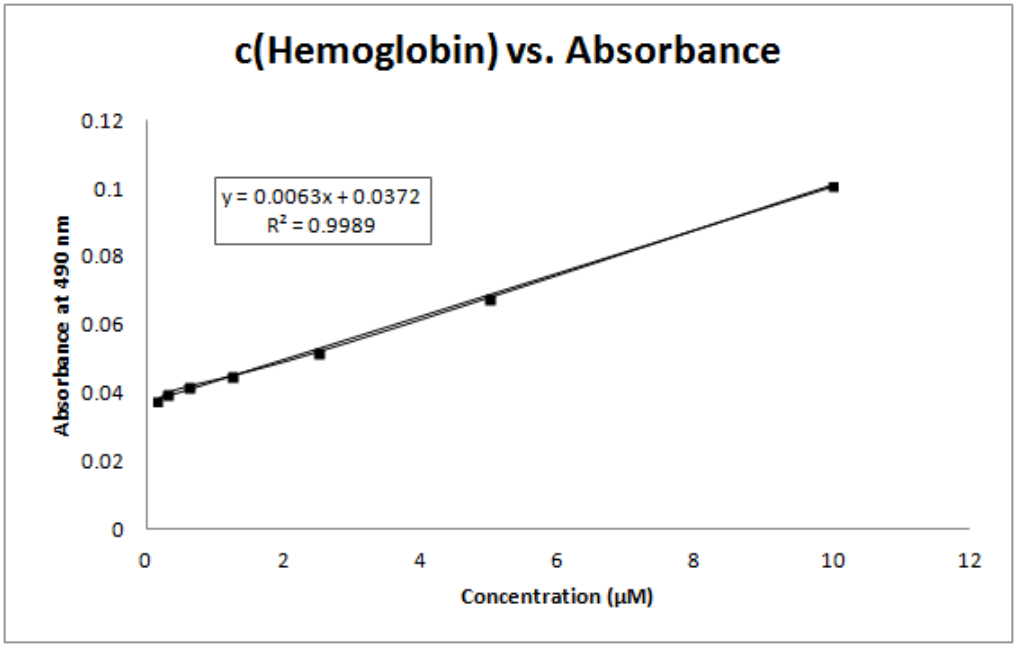

Fig. 8 Calibration curve for hemoglobin, absorbance measured at 490 nm.

Tab. 6 Absorbances at 490 nm for individual groups in hemoglobin quantification. The expected decrease in absorbance due to bonding of Hemoglobin-SMCC to cells was not observed.

	Average absorbance per:		
	No cells	10 000 cells	50 000 cells
Hemoglobin	0.068	0.089667	0.098333
Hemoglobin-SMCC	0.068	0.090667	0.097200

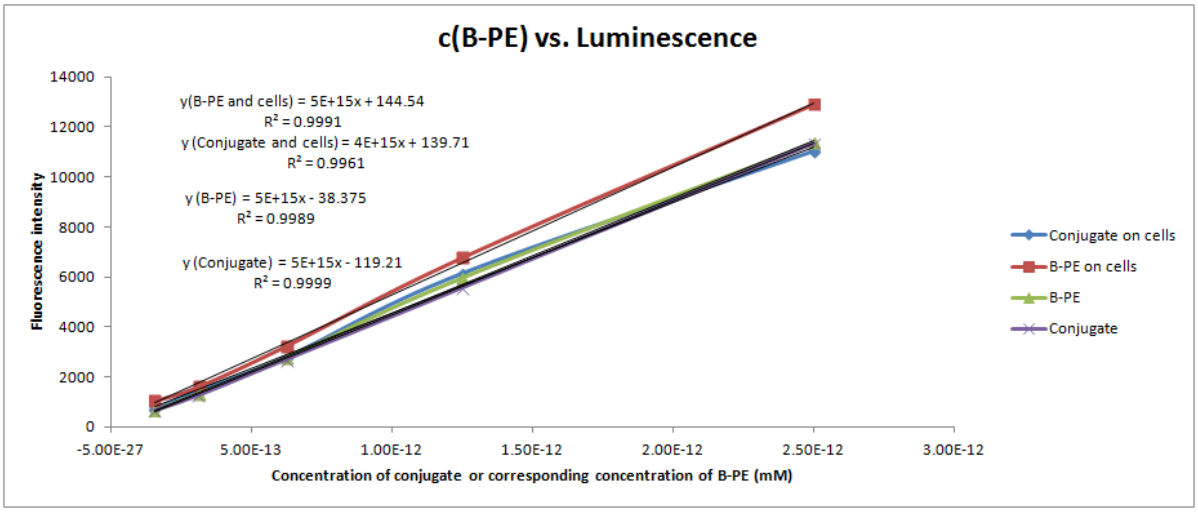

Fig. 9 B-PE determination of conjugate linkage at very low concentrations of B-PE.

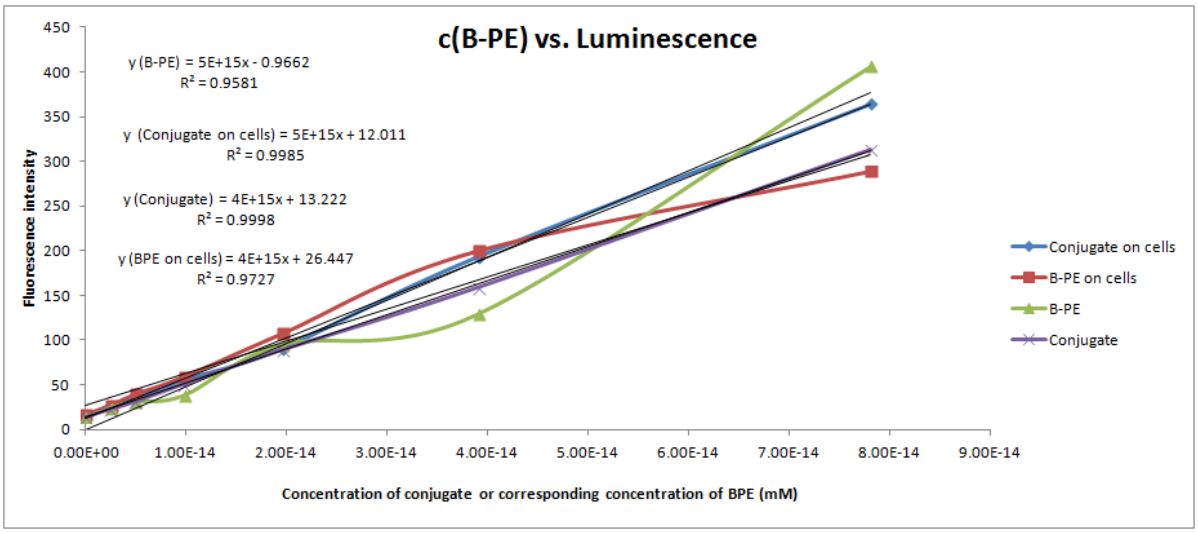

Fig. 10 B-PE determination of conjugate linkage.

7 Discussion

The results obtained for the in vivo experiment showed that IgG-SMCC in combination with LPS caused significant reduction of the tumor volume . Moreover, in the case of two mice treated with this combination of substances, the tumor disappeared temporarily. It has been shown, similarly as in other studies [36], [37] that the combination of effective substance with LPS is the most efficient one. Nevertheless, it has to be taken into account that this conjugate was supposed to cooperate with the immune system in a different way than the other substances and the relevance of such comparisons is therefore limited.

The efficiency of LPS alone may be expected from numerous studies beginning with Coley's bacterial vaccine in which LPS worked as the active component and which is still part of the current research [38], [39]. The unknown was in this case its combination with the effective substance which showed to be successful.

The exact working mechanism of IgG-SMCC is not known yet. It can be deduced, that artificially attached IgG enable start of all mechanisms well known in case of binding of antibodies to antigens expressed on cell surface. These mechanisms are as follows:

1/ direct opsonisation for phagocytes (macrophages, neutrophiles, dendritic cells)

2/ complement activation (classical route), leading to opsonisation on C3b level [21] and to terminal complex formation

3/ ADCC leading to apoptosis

4/ passive attachment is not connected with any direct (specific) effect of antibodies [21]

The mechanism of synergy of IgG-. SMCC with LPS is unknown as well. Combination of massive infiltration of leucocytes caused by LPS with their directing to tumor cells bearing Fc part of immunoglobulin seems to be probable (Ženka, personal communication) For the purpose of revealing it, further studies would be necessary.

When compared to the other studies dealing with quantification of similar types of conjugates [40], the ambiguous results for quantification were surprising. Wachtlová solved the same question using the same methods, but she studied binding of ligands based on anchoring them using hydrophobic chains. In case of BSA and hemoglobin, Wachtlová did

not obtain acceptable results (big fluctuation of values), nevertheless using B-PE she obtained good results. In our case, we applied more complicated two phase binding procedure. High stress applied to cells during the procedure could result in cells destruction and the participation of its compartments in the spectrophotometric and fluorometric answer. As the conjugation procedure was prepared according to already working systems [5], [34] and there is not a reason for suspecting the linkage to fail, it should be possible to prove and quantify it in an improved or alternative way. Further optimization of such quantification method would be therefore necessary focusing on the minimization of steps and stress applied on the cells during procedure.

8 Conclusion

The melanoma progression was studied and artificial opsonization using IgG-SMCC conjugate was applied in order to suppress tumor growth. In vivo opsonization showed that IgG-SMCC in combination with LPS has an ability to reduce significantly tumor growth . In vitro quantification of the bond failed probably due to the amount of steps in the procedure and the mechanical stress applied on the cells.

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11 List of used abbreviations

ADCC	antibody dependent cellular cytotoxicity
APC	antigen presenting cell
B-PE	B-phycoerythrin
BSA	bovine serum albumin
CRP	C-reactive protein
CTLA4	cytotoxic T lymphocyte atigen-4
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
HBS	Hank's buffered saline
HLA	human leukocyte antigen
HPV	human papillomavirus
IFN-α	interferon α
IgG	immunoglobulin G
IL-2	interleukin 2
LPS	lipopolysaccharide
MAC	membrane attack complex
MHC	major histocompatibility complex
NK cells	natural killer cells
PAMPs	pathogen associated molecular patterns

PBS	phosphate buffered saline
PPR	pathogen pattern receptors
ROS	reactive oxygen species
SMCC	succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate
TAA	tumor associated antigen
TCEP	tris (2-carboxyethyl) phosphine hydrochloride
TGF-β	transforming growth factor β