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Faculty of Science

The study of cancer immunotherapy based on installation of phagocytosis stimulating ligands on the tumor cells surface and investigation of underlying mechanisms

Ph.D. Thesis

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ANNOTATION

Immunotherapy became a very promising approach for cancer therapy. Tumor cells are eliminated using the body's own immune system with minimal negative effect on healthy tissue. This thesis is focused on immunotherapy based on activation of innate immunity, specifically on intratumoral application of ligands stimulating phagocytosis and Toll-like receptor ligands. This therapeutic approach was tested in several types of tumor mouse models, such as melanoma B16-F10, pancreatic adenocarcinoma, and pheochromocytoma. The composition of the therapeutic mixture as well as the application schedule were optimized in our studies. Subsequently the underlying mechanisms involved in tumor elimination during this therapy were investigated.

DECLARATION [IN CZECH]

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Veronika Caisova

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LIST OF PAPERS AND AUTHOR'S CONTRIBUTION

The thesis is based on the following papers:

I.

Janotova, T., Jalovecka, M., Auerova, M., Svecova, I., Bruzlova, P., Maierova, V., Kumzakova, Z., Cunatova, S., Vlckova, Z., **Caisova, V.**, Rozsypalova, P., Lukacova, K., Vacova, N., Wachtlova, M., Salat, J., Lieskovska, J., Kopecky, J., Zenka, J., 2014. The use of anchored agonists of phagocytic receptors for cancer immunotherapy: B16-F10 murine melanoma model. PLoS One 9, e85222.

IF (2014): 3.234

Veronika Caisova participated in performing experiments and acquisition of data.

II.

Waldmannova, E., **Caisova, V.**, Faberova, J., Svackova, P., Kovarova, M., Svackova, D., Kumzakova, Z., Jackova, A., Vacova, N., Nedbalova, P., Horka, M., Kopecky, J., Zenka, J., 2016. The use of Zymosan A and bacteria anchored to tumor cells for effective cancer immunotherapy: B16-F10 murine melanoma model. Int Immunopharmacol 39, 295-306.

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Veronika Caisova participated in performing experiments, acquisition of data and data analysis.

III.

Caisova, V., Vieru, A., Kumzakova, Z., Glaserova, S., Husnikova, H., Vacova, N., Krejcova, G., Padoukova, L., Jochmanova, I., Wolf, K.I., Chmelar, J., Kopecky, J., Zenka, J., 2016. Innate immunity based cancer immunotherapy: B16-F10 murine melanoma model. BMC Cancer 16, 940.

IF (2016): 3.288

Veronika Caisova participated in performing experiments, in the design of the experiments, acquisition of data, data analysis and writing manuscript.

IV.

Manuscript:

An effective cancer immunotherapy of melanoma and pancreatic adenocarcinoma based on a combination of TLR agonists and the stimulation of phagocytosis

Veronika Caisová[1], Ondřej Uher[1], Pavla Nedbalová[1], Ivana Jochmanová[2], Karolína Kvardová[1], Kamila Masáková[1], Gabriela Krejčová[1], Lucie Paďouková[1], Jindřich Chmelař[1], Jan Kopecký[1,3], Jan Ženka[1*]

Veronika Caisova participated in performing experiments, in the design of the experiments, acquisition of data, data analysis and writing manuscript.

V.

Manuscript:

Immunotherapy based on intratumoral application of mannan and TLR ligands in pheochromocytoma mouse model

Veronika Caisova[1,2], Garima Gupta[1], Ivana Jochmanova[1,3], Abhishek Jha[1], Liping Li[1], Thanh Huyan[1], Ying Pang[1], Jan Zenka[2] and Karel Pacak[1]

Veronika Caisova participated in performing experiments, in the design of the experiments, acquisition of data, data analysis and writing manuscript.

CONTENT

1. AIMS AND OBJECTIVES

Cancer is a group of diseases characterized by uncontrolled growth of abnormal cells. Nowadays, cancer is becoming the most frequent diagnosis and unfortunately the sufficient therapy is still absent. Therefore, the investigation of new and more effective therapies is crucial in cancer research.

Immunotherapy is considered as a promising and effective cancer treatment and the immunotherapeutic research is extensively expanding. Our studies are focused on one specific type of immunotherapy, namely intratumoral application of phagocytosis stimulating ligands and Toll-like receptor (TLR) ligands. Phagocytosis stimulating ligands with membrane active anchor are attached to tumor cells surface, and marked them for immune infiltrating leukocytes. Phagocytosis stimulating ligands are combined with TLR agonist to improve the effectivity of the treatment. Such a therapeutic mixture results in tumor shrinkage and elimination.

This PhD thesis is a part of a project of the South Bohemia University, Department of Clinical Biology in collaboration with National Institutes of Health, Bethesda, USA.

Aims of this thesis:

1. **Modification and optimization of the therapeutic mixture**
    - Find combination of effective and nontoxic TLR agonists
    - Proper anchor for binding of phagocytosis stimulating ligands
    - Optimization of application schedule
2. **Verification of therapeutic efficiency using several tumor models**
    - Tested tumor models: Melanoma B16-F10
      Pancreatic adenocarcinoma
      Pheochromocytoma/paraganglioma
3. **Investigation of the underlying mechanisms**
    - Adaptive immunity participation
    - Cytotoxic effect of immune cells *in vitro*

2. INTRODUCTION

2.1.CANCER

Cancer is a group of related diseases sharing six main hallmarks (Figure 1): (i) self-sufficiency in growth signals, (ii) insensitivity to growth-inhibitory signals, (iii) evasion of programmed cell death, (iv) limitless replication potential, (v) sustained angiogenesis, and (vi) tissue invasion and metastasis (Hanahan and Weinberg, 2000).

There are many different types of tumors, which can be differentiated based on localization, origin of the tumor cells, or their ability to metastasis. The most common classification is based on invasivity and the ability to metastasis: (i) benign tumors are regarded as less dangerous because of their incapability to invade and spread into different parts of the body. (ii) Malignant tumors, are more dangerous because they can enter the bloodstream or lymphatic system and create secondary tumors in different body locations, called metastases (Chambers et al., 2002).

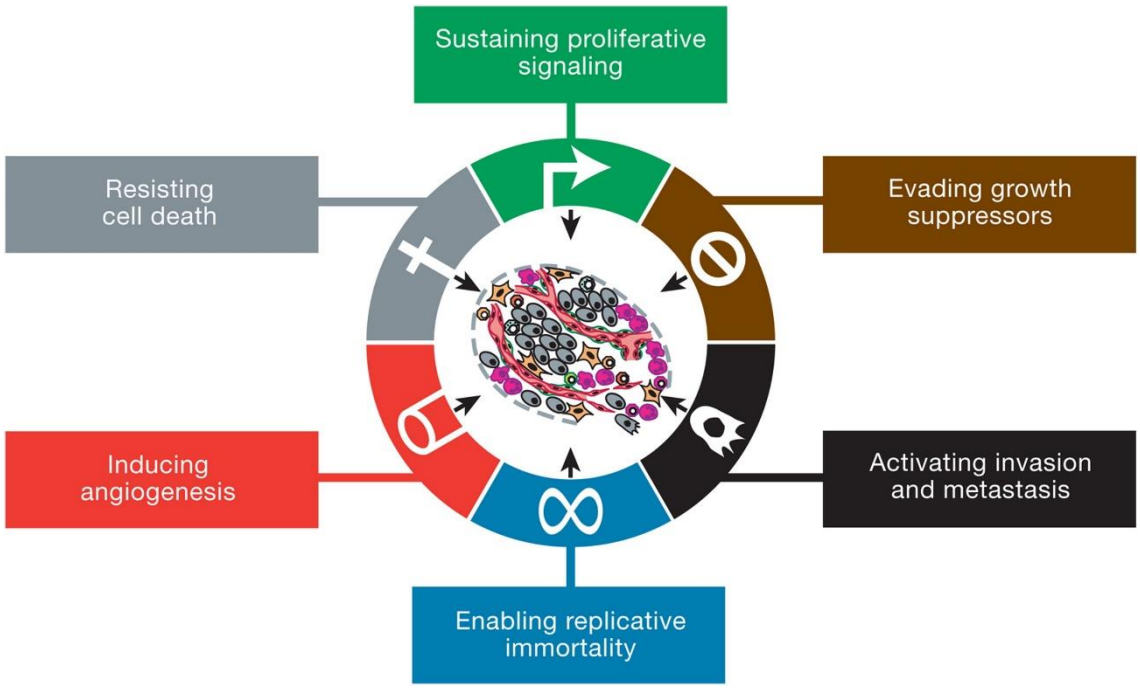

Figure 1 Hallmarks of cancer. Illustration of the original six cancer hallmarks: (i) self-sufficiency in growth signals, (ii) insensitivity to growth-inhibitory signals, (iii) evasion of programmed cell death, (iv) limitless replication potential, (v) sustained angiogenesis and (vi) tissue invasion and metastasis (Hanahan and Weinberg, 2000).

2.1.1. Causes of cancer

Cancer cell transformation is caused by the accumulation of mutations in cells. These mutations can be (i) inherited (people are born with genetic mutation) or (ii) they can occur after birth and are affected by many external factors (Hemminki et al., 2000). These external factors can be divided in three main groups: (i) chemical carcinogens (e.g., nitrosamines and aromatic hydrocarbons), (ii) physical carcinogens (e.g., ultraviolet rays and ionizing radiation) and (iii) biological carcinogens (e.g., Epstein Barr virus and Human Papillomavirus); (Carrillo-Infante et al., 2007; Hemminki et al., 2000; Meinert et al., 1999).

Different types of cancer have different mutation signatures. Certain signatures are related with age of a patient, known defect in DNA, or exposure to mutagens. However, the origin of many mutation signatures is unknown (Alexandrov et al., 2013).

2.1.2. Cancer treatment

There are many different types of cancer treatments. The specific therapy depends on the type of cancer, its progression level, and many other factors. However, there are three standard treatment options. (1) Surgery: if the size and location of the tumor is optimal, then surgery is the first treatment option. During the surgery, the tumor and the surrounding tissue are removed from the patient's body. (2) Chemotherapy: during chemotherapy, drugs inhibiting cell division and growth are used. This therapy can be very effective in some types of tumors, but the cytotoxic effect on healthy cells is still a major concern (DeVita and Chu, 2008). (3) Radiotherapy: during radiotherapy, high doses of radiation are used to destroy the tumor cells. But, there is also significant damage to healthy tissue (Sebag-Montefiore et al., 2009).

In addition, several novel approaches in cancer treatment are emerging, for example a hormone therapy, a targeted therapy, angiogenesis inhibitors, and immunotherapy (Early Breast Cancer Trialists' Collaborative, 2005; Lord and Ashworth, 2008; Sleijfer et al., 2009; Wiezorek et al., 2010). Immunotherapy is one of the most extensively studied cancer therapies, where the immune cells of the patient are used for tumor cells detection and elimination (Rosenberg et al., 2004).

For testing of new therapies, tumor models are essential. In our studies, melanoma, adenocarcinoma and pheochromocytoma tumor models were used.

2.1.3. Tumor models used in our studies

Melanoma

Melanoma is a very aggressive skin tumor rising from melanocytes containing pigment melanin. Incidence rate of this malignancy rapidly increased in the last two decades (Ilic and Ilic, 2016). Based on clinical-histopathological features, melanoma is divided into four main groups: (i) acral lentiginous, (ii) superficial spreading, (iii) nodular, and (iiii) lentigo maligna (Allen and Spitz, 1953; Bandarchi et al., 2010).

The type of melanoma treatment depends on the stage of progression and localization of the tumor. Although the research of therapeutic options for melanoma is in progress, the surgery, chemotherapy, and radiotherapy treatment remain the standards in this type of tumor (Allen and Spitz, 1953; Bandarchi et al., 2010).

In our studies, the B16-F10 mouse melanoma model was used. The B16-F10 model is metastatic melanoma model crucial for melanoma research (Paper 1, Paper 2, Paper 3, Paper 4)

Pancreatic adenocarcinoma

Pancreatic adenocarcinoma is malignancy with an extremely high case-fatality rate. Most of the patients are diagnosed late and thus the stage of the cancer is too progressed for treatment. According to the literature 80-85% of patients have already an incurable tumor at the time of the first diagnosis (Li et al., 2004). Nevertheless, if possible the surgery is still the first-choice therapy. This can be followed by chemotherapy or/and radiotherapy (Kuhlmann et al., 2004; Seufferlein et al., 2012). However, depending on the stage of the tumor, sometimes palliative treatment is the only option.

In our studies the Panc02 mouse pancreatic adenocarcinoma model was used (Paper 4).

Pheochromocytoma/paraganglioma:

Pheochromocytoma/paraganglioma (PHEO/PGL) are rare neuroendocrine tumors with catecholamine secretion. They are originally derived from chromaffin cells of the adrenal medulla and sympathetic ganglia outside the adrenals, respectively (Eisenhofer et al., 2004). This diagnosis is usually connected with certain syndromes: multiple endocrine neoplasia type 2 (MEN2), neurofibromatosis type I (NF1), von Hippel Lindau disease (VHL) and familial paraganglioma/pheochromocytoma syndrome (Shuch et al., 2014). Patients with PHEO/PGL are diagnosed based on symptoms resulting from high production of catecholamines (Scholz et al., 2007).

Approximately 90% of all PHEO tumors and 35% of PGL tumors are benign and relatively well curable. Similar to melanoma, surgery is also the first therapeutic option, and it can be combined with chemotherapy or radiotherapy. The rest of PHEO/PGL tumors are classified as metastatic tumors with lack of treatment options (Scholz et al., 2007).

In our studies, MTT mouse pheochromocytoma cells were used to create pheochromocytoma mouse model (Paper 5).

2.2. IMMUNE SYSTEM AND CANCER

Immune system is a body's defense system that consists of cells, tissue and organs, which all together protect body against pathogens. There are two main parts of immune system: innate immunity and adaptive immunity. The main function of immune cells is recognition of pathogens invading human body and their elimination from organism (Akira et al., 2006; Medzhitov and Janeway, 1998). Interestingly, the immune system has also the ability to recognize and reject tumor cells (Finn, 2012). Even though the immune system has the ability to detect and eliminate tumor cells, the human body is not completely resistant to cancer. The tumor cells regulation is controlled by process called immunoediting.

2.2.1. Cancer immunoediting

Immunoediting is a very dynamic process consisting of three phases (Figure 2): elimination, equilibrium, and escape (Dunn et al., 2002). The first phase (= elimination), innate and adaptive immune cells are able to recognize and eliminate tumor cells. This phase is initiated by inflammation in the location of arising tumor, followed by recruiting immune cells, and synthesis of cytokines and chemokines (Dunn et al., 2002; Dunn et al., 2004b).

The second phase (= equilibrium), the surviving cells from the elimination phase create clones with cumulative numbers of mutations. These mutations can lead to higher resistance to immune system and allow tumor cells to escape from immunosurveilance (Dunn et al., 2004b).

The third phase (= escape), the immune cells will lose control under the tumor cells. Subsequently, tumor cells can grow and create primary tumor and metastases (Dunn et al., 2006; Dunn et al., 2004b; Kim et al., 2007).

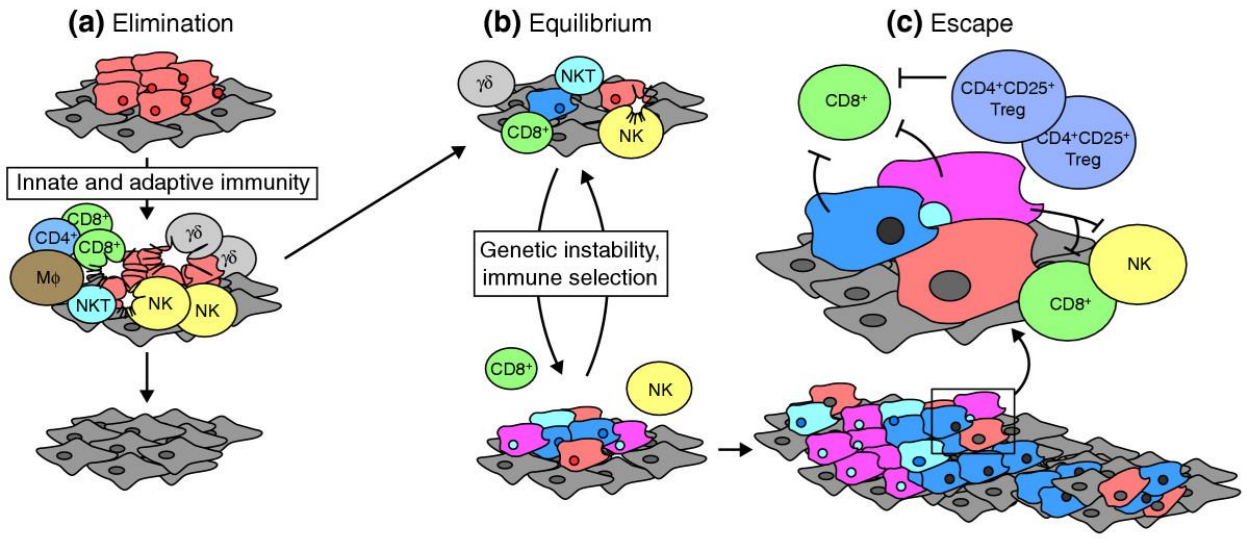

Figure 2 Cancer immunoediting. Three phases of the cancer immunoediting process; elimination, equilibrium, and escape phase. During the elimination phase, the tumor cells are controlled by immune system. During the phase equilibrium, tumor cells are genetically instable and become more resistant to immune cells. This resistance results in tumor cells escape and tumor growth (Dunn et al., 2004a). There are several mechanisms how tumor cell escape from immune control. For example, downregulation of MHC class I antigen expression, immunosuppressive tumor microenvironment, or upregulation of non-classical MHC I antigens.

2.2.2. Innate immunity and cancer

Innate immunity is the first line of immune defense. The most important function of the innate immunity is the inflammation as a response to pathogens invading the body. During the inflammatory reaction, receptors on innate immune cells, called Pattern recognition receptors (PRRs), are able to recognize pathogen structures, called Pathogen associated molecular patterns (PAMPs). This recognition will cause mobilization of immune cells and pathogen elimination (Akira et al., 2006; Medzhitov and Janeway, 1998).

Another important function of innate immunity is the complement activation. Complement is a cascade of plasma proteins and its activation can lead to inflammatory cell attraction, pathogen opsonization, or perforation of pathogen plasmatic membrane (Fujita et al., 2004; Gotze and Muller-Eberhard, 1976; Petersen et al., 2000).

Anatomical barriers, such as skin, gastrointestinal tract or respiratory tract are also important parts of innate immunity. These anatomical barriers are acting as a first mechanical blockage for invading pathogens (Hornef et al., 2002).

Innate immune cells also directly interact with tumor cells and eliminate them from organism. Interestingly, natural killer (NK) cells are primarily responsible for killing cancer cells lacking expression of major histocompatibility complex (MHC) molecules. Activation of stimulatory receptors on the NK cells surface leads to expression of interferon gamma (INFγ), perforins, and inflammatory cytokines. These molecules induce apoptosis of tumor cells (Waldhauer and Steinle, 2008; Zamai et al., 2007). Macrophages are other innate immune cells interacting with tumor cells. Some tumors express "eat me" molecules (e.g. phosphatidylserine and low-density lipoproteins) on their surface. Expression of these molecules leads to activation of macrophage phagocytosis (Mantovani and Sica, 2010). Also, dendritic cells (DC) are crucial innate immune cells interacting with tumor cells. DC interaction with tumor cells via integrins and other receptors causes phagocytosis of apoptotic cancer cells. Moreover, DC as a professional antigen presenting cells represent fundamental connection between innate and adaptive immunity. (Schreiber et al., 2011).

2.2.3. Adaptive immunity and cancer

Adaptive immunity is the second line of immune defense. The power of adaptive immunity lies in immunological memory and high specificity to certain pathogens. T-lymphocytes and B-lymphocytes are the key adaptive immune cells.

T-lymphocytes can be divided in two main groups; Th-lymphocytes and Tc-lymphocytes. Activated Th-lymphocytes, also called CD4+ lymphocytes, produce several types of cytokines. These cytokines are important activators of other immune cells. Tc-lymphocytes, also called CD8+ lymphocytes, have a direct cytotoxic effect on viral infected cells or other abnormal cells (Bonilla and Oettgen, 2010; Chaplin, 2010).

B-lymphocytes are the second crucial adaptive immune cells. B-lymphocytes, professional antigen presenting cells, have the ability to produce antibodies. Antibodies have several functions in the body, such as neutralization of bacterial cells, agglutination of foreign cells, precipitation of serum antigens, or complement activation. Moreover, production of specific type of B-lymphocytes, memory

B-lymphocytes, is crucial for stronger and faster immune reaction during the secondary pathogen invasion (Bonilla and Oettgen, 2010; Chaplin, 2010).

Adaptive immune cells also interact with tumor cells via tumor antigens. Tumor antigens can be divided into two main groups: (1) tumor specific antigens, which are exclusively expressed on the tumor cell surface. (2) Tumor associated antigens, which are expressed predominantly on tumor cells, but their expression is detected on some normal cells as well. Recognition of tumor antigens by adaptive immune cells, following by antigen presentation, can result in tumor cell elimination (Knutson and Disis, 2005; Vesely et al., 2011).

2.2.4. Tumor immune escape mechanisms

Tumor cells can escape from immune recognition via developing of tumor immune escape mechanisms. Tumor immune escape mechanisms play an important role in tumor growth (Becker et al., 1993) and include: (i) modulation of tumor antigens, (ii) masking of tumor antigens, (iii) induction of tolerance, (iv) production of blocking antibodies, and (v) production or expression of immunosuppressants (Becker et al., 1993; Pawelec et al., 2000).

(i) Modulation of tumor antigens: tumor cells can translocate their antigens from surface to cytoplasm and avoid the tumor cell recognition (Khanna, 1998). Some of the tumors can also stop expression of their antigens or make these antigens immunologically invisible (Khanna, 1998).

(ii) Masking of tumor antigens: antigens can be masked by production of mucoproteins and sialomucin (Becker et al., 1993).

(iii) Induction of tolerance: expression of PD-L1 molecules on the surface of tumor cells can induce immune tolerance. For example, PD-L1 protein can bind to PD1receptors on the surface of T-lymphocytes and inhibit their activation (Blank et al., 2005).

(iv) Production of blocking antibodies: blocking antibodies can block complement activation and C3a/C3b production. Some blocking antibodies can even cover tumor cells and protect tumor antigens against immune recognition (Sjogren et al., 1972).

(v) Production or expression of immunosuppressants: tumor cells are able to produce or express immunosuppressants, such as IL10, TGF beta, or VEGF. These

immunosuppressants support escape of tumor cells from immune surveillance and create typical tumor environment (Gabrilovich et al., 1996; Itakura et al., 2011; Yang et al., 2010).

2.3. IMMUNOTHERAPY OF CANCER

Progress in treatment and early detection has led to a significant improvement of cancer management. Nevertheless, most of the currently available treatments are still not effective enough and the toxic effect on healthy cells is still a major concern. Therefore, new therapies are needed.

One of the newly emerging therapy is immunotherapy. Immunotherapy is using a patient's immune system to fight cancer. In the following part, immunotherapies based on cellular immunity, antibody-based immunity, and TLR agonists based immunotherapy are discussed.

2.3.1. Cellular based immunotherapies

Cellular based immunotherapies can be categorized as active or passive. Active cellular based immunotherapies include cell-based vaccines. Passive cellular based immunotherapies include adoptive transfer of NK cells or T-lymphocytes (Borghaei et al., 2009).

2.3.1.1. Cell based vaccines

Cell based vaccines require for their function expression of tumor specific antigens or tumor associated antigens on the surface of tumor cells (Borghaei et al., 2009).

Peptide/Protein Subunit Vaccines **-** peptide/protein vaccines are prepared by the use of one or more amino acid sequences mimicking tumor antigens. Their function is usually boosted by vaccine adjuvants stimulating monocytes and macrophages. After application, these peptides/proteins are presented by antigen presenting cells to CD8+ T-lymphocytes. Subsequently, CD8+ T-lymphocytes lyse tumor cells. Advantages of these peptide/protein based vaccines lies in their low cost and easy manipulation as well as effective function in some types of tumors (Moyle and Toth, 2013). On the other site, the limitations are for example nonspecific binding resulting in reduction effectivity, rapid degradation, and low affinity of protein/peptides to MHC (Azmi et al., 2014).

DNA vaccines – vaccines made up of a bacterial plasmid, which has been genetically modified to express specific tumor antigens after cell invasion. Immune cell recognition of these artificially expressed tumor antigens results in immune activation and tumor cells elimination (Fioretti et al., 2010; Stevenson et al., 2004; Tiptiri-Kourpeti et al., 2016). The advantages of DNA vaccines include flexible design of DNA vectors, innate immune cells activation ability, and CD4+ /CD8+ cells activation. Simultaneously, rapid mass production of DNA vaccines is not a limitation. However, the effect of DNA vaccines is still very limited by low immunogenicity of tumors (Yang et al., 2014).

Whole Cell vaccines – vaccines based on the direct immunization of patients by tumor cells. Tumor cells contain all different types of tumor antigens, which can enhance the immune cell activation (Keenan and Jaffee, 2012). Tumor cells for whole cell vaccination can be autologous or allogenic. Autologous whole cell vaccines are isolated when patient's tumor is removed during the surgery and prepared for immunization. These tumor cells vaccines are effective because of the conformity of the vaccine cell antigens with remaining tumors in the patient's body. However, preparation of this autologous vaccine is a complex process and the surgery for tumor removal is not always possible (Berd et al., 1990). Allogenic whole cell vaccines are prepared from particular tumor cell lines. Tumor cell lines are widely available in unlimited amount. However, variances between vaccine's tumor cell antigens and patient's tumors cell antigens can result in decreased effectivity compared to autologous tumor cell vaccines (de Gruijl et al., 2008; Eaton et al., 2002).

Dendritic cell vaccines - recently emerging cell based vaccine. DC are professional antigen presenting cells representing important communication bridge between innate and adaptive immunity. DC cells are derived from a patient's monocytes and loaded by tumor antigens. These antigens in combination with other stimulating molecules (e.g. LPS and Poly(I:C)) cause DC maturation and activation. Subsequently, backward application of activated DC into the patient's body results in presenting of tumor antigens to T-lymphocytes and inducing anti-tumor response (Palucka et al., 2005; Palucka and Banchereau, 2012; Yu et al., 2004).

2.3.1.2. Adoptive transfer

NK cell adoptive transfer - NK cells, innate immune cells, are able to recognize virus infected cells and cells with unusual expression of MHC molecules. Moreover, their production of IFNγ can polarize T-lymphocytes to Th1 phenotype and initiate DC maturation. The source of NK cells may be either autologous (cells isolated directly from patient) or allogenic (cells donated from healthy relatives). The

isolation of NK cells from patient's body is followed by NK cells cytokine activation (using cytokines: IL-2, IL-12, IL-15, IL-18); (Iliopoulou et al., 2010; Miller et al., 2005). Interestingly, recently identified memory-like NK cells are also considered as a potential source for adoptive transfer (Fehniger and Cooper, 2016).

T-lymphocyte adoptive transfer – T-lymphocytes, adaptive immune cells, have either a direct cytotoxic effect on targeted cells or activate other immune cells by cytokine production. T-lymphocytes are used as an adoptive transfer when isolated tumor infiltrating leukocytes (TIL) are expanded *ex vivo* and backward administered into the patient's body (Besser et al., 2010). If there is no possibility to isolate TIL from tumor tissue, the T-lymphocytes for adoptive transfer are obtained from peripheral blood (Takayama et al., 2000). Innovative way of using T-lymphocytes for cancer treatment is their biological engineering manipulation. T-lymphocyte manipulations result in expression of high affinity antigen-receptors on the surface of T-lymphocytes, effective recognition of specific tumor antigens, and elimination of tumor cells from the patient's body. Viral vectors, such as retrovirus, lentivirus, or transposomes are used for T-lymphocytes transfection. This transfection causes expression of chimeric antigen receptors on T-lymphocyte surface (Grupp et al., 2013). This therapy is called CAR T-cell therapy and is effective in acute lymphoblastic leukemia and advanced lymphoma (Brentjens et al., 2013; Kalos et al., 2011). However, there are still safety concerns about interaction of CAR T-cells with normal cells expressing the same antigens as tumor cells with subsequent autoimmune reactions (Scholler et al., 2012).

2.3.2. Antibody-based immunotherapy

Antibody-based immunotherapy is an extensively studied therapeutic approach including monoclonal antibodies (MoAbs). MoAbs are molecules targeting specific proteins (antigens) expressed on the surface of tumor cells. There are several different types of MoAbs used in cancer treatment such as (i) Naked monoclonal antibodies are the most common MoAbs. These MoAbs are not conjugated with any other kind of drugs and the effect is mostly based on antibody-dependent cellular cytotoxicity (ADCC). The Fc receptor of effector cell interacts with Fc part of antibody attached to tumor cell. This interaction activates immune effector cells such as NK cells. Subsequently, NK cells eliminate tumor cells (Weiner, 2007). Another described mechanism how MoAbs participate in tumor cells elimination is complement-dependent cytotoxicity. During this process, the interaction of antibody and targeted cell activate complement cascade resulted in complement mediated lysis of tumor cells (Rogers et al., 2014;

Zhou et al., 2008). Checkpoint inhibitors are another example of the use of naked monoclonal antibodies. Immune checkpoint proteins are receptors expressed on the surface of T-lymphocytes. Their interaction with specific ligands on the surface of antigen presenting cells leads to protection of antigen presenting cells against T-lymphocytes mediated death. However, some tumors also express checkpoints proteins. In this case, the interaction of receptors and their specific ligands can result in tumor immune escape (Alexander, 2016). Checkpoint inhibitors prevent this receptor interaction and inhibit the protecting signal. PD-1/PD-L1, and CTLA-4 inhibitors are the most frequently used check point inhibitors in cancer treatment (Hodi et al., 2010; Iwai et al., 2017).

(ii) Conjugated monoclonal antibodies are other big group of antibodies used in cancer therapy. These antibodies are conjugated with chemotherapeutic or radiotherapeutic particles. The specificity of conjugated monoclonal antibodies protects healthy cells against serious side effect of chemotherapeutic and radiotherapeutic drugs (Bouchard et al., 2014; Lambert, 2005). Simultaneously, these conjugated monoclonal antibodies are excellent example of beneficial combination of chemotherapy and radiotherapy with immunotherapy (Witzig et al., 2002).

(iii) Bispecific monoclonal antibody is another type of monoclonal antibody. Two different antibodies are designed together and can attach two different antigens. This enhances the therapeutic effect (Chames and Baty, 2009; Marvin and Zhu, 2006; Shen and Zhu, 2008).

2.3.3. Toll-like receptor agonists based immunotherapy

TLRs play a crucial role in innate immunity during the pathogen recognition and immune system activation. They belong to the big family of pattern recognition receptors (PRR). TLRs are able to recognize specific molecules of pathogens called PAMPs (Medzhitov and Janeway, 1998; Takeda and Akira, 2005). TLRs can play either negative or positive role in cancer development (Basith et al., 2012).

TLRs are very conserved transmembrane proteins with an extracellular domain and a cytoplasmic tail. The extracellular domain consists of leucine rich repeats (LRRs). The cytoplasmic domain is called TIR domain. Ten human TLRs (TLR 1 - TLR 10) are known (Figure 3); (Medzhitov and Janeway, 1998; Takeda and Akira, 2005).

TLR 1 (as well as TLR 6) is working in the complex with TLR2 as a heterodimer and recognizes triacyl lipopeptide, lipoteichoic acid (LTA), lipoproteins, peptidoglycan, lipoarabinomannan, lipopolysaccharide, and Zymosan. TLR 3 can recognize double strand RNA produced by viruses. TLR 4 recognizes LPS, cell wall component of Gram-negative bacteria. TLR 5 interacts with flagellin,

molecule presented in bacterial flagella. TLR 7 and TLR 8 can recognize single stranded RNA. For TLR 9, the specific ligand is nonmethylated CpG DNA. Not much is known about TLR 10 ligand (Medzhitov and Janeway, 1998; Takeda and Akira, 2005).

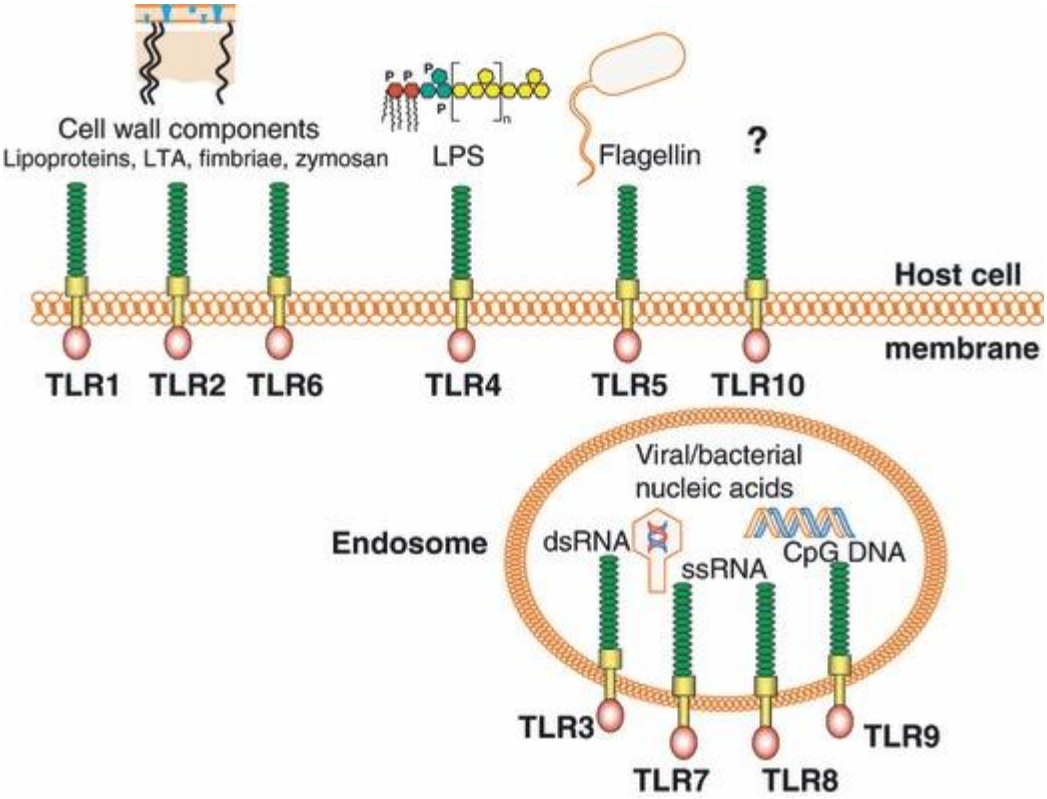

Figure 3 Human TLR receptors and their ligands. TLR receptors are proteins playing an important role in innate immune recognition of pathogens. So far, ten humans TLRs had been discovered. TLR 1, TLR 2, TLR 4, TLR 5, TLR 6 and TLR 10 are extracellular receptors. TLR 3, TLR 7, TLR 8, and TLR 9 are intracellular receptors (Krauss et al., 2010).

When TLRs recognize their specific ligands, two different pathways can be activated and result in different way of immune system action. (i) The first pathway is MyD88 dependent pathway and it results in production of inflammatory cytokines. (ii) The second pathway is MyD88 independent pathway and it results in stimulation of interferon-beta (IFN-beta) production and DC maturation (Kawasaki and Kawai, 2014; Zhu and Mohan, 2010).

Currently, several TLR agonists are being used in cancer treatment: Attenuated ***Mycobacterium bovis*** is effective for TLR 2, TLR 4, and TLR 9 activation. This therapy is called as Bacilus calmete Guerin

vaccine (BCG vaccine) and is successfully used in bladder carcinoma and superficial bladder cancer treatment. (Kawai et al., 2013; Luca and Mihaescu, 2013; Uyl-de Groot et al., 2005).

Another TLR agonist used in cancer treatment is **Imiquimod.** Imiquimod is synthetic TLR agonist activating TLR 7. Imiquimod is administrated as a 5% cream for skin malignancies and skin premalignant conditions. Imiquimod is also used as a vaccine adjuvant (Henriques et al., 2014; Urosevic and Dummer, 2004).

CpG oligodeoxynucleotides are group of TLR ligands used in cancer management. They can be divided into two groups based on their effect on immune system: (i) CpG-A activating NK cells, (ii) CpG-B inducing interferon alpha (IFN-α) and enhancing adaptive immunity. These ligands stimulate predominantly TLR 9 and were tested in several types of tumors such as renal cell carcinoma, melanoma, glioma, and lymphoma (Badie and Berlin, 2013). CpG oligodeoxynucleotides are also used as a adjuvants for cancer vaccines (Shirota et al., 2015).

Polyriboinosinic-polyribocytidylic acid (Poly(I:C)) is a synthetic analog of viral dsRNA activating TLR 3. Mostly, Poly(I:C) is used as a vaccine adjuvant with positive effect on DC cell maturation (Ammi et al., 2015).

The overall effect of TLR agonists in cancer treatment is not as strong as was expected. However, their combination with other type of cancer treatments seems to be very promising. The radiotherapy, chemotherapy, or monoclonal antibodies can be combined with TLR agonists to boost the immune system response (Kang et al., 2016).

2.4. IMMUNOTHERAPY BASED ON APPLICATION OF PHAGOCYTOSIS STIMULATING LIGANDS ON THE SURFACE OF TUMOR CELLS

Immunotherapy based on application of phagocytosis stimulating ligands with mixture of TLR agonists seems to be promising approach in treatment of several types of tumors such as melanoma, pancreatic adenocarcinoma, or pheochromocytoma. Effect of this therapy was evaluated in mouse models as well as *in vitro* in cell lines (Caisova et al., 2016; Janotova et al., 2014; Waldmannova et al., 2016).

This therapy activates innate immunity in three steps: (i) complex of TLR agonists applicated intratumorally enhance tumor immune cells infiltration, (ii) tumor cells covered with anchored

phagocytosis stimulating ligands are attacked by these tumor infiltrating leukocytes, and (iii) this attack leads to tumor cells elimination and subsequently to shrinkage of tumor (Caisova et al., 2016).
In our studies, we investigated three main areas of these therapeutic mechanisms:

(i) Several phagocytosis stimulating ligands were tested in aim to find the most potent one. ***N*-Formyl methionyl-leucyl-phenylalanine** (FMLP) was tested as a first phagocytosis stimulating ligand (Janotova et al., 2014). The origin of this ligand is in bacteria (bacterial proteosynthesis is initiated by formyl-methionine). FMLP activates macrophages as well as stimulates the chemotaxis of leukocytes. FMLP binds to the specific G protein coupled receptors called formyl peptide receptors (FPRs). Three FPRs were described in humans, such as FPR 1, FPR 2, and FPR 3. In mice, the FPR subfamily consists of 8 different receptors. The interaction of these receptors with specific ligands results in immune system activation and pathogen elimination (Wittmann et al., 2002). **Laminarin** was tested as a second phagocytosis stimulating ligand. Laminarin is a polysaccharide isolated from brown algae. Laminarin anchored to cell surface binds to phagocytic receptor Dectin-1, which is a pattern recognition receptor playing an important role in antifungal innate immunity. Activation of Dectin-1 in phagocytic cells leads to phagocytosis of pathogens, activation of NF-kapa B, secretion of proinflammatory cytokines, and production of reactive oxygen species (Huang et al., 2012; Song et al., 2017). **Mannan** was tested as the third phagocytosis stimulating ligand in our therapeutic approach. Mannan is a polysaccharide found in plants and yeasts. Detection of mannan by immune system, specifically by mannan binding lectin, leads to activation of lectin complement pathway. Arising iC3b molecules covalently bound to cell surface (in our case to tumor cells) are recognized by CR3 receptor of immune cells. This interaction results in phagocytosis of targets (in our case of tumor cells). Moreover, mannan can be recognized by immune cells expressing mannose receptor (Kilpatrick, 2002).

(ii) Three different tools for anchoring of phagocytosis stimulating ligands to tumor cell membrane were tested in our studies: **SMCC**, **BAM**, and **DOPE**. **SMCC** anchor is a heterobifunctional protein crosslinker. **BAM** is a biocompatible anchor for cell membranes with one oleyl group. **DOPE** is an anchor with two oleyl groups. N-Hydroxysuccinimide (NHS) group of these anchors reacts with amine (NH_2) group of ligands stimulating phagocytosis (Janotova et al., 2014; Kato et al., 2004).

(iii) The effect of phagocytosis stimulating ligands is enhanced by simultaneous application of TLR agonists. TLR agonists activate immune system and enhance the tumor leukocyte infiltration. In our studies, several different TLR agonists and their combinations were tested. Lipopolysaccharide (**LPS**) was used first as the TLR agonist and promising results were obtained. Using LPS simultaneously with

phagocytosis stimulating ligands resulted in strong tumor immune cell infiltration and tumor elimination (Janotova et al., 2014). However, the LPS was replaced later with another TLR agonists because of its toxicity (Yamamoto et al., 2011). LPS was replaced by combination of three TLR agonists, specifically polyinosinic-polycytidylic acid (**Poly(I:C)**), resiquimod (**R-848**), and lipoteichoic acid (**LTA**) (Caisova et al., 2016). Poly(I:C) is a synthetic analog of double-stranded RNA activating TLR 3. Poly(I:C) has a prominent effect on activation of CD8 cells and NK cells (Ammi et al., 2015). R-848 is an imidazoquinoline compound with potent anti-viral activity. R-848 activates TLR 7/TLR 8, which leads to increasing of proinflammatory cytokine expression and B-lymphocyte activation (Wagner et al., 1999). LTA stimulates TLR 2 and causes cytokine expression and monocyte activation (Schwandner et al., 1999).

This therapeutic approach was tested and modified in our studies (Paper 1 – Paper 5). All the results will be discussed in the following section: Research papers.

3. RESEARCH PAPERS

Paper 1

The use of anchored agonists of phagocytic receptors for cancer immunotherapy: B16-F10 murine melanoma model

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PLOS ONE

The Use of Anchored Agonists of Phagocytic Receptors for Cancer Immunotherapy: B16-F10 Murine Melanoma Model

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Abstract

The application of the phagocytic receptor agonists in cancer immunotherapy was studied. Agonists (laminarin, molecules with terminal mannose, N-Formyl-methioninyl-leucyl-phenylalanine) were firmly anchored to the tumor cell surface. When particular agonists of phagocytic receptors were used together with LPS (Toll-like receptor agonist), high synergy causing tumour shrinkage and a temporary or permanent disappearance was observed. Methods of anchoring phagocytic receptor agonists (charge interactions, anchoring based on hydrophobic chains, covalent bonds) and various regimes of phagocytic agonist/LPS mixture applications were tested to achieve maximum therapeutic effect. Combinations of mannan/LPS and f-MLF/LPS (hydrophobic anchors) in appropriate (pulse) regimes resulted in an 80% and 60% recovery for mice, respectively. We propose that substantial synergy between agonists of phagocytic and Toll-like receptors (TLR) is based on two events. The TLR ligand induces early and massive inflammatory infiltration of tumors. The effect of this cell infiltrate is directed towards tumor cells, bearing agonists of phagocytic receptors on their surface. The result of these processes was effective killing of tumor cells. This novel approach represents exploitation of innate immunity mechanisms for treating cancer.

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Introduction

According to broadly accepted cancer immunoediting hypothesis [1] cancer cells, which overcame elimination and equilibrium phases, generate the critical modifications necessary to circumvent both innate and adaptive immunological defences (escape phase). Numerous escape mechanisms include down-regulation of tumor-specific antigens [2], loss or down-regulation of MHC antigens [3], defects in antigen processing and presentation [4], expression of immune-inhibitory ligands on tumor cells [5], induction of central or peripheral tolerance [6] or generation of an immunosuppressive tumor microenvironment [7].

While the most important component of anti-tumor immunity is represented by cytotoxic T lymphocytes [8], among cells of innate immunity, NK cells seem to play the most significant role [9]. The role of other innate immunity cells is much less explored and almost nothing is known about recognition of tumor cells by unarmed macrophages or granulocytes [10].

Nevertheless, Cui et al. [11] and Hicks et al. [12] showed that mice with a SR/CR mutation, enabling recognition of tumor cells via a so far unknown mechanism, successfully killed tumor cells. *In vitro* experiments demonstrated that cells of innate immunity (NK cells, macrophages, neutrophils) were responsible for cancer cell killing. Exploitation of pattern recognition receptor (PRR) agonists to stimulate innate signalling pathways [13] is another partially successful approach to treatment of cancer. Complex mechanism of PRR agonist action consists in the production of interferon type I and other proinflammatory cytokines, enhanced maturation of dendritic cells, secretion of Th1 cytokines, antigen cross-presentation, activation of NK cells and suppression of regulatory T cells and tumor associated macrophages [14]. Clinical trials focused on usage of synthetic ligands of the Toll-like receptors (TLR) 3,7,9 for tumor treatment [15].

However, besides the fact that activation of signalling receptors (mainly TLR) leads to establishment of strong answer at the level of innate immunity, tumor infiltrating immune cells must recognize tumor cells as the true targets of their attack. We suggest manipulating phagocytic cells (an important component of inflammatory infiltrate) to be able to find their targets by coupling agonists of phagocytic receptors on the surface of tumor cells to obtain a strong antitumor effect. This effect can be dramatically enhanced by simultaneous treatment of TLR receptors with an agonist (e.g., LPS).

Materials and Methods

Ethics Statement

All of the experimental procedures were conducted in accordance with the law of the Czech Republic on the use of experimental animals, safety and use of pathogenic agents. The study was approved by the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic and Institutional and National Committees (protocols no. 138/2008).

Anaesthesia of mice (used during transplantation of melanoma cells) was based on intraperitoneal injection of Ketamine.HCl (75 mg/kg) and Xylazine.HCl (75 mg/kg). For survival analysis mice were monitored twice a day. Where tumor growth restricted an animal's ability to move normally or to eat or drink then mice were sacrificed via cervical dislocation.

Chemicals

Tissue culture media and supplements, laminarin from *Laminaria digitata*, mannan from *Saccharomyces cerevisiae*, lipopolysaccharides (LPS) from *Escherichia coli*, lipoteichoic acid (LTA) from *Bacillus subtilis*, dithiothreitol (DTT), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), DAPI, and f-MLF (N-Formyl-methioninyl-leucyl-phenylalanine) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 4-(*N*-Maleimidomethyl) cyclohexanecarboxylic-acid N-hydroxysuccinimide ester (SMCC) was purchased from Thermo Scientific (Erembodegem, Belgium). Biocompatible Anchor for cell Membrane (BAM, Mw 4000) and N-(Succinimidyloxy-glutaryl)-L-α-phosphatidylethanolamine, Dioleoyl (DOPE) were obtained from NOF EUROPE (Grobbendonk, Belgium). Anti-CD11b-FITC conjugate was obtained from MACS Miltenyi Biotec.

Monomannosyldekalysine was synthesized by Vidia (Prague, Czech Republic). Mannose-$(G)_5$-$(K)_{12}$, mannose-$(G)_5$-$(K)_{10}$-STE (STE means stearic acid), f-MLF-$(G)_5$-$(K)_{12}$, f-MLF-$(G)_5$-$(K)_{10}$-STE, MLF-$(G)_5$-$(K)_{10}$-STE, and f-MLFKK were synthesized by Schafer-N (Copenhagen, Denmark).

Synthesis of laminarin-BAM, mannan-BAM, f-MLFKK-BAM, and f-MLFKK-DOPE

First, both aminated laminarin and mannan were prepared by reductive amination [16]. Laminarin (mannan) solution in an environment of ammonium acetate was reduced by natrium cyanoborohydride at pH 7.5 and 50°C for five days. Solution was further dialyzed using MWCO 3500 dialysis tubing (Serva, Heidelberg, Germany) against PBS at 4°C overnight. Peptide f-MLFKK already contained an amino group.

Binding of BAM (contains one aliphatic chain) or DOPE (two aliphatic chains) on amino group of laminarin (mannan, f-MLFKK) was performed at pH 7.3 according to Kato et al. [17]. During one hour at room temperature N-hydroxysuccinimide (NHS) group of BAM resp. DOPE reacted with amino group of laminarin (mannan), or with ε-amino group of lysine respectively. Solutions obtained (in PBS) were stored frozen at –20°C until use.

Synthesis of laminarin-SMCC, mannan-SMCC, f-MLFKK-SMCC, and their *in vivo* and *in vitro* application

According to manufacturers instructions (Thermo Scientific, Pierce Protein Biology Products), similarly to the previous paragraph, NHS group of SMCC reacted with amino group of aminated laminarin and mannan, or with ε-amino group of lysine in f-MLFKK (equimolar amounts) respectively. To guarantee binding of SMCC containing ligands to tumor cells, it was necessary to ensure existence of –SH groups on the cells. It was accomplished according to Christiaansen et al. [18] by reduction of cystines. In our *in vivo* experiments we used 50 mM solution of TCEP in PBS for this purpose. This solution was injected intratumorally (i.t.) one hour before application of laminarin-SMCC, mannan-SMCC or f-MLFKK-SMCC solutions (in PBS). In our *in vitro* experiments we used 5 mM solution of TCEP in PBS and one hour incubation on ice.

Cell lines and mice

Murine melanoma B16-F10 cells and peritoneal macrophages PMJ2R were purchased from American Type Culture Collection (ATCC, Manassas, VA). Both cell lines were cultivated in RPMI 1640 (Sigma-Aldrich, USA) supplemented with 10% foetal calf serum (FCS, PAA, Austria) and antibiotics. Cells were maintained at 37°C in humidified air with 5% carbon dioxide.

Female SPF C57BL/6 mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Mice were housed in plastic cages with wood-chip bedding situated in a specific-pathogen free room with a constant temperature of 22°C and a relative humidity of 65%. Pellet diet and water were sterilized. Mice were housed in a 12/12-hour photoperiod environment with free access to food and water. Mice weighing 18–20 g were used in experiments.

Tumor transplantation

4×10^5 B16-F10 cells per mouse in 0.1 ml RPMI without FCS were inoculated subcutaneously (s.c.) in a shaved area on the right flank.

Treatment and evaluation of treatment

Mice were randomised in groups twelve days after tumor transplantation. Therapies started immediately (intratumoral applications of 50 µl of corresponding solutions). Since this time, mice were kept individually.

Tumors were measured every second day using callipers. Volume was calculated as previously described [19] using formula $V = \pi/6\ AB^2$ (A denotes the largest dimension of tumor mass and B denotes the smallest dimension).

Mean reduction of tumor growth (%)

Reduction of tumor growth (compared with control) was determined as follows:

$$\frac{(\text{mean tumor volume in control group} - \text{mean tumor volume in treated group}) \times 100}{\text{mean tumor volume in control group}}$$

Mean (in %) of values measured on days 4, 6, 8, 10, 12 and 14 after beginning of therapy was calculated and marked as "mean reduction of tumor growth".

Analysis of cell infiltrate using flow cytometry. Cytokine assay

The tumor was excised from the mouse which had been euthanized via cervical dislocation. It was then gently washed with cold RPMI 1640, cut into small pieces and placed into 1 ml cold RPMI 1640 containing 0.33 mg/ml Liberase DL and 0.2 mg/ml DNase I (both Roche Diagnostics, Germany). After 1 h incubation on a rotary shaker at 37°C, clumps of tissue aggregates were centrifuged at 160 *g* for 10 min at 4°C. Supernatant was used for IL-1 beta, TNF alpha, IL-6, (ELISA, eBioscience), and IL-8 (R&D Systems) determination performed according to manufacturer

recommendations. The resulting pellet was gently passed through a plastic strainer (70 μm, BD Biosciences, USA) into cold PBS (pH 7.3) and washed by centrifugation at 160 *g* for 10 min at 4°C. Cells were then transferred into 96-well plate (Corning Incorporated, USA) and analyzed using flow cytometry.

Cells were incubated with a solution of pre-diluted specific monoclonal antibodies recognizing mouse surface antigens (all eBioscience, USA) in PBS for 20 min at 4°C. In the cell suspension obtained from the tumor, the following leukocyte subtypes were determined: leukocytes (anti-Mouse CD45 PerCP-Cy5.5; clone 30-F11; 0.2 mg/ml), B cells (anti-Mouse CD19 APC; clone eBio1D3; 0.2 mg/ml), T cells (anti-Mouse CD3e FITC; clone 145-2C11; 0.5 mg/ml), CD4+ T cells (anti-Mouse CD4 APC; clone GK1.5; 0.2 mg/ml), CD8+ T cells (anti-Mouse CD8a; clone 53-6.7; 0.2 mg/ml), NK cells (anti-Mouse NK1.1 PE; clone PK136; 0.2 mg/ml), granulocytes (anti-Mouse Ly-6G (Gr-1) Alexa Fluor 700; clone RB6-8C5; 0.2 mg/ml) and monocytes/macrophages (MF cells) (anti-Mouse F4/80 Antigen PE-Cy7; clone BM8; 0.2 mg/ml). Labelled cell samples were washed twice in PBS by centrifugation at 160 *g* for 2 min at 4°C and analyzed using a BD FACSCanto II flow cytometer (BD Biosciences, USA), equipped with two lasers with excitation capabilities at 488 nm and 633 nm. Twenty thousand events were measured in each suspension in three independent repetitions. The labelled cell populations were analysed using BD FACSDiva software 6.1.3. Absolute numbers of leukocyte subsets were quantified using CountBright^{TM} absolute counting beads (Invitrogen, USA). The control of all specific monoclonal antibodies recognizing mouse surface antigens was performed on a sample of splenocytes in each interval of the experiment. Cell count was recalculated and expressed as cells/mm^3 of tumor tissue.

Histology

Tumors were fixed with 4% neutral solution of formaldehyde. Paraffin blocks were prepared. Sections were stained by hematoxylin/eosin.

Lung metastases

Lungs fixed with 4% neutral solution of formaldehyde were examined with the aid of a dissecting microscope. The presence of metastases (black points) was evaluated.

In vitro analysis of the cytotoxic effect of macrophages activated by a TLR ligand on melanoma cells bearing phagocytic receptors

The assay was based on the principle described previously [20]. Murine B16-F10 melanoma cells grown to confluency in 96 well tissue culture plate (Nunc, Roskilde, Denmark) were incubated (30 min, 37°C) with a solution of phagocytic receptor agonists (0.02 mM laminarin–BAM or 0.02 mM mannan-BAM or 0.05 mM f-MLFKK-BAM in culture medium) and subsequently washed. Cells of murine macrophage cell line PMJ2R were preincubated with LPS (1 μg/ml) for 2 hours at 37°C, then they were washed, resuspended in RPMI 1640, 10% FCS and added to B16-F10 in the ratio 5:1. This mixture was incubated for 4 hours at 37°C. After incubation, PMJ2R and dead cells were carefully washed off. Living B16-F10 melanoma cells were released by trypsinisation. Trypan blue excluding cells were quantified with a haemocytometer.

Cell signalling

2×10^5 of each, B16-F10 and PMJ2R cells were seeded together in the presence of laminarin-BAM, or B16-F10 cells with covalently bound laminarin-SMCC were used. After indicated time of incubation the cells were lysed in a modified RIPA buffer (1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.5)) in the presence of protease inhibitors (10 μg/ml aprotinin, 1 μg/ml leupeptin, 1mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin) and phosphatase inhibitors (25 mM sodium fluoride and 2 mM sodium orthovanadate). The cell lysates were mixed with 4x Laemmli sample buffer, than proteins were separated by SDS-PAGE, and transferred to Immobilon-P membrane. The blots were incubated with anti-phospho-NF-κB p65 (Ser536, Cell Signalling) and with anti-β-actin Santa Cruz Biotechnology) antibody at dilution 1:1000. Proteins were visualized by ECL (enhanced chemiluminiscence, Pierce), and their abundance was determined using CCD image system (ChemiDoc^{TM} MP Imaging System, BIO-RAD) and ImageLab software.

Capability of BAM and DOPE to anchor molecules to cell membranes

Conjugation of BAM or DOPE with B-Phycoerythrin (PE) was performed at pH 7.3 in the dark as previously described [17]. One hour lasting interactions of PE-BAM, PE-DOPE and PE with 1×10^5 melanoma cells were performed at 37°C in the dark in triplicates. After centrifugation (2 min. 4°C, 400 g) supernatants were harvested and its fluorescence measured by Infinite M200 reader (Tecan, Switzerland) at 545 nm.

Statistical analysis

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

Results

The effect of laminarin in cancer therapy

The effect of anchored laminarin (laminarin-BAM) on tumor growth and its synergy with LPS. Melanoma B16-F10 was transplanted into 20 C57BL/6 mice. Twelve days after this, mice were randomised in four groups containing five mice each. On this day, tumor volume was measured and tumor therapy started immediately thereafter. As Figure 1A shows, laminarin-BAM did not have a significant effect on tumor growth. The effect of LPS was statistically significant resulting in 63.2% mean reduction of tumor growth (see Materials and Methods for calculation of mean reduction of tumor growth). The combination of laminarin-BAM and LPS showed synergistic and strong reduction of tumor growth (mean reduction of tumor growth was 90.2% compared with the control). We observed that 60% of tumors temporarily disappeared or a shrinkage of tumor volume occurred. Decrease of tumor growth was statistically significant compared with the control and with the effect of individual (laminarin-BAM, LPS) components. Regarding survival, its prolongation in the case of a laminarin-BAM/LPS mixture was not statistically significant.

Synergy of laminarin-BAM with LPS, various regimes of application. A series of experiments similar to the above mentioned one were performed. Optimization of drug application timing was studied. A mixture of 0.2 mM laminarin-BAM and LPS (0.5 mg/ml) in PBS was used. The results are given in Table 1, highlighting the essential significance of short-term but sufficiently effective therapy.

Use of other mode of laminarin binding to the cell surface. Direct covalent *in vivo* binding of laminarin-SMCC to the cells (with prior reduction of cystines by TCEP) was applied.

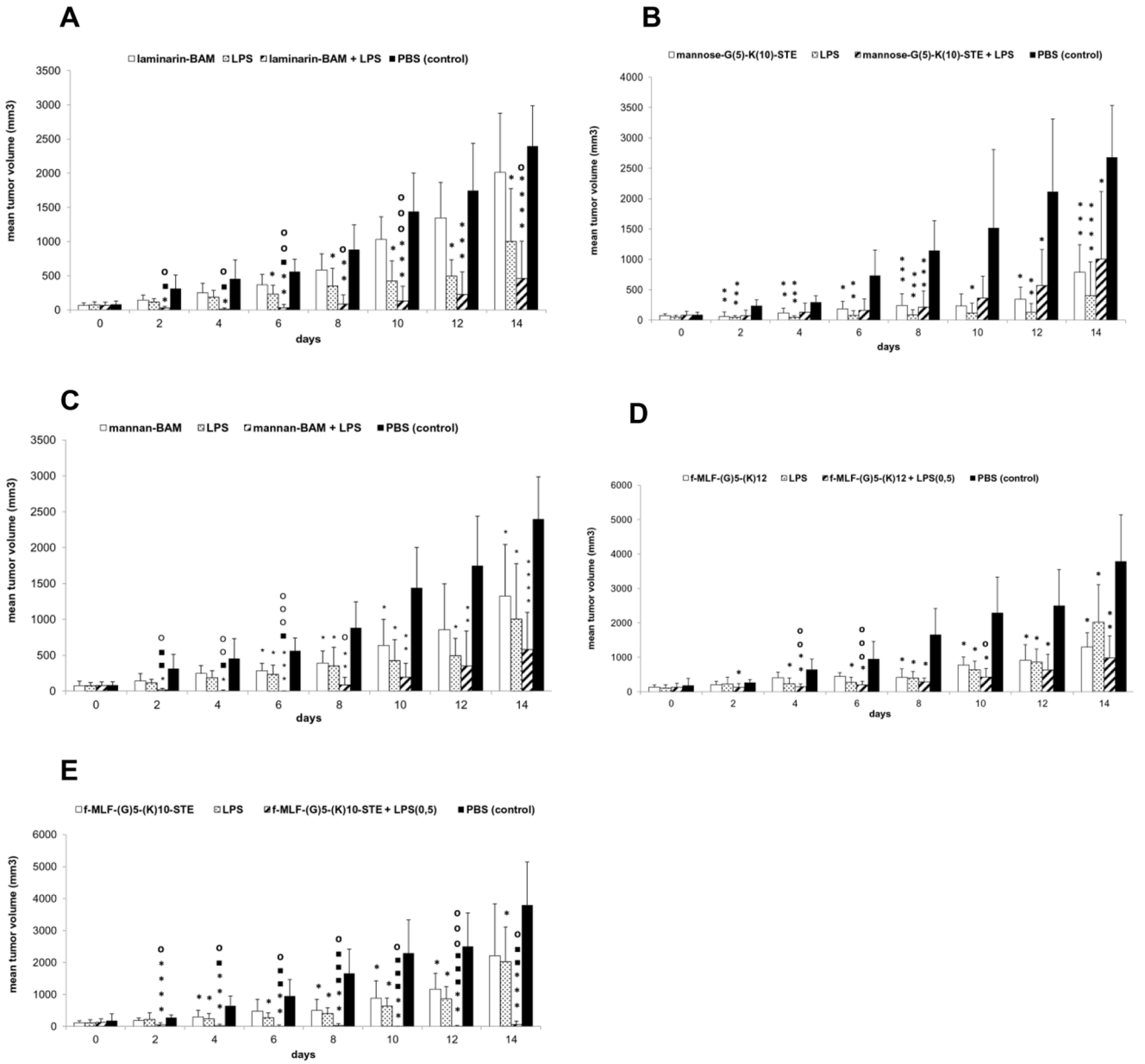

Figure 1. The effect of anchored ligands of phagocytic receptors on tumor growth and their synergy with LPS. C57BL/6 mice (females) were inoculated with 4×10^5 murine melanoma B16-F10 cells per mouse in 0.1 ml RPMI subcutaneously in a shaved area on the right flank. Mice were randomized in groups of 5–6 twelve days after tumor transplantation. Therapies started immediately by intratumoral applications of 50 μl of corresponding solutions and continued every second day for 10 days (together 6 doses). After therapy had commenced, mice were kept individually. Tumors were measured every second day for 14 days and their volume was calculated. (A) Anchored laminarin (laminarin-BAM). Groups of 5 mice obtained 0.2 mM laminarin-BAM in PBS, LPS (0.5 mg/ml PBS), mixture of 0.2 mM laminarin-BAM and LPS (0.5 mg/ml) in PBS, and PBS alone. (B) Anchored mannose. Groups of 6 mice obtained 3 mM mannose-$(G)_{5)}$-$(K)_{10}$-STE in PBS, LPS (0.5 mg/ml PBS), mixture of 3 mM mannose-$(G)_5$-$(K)_{10}$-STE and LPS (0.5 mg/ml) in PBS, and PBS alone. (C) Anchored mannan. Groups of 5 mice obtained 0.2 mM mannan-BAM in PBS, LPS (0.5 mg/ml PBS), mixture of 0.2 mM mannan-BAM and LPS (0.5 mg/ml) in PBS, and PBS alone. (D) Anchored formylpeptide receptor agonist by oligolysin. Groups of 6 mice were injected with 3 mM f-MLF-$(G)_5$-$(K)_{12}$ in PBS, LPS (0.5 mg/ml PBS), mixture of 3 mM f-MLF-$(G)_5$-$(K)_{12}$ and LPS (0.5 mg/ml) in PBS, and PBS alone. (E) Anchored formylpeptide receptor agonist by stearic acid. The same regime as in (D), 3 mM f-MLF-$(G)_5$-$(K)_{10}$-STE used instead of 3 mM f-MLF-$(G)_5$-$(K)_{12}$. *P≤0.05, **P≤0.01, ***P≤0.005, ****P≤0.001 compared to control ■P≤0.05, ■■P≤0.01, ■■■P≤0.005 compared to LPS **o**P≤0.05, **oo**P≤0.01, **ooo**P≤0.005 compared to the ligand.
doi:10.1371/journal.pone.0085222.g001

Laminarin-SMCC (0.2 mM) was administered together with LPS (0.5 mg/ml). This therapy caused stronger reduction of tumor growth than laminarin-BAM/LPS, nevertheless this difference was not statistically significant (data not shown). Reduction (TCEP) and SMCC binding did not influence tumor growth.

Control experiments. To demonstrate the necessity of laminarin anchoring to cancer cells, free laminarin was used instead of laminarin-BAM. Laminarin did not reduce tumor growth and its mixture with LPS did not show any signs of additivity or synergy. Tumor growth reducing activity of this

Table 1. Synergy of laminarin-BAM with LPS, various regimes of application.

Application of 0,2 mM laminarin-BAM and LPS (0,5 mg/ml) in 50 μl i.t.	Mean reduction of tumor growth	Statistical significance of survival prolongation	Survival longer than 100 days from the start of therapy
days 0,2,4,6,8,10	83.0%	no	0/5
day 0	64.0%	no	0/5
days 0,1,2	93.9%	no	1/4
day 0 …. 3 doses one hour apart day 1 …. 2 doses one hour apart day 2 …. 1 dose	93.2%	no	1/5

Groups of 4–5 mice were treated starting the 12th day after tumor transplantation.
doi:10.1371/journal.pone.0085222.t001

mixture corresponded to the activity of LPS alone (data not shown). Anchor alone (lysine-BAM) did not reveal any antitumor activity and its mixture with LPS did not show any signs of additivity or synergy as well.

The effect of molecules with terminal mannose in cancer therapy

Significance of mannose anchoring, the influence of LPS. A 3 mM solution of mannose in PBS did not reduce tumor growth when applied every second day, six injections altogether. Addition of LPS (0.5 mg/ml) did not cause any additivity or synergy, the mixture reduced tumor growth even less than LPS alone. Tumor cells are significantly negatively charged, so we studied their interaction with positively charged mannose-K_{10}, containing ten lysine residues chain. Mannose-K_{10} at 3 mM concentration did not influence tumor growth and addition of LPS (0.5 mg/ml) did not cause additivity or synergy. A low effect (32.7% mean reduction of tumor growth compared with the control) was noted using 3 mM solution of mannose-$(G)_{5)}$-$(K)_{12)}$ in PBS, i.e. compound with 5 glycine residue spacer between the ligand and anchoring part of the molecule. This reduction was statistically significant (compared with the control) only on day 6 of therapy (data not shown).

Addition of a lipophilic anchor (mannose-$(G)_5$-$(K)_{10)}$-STE) led to a further reduction in tumor growth. A solution of this compound in PBS (3 mM) caused a statistically significant reduction of tumor growth (Figure 1B). Mean reduction of tumor growth was 75,6%. Addition of LPS (0.5 mg/ml) did not cause any additivity or synergy, conversely, mean reduction of tumor growth dropped to 71.2%. The effect of LPS alone remained the strongest. Mice were killed 14 days after the beginning of therapy. The solution of mannose-$(G)_5$-$(K)_{10}$-STE fully suppressed appearance of metastases. Incidence and intensity of metastases are summarised in Table 2.

The effect of anchored mannan (mannan-BAM) on tumor growth and its synergy with LPS. Mice were treated with mannan-BAM, LPS and a mixture of the two(Figure 1C). Mannan-BAM caused a weak (50.5%), but statistically significant reduction of tumor growth. The effect of LPS was slightly higher (mean reduction of tumor growth was 63.2%). A combination of both compounds caused a strong synergistic reduction of tumor growth (88.6% compared with the control) and tumors temporally disappeared in 80% of mice. The decrease of tumor growth caused by mannan-BAM/LPS mixture was initially statistically significant compared with both control and both individual components of the mixture, later only with the control. Prolongation of mouse survival, caused by the treatment with the mixture of mannan-BAM/LPS, was not statistically significant.

Synergy of mannan-BAM with LPS, various regimes of application. An optimum regime was best achieved by pulse intratumoral application of 50 μl of 0.2 mM mannan-BAM and LPS (0,5 mg/ml) mixture on days 0, 1, 2. 8, 9, 10.16, 17, 18.24, 25, 26. This regime caused not only significant reduction of tumor growth (94,7%) but also statistically significant prolongation of survival ($P \leq 0.005$), see Figure 2. An 80% survival rate for 100 days was observed.

Use of other mode of mannan binding to the cell surface. Direct covalent *in vivo* binding of 0.2 mM mannan-SMCC to cells (primarily reduced by TCEP) was tested. Mannan-SMCC was administered together with LPS (0.5 mg/ml). As shown in Table 3, high reduction of tumor growth and high ratio of mice with temporary vanishing tumors were observed. The use of four therapeutic pulses of mannan-SMCC/LPS mixture caused

Table 2. Influence of intratumoral application of mannose-$(G)_5$-(K)10-STE, LPS and combination thereof on incidence of metastases of melanoma B16-F10.

Therapy	Incidence of metastases (%)	Intensity of metastases (mean count of metastases in metastases bearing mice)
3 mM mannose-$G_{(5)}$-$K_{(10)}$-STE	0.0	0
3 mM mannose-$G_{(5)}$-$K_{(10)}$-STE + LPS (0.5 mg/ml)	16.7	2
LPS (0.5 mg/ml PBS)	16.7	5
Control – PBS	50.0	4.3

Groups of 6 mice were examined for the presence of metastases 14 days after beginning of the therapy.
doi:10.1371/journal.pone.0085222.t002

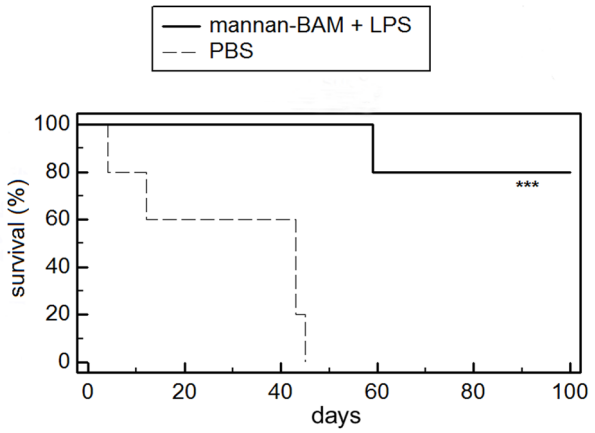

Figure 2. The effect of mannan-BAM/LPS mixture (pulse application) on mouse survival. Mixture of 0.2 mM mannan-BAM and LPS (0,5 mg/ml) in PBS was applied i.t. in pulse regime (days 0,1,2. 8,9,10.16,17,18.24,25,26). Both treated and control group contained 5 mice each. ***indicates P≤0.005 compared to control.
doi:10.1371/journal.pone.0085222.g002

almost statistically significant prolongation of survival. Only in this case, survival longer than 100 days was observed.

Control experiments. As mentioned previously, free mannose did not reduce tumor growth. Its mixture with LPS also did not show any signs of additivity or synergy, all tumor reducing activity of the mixture corresponded to the effect of LPS alone. The same results were obtained with free mannan (data not shown). Testing of new anchoring principles (electrostatic interactions, cell reduction by TCEP and SMCC binding) did not reveal any antitumor activity and combination with LPS did not show any signs of additivity or synergy as well. BAM anchoring did not reveal any anticancer activity as was already described. Regarding $(G)_5$-$(K)_{10}$-STE, as described below, no anticancer activity was connected with this type of anchoring as well.

The effect of formylpeptide receptor agonists in cancer therapy

Significance of anchoring of formylpeptide receptor agonists. The influence of LPS. In the first experiment, agonists of formylpeptide receptors were attached to the tumor cell's surface on the basis of charge interaction as already mentioned above. f-MLF-$(K)_{12}$ was used as an agonist. Even at 3 mM concentration, it did not reduce growth of the melanoma. The agonist effect was enhanced by using a spacer (5 glycine residue chain), which enables higher flexibility of the terminal f-MLF group. The structure of the above mentioned compound was f-MLF-$(G)_5$-$(K)_{12}$. As shown in Figure 1D, the f-MLF-$(G)_5$-$(K)_{12}$ solution caused weak, but nevertheless statistically significant reduction of tumor growth (mean reduction of tumor growth was 59.7%), which was significantly enhanced by addition of LPS to 78.3% mean reduction of tumor growth. The f-MLF-$(G)_5$-$(K)_{12}$/LPS interaction should be considered slightly additive, as their mixture showed only a slightly higher effect than the more effective component of the mixture.

The molecule of formylpeptide agonist was further modified. Charge interactions were coupled with anchoring of aliphatic chain in lipid layer of cytoplasmic membrane. The structure of this compound was f-MLF-$(G)_5$-$(K)_{10}$-STE. As demonstrated in Figure 1E, f-MLF-$(G)_5$-$(K)_{10}$-STE acts comparably (55.0% mean reduction of tumor growth) as the compound without stearic acid, used in previous experiment (59.7%). Combination of f-MLF-$(G)_5$-$(K)_{10}$-STE with LPS led to a strong synergistic effect, showing marked reduction of tumor growth (98.7%). This reduction was statistically significant in comparison with both components of the mixture. Tumors in five of six mice (83.3%) temporarily disappeared. The increase of survival time in this group was statistically significant (P≤0.05).

The use of other modes of binding of f-MLF to the cell surface. A series of experiments revealed that anchored 0.5 mM f-MLF motive in mixture with LPS (0.5 mg/ml) is sufficient for strong reduction of tumor growth. Using these concentrations and various ways of anchoring and timing we performed experiments with the goal to find the best conditions for the strongest antitumor effect.

Results are summarised in Table 4. Experiments confirmed the essential significance of short but sufficiently effective initial therapy, where the mixture of 0.5 mM f-MLFKK-DOPE and LPS (0.5 mg/ml) proved to be the best. 60% of mice treated this way survived 100 days, living further without any pathological symptoms.

Control experiments. Free 3 mM f-MLF did not show any reduction of tumor growth and reduction activity of its mixture with LPS corresponded to the activity of LPS alone. Data not shown. Anchors (DOPE as lysine-DOPE, $(G)_5$-$(K)_{10}$-STE as immunologically inert MLF-$(G)_5$-$(K)_{10}$-STE) did not show any antitumor activity and combinations with LPS did not show any signs of additivity or synergy.

Analysis of the cell infiltrate in tumors using flow cytometry. Cytokine assays

Three experiments of the same design were performed with three different phagocytic receptor ligands: laminarin-BAM, mannan-BAM and f-MLFKK-BAM alone or in combination with LPS.

Table 3. Melanoma therapy based on the use of mannan covalently bound to tumor cell surface, synergy with LPS.

Therapy based on TCEP reduction followed by treatment with 0.2mM mannan-SMCC and LPS (0.5 mg/ml). Days of application	Mean reduction of tumor growth	Statistical significance of survival prolongation	Number of mice where tumors disappeared temporarily	Survival longer than 100 days from the start of therapy
0,1,2	92.6%	no	3/5	0/5
0,1,2,8,9,10	98.3%	no	5/5	0/5
0,1,2,8,9,10,16,17,18,24,25,26	97.6%	P = 0.051	5/5	1/5
0,2,4,6,8,10	98.3%	no	4/5	0/5

Groups of 5 mice were treated starting the 12th day after tumor transplantation.
doi:10.1371/journal.pone.0085222.t003

Table 4. Melanoma therapy using f-MLF bound by various ways to tumor cell surface; synergy with LPS.

Therapy	Mean reduction of tumor growth	Statistical significance of survival prolongation	Number of mice where tumors disappeared temporarily	Survival longer than 100 days from the start of therapy
0.5 mM f-MLFKK-BAM + LPS (0.5 mg/ml), application: day 0 …. 3 doses one hour apart day 1 …. 2 doses one hour apart day 2 …. 1 dose	73.9%	no	3/5	0/5
0.5 mM f-MLFKK-DOPE + LPS (0.5 mg/ml), application: day 0 …. 3 doses one hour apart day 1 …. 2 doses one hour apart day 2 …. 1 dose	79.3%	no	3/5	3/5
0.5 mM f-MLFKK-SMCC + LPS (0,5 mg/ml), (prereduction^{+}), application on days 0,1,2	74.7%	no	2/6	0/6

Groups of 5–6 mice were treated starting the 12th day after tumor transplantation.
$^{+}$reduction of cystins on cancer cells by 50 mM solution of TCEP in PBS.
doi:10.1371/journal.pone.0085222.t004

In all experiments 3 mice from each group (see legend to Figures 3, 4, 5, 6) were killed in 12, 24 and 48 hour intervals (+3 control mice were killed at time 0). Cells for flow cytometry and supernatants for ELISA were prepared and analysed.

Therapy based on the use of laminarin-BAM, LPS and their mixture. Changes in granulocyte count (GR1+) only were observed in the monitored period. A significant increase of their count was detected in laminarin-BAM/LPS and LPS groups (Figure 3). The increase of cell count in the laminarin-BAM/LPS group preceded increase in the LPS group (12 hours difference). These changes were reflected in the total leukocyte count (CD45+) (data not shown). The total number of infiltrating cells during 48 hours was comparable in both groups. No changes in monocyte/macrophage (F4/80+), T lymphocyte (CD3+), CD4+, CD8+, NK, B lymphocyte (CD19+) count were observed.

Therapy based on the use of mannan-BAM, LPS and their mixture. The increase of granulocyte count was detected again mainly in mannan-BAM/LPS and LPS groups (Figure 4A). The increase was synchronous in both groups and was reflected by the increase of total leukocytes (CD45+) (data not shown). No significant differences between mannan-BAM/LPS and LPS groups were found. The increase of B lymphocytes (CD19+) and NK cells in mannan-BAM group and partially in the group mannan-BAM/LPS were demonstrated (Figure 4B, 4C).

Therapy based on the use of f-MLFKK-BAM, LPS and their mixture. The changes observed correspond to the

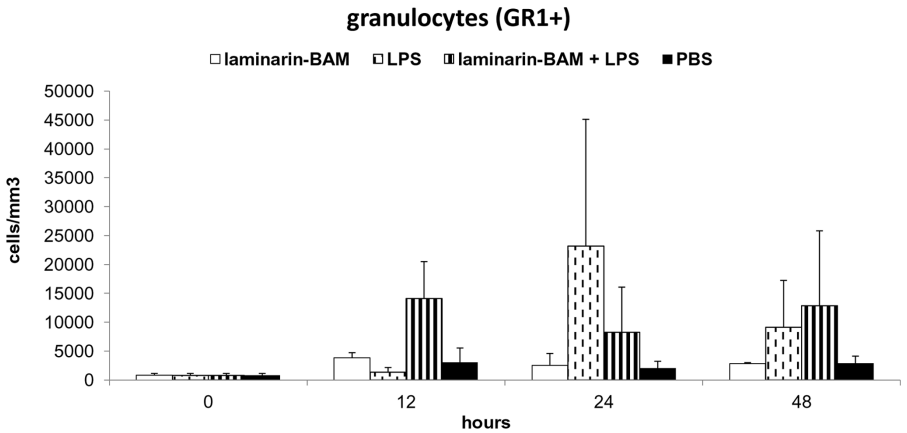

Figure 3. Analysis of cell infiltrate in the tumor during therapy based on the use of laminarin-BAM, LPS and their mixture. Granulocyte detection. Groups of 9 mice received a single dose of 0.2 mM laminarin-BAM in PBS, LPS (0.5 mg/ml PBS), mixture of 0.2 mM laminarin-BAM and LPS (0.5 mg/ml) in PBS, and PBS alone in 50 μl i.t. 3 mice from each group were killed in 12, 24 and 48 hours intervals, cells from excised tumors were prepared by enzymatic treatment (Liberase DL and DNase I) and analysed by flow cytometry. For granulocyte detection anti-Mouse Ly-6G (Gr-1) Alexa Fluor 700 was used.
doi:10.1371/journal.pone.0085222.g003

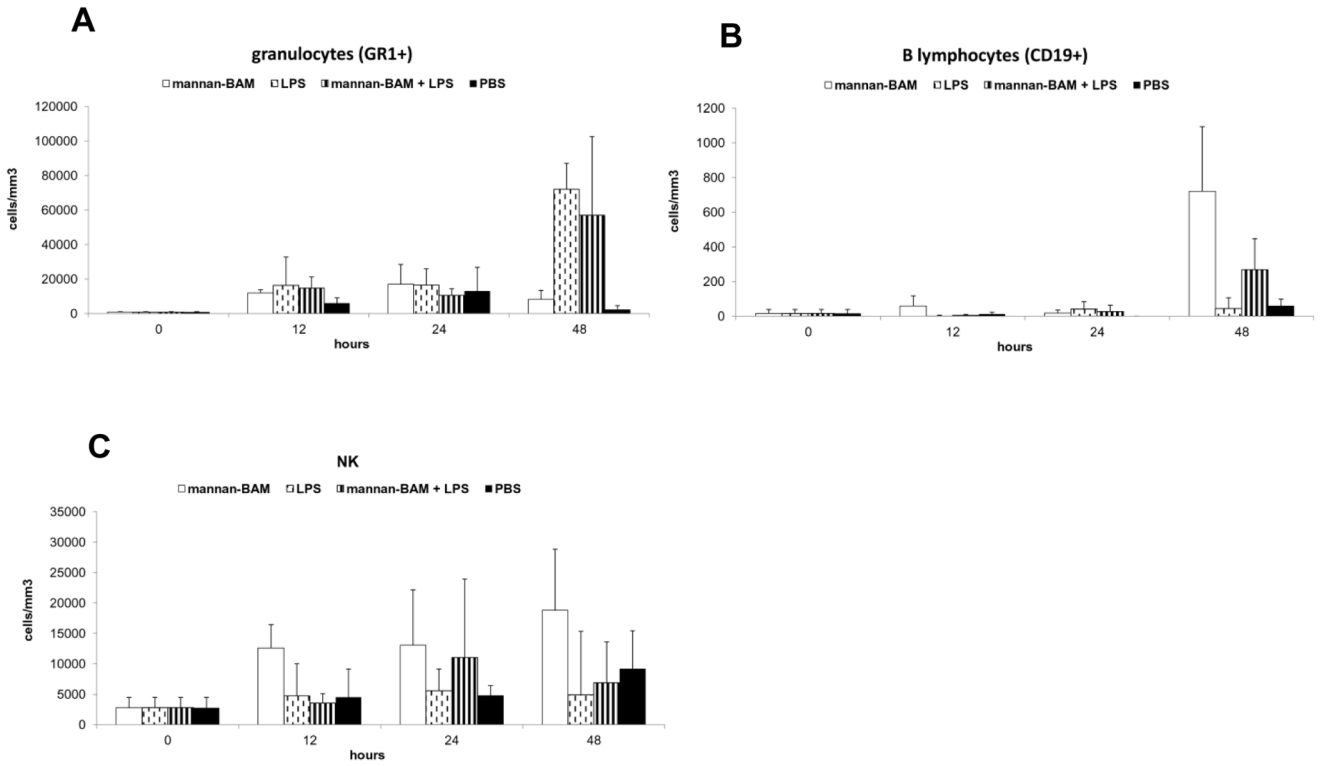

Figure 4. Analysis of cell infiltrate in the tumor during therapy based on the use of mannan-BAM, LPS and their mixture. Groups of 9 mice received a single dose of 0.2 mM mannan-BAM in PBS, LPS (0.5 mg/ml PBS), mixture of 0.2 mM mannan-BAM and LPS (0.5 mg/ml) in PBS, and PBS alone in 50 µl i.t. 3 mice from each group were killed in 12, 24 and 48 hours intervals, cells from excised tumors were prepared by enzymatic treatment (Liberase DL and DNase I) and analysed by flow cytometry. The following labelled antibodies were used: (A) anti-Mouse Ly-6G (Gr-1) Alexa Fluor 700 for granulocyte detection, (B) anti-Mouse CD19 APC for detection of B lymphocytes and (C) anti-Mouse NK1.1 PE for NK cells.
doi:10.1371/journal.pone.0085222.g004

experiment with laminarin-BAM, LPS and their mixture. An increase of granulocyte (GR1+) count in groups f-MLFKK-BAM/LPS and LPS was observed (Figure 5). The increase of cell count in the group f-MLFKK-BAM/LPS preceded that in the group LPS (24 hours difference). The total number of tumor infiltrating cells during 48 hours of experiment was comparable in both groups. Simultaneous presence of agonists of both signalling and phagocytic receptors led to early culmination of granulocyte infiltration only. No changes in monocyte/macrophage (F4/80+), T lymphocyte (CD3+), CD4+, CD8+, NK, B lymphocyte (CD19+) count were observed.

In all three above mentioned experiments the levels of IL-1beta, TNF-alpha, IL-6, and IL-8 were determined. No signs of synergy between LPS and phagocytic ligands causing increased cytokine levels were observed. Ligands alone and LPS alone caused an increase of all cytokines, which corresponds to the onset of

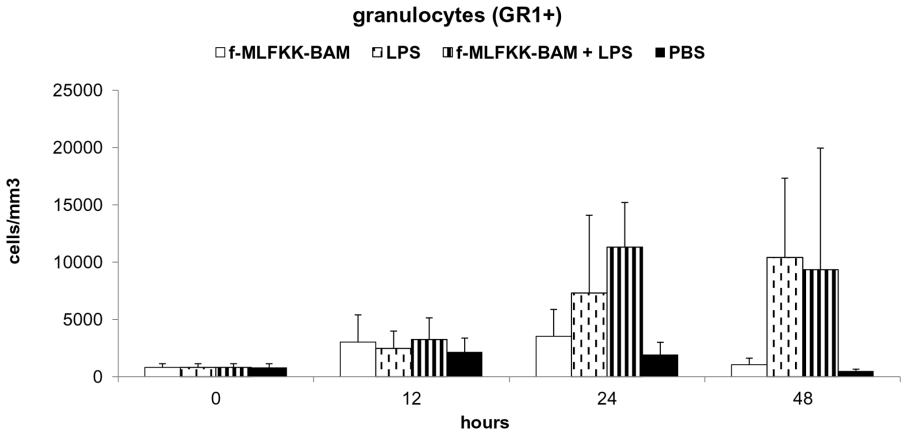

Figure 5. Analysis of cell infiltrate in the tumor during therapy based on the use of f-MLFKK-BAM, LPS and their mixture. Granulocyte detection. Groups of 9 mice received a single dose of 0.5 mM f-MLFKK-BAM, LPS (0.5 mg/ml), mixture of 0.5 mM f-MLFKK-BAM and LPS (0.5 mg/ml), and PBS alone in 50 µl i.t. Preparation of cell suspension and granulocyte staining were performed as in Figure 3.
doi:10.1371/journal.pone.0085222.g005

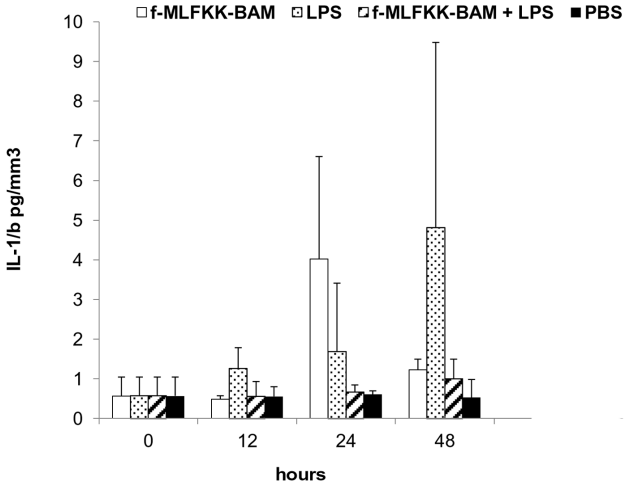

Figure 6. Changes of intratumoral levels of IL-1beta during therapy based on the use of f-MLFKK-BAM, LPS and their mixture. Groups of 9 mice received a single dose of 0.5 mM f-MLFKK-BAM, LPS (0.5 mg/ml), mixture of 0.5 mM f-MLFKK-BAM and LPS (0.5 mg/ml), and PBS alone in 50 µl i.t. 3 mice from each group were killed in 12, 24 and 48 hours intervals. After preparation of cells from excised tumors, corresponding supernatants were used for IL-1beta determination. IL-1beta levels are expressed as pg of IL-1beta/mm^3 of tumor tissue.
doi:10.1371/journal.pone.0085222.g006

inflammatory processes. Levels of typical proinflammatory cytokine IL-1beta are shown in Figure 6.

Histology

Melanoma bearing mice were treated with phagocytic receptor ligands, laminarin-BAM, mannan-BAM and f-MLFKK-BAM alone, or in combination with LPS. Two mice from each group were killed in 24 h intervals (24 h, 48 h, 72 h). Figure 7A shows negligible granulocyte infiltration in the case of PBS application. Application of particular agonists of phagocytic receptors and LPS alone resulted in partial reduction of tumor structures (Figure 7B). The highest reduction was noted for LPS, followed by laminarin-BAM, mannan-BAM and f-MLFKK-BAM. Infiltration constituted by granulocytes (48 h) changed in favour of monocytes/macrophages (72 h). Combinations of LPS with agonists of phagocytic receptors caused a significant reduction of tumor structures (Figure 7C,D).

In vitro analysis of the effect of macrophages activated by a TLR ligand on melanoma cells bearing phagocytic ligands

Anchored laminarin-BAM. As shown in Figure 8A, the effect of resting or LPS-activated PMJ2R macrophages on melanoma cells was similar and low. Anchoring of the phagocytic ligand on melanoma cells enhanced the cytotoxic effect of intact PMJ2R macrophages only slightly. A statistically significant effect was observed when LPS activated PMJ2R macrophages reduced number of laminarin-BAM bearing melanoma cells by 41%.

Anchored mannan-BAM. Neither resting nor LPS activated PMJ2R macrophages caused any effect on melanoma B16-F10 or mannan-BAM bearing melanoma B16-F10 cells (Figure 8B).

Anchored mannan-BAM. Medium with native serum. This experiment was performed as the previous one, but with one modification: foetal calf serum was not heat inactivated; hence complement activity was preserved. Resting PMJ2R cells reduced the number of B16-F10 by 8%. LPS activated macrophages caused 34% statistically not significant reduction of B16-F10 cells. The effect of mannan-BAM binding on tumor cell surface was negligible (3% reduction). Resting PMJ2R macrophages reduced the number of ligand-labelled melanoma cells by 28%. LPS activated PMJ2R macrophages reduced the number of mannan-BAM bearing melanoma cells highly significantly (64% reduction) (Figure 8C). The last two experiments proved the role of complement in killing of mannan-BAM bearing melanoma cells.

Anchored f-MLFKK-BAM. As shown in Figure 8D, resting PMJ2R macrophages showed a statistically not significant reduction of the number of B16-F10 cells (13%). LPS activated macrophages significantly reduced the number of B16-F10 cells (20% reduction). Anchoring of f-MLFKK-BAM on B16-F10 surface caused significant 44% decrease of B16-F10 cell number. Resting PMJ2R reduced the number of f-MLFKK-BAM bearing melanoma cells (B16-F10+ f-MLF) by 17%. LPS activated PMJ2R reduced the number of f-MLFKK-BAM bearing melanoma cells by 33% (statistically significant).

Interaction of macrophages with melanoma cells labelled with phagocytic ligands. Formation of clusters

The influence of laminarin, mannan, and f-MLF (free and bound) on interaction of PMJ2R macrophages with melanoma B16-F10 was studied. Formation of macrophage/melanoma clusters was observed when laminarin-SMCC was covalently bound on melanoma cells. In case of f-MLF, optimal conditions for cluster formation were achieved, when f-MLFKK-BAM was added directly to the mixture of both cells (0.05 mM final concentration), see Figure 9A. Free f-MLF did not show any effect (Figure 9B). Both laminarin-SMCC and f-MLFKK-BAM dependent clusters were composed of PMJ2R and melanoma cells, as proved by immunofluorescence using anti-CD11b-FITC conjugate for PMJ2R staining (all nuclei were stained by DAPI). Mannan-dependent formation of clusters was never observed.

Macrophage activation by laminarin anchored (laminarin-BAM) or covalently bound (laminarin–SMCC) to tumor cells. Cell signalling

To confirm that laminarin anchored to tumor cells activates macrophage cells we measured the phosphorylation of kinase NF-κB p65 (Ser536), a downstream signalling molecule of Dectin-1/SYK signalling pathway [21]. The phosphorylation of NF-κB p65 was determined in coculture of tumor cells and PMJ2R in the presence of laminarin-BAM (0.05 mM final concentration) at indicated times after seeding. Free laminarin at the same concentration was used as a control. As shown in Figure 10A, phosphorylation/activation of NF-κB p65 raised by increasing time of incubation when laminarin –BAM was present in the coculture. Free laminarin did not activate NF-κB p65. Similarly, an increase of NF-κB p65 activation occurred when laminarin-SMCC was covalently bound to B16-F10 cells prior seeding with PMJ2R cells (Figure 10B).

Capability of BAM and DOPE to anchor molecules to cell membranes

Anchoring of BAM and DOPE with covalently bound B-phycoerythrine (PE) to melanoma cells was studied. Fluorimetric method of PE-BAM, PE-DOPE and PE determination was optimised. The amount of bound compounds was calculated from the decrease of fluorescence of their solutions after incubation with cells. Non-specifically bound molecules (PE background) were

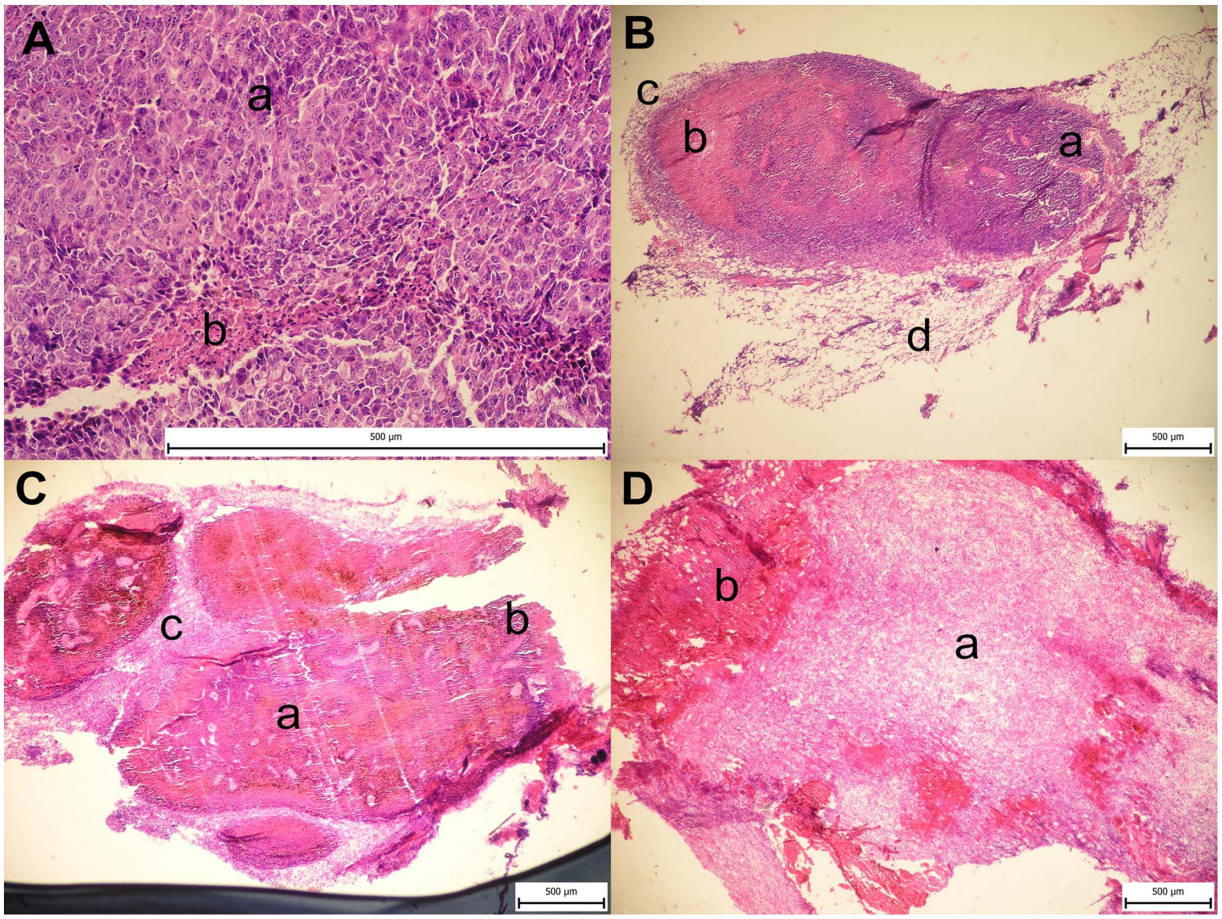

Figure 7. Histology. Melanoma bearing mice were injected i.t. with 50 µl of BAM derivatives of agonists (0.2 mM laminarin-BAM, 0.2 mM mannan-BAM, 0.5 mM f-MLFKK-BAM), their mixtures with LPS (0.5 mg/ml), LPS and PBS alone. Two mice from each group were killed in 24 hours intervals (24, 48, 72 hrs). Excised tumors were fixed with 4% neutral solution of formaldehyde and paraffin blocks were prepared. Sections were stained with hematoxylin/eosin. A– PBS alone; B– effect of particular agonists of phagocytic receptors and LPS alone; C, D– synergistic effect of LPS combinations with particular agonists of phagocytic receptors. Aa – melanoblasts, Ab – necrotic focus with slight granulocyte infiltration, Ba – melanoblasts, Bb – necrotic focus with hemorrhage, Bc – granulation tissue, Bd – slacked edematous ligament, Ca – necrotic tissue with hemorrhage, Cb – negligible residue of tumor, Cc – edematous ligament with inflammatory infiltration, Da – slacked edematous ligament with inflammatory infiltration and hemorrhage foci, Db – bleeding necrosis.
doi:10.1371/journal.pone.0085222.g007

subtracted. As shown in Table 5, at higher original concentrations (before binding) both BAM and DOPE offer similar binding capacity. At lower concentrations BAM is more suitable.

Discussion

The use of agonists of Toll-like receptors in cancer treatment has been tested many times. Chicoine et al. [22] achieved significant tumor regression using intratumorally applied LPS. Nevertheless, their model was considerably artificial (subcutaneously implanted mouse glioblastom) and to obtain tumor regression, high and very toxic doses of LPS (200 µl solution of 2–2.5 mg LPS/ml PBS) were used. In our preliminary experiments (data not shown) we found that LPS solution in concentrations 1 mg LPS/ml and higher is directly cytotoxic, hence, the above mentioned study cannot be considered solely immunological. We are using solution of 0.5 mg LPS/ml. This solution did not show any cytotoxicity either for melanoma or normal not transformed cells (primary culture of guinea pig kidney cells) during 24 hour cultivation (*in vitro* experiments, data not shown). Fifty microliters of the mentioned solution was applied intratumorally in our experiments, which corresponds to 25 µg of LPS only. Mariani at al. [23] achieved inhibition of tumor growth by intratumoral applications of LPS using rat glioma RG-2 cells implanted subcutaneously. It was necessary to use high LPS doses (intratumoral application of 50 µl of LPS at concentration as high as 100 mg LPS/ml saline, hence 5 mg of LPS). Reduction of tumor growth based on intratumoral injection of flagelline (TLR5 agonist) was studied by Rhee et al. [24]. The model used was again artificial (human colon carcinoma transplanted to nu-nu mice), hardly comparable with the fast growing aggressive melanoma B16-F10 exploited in our studies. Synthetic analogues of signal receptors used by pharmaceutical industry have not yielded satisfactory results in cancer treatment [25].

The possibility of using phagocytic receptor agonists in cancer therapy was proved in our study.

First it was necessary to demonstrate that compounds used in our *in vivo* experiments activate innate immunity via activation of phagocytic receptors. It was guaranteed not only by the use of specific well described [26,27] ligands of these receptors. Binding of phagocytic receptor agonists to corresponding receptors has to be strengthened by multiplications of these bonds [28], hence only

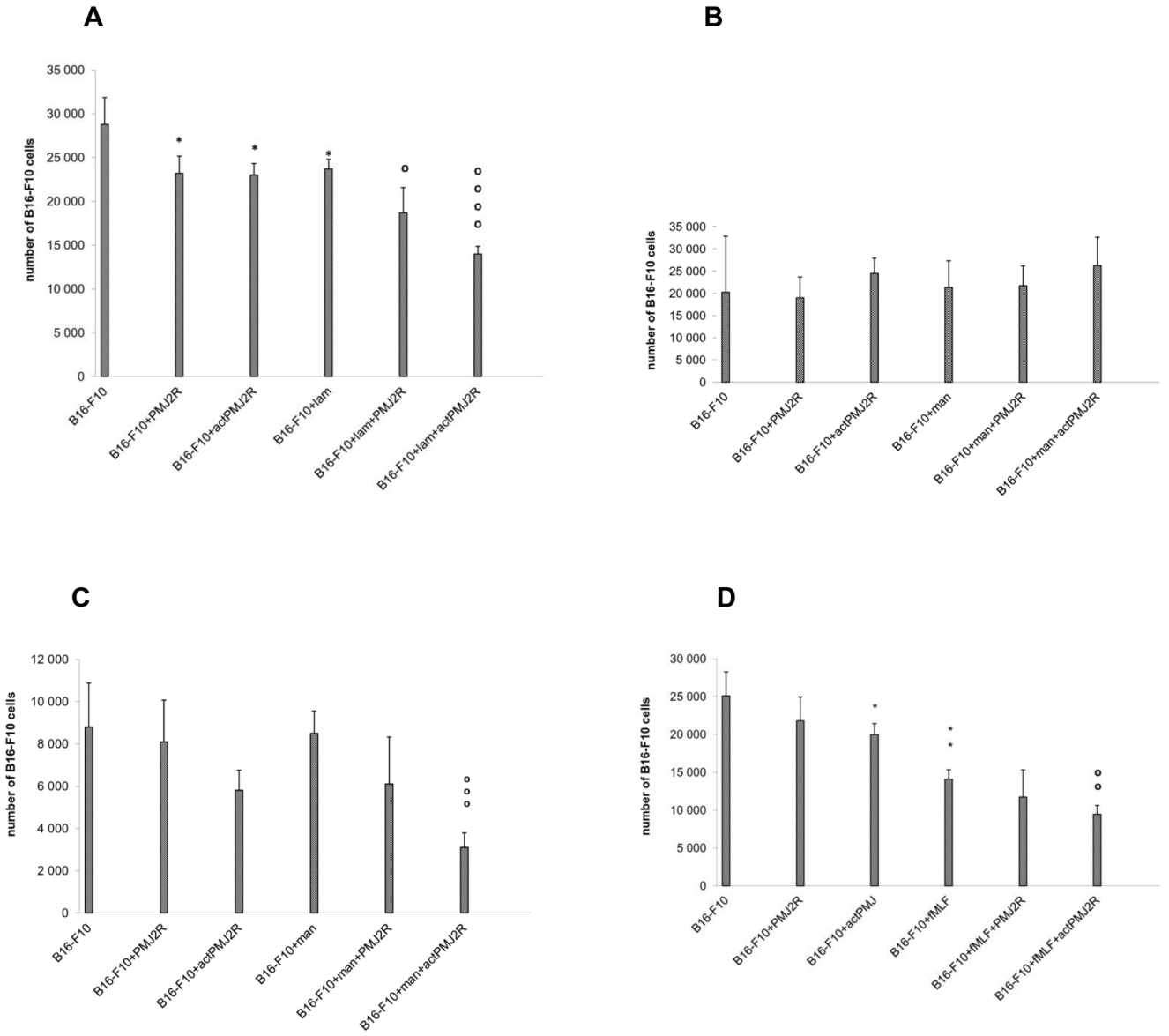

Figure 8. *In vitro* analysis of the effect of macrophages activated by LPS on melanoma cells bearing ligands of phagocytic receptors. Murine B16-F10 melanoma cells grown to confluency in 96 well tissue culture plate were incubated (30 min, 37°C) with solution of phagocytic receptor agonists (0.02 mM laminarin–BAM or 0.02 mM mannan-BAM or 0.05 mM f-MLFKK-BAM in culture medium) and subsequently washed. Cells of murine macrophage cell line PMJ2R were preincubated with LPS (1 μg/ml) for 2 hours at 37°C, washed, and added to B16-F10 in the ratio 5:1. This mixture was incubated for 4 hours at 37°C. After incubation, PMJ2R and dead cells were carefully washed off. Living B16-F10 melanoma cells were released by trypsinisation and calculated. (A) laminarin-BAM, (B) mannan-BAM, (C) mannan-BAM, cells cultured in medium with non-inactivated fetal calf serum, (D) f-MLFKK-BAM. *P≤0.05, **P ≤ 0.005 compared to B16-F10 **o**P≤0.05, **oo**P≤0.005, **ooo**P≤0.0005, **oooo**P≤0.00005 compared to B16-F10+ ligand. The experiment was repeated twice with similar results.
doi:10.1371/journal.pone.0085222.g008

ligands anchored to the cell surface are active. It corresponds well with our experiments. Moreover, the anticancer activity of all compounds tested was fully dependent on molecular motives interacting with phagocytic receptors; no role of anchoring system was detected. Observed onset of inflammation with characteristic picture (increase of proinflammatory cytokines and infiltration of inflammatory cells) indicated participation of considered mechanisms. All *in vitro* experiments with macrophages also supported our opinion of the role of bound phagocytic ligands (ligands which did not bind to B16-F10 cells were washed out). BAM and DOPE are commercial tools for anchoring molecules to cells; nevertheless, we verified their binding capacity to melanoma cells by direct fluorescence based quantitative measurement *in vitro*.

Nevertheless, the effect of anchored agonists of phagocytic receptors caused only partial reduction of tumor growth, comparable with the effect of agonists of TLR. The combination of soluble agonists of TLR and membrane anchored agonists of phagocytic receptors was proven to be the key step. This combination led to huge synergistic reaction, causing strong reduction of tumor growth. Frequent shrinkage and even vanishing of tumors was observed. The vanishing was often temporary. Statistically significant prolongation of survival and frequently complete recovery of mice were achieved by appropriate therapeutic regimes (mice are so far

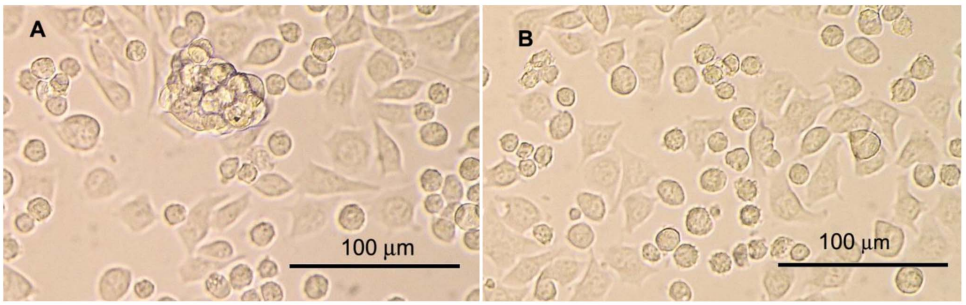

Figure 9. Interaction of macrophages with melanoma cells labelled with phagocytic ligands. Formation of clusters. To the mixture of melanoma B16-F10 cells and macrophage cell line PMJ2R laminarin-BAM (A) or free laminarin (B) were added (0.05 mM final concentrations). Photos were taken after 1 hour incubation at 37°C. The experiment was repeated four times with similar results.
doi:10.1371/journal.pone.0085222.g009

living for hundreds of days). As all interest was concentrated on long lasting survival, the question of metastases was solved only partially. Nevertheless, it is clear that the therapies used suppress metastases as well. Melanoma is a strongly metastasing tumor, and without this suppression mice would be killed by metastases.

Underhill and Gantner [28] described complex interplay of TLR signalization (soluble ligands) and activation of phagocytic receptors (bound ligands), leading to a coordinated inflammatory response. In the area of tumor therapy, this complexity has never been considered and applied.

As the first agonist of phagocytic receptors, low molecular weight soluble laminarin was chosen. Laminarin belongs to β-glucans, whose antitumor properties have been clearly proved in the last 40 years [29]. High molecular weight β-glucans applied per orally are used most frequently. The mechanism of their effect

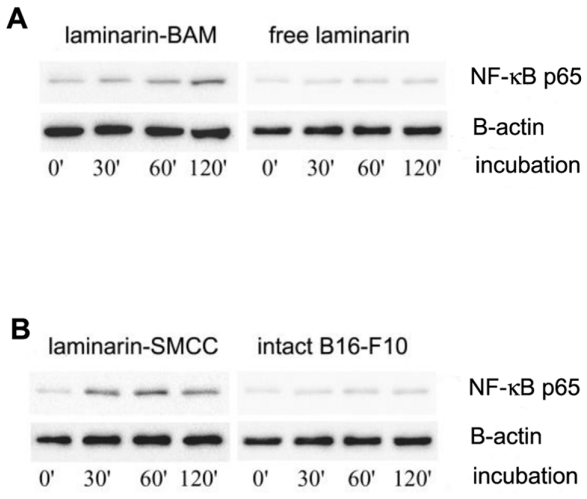

Figure 10. Macrophage activation by laminarin anchored (laminarin-BAM) or covalently bound (laminarin–SMCC) to tumor cells. B16-F10 cells were cultured with PMJ2R cells for indicated time in the presence of 0.05 mM laminarin-BAM. Free laminarin at the same concentration was used as a control. NF-κB kinase activation was assessed by immunoblotting with antibody specifically recognizing only phosphorylated form. β-actin is shown as a loading control. Two independent experiments were performed. Representative blots are shown (A). In further experiment laminarin-SMCC was covalently bound to B16-F10 cells prior seeding with PMJ2R cells. Intact B16-F10 melanoma cells were used as a control. NF-κB kinase activation was assessed as previously described. Resulting blots are shown (B).
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(stimulation of granulocyte, monocyte and macrophage activity) was described by Chan et al. [30]. Nevertheless, detailed knowledge of the mechanism of action is still missing [29]. We were able to achieve as much as 50% reduction of tumor growth with per orally administered high molecular weight β-glucan (*Sacharomyces cerevisiae*) (data not shown). Intratumoral application of high molecular weight β-glucan did not show any effect, similar to intratumoral application of laminarin. Lack of effect of laminarin was not surprising, and corresponded to the knowledge of low molecular weight β-glucans [31]. Soluble laminarin is used as effective inhibitor of Dectin-1 [32]. This is in contrast with our goal to activate Dectin-1, an important phagocytic receptor (expressed on macrophages, neutrophils and dendritic cells). Therefore we anchored laminarin to cancer cells using BAM. This way, a prerequisite for Dectin-1 binding was created. Melanoma cells represent the predominant cell population in the tumor (proved by flow cytometry, data not shown), therefore phagocytic attack was directed against them. When LPS was added, this attack was dramatically enhanced (strong synergy), regardless of partial inhibition of Dectin-1 expression under the influence of LPS described by Willment et al. [33].

As a second agonist of phagocytic receptors, terminal mannose was studied. Terminal mannose is recognised by mannose receptor (MR) occurring mainly on macrophages [34]. Activation of complement by mannose (mannan) binding lectin (MBL) must also be considered. This results in both cell opsonization on C3b level, and formation of cytotoxic terminal complexes. Down-regulation of MR by LPS [35] must also be taken into account. Nevertheless, good therapeutic results were obtained in our experiments.

F-MLF was studied as the last agonist of phagocytic receptors. It stimulates formylmethionine phagocytic receptors (FPRs). Seven FPRs were described in mice, three in humans [36]. In this case, in contrast with MR, no inhibition caused by LPS was described. On the contrary, LPS supports expression of FPRs genes in murine macrophages and neutrophils [37]. Therefore, all experiments with a combination of LPS and anchored f-MLF resulted in strong reduction of tumor growth.

Humans are 1000–10,000 times more sensitive to LPS than mice [38]. The reason is the absence of, so far not well defined, serum proteins, which are able to block the majority of LPS in rodents. In the case of using LPS in combination with phagocytic receptors in human therapy, it will be necessary to work with very low, safe concentrations of LPS. Another way is to replace LPS with other agonists of TLR, like LTA or flagellin.

Using a combination of TLR and phagocytic receptor ligands we achieved 80% (mannan-BAM + LPS) and 60%

Table 5. Proof and quantification of PE-BAM and PE-DOPE conjugates anchored to the cell surface.

Compound	Concentration(nmol/l)	Amount of compounds bound to 100,000 melanoma cells (fmol)	Number of molecules specifically bound (anchored) to one melanoma cell
PE-BAM	2.5	104.84	338,211
	1.25	46.36	150,482
	0.625	27.91	68,889
PE-DOPE	2.5	113.80	392,186
	1.25	33.35	72,164
	0.625	18.99	15,151
PE	2.5	48.68	0
	1.25	21.37	0
	0.625	16.47	0

All measurements were done in triplicates.
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(f-MLFKK-DOPE + LPS) long lasting survival (more than 100 days). To achieve long lasting survival, it is necessary to use well anchored agonists of phagocytic receptors, in an appropriate combination with agonists of TLR and the right timing of therapy. Pulse regime and intensification of therapy at its beginning proved to be very effective.

In the case of combination of LPS with f-MLF-$(G)_5$-$(K)_{10}$-STE or with mannan SMCC (some regimes), we achieved very strong reduction of tumor growth (more than 98%) and temporary disappearance of the majority of tumors. Nevertheless, the treatment did not result in long lasting survival and complete recovery of mice. In case of f-MLF-$(G)_5$-$(K)_{10}$-STE we suppose that the reason for this could be splitting of oligolysine chain by trypsin and trypsin–like proteases of tumor origin, so the agonist had limited lifespan. In the case of mannan SMCC, we suppose that molecules interacting on charge principle or on the basis of hydrophobic anchoring (BAM, DOPE) can be released from damaged cells and attack new cells again. Molecules bound covalently act well but not repeatedly. This hypothesis has to be proven.

Our *in vitro* experiments showed that agonists of phagocytic receptors anchored to tumor cell surface enhance cytotoxic effect of resting phagocytes and especially of phagocytes activated by a TLR ligand. Flow cytometry analysis of cell infiltrate performed in *in vivo* experiments revealed that the presence of a mixture of TLR and phagocytic receptor agonists results in faster commencement of inflammatory infiltration. The overall magnitude of infiltration was the same as when individual agonists were applied. This phenomenon however was not observed in case of mannan (discussed below).

On the basis of these analyses it is not possible to clarify to a full extent the huge antitumor effect of mixtures of TLR and phagocytic ligands observed in *in vivo* experiments. We suppose that this substantial synergy between agonists of phagocytic and Toll-like receptors is based on two events. The TLR ligand induces early and massive inflammatory infiltration of tumors. The effect of this cell infiltrate is directed towards tumor cells, bearing agonists of phagocytic receptors on their surface. As shown by histology, this results in effective killing of tumor cells. The overall antitumor effect could be strengthened by interplay of TLR and phagocytic receptors [28].

Activation of TLR and phagocytic receptors did not always result in synergy. Mannose linked with short peptide and anchored by hydrophobic chain of stearic acid markedly reduced tumor growth, however its administration with LPS was counter-productive. Mannose bound this way probably served as a suitable agonist of the mannose receptor, which is efficiently downregulated by LPS [35]. Mannose as the terminal part of mannan-BAM apparently activated the lectin pathway of complement by means of MBL. This pathway is LPS insensitive. Therefore it was possible to achieve strong synergy between LPS and mannan-BAM. LPS apparently caused massive infiltration of the tumor by phagocytic cells. Opsonization of tumor cells by C3b/iC3b complement components created conditions for the attack of phagocytes against tumor targets. Our *in vivo* experiments correspond well to experiments performed *in vitro*, where the effect of mannan-BAM was dependent on functional complement in culture medium.

Flow cytometry analysis of cell infiltrate did not reveal any signs of mannan-BAM/LPS synergy. As previously described, complement activation and opsonization of tumor cells led to antitumor attack, nevertheless this pathway is probably not connected with interplay of TLR and phagocytic receptors, which could influence inflammatory infiltration.

The strength of binding of phagocytic receptor agonists to tumor cells is very important for the effect of these agonists on tumor growth, and especially for effective synergy with LPS. Tumor cells have a negative surface charge, which is caused by occurrence of sialic acid and phospatidylserine [39,40]. The binding of phagocytic ligands based on charge interaction (positive charged oligolysine chain) seems to be insufficient. Anchoring of agonists based on aliphatic chain of stearic acid (or oleoyl acid as in BAM) proved to be very suitable. Two chains anchoring (DOPE) in pulse regime gave also good results (prolongation of survival). Application of covalently bound agonists of phagocytic receptors (SMCC) resulted in highly significant reduction of tumor volume and even frequent temporary disappearance of tumors. However, the effect on survival was low.

Both *in vivo* and *in vitro* experiments proved killing of tumor cells dependent on binding of phagocytic receptor ligands to tumor cells. To elucidate mechanisms of these processes, we studied first steps of interaction of phagocytes with ligand bearing tumor cells, i.e. clusters formation and cell signalling. Formation of clusters, the first step of interaction of phagocytes with melanoma cells, was observed in case of laminarin and f-MLF. Despite testing various conditions, no clusters were observed in case of bound mannan.

When ligands are bound to the cell surface in sufficient density, then phagocytic receptor- ligand interaction leads to clustering of receptors followed by intracellular signalling [41]. Activation of

Dectin-1 by anchored laminarin was chosen for cell signalling experiments as Dectin-1 is the best-characterized non-opsonic phagocytic receptor [41]. Experiments confirmed that only bound ligand can trigger phagocytic receptors.

In summary, we have found novel principles of effective cancer therapy. The therapy is based on the use of anchored agonists of phagocytic receptors especially in combination with stimulation of cell signalling receptors like TLR4. Further, we would like to design agonists of phagocytic receptors which will bind specifically to tumor cells. The replacement of LPS with human-safe agonists of signalling receptors and various routes of therapeutic mixtures administration will be a matter of further research.

Author Contributions

Conceived and designed the experiments: JŽ TJ MJ JS. Performed the experiments: TJ MJ MA IŠ PB VM ZK ŠČ ZV VC PR NV MW JL JŽ. Analyzed the data: TJ MJ KL JŽ. Wrote the paper: JŽ. Manuscript discussed by: JK JS.

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Paper 2

The use of Zymosan A and bacteria anchored to tumor cells for effective cancer immunotherapy: B16-F10 murine melanoma model

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The use of Zymosan A and bacteria anchored to tumor cells for effective cancer immunotherapy: B16-F10 murine melanoma model

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ABSTRACT

The idea of using killed microorganisms or their parts for a stimulation of immunity in the cancer immunotherapy is very old, but the question of interactions and binding of these preparations to tumor cells has not been addressed so far. The attachment of Zymosan A and both Gram-positive and Gram-negative bacteria to tumor cells was tested in *in vivo* experiments. This binding was accomplished by charge interactions, anchoring based on hydrophobic chains and covalent bonds and proved to be crucial for a strong immunotherapeutic effect. The establishment of conditions for simultaneous stimulation of both Toll-like and phagocytic receptors led to very strong synergy. It resulted in tumor shrinkage and its temporary or permanent elimination. The role of neutrophils in cancer immunotherapy was demonstrated and the mechanism of their action (frustrated phagocytosis) was proposed. Finally, therapeutic approaches applicable for safe human cancer immunotherapy are discussed. Heat killed *Mycobacterium tuberculosis* covalently attached to tumor cells seems to be promising tool for this therapy.

1. Introduction

Positive correlations between infection and the remission of malignant diseases was first observed in the 18th century [1]. This phenomenon was thoroughly studied at the end of the 19th century by W. Coley, who assembled a so-called Coley's toxin, a mixture of inactivated Gram-positive bacteria *Streptococcus pyogenes* and Gram-negative *Serratia marcescens* [2]. The Coley's toxin preparation was not developed as a therapy because of the lack of a standardized preparation, the need for daily applications for extended periods of time, side effects such as fever, and unsatisfactory documentation of therapeutic response [3].

The discovery of pathogen associated molecular patterns (PAMPs) laid the foundation for more exact use of microorganisms and their parts in cancer immunotherapy. This resulted in many studies and culminated in clinical trials at the beginning of the 21st century. These studies were focused on the use of synthetic ligands of signalling receptors (mainly Toll-like receptors 3, 7, 9) in tumor treatment [4]. Unfortunately, the above-mentioned ligands did not provide satisfactory outcomes in cancer treatment [5].

In our previous study we described a novel, innate immunity-based strategy of cancer treatment [6], which was based on the combination of soluble agonists of TLR receptors with ligands of phagocytic receptors attached to tumor cells. TLR activation leads to strong inflammatory infiltration. Agonists of phagocytosis-related receptors direct phagocytic cells to artificially opsonized tumor cells resulting in their killing.

The appropriateness of the use of more complex sets of PAMPs for effective immune response is strongly stressed [7]. Therefore, we used Zymosan A (cell walls of *Saccharomyces cerevisiae*) and both Gram-negative and Gram-positive killed bacteria for tumor immunotherapy. These complex particles offered the possibility to stimulate both signalling receptors (TLR, NLR and others) and phagocytic receptors. We anchored them to tumor cell surface and studied the influence of this binding on tumor immunotherapy. Despite the fact that the idea of using microorganisms in tumor treatment is very old, there is no study that would address their interaction and binding to tumor cells thus far.

2. Materials and methods

2.1. Ethics statement

All experiments with mice were performed according to corresponding laws of the Czech Republic. The design of the study was approved by both the Committee of Biology Centre of the Academy of Sciences of the Czech Republic and the National Committee (protocols

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no. 138/2008). For anaesthesia of mice, intraperitoneal application of a mixture of Ketamine.HCl (75 mg/kg) and Xylazine. HCl (75 mg/kg) was used.

2.2. Materials

Tissue culture media, media supplements, Zymosan A from *Saccharomyces cerevisiae*, lipopolysaccharides (LPS) from *Escherichia coli*, oligolysine (Mw 500–2000), laminarin from *Laminaria digitata*, mannan from *Saccharomyces cerevisiae*, f-MLF (*N*-formyl-methionyl-leucyl-phenylalanine), L-lysine, Tris(2carboxyethyl)phosphine hydrochloride (TCEP), GM-CSF, TNFα, and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4-(*N*-maleimidomethyl) cyclohexanecarboxylic-acid N-hydroxysuccinimide ester (SMCC) was obtained from Thermo Scientific (Erembodegem, Belgium). *N*-Formyl-methioninyl-leucyl-phenylalanine coupled with two lysine molecules (f-MLFKK) was synthesized by Schafer-N (Copenhagen, Denmark). Biocompatible Anchor for cell Membrane (BAM, Mw 4000) was purchased from NOF EUROPE (Grobbendonk, Belgium). Blood agar with 5% of defibrinated ram blood was prepared by Dulab (Dubné, Czech Republic). Polyethylene glycol (M_r 10,000, PEG 10000) was obtained from Aldrich (Milwaukee, WI, USA).

2.3. Cell lines, bacteria and mice

Murine melanoma B16-F10 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Melanoma cells were cultivated in RPMI 1640 (Sigma-Aldrich, USA) supplemented with 10% fetal calf serum (FCS) purchased from PAA (Austria) and antibiotics. Cultivation was performed at 37 °C in a humidified atmosphere with 5% carbon dioxide.

Stenotrophomonas maltophilia (Hugh 1980, Palleroni and Bradbury 1993), strain CCM 1640 and *Serratia marcescens* subsp. *Marcescens* (Bizio 1823), strain CCM 303 were purchased from the Czech Collection of Microorganisms (Brno) and cultivated on blood agar at 37 °C.

Heat killed *Mycobacterium tuberculosis* and *Listeria monocytogenes* were purchased from InvivoGen (Toulouse, France).

SPF C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). All mice (weight 18–20 g) were housed in barrier facilities with free access to sterile food and water; the photoperiod was 12/12.

2.4. Synthesis of laminarin-BAM, mannan-BAM, f-MLFKK-BAM, lysine-BAM, and Zymosan A-BAM

Binding of BAM anchor requires the presence of amino group. Lysine, f-MLFKK and Zymosan-A contain amino groups, laminarin and mannan were aminated by reductive amination as previously described [8] and subsequently dialyzed (MWCO 3500 dialysis tubing, Serva, Heidelberg, Germany) against PBS at 4 °C overnight. Amino groups thereafter reacted at pH 7.3 with N-hydroxysuccinimide group of BAM according to Kato et al. [9]. In case of Zymosan A-BAM synthesis the original Zymosan A was sonicated (sonicator Hielscher UP200S, 10 × 10 s, ice) before the reaction with BAM.

2.5. Synthesis of mannan-SMCC, lysine-SMCC, Zymosan A-SMCC, Mycobacterium tuberculosis-SMCC, and Listeria monocytogenes-SMCC. Their in vivo and in vitro application

Binding of *N*-hydroxysuccinimide group of SMCC to amino groups of aminated mannan, Zymosan-A, lysine, *Mycobacterium tuberculosis* and *Listeria monocytogenes* was performed as recommended by the manufacturer of SMCC (Thermo Scientific, Pierce Protein Biology Products).

Binding of SMCC ligands to tumor cells requires the presence of –SH groups on these cells. It was achieved on the basis of a reduction of cystines as previously described [10]. In experiments *in vivo*, reducing agent (50 mM solution of TCEP in PBS) was injected intratumorally (i.t.). SMCC ligands were applied 1 h later.

As already proved [6], the injection of TCEP solution alone does not have any effect on tumor growth. In the case of *in vitro* experiments, a 5 mM DTT solution was used for reduction instead of TCEP. A reduction of melanoma cells suspension lasting for 1 h was performed on ice. The excess of DTT was subsequently washed away.

2.6. Stenotrophomonas maltophilia and Serratia marcescens therapeutic preparations

Stenotrophomonas maltophilia and *Serratia marcescens* were killed by UV light (1 h exposure). Preparation of *Serratia marcescens*-oligolysine (bacteria with bound oligolysine) was performed according to Christiaansen et al. [10]. Shortly, after 1 h reduction of bacteria by 5 mM DTT on ice, 0.5 mM solution of oligolysine-SMCC in PBS was added and allowed to react for 1 h at room temperature. *Serratia marcescens* with bound oligolysine was purified by centrifugation (10 min, 10,000 × *g*, 4 °C).

Oligolysine-SMCC solution used was prepared according to the instructions of SMCC manufacturer.

2.7. Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) was carried out using a laboratory-made apparatus [11] at a constant voltage (−20 kV on the detector side) supplied by a Spellman CZE 1000 R high-voltage unit (Plainview, NY). The total lengths of the FS capillaries, 100 μm I.D. and 360 μm O.D. (Agilent Technologies, Santa Clara, CA), were 35 cm, with 20 cm long of the separation part of the capillary. The ends of the capillary and the electrodes were placed in 3-mL glass vials filled with background electrolyte (BGE). A LCD 2082 on-column UV–Vis detector (Ecom, Prague, Czech Republic), connected to the detection cell by optical fibers (Polymicro Technologies, Phoenix, AZ, USA), operated at 280 nm. Sample injection was performed by the siphoning action as described previously [11]. The height difference of the reservoirs for the sample injection, Δh, was 15 cm. Bacteria clusters were de-agglomerated by sonication in a Sonorex ultrasonic bath (Bandelin electronic, Berlin, Germany) and then

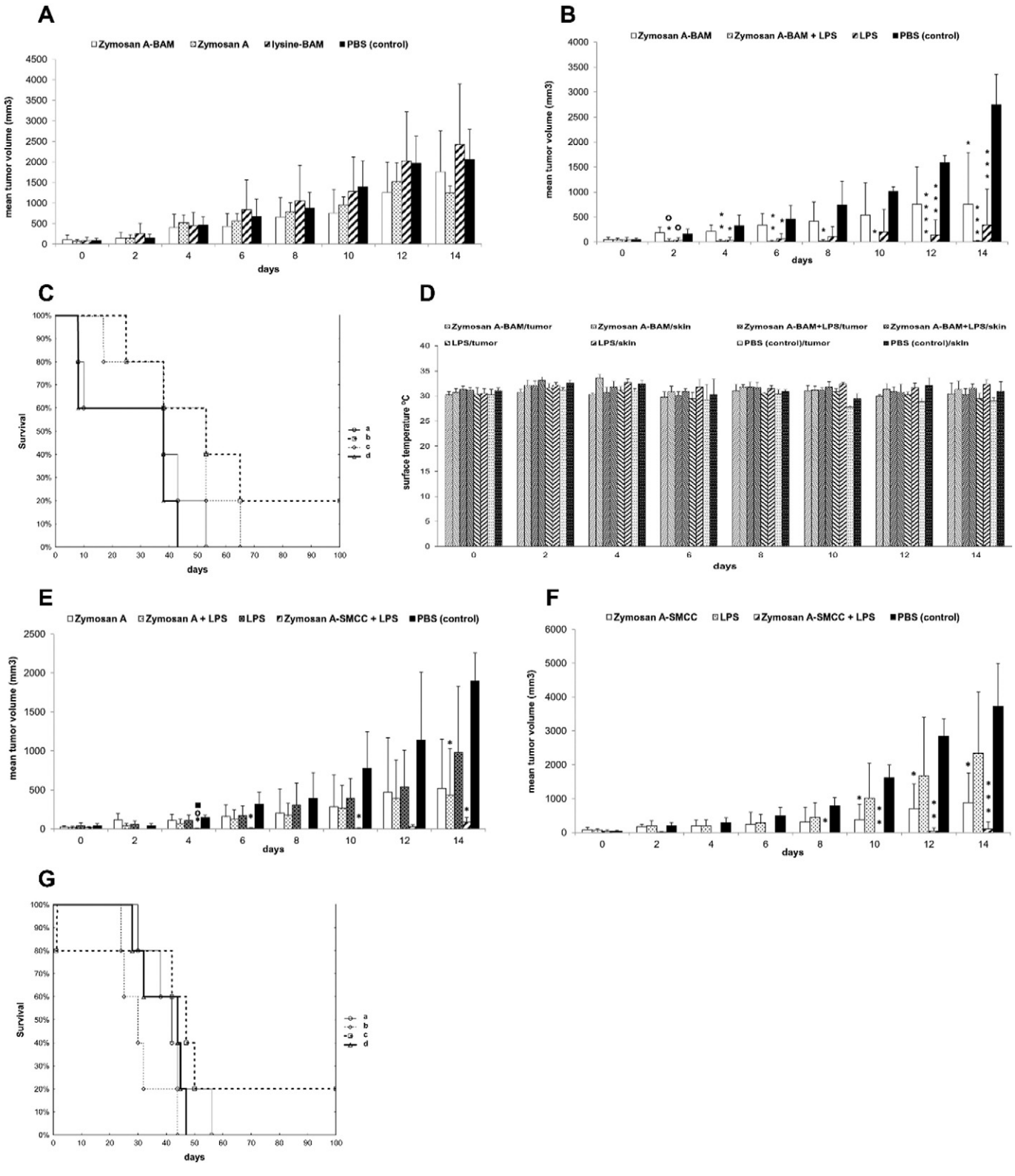
Fig. 1. The influence of Zymosan A (both free and anchored) on melanoma B16-F10 growth. Synergy with LPS. Suspension of B16-F10 melanoma cells in RPMI without FCS was inoculated subcutaneously (s.c.) in the previously shaved right flank of the mice. Each mouse received 4×10^5 melanoma cells in 0.1 ml of medium. Randomization into groups of 5 mice was performed twelve days after transplantation of melanoma cells and was followed immediately by initiation of therapies. These therapies were based on intratumoral application of 50 μl of corresponding preparations and continued every second day for 10 days (6 doses altogether). During the therapy, all mice were housed individually. Measurement of tumors and calculation of their volume were performed every second day for 14 days. In the first two experiments hydrophobic anchoring of Zymosan A (Zymosan A-BAM) was applied. The composition of preparations used was: A- 14 mg Zymosan A-BAM/ml PBS, 14 mg Zymosan A/ml PBS, 0.7 mM lysine-BAM in PBS, and PBS. B- 14 mg Zymosan A-BAM/ml PBS, 14 mg Zymosan A-BAM + 0.5 mg LPS/ml PBS, 0.5 mg LPS/ml PBS, and PBS. $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.005$, $^{****}P \le 0.001$ compared to PBS (control) **o**$P \le 0.05$ compared to Zymosan A-BAM. C- Survival analysis of previous experiment (Fig. 1B) is presented. a- Zymosan A-BAM, b- Zymosan A-BAM + LPS, c- LPS, d- PBS (control). D- Temperature analysis of experiment shown in Fig. 1B. Temperatures of both tumor surface and skin of the left tumor free flank are indicated. E- Effect of covalently bound Zymosan A (Zymosan A-SMCC). The composition of preparations used was: 14 mg Zymosan A/ml PBS, 14 mg Zymosan A + 0.5 mg LPS/ml PBS, 0.5 mg LPS/ml PBS, 14 mg Zymosan A-SMCC + 0.5 mg LPS/ml PBS, and PBS. $^{*}P \le 0.05$ compared to PBS (control). **o**$P \le 0.05$ compared to Zymosan A. ■$P \le 0.05$ compared to LPS. F- In the last experiment with Zymosan A-SMCC more intensive therapy was applied (daily intratumoral application for first three days after randomization in groups). The composition of preparations used was: 14 mg Zymosan A-SMCC/ml PBS, 0.5 mg LPS/ml PBS, 14 mg Zymosan A-SMCC + 0.5 mg LPS/ml PBS, and PBS. $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.005$ compared to PBS (control). G- Survival analysis of experiment shown in Fig. 1F. a- Zymosan A-SMCC, b- LPS, c- Zymosan A-SMCC + LPS, d- PBS (control).

vortexed using a Yellowline TTS 3 Digital Orbital Shaker (IKA Works, Wilmington, DE) immediately before injection of the bacterial sample into the capillary. The sonication was performed at 25 °C and 35 kHz for 1 min. For each sample. The detector signals were acquired and processed with the Clarity Chromatography Station (ver. 2.6.3.313, DataApex, Prague, Czech Republic).

Bacteria were suspended in PBS and their concentrations were adjusted to 5×10^8/ml. The injection time, $t_{inj.}$, of the sample was 10 s.

For CZE separations, 2×10^{-2} mol/l phosphate buffers pH 7 and 10, with addition of 5% (*v*/v) EtOH and 0.5%(w/v) PEG 10000, were used as BGE.

Before each CZE run, the capillaries were rinsed with acetone for 5 min and then back-flushed with the catholyte or BGE for 5 min. For this purpose, a single-syringe infusion pump (Cole-Parmer, Vernon Hills, IL) equipped with a 100 μl syringe (SGE Analytical Science, Victoria, Australia) was used at a flow rate ranging from 3 to 20 μl/min.



2.8. Tumor transplantation

Suspension of B16-F10 melanoma cells in RPMI without FCS was inoculated subcutaneously (s.c.) in the previously shaved right flank of the mice. Each mouse received 4×10^5 melanoma cells in 0.1 ml of medium.

2.9. Treatment and evaluation of treatment

Randomization of mice in groups was performed twelve days after transplantation of melanoma cells and was followed immediately by initiation of therapies. Therapies were based on an intratumoral application of 50 μl of corresponding preparations. All mice were housed individually during therapy.

Tumor size was measured every second day with callipers.

A formula $V = \pi/6\ AB^2$ (A = largest dimension of tumor, B = smallest dimension) was used for tumor volume calculation [12].

2.10. Mean reduction of tumor growth (%)

The calculation was performed as previously described [6]. On days 4, 6, 8, 10, 12 and 14 after beginning of therapy reduction of tumor growth was calculated as follows:

$$\frac{(\text{mean value of tumor volumes in control group} - \text{the same value for treated group}) \times 100}{\text{mean value of tumor volumes in control group}}$$

Mean of calculated reductions in the days indicated was designated as "mean reduction of tumor growth" and expressed in %.

2.11. Measurement of temperature (mice)

Temperature of both right (tumors) and control left murine shaved flanks was measured using IR RODENT THERMOMETER 153 IRB (BIOSEB). On the first day of therapy the temperature was measured every 6 h, then every second day.

2.12. Lung metastases

Murine lungs were carefully removed and fixed with 4% neutral solution of formaldehyde.

The incidence of metastases was evaluated using a dissecting microscope as previously described [6].

2.13. Histology

The excised tumors were fixed with 4% formaldehyde solution as mentioned above. Paraffin blocks were prepared after 7–10 days. Hematoxylin/eosin was used for staining of sections. QuickPHOTOMICRO 3.0 software was used for evaluation of histological findings.

2.14. Analysis of cell infiltrate using flow cytometry

Analysis of cell infiltrate was described in detail previously [6]. Briefly, cells liberated from tumors using Liberase DL and DNase I (both Roche Diagnostics, Germany) were analyzed by using flow cytometry. The following monoclonal antibodies (eBioscience, USA) were used for determining leukocyte subtypes: Total leukocytes (anti-Mouse CD45 PerCP-Cy5.5; clone 30-F11), T cells (anti-Mouse CD3e FITC; clone 145-2C11), CD4+ T cells (anti-Mouse CD4 APC; clone GK1.5), CD8+ T cells (anti-Mouse CD8a; clone 53–6.7), B cells (anti-Mouse CD19 APC; clone eBio1D3), NK cells (anti-Mouse NK1.1 PE; clone PK136), granulocytes (anti-Mouse Ly-6G (Gr-1) Alexa Fluor 700; clone RB6-8C5) and monocytes/macrophages (anti-Mouse *F*4/80 Antigen PE-Cy7; clone BM8).

Analysis was performed using a BD FACSCanto II flow cytometer (BD Biosciences, USA), equipped with two lasers (excitation capabilities at 488 nm and 633 nm). BD FACSDiva software 6.1.3. was used for flow cytometry data analysis.

2.15. Preparation and priming of neutrophils

Neutrophils were isolated from murine bone marrow according to Stassen et al. [13] and purified by using MACS technique (Miltenyi Biotec). Purity was controlled by BD FACSCanto II flow cytometer (BD Biosciences, USA) using anti-Mouse CD45 APC, Clone: 30-F11 and anti-Mouse Ly-6G (Gr-1) Alexa Fluor 700, Clone: RB6-8C5 antibodies (eBioscience). Neutrophils were primed according to Dewas et al. [14] by the mixture of GM-CSF and TNFα (12 ng and 2.5 ng/ml media respectively) for 20 min. The priming solution was enriched with 2 micromolar solution of soluble beta glucan (laminarin) in case of experiments, where CR3 co-stimulation was required (anchored Zymosan A, mannan or *M. tuberculosis*). These experiments were performed in active complement containing medium (FCS was not heat inactivated).

2.16. In vitro analysis of the cytotoxic effect of neutrophils on melanoma cells bearing Zymosan A, M. tuberculosis or agonists of phagocytic receptors

Zymosan A-SMCC (*M. tuberculosis*-SMCC) was covalently bound to B16-F10 melanoma cells reduced by DTT. Binding of agonists of phagocytic receptors was accomplished by incubation (30 min, 37 °C) of melanoma cells with 0.02 mM laminarin-BAM, 0.02 mM mannan-BAM or 0.05 mM f-MLFKK-BAM in culture medium, respectively and subsequent washing. Suspension of bone marrow neutrophils (90% purity) primed with GM-CSF + TNFα (+laminarin in case of Zymosan A-SMCC, *M. tuberculosis*-SMCC, and mannan-BAM) in culture medium was added to B16-F10 in the ratio 5:1. All mixtures were incubated for 2 h at 37 °C. Live, trypan blue unstained melanoma cells were counted using a haemocytometer.

2.17. Preparation and priming of NK cells

NK cells were isolated from murine spleen and purified by using MACS technique (Miltenyi Biotec). Priming (co-stimulation of CR3) was performed using 2 micromolar solution of soluble beta glucan (laminarin). Experiments with anchored mannan were performed in medium containing active complement (FCS was not heat inactivated).

2.18. Statistical analysis

Statistical and survival analysis were performed using one-way ANOVA with Tukey's *post hoc* test and Log-rank test, respectively (STATISTICA 12, StatSoft, Inc., Tulsa, OK 74104, USA).

3. Results

3.1. The use of anchored Zymosan A for tumor immunotherapy

In our first experiment we tested the influence of Zymosan A (both free and coupled with BAM anchor) on melanoma growth. Between days 4 and 12 of the experiment that lasted fourteen days, an anchored Zymosan (Zymosan A-BAM) caused slightly higher reduction of tumor growth than free Zymosan A. Nevertheless, these effects were not statistically significant. Anchor alone (lysine-BAM) did not reduce tumor growth at all (Fig. 1A).

To increase the therapeutic effect of Zymosan A-BAM, we tested a simultaneous addition of strong TLR4 agonists LPS. The combination of Zymosan A-BAM with LPS revealed a strong effect leading to an almost complete temporary elimination of tumors (98.1% mean reduction of tumor growth, Fig. 1B). Despite the fact that one mouse from this group survived >100 days (lived 26 months), the prolongation of

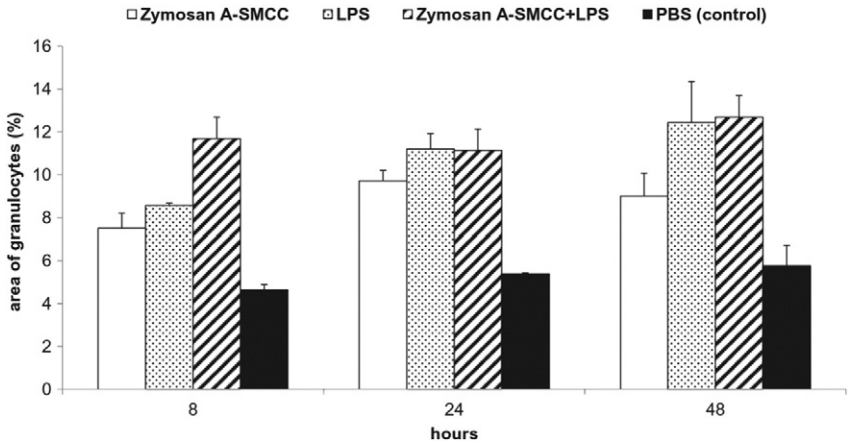


Fig. 2. The influence of Zymosan A-SMCC, LPS and combination thereof on granulocytic melanoma infiltration. Histological analysis. Suspension of B16-F10 melanoma cells in RPMI without FCS was inoculated subcutaneously (s.c.) in the previously shaved right flank of the mice. Each mouse received 4×10^5 melanoma cells in 0.1 ml of medium. Randomization of mice in groups of 6 was performed twelve days after the transplantation of melanoma cells and was followed immediately with a single intratumoral application of 50 μl of corresponding preparations. The composition of preparations used was: 14 mg Zymosan A-SMCC/ml PBS, 0.5 mg LPS/ml PBS, 14 mg Zymosan A-SMCC + 0.5 mg LPS/ml PBS, and PBS. Mice were euthanized 8, 24, and 48 h after the application of therapeutics (2 mice from each group per timepoint). The tumors were excised and fixed with 4% neutral solution of formaldehyde and subsequent histological analysis was performed. Granulocytic infiltration was evaluated.

survival in Zymosan A-BAM/LPS group was not statistically significant (Fig. 1C). In this and every further experiments we measured the temperature of the tumor surface and skin of the left tumor-free flank. We detected only minor changes that did not reflect the way and course of therapy (Fig. 1D).

In the next experiment we used another method of Zymosan A attachment to the tumor cells - covalent binding based on heterobifunctional reagent SMCC. As shown in Fig. 1E, covalently bound Zymosan A (Zymosan A-SMCC) in combination with LPS significantly reduced tumor growth (97.7% mean reduction of tumor growth, on day 8 of therapy we even observed a complete temporary elimination of all tumors). Free Zymosan A in combination with LPS caused 63.6% mean reduction of tumor growth only. Mice in all groups with Zymosan A survived longer than control. Nevertheless, this difference was not statistically significant. TCEP solution used as pre-treatment and SMCC based binding of inert molecules (lysine) did not influence tumor growth.

In the last experiment with Zymosan A we tested the combination of covalently bound Zymosan A (Zymosan A-SMCC) with LPS in another more intensive regime (application on days 0, 1, 2). Mean reduction of tumor growth caused by Zymosan A-SMCC was 61.9%, by LPS 39.1%. The mixture of Zymosan A-SMCC with LPS revealed a very strong synergistic effect with 99.3% mean reduction of tumor growth (Fig. 1F). In days 4–10 we observed a temporary elimination of all tumors with the exception of one mouse that survived and lived for >100 days (Fig. 1G). This mouse lived for 19 months displaying no pathological symptoms. Nevertheless, the prolongation of survival in this group was not statistically significant.

The experiments mentioned above show that in order to achieve a strong reduction of tumor growth, it is important to combine an attachment of Zymosan A to tumor cells with the addition of LPS in a proper application regime.

3.2. The use of anchored Zymosan A for tumor immunotherapy. Histological analysis

In the following experiments we analyzed the course of Zymosan A-SMCC/LPS based therapy of melanoma. Using histology, we evaluated inflammatory infiltration and necrotisation in first 48 h of therapy. As shown in Fig. 2, strong granulocytic infiltration was observed in all treated groups. With the exception of a slightly additive but statistically not significant effect after 8 h, no synergy of Zymosan A-SMCC and LPS was observed; this combination revealed the same effect as LPS alone at 24 and 48 h after treatment. On the contrary, the analysis of necrosis demonstrated synergy of Zymosan A-SMCC and LPS (Fig. 3). Control experiment with an inert molecule (lysine-SMCC) showed that SMCC based binding itself does not stimulate either infiltration or necrosis.

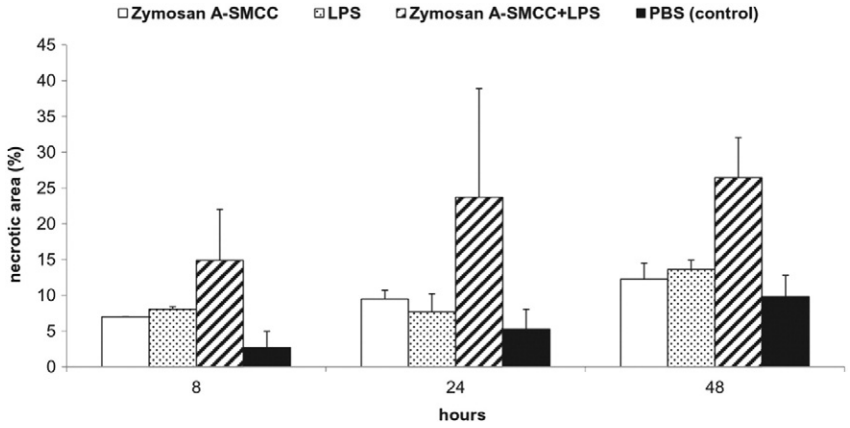


Fig. 3. The influence of Zymosan A-SMCC, LPS and combination thereof on melanoma necrotization. Histological evaluation of necrotization in the previous experiment (Fig. 2) is presented.

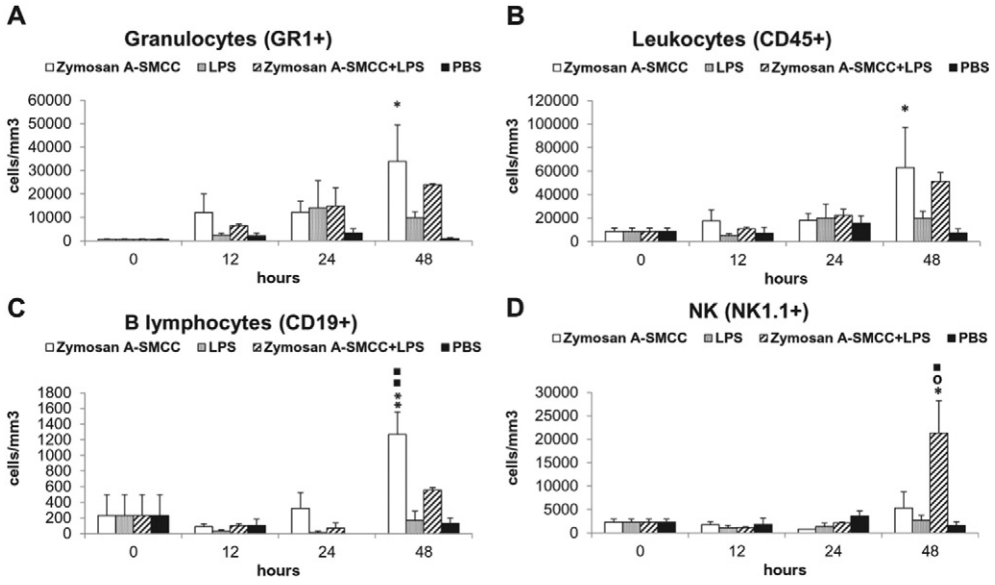


Fig. 4. The influence of Zymosan A-SMCC, LPS and combination thereof on granulocytic melanoma infiltration. Flow cytometry analysis. Suspension of B16-F10 melanoma cells in RPMI without FCS was inoculated subcutaneously (s.c.) in the previously shaved right flank of the mice. Each mouse received 4×10^5 melanoma cells in 0.1 ml of medium. Randomization of mice in groups of 9 was performed twelve days after the transplantation of melanoma cells and was followed immediately with a single intratumoral application of 50 μl of corresponding preparations. The composition of preparations used was: 14 mg Zymosan A-SMCC/ml PBS, 0.5 mg LPS/ml PBS, 14 mg Zymosan A-SMCC + 0.5 mg LPS/ml PBS, and PBS. Mice were euthanized 12, 24, and 48 h after application of therapeutics (3 mice from each group per timepoint). Three mice served as a negative control and were killed without any application at time 0. Analysis of cell infiltrate in excised tumors was performed using flow cytometry and expressed as cells/mm^3 of tumor mass. The following labeled antibodies were used: (A) anti-Mouse Ly-6G (Gr-1) Alexa Fluor 700 for granulocyte detection, (B) anti-Mouse CD45 PerCP-Cy5.5; clone 30-F11 for leukocytes, (C) anti-mouse CD19 APC for detection of B lymphocytes, and (D) anti-mouse NK1.1 PE for NK cells. $^{*}P \le 0.05$, $^{**}P \le 0.005$ compared to PBS (control). **o***P* ≤ 0.05 compared to Zymosan A-SMCC. ■*P* ≤ 0.05 compared to LPS. ■■*P* ≤ 0.01 compared to LPS.

3.3. The use of anchored Zymosan A in tumor immunotherapy. The analysis of inflammatory infiltration by flow cytometry

Flow cytometry analysis of Zymosan A-SMCC/LPS based therapy of melanoma revealed strong granulocytic (GR1+) infiltration in all treated groups (Fig. 4A) corresponding to changes of total leukocyte (CD45+) count (Fig. 4B). An increase of B lymphocytes (CD19+) and of NK cells (NK1.1) in Zymosan containing groups (Fig. 4C, D) was observed at the end of the experiment. No changes in monocyte/macrophage (F4/80+) and T lymphocyte (CD3+, CD4+, CD8+) count were observed.

3.4. Tumor immunotherapy based on the use of Gram-negative bacteria S. maltophilia and S. marcescens

In the following experiments we tested the therapeutic effect of two Gram-negative bacteria, positively charged *Stenotrophomonas maltophilia* and negatively charged *Serratia marcescens*. As shown in Fig. 5, an intratumoral application of *Serratia marcescens* influenced tumor growth only slightly (the mean reduction of tumor growth was 29.2%, not statistically significant). The same amount of positively charged *Stenotrophomonas maltophilia* caused a statistically significant reduction of tumor growth (mean value was 61.3%). When the negative

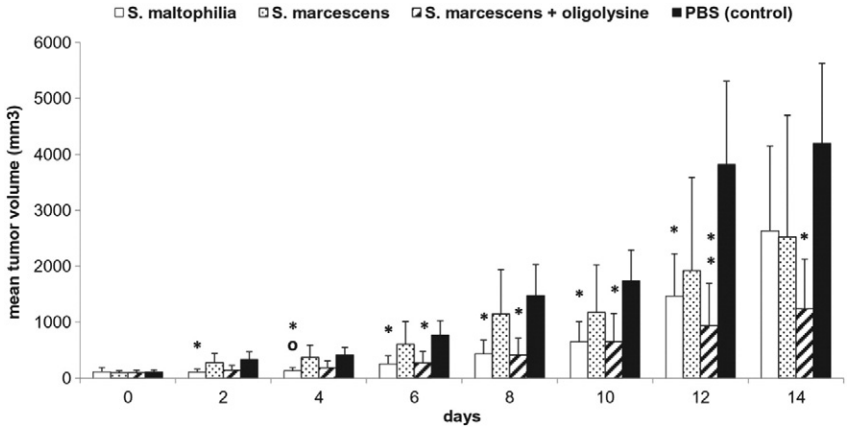


Fig. 5. Melanoma immunotherapy based on the treatment with killed *Stenotrophomonas maltophilia*, *Serratia marcescens*, and *Serratia marcescens*-oligolysine. Suspension of B16-F10 melanoma cells in RPMI without FCS was inoculated subcutaneously (s.c.) in the previously shaved right flank of the mice. Each mouse received 4×10^5 melanoma cells in 0.1 ml of medium. Randomization of mice in groups of 6 was performed twelve days after the transplantation of melanoma cells and was followed immediately with therapies. These therapies were based on intratumoral application of 50 μl of corresponding preparations and continued in pulse regime (together 12 doses on days 0,1,2… 8,9,10,…,16,17,18,…,24,25,26). During the therapy, all mice were housed individually. Measurements of tumors and their volume calculations were performed every second day for 14 days. The composition of preparations used was: *S. maltophilia* (5×10^8/ml PBS), *S. marcescens* (5×10^8/ml PBS), *S. marcescens*-oligolysine (5×10^8/ml PBS), and PBS. $^{*}P \le 0.05$, $^{**}P \le 0.01$ compared to PBS (control). **o***P* ≤ 0.05 compared to the *S. marcescens*.

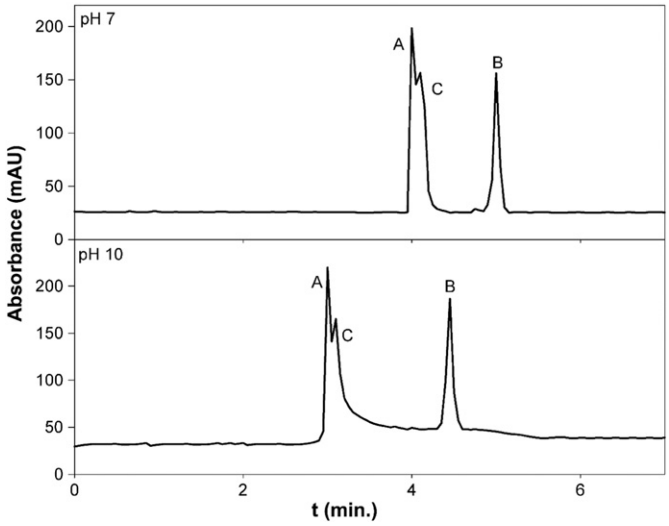


Fig. 6. Capillary zone electrophoresis of killed *Stenotrophomonas maltophilia*, *Serratia marcescens*, and *Serratia marcescens*-oligolysine. Bacteria were suspended in PBS and their concentrations were adjusted to 5×10^8/ml. For capillary zone electrophoresis separations, 2×10^{-2} mol/l phosphate buffers pH 7 and 10, with the addition of 5% (v/v) EtOH and 0.5% (w/v) PEG 10000, were used as background electrolyte. A - *S. maltophilia*, B - *S. marcescens*, C - *S. marcescens*-oligolysine. The relative standard deviation (RSD) of the migration times calculated from a minimum of five independent analyses was always <1.6%.

charge of *Serratia marcescens* was neutralized by covalent binding of polycationic oligolysine, the resulting bacteria reduced tumor growth with the same intensity as *Stenotrophomonas maltophilia* (66.5% mean reduction of tumor growth). Oligolysine alone in comparable concentration did not reveal any effect on melanoma cells.

To verify the success of *S. marcescens* negative charge neutralization, capillary zone electrophoresis was performed. As shown in Fig. 6, *Stenotrophomonas maltophilia* and *Serratia marcescens* with bound oligolysine revealed similar migration rates; *Serratia marcescens* alone differed from them in this aspect significantly.

In all groups, where bacteria were used, we observed prolonged survival. Nevertheless, these differences were not statistically significant.

In further experiments we tried to improve the *Stenotrophomonas maltophilia*-based therapy by simultaneous application of LPS and mannan-BAM respectively. Neither LPS nor mannan-BAM revealed any effect. BAM anchoring also did not improve *Stenotrophomonas maltophilia*-based immunotherapy.

Experiments with intratumoral application of *Stenotrophomonas maltophilia* were performed four times with various application schemes. The best reduction of tumor growth was obtained with intratumoral applications of *Stenotrophomonas maltophilia* in days 0, 2, 4, 6, 8 and 10 (87.5% mean reduction of tumor growth).

3.5. The use of anchored Gram-positive bacteria in tumor immunotherapy

In experiments with Gram-positive bacteria we tested the therapeutic effect of *Mycobacterium tuberculosis* and *Listeria monocytogenes*. Both bacteria were used in free form and in a form, that was able to bind covalently to the tumor cell surface (SMCC). Covalently bound bacteria were tested both alone and in combination with covalently bound mannan (mannan-SMCC). As shown in Fig. 7, free bacteria revealed only a slight and not significant effect on tumor growth. Binding of *Mycobacterium tuberculosis*-SMCC resulted in a strong therapeutic effect (73.7% mean reduction of tumor growth) that was not further influenced by the addition of mannan-SMCC. Binding of *Listeria monocytogenes*-SMCC caused a significant reduction of tumor growth in the first 6 days only. However, the combination with mannan-SMCC resulted in a strong long lasting reduction of tumor growth (74.4% mean reduction of tumor growth). In comparison with the PBS control, the treatment with *Listeria monocytogenes*-SMCC/mannan-SMCC prolonged the survival of mice significantly (mean value of 30.7 to 55.8 days, $P \leq 0.05$).

3.6. The use of anchored Mycobacterium tuberculosis in tumor immunotherapy. Analysis of inflammatory infiltration by flow cytometry

Flow cytometry analysis of *M. tuberculosis*-SMCC-based therapy of melanoma revealed strong granulocytic (GR1+) infiltration in the group treated (Fig. 8A), corresponding to changes of the total leukocyte (CD45+) count (Fig. 8B). No changes in B lymphocyte (CD19+), NK (NK1.1), monocyte/macrophage (F4/80+), and T lymphocyte (CD3+, CD4+, CD8+) count were observed.

3.7. Mechanisms of immunotherapy based on PAMPs attached to tumor cells

Our results show that polymorphonuclear cells, in particular neutrophils, play an important role in immunotherapy based on PAMPs attached to tumor cells. It was verified by *in vitro* experiment with Zymosan A covalently (SMCC) bound to tumor cells. Melanoma cells with Zymosan A attached were killed by neutrophils, while the presence of free Zymosan A caused only a slight reduction of tumor cells count (Fig. 9). Similar results were obtained with *M. tuberculosis* covalently (SMCC) bound to melanoma cells.

Zymosan A and bacteria contain a complex mixture of ligands of both signalling and phagocytic receptors. We assumed that the attachment of ligands of phagocytic receptors is crucial for effective tumor cell killing. To prove this hypothesis we used anchored laminarin (beta glucan) and mannan (beta glucans and mannans are important

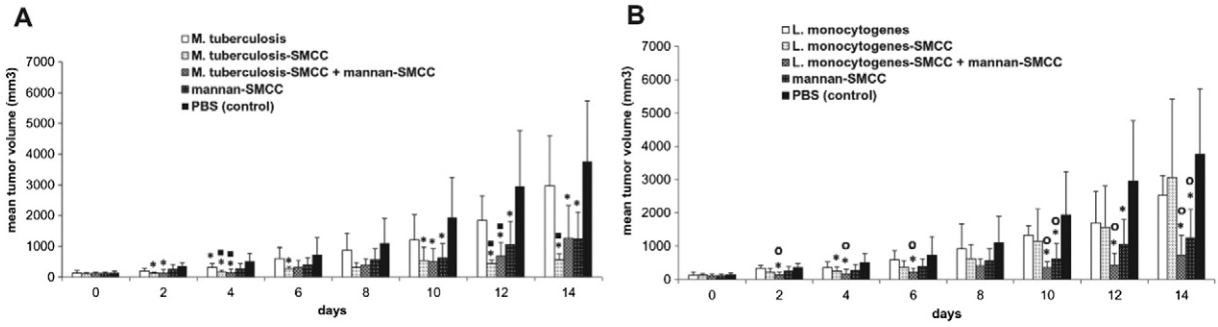


Fig. 7. The use of anchored Gram-positive bacteria in tumor immunotherapy. Experimental design was the same as in Fig. 5. The composition of preparations used was: A - *M. tuberculosis* (5 mg/ml PBS), *M. tuberculosis*-SMCC (5 mg/ml PBS), *M. tuberculosis*-SMCC + mannan-SMCC (5 mg/ml 0.2 mM mannan-SMCC in PBS), 0.2 mM mannan-SMCC in PBS, and PBS. B - *L. monocytogenes* (1×10^9/ml PBS), *L. monocytogenes*-SMCC (1×10^9/ml PBS), *L. monocytogenes*-SMCC + mannan-SMCC (1×10^9/ml 0.2 mM mannan-SMCC in PBS), 0.2 mM mannan-SMCC in PBS, and PBS. *$P \leq 0.05$ compared to PBS (control). ■$P \leq 0.05$ compared to *M. tuberculosis*. o$P \leq 0.05$ compared to L. *monocytogenes*.

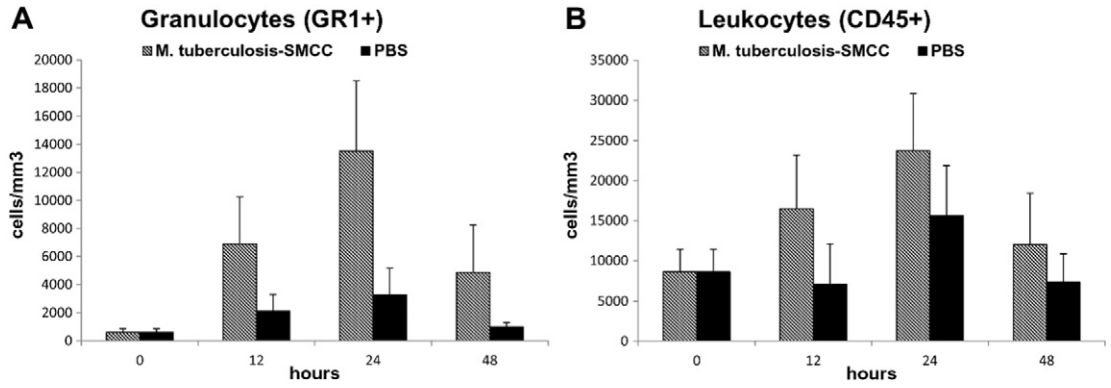


Fig. 8. The use of anchored *Mycobacterium tuberculosis* in tumor immunotherapy. Analysis of inflammatory infiltration by flow cytometry. Suspension of B16-F10 melanoma cells in RPMI without FCS was inoculated subcutaneously (s.c.) in the previously shaved right flank of the mice. Each mouse received 4×10^5 melanoma cells in 0.1 ml of medium. Randomization of mice in groups of 9 was performed twelve days after the transplantation of melanoma cells and was followed immediately with single intratumoral application of 50 μl of corresponding preparations. The composition of preparations used was: 5 mg *M. tuberculosis*-SMCC/ml PBS and PBS alone as control. Mice were euthanized at 12, 24, and 48 h after the application of therapeutics (3 mice from each group per timepoint). Three mice served as negative control and were killed without any application at time 0. Analysis of cellular infiltrate in the excised tumors was performed using flow cytometry and expressed as cells/mm^3 of tumor mass. The following fluorescently labeled antibodies were used: (A) anti-Mouse Ly-6G (Gr-1) Alexa Fluor 700 for granulocyte detection, (B) anti-Mouse CD45 PerCP-Cy5.5; clone 30-F11 for leukocytes.

parts of Zymosan A, see below) as phagocytic ligands instead of Zymosan A. Anchored f-MLFKK was used instead of bacteria, as formylmethione peptides are characteristic bacterial ligands of formyl peptide receptors (FPR), taking part in phagocytosis [15–17]. Neutrophils significantly reduced the count of melanoma cells with attached laminarin, mannan or f-MLFKK, respectively (Fig. 10). Free ligands did not show any effect.

Direct phagocytosis of relatively big melanoma cells by small neutrophils does not seem possible. In this case, a process called "frustrated phagocytosis" is considered [18]. *In vitro* experiments allowed showing the interactions of neutrophils with melanoma cells covered by ligands of phagocytic receptors (laminarin-BAM, mannan-BAM, f-MLFKK-BAM). As shown in Fig. 11, frequent interactions, flattening and adherence of neutrophils to melanoma cells were observed. These interactions resulted in the killing of melanoma cells (S1 Movie). Intact (control) melanoma cells did not interact with neutrophils.

Table 1 reports the intensity of neutrophils' interactions with melanoma cells with attached ligands of phagocytic receptors (laminarin-BAM, mannan-BAM, f-MLFKK-BAM). The effects of ligands, unable to anchor to melanoma cells (free forms of laminarin, mannan, f-MLF) were studied as well. The fundamental role of the attachment of phagocytic receptors agonists to the surface of tumor cell for neutrophil/tumor cell contacts and interactions was proved for all ligands.

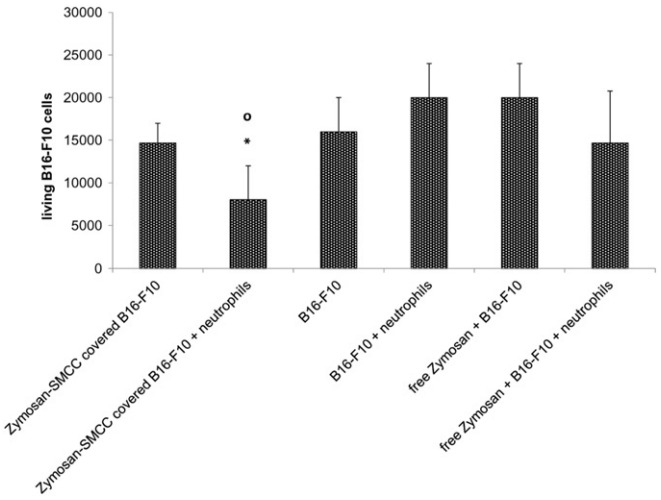


Fig. 9. Cytotoxic effect of murine neutrophils on melanoma B16-F10 cells with covalently attached Zymosan A. Zymosan A-SMCC was covalently bound to B16-F10 melanoma cells reduced by DTT. Suspension of bone marrow neutrophils (90% purity) primed with GM-CSF, TNFα and laminarin in culture medium was added to B16-F10 in the ratio 5:1 followed by an incubation for 2 h at 37 °C. Subsequently, living trypan blue excluding melanoma cells were quantified with a haemocytometer. *$P \leq 0.05$ compared to B16-F10 + neutrophils. **o**$P \leq 0.05$ compared to free Zymosan + B16-F10.

In our *in vivo* experiments with Zymosan A-SMCC in combination with LPS we observed the increase of NK cells count (Fig. 4D). NK cells are not phagocytes, nevertheless they express complement receptor 3 (CR3). It creates condition for cytotoxic attack of cells opsonized by mannan or mannan containing Zymosan A. This attack was confirmed by *in vitro* experiment using mannan anchored to melanoma cells (Fig. 12).

4. Discussion

In our experiments we demonstrated an effective therapy of very aggressive B16-F10 melanoma based on binding of phagocytic receptor ligands on tumor cells in combination with intratumoral administration of TLR ligands. Zymosan A anchored to melanoma cells using SMCC-based covalent binding (Zymosan A-SMCC) revealed strong synergy with LPS, leading to the shrinkage of tumors and their temporary or permanent elimination. The observed therapeutic effects were fundamentally dependent on anchoring of Zymosan A to tumor cells and corresponded well with our experience with the synergy of anchored laminarin (laminarin-BAM, laminarin-SMCC) or mannan (mannan-BAM, mannan-SMCC) with LPS [6]. This similarity can be explained by the fact, that Zymosan A contains a high amount of both beta glucans (laminarin belongs to this group) and mannans [19].

In order to better understand the synergy of anchored Zymosan with LPS, we performed both histological and flow cytometry analysis. Both analyses revealed strong granulocytic infiltration in all treated groups. No synergy of anchored Zymosan and LPS was based on cell infiltration. We propose that infiltrating immune cells (primarily granulocytes) attack Zymosan A-labeled tumor cells that leads to a massive necrotisation of tumor tissue. Hence, the synergy reflects the effect of immune cells and cannot be attributed solely to their count. These observations are in accordance with our histological and flow cytometry analysis of the synergy of anchored laminarin (mannan) with LPS [6].

Suppression of tumor growth by LPS or Zymosan A was described by Mariani et al. [20]. He used glioma RG-2 cells implanted subcutaneously into rats and very high doses of substances, in particular LPS, so their results can hardly be compared with ours. Synergy of LPS with Zymosan A and Zymosan A anchoring were not studied.

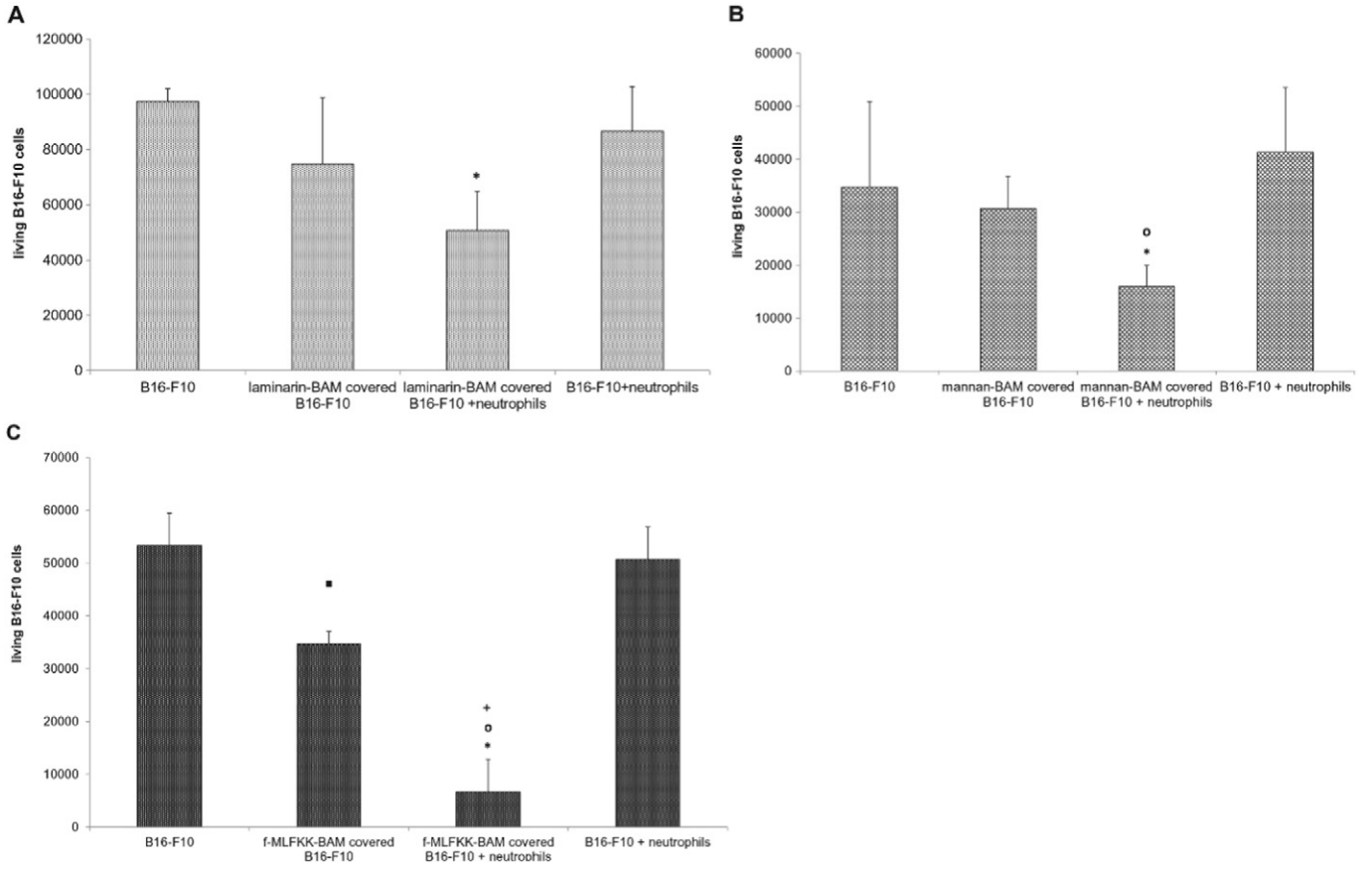


Fig. 10. Cytotoxic effect of murine neutrophils on melanoma B16-F10 cells bearing agonists of phagocytic receptors. B16-F10 melanoma cells were incubated (30 min, 37 °C) with 0.02 mM laminarin-BAM, 0.02 mM mannan-BAM or 0.05 mM f-MLFKK-BAM in culture medium, respectively and subsequently washed. Suspension of bone marrow neutrophils (90% purity) primed with GM-CSF + TNFα (+laminarin in case of mannan-BAM) in culture medium was added to B16-F10 in the ratio 5:1. All mixtures were incubated for 2 h at 37 °C. After incubation, living trypan blue excluding melanoma cells were counted with a haemocytometer. A – laminarin-BAM $^{*}P \le 0.05$ compared to laminarin-BAM covered B16-F10. B – mannan-BAM $^{*}P \le 0.05$ compared to mannan-BAM covered B16-F10 **o***P* ≤ 0.05 compared to B16-F10 + neutrophils. C – f-MFKK-BAM $^{*}P \le 0.005$ compared to f-MLFKK-BAM covered B16-F10. **o***P* ≤ 0.0005 compared to B16-F10. +*P* ≤ 0.0005 compared to B16-F10 + neutrophils. ■*P* ≤ 0.05 compared to B16-F10.

In further research we focused on another natural complex of agonists of pattern recognition receptors (PRRs), bacteria. We again analyzed how significant their attachment to tumor cells for effective cancer immunotherapy is. The simplest way is the attachment based on electrostatic interactions. Tumor cells have a significant negative surface charge due to the presence of phosphatidylserine [21] and chondroitin sulphate [22]. This negative charge is considered to be the main reason of specific and effective binding of cationic antimicrobial proteins [23]. Therefore, we searched for positively charged bacteria. From the total of 156 strains, only two positively charged bacterial strains at neutral pH have been described [24], namely *Streptococcus thermophilus* (positively charged in the buffer of specific composition only) and *Stenotrophomonas maltophilia*. For our experiments we chose *Stenotrophomonas maltophilia*, as its strong adhesion to negatively charged surfaces was described [24].

We supposed that this positive charge is responsible for binding of *Stenotrophomonas maltophilia* to the surface of tumor cells and subsequent killing of these cells by innate immunity. Negatively charged *Serratia marcescens* [25] revealed a considerably lower effect. Nevertheless, it was possible to enhance the effect of this bacterium by influencing its surface charge by polycations. *Serratia marcescens* was used for decades as an important part of the above-mentioned Coley's toxin. We suppose that the analysis of interactions of this preparation with tumor cells could improve its efficacy.

It seems that *Stenotrophomonas maltophilia* contains a sufficient amount of LPS as its further addition did not improve the effect of the bacteria itself. Also, the attachment of this positively charged bacterium to negatively charged tumor cells was sufficient for the stimulation of an immune attack, because stronger BAM anchoring did not reveal any therapeutic improvement.

Our studies with Gram-positive bacteria once again confirmed the significance of bacteria binding to the tumor cell surface. It should be highlighted that no LPS and no LPS containing bacteria were used in these experiments, so it brings possibilities for safe treatment in the area of human medicine. LPS is well tolerated by rodents, dogs and cats [26], but it is very dangerous for humans, causing septic shock [27].

Bacillus Calmette-Guerin (BCG), attenuated bovine tuberculosis bacteria (*Mycobacterium bovis*), in the form of repeated intravesical instillations, has already been in use for over 30 years as a standard method of preventing cancer recurrence after endoscopic surgery of intermediate- and high-risk non-muscle invasive bladder cancer [28]. It is also effective *in situ* against inoperable bladder carcinoma resulting in a 70–75% complete response rate [29]. Unfortunately, in other tumor types BCG application has not proven to be more effective than conventional therapies [29]. Based on our experience with anchoring of *M. tuberculosis*, we propose this approach in the case of BCG treatment as well.

Attenuated *Listeria monocytogenes* is considered to be a promising therapeutic vaccine vector for tumor immunotherapy [30]. Our approach offers an opportunity of simple and safe use of this bacterium for cancer treatment.

The immunoreactivity of phagocytic receptors ligands is conditioned by their expression on solid surfaces in sufficient density [31]. Anchoring of these ligands to tumor cell surface leads to tumor suppressive effects [6]. Ligands of TLR receptors are able to signalize in soluble form [31]. Nevertheless, Tom et al. [32] and Liu et al. [33] described that their binding to tumor cells surface could be suitable. Bound agonists

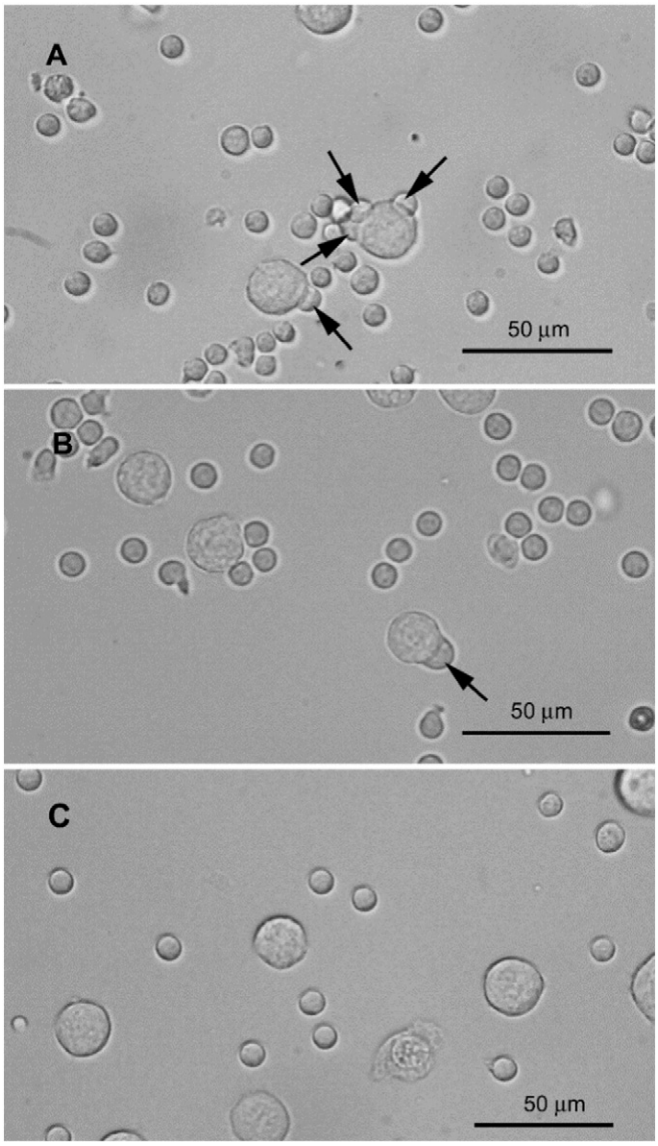


Fig. 11. Interactions of neutrophils with melanoma cells bearing ligands of phagocytic receptors. Both coverage of melanoma cells with ligands of phagocytic receptors and isolation and priming of neutrophils were performed as previously described (Fig. 10). Neutrophils and B16-F10 cells in 2:1 ratio were incubated for 60 min. and photographic documentation was made every 10 min. The considerable difference in size allowed to distinguish melanoma cells (big) and neutrophils (small cells). (A) Rosette formation and adherence of neutrophils to mannan-BAM covered B16-F10 cells, 40 min, (B) neutrophil adhered to laminarin-BAM covered melanoma cell, 20 min, (C) intact melanoma cells (control), 30 min. Arrows – neutrophils adhered to melanoma cells in the process of "frustrated phagocytosis".

of TLR2 and TLR9 receptors revealed a higher immuno-stimulatory effect than their free forms. Bacteria and Zymosan A particles contain a mixture of PAMPs and are able to stimulate both signalling and phagocytic receptors. However, they have only negligible effect on tumor cells unbound. As apparent from our experiments, in order to achieve a strong immunotherapeutic effect, binding of bacteria and Zymosan A to tumor cell surface is of key importance.

Experiments with Zymosan A and both Gram-positive and Gram-negative bacteria addressed not only the question of attachment of ligands to the tumor cell surface, but also the question of the synergy between TLRs and phagocytic receptors ligands that was underpinned by Underhill and Gantner [31] as an important condition for an effective immune response. *Stenotrophomonas maltophilia* and *Mycobacterium tuberculosis* contain all necessary components and no other additives are required. LPS content in *Stenotrophomonas maltophilia* has already been discussed. *Mycobacterium tuberculosis* possesses not only a large repertoire of TLR ligands [34] but also interacts with many phagocytic receptors, including Fc receptor (FcR), mannose receptor (MR), complement receptors (CR1, CR3, CR4), surfactant protein receptors (SPR), and scavenger receptors (SR) [35].

On the other hand, anchored Zymosan A and *Listeria*-based therapies required further co-stimulation. Zymosan A contains a high amount of beta glucans and mannans, therefore it stimulates the receptors of phagocytic ligands like Dectin-1 and CR3 quite well (lectine pathway of complement activation, iC3b opsonisation). Beta glucan dependent TLR2 signalling seems to be insufficient, as the strong improvement of Zymosan A based tumor therapy by LPS stressed the necessity to enhance TLR signalling. *Listeria monocytogenes* seems to be good stimulator of TLR receptors but to achieve a strong therapeutic effect it is necessary to combine it with phagocytic ligand (anchored mannan).

Regarding the mechanisms of immunotherapy based on PAMPs attached to tumor cells, we propose in accordance with Janotová et al. [6] that there is a synergy of TLR signalling (responsible for inflammatory cell infiltration) with phagocytosis triggered by ligands of phagocytic receptors. The processes of TLR signalling and inflammatory cell infiltration were thoroughly studied by Mogensen [36]. Histological and flow cytometry analyses of our experiments revealed strong granulocyte infiltration. Therefore, we suppose that neutrophils play an important role in the killing and phagocytosis of tumor cells with attached PAMPs (Zymosan A, bacteria). This role is also supported by fast onset of therapeutic effects which corresponds with the role of neutrophils as defenders in the first line and was confirmed by *in vitro* experiments. In these experiments we proved the key role of attachment of ligands of phagocytic receptors to tumor cells for their effective neutrophil mediated killing. We used neutrophils isolated from bone marrow, therefore the priming with GM-CSF and TNFα was essential for their full activity. Anchored mannan (mannan-BAM, mannan containing Zymosan A-SMCC) initiates a lectin pathway of complement activation [6]. It creates the conditions for a CR3 mediated neutrophil attack (on the basis of iC3b formation). As already mentioned, *M. tuberculosis* is recognized by complement receptors including CR3 directly. For cytotoxic activity of neutrophils, it is necessary to co-stimulate CR3 by binding of appropriate molecules to its lectin domain [37]. We found that soluble laminarin represents a suitable and essential compound for this purpose.

Targets (melanoma cells with attached PAMPs) are bigger than neutrophils. It indicates the participation of "frustrated phagocytosis" [18] in the killing of tumor cells. In this process, tight contact of neutrophils and targets, flattening of neutrophils and the creation of pockets between neutrophils and target cells is described. Targets are killed by the release of cytotoxic components of neutrophil armentarium (granule content, ROS) into the pockets [38]. In our experiments, we observed frequent interactions of neutrophils with B16-F10 cells (rosette

Table 1
The interactions of neutrophils with free melanoma cells and cells with anchored ligands of phagocytic receptors.

	Mean count of neutrophils adhered to one melanoma cell		
Ligand	20 min	30 min	40 min
Laminarin-BAM	0.298	0.204	0.298
Laminarin free	0.225	0.014	0.097
Mannan-BAM	0.383	0.557	0.917
Mannan free	0.125	0.032	0.068
f-MLFKK-BAM	0.560	0.616	0.644
f-MLF free	0.189	0.202	0.083
PBS (control)	0.076	0.041	0.041

Both coverage of melanoma cells with ligands of phagocytic receptors and isolation and priming of neutrophils was performed as described in the legend for Fig. 10. Free ligands were added in a concentration of 0.02 mM (laminarin, mannan) and 0.05 mM (f-MLFKK). Neutrophils and melanoma cells were incubated at a 2:1 ratio. The calculation of the mean count of neutrophils adhered to one melanoma cell was based on the observation of 40.04 ± 17.84 melanoma cells on average for each ligand and time.

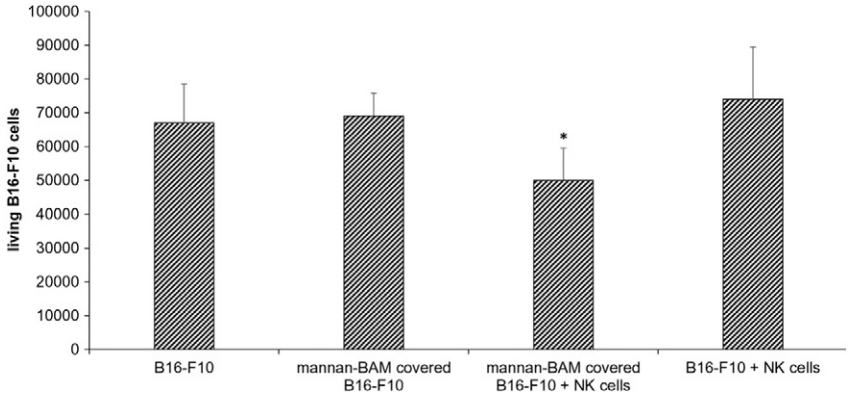


Fig. 12. Cytotoxic effect of murine NK cells on melanoma B16-F10 cells with anchored mannan. B16-F10 melanoma cells were incubated (30 min, 37 °C) with 0.02 mM mannan-BAM in culture medium and subsequently washed. Suspension of laminarin primed spleen NK cells in culture medium was added to B16-F10 in the ratio 5:1. Cells were then incubated for 2 h at 37 °C. After incubation, living trypan blue excluding melanoma cells were counted with a haemocytometer. $^{*}P \leq 0.05$ compared to B16-F10 + NK cells.

formation), flattening and the adherence of neutrophils to these cells resulting in killing of melanoma cells. All these processes were fully dependent on the presence of ligands of phagocytic receptors attached to melanoma cells.

Our observations are in accordance with the study of Dallegri et al. [39] highlighting the significance of neutrophil-target adherence for effective killing of tumor target cells primarily by hypochlorous acid (HOCl). Also antibody-dependent cellular cytotoxicity (ADCC) killing of antibody-coated tumor cells requires the intervention of adhesion-promoting glycoproteins belonging to the CD11-CD18 complex [40].

Neutrophils can play both pro- and anti-tumor roles [41,42]. This controversial role can be explained by the fact that neutrophils exhibit substantial plasticity and can be polarized to an N1 antitumoral or N2 protumoral phenotype in response to microenvironment [43]. Ligation of TLR receptors significantly stimulates neutrophil functions including phagocytosis [44]. This activation of recruited neutrophils together with anchoring of phagocytosis stimulating ligands on tumor cells could be at least partially responsible for observed synergy of TLR agonists and ligands stimulating phagocytosis.

As already discussed, we suppose that neutrophils play key role in therapies based on phagocytosis stimulating ligands. NK cells are not phagocytes, nevertheless they have CR3, and therefore they are able to recognise tumor cells bearing proper agonist. Resulting cytotoxicity can increase overall therapeutic effect.

Zymosan A and most of the bacteria that were used in our study were thoroughly tested in the past and both immunostimulatory and antitumor effects were recorded. We would like to highlight that their efficacy in tumor immunotherapy can be dramatically enhanced by their attachment to tumor cells. Requirement for the presence of both signalling and phagocytic stimuli should be considered as well. LPS-free preparations (*Mycobacterium tuberculosis*-SMCC) offer the opportunity of safe use in human cancer immunotherapy.

5. Conclusion

Simultaneous stimulation of both Toll-like and phagocytic receptors can be used for very effective cancer immunotherapy.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.intimp.2016.08.004.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Paper 3

Innate immunity based cancer immunotherapy: B16-F10 murine melanoma model

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RESEARCH ARTICLE Open Access

Innate immunity based cancer immunotherapy: B16-F10 murine melanoma model

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Abstract

Background: Using killed microorganisms or their parts to stimulate immunity for cancer treatment dates back to the end of 19th century. Since then, it undergone considerable development. Our novel approach binds ligands to the tumor cell surface, which stimulates tumor phagocytosis. The therapeutic effect is further amplified by simultaneous application of agonists of Toll-like receptors. We searched for ligands that induce both a strong therapeutic effect and are safe for humans.

Methods: B16-F10 murine melanoma model was used. For the stimulation of phagocytosis, mannan or N-formyl-methionyl-leucyl-phenylalanine, was covalently bound to tumor cells or attached using hydrophobic anchor. The following agonists of Toll-like receptors were studied: monophosphoryl lipid A (MPLA), imiquimod (R-837), resiquimod (R-848), poly(I:C), and heat killed *Listeria monocytogenes*.

Results: R-848 proved to be the most suitable Toll-like receptor agonist for our novel immunotherapeutic approach. In combination with covalently bound mannan, R-848 significantly reduced tumor growth. Adding poly(I:C) and *L. monocytogenes* resulted in complete recovery in 83% of mice and in their protection from the re-transplantation of melanoma cells.

Conclusion: An efficient cancer treatment results from the combination of Toll-like receptor agonists and phagocytosis stimulating ligands bound to the tumor cells.

Keywords: Cancer immunotherapy, Innate immunity, Melanoma, Neutrophils, Resiquimod, Mannan, Phagocytosis

Background

Cancer immunotherapy based on the stimulation of innate immunity has a long history. W. Coley initiated the first studies at the end of 19th century, using a mixture of inactivated bacteria, Gram-positive *Streptococcus pyogenes* with Gram-negative *Serratia marcescens* - so called Coley's toxin [1]. Further improvement of cancer immunotherapy based on the use of microorganisms and their parts was significantly influenced by the discovery of pathogen associated molecular patterns (PAMPs). PAMPs allowed for the understanding of mechanisms, how innate immunity recognizes foreign microorganisms, and how the immune response is triggered. Synthetic PAMPs analogues (mainly agonists of Toll-like receptors) were synthesized and tested in cancer therapy [2]. However, the impact of these therapies was not as strong as expected [3]. Even though agonists of Toll-like receptors (TLR) stimulate inflammation, we hypothesize that the infiltrating cells cannot recognize tumor cells as a target of their attack, because they do not have any PAMPs on their surface.

This problem was solved in our previous studies [4, 5], where we described the use of phagocytic receptors agonists anchored to the surface of tumor cells for cancer immunotherapy. To achieve a sufficiently strong therapeutic effect, it was necessary to combine this therapy

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with simultaneous application of lipopolysaccharide (LPS) – the agonist of TLR4. The obtained synergy resulted in shrinkage and temporary or permanent disappearance of the tumors.

LPS is well tolerated by rodents, dogs and cats [6], but causes septic shock in humans [7]. Therefore we searched for an effective TLR agonist that would be safe for humans and shows strong synergy with phagocytosis stimulating ligands attached to tumor cells. Anchored mannan was selected for the stimulation of phagocytosis, as it gave the best results (mainly survival prolongation) in previous experiments [4, 5]. Resiquimod (R-848) proved to be the best TLR agonist demonstrating synergy with mannan anchored to tumor cells. This innate immunity based cancer immunotherapy was further improved by our search to find the optimal therapeutic mixture, concentration, and timing.

Methods

Chemicals

Tissue culture media and media supplements, mannan (*Saccharomyces cerevisiae),* laminarin (*Laminaria digitata)*, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), f-MLF (N-Formyl-methionyl-leucyl-phenylalanine), epicatechin, polyinosinic:polycytidylic acid, sodium salt (poly (I:C)), lipopolysaccharide (LPS) from *Escherichia coli*, TNF-alpha, and GM-CSF were obtained from Sigma-Aldrich (St. Louis, USA). 4-(*N*-Maleimidomethyl) cyclohexanecarboxylic-acid N-hydroxysuccinimide ester (SMCC) was provided by Thermo Scientific (Erembodegem, Belgium). Biocompatible Anchor for cell Membrane (BAM, Mw 4000) was obtained from NOF EUROPE (Grobbendonk, Belgium). N-Formyl-methionyl-leucyl-phenylalanine with two lysine molecules (f-MLFKK) was synthesized by Schafer-N (Copenhagen, Denmark). Imiquimod (R-837) was delivered by Merck Millipore (Billerica, USA), monophosphoryl lipid A (MPLA) by Avanti Polar Lipids (Alabaster, USA), and resiquimod (R-848) by Tocris Bioscience (Bristol, UK).

Cell lines, bacteria and mice

Murine melanoma B16-F10 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and were cultivated in RPMI 1640 (Sigma-Aldrich, USA) supplemented with 10% fetal calf serum (FCS, PAA, Austria) and antibiotics. Cells were maintained at 37 °C in a humidified atmosphere with 5% carbon dioxide.

Heat killed *Listeria monocytogenes* was purchased from InvivoGen (Toulouse, France). SPF C57BL/6 mice (female, 18–20 g) were obtained from Charles River Laboratories (Sulzfeld, Germany). Mice were housed in barrier facilities with free access to sterile food and water. Photoperiod was 12/12, temperature 22 °C. All experimental mouse procedures were performed in accordance with the laws of the Czech Republic. Experimental project was approved by the Ministry of Education, Youth and Sports (protocol no. 28842/2014-3).

Synthesis of mannan-BAM and f-MLFKK-BAM

First, aminated mannan was prepared by reductive amination according to Torosantucci et al. [8]. A mannan solution in an environment of ammonium acetate was reduced by natrium cyanoborohydride at pH of 7.5 and 50 °C for 5 days. The solution was subsequently dialyzed (MWCO 3500 dialysis tubing, Serva, Heidelberg, Germany) against PBS at 4 °C overnight. Peptide f-MLFKK already contained an amino group required for binding of BAM.

Binding of BAM anchor on amino group of mannan (f-MLFKK) was performed at pH of 7.3 [9]. During one hour at room temperature, N-hydroxysuccinimide (NHS) group of BAM reacted with amino group of mannan or with ε-amino group of lysine, respectively.

Synthesis of mannan-SMCC and *Listeria monocytogenes*-SMCC. In vivo application

Binding of NHS group of heterobifunctional compound SMCC to amino groups of aminated mannan and *Listeria monocytogenes* was performed according to SMCC manufacturer's instructions (Thermo Scientific, Pierce Protein Biology Products). Binding of mannan-SMCC or *Listeria monocytogenes*-SMCC to tumor cells requires the presence of –SH groups on these cells. Addition of –SH groups on tumor cells was accomplished by the reduction of cystines as previously described [10]. A reducing agent (50 mM solution of TCEP in PBS) was injected intratumorally (i.t.) 1 h prior to application of SMCC ligands. The injection of TCEP alone does not have any effect on tumor growth [4].

Tumor transplantation

B16-F10 melanoma cells, suspended in serum free RPMI 1640, were inoculated subcutaneously (s.c.) in the previously shaved right flank of mice. Each mouse received 4×10^5 melanoma cells in 0.1 ml of medium.

Treatment and evaluation of treatment

Randomization of mice in groups was performed 12 days after transplantation of melanoma B16-F10 cells and was immediately followed by initiation of therapies based on intratumoral application of 50 microliters of corresponding preparations (day 0). All mice were housed individually during therapy.

Tumor size was measured with callipers every other day. Tumor volume was calculated, as previously described [11], using the formula $V = \pi/6\ AB^2$; A = the largest dimension of tumor mass (length), B = the smallest dimension of the tumor mass (height).

Mean reduction of tumor growth (%)

The calculation of mean reduction of tumor growth was performed as previously described [4]. After therapy began, on days 4, 6, 8, 10, 12 and 14, the reduction of tumor growth was calculated using the following formula:

$$\frac{(\text{mean tumor volume in control group} - \text{mean tumor volume in treated group}) \times 100}{\text{mean tumor volume in control group}}$$

The average of calculated reductions in the indicated days is regarded as "mean reduction of tumor growth".

Analysis of cell infiltrate using flow cytometry. Cytokine assay

Analysis of cell infiltrate was performed as previously described [4]. Mice were euthanized via cervical dislocation, and the tumors were excised. Subsequently, each tumor was gently washed with cold RPMI 1640 medium, cut into small pieces, and placed into 1 ml cold RPMI 1640 containing 0.33 mg/ml Liberase DL and 0.2 mg/ml DNase I (both Roche Diagnostics, Germany). After a 1 h incubation on a rotary shaker at 37 °C, clumps of tissue aggregates were centrifuged at 160 *g* for 10 min at 4 °C. Supernatant was used to determine IFN-gamma and IL-10 using the ELISA kit (eBioscience and LSBio, respectively), performed according to manufacturers recommendations. The resulting pellet was gently passed through a plastic strainer (70 μm, BD Biosciences, USA) into cold PBS (pH 7.3) and washed by centrifugation at 160 *g* for 10 min at 4 °C. Cells were then transferred into a 96-well plate (Corning Incorporated, USA) and analyzed by flow cytometry. Particular leukocyte subtypes were determined using the following monoclonal antibodies (eBioscience, USA): a) Total leukocytes - anti-mouse CD45 PerCP-Cy5.5; clone 30-F11, b) T cells - anti-mouse CD3e FITC; clone 145-2C11, c) CD4+ T cells - anti-mouse CD4 APC; clone GK1.5, d) CD8+ T cells - anti-mouse CD8a; clone 53–6.7, e) B cells - anti-mouse CD19 APC; clone eBio1D3, f) NK cells - anti-mouse NK1.1 PE; clone PK136, g) granulocytes (anti-mouse Ly-6G (Gr-1) Alexa Fluor 700; clone RB6-8C5, h) macrophages - anti-mouse F4/80 Antigen PE-Cy7; clone BM8, and i) dendritic cells - anti-Mouse CD11c PE; clone N418, anti-Mouse MHCII (I-A/I-E) Alexa Fluor 700; clone M5/114.15.2). Analysis was performed using a BD FACSCanto II flow cytometer (BD Biosciences, USA), equipped with two lasers (excitation capabilities at 488 nm and 633 nm). BD FACSDiva software 6.1.3. was used for the analysis of flow cytometry data.

Preparation and priming of neutrophils

Neutrophils were isolated from murine bone marrow according to Stassen et al. [12] and subsequently purified using MACS technique (Miltenyi Biotec). Purity was checked by BD FACSCanto II flow cytometer (BD Biosciences, USA) using anti-mouse CD45 APC, Clone: 30-F11 and anti-mouse Ly-6G (Gr-1) Alexa Fluor 700, Clone: RB6-8C5 antibodies (eBioscience). Neutrophils were primed according to Dewas et al. [13] by the mixture of GM-CSF and TNF-alpha (12 ng and 2.5 ng/ml respectively) for 20 min. The priming solution was enriched with 2 micromolar solution of soluble beta glucan (laminarin) as previously described [5]. Experiments were performed in complement containing medium (FCS was not heat inactivated).

Statistical analysis

Statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test and Log-rank test, respectively (STATISTICA 12, StatSoft, Inc., Tulsa, OK 74104, USA). Error bars indicate SEM.

Results

Searching for proper combination of TLR agonist and phagocytosis stimulating ligand leading to effective melanoma B16-F10 immunotherapy

The main goal of our study was to find proper TLR agonist(s), which, in combination with phagocytosis stimulating ligands, would result in tumor shrinkage and elimination. Previously, we discovered that mannan attached to tumor cells (hydrophobic BAM anchor or SMCC) was a good stimulator of phagocytosis [4, 5]. Thus, we used this finding throughout the present study. TLR ligand replacement was necessary due to the previously used LPS, a TLR4 agonist, which poses a dangerous threat to humans [4, 5]. Overall, three different TLR agonists were tested as possible LPS replacements.

First, we tried monophosphoryl lipid A (MPLA) which is an LPS derivative and TLR4 agonist with very low toxicity for humans. However, MPLA, mannan-BAM, or their mixture, lead to only slight, non-significant tumor growth reduction (Fig. 1). No signs of MPLA and mannan-BAM synergy were observed. Similar results were observed with the use of another tested compound, TLR7 agonist imiquimod (R-837) (data not shown).

Resiquimod (R-848), a TLR7 agonist in mice and TLR7 and 8 agonists in humans, was likewise studied. The R-848 + mannan-BAM combination revealed a strong synergistic effect resulting in 75.4% mean reduction of tumor growth (Fig. 2a). As shown in Fig. 2b, mice treated with this combination survived longer than PBS treated control group. However, the difference was not statistically significant. This experiment was repeated twice with similar results, including the observation of more than 100 days survival.

Therapy based on combination of R-848 with anchored f-MLF motif

The effect of therapeutics based on anchored mannan depends on the presence of mannan binding lectin (MBL) in

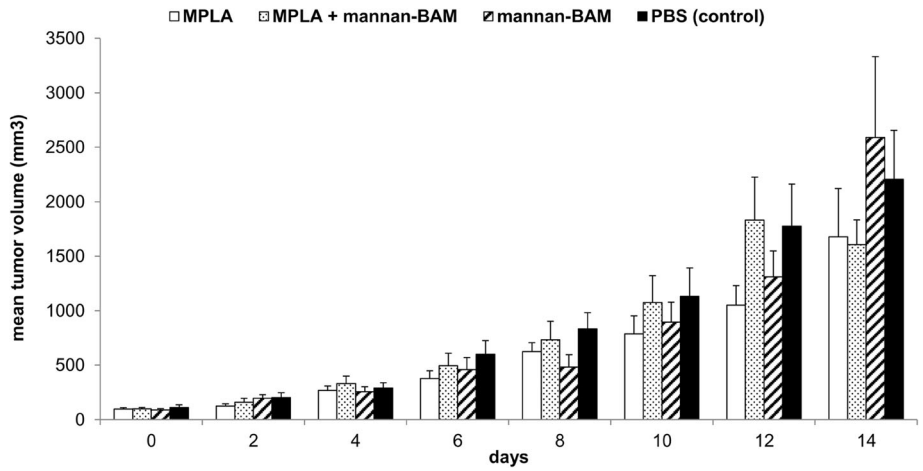

Fig. 1 Immunotherapy of melanoma B16-F10 using MPLA alone or in combination with mannan-BAM. C57BL/6 mice (females) were inoculated subcutaneously in a shaved area of the right flank with 4×10^5 murine melanoma B16-F10 cells per mouse in 0.1 ml RPMI. Twelve days after tumor transplantation, mice were randomized in groups of six. Therapies started immediately. The preparations were applied intratumorally (50 microliters/mouse) in pulse regime (days 0, 1, 2…8, 9, 10). After therapy commenced, mice were kept individually. Tumors were measured every second day for 14 days and their volume was calculated. The composition of preparations used was: 0.5 mg MPLA/ml PBS, 0.5 mg MPLA/ml 0.2 mM mannan-BAM in PBS, 0.2 mM mannan-BAM in PBS, PBS

serum. As 5–10% of humans lack MBL, it is necessary to have mannan independent therapeutic system. Therefore, we tested the combination R-848 + f-MLFKK-BAM and compared it to R-848 + mannan-BAM. Both mixtures caused comparable reduction of tumor growth (Fig. 3).

Immunotherapy of melanoma B16-F10 based on the synergy of R-848 and mannan-SMCC. Further improvement using poly(I:C) and anchored *L. monocytogenes*

The combination of R-848 with anchored mannan showed the best therapeutic effect from all studied combinations. Thus, we focused on further improvement of this therapy. Specifically, we tested a stronger binding of mannan-SMCC (covalent binding) together with the addition of other TLR agonists. *Listeria monocytogenes*, a predominant agonist of TLR2, was added alone and/or in the combination with TLR3 agonist poly(I:C). As shown in Fig. 4a, the mixture of R-848 with mannan-SMCC, resulted in strong inhibition of tumor growth. Further addition of *L. monocytogenes* alone or in the combination with poly(I:C) did not significantly improve its therapeutic effect. The major effects of additives were

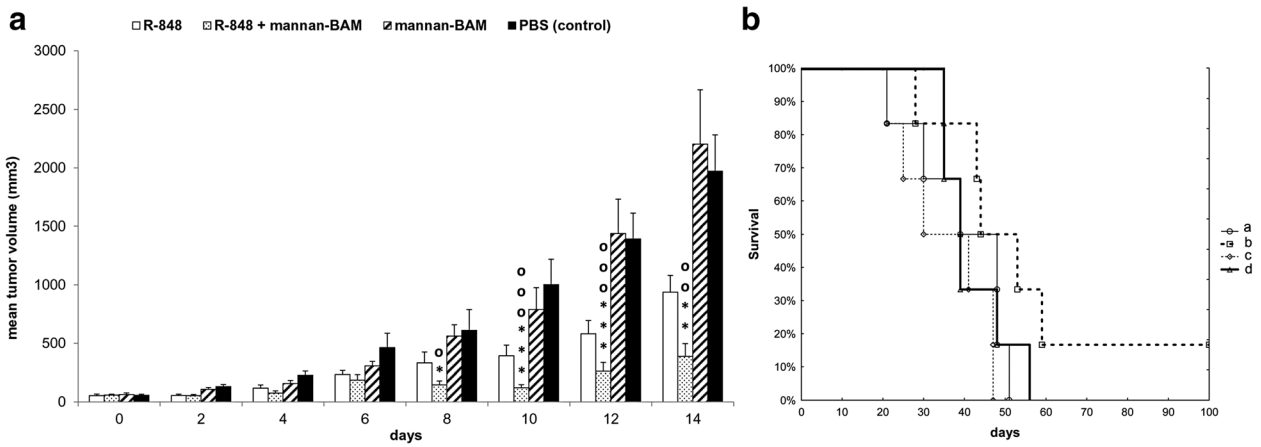

Fig. 2 Immunotherapy of melanoma B16-F10 based on the synergy of R-848 and mannan-BAM. The experimental design was the same as described in Fig. 1. Six mice were used per group. The composition of therapeutic mixture was: 0.5 mg R-848, HCl form/ml PBS, 0.5 mg R-848, HCl form/ml 0.2 mM mannan-BAM in PBS, 0.2 mM mannan-BAM in PBS, PBS. **a** The effect of therapy on tumor growth. * $P \leq 0.05$ * * $P \leq 0.005$ * * * $P \leq 0.0005$ compared to control (PBS). o $P \leq 0.05$ o o $P \leq 0.005$ o o o $P \leq 0.001$ compared to mannan-BAM. **b** Survival analysis. a – R-848, b – R-848 + mannan-BAM, c – mannan-BAM, d- PBS (control)

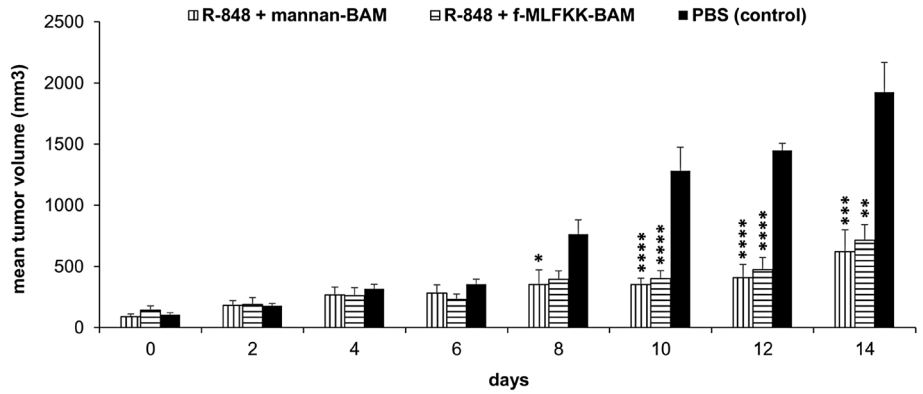

Fig. 3 Therapy based on the combination of R-848 with anchored f-MLF motif. The experimental design was the same as described in the Fig. 1. Six mice were used per group. The composition of therapeutic mixture was: 0.5 mg R-848, HCl form/ml 0.2 mM mannan-BAM in PBS, 0.5 mg R-848, HCl form/ml 0.5 mM f-MLFKK-BAM in PBS, PBS was used as a control. * $P \leq 0.05$ ** $P \leq 0.005$ *** $P \leq 0.001$ **** $P \leq 0.0005$ compared to control (PBS)

observed in the survival experiments. As shown in Fig. 4b, groups treated with *L. monocytogenes* exhibited significantly higher survival rates than untreated groups. An 83.3% survival rate was observed, independent of the presence or absence of poly(I:C). Furthermore, all surviving mice were re-transplanted again with B16-F10 on day 120. As shown in Table 1, re-transplantation was successful in one mouse in the group b and in two mice in group d. This resulted in death of animals. In the groups treated with the mixture containing poly(I:C) (c and e), the mice were fully protected against re-transplantation. All mice lived without any pathological symptoms for more than 1 year after treatment.

Flow cytometry analysis of cell infiltrate in R-848 + poly(I:C) + *L. monocytogenes*-SMCC + mannan-SMCC melanoma treatment. Cytokine assay

During the course of therapy with the complex therapeutic mixture (R-848 + poly(I:C) + *L. monocytogenes*-SMCC + mannan-SMCC), which showed the best therapeutic effect in the previous experiment, we analyzed tumor infiltrate from treated mice and compared it with a PBS control. In treated group, a strong granulocytic infiltration was observed. In particular, infiltration was higher between days 7 and 15, reaching statistical significance on day 7 (Fig. 5a). Minor, but not significant, increase of CD4+ Th lymphocytes was observed in the treated group, which contrasts with the no change observed in the control group (Fig. 5b). The levels of Tc lymphocytes (CD8+) were low in both groups throughout the monitored period (Fig. 5c). No dramatic changes in the count of dendritic cells were observed. However, a non-significant, higher quantity of these cells was observed throughout the monitored period when compared to the control group (Fig. 5d). No changes in B lymphocyte, NK, and monocyte/macrophage counts were observed.

Cytokines measurement revealed high levels of IFN-gamma (Fig. 6a), low levels of IL-10 (Fig. 6b), and high IFN-gamma/IL-10 ratio (Fig. 6c) in tumor environment of treated mice indicating initiation of Th1 response.

At the beginning of the therapy, mean tumor volume was 155.4 + −93.2 mm^3. Analysis of tumor infiltrating cells and cytokines was terminated on day 19 of treatment, as 9 surviving mice were tumor free.

Interaction of neutrophils with opsonized tumor cells – frustrated phagocytosis and oxidative burst

The role of phagocytes (granulocytes) in the herein described cancer treatment approach based on artificial opsonization of tumor cells is supported in Fig. 5. Depletion of neutrophils by Ly6G antibody reduced the effect of R-848 + mannan-BAM therapy [unpublished results]. An attempt of phagocytes (especially neutrophils) to phagocyte relatively large melanoma cells was described as a specific type of frustrated phagocytosis [5, 14]. This idea was supported by the estimation of the frequency of frustrated phagocytosis events in neutrophil-melanoma interaction, observed during in vitro experiments (Table 2). The key role of mannan and f-MLF attachment to tumor cell surface for the stimulation of frustrated phagocytosis was demonstrated.

Frustrated phagocytosis is initiated by tight contact between neutrophils and melanoma cells and is followed by the release of granule content into the pockets formed between neutrophils and tumor cells [5, 14]. Granules contain components involved in killing target melanoma cells either directly (hydrolases, defensins) or indirectly (myeloperoxidase dependent HClO formation connected with oxidative burst). We analyzed the cytotoxic effect of these processes and the participation of oxidative burst dependent mechanisms. The latter was analyzed

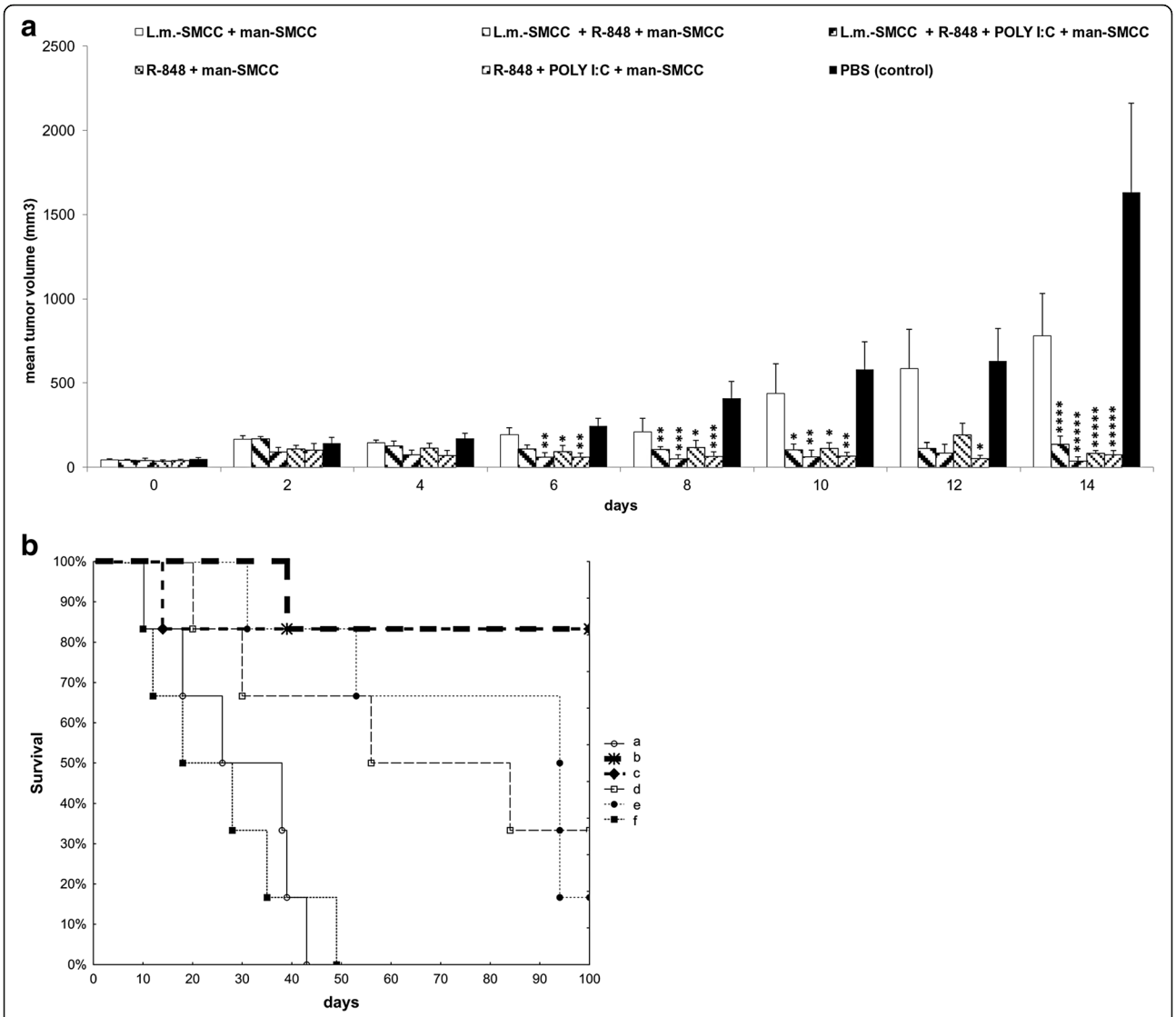

Fig. 4 Immunotherapy of melanoma B16-F10 based on the synergy of R-848 and mannan-SMCC. Further improvement using poly(I:C) and anchored *L. monocytogenes*. Tumor transplantation and measurement was performed as described in Fig. 1. Six mice were used per group. Therapies, based on intratumoral application of corresponding preparations (50 microliters/mouse), started 12 days after tumor transplantation. Four therapeutic pulses were applied on days 0, 1, 2…8, 9, 10…16, 17, 18…24, 25, 26. Therapeutic mixture contained following concentrations of active parts dissolved in PBS: 1 billion *L. monocytogenes*-SMCC/ml, 0.5 mg R-848, HCl form/ml, 0.5 mg poly(I:C)/ml, 0.2 mM mannan-SMCC, PBS was used as control. **a** The effect of therapy on tumor growth. * $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.005$ **** $P \leq 0.001$ ***** $P \leq 0.0005$ compared to control (PBS). **b** Survival analysis. a – L.m.-SMCC + man-SMCC. b – L.m.-SMCC + R-848 + man-SMCC. c – L.m.-SMCC + R-848 + poly(I:C) + man-SMCC. d – R-848 + man-SMCC. e – R-848 + poly(I:C) + man-SMCC. f – PBS (control). b versus f ….. $P \leq 0.005$. c versus f ….. $P \leq 0.01$. d versus f ….. $P \leq 0.05$. e versus f ….. $P \leq 0.05$

Table 1 Immunotherapy of melanoma B16-F10 based on the synergy of R-848 and mannan-SMCC. Further improvement using poly(I:C) and anchored *L. monocytogenes*. Re-transplantation

Initial treatment	Number of survived mice	Group (see Fig. 4b)	Number of successful re-transplantations
L. monocytogenes-SMCC + R-848 + mannan-SMCC	5	b	1
L. monocytogenes-SMCC + R-848 + poly(I:C) + mannan-SMCC	5	c	0
R-848 + mannan-SMCC	2	d	2
R-848 + poly(I:C) + mannan-SMCC	1	e	0

Re-transplantation of mice that survived in experiment shown in Fig. 4 was performed on day 120. All surviving mice were inoculated again with B16-F10 (4×10^5 melanoma cells/mouse s.c.)

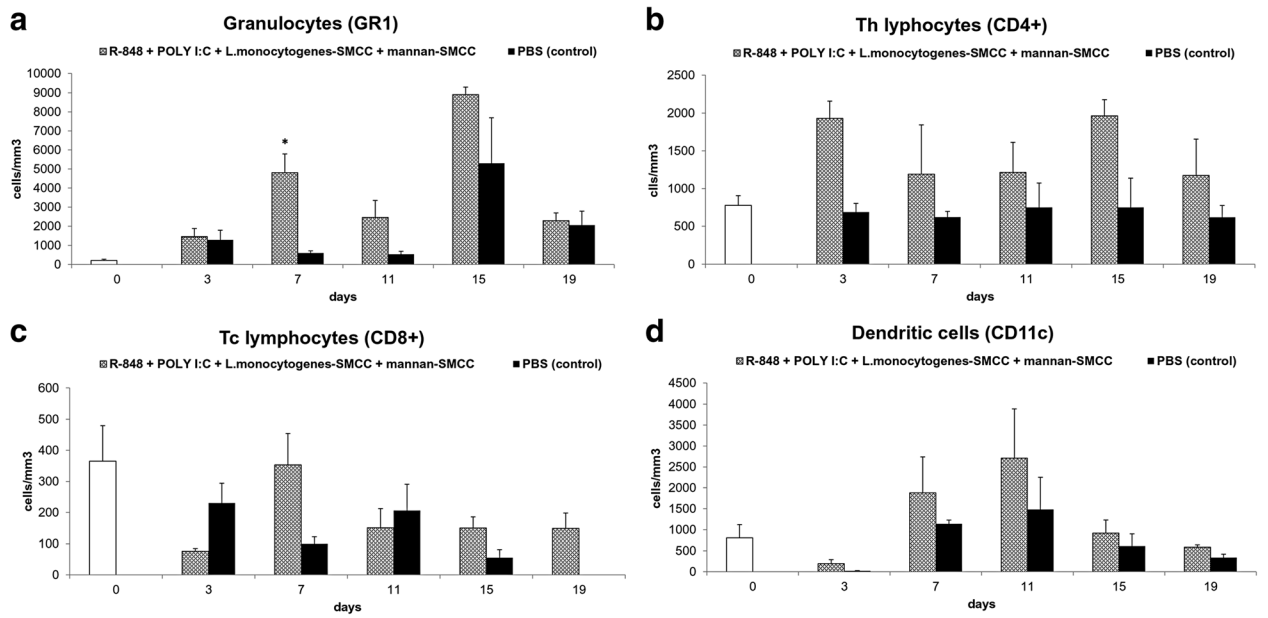

Fig. 5 Flow cytometry analysis of cell infiltrate in R-848 + poly(I:C) + *L. monocytogenes*-SMCC + mannan-SMCC melanoma treatment. The transplantation of melanoma B16-F10 was performed as described in Fig. 1. Twelve days after tumor transplantation, mice were randomized in two groups of 24. Therapy based on intratumoral application of corresponding therapeutic mixtures (50 microliters/mouse) started immediately. Four therapeutic pulses were applied on days 0, 1, 2…8, 9, 10…16, 17, 18…24, 25, 26. The composition of therapeutic mixture was: 1 billion *L. monocytogenes*-SMCC + 0.5 mg R-848, HCl form + 0.5 mg poly(I:C)/ml 0.2 mM mannan-SMCC in PBS, PBS was used as a control. Three mice from each group were euthanized on days 3, 7, 11, 15, 19 after the start of the therapy. Three mice were killed without any application at time 0 (negative control). The analysis of cell infiltrate of excised tumors was performed using flow cytometry and expressed as cells/mm^3 of tumor mass. The following labeled antibodies were used: **a** anti-mouse Ly-6G (Gr-1) Alexa Fluor 700 (granulocyte detection), **b** anti-mouse CD4 APC; clone GK1.5 (CD4+ Th lymphocytes), **c** anti-mouse CD8a; clone 53–6.7 (CD8+ Tc lymphocytes), **d** anti-Mouse CD11c PE; clone N418, anti-Mouse MHCII (I-A/I-E) Alexa Fluor 700; clone M5/114.15.2 (dendritic cells). * $P \leq 0.05$ compared to control (PBS)

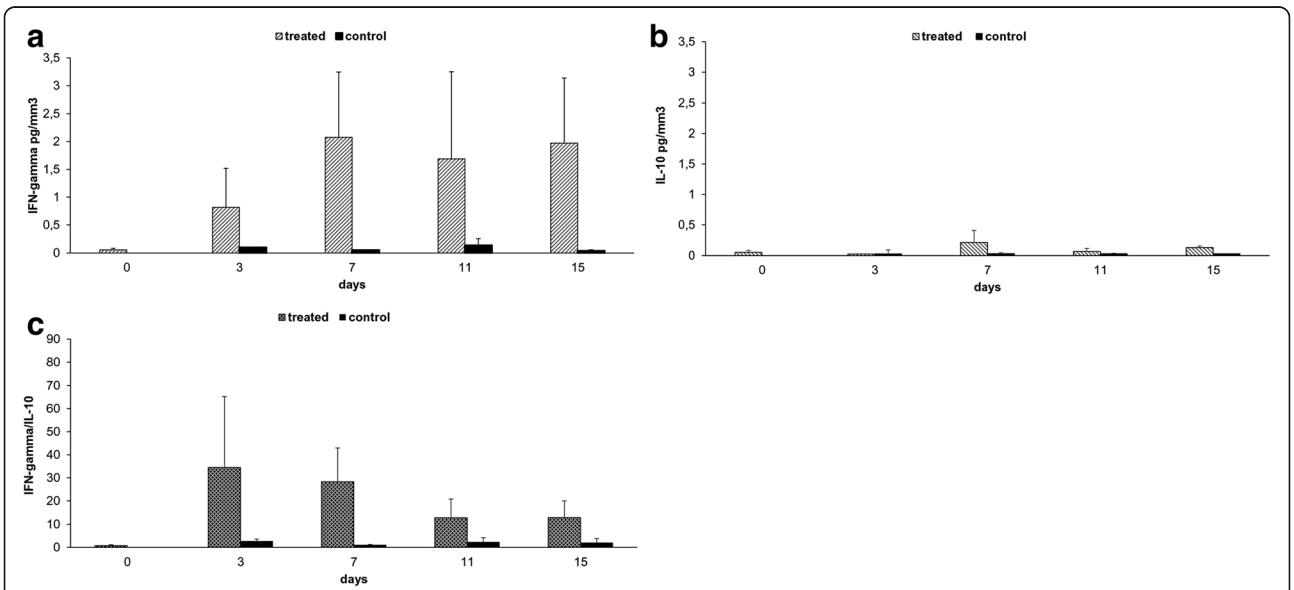

Fig. 6 R-848 + poly(I:C) + *L. monocytogenes*-SMCC + mannan-SMCC melanoma treatment. Cytokine assay. After separation of cells from tumors excised in previous experiment (Fig. 5), cytokine analysis of corresponding supernatants was performed: **a** IFN-gamma, **b** IL-10, **c** calculated ratio of IFN-gamma/IL-10

Table 2 Interactions between neutrophils and melanoma cells with or without anchored ligands of phagocytic receptors

	Mean count of neutrophils attached to one melanoma cell by mechanism of frustrated phagocytosis		
Ligand	20 min.	30 min.	40 min.
Mannan-BAM	0.333	0.556	0.733
Mannan free	0.111	0.000	0.000
f-MLFKK-BAM	0.589	0.311	0.360
f-MLF free	0.480	0.111	0.000
PBS (control)	0.000	0.000	0.000

B16-F10 melanoma cells were incubated (30 min, 37 °C) with 0.02 mM mannan-BAM or 0.05 mM f-MLFKK-BAM in culture medium and then subsequently washed. Suspension of bone marrow neutrophils (90% purity) primed with GM-CSF + TNF-alpha (+ laminarin in case of mannan-BAM) in culture medium was added to B16-F10 cells. Free ligands were added in a concentration of 0.02 mM (mannan) and 0.05 mM (f-MLFKK). Neutrophils and melanoma cells were incubated at a 2:1 ratio

The rate of frustrated phagocytosis events was estimated by light microscopy: Frustrated phagocytosis was defined as neutrophil/melanoma cell contact, where neutrophil adhere tightly to B16-F10 cell. Such contact is further characterized by neutrophil flattening and gaining of waning moon shape [5]

using epicatechin - an inhibitor of oxidative burst. As shown in Fig. 7a, neutrophils killed 50% of opsonized melanoma cells and the inhibition of oxidative burst resulted in 50% reduction of cytotoxicity. This result supports the hypothesis that oxidative burst participates on cytotoxic effects resulting from the frustrated phagocytosis.

Neither melanoma cells (Fig. 7a) nor neutrophils (Fig. 7b) were directly affected by epicatechin alone.

Discussion

Our previous study demonstrated that TLR agonists combined with phagocytic receptors ligands may act as an effective cancer therapy [4, 5]. In the present follow-up study, we focused on searching for ligands that could be applied in humans. To stimulate phagocytosis, mannan was attached to tumor cells either covalently by an SMCC anchor or through a hydrophobic BAM anchor. As we described previously, mannan stimulates MBL-dependent phagocytosis, which kills tumor cells [4, 5]. This is based on the initiation of the lectin pathway of complement activation with MBL-mannan complex, leading to iC3b opsonization of target cells.

The TLR4 agonist LPS, which was used in our previous studies [4, 5] cannot be utilized in humans due to the risk of developing septic shock. We tested several possible replacement compounds, the first being MPLA, a low-toxicity derivate of the lipid A region of LPS [15]. MPLA alone showed only negligible effect on tumor growth. When combined with mannan, no synergy resulted. Mata-Haro and colleagues [16] reported that low toxicity of MPLA, in comparison to LPS, is caused by the active suppression of pro-inflammatory activity. This could explain the failure of MPLA + mannan-BAM

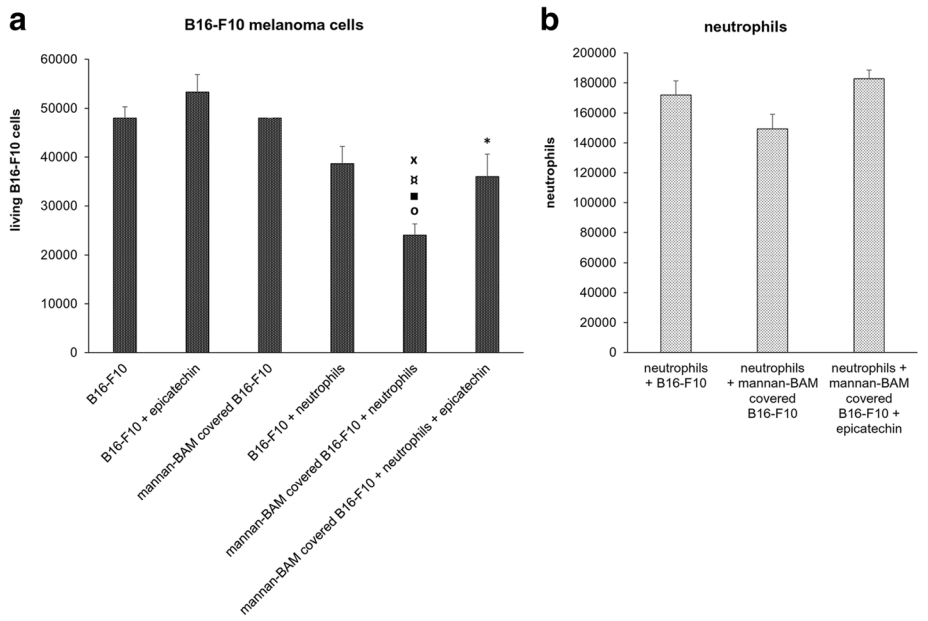

Fig. 7 The interaction of neutrophils with opsonized melanoma cells. Oxidative burst. B16-F10 melanoma cells were incubated (30 min, 37 °C) with 0.02 mM mannan-BAM in culture medium and subsequently washed. The suspension of bone marrow neutrophils (90% purity), primed with GM-CSF + TNF-alpha + laminarin in culture medium was added to B16-F10 (both free and mannan-BAM covered) in the ratio 5:1. Where indicated, 0.1 mM epicatechin was added. All mixtures were incubated for 2 hours at 37 °C. After the incubation, living, trypan blue excluding melanoma cells (**a**) and neutrophils (**b**) were counted with a haemocytometer. *$P \leq 0.05$ compared to B16-F10 + epicatechin. o$P \leq 0.005$ compared to B1-F10. ■$P \leq 0.0005$ compared to B16-F10 + epicatechin. ¤$P \leq 0.005$ compared to mannan-BAM covered B16-F10. x$P \leq 0.05$ compared to B16-F10 + neutrophils

therapy, as infiltration of inflammatory cells is crucial for the presented therapy [4, 5].

R-837, another TLR agonist, has been shown to induce an anti-tumor immune response and is being used to treat skin tumors [17]. However, in our experiments, R-837 only exhibited a weak effect, and was thus not involved in further treatments. An explanation for the insufficient impact of R-837 on tumor cells could be that it induces less pronounced production of cytokines and enhancement of cellular immunity than R-848, for a review see [18].

The last tested LPS replacement substance was R-848. R-848 alone caused visible, but not statistically significant tumor growth reduction. Complete recovery was not observed. However, R-848 combined with anchored mannan resulted in significant synergy and partial recovery of treated mice. Regarding the mechanisms of action, we are considering the important role of granulocytes (neutrophils), as their strong infiltration was noticed. In our previous in vitro experiments, we observed a significant cytotoxic effect of neutrophils against the tumor cells opsonized with mannan. These tumor cells were killed by frustrated phagocytosis [5]. In herein present study, we confirmed our previous observations and by using epicatechin, revealed significant participation of oxidative burst in killing mechanisms.

The synergy between R-848 and anchored mannan corresponds to our therapeutic concept based on inflammatory infiltration of tumors and the direction of recruited phagocytes to opsonized tumor cells [4, 5]. Ensuring proper timing of drug delivery is vital for an effective therapy. For R-848 + mannan based therapies we used the same pulse regime as previously described [4]. The optimal therapeutic scheme corresponds well with the observation from Bourquin et al. [19], who based their tumor treatment strategy on the repeating cycles of R-848 injections, separated by treatment-free intervals. Treatment free intervals are necessary for the recovery of sensitivity to R-848. R-848, like other TLR agonists, induces TLR tolerance, which should be circumvented by proper timing of therapy [20, 21].

The induction of synthesis of pro-inflammatory cytokines in the tumor environment is important for the recruitment of inflammatory cells. Simultaneously, conditions for the shift of tumor-associated macrophages (TAMs) towards an anti-tumor, pro-inflammatory M1 phenotype and reduction of the activity of tumor protecting immunosuppressive T regulatory lymphocytes (Tregs) and myeloid derived suppressor cells (MDSC) are created [22]. The direct effect of R-848 on MDSC count reduction [23] and the stimulation of phagocytic activity of infiltrating cells by TLR agonists should be taken into account [24].

R-848 also induces the maturation of plasmacytoid dendritic cells (pDC) [25] and promotes the production of antibodies [26]. R-848 was described as potential vaccine adjuvant enhancing Th1 response in mice [27]. Furthermore, R-848 also had a direct effect on tumor cells - it upregulates the expression of opioid growth factor receptor, which leads to the anti-proliferative and cancer suppressive effects, independent of immune function [28]. All these mechanisms can contribute to the effect of therapy.

Since a small percentage of humans are MBL deficient, f-MLF was tested as an alternative ligand of phagocytic receptors. When anchored, f-MLF was able to stimulate phagocytosis and kill tumor cells [5]. Positive results of the treatment with R-848 + anchored f-MLF supported the possibility of using this ligand for the treatment of patients with an MBL deficiency.

To enhance the effect of R-848 + mannan based therapy, we tested the the effects of adding of heat killed *L. monocytogenes* into the treatment mixture. Introduction of *L. monocytogenes* did not accelerate the shrinkage of tumors, but had a strong effect on survival rate of mice. Heat killed *L. monocytogenes* is able to induce Th1-dominated immune response [29]. We hypothesize that cell-mediated adaptive immunity joined the innate immune response and eliminated the remaining melanoma cells. This is supported by the observed Th1 response initiation. Moreover, 80% of mice were protected against re-transplantation of melanoma cells, which suggests that acquired immunity response was directed against melanoma specific antigens and that tumor antigen-specific memory cells were involved.

The addition of poly(I:C) into the therapeutic mixture (with and without *L. monocytogenes*) also increased the resistance of treated mice against re-transplantation. Poly(I:C) works in synergy with R-848 at the level of stimulation of pro-inflammatory cytokines synthesis [30, 31] and is frequently used as vaccine adjuvant. Additionally, poly(I:C) stimulates both human [32] and murine [33] dendritic cells maturation, so it can enhance antigen presentation to the cells of adaptive immunity.

Survival of all treated mice for more than 1 year after treatment serves as indirect proof that the presented combined therapy (*L. monocytogenes* + R-848 + poly(I:C) + mannan-SMCC) may eliminate metastases as well, because B16-F10 tumors metastasize very early (before the day 10 after transplantation as described by Wald et al. [34], i.e. prior to the initiation of our therapy). However, this aspect needs further investigation.

In summary, we have demonstrated the strong therapeutic effect when the TLR agonist R-848 is combined with anchoring mannan to the tumor cells. This effect was further enhanced by addition of another TLR agonists (poly(I:C), *L. monocytogenes*) into the therapeutic mixture. Innate immunity cells, particularly neutrophils, seem to play a key role in the presented treatment mechanism. Evaluating the role of adaptive immunity in the above described therapy will be the main goal as we continue our research.

Conclusions

Therapy based on R-848 + mannan-SMCC with supportive *L. monocytogenes* and poly(I:C) is much too complex to provide a detailed description of all involved mechanisms. Nevertheless, the acting components play important roles and perform in synergy. We assume that this therapy can be used for cancer treatment in humans, as the majority of the components in the therapeutic mixture have already been used or tested in clinical trials. The presented treatment of fast growing, aggressive and low immunogenic B16-F10 melanoma, represent a base for promising future research in the field of human cancer immunotherapy.

Additional files

Additional file 1: Fig1-4,6,7_DATA. (XLSX 45 kb)

Additional file 2: Fig5_DATA. (XLSX 151 kb)

Abbreviations

f-MLF: N-Formyl-methionyl-leucyl-phenylalanine; f-MLFKK: N-Formyl-methionyl-leucyl-phenylalanine with two lysine molecules; LPS: Lipopolysaccharide; PAMPs: Pathogen associated molecular patterns; poly(I:C): Polyinosinic:polycytidylic acid, sodium salt; SMCC: 4-(*N*-Maleimidomethyl) cyclo-hexanecarboxylic-acid N-hydroxysuccinimide ester; TCEP: Tris(2-carboxyethyl)phosphine hydrochloride; TLR: Toll-like receptor

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its Additional file 1: Figure S1–S4, S6, S7 and Additional file 2: Figure S5. These data files contain all data used in Figs. 1, 2, 3, 4, 5, 6 and 7.

Authors' contributions

Conceived and designed the experiments: JŽ VC JK JC. Performed the experiments: VC AV ZK SG HH NV GK LP JŽ. Analyzed the data: VC JŽ. Wrote the paper: VC JŽ IJ KIW. Manuscript discussed by: VC JC JK IJ KIW. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval

All experimental procedures with mice were performed in accordance with the laws of the Czech Republic. Experimental project was approved by Ministry of Education, Youth and Sports (protocol no. 28842/2014-3).

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Paper 4

An effective cancer immunotherapy of melanoma and pancreatic adenocarcinoma based on a combination of TLR agonists and the stimulation of phagocytosis

An effective cancer immunotherapy based on a combination of TLR agonists and the stimulation of phagocytosis

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Abstract

Immunotherapy represent effective cancer treatment. Many different types of immunotherapies are currently used in cancer management. Immunotherapy based on activation of innate immunity via Pathogen-associated molecular patterns (PAMPs) seems to be a promising concept for cancer treatment. This therapy is based on the combination of two groups of PAMPs, phagocytosis stimulating ligands (mannan) and TLR agonists (resiquimod (R-848), poly(I:C), *L. monocytogenes).* Intratumoral application of mixture of these compounds resulted in strong reduction of tumor growth in the B16-F10 melanoma model. In current study, we focused on optimization of the composition as well as the timing of this therapeutic mixture. These modifications resulted in eradication of tumors in most of the experimental mice, resistance to B16-F10 cells re-transplantation, and eradication of metastases in some of the experimental animals. Optimized therapy was also tested in pancreatic adenocarcinoma mouse model (Panc02 model) with promising effect on tumor elimination. Moreover, the combination of this therapy with checkpoint inhibitors or immune activators was tested. The results of our study suggest that

immunotherapy based on application of PAMPs has strong tumor eliminative effect in several tumor models with potential anti-metastatic effect.

Paper 5

Immunotherapy based on intratumoral application of mannan and TLR ligands in pheochromocytoma mouse model

Immunotherapy based on intratumoral application of mannan and TLR ligands in pheochromocytoma mouse model

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Preliminary data

Abstract

Pheochromocytoma and paraganglioma (PHEO/PGL) are rare neuroendocrine tumors localized in adrenal gland or extra-adrenally, respectively. Around 10%-30% of these tumors are malignant with limited amount of treatment. In the current study, we focused on investigation of recently emerging immunotherapy approach based on activation of innate immunity via Pathogen-associated molecular patterns (PAMPs) in PHEO. Suitable PHEO mouse tumor model was established. Subsequently, intratumoral application of PAMPs (mannan-BAM + R-848 + LTA + POLY(I:C)) resulted in a strong reduction of tumor growth in the PHEO mouse model. Tumor growth reduction was also associated with prolongation of survival. The participation of innate immunity in PHEO elimination was verified using mice lacking functional adaptive immune cells. The mechanisms involved in PHEO elimination were investigated using neutrophils and MTT-luciferase PHEO cells *in vitro*. Based on our preliminary data, innate immunity activation in PHEO mouse model leads to the reduction or the elimination of tumors and thus represents a potential future therapy for PHEO patients.

4. SUMMARY OF THE RESULTS

This thesis is based on five research papers (three of them are published in journals with the impact factor, two of them are in a manuscript version). The whole research is focused on application of immunotherapy based on combination of phagocytosis stimulating ligands and TLR agonists in melanoma, pancreatic adenocarcinoma, and pheochromocytoma mouse models.

Testing new anticancer therapies is crucial since there is no effective treatment for many type of tumors. Immunotherapy seems to be a promising therapeutic approach by using a body´s own immune system to eliminate tumor cells from the organism. Moreover, this therapy has a minimal harmful effect on healthy cells.

PAMPs are potent molecules in cancer treatment because of their ability to activate innate immune system (Hobohm et al., 2008). In our first study **(Paper 1)**, new therapeutic concept based on combination of phagocytosis stimulating ligands and TLR agonists was proposed. Three different phagocytosis stimulating ligands, specifically laminarin, mannan, and formylpeptide receptors ligand were tested in the B16-F10 mouse model. Two types of anchors (BAM anchor, SMCC anchor) were tested for effective binding of ligands stimulating phagocytosis to tumor cell membranes.

LPS was used as a TLR agonist. Laminarin-BAM combined with LPS resulted in 90.2% reduction of tumor growth compared to the control group. Mannan-BAM combined with LPS resulted in 88.6% reduction of tumor growth. Formylpeptide receptors ligand combined with LPS resulted in 78.3% reduction of tumor growth. Combinations of mannan-BAM/LPS and f-MLF-BAM/LPS resulted in an 80% and 60 % recovery for mice, respectively (Janotova et al., 2014).

The analysis of tumor infiltrating leukocytes during the therapy revealed changes in granulocytes count caused predominantly by LPS. No dramatic changes in count of other immune cells were observed. *In vivo* results were also verified in *in vitro* experiments (Janotova et al., 2014).

In summary, both *in vivo* and *in vitro* experiments revealed a reduction of tumor cells as a result of activation of immune cells by anchored phagocytosis stimulating ligands. The underlying mechanisms involved in tumor cell elimination were investigated by tumor infiltrating leukocytes analysis, measuring activation of macrophages by western blot, and observation of interaction of tumor cells and macrophages (Janotova et al., 2014).

In the second study **(Paper 2)**, Zymosan A, gram-positive and gram-negative bacteria were tested for their potential to be used in cancer treatment. Bacteria can represent an important tool for cancer treatment because of their complexity in PAMPs expression (Waldmannova et al., 2016).

Zymosan A was covalently or by BAM anchor bound to the surface of tumor cells with simultaneous application of LPS. The therapeutic mixture with BAM anchor resulted in temporary elimination of tumors with 98.1% reduction of tumor growth compared to the control. The therapeutic mixture with covalent anchor resulted in temporary elimination of tumors with 97.7% reduction of tumor growth compared to the control. The application schedule was also modified to enhance effectivity of the treatment. To uncover immune mechanisms involved in tumor elimination during the therapy, histological analysis and flow cytometry analysis of the tumors were performed. The results, similarly to the previous paper, revealed strong granulocyte infiltration in all treated groups. This strong infiltration was also linked to LPS application (Waldmannova et al., 2016).

Subsequently, gram-negative bacteria *S. maltophilia* (positive charged) and *S. marcescens* (negatively charged) were tested for their possible positive therapeutic effect in a melanoma model. The intratumoral application of positive charged bacteria *S. maltophilia* resulted in 61.3% reduction of tumor growth, however, the negative charged bacteria *S. marcescens* resulted only in slight reduction of tumor growth. After neutralization of the negative charge and positive depolarization, intratumoral application of *S. marcescens* resulted in a 66.5% reduction of tumor growth (Waldmannova et al., 2016).

Mycobacterium tuberculosis and *L. monocytogenes* were studied as well. Covalent binding of these bacteria to tumor cells resulted in strong reduction of tumor growth.

The mechanism of tumor cells elimination during the therapy was investigated. *In vitro* experiments revealed activation of neutrophils as a result of their interaction with tumor cells covered by anchored phagocytosis stimulating ligands. As a consequence of the neutrophil activation, frustrate phagocytosis of tumor cells by neutrophils was observed (Waldmannova et al., 2016).

In the third study **(Paper 3),** we focused mainly on modification of previously published therapeutic mixture. In the previous study, laminarin, mannan, and N-Formyl-methionyl-leucyl-phenylalanine (FMLP) were tested as phagocytosis stimulating ligands. For the current study, only mannan and FMLP were chosen as the most effective phagocytosis stimulating ligands. The first step in the modification process was LPS replacement. LPS is well tolerated in mouse (Warren et al., 2010), nevertheless, the equivalent doses can cause septic shock in humans (Yamamoto et al., 2011).

Monophosphoryl lipid A (MPLA), imiquimod (R-837), and resiquimod (R-848) were tested as a potential replacement of LPS. Only resiquimod in combination with mannan-BAM resulted in significant synergy in reduction of tumor growth. The second step was to enhance the therapeutic effect of the mixture. This was accomplished by a combination of several TLR agonists. *L. monocytogenes* and poly(I:C) were combined with R-848. This combination resulted in a complete recovery in 83% mice and their protection from re-transplantation of melanoma cells. Also, Flow cytometry analysis of tumor infiltrating leukocytes revealed strong granulocyte infiltration in the treated group compared to the control group (Caisova et al., 2016).

In the fourth study **(Paper 4)**, we focused on final optimization of the therapeutic mixture (mannan-BAM + R-848 + *L. monocytogenes* + Poly (I:C)) successfully used in Paper 3. Moreover, the optimized therapy was tested in another type of tumor, specifically in pancreatic adenocarcinoma mouse model. First, the composition of the therapeutic mixture was optimized. *L. monocytogenes* was replaced by more defined substance called lipoteichoic acid (LTA, TLR 2 agonist). This optimized therapy resulted in the eradication of melanoma tumors in 83% of mice. More importantly, optimized therapeutic mixture caused resistance to tumor cells re-transplantation and potential anti-metastatic effect. As a second step, we optimized the therapeutic schedule. Therapy based on four therapeutic pulses resulted in the most effective eradication of tumors.

During the investigation of underlying mechanisms, we found that the activation of innate immunity is followed by activation of adaptive immunity.

To investigate the effect of the therapy in another tumor types, we tested application of therapeutic mixture in very aggressive pancreatic adenocarcinoma. The therapy of pancreatic carcinoma resulted in less significant reduction of tumor growth compared to melanoma. However, the therapy was significantly improved by combination with anti-CD40 antibody. Subsequently, the synergistic effect with checkpoints inhibitors was investigated.

The last manuscript with preliminary data **(Paper 5)** is focused on testing immunotherapy based on combination of phagocytosis stimulating ligands and TLR agonists (mixture of mannan-BAM + R-848 + Poly (I:C) + LTA) in mouse pheochromocytoma model. Pheochromocytoma is a rare neuroendocrine tumor with catecholamine secretion. Most of these tumors are benign and curable. However, the treatment for malignant pheochromocytoma is missing (Scholz et al., 2007). Therefore, investigation of new therapeutic strategies is crucial.

First, the new mouse model for immunotherapy testing in pheochromocytoma was established. The immunotherapy based on combination of phagocytosis stimulating ligands and TLR agonists was tested. This therapy resulted in a strong reduction of tumor growth compared to the control group. The predominant role of innate immunity was verified using mouse lacking functional T- and B-lymphocytes. In these mice immunotherapy resulted in similar reduction of tumor growth as in immunocompetent mice.

The underlying mechanisms involved in the pheochromocytoma tumor elimination were also investigated. The flow cytometry analysis and histological analysis revealed strong tumor infiltration with leukocytes during the immunotherapy. In addition, the effect of neutrophils on mouse pheochromocytoma MTT cells covered with attached mannan-BAM was studied.

5. CONCLUSION AND FUTURE PERSPECTIVES

Immunotherapy based on intratumoral application of phagocytosis stimulating ligands and TLR agonists seems to be promising therapeutic approach for cancer treatment (Caisova et al., 2016; Janotova et al., 2014; Waldmannova et al., 2016). This therapy tested in melanoma mouse model, pancreatic adenocarcinoma mouse model, and pheochromocytoma mouse model revealed significant reduction of tumor growth. The tumors were completely eliminated in most of the experimental animals. Moreover, the therapy resulted in a resistance against tumor cells re-transplantation. Innate immunity was identified as the critical immune part involved in the tumor elimination. Furthermore, after innate immunity activation antigens were presented to adaptive immune cells, which resulted in a long term anticancer immunity. Possible combination of this therapy with other therapies, such as checkpoint inhibitors and immune activators, opens new perspectives for cancer management (Caisova et al., 2016; Janotova et al., 2014; Waldmannova et al., 2016).

Despite the positive results of the immunotherapy in mouse tumor models, further studies are needed to prove the efficiency for human use.

The future research will be focused on

I. Possible anti-metastatic effect of the therapy

We aim to intensify the activation of adaptive immunity during the therapy based on intratumoral application of phagocytosis stimulating ligands and TLR agonists. This can result in stronger anti-metastatic effect.

II. Investigation of mechanisms involved in tumor elimination

The mechanisms involved in the therapy based on intratumoral application of ligands stimulating phagocytosis and TLR agonists will be deeply investigated.

Contribution of adaptive immunity and detailed mechanisms will be studied *in vivo* using knock-out mice and blocking antibodies.

III. Testing of combination of presented immunotherapy with other available cancer therapies for enhancement of the anticancer effect

Combined therapy (combination two or more therapeutic agents) is fundamental for cancer therapy. Based on our study, the combination of presented immunotherapy with checkpoint inhibitors/immune activators has a beneficial effect on the therapeutic effectivity. This combination will be studied in more detail as well as combination with other available tumor therapies.

6. LIST OF ABREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
BAM	biocompatible Anchor for Membrane
BCG	bacillus Calmette-Guerin
CAR T-cells	chimeric antigen receptor Tcells
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CR3	complement receptor 3
DC	dendritic cell
DNA	deoxyribonucleic acid
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
dsRNA	double-stranded RNA
FMLP	N-Formyl methionyl-leucyl-phenylalanine
IL	interleukin
INF	interferon
LPS	lipopolysaccharides
LRRs	leucine-rich repeats
LTA	lipoteichoic acid
MEN2	multiple endocrine neoplasia type 2
MHC	major histocompatibility complex
MoAb	monoclonal antibody
MPLA	monophosphoryl lipid A
MR	mannose receptor
mRNA	messenger ribonucleic acid
MTT cells	mouse tumor tissue cells
MyD88	myeloid differentiation primary response 88
NF1	neurofibromatosis type 1
NF-kappa B	nuclear factor-κB
NH_2	amino group
NHS	N-Hydroxysuccinimide
NK cells	natural killer cells

PAMPs	pathogen-associated molecular patterns
PD1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PHEO/PGL	paraganglioma-pheochromocytoma (PGL/PCC)
Poly(I:C)	polyinosinic-polycytidylic acid
PRRs	pattern recognition receptors
R-848	resiquimod
R-837	imiquimod
TIL	tumor-infiltrating lymphocytes
TLR	toll-like receptors
TGF	transforming growth factor
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
VHL	von Hippel–Lindau disease

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2014 – present University of South Bohemia, Faculty of Biological Sciences: Ph.D. studies, Infection biology, Ph.D. thesis, Studium nádorové imunoterapie založené na instalaci ligandů fagocytárních receptorů na nádorové buňky a objasnění probíhajících procesorů [The study of cancer immunotherapy based on installation of phagocytosis stimulating ligands on the tumor cells surface and investigation of underlying mechanisms]; supervisor: RNDr. Jan Zenka CSc.

2012 – 2014 University of South Bohemia, Faculty of Biological Sciences: MSc. studies, Clinical Biology, diploma thesis, Pouziti agonistu formyl peptidovych receptoru v terapii nadorovych onemocneni [The use of agonists of formyl peptide receptors for cancer therapy]; supervisor: RNDr. Jan Zenka CSc. (graduate with honors)

2008 – 2012 University of South Bohemia, Faculty of Biological Sciences: Bc. studies, Biology, bachelor thesis, Aplikace patogennich organismu a jejich casti v terapii nadorovych onemocneni [The application of the pathogen organisms and their parts in tumor immunotherapy]; supervisor: RNDr. Jan Zenka CSc.

Research Internship

2016 – present Visiting fellow; National Institutes of Health, Bethesda, Maryland 20892, USA

Postgraduate Courses

2014 Institute of Postgraduate Education in Medicine, Prague: Methods in Laboratory Medicine (accredited qualification course)

2014 Prague school of flow cytometry: Flow cytometry in hematological malignancies

International Conferences

2016 3th Immunotherapy of cancer conference 3 ITOC, Munich
Poster presentation

Publications

Janotova, T., M. Jalovecka, M. Auerova, I. Svecova, P. Bruzlova, V. Maierova, Z. Kumzakova, S. Cunatova, Z. Vlckova, **V. Caisova**, P. Rozsypalova, K. Lukacova, N. Vacova, M. Wachtlova, J. Salat, J. Lieskovska, J. Kopecky and J. Zenka (2014). "The use of anchored agonists of phagocytic receptors for cancer immunotherapy: B16-F10 murine melanoma model." PLoS One 9(1): e85222. **IF** (2014): 3.234

Waldmannova, E., **V. Caisova**, J. Faberova, P. Svackova, M. Kovarova, D. Svackova, Z. Kumzakova, A. Jackova, N. Vacova, P. Nedbalova, M. Horka, J. Kopecky and J. Zenka (2016). "The use of Zymosan A and bacteria anchored to tumor cells for effective cancer immunotherapy: B16-F10 murine melanoma model." Int Immunopharmacol 39: 295-306.
IF (2016): 2.956

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