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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

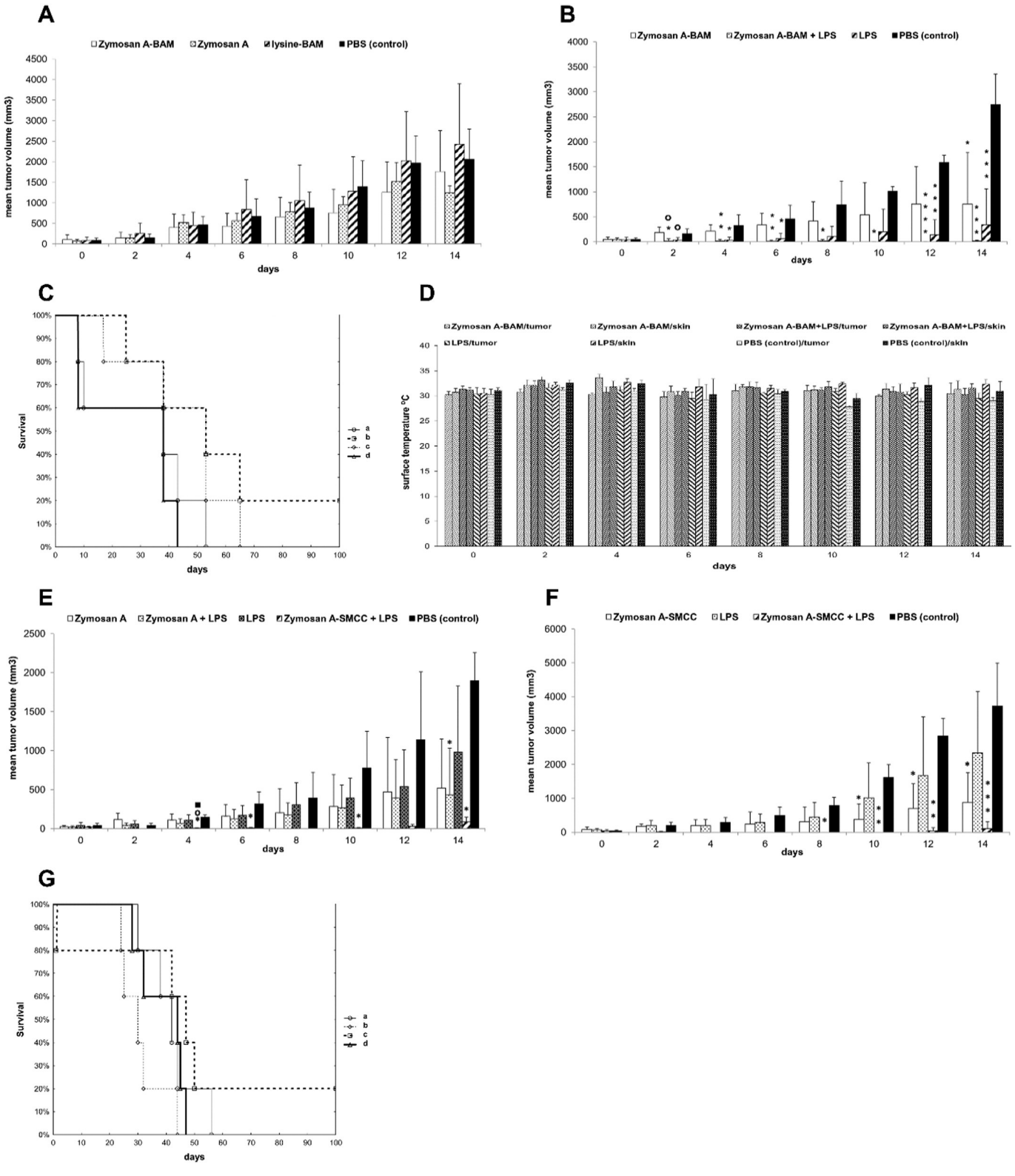
Fig. 1. The influence of Zymosan A (both free and anchored) on melanoma B16-F10 growth. Synergy with LPS. Suspension of B16-F10 melanoma cells in RPMI without FCS was inoculated subcutaneously (s.c.) in the previously shaved right flank of the mice. Each mouse received 4×10^5 melanoma cells in 0.1 ml 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

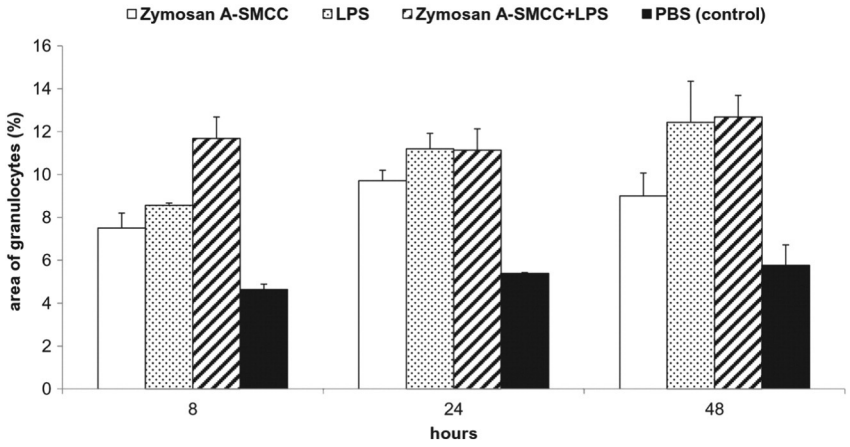


Fig. 2. The influence of Zymosan A-SMCC, LPS and combination thereof on granulocytic melanoma infiltration. Histological analysis. Suspension of B16-F10 melanoma cells in RPMI without FCS was inoculated subcutaneously (s.c.) in the previously shaved right flank of the mice. Each mouse received 4×10^5 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.

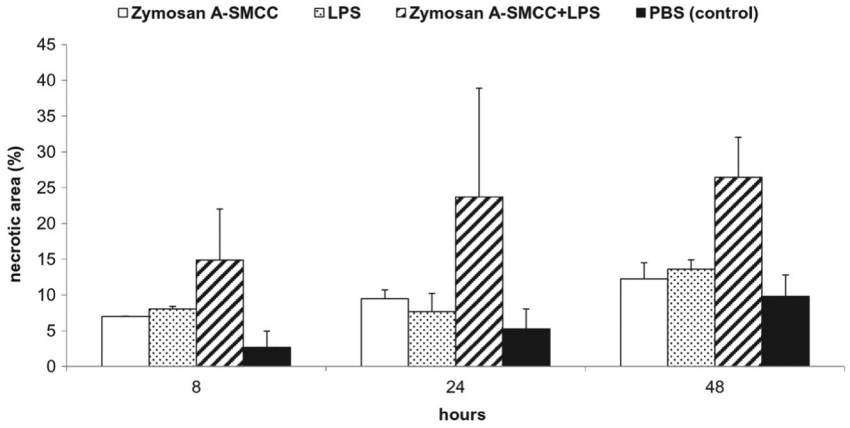


Fig. 3. The influence of Zymosan A-SMCC, LPS and combination thereof on melanoma necrotization. Histological evaluation of necrotization in the previous experiment (Fig. 2) is presented.

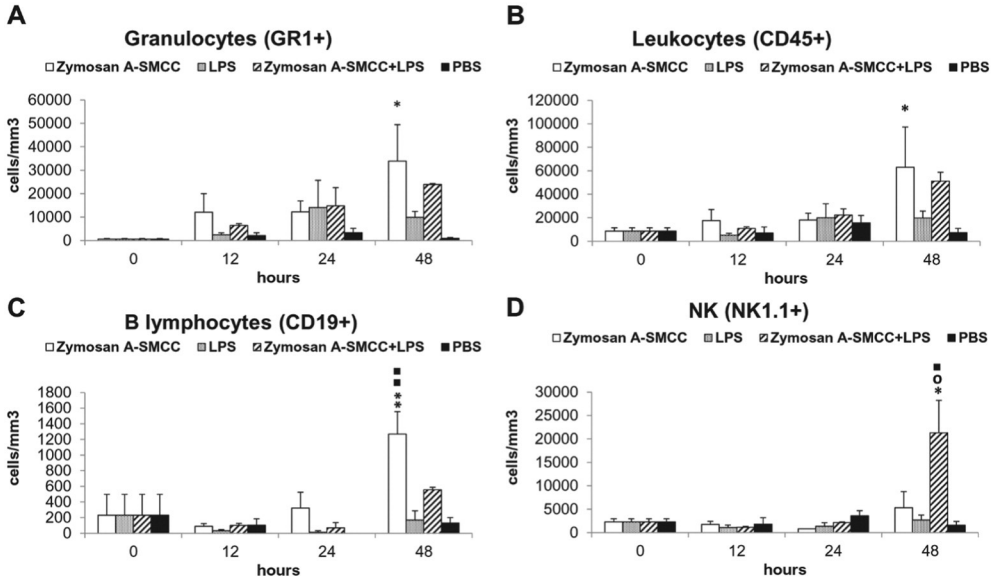


Fig. 4. The influence of Zymosan A-SMCC, LPS and combination thereof on granulocytic melanoma infiltration. Flow cytometry analysis. Suspension of B16-F10 melanoma cells in RPMI without FCS was inoculated subcutaneously (s.c.) in the previously shaved right flank of the mice. Each mouse received 4×10^5 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

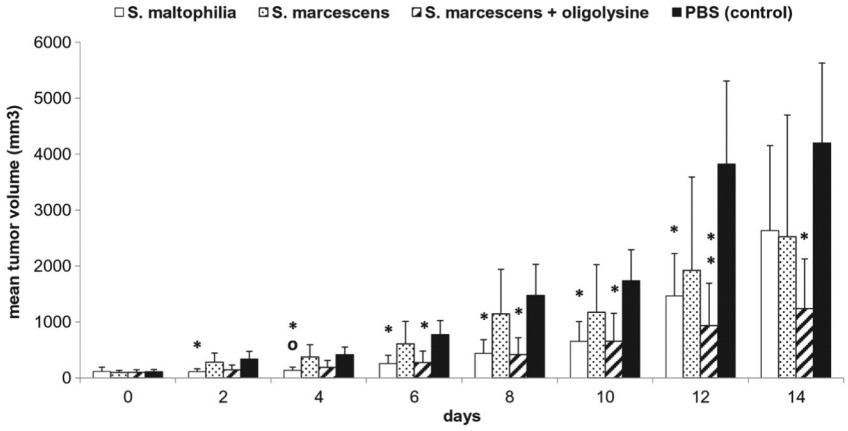


Fig. 5. Melanoma immunotherapy based on the treatment with killed *Stenotrophomonas maltophilia*, *Serratia marcescens*, and *Serratia marcescens*-oligolysine. Suspension of B16-F10 melanoma cells in RPMI without FCS was inoculated subcutaneously (s.c.) in the previously shaved right flank of the mice. 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*.

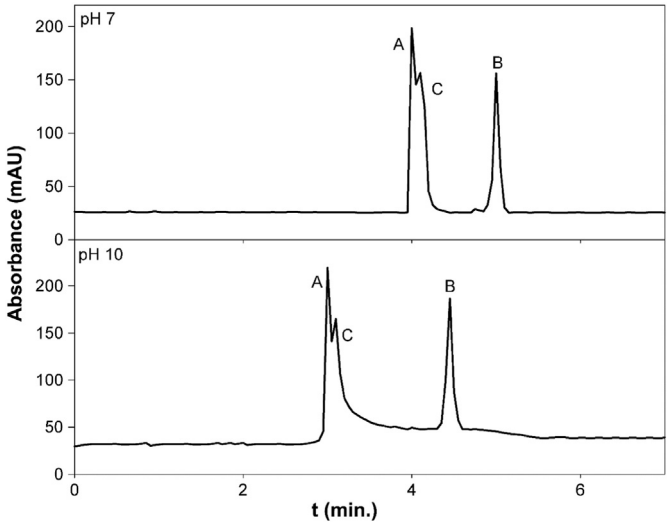


Fig. 6. Capillary zone electrophoresis of killed *Stenotrophomonas maltophilia*, *Serratia marcescens*, and *Serratia marcescens*-oligolysine. Bacteria were suspended in PBS and their concentrations were adjusted to 5×10^8/ml. For capillary zone electrophoresis separations, 2×10^{-2} mol/l phosphate 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

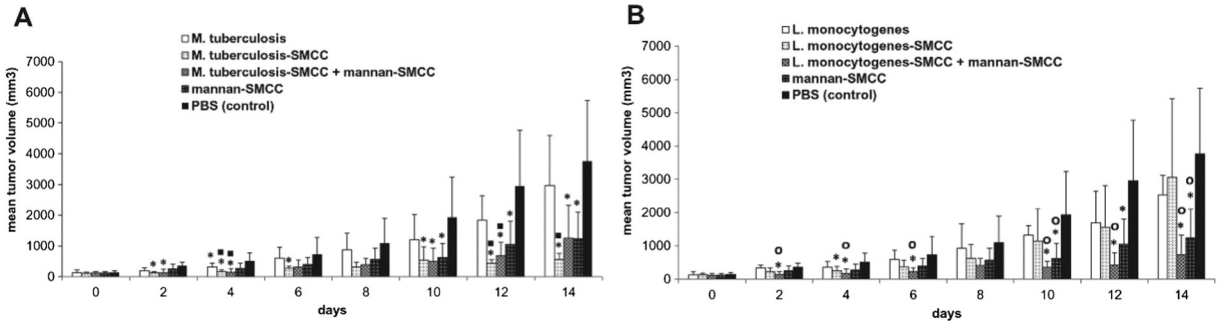


Fig. 7. The use of anchored Gram-positive bacteria in tumor immunotherapy. Experimental design was the same as in Fig. 5. The composition of preparations used was: A - *M. tuberculosis* (5 mg/ml PBS), *M. tuberculosis*-SMCC (5 mg/ml PBS), *M. tuberculosis*-SMCC + mannan-SMCC (5 mg/ml 0.2 mM mannan-SMCC 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*.

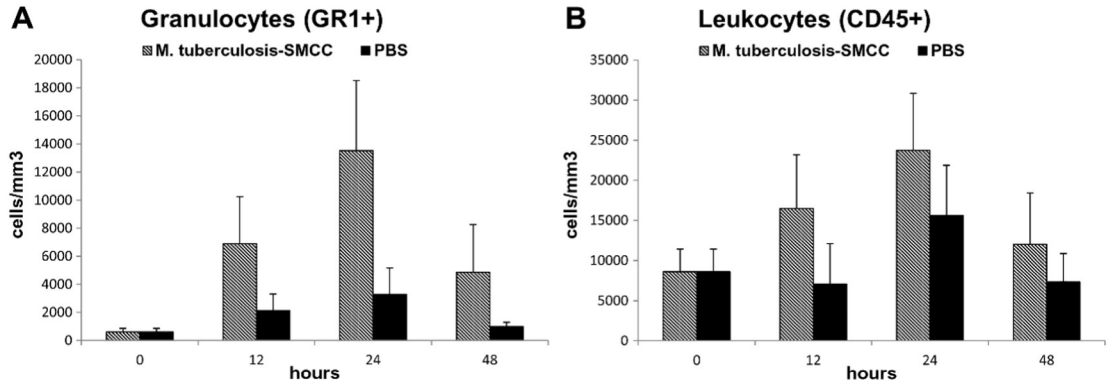


Fig. 8. The use of anchored *Mycobacterium tuberculosis* in tumor immunotherapy. Analysis of inflammatory infiltration by flow cytometry. Suspension of B16-F10 melanoma cells in RPMI without FCS was inoculated subcutaneously (s.c.) in the previously shaved right flank of the mice. Each mouse received 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.

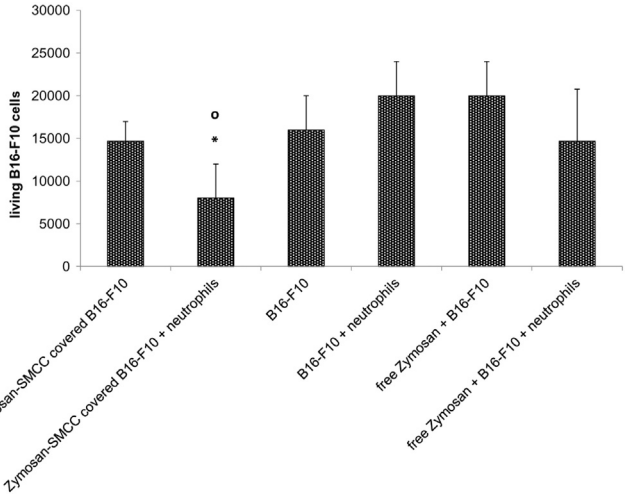


Fig. 9. Cytotoxic effect of murine neutrophils on melanoma B16-F10 cells with covalently attached Zymosan A. Zymosan A-SMCC was covalently bound to B16-F10 melanoma cells reduced by DTT. Suspension of bone marrow neutrophils (90% purity) primed with GM-CSF, TNFα and laminarin in culture medium was added 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.

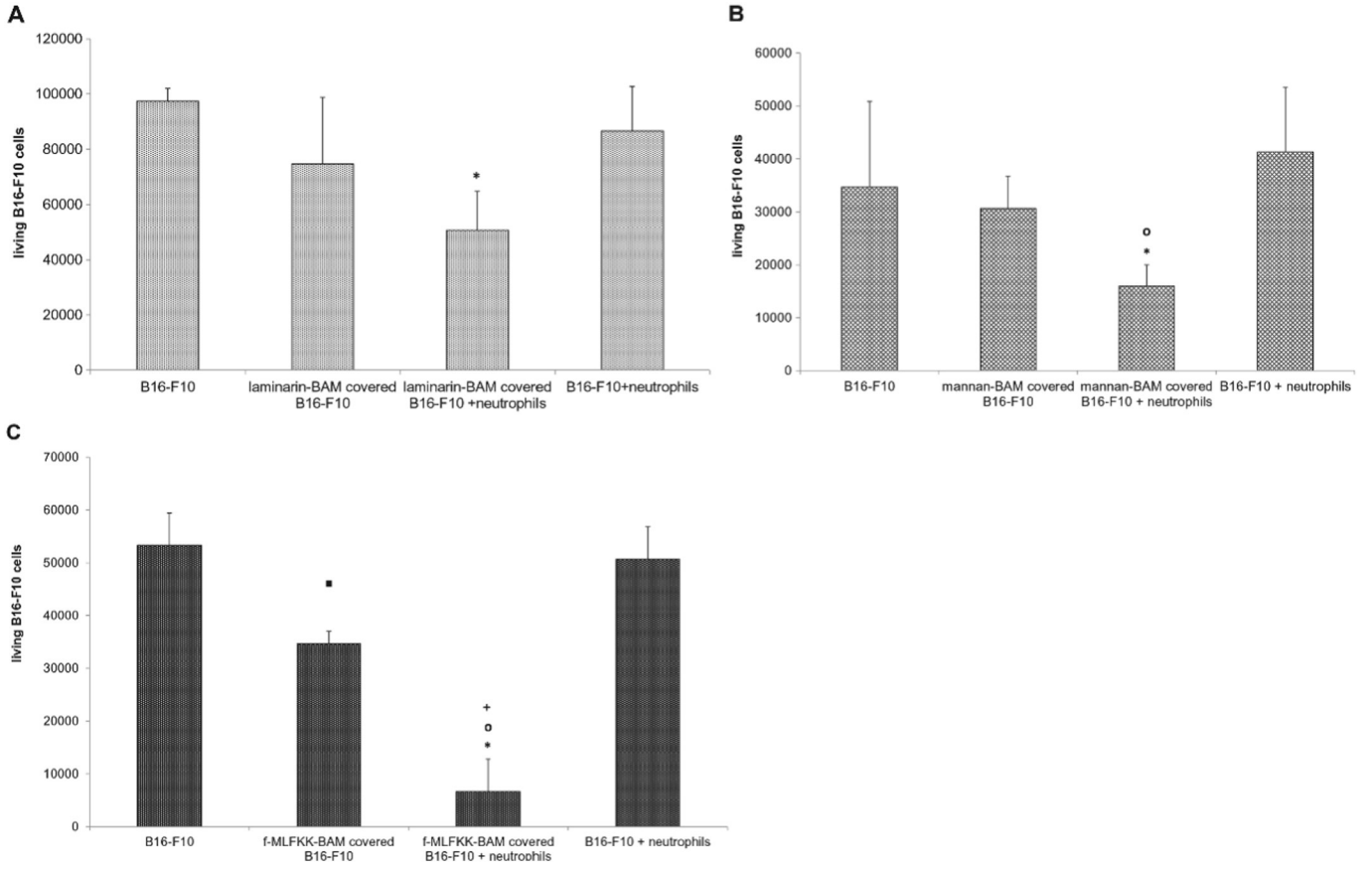


Fig. 10. Cytotoxic effect of murine neutrophils on melanoma B16-F10 cells bearing agonists of phagocytic receptors. B16-F10 melanoma cells were incubated (30 min, 37 °C) with 0.02 mM laminarin-BAM, 0.02 mM mannan-BAM or 0.05 mM f-MLFKK-BAM in culture medium, respectively and subsequently washed. Suspension 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 $^{*}P \leq 0.05$ compared to laminarin-BAM covered B16-F10. B – mannan-BAM $^{*}P \leq 0.05$ compared to mannan-BAM covered B16-F10 **o***P* ≤ 0.05 compared to B16-F10 + neutrophils. C – f-MFKK-BAM $^{*}P \leq 0.005$ 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

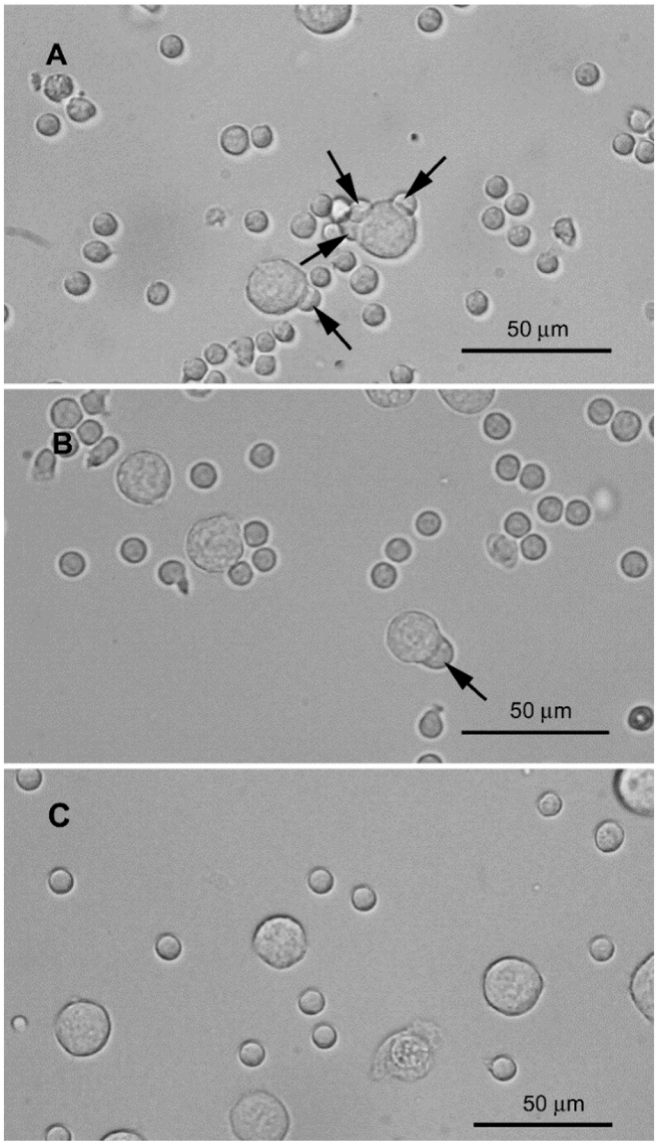


Fig. 11. Interactions of neutrophils with melanoma cells bearing ligands of phagocytic receptors. Both coverage of melanoma cells with ligands of phagocytic receptors and isolation and priming of neutrophils were performed as previously described (Fig. 10). Neutrophils and B16-F10 cells in 2:1 ratio 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.

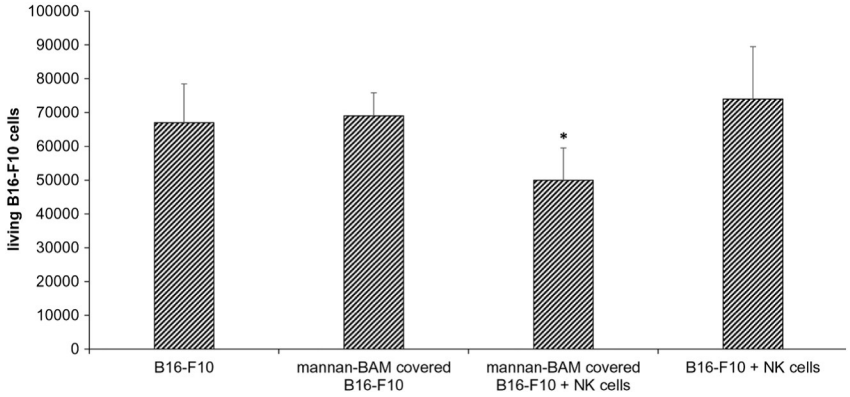


Fig. 12. Cytotoxic effect of murine NK cells on melanoma B16-F10 cells with anchored mannan. B16-F10 melanoma cells were incubated (30 min, 37 °C) with 0.02 mM mannan-BAM in culture medium and subsequently washed. Suspension of laminarin primed spleen NK cells in culture medium was added to B16-F10 in 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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