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Effective cancer immunotherapy based on combination of TLR agonists with stimulation of phagocytosis

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ABSTRACT

Immunotherapy emerges as a fundamental approach in cancer treatment. Up to date, the efficacy of numerous different immunotherapies has been evaluated. The use of microorganisms or their parts for immune cell activation, referred to as Pathogen-Associated Molecular Patterns (PAMPs), represents highly promising concept. The therapeutic effect of PAMPs can be further amplified by suitable combination of different types of PAMPs such as Toll like receptor (TLR) agonists and phagocytosis activating ligands. Previously, we used the combination of phagocytosis activating ligand (mannan) and mixture of TLR agonists (resiquimod (R-848), poly(I:C), inactivated *Listeria monocytogenes*) for successful treatment of melanoma in murine B16-F10 model. In the present study, we optimized the composition and timing of previously used mixture. Therapeutic mixture based on well-defined chemical compounds consisted of mannan anchoring to tumor cell surface by biocompatible anchor for membranes (BAM) and TLR agonists resiquimod, poly(I:C), and lipoteichoic acid (LTA). The optimization resulted in (1) eradication of advanced stage progressive melanoma in 83% of mice, (2) acquisition of resistance to tumor re-transplantation, and (3) potential anti-metastatic effect. After further investigation of mechanisms, underlying anti-tumor responses, we concluded that both innate and adaptive immunity are activated and involved in these processes.

We tested the efficacy of our treatment in Panc02 murine model of aggressive pancreatic tumor as well. Simultaneous application of agonistic anti-CD40 antibody was necessary to achieve effective therapeutic response (80% recovery) in this model.

Our results suggest that herein presented immunotherapeutic approach is a promising cancer treatment strategy with the ability to eradicate not only primary tumors but also metastases.

1. Introduction

Immunotherapy has a long history in cancer treatment. Many different approaches focusing on immune cells activation and (subsequent) tumor eradication have been evaluated. The oldest immunotherapeutic concept is based on the use of pyogenic bacteria. This concept was described in the late 19th century by William Coley [1] and it is based on the ability of innate immunity to recognize pathogens through germline-encoded pattern-recognition receptors (PRRs) resulting in immune system activation and pathogen elimination [2]. This mechanism can simultaneously increase the effectivity of tumor cell recognition by immune system and, thus, it designates pathogens as a potential tool for cancer treatment [3]. Subsequently, isolation and synthesis of PAMPs opened new perspectives for this therapeutic approach. This research has primarily been focused on a specific group of PAMPs, so-called agonists of Toll-like receptors (TLR) [4]. TLR agonists are very effective in activation of immune cells and their attraction into the tumor [5]. Recently, we have shown that application of TLR agonists along with simultaneous labelling of tumor cells with ligands activating phagocytosis can significantly increase the ability of immune cells to recognize tumor cells [6,7]. The combination of resiquimod (TLR7 and TLR8 agonist, in case of mice TLR7 agonist only) and mannan (ligand activating phagocytosis) was shown to be the most effective formula. Moreover, the effect of therapy was significantly improved by addition of other TLR agonists, specifically poly(I:C) (TLR3) and *L. monocytogenes* (TLR2) [8].

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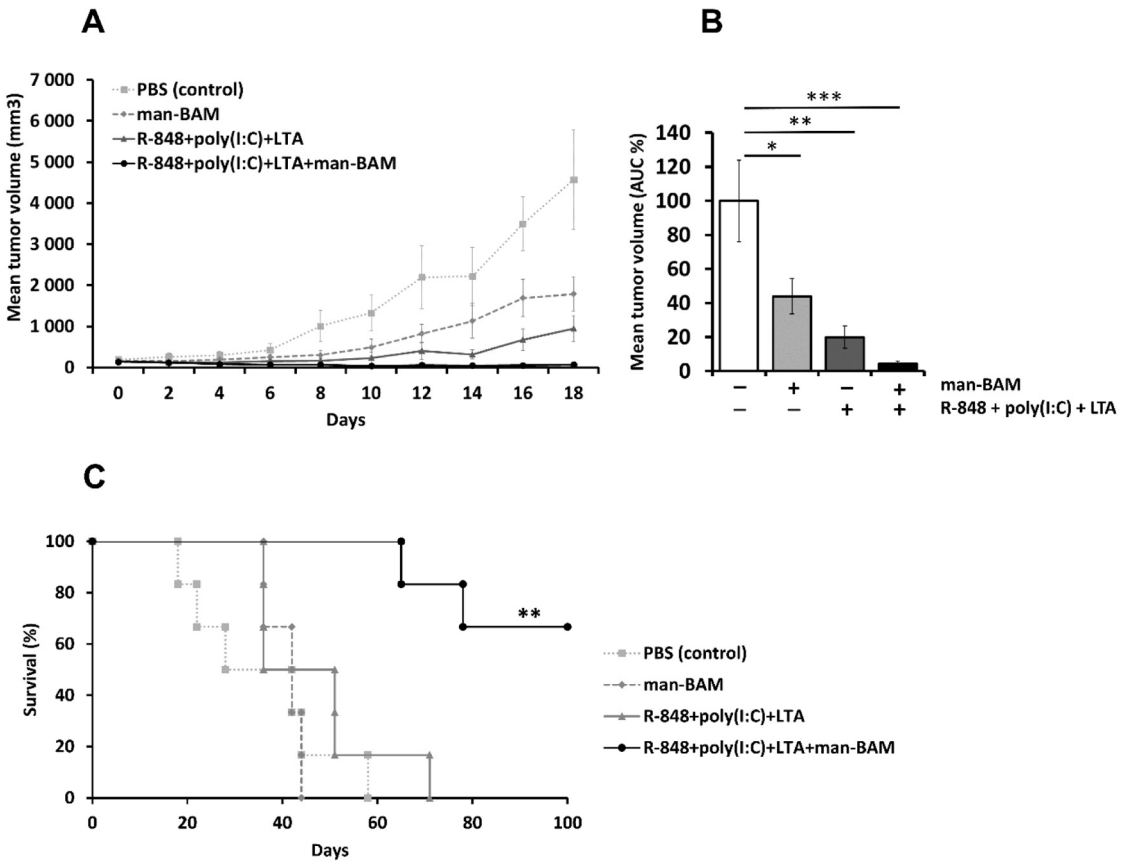

Fig. 1. Cancer immunotherapy based on the combination of TLR agonists and phagocytosis stimulation. Murine B16-F10 model. Therapeutics were applied in pulse regime (days 0, 1, 2, … 8, 9, 10). Tumor growth is presented by curves (A) with corresponding area under curve (AUC) analysis for each group (B). PBS treated control was considered as 100%. Statistical analysis was performed on AUC values by one-way ANOVA with Tukey's *post hoc* test. (C) Survival analysis, Long rank test, compared to PBS (control). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

In the present study we aimed to enhance the efficacy of this therapy. We focused on (1) the optimization of the therapeutic mixture composition and therapeutic timing, (2) analysis of mechanisms involved in anti-tumor responses in B16-F10 melanoma model, and (3) the possibility to combine this therapy with the use of checkpoint inhibitors and immune activators. Furthermore, we investigated the effectiveness of the present therapy in murine model of pancreatic adenocarcinoma (Panc02).

2. Materials and methods

2.1. Ethics statement

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

2.2. Materials

Tissue culture media, media supplements, laminarin from *Laminaria digitata*, mannan from *Saccharomyces cerevisiae*, GM-CSF, TNF-α, lipoteichoic acid (LTA) from *Bacillus subtilis*, and polyinosinic: polycytidylic acid, sodium salt (poly(I:C)) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Resiquimod (R-848) was obtained from Tocris Bioscience (Bristol, UK). Biocompatible Anchor for cell Membrane (BAM, Mw 4000) was purchased from NOF EUROPE (Grobbendonk, Belgium). Mannan-BAM synthesis was performed as previously described [8]. Monoclonal antibodies anti-PD-1 (rat IgG2a, clone RMP1-14), anti-CTLA-4 (hamster IgG, clone 9H10), and anti-CD40 (rat IgG2a, clone FGK4.5/FGK45) were purchased from BioXCell (West Lebanon, NH, USA).

2.3. Cell lines and mice

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

The murine pancreatic adenocarcinoma cell line Panc02 was obtained from Prof. Lars Ivo Partecke (Greifswald, Germany). These cells were cultivated in DMEM supplemented with 10% fetal calf serum and antibiotics under the same conditions as melanoma cells.

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. Tumor transplantation

C57BL/6 mice (females) were subcutaneously injected with 4×10^5 B16-F10/Panc02 cells (suspended in 0.1 ml serum free of RPMI 1640/DMEM) into the previously shaved right flank. For the melanoma B16-F10 metastasis model, mice were injected with 1×10^5 B16-F10 cells (suspended in 50 μL PBS) into the lateral tail vein.

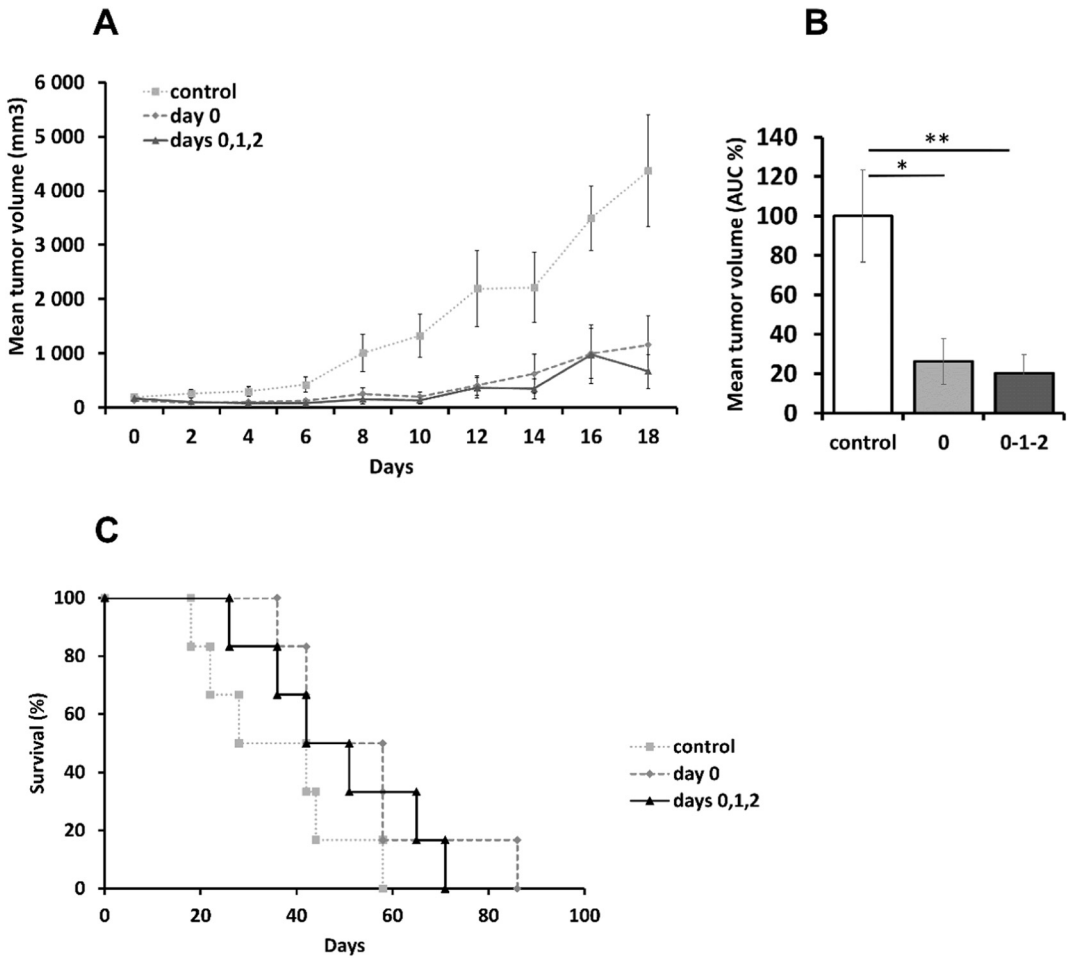

Fig. 2. Shortened cancer immunotherapy, B16-F10 model. Therapeutic mixture (R-848 + poly (I:C) + LTA + mannan-BAM) was applied in two different application regimes (day 0 and days 0, 1, 2). Control group did not receive any treatment. Tumor growth is presented by curves (A) with corresponding area under curve (AUC) analysis for each group (B). Control group was considered as 100%. Statistical analysis was performed on AUC values by one-way ANOVA with Tukey's *post hoc* test. (C) Survival analysis, Log rank test, compared to control. * = $p < 0.05$, ** = $p < 0.01$.

2.5. Treatment and evaluation

Twelve days after tumor cell transplantation mice were randomly divided into groups of six (unless otherwise indicated). Immediately after randomization the therapy was started (day 0). Therapeutic substances were applied intratumorally (50 µl/mouse) in particular regime. During the treatment, all animals were housed individually. Tumor size was measured every other day by a caliper. A formula $V = \pi/6\ AB^2$ (A = largest dimension of tumor, B = smallest dimension) was used for tumor volume calculation [9].

Therapeutic mixtures used for immunotherapy consisted of active compounds – 0.5 mg R-848, HCl form, 0.5 mg poly(I:C), 0.5 mg LTA/ml, and 0.2 mM mannan-BAM – dissolved in PBS. Plain PBS was used as a control in each experiment.

2.6. Evaluation of metastases

In the B16-F10 mouse model, lungs of experimental animals were carefully removed and fixed with 4% neutral solution of formaldehyde. The presence of metastases was evaluated using dissection microscope as previously described [6].

In the Panc02 mouse model, the examination of all inner organs (lung, heart, liver, stomach, intestine, kidney, spleen) was performed.

2.7. Flow cytometry analysis of tumor-infiltrating leukocytes

Analysis of tumor-infiltrating leukocytes was performed as described previously [8]. Briefly, tumors were digested with Liberase DL and DNase I (both Roche Diagnostics, Mannheim, Germany). Subsequently, particular leukocyte subtypes were determined using BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) and following monoclonal antibodies (eBioscience, San Diego, CA, USA) were used in this experiment: 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 Gr-1 Alexa Fluor 700; clone RB6-8C5, and h) macrophages - anti-mouse F4/80 Antigen PE-Cy7; clone BM8. BD FACSDiva software 6.1.3. (BD Biosciences, San Jose, CA, USA) was used for the flow cytometry data analysis.

2.8. Preparation and priming of neutrophils

Suspension of murine bone marrow cells was prepared as described by Stassen et al. [10]. This suspension was used for preparation of neutrophils purified by magnetic-activated cell sorting (MACS) technique (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of neutrophils was evaluated by BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) using anti-mouse CD45 APC, clone 30-

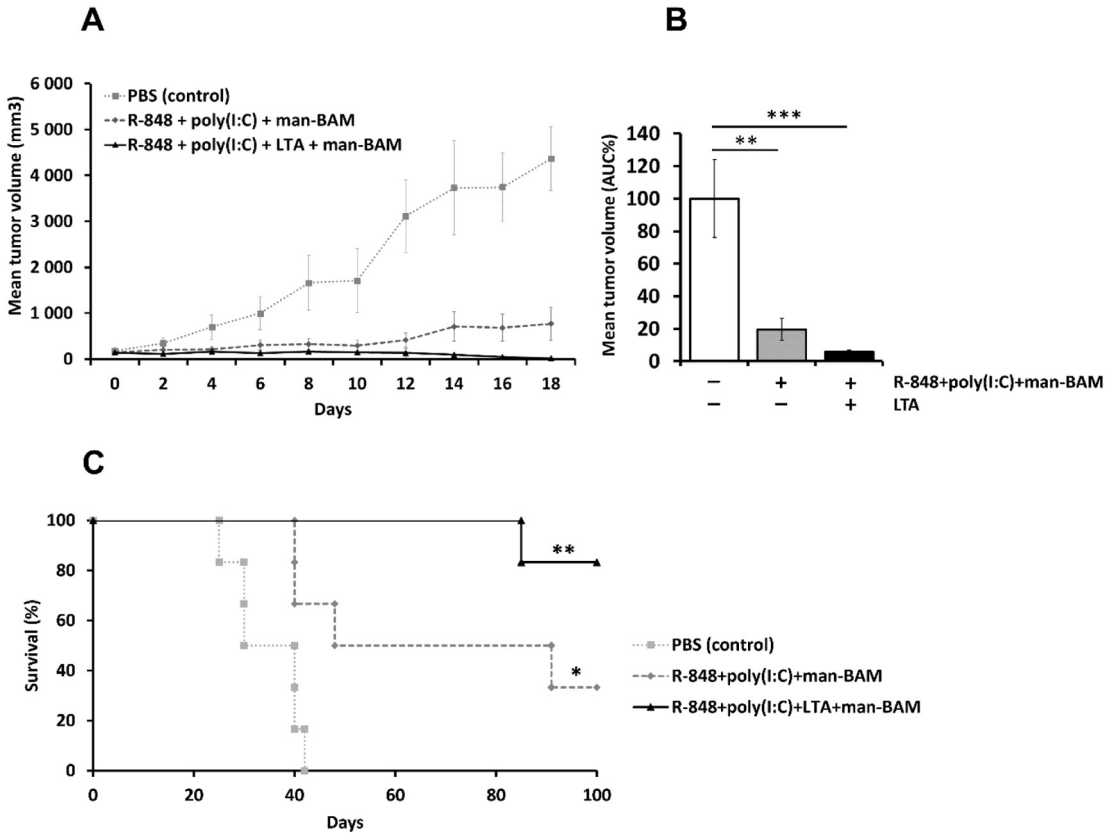

Fig. 3. Efficacy of cancer immunotherapy based on four therapeutic pulses, B16-F10 model. Therapeutic preparations were applied in pulse regime (days 0, 1, 2… 8, 9, 10… 16, 17, 18… 24, 25, 26). Tumor growth is presented by curves (A) with corresponding area under curve (AUC) analysis for each group (B). PBS treated control was considered as 100%. Statistical analysis was performed on AUC values by one-way ANOVA with Tukey's *post hoc* test. (C) Survival analysis, Log rank test, compared to PBS (control). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

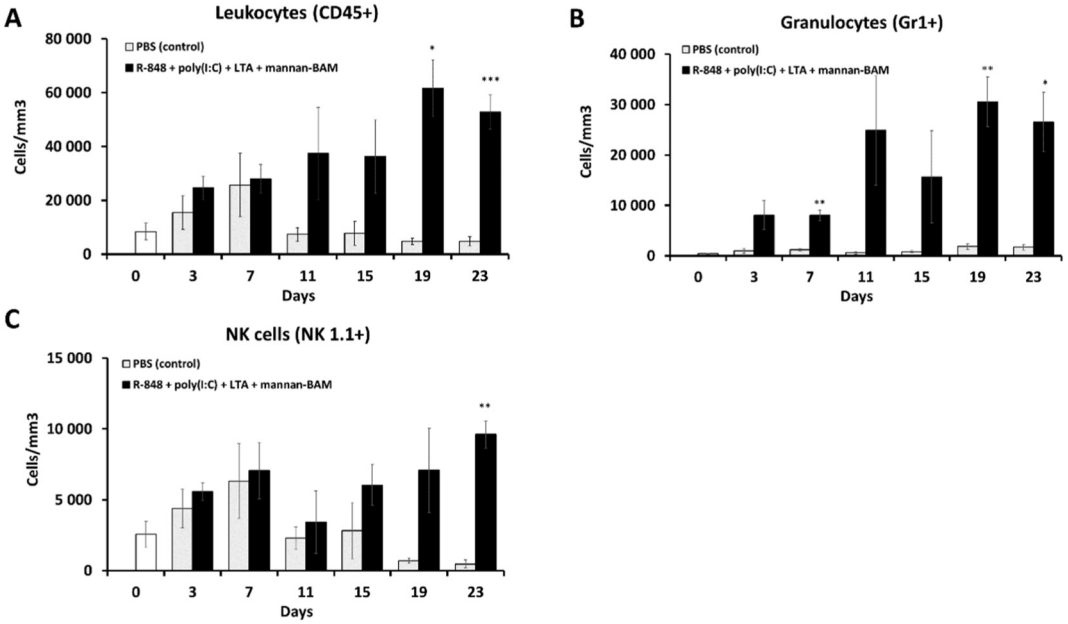

Fig. 4. Changes in tumor-infiltrating lymphocytes during the immunotherapy of murine melanoma (B16-F10). Twelve days following tumor cell transplantation, mice were randomly divided into two groups of 24. The therapeutic mixture (50 μL/mouse) was administered in four pulses on days 0, 1, 2…8, 9, 10…16, 17, 18… 24, 25, 26. Three mice from each group were euthanized on the 3rd, 7th, 11th, 15th, 19th, and 23rd day of the therapy. Another 3 mice were euthanized on day 0 without any treatment. Tumors were excised and analyzed for immune cell infiltrate using flow cytometry. Cell infiltration is expressed as number of cells per mm^3 of tumor mass. Following populations were analyzed: (A) leukocytes (CD45+), (B) Neutrophils (CD45 + Gr-1+), and (C) NK cells (CD45 + NK1.1+). Statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$ compared to PBS (control).

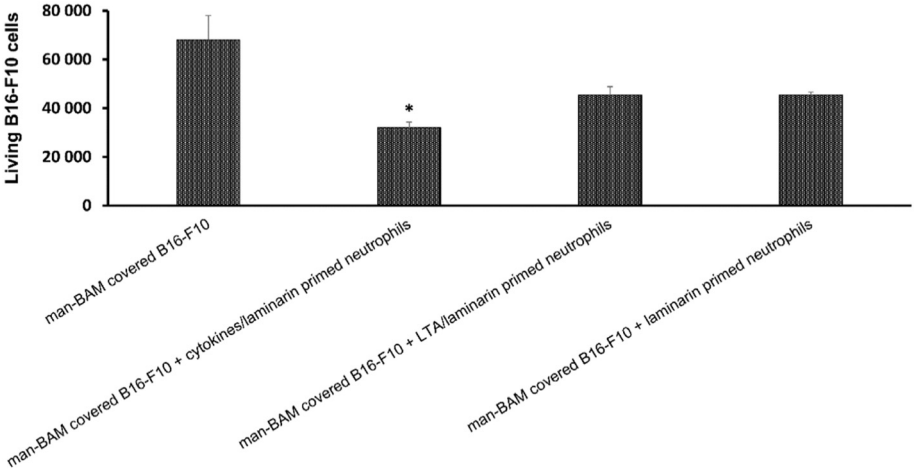

Fig. 5. In vitro analysis of cytotoxic effect of neutrophils on melanoma cells covered by anchored mannan. B16-F10 melanoma cells were incubated (30 min, 37 °C) with 0.02 mM mannan-BAM in culture medium and subsequently they were washed. Suspension of bone marrow neutrophils (90% purity) primed with cytokines (GM-CSF + TNF-alpha) or with LTA in culture medium was added to B16-F10 cells in 5:1 ratio. All mixtures were incubated at 37 °C for 2 h. Afterwards, trypan blue exclusion staining was performed, and viable melanoma cells were counted with a hemocytometer. Statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test,* = $p < 0.05$ compared to man-BAM covered B16-F10 cells.

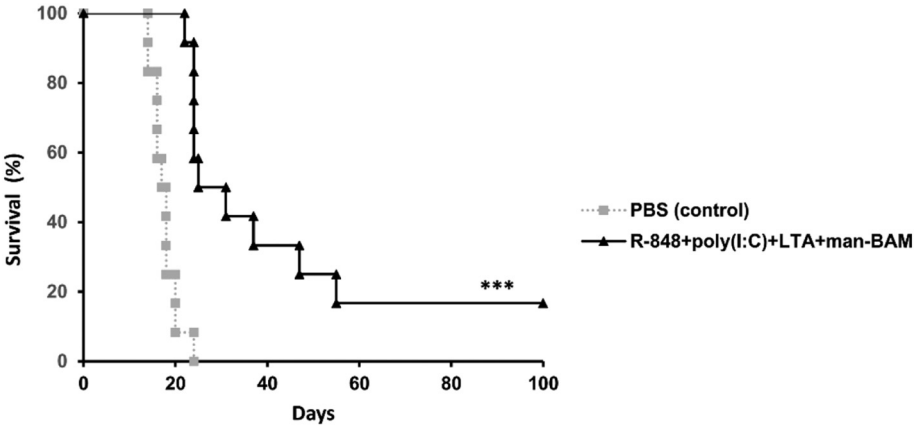

Fig. 6. Potential anti-metastatic effect of cancer immunotherapy. Survival analysis. Subcutaneous transplantation of 4×10^5 of melanoma B16-F10 cells along with intravenous administration of 1×10^5 of the same cells (per mouse) was performed. On day 12 after tumor transplantation, mice were randomized in two groups of 12, and therapy based on intratumoral application of corresponding preparations (50 µL/mouse) was started. Four therapeutic pulses were applied on days 0, 1, 2…8, 9, 10…16, 17, 18…24, 25, 26. Survival analysis and Log rank test were performed. *** = $p < 0.001$ compared to PBS (control).

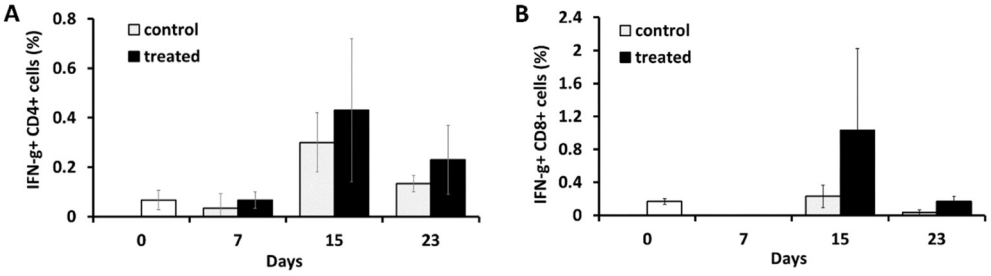

Fig. 7. Activation of adaptive immunity during the course of therapy. The transplantation of melanoma B16-F10 was performed as described in Fig. 1. Twelve days after tumor transplantation, mice were randomized in two groups of 9. Three more mice were euthanized for analysis before onset of treatment. Intratumoral application of the PBS (grey bars, 50 µl/mouse) or therapeutic mixture (R-848 + poly (I:C) + LTA + mannan-BAM, black bars, 50 µl/mouse) was performed in three pulses on days 0, 1, 2… 8, 9, 10… 16, 17, 18. Three mice from each group were euthanized on days 7, 15, and 23. Splenocytes were isolated and cultivated in the presence (or not) of UV killed melanoma cells. The production of intracellular IFN-γ was measured by flow cytometry. Control values (without antigen) were subtracted. (A) Intracellular IFN-γ production in CD4+ cells, (B) intracellular IFN-γ production in CD8+ cells.

F11 and anti-mouse Ly-6G (Gr-1) Alexa Fluor 700, clone RB6-8C5 antibodies (eBioscience, San Diego, CA, USA). Neutrophils were primed with a mixture of GM-CSF and TNF-α (12 ng and 2.5 ng/ml media, respectively) as reported by Dewas et al. [11], or by lipoteichoic acid (LTA, 0.05 mg/ml) for 20 min. Additionally, the priming solution was supplemented with 2 µM solution of soluble beta glucan (laminarin). Non-heat inactivated FBS was used as a source of complement proteins during the procedure.

2.9. Analysis of the cytotoxic effect of neutrophils on B16-F10/Panc02 cells labelled with mannan-BAM

B16-F10/Panc02 tumor cells were incubated with 0.02 mM mannan-BAM for 30 min at 37 °C. After incubation, non-bound mannan-BAM was washed away by centrifugation. Neutrophils obtained from murine bone marrow were prepared and primed as described above. Subsequently, neutrophils were co-cultured with tumor cells (5:1 ratio) for 2 h in 37 °C. Following the incubation, cells were mixed with trypan blue and the live/dead cell counts were calculated using hemocytometer. Non-heat inactivated FBS was used as a source of complement proteins during the procedure.

2.10. IFN-γ intracellular staining in T cells

Mice were euthanized by cervical dislocation. Single cell suspension of splenocytes was prepared using a plastic strainer (70 µm, BD Biosciences, San Jose, CA, USA). Red blood cells were eliminated by lysis buffer (TRIS buffered 0.84% NH_4Cl solution). Splenocytes were co-cultured with UV-killed-B16-F10 cells (6:1 ratio) for 6 h at 37 °C and 5% CO_2. For each spleen, a sample prepared without UV-killed-B16-F10

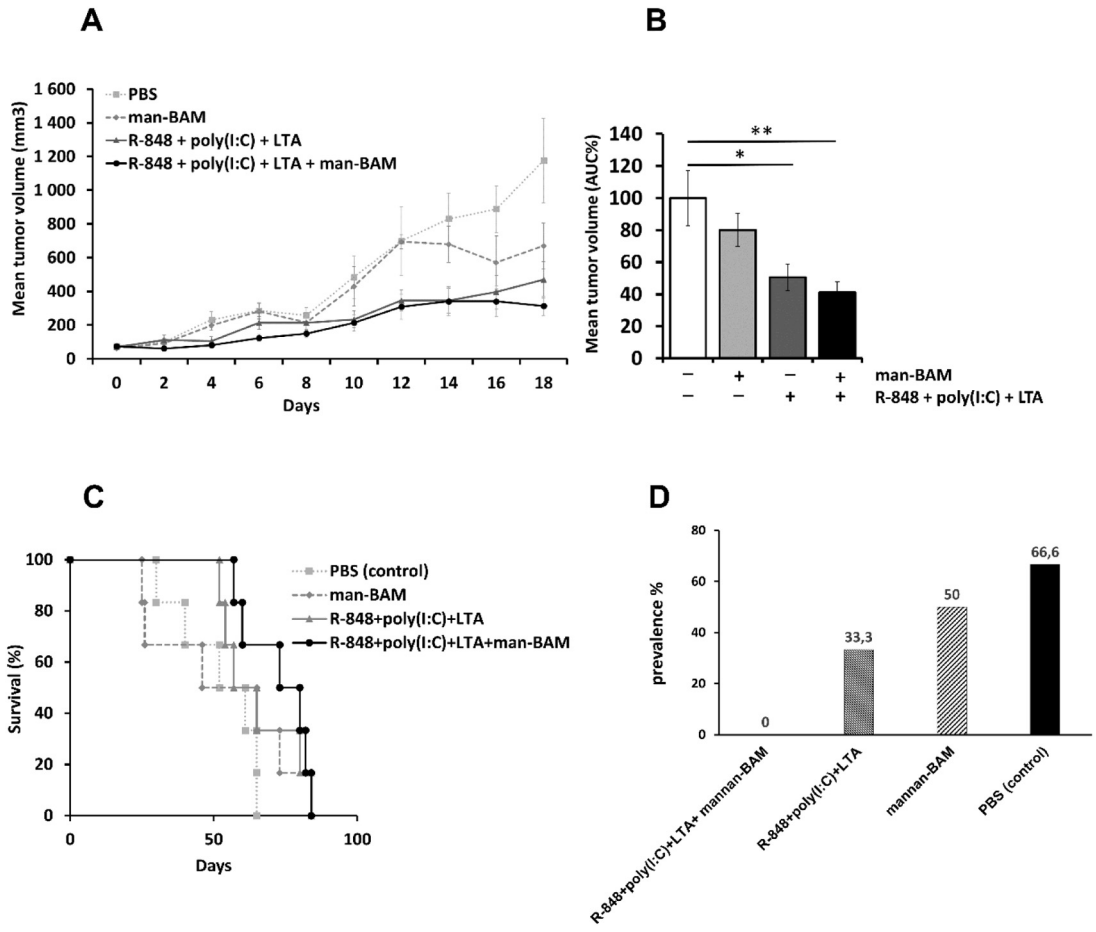

Fig. 8. Immunotherapy of pancreatic adenocarcinoma. Therapeutic preparations were applied in pulse regime (days 0, 1, 2… 8, 9, 10… 16, 17, 18… 24, 25, 26). Growth of tumors is presented as growth curves (A) with corresponding area under curve (AUC) analysis for each group (B), PBS treated control was considered as 100%. Statistical analysis was performed on AUC values by one-way ANOVA with Tukey's *post hoc* test, * = $p < 0.05$, ** = $p < 0.01$. (C) Survival analysis, Long rank test. R-848 + poly(I:C) + LTA + mannan-BAM compared to PBS (control) was near to statistical significance, $p = 0.063$. (D) Prevalence of metastases.

cells was used as a control.

Monensin (1 μl/ml, eBioscience, San Diego, CA, USA) was added to splenocytes for the last 5 h of co-cultivation. Afterwards, surface staining of CD markers was performed with anti-mouse CD3e FITC, anti-mouse CD4 APC, and anti-mouse CD8 PECy7 (eBioscience, San Diego, CA, USA). Cells were washed, fixed, and cell membranes were permeabilized (Foxp3/Transcription Factor Staining Buffer Set, eBioscience, San Diego, CA, USA). Intracellular staining of IFN-γ was performed using anti-mouse IFN-gamma PE (eBioscience, San Diego, CA, USA). Cells were analyzed using BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) and results were evaluated using BD FACSDiva software 6.1.3. (BD Biosciences, San Jose, CA, USA).

2.11. Statistical analysis

Area under curve (AUC) was calculated and statistical analysis was performed on AUC values by one-way ANOVA with Tukey's *post hoc* test. For survival analysis Log-rank test was used (STATISTICA 12, StatSoft, Inc., Tulsa, OK, USA). Error bars indicate SEM.

3. Results

3.1. The synergy between TLR agonists and phagocytosis stimulating ligand in cancer immunotherapy

The use of either mixture of three TLR agonists (R-848, poly(I:C), LTA) or anchored phagocytosis stimulating mannan-BAM resulted in significant reduction of tumor growth without any effect on the survival of treated mice. However, when used in combination (TLR agonists + mannan-BAM), the treatment led to tumor shrinkage and elimination (Fig. 1A, B) along with 66% survival rate of treated mice (Fig. 1C). Mice, surviving for 100 days, did not show any pathological signs. The site of healed tumor was covered by hair and no metastases were found in lungs.

3.2. Design and optimization of dosage regimen

As described in Section 2.1, therapeutic mixture applied in two 3-day pulses separated by five-day gap, resulted in significant reduction of tumor growth with survival time prolongation. Despite that promising results, it was necessary to establish the optimal number of applications. First, we tested the effects of shortening of the duration of therapy. Not only one therapeutic 3-day pulse (0,1,2), but single injection of therapeutic mixture significantly suppressed tumor growth (Fig. 2A, B). Nevertheless, the shortened therapy was not sufficient to prolong survival of the mice (Fig. 2C). This indicates that at least two therapeutic pulses of the complete mixture R-848 + poly (I:C) + LTA + mannan-BAM are necessary to achieve prolonged survival.

Subsequently, we tested the efficacy of four therapeutic pulses. The use of mixture of three TLR agonists (R-848, poly(I:C), LTA) with anchored mannan (mannan-BAM) resulted in the elimination of tumors (Fig. 3A, B) and led to 83% survival in the treated group of mice (Fig. 3C). Moreover, the therapy induced resistance against re-

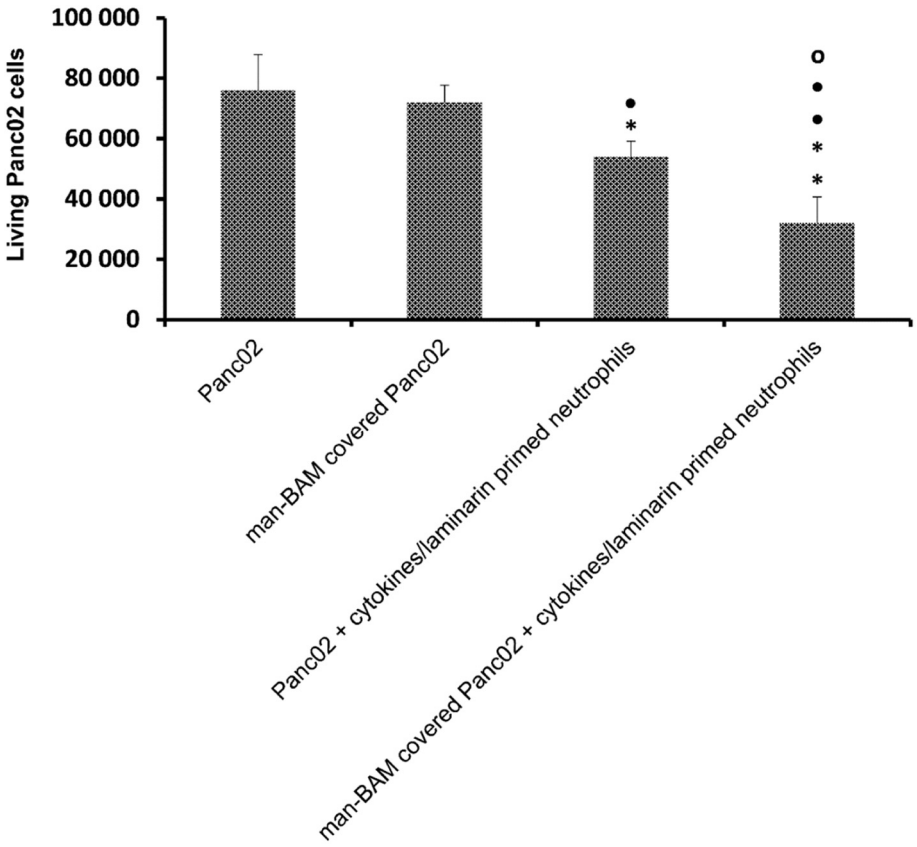

Fig. 9. In vitro analysis of cytotoxic effect of neutrophils on Panc02 cells covered by anchored mannan. The suspension of primed bone marrow neutrophils (90% purity) was added to Panc02 cells (both free and mannan-BAM covered) in a 5:1 ratio. After subsequent incubation, living cells were counted using hemocytometer and trypan blue exclusion staining. Statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test,* = $p < 0.05$ compared to Panc02, ** = $p < 0.001$ compared to Panc02, • = $p < 0.05$ compared to man-BAM covered Panc02, •• = $p < 0.001$ compared to man-BAM covered Panc02, o = $p < 0.05$ compared to Panc02 + neutrophils.

transplantation of cancer cells. Using scheme identical to the primary transplantation, re-transplantation of melanoma cells was performed on day 120 after therapy initiation (4×10^5 melanoma B16-F10 cells/mouse, subcutaneous application). None of the mice regrew the tumor.

Furthermore, this experiment proved the necessity of using the complex TLR agonists mixture. Omission of TLR2 agonist (LTA) decreased the therapeutic effect.

3.3. Tumor leukocyte infiltration changes in the course of immunotherapy

Flow cytometry analysis of tumor infiltrate during the therapy (as described in Fig. 3) showed strong leukocytic infiltration (Fig. 4A) with predominantly granulocytic content (Fig. 4B). Increased counts of NK cells were detected as well (Fig. 4C). The counts of CD4+ and CD8+ T-lymphocytes, B-lymphocytes, and macrophages were low, and no changes in their quantity were observed during the treatment (data not shown).

Evaluation of leukocyte infiltration was terminated on 23rd day of the therapy due to disappearance of all tumors in the treated group. Surviving mice (6 in treated group, 5 controls) were euthanized on day 31 by cervical dislocation and the evaluation of lung metastases was performed. No metastases were found in the treated group, while in the control group (PBS), metastases were present in 80% of mice with average of 4 metastases per mouse.

3.4. Cytotoxic effect of neutrophils on melanoma cells covered by anchored mannan in vitro

Substantial enhancement of the immunotherapy efficacy by LTA (Fig. 3) led us to investigate whether it was caused by direct priming of neutrophils by LTA. Our results (Fig. 5) did not support this presumption. To prime neutrophils, it was necessary to use cytokines (GM-CSF + TNF-alpha), which mimic the natural way of activation.

3.5. Combination of TLR agonists with stimulation of phagocytosis revealed potential anti-metastatic effect

We also evaluated the effect of our therapeutic mixture on metastases. Simultaneous subcutaneous transplantation of melanoma B16-F10 cells (4×10^5 cells per mouse) along with their intravenous administration (1×10^5 cells per mouse) was performed. After 12 days, subcutaneous tumors were treated by intratumoral application of the therapeutic mixture in treated group or by PBS application in control group. The therapy significantly prolonged survival of treated mice and two out of twelve mice were completely cured (Fig. 6). All surviving mice were euthanized 100 days after initiation of the therapy. No metastases were found in lungs of these mice. In contrast, there was a massive occurrence of lung metastases in the control group (78.9 ± 36.4/mouse), resulting in shortened survival (compare to control group in Fig. 3C).

3.6. Onset of innate immunity effectors was followed by activation of adaptive immunity

We consider the activation of innate immunity to be central mechanism of action in the herein presented cancer immunotherapy based on the combination of TLR agonists (R-848, poly(I:C), LTA) and phagocytosis stimulating ligand (mannan). Moreover, stimulation of phagocytosis, support of dendritic cells maturation, and establishment of proinflammatory Th1 millieu [8] create optimal conditions for subsequent activation of adaptive immunity.

In order to investigate the stimulation of adaptive immunity, we analyzed antigen specific activation of splenic T lymphocytes by measuring of IFN-γ intracellular production of in response to melanoma antigens exposure. Adaptive immunity response culminated on day 15 of the therapy at the level of both CD4+ (Fig. 7A) and CD8+ (Fig. 7B) splenocytes. The contribution of adaptive immunity to therapeutic effect was further supported by experiments involving SCID mice, which

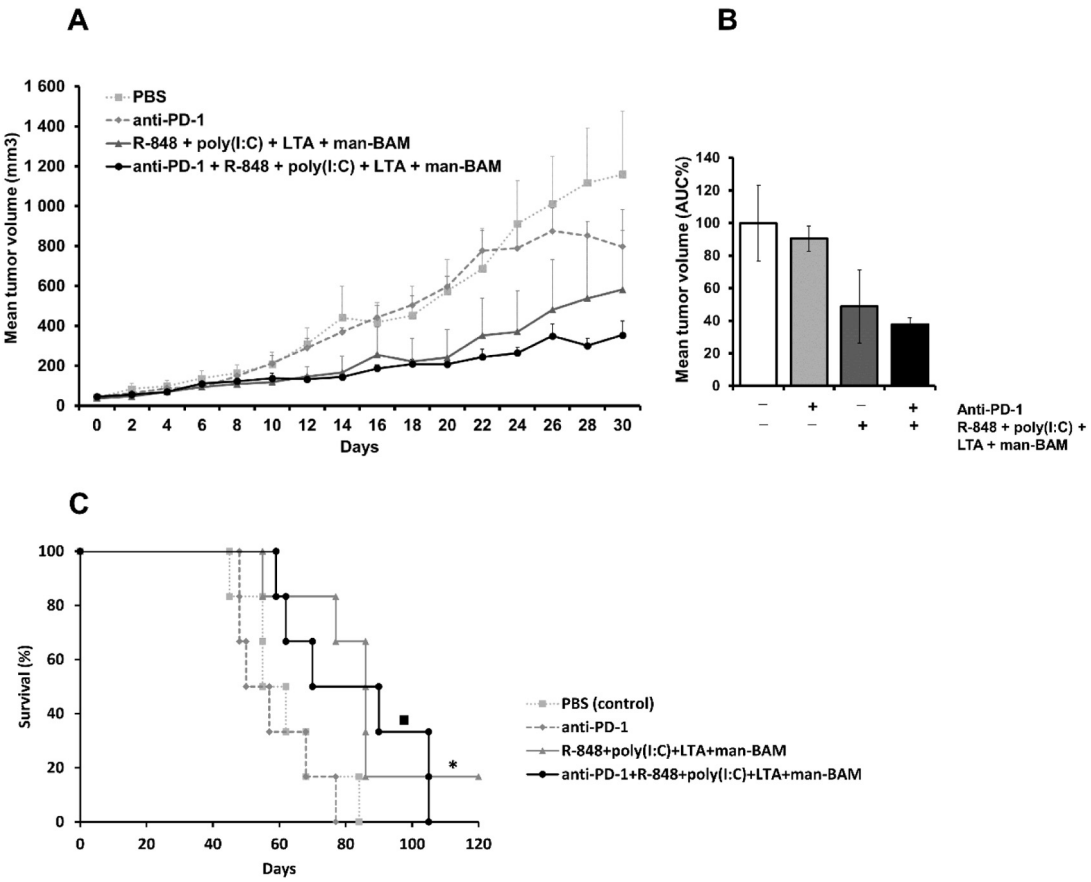

Fig. 10. Combination of TLRs/man-BAM therapy with anti-PD-1. Experimental design was the same as described in the Fig. 8. Where indicated, anti-PD-1 antibody was used in final concentration of 0.4 mg/ml of PBS or therapeutic mixture, respectively. Growth of tumors is presented as curves (A) with corresponding area under curve (AUC) analysis for each group (B). PBS treated control was considered as 100%. Statistical analysis was performed on AUC values by one-way ANOVA with Tukey's *post hoc* test. (C) Survival analysis, Long rank test, * = $p < 0.05$ compared to PBS (control) and anti-PD-1, ■ = $p < 0.05$ compared to anti-PD-1.

revealed partial inhibition of tumor growth but not survival prolongation (data not shown).

3.7. Immunotherapy of pancreatic adenocarcinoma

Therapy of pancreatic adenocarcinoma poses one of the greatest challenges in oncology. Thus, we examined the effect of our therapy based on intratumoral application of R-848, poly(I:C), LTA, and mannan-BAM in the Panc02/mouse model. We evaluated the effects of the TLR agonists alone (R-848, poly(I:C), LTA), the phagocytosis stimulating compound (mannan-BAM), and the complete therapeutic mixture (R-848, poly(I:C), LTA, mannan-BAM). The use of the complete therapeutic mixture exhibited the highest efficacy, as indicated by reduction of tumor growth (Fig. 8A, B), survival prolongation (Fig. 8C), and suppression of metastases (Fig. 8D).

As demonstrated above (Fig. 4), therapy based on the combination of R-848, poly(I:C), LTA, and mannan-BAM significantly stimulates granulocytic inflammatory infiltration. Therefore, we studied the cytotoxic effect of neutrophils on Panc02 cells covered by mannan-BAM *in vitro*. This effect (Fig. 9) was comparable to the effect of neutrophils on B16-F10 cells described above and previously [7]. Thus, the lower efficacy of the therapeutic mixture in the Panc02/mouse model compared to the B16-F10/mouse model cannot be explained by higher resistance of Panc02 cells to neutrophil attack. Furthermore, we examined the cytotoxic effect of NK cells on Panc02 cells covered by mannan-BAM. Killing of 32% of Panc02 cells correspond well to effects of NK cells on melanoma B16-F10 cells (29%) described previously [7].

3.8. Simultaneous administration of checkpoint inhibitors/agonist enhanced antitumoral activity of therapeutic mixture

To achieve higher efficacy of our immunotherapy, we decided to involve checkpoint inhibitors (anti-PD-1 and anti-CTLA-4 antibodies, respectively) and one activator (agonistic anti-CD40 antibody). Anti-PD-1 antibody itself had only negligible effect on the growth of Panc02 tumors (Fig. 10A, B). Combination of anti-PD-1 with mixture of R-848, poly(I:C), LTA, and mannan-BAM resulted in additive effects on reduction of tumor growth (Fig. 10A, B), although not statistically significant.

The combination of our immunotherapeutic mixture with anti-CTLA-4 antibody showed strong synergy leading to reduction of tumor growth (Fig. 11A, B) and prolongation of survival (Fig. 11C).

The strongest synergy was achieved by combination of R-848 + poly(I:C) + LTA + mannan-BAM mixture with agonistic anti-CD40 antibody (Fig. 12A, B, C). Anti-CD40 antibody itself showed an inhibiting effect (though not significant) on the tumor growth, but the synergy with R-848 + poly(I:C) + LTA + mannan-BAM dramatically affected both tumor growth (Fig. 12A, B) and mice survival (Fig. 12C). Importantly, the addition of anti-CD40 antibody significantly prolonged the survival of mice, when compared with R-848 + poly (I:C) + LTA + mannan-BAM mixture alone (Fig. 12C). Mice remained resistant to this type of tumor, as assessed by re-transplantation of Panc02 cells (s.c. application of 4×10^5 cells/mouse) on day 120 after the therapy initiation.

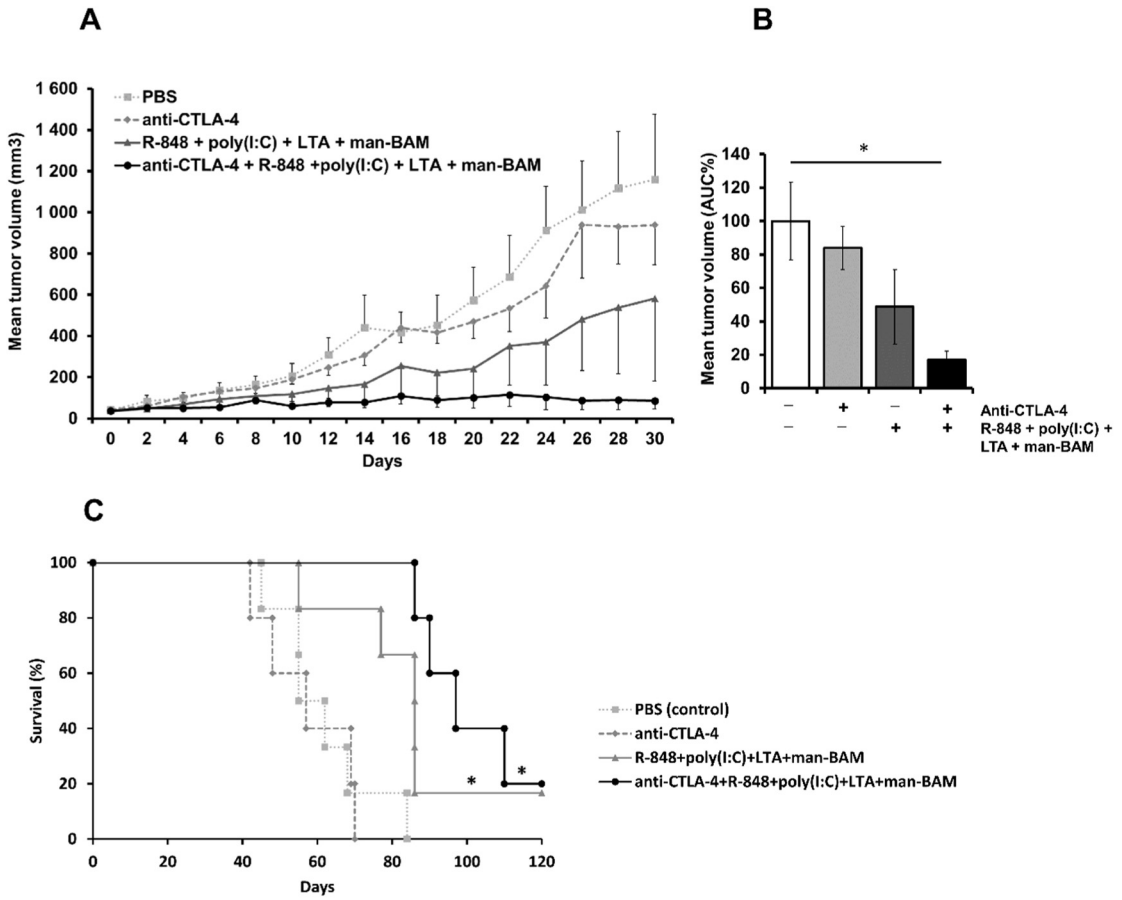

Fig. 11. Combination of TLRs/man-BAM therapy with anti-CTLA-4 therapy. Experimental design was the same as described in the Fig. 8. Where indicated, anti-CTLA-4 antibody was used in final concentration of 0.4 mg/ml of PBS or therapeutic mixture, respectively. Growth of tumors is presented as curves (A) with corresponding area under curve (AUC) analysis for each group (B). PBS treated control was considered as 100%. Statistical analysis was performed on AUC values by one-way ANOVA with Tukey's *post hoc* test, * = $p < 0.05$. (C) Survival analysis, Long rank test, * = $p < 0.05$ compared to PBS (control) and anti-CTLA-4.

4. Discussion

Recently, we demonstrated a strong synergy between the therapeutic mixture containing three TLR agonists (R-848 + poly (I:C) + heat-killed *L. monocytogenes*-SMCC) and anchored phagocytosis stimulating mannan (mannan-SMCC), which resulted in eradication of advanced melanoma [8]. *L. monocytogenes* significantly increased the efficacy of the treatment; however, the use of whole microorganisms is inconvenient for pertinent clinical applications. Thus, we replaced *L. monocytogenes* by another TLR2 agonist, lipoteichoic acid (LTA). Furthermore, an effective, but complicated anchoring of mannan to tumor cells with SMCC was replaced by BAM anchor (mannan-BAM, one-step application). Our new cancer immunotherapeutic mixture was highly effective, even if applied in only two therapeutic pulses. Four pulses resulted in 83% cure rate, which is the same efficacy as described previously [8]. The advantage of this improved treatment is the simple one-step anchoring of mannan and the use of well-defined chemical compounds including LTA. Moreover, LTA is not only TLR2 agonist; it is important ligand of scavenger receptors. LTA is able to interact with cell membranes [12] so its binding to tumor cells and fragments of tumor cell membranes can support scavenger receptors-mediated recognition of tumor antigens by dendritic cells. If significant contribution of LTA to efficacy of treatment is based on mentioned mechanisms will be matter of further studies.

We found four pulses regime as an optimal timing schedule. Separation of treatment pulses by treatment free intervals is necessary for recovery of sensitivity of TLR receptors to their agonists [8,13]. Moreover, as will be discussed below, our treatment initiated by innate immunity attack is followed by activation of adaptive immunity. So four pulses lasting treatment resembling vaccination scheme creates better conditions for activation of adaptive immunity than shorter one.

Investigation of mechanisms underlying the action of present experimental immunotherapy confirmed our previous observations and conclusions [6–8]. The major effects of the treatment seem to be massive inflammatory infiltration with dominant granulocytic fraction and immune destruction of tumor cells covered by anchored mannan. Mannan activates complement with a subsequent stimulation of phagocytosis (iC3b formation) [6,7]. Granulocytes most likely stand in the centre of cytotoxic anti-cancer effect of the treatment. Based on their counts, we consider neutrophils to be key phagocytic cells involved in the tumor eradication. Higher amount of NK cells, especially in the later phase of the treatment, may contribute to the general effect of experimental immunotherapy, as previously described [7]. Contribution of macrophages should be considered as well [6].

We presume that the attack of innate immunity cells on tumor cells is followed by activation of adaptive immunity. Presented experimental immunotherapy stimulates phagocytosis and thus supports subsequent antigen presentation by phagocytes. The function of dendritic cells, the main antigen presenting cells, is dependent on their proper maturation and presence of costimulatory molecules. This maturation is significantly supported by our therapeutic mixture, specifically by R-848 and poly(I:C) [14,15]. Activation of adaptive immunity is proved by long lasting resistance to re-transplantation of melanoma cells and antigen specific response of CD4+ and CD8+ T-cells.

Herein presented immunotherapeutic approach was effective also in the treatment of murine model of pancreatic adenocarcinoma. Similarly

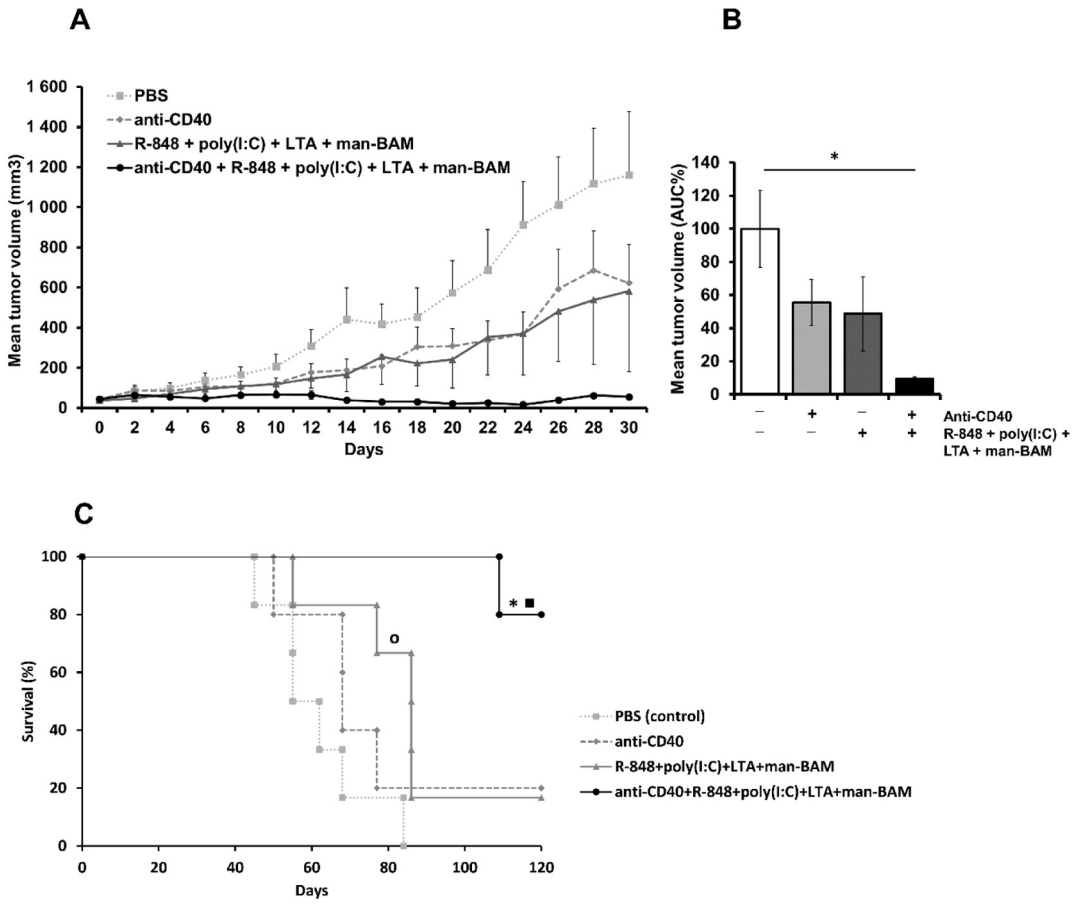

Fig. 12. Combination of TLRs/man-BAM therapy with anti-CD40 therapy. Experimental design was the same as described in the Fig. 8. Where indicated, anti-CD40 antibody was used in final concentration of 0.4 mg/ml of PBS or therapeutic mixture, respectively. Growth of tumors is presented as curves (A) with corresponding area under curve (AUC) analysis for each group (B). PBS treated control was considered as 100%. Statistical analysis was performed on AUC values by one-way ANOVA with Tukey's *post hoc* test, * = $p < 0.05$. (C) Survival analysis, Long rank test, * = $p < 0.05$ compared to anti-CD40 and R-848 + poly(I:C) + LTA + man-BAM groups, ■ = $p < 0.005$ compared to PBS (control), o = $p < 0.05$ compared to PBS (control).

to the melanoma model, synergy between TLR agonists and stimulation of phagocytosis was observed during the treatment of pancreatic adenocarcinoma as well as the suppression of metastasis. However, the outcomes of the treatment were not as significant as in melanoma. Since there is no evidence of resistance of pancreatic adenocarcinoma cells against attack of innate immune cells (based on our *in vitro* results), there has to be another mechanism responsible for lower efficacy of our experimental immunotherapy. The lower efficacy of our experimental immunotherapy can be partially explained by low infiltration of effector T- cells and simultaneous massive infiltration by immunosuppressive leukocytes in pancreatic adenocarcinoma. Dense desmoplastic stroma supports angiogenesis while evading from immune cells. Stroma is not only a passive barrier for the immune system; it also interacts with tumor cells stimulating their progression and invasiveness [16]. In effort to improve effect of our experimental immunotherapy in pancreatic adenocarcinoma, we combined the TLRs/mannan-BAM treatment with anti-CTLA-4 and anti-CD40 antibodies, respectively. Depletion of T-regulatory lymphocytes (Tregs) is considered to be the major outcome of anti-CTLA-4 therapy. Synergy between anti-CTLA-4 and TLRs/mannan-BAM therapy indicates the role of adaptive immunity in our immunotherapeutic approach, as in pancreatic adenocarcinoma Tregs play important role in suppression of adaptive immunity [16]. The synergy of anti-CD40 antibody with our treatment was expected since TLR agonists/mannan-BAM therapy is based on the primary stimulation of phagocytosis. Agonistic anti-CD40 antibody mimics CD40L on Th cells, resulting in activation of phagocytes and licensing of APC to induce anti-tumor T cell response.

Our treatment joins innate immunity based attack with activation of adaptive immunity. As own tumor is utilized for vaccination, we suppose that this treatment corresponds to the new paradigm for cancer therapy, *intratumoral immunization* [17]. Successful intratumoral immunization has to combine release of tumor antigens with support of antigen presentation, reduction of immune suppression and activation of cytotoxic cells [17–22]. Our treatment fulfils main criterions of this approach. The primary innate immunity based attack of tumors mimics natural elimination of pathogens and leads to both tumor shrinkage and release of tumor antigens. Moreover, the maturation of dendritic cells and the antigen presentation is supported by TLR agonists and can be further amplified by agonistic anti-CD40 antibody [23]. Anti-CTLA-4 can be used for Tregs depletion, if desirable [21,22].

5. Conclusion

The presented immunotherapeutic approach uses mechanisms of innate immunity for direct elimination of tumors by application of therapeutic mixture directly into the tumor. Consequently, due to support of T-cells by antigen presentation, the whole organism is vaccinated against the tumor. This is demonstrated by suppression of metastasis and acquisition of resistance to cancer recurrence.

Conflict of interests

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

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