

Antitumor effect of the combination of manumycin A and Immodin is associated with antiplatelet activity and increased granulocyte tumor infiltration in a 4T1 breast tumor model

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Abstract. Manumycin A is a natural antibiotic isolated from *Streptomyces parvulus* with broad range of biological activities including antineoplastic activity in several *in vitro* and *in vivo* cancer models. Immodin [dialyzable leukocyte extract (DLE)] is a dialysate released from disintegrated blood leukocytes of healthy donors which exerts immunonormalizing effects on cell-mediated immune responses. The aim of the present study was to explore the antitumor potential of the combination of manumycin A and Immodin in an experimental breast cancer model. Experiments were carried using a 4T1 tumor-bearing BALB/c mouse model. Survival analysis, tumor growth, hematological and biochemical profiles, leukocyte differential, phagocytic activity of leukocytes and histology of the primary tumor were examined. The combination treatment suppressed the tumor growth and prolonged the survival of tumor-bearing mice, decreased the number of monocytes, platelets and plateletcrit in peripheral blood of the tumor-bearing mice and increased the infiltration of neutrophils and eosinophils in the primary tumor. Moreover, individual therapies enhanced the phagocytic activity of monocytes and neutrophils. These findings demonstrate the antitumor effect of the combination of manumycin A and Immodin in 4T1 tumor-bearing mice

associated with strong antiplatelet activity and innate immunity activation.

Introduction

Manumycin A is a natural antibiotic isolated from *Streptomyces parvulus* with various biological activities that can act as a competitive Ras farnesyltransferase inhibitor or irreversible inhibitor of neutral sphingomyelinase. Manumycin A as a single agent and also in two- or triple-drug combinations showed *in vitro* and *in vivo* anticancer activity based on its cytotoxic, antiproliferative, proapoptotic, proautophagic, antiangiogenic and antimetastatic properties in various experimental models of fibrosarcoma (1), pancreatic (2) and anaplastic thyroid cancer (3), glioma (4), lymphoma (5) and others. Manumycin A has been shown to be effective against aggressive and therapy-resistant triple-negative breast cancer (6). However, in addition to its anticancer properties, manumycin A also exerts beneficial effects on atherosclerosis (7) and immunomodulatory properties including modification of pro-inflammatory responses in glioma and non-glioma tumor cells through abrogation of IL-1 β -induced HIF-1 α activation in tumor cells (8), suppression of interferon- γ and tumor necrosis factor- α in BALB/c-mice (9) and downregulation of mRNA for IL-6, TLR-8, IL-1 β and inhibition of IL-1 β , IL-6 and IL-8 production in human monocytes (10).

Immodin [also known as DLE or transfer factor (TF)] is a leukocyte immunomodulator and represents dialyzable leukocyte extracts (DLEs) released from disintegrated blood leukocytes of healthy human donors which are able to transfer cell-mediated immunity from sensitized donors to naive recipients. The benefits of TF were discovered ~50 years ago and the clinical experience is based on the extensive research conducted by numerous groups worldwide (11,12). DLE or TF preparations have been reported as beneficial in a broad spectrum of pathologies caused by microbial agents (13; reviewed in ref. 14) and in various diseases including allergies,

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rhinitis (15), asthma and atopic dermatitis (16), also sepsis (17) and rheumatoid arthritis (18). In the context of cancer therapy, DLE was found to be effective as an adjuvant to chemotherapy in patients with osteosarcoma (19), non-small cell lung (20,21) and breast cancer (22), in experimental glioma (23), and DLE improved survival in patients with bronchogenic carcinoma (24) and prostate cancer (25). In regard to cancer, the mechanisms that underlie the immunomodulatory effects of DLE have not been elucidated.

The key steps in breast tumor progression, including cellular transformation, proliferation, tumor cell survival and angiogenesis, can be mediated by components of the hemostatic system (26). Platelets as part of the circulatory system play a fundamental role in maintaining hemostasis. However, in addition to this role, they are also important factors in inflammation, atherosclerosis and cancer dissemination. Complex interactions between tumor cells and circulating platelets play an important role in cancer growth and dissemination (27). Platelets guard tumor cells from immune elimination and promote their arrest at the endothelium (28). The growing tumor can enhance the production and activation of platelets, thereby potentially creating a positive feedback loop to fuel tumor growth (29). In addition, a tumor produces high levels of cytokines and growth factors known to promote tumor growth and metastasis and these factors can be sequestered in and released by platelets (30). These pro-tumorigenic effects make platelets a rational target for anticancer therapy.

The other components of the circulatory system, neutrophils and eosinophils, are essential parts of innate immunity. However, in addition to their important role in fighting infections and inflammation, they play a critical role in the development of effective antitumor immunity (31). The infiltration of neutrophils and/or eosinophils in colorectal and primary small cell esophageal carcinoma was found to be associated with a good prognosis and enhanced survival of patients (32-34). Studies using *in vivo* models reported that tumor-homing neutrophils and/or eosinophils were essential for tumor rejection by initiation of changes in the tumor microenvironment (35-37).

In the present study, we used a strongly immunogenic 4T1 mouse model as a well characterized system to replicate stage IV breast cancer for evaluation of the antitumor potential of a novel combination of the natural drug manumycin A and the immunomodulator Immodin.

Materials and methods

Ethical approval. The State Veterinary and Food Administration of the Slovak Republic approved the experimental protocol (no. 4296/12-221e), and the animals were handled and sacrificed in a humane manner in accordance with the guidelines established by the relevant commission.

Reagents. Manumycin A and Immodin were purchased from Sigma-Aldrich (St. Louis, MO, USA) and IMUNA PHARM (Šarišské Michaľany, Slovakia), respectively. The drugs were freshly prepared on the day of use. Immodin was prepared by dissolving the lyophilized dialysate of 200×10^6 leukocytes in water for injection. Manumycin A was resuspended

in vehiculum (1% Cremophor oil in deionized water; Sigma-Aldrich) to reach the concentration of 0.5 mg/ml.

Cell line. 4T1 mouse mammary carcinoma cell line was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were maintained in RPMI-1640 medium enriched with 5 mM glutamate and 10% heat non-inactivated fetal bovine serum (FBS) (both from Thermo Fisher Scientific, Waltham, MA, USA) and maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂. Media were gentamicin- and antibiotic-free.

Animal model, cancer cell inoculation and experimental design/drug treatment. Female BALB/c mice (Velaz, Prague, Czech Republic) at 10 weeks of age were used in the experiments. The animals were adapted to standard vivarium conditions with temperature 22±2°C, relative humidity 45-60%, artificial regimen (L/D 12:12 h). During the experiment, the animals were fed with a standard MP-08-06 diet (Biofer, Veľký Šariš, Slovakia) and water *ad libitum*. Mouse mammary adenocarcinoma 4T1 cells (1×10^5 cells/mouse) were inoculated subcutaneously in the abdominal mammary gland of syngeneic BALB/c mice on day 0. The growth of tumors was monitored from the third day after 4T1 cell inoculation and the size of palpated tumors for each mouse individually was recorded. Animals were randomized into 6 experimental groups (25 animals/group): untreated control (C); Immodin-treated (IM); tumor control (4T1); 4T1 treated with Immodin (4T1 + IM); 4T1 treated with manumycin A (4T1 + MANU); 4T1 treated with Immodin and manumycin A (4T1 + IM + MANU). Drug administration was initiated on day 5 after cell inoculation corresponding to the onset of palpable tumors. Immodin was administered intraperitoneally to mice 12 times at the concentration of 0.05 IU/mouse alone or along with manumycin A administered perorally at the dose of 100 µg/mouse (6 times, every second day) after the onset of palpable tumors. Seventy-two hours after the final Immodin and/or manumycin A administration, 10 animals from each group were sacrificed by cervical dislocation under anesthesia. Details of the experimental scheme are shown in Fig. 1.

Tumor weight, tumor volume and animal survival. During the autopsy of 10 mice/group, each primary tumor was isolated, measured and weighed on digital scales and 5 of the tumors were processed for histological analysis. The volume (V) of tumors was calculated based on its diameters S_1 and S_2 ($S_1 < S_2$) using the formula $V = \pi \times (S_1)^2 \times S_2 / 12$. The remaining 15 animals/group were used for the evaluation of the survival of the animals by recording the number of dead animals each day. Starting the day after analysis, the survival times of 15 animals/group were reported as the percentage of animals surviving the following 27 days. Animals in this experiment were sacrificed when moribund. On day 28, the surviving mice were euthanized by cervical dislocation. Survival data were analyzed by the program MedCalc (<http://www.medcalc.org/manual/kaplan-meier.php>) as well as by GraphPad Prism software (version 5.01) (GraphPad Software, Inc., San Diego, CA, USA) and are presented in the Kaplan-Meier survival curves.

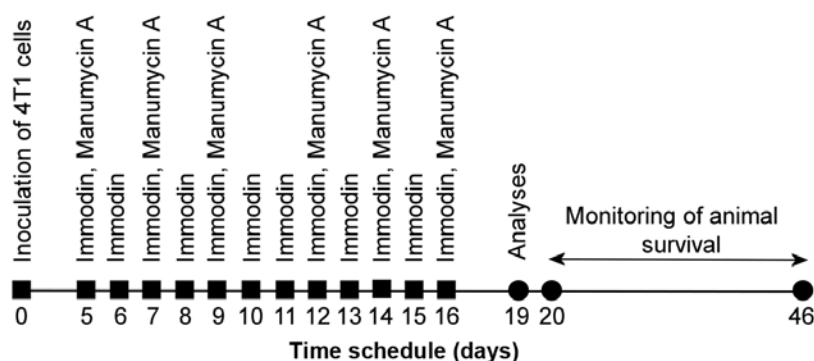


Figure 1. The experimental scheme.

Blood and serum analysis. Blood samples were collected shortly before cervical dislocation by retro-orbital bleeding in 1-ml K₃EDTA-containing tubes, 2-ml heparin-containing tubes and 2-ml serum collection tubes and stored at 4°C. Samples with whole blood in K₃EDTA tubes (both from Sarstedt, Nümbrecht, Germany) were analyzed using an automated veterinary hematology analyzer (Mindray BC 2800VET; Mindray, Shenzhen, China). Refrigerated samples were warmed to room temperature (RT) for 30 min before analysis. Plasma/serum tubes were placed on ice, blood for serum collection was allowed to clot for at least 30 min and subsequently both tubes were centrifuged at 2,400 x g at 4°C for 15 min. Serum samples were stored at 4°C and biochemical analyses were performed over 2 consecutive days using an automated clinical chemistry analyzer (ELLIPSE; AMS SpA, Rome, Italy) according to the manufacturer's instructions.

Leukocyte differential count. Air-dried smears of blood were stained with May-Grünwald/Giemsa stain and scanned using a light microscope (Leica DM500; Leica, Wetzlar, Germany) at a magnification of x1,000 using oil immersion following standard routines. In each microscopic field, the leukocytes were classified as lymphocytes, monocytes, eosinophils, neutrophils and basophils. In each smear, we counted 150-200 leukocytes/sample/mouse/group. The mean cell numbers were calculated after scoring smears from 10 mice/group. The neutrophil-lymphocyte ratio (NLR) was calculated from the white cell differential count.

Flow cytometric analysis of leukocyte phagocytic activity. The phagocytic activity of blood monocytes and neutrophils was quantitatively determined using a commercial test kit (FagoFlowEx kit; EXBIO, Praha, Czech Republic) which is based on the ingestion of FITC-labeled *Escherichia coli* (*E. coli*) bacteria by phagocytes. Briefly, 100 µl of heparinized whole blood was incubated at 37°C for 10 min with 20 µl *E. coli* bacteria that were opsonized with immunoglobulin and complement and fluorescein (FITC)-labeled. Control samples were incubated in an ice bath. Phagocytosis was stopped by placing the sample on ice and adding a solution that quenches the FITC fluorescence of surface bound bacteria, leaving the fluorescence of internalized particles unaltered. After two washing steps, erythrocytes were lysed by adding a BD FACS lysing solution (Becton-Dickinson Biosciences, San Jose,

CA, USA) and incubating for 20 min at RT, followed by an additional washing. A DNA staining solution was added prior to flow cytometric analysis, to exclude aggregation artefacts of bacteria or cells. Samples were kept on ice, and analyzed within 30 min of preparation using a BD FACSCanto™ flow cytometer (Becton-Dickinson Biosciences) and analyzed by BD FACSDiva™ software. Fluorescence measurements were carried out using a 488 nm blue excitation laser. Bacteria were excluded using the red fluorescence histogram (FL2) where leukocytes have higher DNA content as compared to bacteria. Neutrophils and monocytes were gated separately on FSC vs. SSC dot plot and their green fluorescence histograms (FL1) were analyzed. The results are expressed as a percentage.

Histological analysis of tumor sections. Tumors isolated from 5 animals from each experimental group were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.2) for 24 h at 4°C, washed in tap water for 5 h and processed for preparation of paraffin sections according to the standard protocol. Tumors were embedded in low melting paraffin (Paraplast; Sigma-Aldrich) and the sections (7-µm thick) were used for a set of staining procedures. Using the standard protocol, some slides were stained with Mayer's hematoxylin/eosin and other slides were used to localize granulocytes after modified Sirius red staining protocol originally described by Llewellyn (38). Briefly, dehydrated sections were placed in Harris hematoxylin (pH ≤5.0) for 3 min, were rinsed in running tap water and briefly immersed in 100% ethanol. This nuclear staining was followed by the staining of eosinophilic granules by alkaline Sirius red stain (without sodium chloride, pH 8.5) (Sigma-Aldrich), in which slides were immersed for 1 h. After rinsing the slides in tap water for 5 min, sections were dehydrated in a set of graded alcohols and cleared in HistoChoice clearing solution (Amresco, Solon, OH, USA). Finally, sections were mounted into permanent medium HistoChoice mounting fluid (Amresco). After staining, granules of eosinophils were observed as bright red, whereas granules of neutrophils were not visible and both cell types could also be discriminated by the shape of nuclei stained dark blue. The morphometric analysis of both cell types was carried out at a magnification of x1,000 using Olympus Microscope BX51 and a digital analysis imaging system 'Analysis Docu' (Soft Imaging Systems 3.0; Prague, Czech Republic). After analysis of an average 30 screen fields on the sections of

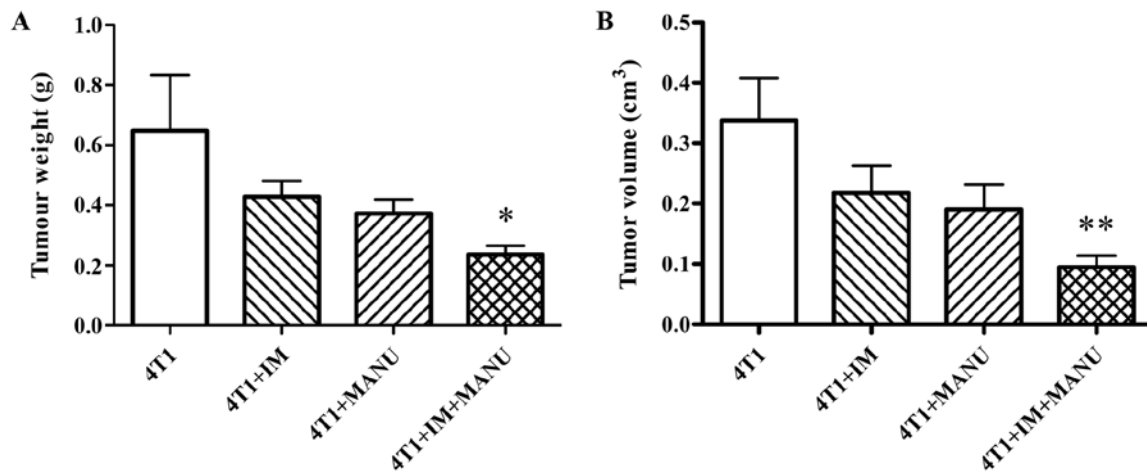


Figure 2. Effect of the treatments on (A) tumor weight and (B) tumor volume. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. 4T1, tumor control (n=11); 4T1 + IM, 4T1 treated with Immodin (n=14); 4T1 + MANU, 4T1 treated with manumycin A (n=14); 4T1 + IM + MANU, 4T1 treated with Immodin and manumycin A (n=14). Data are expressed as mean \pm SEM (* P <0.05, ** P <0.01 as compared to the 4T1 group).

individual tumors the mean number of counted cells for each section was calculated for 0.1 mm² of tissue area. Finally, the mean number of the cells recorded for the sections of tumors from 5 mice were calculated showing data for 0.1 mm².

Statistical analyses. All results were analyzed using GraphPad Prism software (version 5.01) for normal distribution. All data were examined for normal distribution and then the appropriate tests were applied. Statistical differences between groups were analyzed using ANOVA followed by Tukey's post hoc test or ANOVA (Kruskal-Wallis test) followed by pairwise multiple comparison procedures (Dunn's method) or Mann-Whitney rank-sum test. For Kaplan-Meier survival analysis, a log-rank (Mantel-Cox) test was applied. All differences were considered statistically significant at P <0.05. For ethical reasons, when possible and appropriate, different tissues, organs and whole blood samples from one animal were used for several measurements (i.e., samples for determination of different parameters in whole blood were obtained from the same animals).

Results

Tumor weight and tumor volume. Single agent treatments with Immodin and manumycin A resulted in a non-significant decrease in tumor weight and volume compared to the untreated 4T1 group. The combination treatment more efficiently reduced the tumor weight (P <0.01; Fig. 2A) and volume (P <0.05; Fig. 2B) compared to the untreated 4T1 group.

Animal survival. Every experimental group of tumor-bearing mice had a decreased survival compared to the survival noted in the control and Immodin group (P <0.0001). All the treatments prolonged the survival of the tumor-bearing mice compared to that of the untreated 4T1 group (P <0.0001), but the most pronounced effect was observed after combined therapy. The Kaplan-Meier curves for all treatment groups showing the percentage of animals surviving vs. time are presented in Fig. 3.

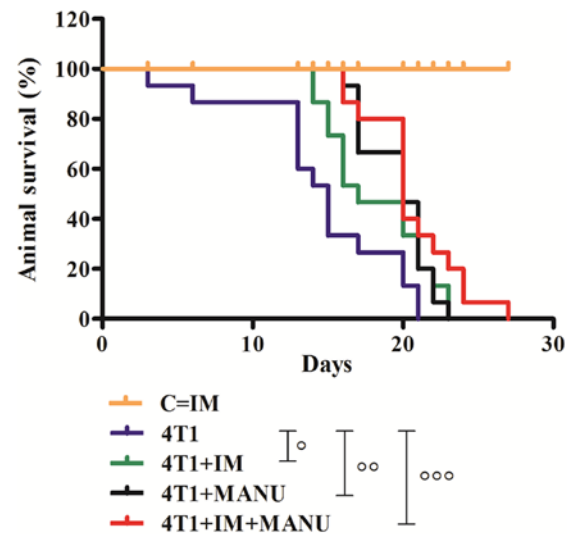


Figure 3. Effect of the treatments on animal survival. Kaplan-Meier survival curves (n=15) were compared using log-rank test (Mantel-Cox); P =0.0312 (4T1 vs. 4T1 + IM), P =0.0019 (4T1 vs. 4T1 + MANU), P <0.0007 (4T1 vs. 4T1 + IM + MANU); ° P <0.05, °° P <0.01, °°° P <0.001. C, untreated control; IM, Immodin treatment; 4T1, tumor control; 4T1 + IM, 4T1 treated with Immodin; 4T1 + MANU, 4T1 treated with manumycin A; 4T1 + IM + MANU, 4T1 treated with Immodin and manumycin A.

Hematological parameters. The number (both the percentage and count) of monocytes was significantly increased in the untreated as well as this number in the treated 4T1 groups compared to the control (P <0.001 for 4T1 and 4T1 + IM; P <0.01 for 4T1 + MANU; P <0.05 for 4T1 + IM + MANU). While the monocyte count was significantly decreased after treatment with the drug combination when compared to the count noted in the untreated 4T1 group (P <0.05), the percentage of monocytes was not significantly different between the untreated and treated 4T1 groups. The platelet count was significantly increased in the 4T1 group compared to that in the untreated control (P <0.05). The combined treatment prevented this increase; the platelet count in this group

Table I. Effect of the treatments on hematological parameters.

	C	IM	4T1	4T1 + IM	4T1 + MANU	4T1 + IM + MANU
Total WBCs ($\times 10^9/l$)	6.19 \pm 0.47	14.33 \pm 2.66	31.34 \pm 9.37 ^a	43.10 \pm 6.00 ^c	35.50 \pm 6.79 ^b	31.38 \pm 2.51 ^b
Monocytes ($\times 10^9/l$)	0.17 \pm 0.03	0.88 \pm 0.26	2.32 \pm 0.46 ^c	1.85 \pm 0.19 ^c	1.58 \pm 0.20 ^b	1.13 \pm 0.08 ^{a,d}
Monocytes (%)	2.79 \pm 0.25	2.70 \pm 0.14	4.92 \pm 0.34 ^c	4.20 \pm 0.39 ^a	4.14 \pm 2.6 ^a	4.10 \pm 0.35 ^a
Lymphocytes ($\times 10^9/l$)	4.74 \pm 0.38	9.23 \pm 1.71	13.72 \pm 2.43 ^b	11.84 \pm 1.11 ^b	9.2 \pm 1.25	10.3 \pm 0.82
Lymphocytes (%)	76.63 \pm 2.04	69.25 \pm 4.78	38.00 \pm 6.47 ^c	29.74 \pm 2.65 ^c	32.63 \pm 4.62 ^c	34.28 \pm 0.61 ^c
Granulocytes ($\times 10^9/l$)	1.3 \pm 0.16	4.21 \pm 1.15	22.88 \pm 8.53 ^b	30.23 \pm 5.14 ^c	24.34 \pm 5.12 ^b	19.50 \pm 1.62 ^a
Granulocytes (%)	20.59 \pm 1.82	27.69 \pm 3.97	65.10 \pm 6.90 ^c	71.14 \pm 3.98 ^c	65.97 \pm 4.67 ^c	60.11 \pm 1.90 ^c
Platelets ($\times 10^9/l$)	377.0 \pm 24.25	399.8 \pm 53.96	631.6 \pm 73.14 ^a	586.2 \pm 54.22	431.2 \pm 52.07	321.6 \pm 42.14 ^c
PCT (%)	0.32 \pm 0.02	0.36 \pm 0.03	0.50 \pm 0.05 ^b	0.41 \pm 0.03	0.33 \pm 0.04 ^d	0.23 \pm 0.03 ^f
PDW	16.54 \pm 0.09	16.41 \pm 0.11	16.12 \pm 0.08	16.14 \pm 0.09	16.11 \pm 0.13	16.19 \pm 0.18
RBC ($\times 10^{12}/l$)	10.78 \pm 0.23	12.36 \pm 0.43	13.73 \pm 0.51 ^c	12.69 \pm 0.4 ^a	12.94 \pm 0.57 ^a	13.04 \pm 0.40 ^b
HCT (%)	58.31 \pm 1.37	67.81 \pm 2.40	73.66 \pm 2.56 ^a	69.54 \pm 2.53	63.90 \pm 6.17	70.99 \pm 2.10
HGB (g/dl)	17.49 \pm 0.46	21.23 \pm 0.71 ^a	23.04 \pm 1.01 ^c	21.27 \pm 0.75 ^a	21.62 \pm 1.04 ^a	22.01 \pm 0.62 ^b
MCV (fl)	54.1 \pm 0.32	54.9 \pm 0.39	53.70 \pm 0.26	54.77 \pm 0.47	53.92 \pm 0.45	54.47 \pm 0.36
RDW (%)	15.03 \pm 0.11	14.97 \pm 0.15	15.74 \pm 0.13 ^a	15.98 \pm 0.15 ^c	15.67 \pm 0.16 ^a	15.80 \pm 0.09 ^b
MCHC (g/l)	299.29 \pm 1.97	312.71 \pm 1.85 ^b	311.86 \pm 3.51 ^b	305.57 \pm 1.65	309.71 \pm 2.24 ^a	309.71 \pm 1.78 ^a
MCH (pg)	16.14 \pm 0.11	17.13 \pm 0.11 ^c	16.71 \pm 0.16 ^a	16.67 \pm 0.09	16.66 \pm 0.14	16.83 \pm 0.12 ^b
MPV (fl)	8.10 \pm 0.23	8.03 \pm 0.19	7.51 \pm 0.12	7.51 \pm 0.10	7.69 \pm 0.18	7.76 \pm 0.15

Data are expressed as mean percentage \pm SEM (n=7); ^aP<0.05 as compared to the control; ^bP<0.01 as compared to the control; ^cP<0.001 as compared to the control; ^dP<0.05 as compared to 4T1; ^eP<0.01 as compared to 4T1; ^fP<0.001 as compared to 4T1. C, untreated control; IM, Immodin treatment; 4T1, tumor control; 4T1 + IM, 4T1 treated with Immodin; 4T1 + MANU, 4T1 treated with manumycin A; 4T1 + IM + MANU, 4T1 treated with Immodin and manumycin A; WBC, white blood cell count; RBC, red blood cell count; HGB, hemoglobin; HCT, hematocrit; PCT, plateletcrit; MCV, mean corpuscular volume; RDW, red cell distribution width; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin; PDW, platelet distribution width; MPV, mean platelet volume.

was significantly decreased compared to the count in the untreated 4T1 group (P<0.01) and the count reached the level similar to that of the control group. In addition, the platelet count decreased following single treatments compared to the count in the untreated 4T1 group, although these differences were not significant. There was also a significant increase in plateletcrit (PCT) in the 4T1 group compared to that in the control (P<0.01). Treatment with manumycin A (P<0.05) or the drug combination (P<0.001) caused a decrease in plateletcrit compared to that in the untreated 4T1 group. While the percentage of peripheral blood lymphocytes decreased in all 4T1 groups compared to the control (P<0.001), the lymphocyte count was elevated in these groups compared to the count noted in the control (P<0.01). Comparison of the number or percentage of granulocytes and lymphocytes revealed a reversal of granulocyte-lymphocyte ratio in the tumor-bearing mice, with an 18- and 3-fold increase in the number and percentage of granulocytes. In addition, almost all hematological parameters in the 4T1 group showed a significant difference compared to the control (increase in red blood cell parameters RBC, HCT, HGB, RDW, MCHC and MCH) but neither the single nor the combination treatment affected these changes (Table I).

Biochemical parameters. Analysis of biochemical parameters revealed a significant decrease in the serum concentration

of total cholesterol (P<0.05), LDL-cholesterol (P<0.05), HDL-cholesterol (P<0.01), triglycerides (P<0.001) and total proteins in the untreated 4T1 group compared to these values in the control (P<0.001). All the treatments resulted in a significant decrease in serum concentration of LDL-cholesterol compared to both the control (P<0.001 for all treated groups) and 4T1 group (P<0.05 for 4T1 + IM and 4T1 + MANU; P<0.01 for 4T1 + IM + MANU). The combined treatment prevented the decrease in serum concentration of HDL-cholesterol which was significantly increased compared to that in the 4T1 group (P<0.05) reaching a value similar to that of the control group. The serum concentration of total proteins was increased in the 4T1 + MANU (P<0.05) and 4T1 + IM + MANU (P<0.05) groups compared to the serum concentration in the 4T1 group (Table II). There were no significant changes in the levels of urea and creatinine or liver transaminases AST, ALP and ALT between the treated and untreated 4T1 groups (data not shown).

Leukocyte differential count. Evaluation of the white blood cell differential counts revealed a marked increase in the number of neutrophils (70.43 \pm 6.47 vs. 18.00 \pm 1.88; P<0.001) accompanied by a decreased number of lymphocytes (27.71 \pm 8.06 vs. 73.07 \pm 2.51; P<0.001) and eosinophils (0.36 \pm 0.28 vs. 1.50 \pm 0.27; P<0.05) in the 4T1 group compared to these numbers in the control group. The number of monocytes was

Table II. Effect of the treatments on biochemical parameters in serum.

	C	IM	4T1	4T1 + IM	4T1 + MANU	4T1 + IM + MANU
Total cholesterol (mmol/l)	4.41±0.12	4.00±0.09	3.53±0.26 ^a	3.63±0.16 ^a	3.80±0.17	3.90±0.10
LDL-cholesterol (mmol/l)	2.64±0.03	2.38±0.03 ^c	2.54±0.00 ^a	2.44±0.03 ^{c,d}	2.44±0.02 ^{c,d}	2.43±0.01 ^{c,e}
HDL-cholesterol (mmol/l)	2.26±0.06	2.15±0.08	1.68±0.15 ^b	1.81±0.02 ^b	1.88±0.10	2.11±0.06 ^d
Triglyceride (mmol/l)	2.99±0.00	2.08±0.09 ^c	2.09±0.08 ^c	2.28±0.09 ^c	2.10±0.06 ^c	1.90±0.02 ^c
Total protein (g/l)	51.30±0.10	46.90±1.10	42.05±0.18 ^c	45.33±0.54 ^b	45.93±1.21 ^{b,d}	46.35±0.63 ^{a,d}

Data are expressed as mean percentage ± SEM (n=7); ^aP<0.05 as compared to the control; ^bP<0.01 as compared to the control; ^cP<0.001 as compared to the control; ^dP<0.05 as compared to 4T1; ^eP<0.01 as compared to 4T1. C, untreated control; IM, Immodin treatment; 4T1, tumor control; 4T1 + IM, 4T1 treated with Immodin; 4T1 + MANU, 4T1 treated with manumycin A; 4T1 + IM + MANU, 4T1 treated with Immodin and manumycin A.

Table III. Effect of the treatments on white blood cell differential leukocyte count.

	C	IM	4T1	4T1 + IM	4T1 + MANU	4T1 + IM + MANU
Neutrophils (band)	0.36±0.18	0.71±0.24	0.21±0.10	0.14±0.09	0.36±0.14	0.43±0.17
Neutrophils (segment)	18.00±1.88	23.21±2.21	70.43±6.47 ^c	75.64±3.25 ^c	72.43±4.86 ^c	68.43±2.77 ^c
Lymphocytes	73.07±2.51	67.29±2.14	27.71±8.06 ^c	16.71±2.98 ^c	17.57±3.82 ^c	22.36±2.30 ^c
Eosinophils	1.50±0.27	1.21±0.26	0.36±0.28 ^a	0.29±0.29 ^a	0.36±0.09 ^a	0.21±0.15 ^b
Monocytes	7.41±1.60	7.50±0.44	10.17±0.98	9.25±1.16	8.08±1.39	9.33±1.63

Data are expressed as mean percentage ± SEM (n=7). ^aP<0.05 as compared to the control; ^bP<0.01 as compared to the control; ^cP<0.001 as compared to the control. C, untreated control; IM, Immodin treatment; 4T1, tumor control; 4T1 + IM, 4T1 treated with Immodin; 4T1 + MANU, 4T1 treated with manumycin A; 4T1 + IM + MANU, 4T1 treated with Immodin and manumycin A.

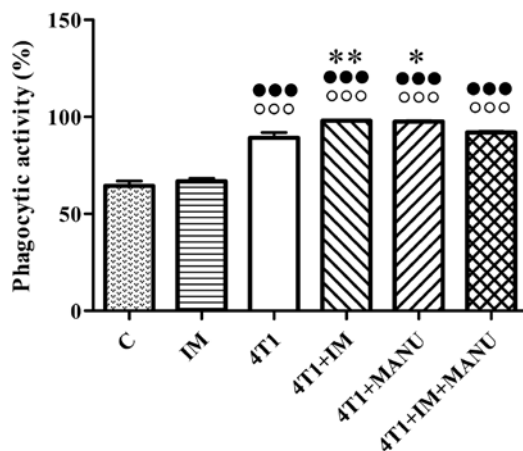


Figure 4. Total phagocytic activity of monocytes and neutrophils. Data are expressed as mean ± SEM (n=7). ***P<0.001 as compared to C; **P<0.01 as compared to IM; *P<0.05, **P<0.01 as compared to 4T1. C, untreated control; IM, Immodin treatment; 4T1, tumor control; 4T1 + IM, 4T1 treated with Immodin; 4T1 + MANU, 4T1 treated with manumycin A; 4T1 + IM + MANU, 4T1 treated with Immodin and manumycin A.

not significantly different between the tested groups. The basophile counts were 1±1 and are not shown in Table III. The NLR calculated from the white cell differential count revealed a 14-fold increase in NLR in the 4T1 group compared to that

of the control (3.56±1.28 vs. 0.25±0.03; P<0.001). None of the therapies affected this ratio.

Phagocytic activity. Analysis of phagocytic activity of peripheral blood monocytes and neutrophils revealed a significant increase in the percentage of phagocytosing cells in all 4T1 groups compared to the controls (P<0.001). Administration of Immodin or manumycin A to the 4T1 group enhanced the phagocytic activity compared to the activity in the untreated 4T1 group (P<0.01 for IM; P<0.05 for MANU). The phagocytic activity in the 4T1 + IM + MANU group was not significantly different from the untreated 4T1 group. Due to the same trend of phagocytic activity of monocytes and neutrophils, we decided to demonstrate the total phagocytic activity (Fig. 4).

Neutrophils and eosinophils in primary tumor tissue. Histological analysis of tumor tissue revealed an increased infiltration of neutrophils (Fig. 5A) and eosinophils (Fig. 5B) in primary tumors of the animals treated with the drug combination. The number of neutrophils was significantly higher in the 4T1 + IM + MANU group in comparison with all the other groups (P<0.05 for 4T1 and 4T1 + MANU; P<0.001 for 4T1 + IM). The number of eosinophils was significantly higher in the 4T1 + MANU group in comparison with the 4T1 group (P<0.01) and in the 4T1 + IM + MANU group in comparison with all other groups (P<0.001).

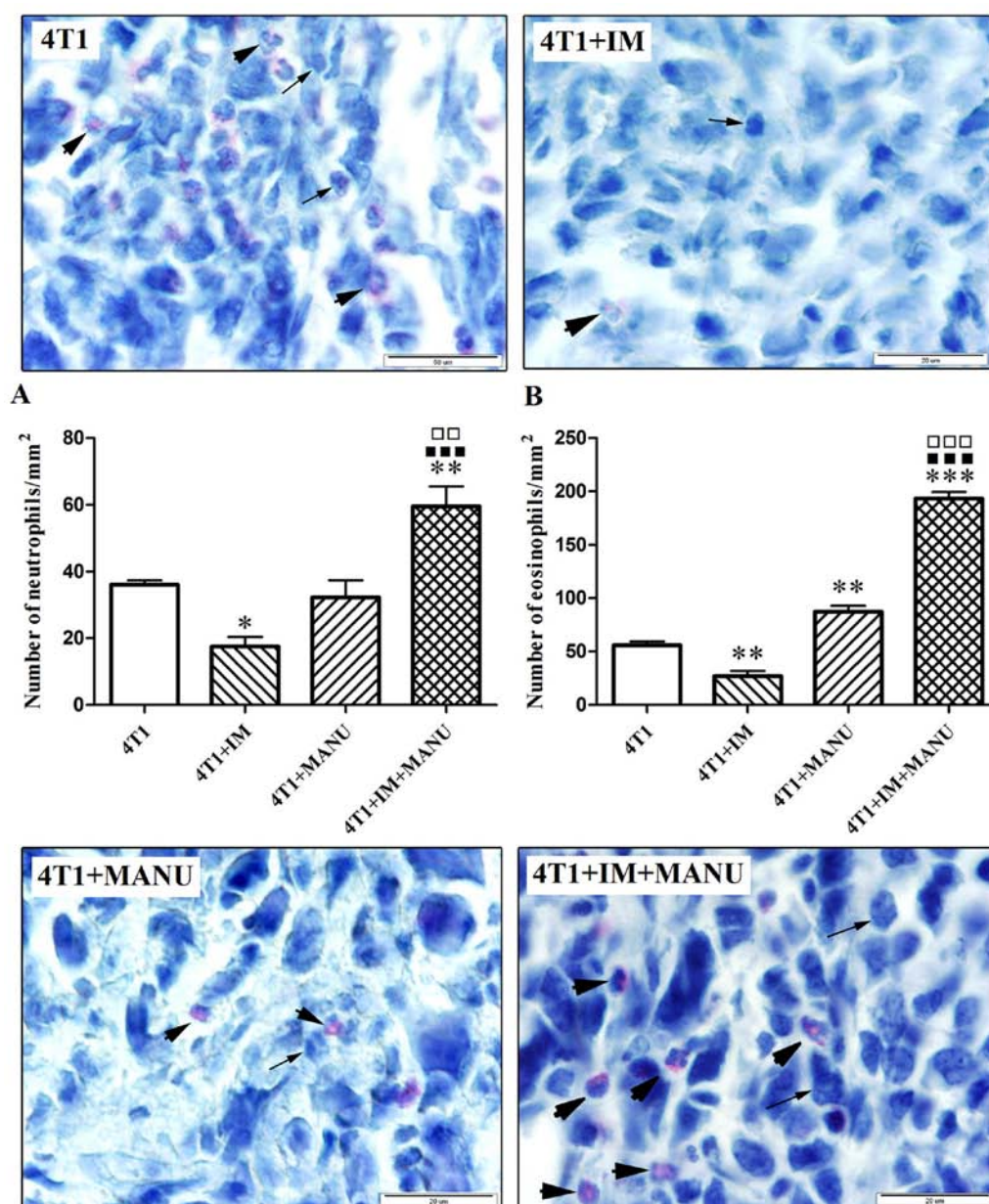


Figure 5. Effect of the treatments on the number of (A) neutrophils and (B) eosinophils in the primary tumor. Data are expressed as mean \pm SEM (n=5). 4T1, tumor control; 4T1 + IM, 4T1 treated with Immodin; 4T1 + MANU, 4T1 treated with manumycin A; 4T1 + IM + MANU, 4T1 treated with Immodin and manumycin A. *P<0.05, **P<0.01 as compared to 4T1; ***P<0.001 as compared to 4T1 + IM; □□P<0.01, □□□P<0.001 as compared to 4T1 + MANU. Representative images from each group are shown. Arrows and arrowheads indicate neutrophils and eosinophils, respectively.

Discussion

In the present study, we reported on the markedly reduced tumor growth and prolonged survival of 4T1 tumor-bearing mice receiving the combination treatment of manumycin A and Immodin. Since cancer-associated inflammation is a key determinant of disease progression and the inflammatory response involves systemic alterations, whole blood cell analyses were carried out to reveal alterations in the hematological profile in both untreated and treated tumor-bearing mice. Our results showed that almost all hematological parameters were affected by the tumor presence. Regarding leukocytes, an elevated white blood cell count including both the number of monocytes and granulocytes as well as an increased count of lymphocytes were detected in the

peripheral blood of the tumor-bearing mice (number means both the absolute count and percentage). These changes indicate 4T1 cell-triggered activation of an inflammatory response involving systemic alterations. However, besides monocytes, none of the parameters were affected by the treatments. Monocyte counts dropped significantly after the combination treatment but were still slightly higher than in the control. On the basis of our complete results showing an antitumor effect of the combination treatment, we speculated that it may be caused by the recruitment of circulating monocytes from the blood into a tumor and their differentiation into M1-like macrophages with pro-inflammatory, cytotoxic and antitumor properties (39-41).

The analysis of the white blood cell differential counts revealed that the most abundant circulating blood leukocytes

in the 4T1-bearing mice were neutrophils. In the same cancer model, circulating neutrophil numbers continuously increased with tumor progression (42). Increasing levels of WBC and neutrophils as a result of progressive increase in extramedullary hematopoiesis in the spleen and the liver were observed by week 2 after 4T1 tumor inoculation (43). Regarding granulocytes and lymphocytes, our results are partially in agreement with a report on leukemoid reaction caused by 4T1. Whereas DuPre' and Hunter (44) observed a profound granulocytosis associated with a decrease in peripheral blood lymphocytes as the percentage of total leukocytes and constant numbers of lymphocytes in 4T1 tumor-bearing mice (by day 15 post-tumor transplant vs. by day 19 post-tumor transplant in the present study), our results demonstrated an elevated number of granulocytes associated with a strong decrease in the percentage of peripheral blood lymphocytes and elevated lymphocyte counts. Despite the fact that the lymphocyte counts were higher in tumor-bearing mice compared to the control, comparison of the number or percentage of both the granulocytes and lymphocytes revealed a reversal of the granulocyte-lymphocyte ratio (G/L) in the tumor-bearing mice. It has been reported that with the progression of cancer, the proportion of granulocytes increases in peripheral blood and the G/L ratio is associated with tumor progression and shorter survival (45). The high levels of granulocyte colony-stimulating factor (G-CSF) protein in the serum of tumor-bearing mice suggests that this factor is responsible for the increase in granulocytes (44). Absolute lymphocyte counts can be considered a biomarker of immune status in the presence of a malignancy (46). The rapid growth observed during the first two weeks after the inoculation of tumor cells was followed by regression of growth between weeks 2 and 4 associated with necrosis and infiltration of leukocytes (43). Our findings demonstrate the increased infiltration of primary tumors with neutrophils and eosinophils. Based on the inhibitory effect of the combination treatment on tumor growth and prolonged survival of animals, we assume the antitumor potential of granulocytes which has been reported in several studies. Carretero *et al* (37) demonstrated an essential role of tumor-homing eosinophils in tumor eradication and survival. Antitumor effect of Virulizin® was associated with the recruitment of eosinophils into tumors (47). Tumor-infiltrating neutrophils played an essential role in establishment of antitumor immunity following PDT (48), in stimulation of an antitumor response in early-stage human lung cancer (49) and acted as potential mediators of zoledronate-induced antitumor activity (50).

Injection of the 4T1 mammary carcinoma cell line into BALB/c mice was found to induce a large increase in peripheral blood neutrophils that correlated with tumor growth (51). Numerous pre-clinical and clinical studies have demonstrated the association between the increased number of circulating neutrophils and tumor progression (52-54). The high neutrophil-lymphocyte ratio (NLR), one of the peripheral blood-derived indicators of the systemic inflammatory response, was found to be associated with adverse survival in many solid tumors including breast cancer (55-59). Our data also showed an increased neutrophil counts accompanied by a decreased number of lymphocytes and high levels of NLR in tumor-bearing mice. Notably, despite the fact that these

changes were not modified by the treatments, they were not associated with tumor progression.

The evaluation of phagocytosis as a key mechanism of the innate immune response revealed an increased phagocytic activity of peripheral blood leukocytes in all tumor-bearing mice with a profound increase in mice treated with single manumycin A or Immodin indicating the activation of phagocytic capacity of leukocytes in the presence of the tumor and its further potentiation by single treatments. It has been revealed that the phagocytic capacity of leukocytes is significantly activated in the presence of a pathological process in the organism and this activation is much more evident in cancer patients than in patients with non-malignant disorders. In contrast, surgery, chemoradiotherapy (60) and chemotherapy (61) caused a significant decrease in the phagocytosis of peripheral blood neutrophils in breast cancer and macrophages in oral cancer (62), thus interfering with the defense reactions. In this regard, it is important that none of the treatments negatively affected the phagocytic function, since it has been suggested that such therapeutical procedures must be chosen which do not interfere with the course of defense reactions (63).

Furthermore, we observed a high number of platelets and elevated plateletcrit in the untreated tumor-bearing mice. The PDW and MPV were unchanged in all experimental groups. The same alterations in platelet parameters have been documented in patients with colorectal cancer (64). Notably, both the platelet counts and plateletcrit returned to the normal range in the tumor-bearing mice treated with the combination of manumycin A and Immodin indicating its marked antiplatelet action. This is of great importance since an elevated blood platelet count correlates with poor survival and prognosis in a large variety of cancers including breast cancer (65-69). There is evidence for the positive antitumor effect of circulating platelet reduction. Lowering platelet counts in various experimental and clinical models decreased tumor lung invasion (70,71), inhibited gastric and ovarian tumor growth (72,73), prevented or delayed the development of hepatocellular carcinoma (74) and resulted in better survival of patients with head and neck squamous cell carcinoma (75). Stone *et al* (73) suggested the existence of a paracrine circuit wherein increased production of thrombopoietic cytokines in tumor and host tissue leads to paraneoplastic thrombocytosis, which fuels tumor growth. In their study anti-interleukin (IL)-6 antibody treatment significantly reduced platelet counts in tumor-bearing mice and in patients with epithelial ovarian cancer. An increased plasma IL-6 in patients with metastatic prostate carcinoma was found to be correlated with tumor burden (76). It has been reported that the presence of a tumor increases both tumor- and host-derived IL-6 in plasma and significant amounts of IL-6 are stored in platelets (30). Recently, it has been observed that manumycin A decreased interleukin levels involved in various types of malignancies in colorectal adenocarcinoma Caco-2 cells (77) and downregulated the mRNA for IL-6 and inhibited IL-6 production in TNF- α stimulated human monocytic leukemia THP-1 cells and peripheral blood monocytes (10). Based on these data, we speculated that the antiplatelet effect of single manumycin A or the combination treatment may involve the inhibition of IL-6.

In the present study, we further demonstrated that the red blood cell markers were also affected by the tumor showing

an increase in RBC, HCT, HGB, RDW, MCHC and MCH. All of these parameters were increased in the tumor-bearing mice. We suppose that these changes which were unaffected by either treatment, may be a consequence of tumor-stimulated stress erythropoiesis in the spleen. According to a recent study, 4T1 tumor development is associated with the suppression of medullary erythropoiesis by GCS-F, whereas tumor stress promotes erythropoiesis in the spleen. The average spleen weight and splenocyte numbers started to increase one week after 4T1 tumor cell implantation (78). Our results also showed a significant increase in mean spleen weight in tumor-bearing mice which was slightly suppressed by the combination treatment (data not shown). Yilmaz *et al* (79) suggested that the increase in RDW is the result of complex factors, including inflammation, oxidative stress and the immune response.

Alterations in cholesterol metabolism including decreased total serum cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides have been documented as a preclinical or consequential effect of some types of cancer (80,81). In contrast, an inverse relationship has been demonstrated between HDL cholesterol and cancer risk (82-84). Low HDL-cholesterol levels may simply be a reflection of chronic conditions that increase inflammation and insulin resistance, which may directly influence atherosclerosis and carcinogenesis (85). Our results showed the ability of the combination treatment to decrease LDL-cholesterol and also to restore the concentration of HDL-cholesterol and the total protein content in tumor-bearing mice indicating the positive effect of the treatment on cholesterol and protein metabolism as part of its anticancer action. These results are in accordance with the data from another study, which showed a decrease in LDL-cholesterol in quercetin-treated S-180-bearing mice, whereas the HDL-cholesterol increased compared with levels in the S-180-bearing mice (86).

In summary, our results revealed the most potent antitumor effect of the novel combination of manumycin A and Immodin in a 4T1 breast carcinoma mouse model demonstrated by reduced tumor growth and prolonged survival associated with strong antiplatelet effect and enhanced granulocyte infiltration in the primary tumor indicating that various mechanisms are involved in the antitumor activity of the combination treatment.

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