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ORIGINAL ARTICLE

Poliglusam Nanoparticles Activate T Cell Response in Breast Cancer Cell: an In Vivo and In Vitro Study

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Abstract

Poliglusam nanoparticles are potential therapeutic agents for the treatment of cancer. In particular, their efficacy has been reported as delivery systems in breast cancer. The aim of this study is to propose a new immunotherapeutic strategy, using Poliglusam nanoparticles as activators of the human immune response. Poliglusam nanoparticles were synthesized and characterized using both dynamic light scattering and electron microscopy. Whilst, their effectiveness in immune stimulation and detection of apoptosis was evaluated by cytokine and TUNEL assays. Finally, the cytokines pattern in splenocytes revealed an increase in IFN-γ production. The results of cytotoxicity on 4 T1 cells show an increase in the mortality rate with respect to the control cell line. The rate of apoptosis induced by Poliglusam nanoparticles on 4 T1 mouse breast cancer cell line is about 45% higher compared to MCF-7 human cells line, revealing the natural tendency of Poliglusam in increasing the production of IFN-γ in cancer cells. At the state-of-art of the knowledge, very few information have been achieved on the immunological effects of Poliglusam. This work is one of the first studies for the identification of non-functionalized Poliglusam nanoparticles impact on breast cancer. Thus, their immunotherapeutic effect, combined with an anticancer drug, can be employed as potential effective drug for eliminating breast cancer cells in the future.

Keywords Poliglusam · Nanoparticles · Breast cancer · Immunotherapy and apoptosis

Introduction

Breast cancer is one of the most important metastatic cancers in women and is the second most common tumor after lung cancer worldwide, causing 450,000 of annual deaths [17]. According to the seriousness of the malignancy, the tumor could be treated in situ or by the use of more invasive techniques, such as surgery, radiotherapy, and drug-treatment [23]. Nevertheless, these methods usually provide a decrease in the tumor size but cancer elimination is still the major challenge. In addition, the current therapies are largely dependent on the tumor stage and are based on the assumption that the cancer is composed by homogeneous population [24] of target cells with rapid proliferation and differentiation [4]. To overcome the limitations that affect the current techniques, new cancer treatments have been explored during the last decades, including hormonal, gene and immune-therapy [15].

Although several methodologies are increasing the opportunities to defeat the malignancy, none of these techniques is completely efficient. According to the authors, one of the most promising direction to improve cancer therapy might involve the strengthening of the immune system. Polymer–drug conjugates are one of the most effective and investigated types of nano-carriers among the clinical methods to deliver cancer drugs. Several polymeric nanoparticles are now at different stages of preclinical and clinical development. Among these, Poliglusam nanoparticles have been reported as potential candidate thanks to their non-toxic, abundant, renewable and biodegradable nature [18, 11]. Moreover, the presence of positive charges caused by Poliglusam protonation, increases the possibility to permeate the cell membrane [6]. In the past, they have been applied in the pharmaceutical industry [1] for biological purposes as antimicrobial and antioxidant agents. In

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this study, the evaluation of immuno-enhancing effects and antitumoral proprieties of Poliglusam nanoparticles (Chitosan) tested in a mouse breast cancer model is presented.

Materials and Methods

Ethics Statement

Animal maintenance and protocols were approved and performed in accordance with the regulation provided by the Animal Ethics Committee of Tarbiat Modares University of Medical Sciences as well as the United States NIH guidelines. All surgeries were performed under deep anesthesia and all efforts were made to minimize suffering.

Characterization of Pol Nanoparticles

Preparation of Low-Molecular-Weight Poliglusam

Depolymerization of Poliglusam was described according to the method by Moghaddam et al. [16, 27].

Preparation of Poliglusam Nanoparticles

Poliglusam were prepared according to the ionic gelation method reported by Calvo et al [3, 5]. Briefly, a Pol solution (0.1% *w*/*v*) was obtained by dissolving low-molecular-weight Pol in 0.5% *v*/v acetic acid. A solution of Pol (1.0 mg/mL) was prepared in distilled water. Pol nanoparticles were prepared spontaneously upon addition of various concentrations of sodium tripolyphosphate (TPP) (0.015%, 0.020%, 0.025%, and 0.030% *w*/*v*) to the Pol solution under gentle magnetic stirring at room temperature for one hour. In all cases, the volume ratio of Pol:TPP solution were 2:1. Lately, the opaque suspension was assigned to the formation of nanoparticles. The Pol nanoparticles were separated from the aqueous medium by Amicon Ultra-15 centrifugal filters (Millipore, Billerica, MA) at 4 °C for 30 min. After their removal from the filter, they were resuspended in 2 mL of deionized water. Finally, the suspension was freeze-dried and stored at 4 °C until use.

Morphology of Nanoparticles

Measurement of Particle Size, Polydispersity and Zeta Potential Poliglusam nanoparticles mean size, polydispersity, and zeta potential were measured as described previously [20] by dynamic light scattering (DLS) and laser Doppler electrophoresis using a Zetasizer Instrument (Nano-ZS, Malvern, Worcestershire, UK). All data measurements were performed at a wavelength of 633 nm at 25 °C with a detection angle of 90°. Each sample was measured three times.

Scanning Electron Microscopy (SEM) Surface morphology of the prepared nanoparticles was studied using SEM (MIRA TESCAN, Czechia). Poliglusam nanoparticles were dried on an aluminum disk at room temperature and nanoparticle-treated and untreated cells were fixed with gold using a sputter coater (SCD 005; Bal-Tec, Balzers, Liechtenstein).

Cell Line and Cell Culture

Mice and human breast tumor cells were obtained from the Pasteur Institute (Tehran, Iran). Cells were grown at 37 °C in an atmosphere of 5% CO2, 95% air. MCF-7 cells were cultured in DMEM F12 supplemented with 10% fetal bovine serum (FBS). 4 T1 cell lines were cultured from RPMI 1640 medium. MCF-10A cells were grown in DMEM F12 supplemented with insulin 10 mg/ml, hydrocortizone 1 mg/ml, EGF 100 mg/ml and 5% horse serum [2].

Cytotoxicity Assay

3-(4, 5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide blue-indicator dye (MTT) -based assay was used to measure cell cytotoxicity. 4 T1 cells were seeded in clear 96-well plates at a density of 7500 cells/well and 10,000 cells/well of MCF-7 and MCF-10A. Different concentrations of Poliglusam (3.65, 7, 15, 30, 60, 125 and 500 μg) were added to each well and only culture medium was used as negative control. The cell number was evaluated using the MTT cell proliferation assay [28]. The absorbance was measured at 570 nm on a microplate spectrophotometer reader (Benchmark, Bio-Rad, Hercules, CA, USA). All the compounds were evaluated in three independent experiments, with eight triplicates per each experiment.

Viability of Cells

After their collection, viable and non-viable cells were counted on a hemocytometer plate under a microscope by a solution of 0.4% of Trypan blue (Sigma-Aldrich) diluted in PBS. The percentage of mortality was determined after repeating the experiment three times [19].

Apoptosis Assay

Annexin V-FITC was used as a marker of phosphatidylserine exposure and propidium iodide (PI) as a marker for dead cells (APOAF Annexin V-FITC Apoptosis Detection Kit; Sigma-Aldrich). The assay was performed on 4 T1, MCF-7 and

MCF-10A cell lines. Cells were seeded and treated with the indicated compounds or solvent as for the cell death analysis. After 24 h of treatment, cells were harvested by centrifugation (1100 rpm, 10 min) and aliquots of 5×10^5 cells were washed with PBS and resuspended in 500 μl of binding buffer, provided with the kit. A volume of 2.5 μl of Annexin V-FITC and 2 μl of PI were added and cells were incubated at room temperature in the dark for 30 min. Approximately 20,000 cells were analyzed by flow cytometry using a Cytomics FC 500 flow cytometer with CXP software (Beckman Coulter). The percentage of cells in each category was determined. The experiments were performed twice and gave similar results [5, 12].

In Vivo Antitumor Efficacy of Poliglusam Nanoparticles in Mice

BALB/c inbred female mice (Pasteur Institute, Iran) were used housed tow to a cage, with access to autoclaved standard mouse chow at libitum. All animals received humane care. The animal protocol was reviewed and approved by the Animal Care and Research Committee of the Tarbiat Modares University.

Breast Cancer Model

Seven-week-old, BALB/c mice were transplanted in the left flank with 7×10^5–10^6 cells of 4 T1 breast cancer cell line (Pasteur Institute, Tehran, Iran). The tumors developed on the 10th day, after which we started the therapy at the 15th day. Tumor volume was measured using a digital Vernier caliper every day (Mitutoyo, Japan). The animals were sacrificed at the 30th day. All of spleens and tumors of mouse were separated for immunological and molecular test.

Mononuclear Cell (MNC) Separation

Tumor-bearing mice were treated with Poliglusam nanoparticle and PBS as control for 30 consecutive days. On day 30, after tumor transplantation, mice were sacrificed by cervical dislocation and spleen was resected. Spleen was removed under sterile conditions and suspended in PBS containing 2% FBS. Red blood cells (RBCs) were lysed with lysis buffer. Mononuclear cell suspension was prepared and adjusted to 3×10^6 cells/ml in RPMI 1640 (Gibco) supplemented with 5% FCS, 4 mM L-glutamine, 25 mM HEPES, 0.1 mM non-essential amino acid, 1 mM sodium pyrovate. The number of isolated cells from the spleen was in a similar range among different samples (3–5×10^6 cell/ml), and the viability of cells was an average of 95% according to Trypan blue staining.

Tumor Antigen Preparation

Tumors cell line suspension was washed with saline and then subjected to three rounds of freezing (−170 °C) and thawing (37 °C). In order to inhibit serine proteases, 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to the cell lysates. Finally, the extract was dialyzed and filtered. The protein concentration of the extract was determined using spectrophotometry method and then stored at −20 °C until use.

Splenocytes Cytokine Assay

Isolated spleen MNCs were cultured in 24-well plates (Nunc, Denmark). 20 μl of purified tumor antigen was added to each well and the plates were centrifuged after 72 h (Eppendorf 300 g, 10 min). Lately, the supernatants were collected. IFN-γ concentration was measured using R&D DuoSet ELISA Development kit, according to the manufacturers' protocols. Each sample was analyzed in duplicates.

Immuno-Histological Examination

Histological examinations included TUNEL assays using a commercial apoptosis detection kit (In Situ Cell Death Detection kit (ISCDD), Roche, Germany) with the following minor modifications to the manufacturer's instructions. Samples were fixed in 4% (*v*/v) paraformaldehyde for 10 min at room temperature. The samples were then washed with PBS twice, for 5 min each time, and incubated with 0.2% (v/v) Triton X-100 for 15 min at room temperature. After the samples were washed twice more with PBS, for 5 min per time, samples were incubated with buffer from the In Situ Cell Death Detection (ISCDD) kit for 10 min at room temperature. The equilibration buffer was drained and the reaction buffer, containing equilibration buffer, nucleotide mix and TdT enzyme, was added to the tissue sections, incubated in a dark humid atmosphere at 37 °C for 1 h. The reaction was stopped soaking the samples in standard saline citrate for 15 min. Lately, they were washed three times for 5 min each to remove unincorporated fluorescein- dUTP. TUNEL data were analyzed by a researcher blind to the nature and dose of the drug, at 100X magnification, using a fluorescent microscope.

Statistical Analysis

Results are expressed as the mean ± SD (standard deviation). The statistical significance of differences between treatment groups was evaluated by a Student's *t* test for unpaired observations or a Chi-test using the Analysis Toolpak of Microsoft Excel. In all analyses, differences with $p < 0.05$ were considered significant.

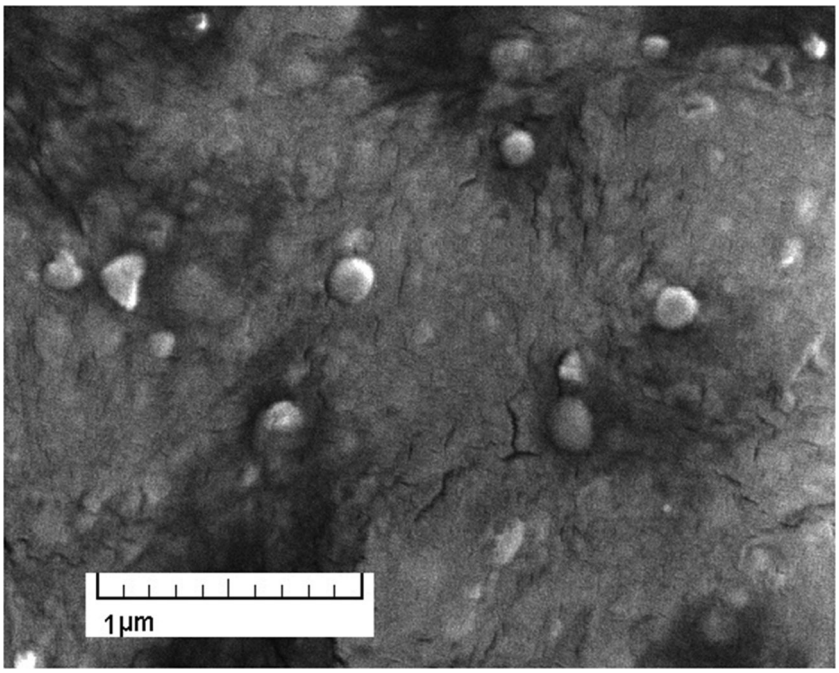

Fig. 1 Poliglusam nanoparticle observed by SEM. The scale bar of 1 μm is reported at the bottom left-hand corner of the panel

Results

Poliglusam Characterization

Nanoparticle size was checked by both DLS and electron microscopy, confirming that the diameter was in the range of 200 ± 10 nm (Fig. 1). The main parameters affecting the size of nanoparticles are the concentrations of Poliglusam and TPP [9]. The dimension of the nanoparticles increases at higher concentration of TPP (over 0.03% *w/v*), since it behaves as a linker involved in the growth and aggregation of particles. Indeed, at concentration lower than 0.02% w/v, no nanoparticle has been detected, as already reported by Calvo et al. Moreover, the electric charge of the nanoparticles is also affected by TPP, since its negative charged group can interact with positively charged amino groups, modifying the nanoparticles surface density [3].

Cytotoxicity Assay

To verify how Poliglusam affect 4 T1 cell line, a MTT assay was performed for 24 h. Analytical statistic test suggests that tumor cell viability depends on the concentration of Poliglusam nanoparticles and on there incubation time in cell culture. The assay reported that the highest risk of toxicity is verified at concentration of nanoparticles higher than 250 μg/mL, with IC50 (p <0.001). Furthermore, the data report a significance difference in cell viability during the treatment with different doses of Poliglusam nanoparticles (results are reported in Fig. 2). For instance, Cells treated with 62 μg/mL of Poliglusam, were found dead compared to the control group (viability by 97%). In order to confirm the study of cell viability, 4 T1 cells were exposed to Trypan blue staining and comparable results with MTT assay were reported, proving the efficiency of Poliglusam nanoparticles

Cell Apoptosis

According to the results of the MTT test, a specific concentration IC50 of Poliglusam was used for flow-cytometry test. The results showed that cells treated with the nanoparticles present a significant increase in the number of necrosis and apoptosis cells compared to no-treated ones (control group). Figure 3a shows that the 30.4% of the 4 T1 cells, treated with Poliglusam, died of apoptosis, whilst 22.7% of necrosis, compared to the control group. The same experiment was performed in MCF-7 human cell line, reporting 45% and 12.3% of apoptosis and necrosis rate, respectively, compared to the control group (Fig. 3b). On the contrary, MCF-10A normal breast cells line is resistant to Poliglusam treatment (Fig. 3c), confirming that nanoparticles are only effective on cancer cells and do not damage the healthy ones. Cytotoxicity operates on the cancer cells by inducing apoptosis, as revealed by Annexin staining. In a similar way, some other death events may take place (i.e. autophagy), but they have not been taken into account in this study.

Immuno-Histological Examination (TUNEL Assay)

Effects of Poliglusam antitumoral effect was investigated on 4 T1 breast tumor cells in mice Balb/C. TUNEL Immuno-histo Fluorescence Method (TIM) was used to analyze

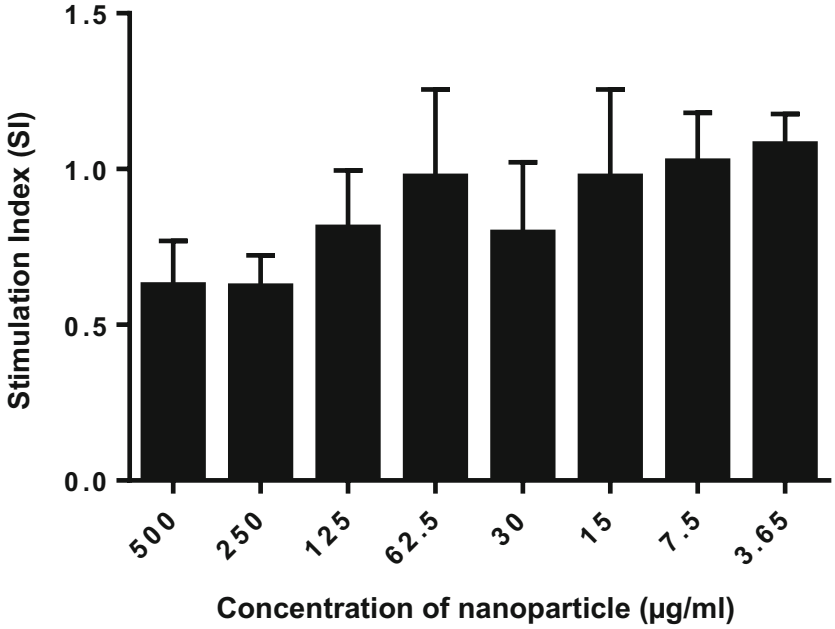

Fig. 2 Cytotoxic effects of Poliglusam in 4 T1 cells, cultured with different concentration (from 3.65 to 500 µg/mL) of Poliglusam nanoparticles for 24 h. On the x-axis, the concentration of the Poliglusam nanoparticles is reported in decreasing order. On the y-axis, the stimulation index of each treatment is reported

apoptosis in tumor tissue. Positive Cells to apoptosis are recognized by green fluorescence (Fig. 4A) using Fluorescein isothyocianate (FITC). 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI, blue stain) is used to check the location of nucleus (Fig. 4B), in order to reveals the nature of apoptosis induced by Poliglusam on 4 T1 breast tumor cells.

Hematoxylin and Eosin (HE) staining were used to identify the apoptotic area in tumor cells of treated mice and confirmed the occurrence of cell death. Furthermore, samples from other mice tissues (lung, liver and kidney) were also analyzed without revealing the diffusion of cancer cells in different area of the organism, excluding both the development of metastasis and tumor contamination (data not shown).

Splenocytes Cytokine Assay

The cytokine pattern of splenocytes in tumor-bearing animals treated with Poliglusam, was evaluated and compared to control (as described in the paragraph 2.11). Results revealed that the increase in IFN-γ production appears statistically significant compared to the control. On the other side, no differences were observed between the treated groups (*p* value 0.04, 0.08 and 0.7 for mice treated with standard kit, respectively) (Fig. 5).

Discussion

During the last few years, particular special attention has been paid to the development of new anticancer therapeutical strategies. Several nano-carries have been tested and already reported in literature [19]; among the novel nano-systems for clinical and biomedical application, Poliglusam nanoparticles have been considered an innovative delivering system and their efficiency in drug-release has been already investigated [26]. In this study, we propose Poliglusam as a specific immunogenic treatment in cancer breast cells. Thanks to their similarity to human mammary carcinoma cells, 4 T1 breast cancer cell line was developed in BALB/c mice and used as a model for human breast cancer. The tumor cell line spontaneously spread to various distant organs by the time the primary tumor is palpable. Pulmonary metastasis are the major evidence of their migration, inducing the death of 4 T1-bearing mice. For these reasons, 4 T1 in the immune-competent and syngeneic BALB/c mouse is recognized as the most challenging breast tumor model for the evaluation of the efficacy of novel immunotherapy, and for the determination of the immuno-stimulation. As demonstrated by the cytokine pattern in splenocytes, Poliglusam nanoparticles induce an increase in IFN-γ production in cellular immune activity, confirming its protective role in tumor onset. It plays an anti-proliferating role, which induces cell death by apoptosis in a large number of tumor cells and is able to activate the signal transducer and activators of transcription, STAT1 [7, 10]. It is produced in stimulated T-cells and enhances the shift to a protective Th1 pattern, which induces tumor immunity [8, 13, 14, 21]. The balance between the production of Th1 and Th2 cytokines can determine the direction and the consequences of an immune response system. In fact, IFN-γ can prevent the spread of tumors with the inhibition of angiogenesis, causing the induction of Chemokine 10 (CXCL10) secretion and Monokine MIG as a response to interferon γ production, which has role as containment vessel of the tumor [22]. Moreover, IFN-γ can induce the increase of

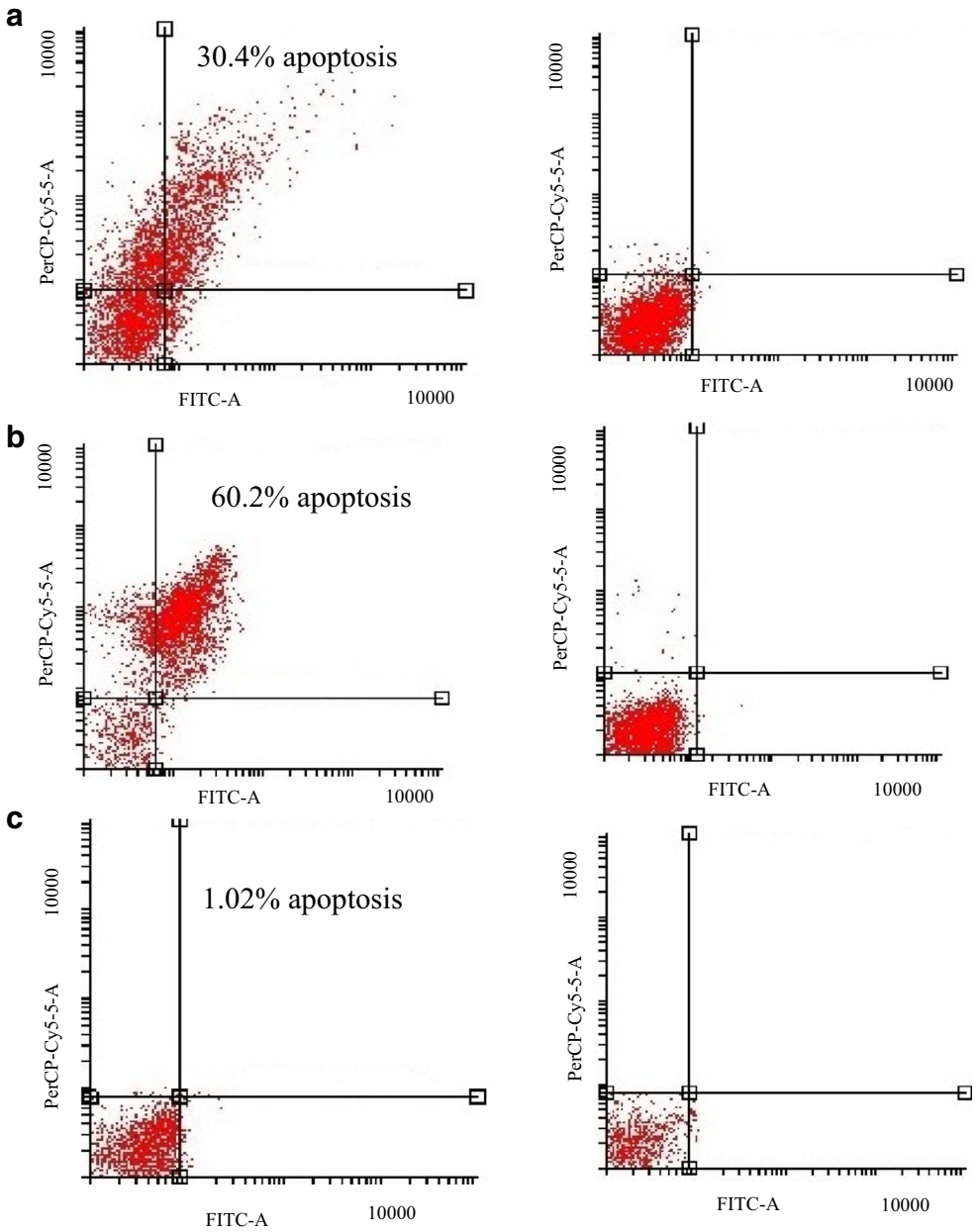

Fig. 3 Flow-cytometric monitoring of apoptosis (Annexin V+, PI−) and necrosis (Annexin V+, PI+) in cell treatment by nanoparticle in vitro for 24 h (FITC-A: Annexin V, perCP-Cy5–5-A: PI). **a**) 4 T1 cell line; **b**) flow cytometry of MCF-7 human cells; **c**) MCF-10A normal breast cell line treated with Poliglusam nanoparticles (Poliglusam) and without treatment (control group)

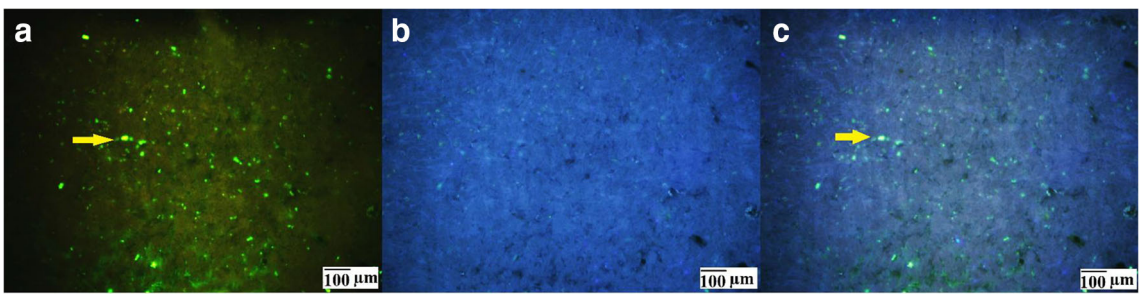

Fig. 4 TUNEL Assay: The level of staining indicates the degree of DNA damage induced by nanoparticle treatment in tumor tissue. The more positively stained cells are in the final stages of apoptosis. **a**) Green fluorescence is due to FITC staining. **b**) DAPI staining for cytoplasm is observed blue color. **c**) Marge of A and B. Magnification: 100 ×

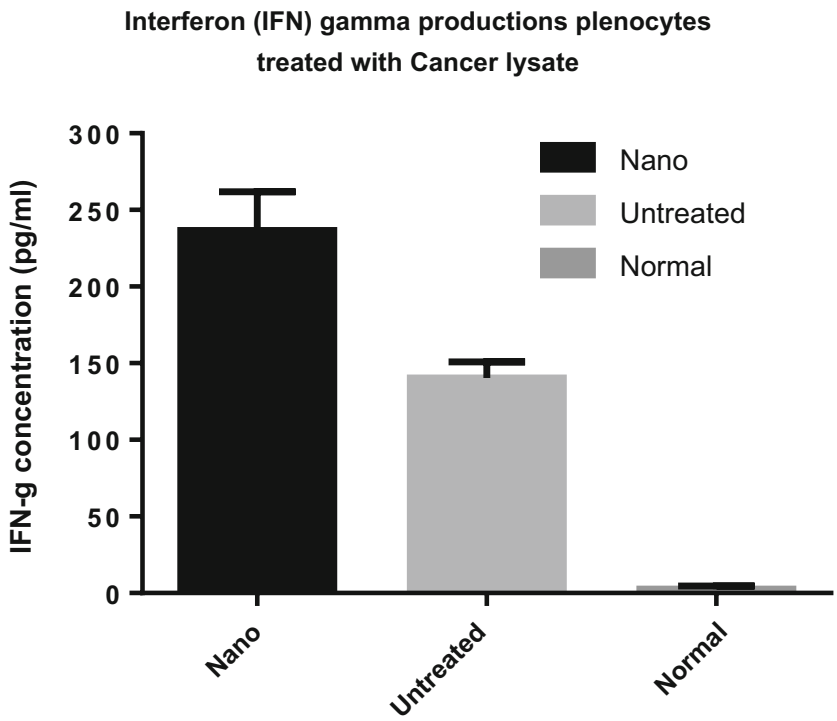

Fig. 5 The level of IFN- γ production in different groups of mice under study ($p > 0.05$)

phagocytes activity by enabling macrophages [25]. The TUNEL assay clearly confirms the results of cytokine assay, revealing an apoptotic pathway that enables cell death by systematic effect and stimulation of immune system, induced by Poliglusam. To conclude, our findings suggest that Poliglusam nanoparticles might play a role in attenuating tumor growth by altering the cytokine milieu and anti-tumoral cell activation during 4 T1 cell carcinogenesis.

Conclusion

It is possible to speculate that Poliglusam nanoparticles and derivatives could be used as an adjuvant treatment during anticancer chemotherapy. Thus, the Poliglusam nanoparticles from immunotherapy combined with an anticancer drug can be employed as a potential factor in the treatment of breast cancer in the future.

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Author Contributions NS conceived the study. NS conducted the experiments and analyzed the results. NS and AV drafted the manuscript and made substantial contributions to the design of the study. NS and AV critically reviewed the manuscript and participated in data analysis. All the authors studied and approved the final manuscript.

Compliance with Ethical Standards

Ethical Issues None to be declared.

Competing Interests The authors declare that there are no conflicts of interest.

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