How to Cite:
Mannoush, S. H., Jabir, M. S., & Thaker, A. A. (2022). Gold and silica gold nanoparticles enhances macrophages kill tumor cells via granzyme-perforin pathway. *International Journal of Health Sciences*, *6*(S6), 8732–8745.
https://doi.org/10.53730/ijhs.v6nS6.12272

Gold and silica gold nanoparticles enhances macrophages kill tumor cells via granzyme: Perforin pathway

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Abstract---The goal of the study was to see if gold nanoparticles (GNPs) and silica gold nanoparticles (Si-GNPs) could help cancer cells phagocytes. Scanning electron microscopy (SEM) and Ultraviolet-visible spectroscopy (UV-VIS) absorptions were used to characterize the nanomaterial's. In both in vitro and in vivo conditions, the GNPs and Si-GNPs enhanced macrophage phagocytosis capabilities, resulting in the annihilation of cancer cells. The effect of GNPs and Si-GNPs in Tumor necrosis factor alpha (TNF-α) and Interleukin-8 (IL-8) levels was investigated in Ehrlich tumor cells using Enzyme linked immunosorbent assay (ELISA) assay. The results shows the ability of GNPs and Si-GNPs to increase TNF-α and IL-8 in treated Ehrlich Ascites Carcinoma (EAC). It was found that the most effect was in Si-GNPs group. GNPs and Si-GNPs enhance phagocytic cells for increased granzyme and perforin-mediated cancer cell death on a regular basis. Overall, the findings showed that GNPs and Si-GNPs improved phagocytic cell activation, which might be employed as a promising technique for managing cancer cells by enhancing cell death via a Granzyme-perforin dependent mechanism.

Keywords---GNPs, Si-GNPs, phagocytosis, SKOV-3, EAC, granzyme, perforin.

Introduction

Tumor cell death triggered by various substances can stimulate the immune system and trigger an Ag-specific response against the tumor, which is referred to as immunogenic cell death. Chemotherapeutic medicines, Ab therapy, irradiation, and repeated freeze–thaw cycles are all used to induce immunogenic cell death [1]. Dead cells are primarily cleared in vivo by macrophages, although their engulfment by dendritic cells (DC) and subsequent Ag processing is crucial for cellular Ag cross-presentation. According to in vitro and in vivo studies, the CD8a+ DC fraction plays a critical role in phagocytosis and cross-presentation of cell-associated Ags to activate a particular CTL response [2-5]. Macrophages are a type of leukocyte that are formed when monocytes in tissues differentiate. They can be found in almost all tissues [6]. Macrophages are important regulators of an organism's host defense. Resting macrophages (M0) can be polarized into pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes in response to diverse local microenvironments and play different roles in different physiological or pathological circumstances.

Polarized macrophages can also be reprogrammed by reversing their phenotype in response to changes in the environment. Macrophage polarization and reprogramming are engaged in the processes of many diseases and play an important role in maintaining the immune system's stable state [7]. In processes known as phagocytosis, pinocytosis, or receptor-mediated endocytosis, macrophages consume and digest cellular debris, foreign substances, bacteria, and cancer cells [8]. Macrophages are crucial members of the innate immune system because they not only serve an innate defense role through phagocytosis, but they also initiate adaptive immunity by recruiting and activating other immune cells such as lymphocytes through a process known as antigen presentation [9]. Macrophages play crucial pro- and anti-inflammatory roles through the secretion of cytokines, in addition to boosting the immune system [10]. M1 macrophages secrete pro-inflammatory mediators like tumor necrosis factor (TNF-), interleukin 6 (IL-6) and IL-12 to promote inflammation, whereas M2 macrophages secrete anti-inflammatory cytokines like transforming growth factor (TGF) and IL-10 to reduce inflammation and promote tissue healing [11].

The polarization and reprogramming phases of these cells are crucial to their function. In response to diverse micro-milieu settings, M0 macrophages can polarize into pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages. A reversal of the phenotypic, on the other hand, can result in the reprogramming of an already polarized cell [12]. Immune regulatory, inflammatory, and proliferative capabilities, as well as cellular metabolism and tissue remodeling, are among the most important functions of macrophages of various polarization states. For example, M1 macrophages, for example, are important effector cells in the resistance to intracellular infections and tumor progression [13]. Nanoparticles based on gold chemistry have recently gained a lot of scientific and practical attention. GNPs are versatile agents that may be used in a variety of biomedical applications, including very sensitive analytical testing, ablation heat and radiotherapy development, and drug and gene transfer.

For biomedical applications, gold nanoparticles (GNPs) must be externally functionalized in order to be targeted to specific disease areas and interact selectively with cells or biomolecules. The resulting GNPs have distinctive characteristics like shape, size, electrical properties, a high surface area to quantity ratio, and surfaces that may be easily changed with ligands containing functional groups such as thiols, phosphines, and amines, which have affinity for gold surfaces, This is a common misunderstanding [14]. The cellular uptake of gold nanoparticles (GNPs) was also reported [15], with significant effects on cell proliferation activity in ovarian cancer cells, including SKOV3 and others, OVCAR5, OVCAR8, and others. The pristine carbon quantum dots and their metal composite are also known to target cytokines, metalloproteinase, and the cytoskeleton in ovarian cancer SKOV3 cells [16].

With substantial study in oncology, GNPs have attracted the most attention in biological applications. As a result, GNPs have emerged as a promising research topic in cancer theranostics. Due to their unique physicochemical features, GNPs have been studied for a variety of cancer-related applications, including gene therapy, targeted drug administration, radiation tumor detection, and cellular bio imaging [17,18]. Silicon, or Si, is a chemical element that can be found in the Earth's crust. Silicate (SiO4) and silica are its oxide forms (silicon dioxide, SiO2). Si is frequently employed in industry, and its oxide forms are frequently used in biological applications. SiNPs have a number of unique characteristics, including being easily manufactured, having a changeable surface, having strong mechanical capabilities, and having a chemical composition that is generally innocuous. For decades, they've been employed as biomaterials. Silica comes in two fundamental forms: crystalline and amorphous.

The molecular formula is identical in both versions [19]. The role of the granzyme and its T-cell mediated releases has been studied in tumor-bearing responsive and muted-response mouse models [20]. CD68 is a glycoprotein intracellular membrane known by other names such as Macrosialin and KP1. It has a molecular weight of 110 kDa and belongs to the family of conjugated membrane proteins that regulate body condition due to its intracellular location, primarily within cytoplasmic granules such as endosomes and lysosomes, as well as structural properties [21]. CD68 is the traditional immunohistochemical marker for macrophages and mononuclear cells, but it has recently been identified as a surface marker for neutrophils. In pluripotent T cells, natural killer cells, and non-blood tissues such as the liver and renal tubules [22]. In addition to its expression in many types of tumor cells, this protein allows these cells to adhere to selectins and lectins molecules of vascular endothelial cells, which facilitates tumor cells to spread to secondary sites, Al-Saadi (2014) pointed out the important role of CD68 protein in the formation of tumors and inflammation as an initiating factor for inflammation in colorectal cancer.

It can also be considered as a prognostic factor for the progression of some types of tumors, such as the squamous cell carcinoma of the head and neck, where large macrophages (CD68 +) were seen infiltrating and associated with the transformation from normal epithelial tissue to neoplastic tissue [23]. Tumor necrosis factors (TNF family) are a group of cytokines produced mostly by macrophages and capable of causing hemorrhagic necrosis in certain tumor cell

types. Tumor Necrosis Factor Alpha (TNF Alpha) and Tumor Necrosis Factor-beta (TNF-), also known as Lymphotoxin-alpha), were the first two members of the family to be identified [24]. TNF-alpha, also known as TNF, TNFA, or TNFSF2, is a multifunctional cytokine involved in the control of a wide range of biological processes, including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. TNF-R1 (TNF receptor type 1, CD120a; p55/60) and TNF-R2 (TNF receptor type 2, CD120b; p75/80) are two TNF-alpha receptors that bind to TNF-alpha. TNF-alpha is mostly generated by macrophages, TNF-alpha plays a role in carcinogenesis and is hence recognized as a molecular insight in cancer treatment [25]. Interleukin-8 is a pro-inflammatory cytokine that appears to recruit and activate circulating and tissue neutrophils to the site of tissue injury in infection and inflammation.

Macrophages and other cell types, such as vascular endothelium, produce IL-8, which can be secreted by any cell having toll-like receptors, which are implicated in the innate immune response [26]. IL-8 is produced by endothelial cells at the inflammatory site and is responsible for guiding activated leukocytes to the inflammatory site. By changing the production of adhesion molecules, IL-8 makes it easier for activated leukocytes to traverse the endothelium. The 72 amino acid IL-8 variation inhibits leukocyte adherence to activated endothelium cells considerably more effectively than the 77 amino acid IL-8 version. The interaction of IL-8 with particular receptors, CXCR1 and CXCR2, expressed on neutrophils and T cells, causes antigenic effects. GCP2 and IL-8 stimulate CXCR1 (granulocyte chemotactic protein-2). CXCR2 binds to IL8, NAP1 (neutrophil attractant/activation protein-1) and other proteins [27]. One important chemokine that plays a vital role in most inflammatory pathways is interleukin (IL)-8 [28]. When neutrophils arrive at the site of inflammation, IL-8 induces them to carry out phagocytosis, which improves tissue healing efficiency [29]. IL-8 gene expression in human ovarian cancer subtypes [30]. The goal of this study was to assess phagocytic cells' anti-cancer efficacy, which was mediated by GNPs and silica - GNPs. This research shows that GNPs and silica - GNPs have a potent tumoricidal effect on cancer cells via a granzyme-perforin dependent mechanism.

Materials and Methods

Characterization and preparation of GNPs by UV-Spectrophotometer analysis

GNPs and Si-GNPs were supplied from Sigma-USA. The samples were prepared and scanned using a UV spectrophotometer with absorbance range of 200-1000 nm as mentioned by [31].

Characterization and preparation of GNPs by Scanning Electron Microscopy

By prepared a thin film of the sample on a cover slide by dropping only 5 µL on the cover slide, then the film allowed to dry by putting it at room temperature for a period and then subjected to examination on the SEM as described by [32].

Experimental animals

In this study, white mice obtained from the animal house of the Iraqi Center for Cancer Research. They are adult males, aged 6–8 weeks. All animals were housed in individual cages intended for changing sawdust and feed, and were divided into four groups, each including six mice in identical cages, with free access to water and diet. In addition, the temperature in the animal house was kept between 20 - 30 ℃ and light: dark period of 12 hrs. As mentioned by [33].

Cell line

The cell bank unit, Department of experimental therapy, Iraqi Center for Cancer and Medical Genetic Research, Mustansiriyah University, Baghdad, Iraq, supplied the SKOV3 ovarian cancer cell line. The cells were maintained in 10% fetal bovine serum RPMI-1640 supplemented with 100 units/ml of antibiotic solution (penicillin, and 100 g/mL streptomycin). Cells were subcultured by trypsinization using trypsin-EDTA solution 0.25% (US Biological, USA) once a week and incubated in a CO2 incubator at 37°C [34].

Macrophages Derived from Bone Marrow

Male albino mice (6–8 weeks old) were used to isolate primary bone marrow-derived macrophages (BMDMs) [35].

Isolation of splenic macrophage

Ten-week-old mice were immunized with the adjuvant containing killed tuberculosis germs. After 7 days, phagocytic cells were isolated from the spleen using Histopaque-1083 and incubated in plastic dishes for 1 hr. at 37°C. Then, adherent cells were collected for next experiment.

In Vivo Tumoricidal Activity of Macrophage Cell

The tumoricidal activity of macrophage in the peritoneal cavity was done according to [36]. briefly, GNPs, Silica-GNPs was given at a dose of 20 µg/mice for three days. Ehrlich cancer cells were intraperitoneal injected in mice at (2×10^6 cells). The negative group was injected with 200 µL of PBS, and the positive control was injected with Ehrlich cancer cells at a concentration of 2×10^6 cells. On day 14, mice were sacrificed and 3 mL of sterile saline was injected into the external abdomen region. Following the collection of peritoneal cells, they were fixed with 4% paraformaldehyde and stained with Giemsa stain [37].

The phagocytic index was measured by the equation below:

$$\text{Phagocytic index\%} = \frac{\text{phagocytic cells}}{\text{Phagocytic + non- phagocytic cells}}$$

Blood samples

Using cardiac heart puncture, blood samples were taken directly from the heart. Approximately 1–1.5 mL of blood was taken and deposited in special anticoagulant-free test tubes, which were left at room temperature for about a quarter of an hour before the serum was swiftly separated using a centrifuge. For 3000 r/min for 5 minutes, then store the serum in clean new plastic tubes at -20°C until the ELISA tests are done.

ELISA assay

Three groups mice were used, three mice for each group. The first group Ehrlich cancer cells are injected intraperitoneal of mice at a dose of 25 µg/mice. The second group Ehrlich cancer cells (25 µg/mice) with GNPs (20µg/mice) injected intraperitoneal. The third group 25 µg/mice from Ehrlich cancer cells with 20µg/mice Si-GNPs injected intraperitoneal. It must be mentioned that other group of mice each containing three mice also, as negative control was injected with 250µl PBS, Injections three times a week for two weeks then the blood is taken from the heart and collected in tubes, and then the serum is taken in a new tube store -80 ℃ for future use. The supernatant produce was assessed by using mouse cytokine IL-8 and TNF-α assay kits, Optical densities (OD) were measured with a microplate reader set at 450 nm [38].

In Vitro Tumoricidal Activity of Macrophage Cell
Immunofluorescent staining

In this assay, splenic macrophages cells were treated and then fixed using 4% formaldehyde for 30 minutes at 4°C. Then, cells were stained with primary antibody anti-mouse CD68 Ab and goat anti-serum for granzyme B (N-19) at concentration 1µg/ml in 4°C overnight. Cells were washed three times using FPS for removing the unbound primary antibody. Then, the cells were treated with secondary antibody Alexa 488-labeled anti-mouse IgG and Alexa 568-labeled anti-goat IgG Abs (Invitrogen Life Technologies) at concentration 2 µg/ml at room temperature for 2 hrs. The results of were detected using fluorescent confocal microscope.

Statistical analysis

Graph-Pad Prism was applied to analyze the data (three replicates). The results are represented as mean ± SD Differences were regarded as significant at $p \leq 0.05$ [39].

Results and Discussion

Characterization of Gold Nanoparticles

The GNPs and Si-GNPs were confirmed using a UV-visible spectrophotometer (Figure 1) depicts the picture of gold nanoparticles acquired from UV-spectrum. In a UV-spectrum test, the peak of GNPs was identified at roughly 530 nm. The SEM image exhibits that the morphology of the GNPs is the regular spherical

shape with smooth surface and the particles size were about 10-20 nm as seen in (Figure 2).

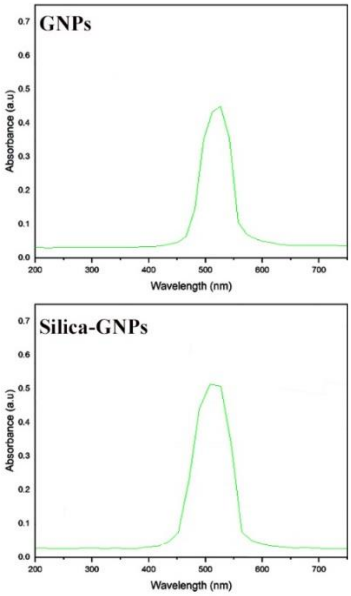

Figure 1. Uv-spectrum of GNPs, and Si-GNPs

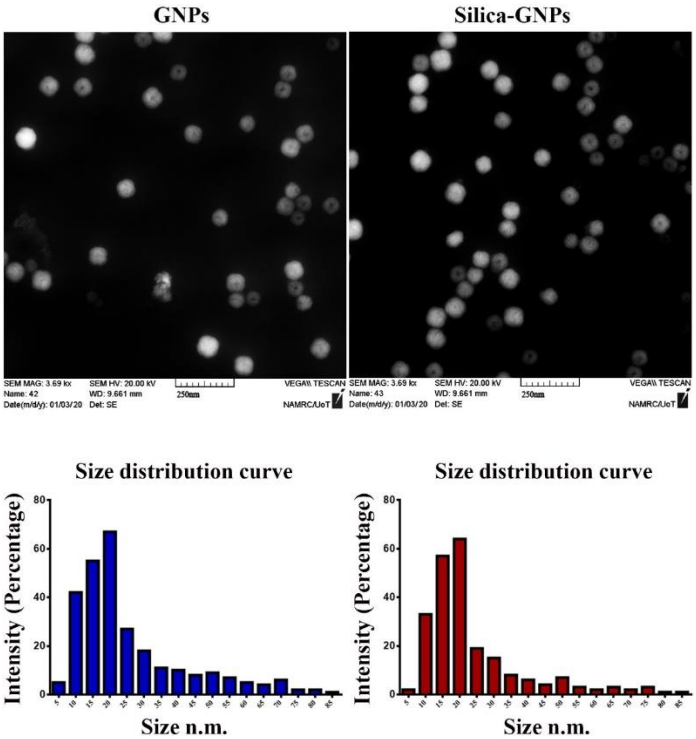

Figure 2. SEM images of GNPs and Si-GNPs

GNPs, Si- GNPs increases tumoricidal activity of macrophage

An animal model was used to confirm the tumoricidal activity. Figure 3 and 4 the distribution of peritoneal macrophages which are pre-treated with GNPs, Si-GNPs containing many cytoplasmic vacuoles. The peritoneal macrophages, were showed changed in size. Pre-treated with GNPs and Si-GNPs, induced the biological, physiological, and functional activities of Bone marrow-derived macrophage (BMDMs) against in Ehrlich ascites tumor cells. The findings of this study revealed that using GNPS and Si-GNPs as phagocytosis inducer materials can be the most powerful, effector, and anticancer strategy, In vivo model as in (Figure 3). The capacity of LPS and GNPS to activate phagocytic cells could be linked to their ability to produce and enhance reactive oxygen species release (ROS). ROS can enter mitochondria, causing macrophage activation as well as the generation and stimulation of metabolic and inflammatory processes [39].

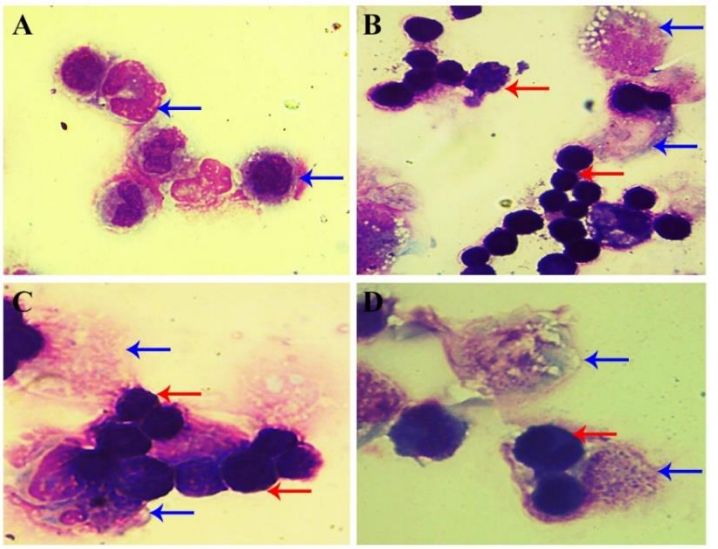

Figure 3. GNPs and Silica-GNPs increased tumoricidal *in vivo*. (A) Control peritoneal macrophages. (B) Peritoneal macrophages + Ehrlich cancer cells). (C) Peritoneal macrophages + Ehrlich cancer cells + GNPs (Dose: 20 mg/Kg). (D) Peritoneal macrophages + Ehrlich cancer cells + Silica-GNPs at concentration 20 mg/Kg. The Blue arrow represents macrophage cells and the Red arrow represents Ehrlich ascites tumor cells. Magnification power 100x.

In the presence of GNPs and Si-GNPs as enhancers for phagocytic cells, macrophage increased their phagocytosis of Ehrlich tumor cell. The phagocytic index of macrophage was then determined in the presence and absence of GNPs, and Si-GNPs was calculated (Figure 4). The Macrophages showed more potent phagocytosis of Ehrlich cancer cells in the presence of nanoparticle materials.

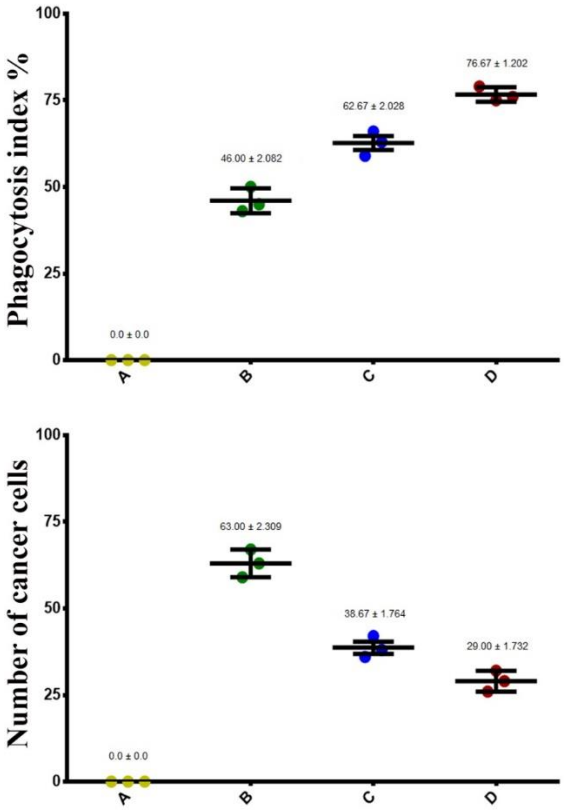

Figure 4. GNPs and Silica-GNPs augmented phagocytic index and reduced the number of tumor cells in the peritoneal cavity. (A), Control peritoneal macrophages (B), Macrophages cells + Ehrlich tumor cells. (C) macrophages + Ehrlich tumor cells + GNPs at concentration 20 mg/Kg. (D) Macrophages + Ehrlich tumor cells + Silica- GNPs at concentration 20 mg/Kg trastuzumab. The data are represented as mean + SD.

GNPs and Si-GNPs increased proinflammatory cytokines in serum

Compared to control, the results showed that the GNPs and Si-GNPs ability to increase the secretion TNF-α and IL-8 in all mice groups , in treated GNPs was 271.6±19.85, 208.4±13.24 While Si-GNPs was the most effect level 397.3±10.71, 305.4±10.61, but there were no TNF-α and IL-8 expression levels in untreated cell line as shown in (Figure 5).

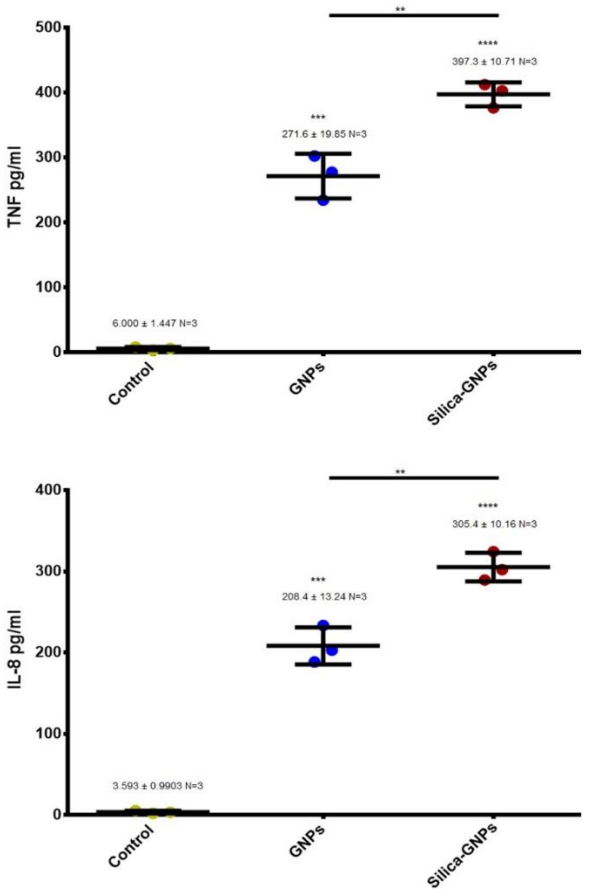

Figure 5. GNPs and Silica-GNPs increased TNF- α and IL-8 production *In-vivo*.

The data are represented as mean + SD. TNF levels in MCF-7 cells were measured using an ELISA assay after 24 hours of treatment with various therapeutic materials. The increased TNF-levels that arose after using LPS are primarily due to increased NF-kB protein expression in breast cancer cells, which leads to increased TNF-expression [40].

GNPs and Si-GNPs augmented Macrophages kill tumor cells through granzyme-preforin pathway

The results of this study showed that the granzyme B was spread in the cytoplasm of splenic macrophages as seen in (Figure 6). Splenic phagocytic cells which are pre-treated with GNPs, and Silica/GNPs exhibited more Granzyme B signaling.

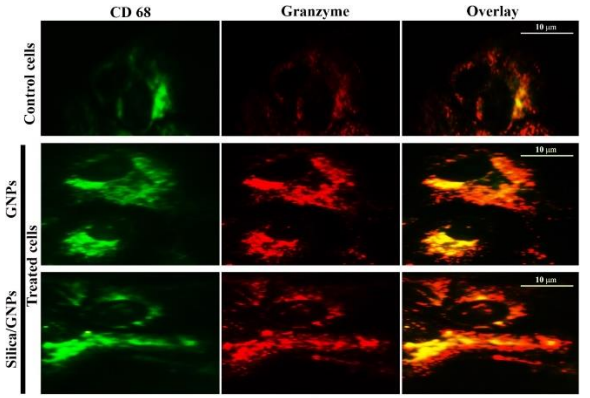

Figure 6. GNPs and Silica-GNPs increased killing of cancer cells through Granzyme- Perforin pathway. Control cells represented BMDMs + SKOV-3 cells. Other conditions as in control in presence of GNPs and Silica-GNPs at concentration 10 µg/ml. green color represented CD68, Red color represented Granzyme. While, Yellow represented Colocalization

Conclusions

In the current work, the role of GNPs and Si-GNPs as phagocytosis inducers was examined using in vitro and in vivo models. The cancer cells were killed by phagocytosis, which was mediated by GNPs and Si-GNPs. The current study establishes that GNPs and Si-GNPs are mediated by each other. A potential solution is granzyme -perforin dependent macrophage tumoricidal action. The research has the potential to grow into something more. The role of nanoparticles in cancer immunotherapy should be investigated further. The process' immunological components Furthermore, the individual molecular functions of perforin and granzyme must be understood. Details on the molecular mechanism nanomaterials, and the trigger for choosing the granzyme-perforin pathway.

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