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EVALUATION OF IMMUNE SYSTEM STIMULATION WITH VACCINE PREPARED AGAINST INDUCED BREAST CANCER IN ALBINO MICE

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ABSTRACT

This study was designed to prepare vaccines. Cancer vaccines promote the destruction of cancer cells, and the cancer cells contain special antigens on their surface when the vaccine is given, it acts as an antigen to activate the immune system. The IL-2 stimulates the response to a type of cells called T-cell, and outer membrane vesicle (OMV) targeting cancer cells by stimulating the immunity to respond with it two types including innate and adaptive immunity, that lead to stimulating the immune system to reduce mammary adenocarcinoma induction in lab mice using T4-1 cell line breast cancer by taking blood and serum to evaluate the immune system efficacy. The tumor induction success by monitoring mice body weight loss showed the lost weight began in the third week after tumor induction, so 23.2 g at first and second week to 14.35 g at the end of the fourth week, whereas the control animals were weighed 32.37 g. The immunity system efficacy results appear a difference in blood and serum parameters after cancer induction. The result shows an increase in total WBC and monocytes (5900, 0.2 cells/mm respectively) but non significantly decreased in neutrophils and lymphocytes count (2.6, 5.9 cells/mm, respectively). Therefore, the first and second doses of vaccines increased the antibody and complement of the immune system compared with control. While Eliaza data for cytokines profile referred to elevated IL2 (26.5 pg/ml) in the serum of vaccination mice but only significantly decreased in IL6 and IL-22 amount (20.4 and 19.6 pg/ml respectively) comparing with control.

Keywords: ***anti-tumor cytokines, murine cell line, immunotherapy, IL2, outer membrane vesicles***

1. INTRODUCTION

The common treatment methods are surgical treatment, radiotherapy, and chemotherapy (McCune, 2018). Most of the malignant growth drugs are exceptionally toxic. Therefore, the need to search for a new drug is required, preventing or slowing cancer progression and is less toxic and safer (Aziz *et al*., 2017). A non-specialist reader could understand the significance of the presented results. Another restorative methodology is to treat malignant growth through bacterial outer membrane vesicles (OMV) as therapeutic agents to treat cancer via immunotherapy. The ability of bacterial outer membrane vesicles to effectually make lasting anti-tumor immune reactions can destroy recognized tumors without distinguished adversative properties. Furthermore, steadily controlled bacterial outer membrane vesicles collect in the tumor tissue and help produce anti-tumor cytokines (Kim *et al*., 2017).

This system includes supporting and remaining so far weak immune response against cancer cells (Xu *et al*., 2019). These days, immunotherapy is acknowledged as the fourth mainstay of standard cancer treatment (McCune, 2018). In the malignancy immunotherapy field, the cancer vaccine inspires the most excitement. They can effectively produce a resistant reaction explicit to tumor cells (TCs) and a dependable immunological memory (Rosenberg *et al*., 2004). The cancer vaccine helps re-teach the immune system to recognize and eliminate tumor cells (Lasaro *et al*., 2010). There is proof that both

humoral and cell-mediated reactions are significant in anticancer immunotherapy. Antibodies can viably dispose of malignant growth cells through immune response intervened cell cytotoxicity (Karagiannis *et al*., 2013). Expanded anticancer humoural reactions are associated with diminished cancer repeat and enhanced survival (Vermaelen, 2019). For the readiness of complete cancer cell vaccines, cancer cells from cell lines or essential tumors can be utilized (Ning *et al*., 2012). Moreover, malignant growth cells can be given by the patient (autologous) or a contributor (allogeneic) (Li *et al*., 2009).

At the point as soon as a cell cancer dies, it very well may be evident by (immunogenic) or without (nonimmunogenic) inspiring, an immune reaction in contrast to its dead cell antigens, in cancer cell vaccine produce, immunogenic cell death (ICD) is the target. An immunogenic death relies upon ICD-instigating improvement (Kroemer *et al*., 2013). Different ICD- inducing motivations have been defined, and their crucial characteristic is the capability to make expression and release of a damage-associated molecular pattern (DAMP) from the cells they were killed. The DAMPs that are expressed and discharged by the cancer cells afterward, ICD can cooperate with design recognition receptors on several immune cells affording to a distinct spatiotemporal design. DAMPs tie to pattern recognition receptors transports around the coming of cytokines and chemokines by the immune cell. Therefore, APCs are animated to effectively take up and progress tumor antigens and cross-prime T cells (Kepp *et al*., 2011). Different elements can impact the adequacy of DAMPs (Krysko *et al*., 2012). The death-inducing stimulus, the created DAMPs themselves, the DAMP location, and the mix of cancer and host-related components affect the immunogenicity of the entire cancer cell vaccine. The malignant growth cell has to express the DAMPs, which thus must bind immune cells and not cancer cells (Cicchelero *et al*., 2014). For sure, the binding of DAMPs to toll-like receptors (TLRs) expressed on malignant growth cells can advance cell endurance and chemoresistance (Krysko *et al*., 2012). Conversely, the patient must be immunodeficient. So line with this theory. This research aimed to assay the efficacy of the death cell line with the OMV vaccine on mice with induction breast cancer (Kroemer *et al*., 2013).

2. MATERIALS AND METHODS

2.1. Extraction of outer membrane vesicles (OMVs) from *E. Coli*

The *E. coli* were grown up overnight in LB medium with carbenicillin (50 µg/mL). Later overnight culture, the *E. coli* solution (200 µL) was washed away with new LB media, and new Luria-Bertani media with the antibacterial drug (carbenicillin, 20 mL) was added. The solution was put in an incubator at 37 °C with strong shaky (200 rpm) awaiting the optical density reached OD578 nm 0.7. Formerly, Isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM) was added to cells, and the solution was incubated for 1 h at 30 °C with strong shaking (200 rpm). The collected E. coli cells were put off with 0.2 M Tris–HCl buffer (pH 8.0) and lysed by lysozyme (200 µg/mL in an ending concentration with 20 mM sucrose and 0.2 mM EDTA) for 10 min at RT. Formerly, PMSF (1 mM) and aprotinin (20 µg/mL) were additional. The OMs of E. coli cells were secluded by adding a similar capacity of extraction buffer (2% Triton X-100, 50 mM Tris–HCl, 10 mM $MgCl_2$) with DNAse (10 µg/mL). Afterward, incubation for 30 min on freeze, the lysate was centrifuged at 4,000 rpm for 5 min. The OM-containing supernatant was centrifuged at 18,000 rpm for 10 min and rinse away with PBS 2 times (Park *et al*., 2015).

2.*2.* Cell lines:

4T1 murine breast cancer cell line, It was received from the consultation office in the medicine college Babylon university that made in National cell bank-Pasteur Institute (NCBI), Iran, and cultured in RPM1-1640 medium enhanced with 20%FBS (Sigma –Aldrich), 2 mmol/L GlutaMax,100 µ/ml penicillin, and 100 µg/ml streptomycin.

2.3 Vaccine preparation*:*

To generate breast cancer vaccine, 4T1 cell line was incubated in whole media enhanced with 200 ng/ml IL2 (Elabscience, USA) through 24 h, γ-irradiated (150 Gy) on snow for killing cells, then wash away 3 times in PBS and resuspended in PBS for inoculation. Then 0.05 µmol/l/L doxorubicin (DOX) and 5 µg/ml OMVs were additional to the ending 24 hours of culture and store at 4 °C (Park *et al*., 2015).

2.4. Experimental Design:

In the experimental design, mice (n=30) were assigned randomly to 3 experimental groups:

***2.4.1.Cancer implantation*:** (25 mice), 4T1 Murine breast cancer cell line were harvested at the mono confluent stage and wash away. Subcutaneous implantation 5×105 cells in 100µL of RPM1-1640 were inoculated in the back area of mice (Hunn *et al*., 2012).

Two doses of immune suppressants (hydrocortisone) were given to mice, one before 24 h of tumor implantation and the other after 24 h after tumor implantation. All animals give gentamycin orally in drinking distilled water for 24 hours after implantation. Period to sign look was definite as a period to mass loss further than 10% obvious interactive signs (reduced activity, hunching).

2.4.1. First group: Vaccination group (15 mice of cancer implantation): This group was injected intraperitoneally with 200µL of a vaccine in PBS weekly for two months.

2.4.2 Second group: Cancer implantation positive group: remained 10 mice with cancer implantation preserved without a vaccine.

2.4.3. Third group: Control group (5mice): This group was injected intraperitoneally with 200µL of PBS weekly for two months.

All groups were killed after two months. Analysis of cytokine released: All animals autopsied for blood analysis. The blood samples were put in an anticoagulant tube for measuring the WBC and differential count by hematology analyzer system (Lab. tech, Korea). The serum was taken for quantities estimation of IL15, IL22, and IL-2 were performed by ELISA, which based on using the cytokines in Rate sera, plasma, and other body fluids. This test has been achieved according to the manufacturing company (Elabscience, USA).

2.5 Ethical Approved

This study was appropriate by the ethical and research committee of the College of Science / Kufa University / Iraq. protocol number (15788/RE)

2.6 Arithmetical study

The results are obtainable as income and statistical with standard error (S.E.) and analyzed using a one-way analysis of variance (ANOVA) test, using Graph pad prism 35.04. $P < 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

3.1 Induction Tumor in Albino Mice:

Mass Loss Convinced by Tumors. Assorted murine transplantable tumors were examined for their faculties to make mammary adenocarcinoma tumors in mice founded on how much consider had been absorbed during tumor growth. The lost weight started in the third week after tumor induction, so 23.2 g at the first and second week to 14.35 g at the end of the fourth week while the control animals were weighed 32.37 g (Figures 1 and 2)

The result approves with (Tanaka *et al*., 1990), who discussed that later 3 weeks of tumor induction, the adipose tissue was virtually completely depleted and compositional analysis of the body components further confirmed that this substantial tissue wasting was due to the damage of whole-body fat and slim body mass but not that of water content. Implanted tumor models have been informed to result in key features of anorexia, heaviness loss, and loss of lean and fat mass, and increased energy spending (Marks *et al*., 2003). Among the features used to evaluate animal models is anorexia leading to losing weight. Dependent on the model used, this decrease in food intake occurs following 4–14 days of tumor burden, resulting in at first a slight decrease in food intake before revealing a severe decrease in food intake shortly afterward (Valenzano *et al*., 2005).

Figure 1. *Death albino mouse after a month of cancer induction by Murine breast cancer 4T1 cell line*

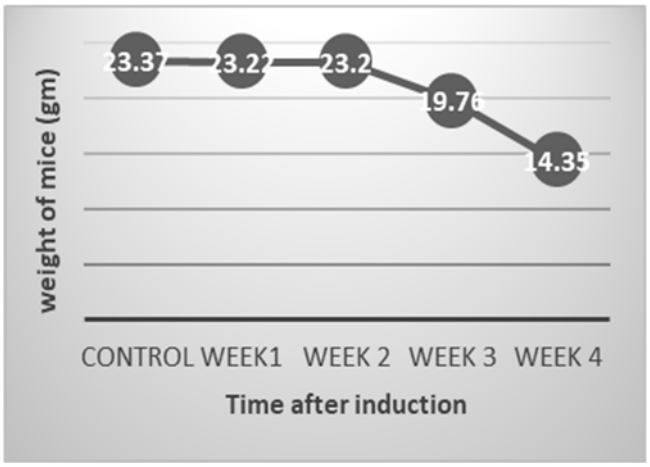

Figure 2. *Weight monitoring in cancer induction mice group*

3.2 Immunological parameters profile

When one month of murine tumor stimulation by T4-1 cell line, groups of induction mice were killed for assaying the immunity system ability. The results showed a decrease in hematology parameters. In the long run, a decrease in total WBC and monocytes but non significantly decreased in neutrophils and lymphocytes count.

Furthermore, there a significant decrease in humoral immunity parameters presented in IgG, IgM, C3, and C4 that is assaying by immunodiffusion agar. While Elizas data for cytokines profile referred to elevate of IL22 and IL6 in the serum of stimulation mice but only significantly decreased in IL2 amount (Table1). The reasons for these results related to exposing the animals to immunosuppression drugs dexamethasone previously tumor stimulation that causes the decreased immunity for a short period until development tumor that too contributed in this decreasing through that tumors are skilled at flaking external antigens or down-regulating expression of key particles essential for associates by immune cells (Whiteside, 2006). Here means tumors can escape the host's immune reaction by the existence of deprived stimulators of T cells or reduced goals for tumor-specific T cells (CTL). The appearance of molecules such as TAA, HLA class I molecules, or antigen processing mechanism components (APM) is generally miserable planned or altered in tumor cells (Ferris *et al*., 2005)

This variety shows that tumors are very skilled in their capacity to weaken host immune reactions which by an extensive collection of biological effector molecules such as many different adjacent receptor systems, small molecular types, cell enzymes, soluble cell components and cytokines / chemokines, This variety made cancer cells weakens the immune system by suppressing insoluble immune immunity (TGFβ, IL-10, ROS, enzymes, inhibitory lignans such as FasL or TRAIL) that tumor cells or additional cells release into the micro-tumor environment, Suppressive cell groups, i.e., regulatory T cells (CD4 + CD25) or marrow-derived marrow cells have established a major role in depressing the regulation of host anti-tumor immunity [20] and there are several mechanisms for performance these complicated functions: by increasing cAMP, increasing or Programmed cell death by FAS is stopped by planar regulation NO, has pro-oxidant activity, increases cAMP levels, reasons programmed cell death in NK cells, inhibits tumor CTL (Uotila, 1996). prevents T cell responses weaken T cell functions, reduces expression ζ series (Rodriguez *et al*., 2004), prevents ointment and grenase expression MRNA; stops multiplying of lymphocytes (Mocellin *et al*., 2004), inhibits making of IL-1_, IFN-_, IL-12, and TNF_ (Vicari and Trinchieri, 2004; Young, 2004), promotes macrophage expansion linked with immunosuppressive tumors (McKallip and Ladisch, 1999), prevents proliferation of IL-dependent lymphocytes 2 or instigates apocalyptic signs (Aparicio-Domingo *et al*., 2015)

About the raised levels of IL22 and IL6 in tumor stimulation mice related, several populations of immune cells at a site of inflammation create IL-22. Producers are αβ T cells classes Th1, Th22, and Th17 along with γδ T cells, NKT, ILC3, neutrophils, and macrophages. IL-22 incomes influence non-hematopoietic cells – chiefly stromal and epithelial cells (Khosravi *et al*., 2018) IL-22 natural action is begun by binding to a cell-surface complex composed of IL-22R1 and IL-10R2 receptor chains and additionally planned by contacts through a solvable binding protein, IL-22BP, which dividends sequence likeness by an extracellular region of IL-22R1 (sIL-22R1). IL-22 and IL-10 receptor chains play a portion in cellular directing and signal transduction to selectively recruit and normalize immune reactions (Aparicio-Domingo *et al*., 2015). The role of IL-22 in cancer is composite, and cancer-promoting and restrictive functions of IL-22 have been termed. The exact details why IL-22 can be a barricade in contradiction of cancer progress then below sure situations are likewise capable of helping tumor growth are indefinite (Hernandez *et al*., 2018).

Though IL-6 is a multifunctional cytokine that was initially categorized as a regulator of

immune and inflammatory responses; still, raised expression of IL-6 has been noticed in multiple epithelial tumors (Kishimoto, 2005), IL-6 signing has also been involved in tumorigenesis (Jung *et al*., 2015) Yet, the nature of IL-6's contribution in cancer has been fairly contentious, such as dichotomous roles for IL-6 in both tumor-promoting and -oppressive doings have been described. For example, IL-6 signaling has been connected to together pro-and antiapoptotic action in breast cancer cells (Conze *et al*., 2001; Alkayaty *et al.,* 2017), since normal and tumor tissue from the identical patient exposed that IL-6 mRNA is stated at meaningfully advanced stages in mammospheres resulting from tumor tissue. In accumulation, spheroids cultured from the MCF-7 breast cancer cell line also enclosed high IL-6 levels, and action with IL-6–blocking antibodies repressed spheroid creation. In height, expression of IL-6 was too distinguished in basal-like breast carcinoma tissues, which are supplemented in mammosphere and stem cell markers. (Dittmer *et al*., 2020)

3.3 Evaluation of Immunological parameters after vaccination:

The results showed that later vaccination indicated elevated in all tested parameters Total WBC, Monocytes, neutrophil, and lymphocytes (9.700, 0.5, 3.1, and 6.2 cells/mm respectively). The highest increase of IgG, IgM, C3, C4, and IL-2 (65.7, 16.6, 14.7, 9.7 cells/cu.mm and 26.5 pg/ml respectively), while a decrease of IL-6 and IL-22 (20.4 pg/ml and 19.6 pg/ml respectively). Alkayaty *et al.* (2017) suggested that the mixture of interleukin-2, tumor cell, and VOM vaccine induces long-lasting anti-tumor immunity that can protect a system against tumor recurrence; also, it can preserve a modulate host immune system and increased phagocytic activity. The contact between neutrophils and the mixture has the probability of being helpful in the treatment of cancer disease. Neutrophils have been reflected the "kamikaze" cells that reach initial at the place of damage and sacrifice themselves capable of using an anti-tumor action straight a diversity of mechanisms that include phagocytosis secretion of Tumor Necrosis Factor-α TNF-Related Apoptosis-Inducing Ligand; TRAIL, anti-microbial protein or antibody-dependent cell-mediated cytotoxicity (Zhang *et al*., 2019). Stimulation of cytotoxic T-cells (CTL) is an important factor of the immunological response to cancer cells. CTL is created by cancer in treating lymphocytes. It distinguishes TAAs, which currently on the MHC Class I molecules on the tumor cell surface. Later CTL and recruit the programmed-cell death of the malignant cells will stimulate the Fas/FasL pathway (Zilio *and* Serafini*,* 2016).

Weiner *et al*. (2010) found the binding IgG molecules to the cell surface primes to high-affinity binding of C1q to the Fc domain, trailed through stimulation of C1r enzymatic action then subsequent stimulation of downstream complement proteins. The end of this cascade is creating holes by the membrane attack complex (MAC) on the tumor cell surface and following tumor cell lysis. Also, the making of the extremely chemotactic complement molecules C3a and C5a leads to the recruitment and stimulation of immune effector cells, such as macrophages, neutrophils, basophils, mast cells eosinophils. Cross-linking of FcγRs on these cells helps ADCC and tumor cell obliteration next tumor cell lysis. Antigen-presenting cells can present tumor-derived peptides on MHC class II molecules and promote CD4+ T cell stimulation. Moreover, in a progression identified as cross-presentation, tumor-derived peptides can be accessible on MHC class I molecules, causing the stimulation of CD8+ cytotoxic T cells (Roopenian and Akilesh, 2007).

OMVs of *E. Coli* can cooperate with pattern recognition receptors, such as TLR4 to encourage cytokines and chemokines. OMV might stay accepted via DCs that are directed to the enrolment of immune cells, then motivated antigen-presenting cells (APC) concluded (TLR) documented (PAMPs). This progression heightened T helper cell creation (with Th1 and Th2) and completely improved cellular and humoral immunity. Also, OMVs can too circulate interested in non-immune cells and weight on MHC class II molecules. Stimulated antigen-presenting cells rapid MHC class II molecules that relate with the T cell receptor (TCR) on CD4+ T cells to initiative antigen-specific T cell responses, ensuing in T helper cell propagation, thus producing antigen-specific antibodies in many tissues (Zhang *et al*., 2019) (IL-2) stimulates the development and action of T cells. IL-2 prevents the progress and production of cancer cells and creates additional observable to the immune system. The medication encourages the growth of killer T cells and additional immune cells. IL-2 does not spasm cancer cells straight – it supports the immune system do the profession by improving the capability of white blood cells, called T cells, to target and destroy cancer cells. It created that Blocking IL-1 activity by IL-1R antagonist or anti-IL-1R neutralizing antibody directed to a decrease in the number of IL-22 generating cells associated

with reducing tumor development decrease (Markota *et al*., 2018). (IL-2) stimulates the development and action of T cells.IL-2 stops the growth and increase of cancer cells and makes them more visible to the immune system. The drug encourages the development of killer T cells and other immune cells. IL-2 does not directly attack cancer cells, so when stimulating the immune system, IL-2 helps do the work (Jubori *et al*., 2018) by improving the capability of certain leukocytes called T cells to destroy cancer cells. The blocking of the IL-1 axis will disrupt IL-22 making and reduction its effect on cancer cells leading to improved tumor control (Voigt *et al*., 2017)

It is attractive to venture that obstruction of IL-1 reduced IL-22 production in these patients and eventually stopped or slowed cancer growth and increase. Likewise, an additional way of manipulating the IL-1and IL-22 axis would be to avoid the processing of IL-1β by exact inhibitors of the IL-1-processing mechanism such as the inflammasome. The growth of inflammasome inhibitors is presently started for the treatment of inflammatory diseases. These combinations might also show of use in cancer treatment as soon as their in vivo efficiency in reducing IL-1β production has been proved (Markota *et al*., 2018)

Another way to target IL-22 in cancer is direct inhibition to signs of IL-22-IL-22-R1, using antibody neutralization or IL-22-BP. Targeting chemokines that penetrate cell producing IL-22 or by inhibiting transcription factors that induction IL-22 signaling (Sabat *et al*., 2014). IL-21 is a type I cytokine with close homology to IL-2, IL-4, and IL-15 that shares with these cytokines and IL-7 and IL-9 the γc receptor subunit cytokine-specific-receptor (Gajewski and Hodi, 2011).

4. CONCLUSIONS:

The use of cancer immunotherapy agents to stimulate the immune system to identify and attack malignancies has provided new potentials for active cancer treatment. Additional cytokine IL2 with OMV to completely dead cancer cell line for generating cancer vaccine product showed substantial improvement in the efficient immune system parameters so, Combination therapy might be a promising therapeutic strategy to treat cancer in the future. Something in the related field.

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***Table 2*.** A table showing the immunological parameters of the mice in which the cancer was induced and the mice that received the vaccine

Parameters	**mean**					
	Control	**±Sd**	**Cancer induction**	**±Sd**	**Cancer vaccine**	**±Sd**
Total WBC	7.800	±0.2	5.900	±1153.25	9.700	±1374.77
Monocytes	0.4	±0.2	0.2	±0.15	0.5	±0.28
neutrophil	2.9	±0.2	2.6	±1.33	3.1	±1.43
lymphocytes	6	±2.64	5.9	±1.94	6.2	±2.12
IgG	57.3	±1.51	49.6	±9.95	65.7	±5.001
IgM	12.1	±1.05	8.7	±0.90	16.6	±1.82
C3	10.3	±1.18	6.7	±1.80	14.7	±2.56
C4	6.4	±1.56	4.2	±1.65	9.7	±2.95
IL-22	15.6	±2.38	23.1	±3.75	19.6	±3.22
IL-6	18.9	±3.37	27.8	±1.95	20.4	±2.47
1L-2	17.4	±2.90	15.6	±3.82	26.5	±3.84