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Evaluation of Microparticulate Ovarian Cancer Vaccine via Transdermal Route of Delivery

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Abstract
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Evaluation of Microparticulate Ovarian Cancer Vaccine via Transdermal Route of Delivery

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Abstract: Ovarian cancer is the fifth most commonly occurring malignancy in women, with the highest mortality rate among all the gynecological tumors. Microparticulate vaccine can serve as an immunotherapeutic approach with a promising antigenic delivery system without a need for conventional adjuvants. In this study, a microparticulate vaccine using whole cell lysate of a murine ovarian cancer cell line, ID8 was prepared by spray drying. Further, the effect of interleukins (ILs) such as IL-2 and IL-12 was evaluated in a separate study group by administering them with vaccine particles to enhance the immune response. The vaccine microparticles were administered to C57BL/6 female mice via transdermal alone and in combination with the oral route. The transdermal vaccine was delivered using a metallic microneedle device, AdminPen™. Orally administered microparticles also included an M-cell targeting ligand, Aleuria aurantia lectin, to enhance the targeted uptake from microfold cells (M-cells) in Peyer's patches of small intestine. In case of combination of routes, mice were given 5 transdermal doses and 5 oral doses administered alternatively, beginning with transdermal dose. At the end of vaccination, mice were challenged with live tumor cells. Vaccine alone resulted in around 1.5 times tumor suppression in case of transdermal and combination of routes at the end of 15th week when compared to controls. Inclusion of interleukins resulted in 3 times tumor suppression when administered with transdermal vaccine and around 9 times tumor suppression for the combination route of delivery in comparison to controls. These results were further potentiated by serum IgG, IgG1 and IgG2a titers. Moreover, CD8+ T-cell, CD4+ T-cell and NK (natural killer) cell populations in splenocytes were elevated in case of vaccinated mice. Thus, vaccine microparticles could trigger humoral as well as cellular immune response when administered transdermally and via combination of route of delivery. However overall, vaccine administered with interleukins, via combination of route, was found to be the most efficacious to suppress the tumor growth and lead to a protective immune response.

1. Introduction:

Ovarian cancer is the most lethal gynecological cancer and the fifth most leading cause of cancer related deaths in women in the US [1, 2]. The National Cancer Institute (NCI) has estimated 21,290 new cases and 14,180 deaths due to ovarian cancer in the US in 2015. When cancer incidences are compared worldwide, the mortality rate associated with ovarian cancer was found to be relatively high in the US and Europe [3]. Since it is very difficult to detect an ovarian cancer, especially in the early stages, it is referred to as a 'silent killer'. Only about 10% of

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ovarian cancers are usually found in the early stages. Patients with epithelial tumors, which account for approximately 90% of ovarian cancer, generally have poor overall survival and the 5-year survival for stages III–IV of these tumors is about 29.1% [4]. The first-line treatment for advanced ovarian cancer involves surgery to remove the tumor, followed by chemotherapy. However, the cancer relapses within relatively short periods of time even after treatment. It has been reported that up to 75% of patients responding well to the initial treatments face tumor relapse within 18–28 months [5]. Moreover, chemotherapeutic treatments for cancer are toxic and/or of minimal therapeutic value. Therefore, alternative approaches such as immunotherapy is being investigated to prevent relapse of cancer. Several vaccines are underway in clinical trials and most of them have not progressed beyond phase I/II studies [6, 7].

Various proteins and peptides have been approved or are being evaluated in clinical trials for treatment of cancer. Due to limited oral bioavailability of such antigens, injectable routes of administration are currently being used. Scientists have been exploring the potential of delivering vaccine antigens orally or transdermally as these delivery routes have ease of administration, are non-invasive and patient compliant. Transdermal delivery is considered as the best route for vaccine administration because of the skin-associated lymphoid tissue which comprises of Langerhans cells, dermal dendritic cells, lymph nodes and subsets of T-lymphocytes. Microneedles have been used to pierce the upper layer- Stratum corneum of the skin to enhance transdermal delivery by promoting the transport of macromolecules that cannot be delivered across the skin by passive diffusion alone [8, 9]. Microneedles are micron-sized needles, which upon insertion into the skin result in formation of aqueous conduits forming a passage for the vaccine antigens towards the immune-competent skin layers. Due to their short needle length, they avoid contact with the nerve endings in the dermis thus remain to be a painless mode of immunization [10-12].

In addition, the microparticulate delivery system has several advantages over the usage of the antigens alone. Particulate antigens have been proven to be more immunogenic than soluble antigens [13, 14]. Improved uptake of the particles compared to the solution results in higher cytotoxic T-lymphocytes (CTLs) response against the cancer cells. The antigen presenting cells (APCs) in the body easily phagocytose these microparticles recognizing them as an antigen and generate an immune response [15]. Further, they are drained into the nearby lymph nodes where they activate various other immune cells. Thus, the particulate delivery systems may mimic pathogens that are commonly recognized, phagocytosed and processed by professional antigen-presenting cells (APC) [16, 17]. When administered transdermally, the microparticles are taken up by the immune cells in the skin, which trigger mucosal as well as systemic immune response [10]. Langerhans cells are dendritic cells that activate T cells and induce a strong immune response and occupy around 20% of the skin’s area. On the other hand, M-cells are the microfold cells, which act as sampling ports for any foreign entities encountered in the small intestine upon oral administration [18-23]. These M cells house various dendritic cells and immune cells in them. Once the oral vaccine particle is sampled by M-cells, it is processed by a dendritic cell/antigen presenting cell (APC) and presented on MHC (major histocompatibility complex) Class I or MHC Class II molecules [24, 25]. The antigens are further recognized by the immune cells in the vicinity leading to the cascade of an immune response. The immune response also includes humoral response by plasma B-cells, which leads to production of antibodies and their class switching. The role of B-cells has been debatable in past but a recent study by Mahmoud

SM et al. shows that the humoral immunity is important in addition to cell-mediated immunity in prognosis of breast cancer [26]. Thus, we aim to trigger both humoral and cell-mediated immune response through this prophylactic cancer vaccine, which can impart resistance against tumor challenge. Moreover, the microparticulate drug delivery system can be used to assimilate various antigens in one delivery system that can reduce the number of doses as well as reduce the different vaccination regimen [13, 14].

In this study, we have investigated whether vaccination with microparticles containing the ovarian cancer antigens can prevent/ retard ovarian cancer growth. A murine ovarian cancer cell line, ID8 was used as a source of antigens for vaccine preparation. The cell line correlates closely to human ovarian cancer cell lines in signaling pathways and results in development of tumor in mice models similar to human ovarian cancer. Thus, ID8 cell line provides a unique model to study the immune response developed by the vaccine against the initiation and progression of ovarian cancer in mice with an intact immune system [2]. Therefore, we proceeded with a whole cell lysate of ID8 cells to prepare the vaccine for this study. Despite of advancement in recombinant technology and gene expression, the whole cell lysate vaccine still remains a very promising approach. Whole cell lysate provides a pool of tumor-associated antigens (TAAs) which can induce both CD8+ and CD4+ T cells [27].

In our previous study, microparticulate vaccine was found to be efficacious when administered orally [23]. Therefore, we aimed to evaluate the microparticulate vaccine via transdermal route alone and in combination with oral route. By combination route of administration, aim was to achieve merits of both oral and transdermal immunization [28]. The vaccine particles were administered for this purpose using a microneedle device called as AdminPen™. For this purpose, microparticles were prepared by spray drying technique using methacrylic copolymer Eudragit® FS 30 D and hydroxyl propyl methyl cellulose acetate succinate (HPMCAS) as described elsewhere [20, 23]. These polymers have been reported their applications for transdermal delivery in form of patches as well as particulates [29, 30]. Several others have mentioned their usage for oral sustained or controlled release delivery [31, 32]. To target the vaccine formulation to M-cells in the Peyer's patches of the intestine upon oral delivery, M-cell targeting agent, Aleuria aurantia lectin (AAL) was used in the formulation [15, 20, 21]. In addition, immunostimulatory molecules such as IL-2 and IL-12 were added in order to enhance the overall potency of the formulated vaccines. Oral delivery was performed by using an oral gavage. Transdermal delivery was achieved using an AdminPen™ device comprised of an array of 43 metallic microneedles of 1100 nm length in 1 cm sq area of circular microneedle array made of SS316 stainless steel (as shown in figure 1). In the present study, we demonstrate and compare the efficacy of the vaccine formulation which was administered via two different approaches based on route of administration: (1) transdermal and (2) combination of transdermal and oral route *in vivo* in mouse tumor model.

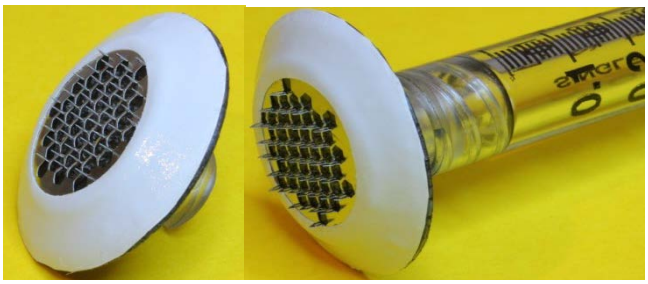


Fig. 1: AdminPen™ device comprised of an array of 43 metallic microneedles of 1100 nm length in 1 cm sq area of circular microneedle array made of SS316 stainless steel, attached to a syringe (Image produced with a permission from AdminMed / nanoBioSciences LLC)

2. Materials and Methods

2.1. Materials

ID8 cell line was kindly provided by Dr. Katherine Roby, Kansas University Medical Center, Kansas City, KS. Six to eight week-old C57BL/6 female mice were purchased from Charles River Laboratories, Wilmington, MA. HPMCAS was purchased from AQOAT, FMC Biopolymers, Philadelphia, PA. Eudragit® FS 30 D was generously gifted by Evonik industries, Parsippany, NJ. Mouse plasma was obtained from Biochemed, Winchester, VA. AAL was obtained from Vector Labs, Inc., Burlingame, CA. Recombinant murine interleukins, IL-2 (5 X 10^6 units/mg) and IL-12 (1 X 10^7 units/mg) were purchased from Peprotech Inc., Rocky Hill, NJ. Flow cytometry cell markers were purchased from eBioscience, San Diego, CA. Goat anti-mouse HRP-IgG and anti-IgG subtypes were purchased from Bethyl Laboratories, Montgomery, TX and Sigma, St. Louis, MO respectively. AdminPen™ device was purchased from nanoBioSciences LLC. All other materials used were of analytical grade.

2.2. Preparation and characterization of whole cell lysate of ID8 ovarian cancer cell line

The whole cell lysate of the murine ovarian cancer ID8 cells was prepared using hypotonic buffer and freeze-thaw cycles as described elsewhere [23, 33, 34]. The lysate obtained was stored at -80^0 C until used. The whole cell lysate of ID8 cell line was characterized for total protein content using Bio-Rad DC™ protein assay. The lysate was also screened for presence of the only known marker, by western blot analysis as described elsewhere [23, 35].

2.3. Preparation and characterization of vaccine microparticles

The vaccine formulation was prepared by using spray drying technique as described elsewhere [20]. Briefly, hydroxyl propyl methyl cellulose acetate succinate (HPMCAS) and Eudragit® FS 30D were dissolved in an alkaline solution, followed by addition of chitosan glycol. Mouse plasma, trehalose, and tween 20 were added to the solution. Whole cell lysate obtained from ID8 cells (5%w/w) was added to this feed mixture and temperature was maintained at $4^0 \pm 2^0$ C throughout the spraying. This aqueous solution was spray dried using Buchi B-191 Mini Spray Dryer (Buchi Corporation, New Castle, DE) at inlet temperature 125° C, outlet temperature 80° C, 500Nl/h, and 2% spray flow feed rate (10 mL per 30 min) of peristaltic pump, and nozzle diameter 0.7 mm . The particles were characterized for size and charge, using laser particle counter (Spectrex PC -2000) (n=3) and Malvern zeta sizer (ZEN 1600) (n=10) respectively. Loading efficiency was determined by Biorad DC™ protein assay by extracting the lysate in phosphate buffered saline.

2.4. Immunization

The immunogenicity of microparticulate vaccine was evaluated using C57BL/6 female mice model. The animal experiments were carried out as per approved protocols by Mercer University's Institutional Animal Care and Use Committee (IACUC). Animals (n=8) were administered with microparticles as one prime dose followed by one booster after one week and thereafter by 8 boosters with an interval of two weeks. In case of vaccine with interleukins formulations, 4×10^5 U of IL-2 and 8×10^5 U of IL-12 were added to this particulate suspension. For delivering microparticles via transdermal route, mice skin was shaved two days prior to vaccination. Around 5 mg of microparticles were suspended in citrate buffer, pH 4.0 containing PEG 8000 as a viscosity modifier [11]. These mice were vaccinated by delivering this microparticulate suspension through AdminPen™ 1200 microneedle liquid injection system (refer to figure 1) which allows particles to seep into microchannels created by microneedles. For oral route, 5 mg of microparticles was suspended in citrate buffer and administered orally using oral gavage. In case of combination of routes, mice were given 5 transdermal doses and 5 oral doses administered alternatively, beginning with transdermal dose. In a study by Chiriva-Internati et al., SP17 protein has been evaluated as an ovarian cancer vaccine in murine models, where the mice were immunized intramuscularly with SP17 in ten doses of vaccination. It has shown to be a promising approach prophylactically as well as therapeutically [35]. The number of doses were decided based on the previous successful particulate vaccine studies conducted by the group [23]. The booster doses were intended to potentiate the immune response by repeated exposure of the antigen every alternate week. Three different formulations such as placebo, vaccine and vaccine with interleukins were evaluated for this purpose.

2.5. Tumor challenge study

One week after the last vaccination, the mice were challenged s.c. with 1×10^7 live ID8 cells as described elsewhere [36, 37]. The cells were injected into the right back flank of mice. Tumor development was monitored using digital Vernier calipers. The mice were euthanized whenever the tumor ulcerated or tumors exceeded a size of 15 mm in any of the perpendicular diameters. The tumor volume (V) was determined by using the formula, $V=1/2(\text{Length})(\text{Width})^2$ [36, 38].

2.6. Assessment of humoral (B-cell mediated) immune response in serum

The blood samples were collected prior to each dose of vaccination. Serum was analyzed by ELISA as described elsewhere. Briefly, a 96 well plate coated with the lysate (100µg/well) was incubated with 1: 10 dilution of serum samples. HRP-tagged secondary anti-mouse goat IgG was then added to each well, and incubated for 1 hr. TMB substrate reagent (3,3', 5,5"-tetramethyl benzidine) (BD OptEIA™, BD Biosciences, CA) was added and the plate was again incubated for 30 min. The reaction was stopped by addition of 4N H_2SO_4. The plate was read using microplate reader (BioTek instruments Inc., Winooski, VT) at 450 nm. In case of IgG subtypes analysis, serum samples were analyzed in dilution of 1:400. The plate was then incubated with goat anti-mouse IgG subtype IgG1 or IgG2a, followed incubation with HRP-conjugated anti-goat IgG. Thereafter, same procedure as described above for IgG titers analysis was followed.

2.7. Determination of T-cell based/ cellular immune response

A separate group of mice was vaccinated in similar way as described in section 2.4. At the end of vaccination, mice were euthanized and the spleens were harvested. The single cell suspension of pooled splenocytes were stimulated for 5 days at 37^0C with mitomycin-treated tumor cells in a

ratio 10:1 with 10 U/mL of recombinant murine IL-2. At the end of 5-days, the cells were washed with Hank's balanced salt solution and labeled with anti-mouse CD8a FITC (for CD8+ T-cells), anti-mouse CD4 PE (for CD4+ T-cells), and anti-mouse NK PE (for NK cells). The cells were analyzed for the specific cell populations by flow cytometric analysis using BD accuri® C6 flow cytometer [23].

2.8. Statistical analysis

Serum IgG subtype titers and flow-cytometry results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Tumor volume measurements and serum IgGs were analyzed by two-way ANOVA followed by Bonferroni multiple comparisons. All statistical analyses were performed using GraphPad Prism5 (trial version 5.04, GraphPad Software, Inc., La Jolla, CA). For each test, p value less than 0.05 was considered significant. Data are expressed as mean ± standard error.

3. Results and Discussion

3.1. Preparation and characterization of the whole cell lysate

The total protein concentration of lysate was 1.56 ± 0.5 mg/mL. The western blot analysis of the lysate showed the presence of SP17 antigen in the lysate prepared (shown elsewhere) [23]. The only protein which is expressed by ID8 cells and that has been studied is the sperm protein (SP17, molecular weight 15kD). It is one of the cancer/testis antigens, a sub- class of TAAs. It is non-mutated self-antigen which is reported to be recognized by CD8+ T-cells. This protein has been found to be expressed or over-expressed in ovarian cancers. Moreover, human SP17 shows 70% homology with murine SP17. Thus, SP17 is a promising antigen for immunotherapy in ovarian cancer [35]. Therefore, the presence of SP17 in the lysate ensured that the protein cocktail obtained was antigenic when given as a vaccine.

3.2. Preparation and characterization of vaccine microparticles

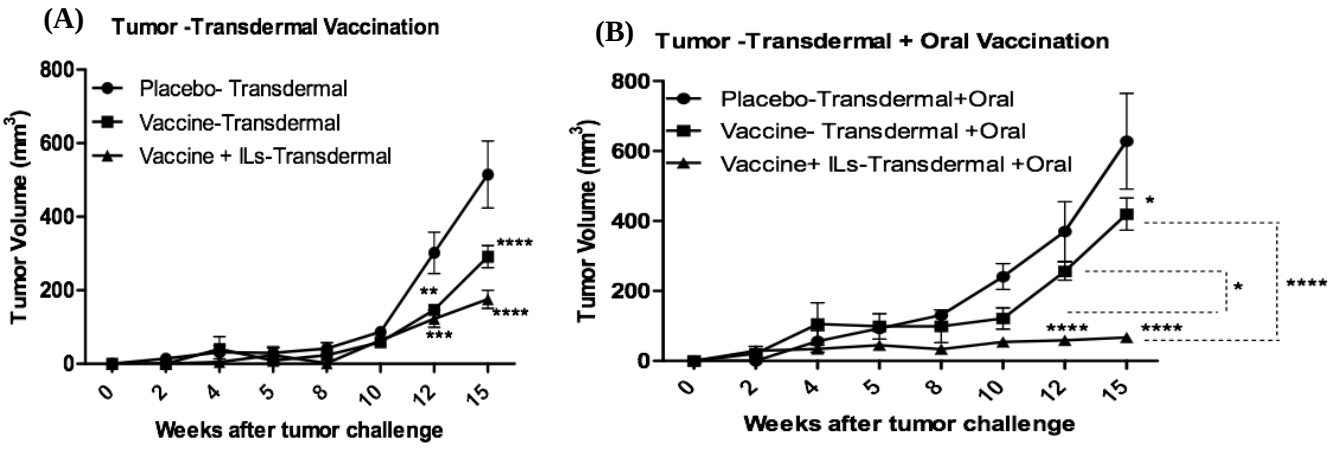
The particles obtained were of 1.58 ± 0.62 μm size with a charge of 12.48 ± 2.32 mV. There was no significant change in size and charge upon loading these particles with the lysate. The production yield was 72.58 ± 3.41 % w/w. The loading efficiency of the particles was 92.68 ± 4.77% w/w. These microparticles were within the size range to mimic pathogenic species; thereby enhancing the potential of naturally being phaygocytosed by dendritic cells. Thus, the uptake would allow processing of antigen-loaded microparticles leading to MHC presentations to the immune cells.

3.3. Immunization suppresses tumor growth

At the end of vaccination, 10^7 ID8 cells were injected subcutaneously to the animals of each study group and the tumor volumes were measured every week using digital Vernier calipers. In the control group, mice were treated with placebo particles and the tumor developed rapidly. However, vaccinated mice showed tumor suppression when compared to the control/ placebo group as shown in figure 2.

Vaccine alone resulted in around 1.5 times tumor suppression in case of transdermal and combination of routes at the end of 15^{th} week. In case of interleukins, transdermal route showed around 3 times of tumor suppression and combination of routes resulted in around 9 times of tumor suppression, when compared to control mice. Transdermally vaccinated mice showed significant retardation of tumor volume in comparison to control animals at 12^{th} week after the

tumor challenge ($p<0.05$), while mice vaccinated with combination of two routes showed significant retardation at 15th week after tumor challenge. The tumor volume measurements



obtained are shown in figure 2.

Fig.2 Immunization with vaccine microparticles suppresses tumor growth: Mean tumor volumes for mice groups treated with two different approaches: (A) Transdermal (B) Transdermal + Oral vaccination: Placebo, vaccine microparticles with and without interleukins. The tumor volume was monitored with the aid of Vernier calipers on a weekly basis. Vaccinated mice showed higher tumor suppression as compared to non-vaccinated/placebo treated mice for (A) Transdermal (B) Transdermal + Oral vaccination groups ($p<0.05$), *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$

In case of transdermal route, there was no significant difference between vaccine and vaccine with interleukin groups in terms of tumor volumes. This can be due to the interleukin concentration used in vaccine which was not high enough to retard tumor growth more than that seen with vaccine alone. Higher concentration of interleukins might be needed to show additional tumor suppression. However, in case of combination routes, a significant difference was seen in tumor volumes of vaccine with and without interleukins at 12th week ($p<0.05$) and 15th week ($p<0.0001$). The interleukins were found to contribute to even more tumor suppression when administered via oral as well as transdermal route.

3.4. Immunization with vaccine microparticles generates humoral immune response and interleukins influence Th1/Th2 response

In order to determine B-cell response, the serum samples collected in between doses were analyzed by ELISA. Immunized mice showed elevated IgG titers as compared to control ones at the end of transdermal vaccination ($p<0.01$) as shown in figure 3(A). For combination route (figure 3(B)), mice vaccinated with interleukins showed elevated response as compared to control mice ($p < 0.01$) and mice treated with vaccine particles alone ($p < 0.05$) at the end of second dosing. At the end of vaccination, it resulted in elevated titers in vaccinated mice as compared to control mice and incorporation of interleukins resulted in further increase in titers.

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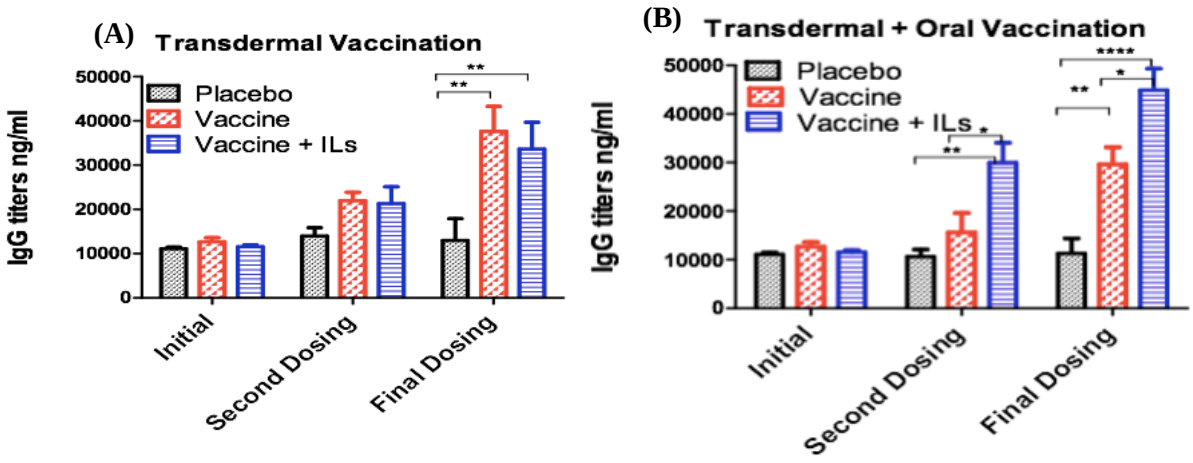

Fig. 3: Humoral response obtained with Immunization with vaccine microparticles for mice groups treated with two different approaches: (A) Transdermal (B) Transdermal + Oral vaccination: Placebo, vaccine microparticles with and without interleukins. Serum IgG titers determined by ELISA, showing higher titers at the end of final vaccination in case of mice in (A) Transdermal (B) Transdermal + Oral vaccination groups with and without interleukins when compared to mice treated with placebo microparticles, while only in case of combination routes, the IgG titers were elevated even further upon addition of interleukins. ($p<0.05$), *$p<0.05$, **$p<0.01$, ****$p<0.0001$

Serum IgG1 titers (indicative of Th2 response) and IgG2a titers (indicative of Th1 response) were elevated in mice vaccinated with interleukins when compared to control mice in case of both study approaches as shown in figure 4 (A, B) and 5(A, B) respectively. Thus, both subtypes

of IgGs indicate that mixed Th1 and Th2 immune response was generated in case of vaccine with interleukins group.

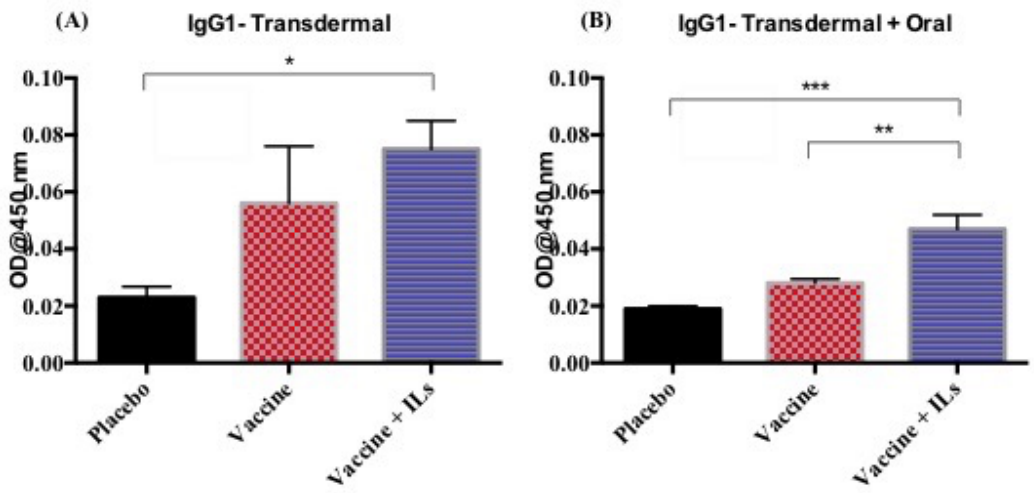

Fig. 4: IgG1 humoral immune response (indicative of Th2 response) for mice groups treated with two different approaches: (A) Transdermal (B) Transdermal + Oral vaccination: Placebo, vaccine microparticles with and without interleukins. Serum IgG1 titers determined by ELISA, showing higher titers in case of mice vaccinated with interleukins as compared to placebo treated/ non-vaccinated mice. Vaccine with interleukins showed even further elevation in IgG1 titers in case of combination route of vaccination. ($p<0.05$), *$p<0.05$, **$p<0.01$, ***$p<0.001$

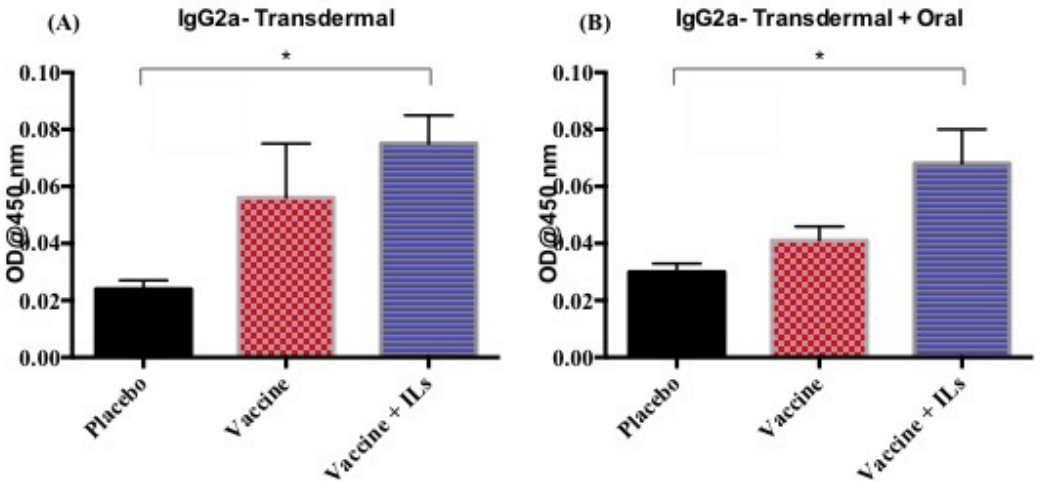

Fig.5. IgG2a humoral immune response (indicative of Th1 response) for mice groups treated with two different approaches: (A) Transdermal (B) Transdermal + Oral vaccination: Placebo, vaccine microparticles with and without interleukins. Serum IgG2a titers determined by ELISA, showing higher titers in case of mice vaccinated with interleukins as compared to placebo treated/ non-vaccinated mice. ($p<0.05$), *$p<0.05$

Stimulation of immune system as a function of a vaccine is mainly based on humoral and cellular response reflected in serum and lymphatic organs of the body. Humoral response is majorly read as IgG antibodies secreted by B-cell activation. Moreover, IgG subtypes such as IgG1 and IgG2a were determined in serum samples which are indicative of Th2 and Th1 response respectively. The Th1 response indicates that T-helper cells (CD4+ T-cells) are activated to trigger macrophages and cytotoxic (CD8+) T-cells, hence further cascade of immune pathway. On the other hand, Th2 response depicts that CD4+ T-cells are triggered to activate B- cells (humoral arm of immune response).

Serum IgG levels were found to be elevated at the end of dosing in case of vaccinated mice via both study approaches and interleukins showed further rise in levels of IgG only in case of combination routes. Thus, humoral response was triggered by these particulate vaccines upon oral and transdermal administration. Further, there was an elevation in IgG1 and IgG2a titers in mice vaccinated with interleukins in case of both study approaches. This indicates the activation of Th1 and Th2 response only in presence of interleukins administered via transdermal and combination route. Vaccine treated mice did not show any elevation in titers which again indicated the influence of interleukins mediating Th1 and Th2 pathway. Thus vaccine with interleukins resulted in mixed response of Th1 and Th2 pathways. This humoral response complied with tumor challenge study results where interleukins group showed further suppression of tumor for combination routes of vaccination. Only combination route showed increase in IgG1 and IgG2a titers along with elevated IgG titers upon vaccination with

interleukins when compared to control and mice treated with vaccine particles alone. This complied with the findings on tumor suppression.

3.5. Immunization generates T-cell based/ cellular immune response

The second array of cancer immune response comprises of activation of T-cytotoxic (CD8+) and T-helper (CD4+), Natural Killer (NK) cells which contribute to cellular response. For this purpose, splenocytes were screened for specific cell markers by flow cytometry to determine CD8+, CD4+ T-cell and NK-cell populations and the results obtained are shown in figure 6.

In case of transdermal immunization, CD8+ T-cell populations were found to be elevated in all vaccinated mice when compared to control group as shown in figure 6 (A). Moreover, the inclusion of interleukins in the vaccine resulted in further elevation in this cytotoxic T-cell population ($p<0.05$). In case of immunization via combination of two routes, vaccine alone did not result in any significant increase in this cell count as shown in figure 6 (B). However, vaccine with interleukins resulted in higher population than vaccine alone as well as control group. This difference in CD8+ T-cells response in terms of route can be attributed to the dosing regimen followed for transdermal (10 doses of vaccine via skin) and combination route (5 doses of vaccine via skin and 5 doses via oral route).

In case of CD4+ T-cell titers, all vaccinated groups showed significant rise in level of these cells as shown in figure 6 (C, D). However, further elevation in CD4+ T-cell population was not seen upon inclusion of interleukins ($p<0.05$).

We also analyzed NK-cell populations in splenocytes. We found that all vaccinated mice demonstrated higher levels of NK-cells when compared to control mice. On the other hand, in case of transdermal vaccination, interleukins group showed lower NK-cell titers than the one obtained in vaccine alone group. However, the incorporation of interleukins resulted in even further elevation in case of combination routes other than transdermal vaccination as shown in figure 6 (E and F) ($p<0.05$). Transdermally vaccinated mice received vaccine particles via skin for 10 times; while combination route delivered 5 doses via skin and 5 oral doses administered alternatively. This NK-cell activity seen in combination appeared to play a major role in tumor suppression based upon overall analysis of immune response.

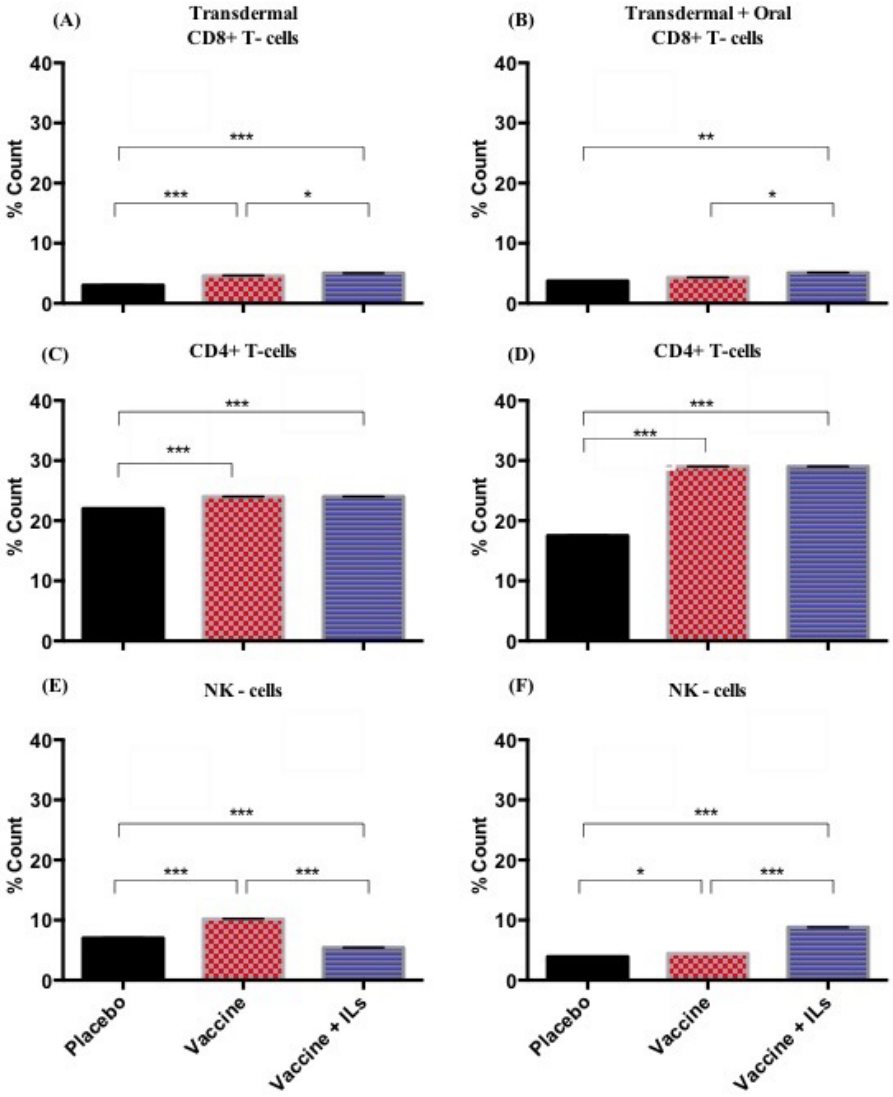

Fig. 6. T-cell based/ cellular immune response: CD8+ T-cells population determined by flow cytometry for mice treated with (A) Transdermal (B) Transdermal + Oral vaccination; CD4+ T-cells population determined by flow cytometry for mice treated with (C) Transdermal (D) Transdermal + Oral vaccination and NK-cells population determined by flow cytometry for mice treated with (E) Transdermal (F) Transdermal + Oral vaccination. *$p<0.05$, **$p<0.01$, ***$p<0.001$

The overall results obtained are summarized in table 1 to provide a statistical comparison between the two routes of vaccine delivery. The symbol '*' is used to indicate the statistically significant difference compared to control/ placebo mice (PL); while this difference is represented with letter 'a' for comparison between vaccine (V) and vaccine with interleukins group (V + IL). When data was analyzed for vaccinated and control/placebo mice, tumor suppression was seen in case of both transdermal and combination routes as indicated by '*'. Interleukins were found to result in further suppression only in case of combination routes as

indicated by ‘a’. Further the level of significance for each comparison is described in the table 1 caption. In case of interleukins, transdermal route showed around 3 times of tumor suppression and combination of routes resulted in around 9 times of tumor suppression, when compared to control mice. Thus, the combination route was found to the more effective route for suppression of tumor when vaccine was administered along with interleukins. In order to understand the mechanism behind this effect, we performed analysis of humoral as well as cellular response. As indicated in Table 1, for combination route, the vaccine with interleukins group showed elevated response in almost all humoral and cellular responses as represented by level of significance with letter ‘a’ when compared to vaccine alone group. This effect (indicated by letter ‘a’ in table 1) was not seen in case of transdermal vaccination to the extent seen in combination route. This correlated with the tumor retardation response seen in case of combination route when vaccinated with interleukins. Thus, the study concludes that the combination route of vaccine delivery in presence of interleukins provides an improved humoral/cellular immune response that is capable of providing protection against tumor growth, as seen with this murine model for ovarian cancer.

The overall stimulation of humoral and cellular response upon vaccination indicates the efficacy of the vaccine microparticles. Moreover, the immune stimulation in terms of humoral and cellular response obtained correlated with tumor volume retardation. This study indicated that humoral response as well as cellular response was needed for tumor suppression. This finding correlated with the study reported by Mahmoud SM et al., where it was found that the humoral immunity is important in addition to cell-mediated immunity in prognosis of breast cancer [26].

Table 1: Summary of results obtained from *in-vivo* study of ovarian cancer vaccine microparticles via two different study approaches based on route of administration
[1]at the end of 15th week, PL=Placebo, V= Vaccine, V + ILs= Vaccine with interleukins, *p<0.05**,** **p<0.01**,** ***p<0.001**,** ****p<0.0001, results expressed in comparison to mice treated with placebo particles within each study approach based on route of administration
[a]p<0.05**,** [aa]p<0.01**,** [aaa]p<0.001**,** [aaaa]p<0.0001 results expressed for comparison between vaccine and vaccine with interleukins groups within each study approach based on route of administration

In-vivo study	**Transdermal**			**Transdermal + Oral**		
	PL	V	V+ ILs	PL	V	V+ ILs
Tumor volume reduction[1]	-	****	****	-	* -	**** aaaa
IgG titers	-	**	**	-	** -	**** a
IgG1 titers (Th2 type response)	-	-	*	-	- -	*** aa
IgG2a titers (Th1 type response)	-	-	*	-	-	*
CD8+ T-cell population	-	*** -	*** a	-	- -	** a
CD4+ T-cell population	-	***	***	-	***	***
NK-cell population	-	*** aaa	*** -	-	* -	*** aaa

4. Conclusion

The efficacy of vaccine microparticles containing whole cell lysate of ID8 ovarian cancer cells in retarding tumor growth in murine models was demonstrated in this study via two different study approaches based on routes of administration. Thus, the microparticulate vaccine when given via combination of oral and transdermal routes provides a promising approach in terms of cost-effectiveness, ease of production and patient compatibility. Vaccine with interleukins when administered via combination of two routes was found to result in higher tumor suppression in correlation to cellular and humoral response as compared to other routes. This study was performed in prophylactic setting to check the efficacy of particles, which forms the strong basis for further studies to evaluate therapeutic efficacy of the particles in tumor bearing animals.

5. Conflict of Interest statement

The authors report no conflict of interest.

6. Acknowledgement

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