Original Article

Dendritic cells generated from naïve and tumor-bearing mice uniquely restores different leukocyte subpopulations in chemotherapy-treated tumor-bearing mice

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ABSTRACT

Background: Dendritic cell (DC)-based vaccination has shown promising application in tumor immunotherapy. However, it is not clear whether the presence of tumor impacts the efficacy of generation and functionality of DCs. **Aim:** To compare the phenotype of DCs generated from naïve or tumor bearing mice and their capability to restore leukopenia-associated chemotherapy. **Materials and Methods:** DCs were generated from bone marrow (BM) of naïve or Ehrlich ascites carcinoma (EAC) bearing mice. EAC is an undifferentiated breast cancer cell line with the high transplantable capability and rapid proliferation. BM cells were cultured *in vitro* for 7 days with granulocyte macrophage colony-stimulating factor and interleukin-4 (20 ng/ml each), loaded with different concentrations of EAC cell lysate (0.5, 1, 3 and 5 mg/10^6) DCs followed by activation with the toll-like receptor 3 ligand poly(I:C). For DC-based vaccination, CD1 mice (n = 5/group) were inoculated with an intraperitoneal (i.p.) injection of 0.25 × 10^6 EAC cells to form ascites, treated on day 14 with an i.p. injection of cyclophosphamide (4 mg/mouse) and on day 15 with subcutaneous injection of 2 × 10^6 DCs from control or EAC bearing mice. Injected DCs were loaded with or without EAC lysate followed by i.p. injection of 50 µg/mouse poly(I:C). On day 21, mice were bled and sacrificed for peripheral blood count and spleen and BM cellularity. **Results:** Yield of DCs generated from naïve or EAC bearing mice, as well as their phenotype ($CD11c^+$ $CD11b^+$) and activation (CD40 and CD80) with poly(I:C) were similar. Loading DCs with 1 mg EAC lysate induced better viability and activation phenotype as compared with the other concentrations. Regardless the source of DCs, DCs vaccination restored the total numbers of leukocytes in blood but not in the spleen and BM. The effect on peripheral blood leukocytes was coincided with the restoration of the relative numbers of lymphocytes, monocytes, and granulocytes. **Conclusion:** These data support the use of allogenic DCs from healthy donors in anticancer DC-based vaccination.

Key words: Cancer, chemotherapy, dendritic cells

INTRODUCTION

Dendritic cells (DCs), the most potent antigen-presenting cells (APCs), bridge innate and adaptive immunity,[1] with the efficient capability to prime naive T cells.[2] DCs respond to antigens and molecules coding for pathogen-associated molecular patterns or damage-associated molecular patterns, together are called danger signals, which induces a plethora of proinflammatory cytokines.[3] DCs respond to these harmful molecules by efficient antigen uptake, processing, and presentation resulting in robust induction and maintenance of tumor-specific cytotoxic T lymphocyte (CTL) responses.[4] Given these features of DCs, they have been extensively used in tumor immunotherapy

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in preclinical studies which resulted in several clinical trials with the goal to elicit or amplify immune responses against cancer and chronic infectious diseases.[5] Although a remarkable amount of data has been obtained from these studies, defining approaches that can further augment this cell-based therapy is required.[6-8]

A critical issue in the development of DC-based vaccines and their ability to stimulate immune responses depends largely on the activation status of DCs. It is not clear, however, whether tumor microenvironment impacts the phenotype, activation, and functionality of DCs generated from this microenvironment. Our and others recent studies have reported that preconditioning a recipient host with cyclophosphamide (CTX) creates host microenvironment that can boost T-cell responses to active vaccination, including DC-based vaccination.[9-12]

These beneficial effects of CTX preconditioning regimen were found to be associated initially with rapid induction of leukopenia (from days 1 to 7) followed by the restoration of the host cellularity which reaches the normal values by day 21.[13] Interestingly, the restoration phase was accompanied by increases in the numbers of DCs in the peripheral blood from days 9 to 16 which were responsive to stimulation with the toll-like receptor 3 (TLR3) agonist poly(I:C).[14]

How this host cellularity responds to active vaccination, in particular, DC-based vaccine, needs to be addressed. Further, how the tumor microenvironment shapes the DC vaccine also needs to be investigated. In this study, we investigated the effects of antigen loading and DC stimulation on the yield, phenotype, and activation of DCs generated from mice with or without tumor using Ehrlich ascites experimental model. We also investigated the effects of the generated DCs on the recovery of the host from CTX-associated leukopenia. The obtained results concluded that yield, phenotype, and activation status of DCs generated *in vitro* from bone marrow (BM) are not altered by the tumor microenvironment. Further, these cells are capable of inducing restoration of chemotherapy-associated leukopenia. This study opens a new avenue in the application of allogenic DCs in vaccination in cancer immunotherapy.

MATERIALS AND METHODS

Mice

Adult female Swiss albino mice (CD1 strain; from National Research Center, Cairo, Egypt) weighting 20 ± 2 g were used in this study were purchased. Mice were housed (5 animals per cage) at the animal facility, Zoology Department, Faculty of Science Tanta University, Tanta, Egypt in clean and dry plastic cages and at in 12 h/12 h dark/light cycle under laboratory condition of temperature and humidity. The mice were fed with rodent pellets and tap water *ad libitum*. This study was performed in accordance to guidelines of the use of experimental animals in research at Zoology Department, Faculty of Science, Tanta University, Tanta, Egypt.

Cell lines

Ehrlich ascites carcinoma (EAC) is referred to as an undifferentiated breast carcinoma cell line that is originally generated from female CD1 mice with the high transplantable capability and rapid proliferation, making them sensitive to chemotherapy. EAC bearing mice have short life span (~21 days).[15] The tumor cell line was maintained by means of bi-weekly serial intraperitoneal (i.p.) transplantation of 2.5 × $l0^6$ viable tumor cells in 0.3 ml of saline into female Swiss albino mice (8–10 weeks old). The cell viability was assessed using Trypan blue assay and counted by hemocytometer before injection into naïve CD1 mice for experimentation.

Reagents

CTX (CTX; Sigma-Aldrich Co., USA) was dissolved in phosphate-buffered saline (PBS) and frozen until used. The following monoclonal antibodies were used in this study: PE-labeled anti-mouse CD11c and APC-labeled anti-mouse CD11b were purchased from eBioscience (San Diego, CA, USA). Murine recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) cytokines were purchased from R and D systems (Minneapolis, MN, USA). Roswell Park Memorial Institute medium (RPMI) 1640 (Invitrogen) was supplemented with heat-inactivated fetal bovine serum (FBS) (10% v/v) and 2-mM L-glutamine (Invitrogen), penicillin-streptomycin mixture with 100 IU/ml penicillin and 100 μg/ml streptomycin, 1-mM sodium pyruvate, and nonessential amino acids (Invitrogen). ACK Lysis buffer was purchased from Lonza, BioWhittaker, USA. Antibodies were used at concentrations recommended by their manufacturers.

Generation of dendritic cells *in vitro*

DCs were generated from BM precursors of CD1 mice. BM cells were flushed from tibia, and femur and single cell suspension were suspended in complete RPMI-1640 medium (1 × 10^6/ml) with 10% FBS and 1% P/S, 20 ng/ml of recombinant murine GM-CSF and 20 ng/ml of recombinant murine IL-4 were added. Three milliliters of the cell suspension was plated in each well of a 6-well plate followed by a 7 days culture at 37°C, 5% CO_2, and 95% humidity. On day 4, 1.5 ml of medium from each well were carefully removed and replaced by freshly prepared complete RPMI media containing GM-CSF and IL-4 (each 20 ng/ml). On day 7, DCs were pulsed with PBS or different concentration of tumor lysate for 24 h. Then, the cells were activated with

25 μg/ml of poly(I:C) for 24 h. Then, DCs were harvested by carefully pipetting and collected in a 50 ml Falcon tube for counting and phenotypic analysis of generated DCs was confirmed by flow cytometry. The cell number was determined using a Neubauer counting chamber. A sample of 10 μl was diluted with Trypan blue in a ratio 1:10 and four quadrates were counted and divided by four to attain a precise number of cells. The following formula was used:

$N \times 10^4 \times$ dilution factor (10) = cell number/ml

Flow cytometry

Single-cell suspensions were prepared and counted as has been previously described. DCs were labeled with anti-mouse PE-CD11c, FITC-Ly6-G, and APC-CD11b antibodies (eBioscience) for 30 min at dark incubation 4°C. The cells were washed twice with PBS and then resuspended in 0.3 ml of PBS supplemented with 0.5% BSA and 0.02% sodium azide. Cells were then washed and acquired by Partec flow cytometer (Sysmex-Partec Company, Germany). The analysis was performed with FlowJo software (Treestar, Ashland, OR, USA).

Preparation of tumor cell lysate and pulsing dendritic cells

In order to make intracellular proteins accessible for DCs pulsing with tumor antigens. The ascetic fluid was collected using a syringe, and the EAC cells were counted using a Neubauer hemocytometer, and the cell viability was determined by using Trypan blue dye exclusion assay. EAC cells were washed twice, resuspended in PBS at a density of 5×10^6 cells/ml, and stored in liquid nitrogen until use. Frozen tumor cells were lysed by four repetitive freezing–thawing cycles (freezing at −80°C and thawing at 37°C). Lysis was monitored by light microscopy, and the cell debris were centrifuged (2000 rpm, 7 min, 4°C) and the supernatants were filtered through 45 μm cell strainers and collected in a sterile Falcon tube with 2 ml of ice-cold PBS.[16] Protein concentration was determined by Bradford assay according to manufacturers' instruction.[17] The cell lysate was kept in small aliquots (1 ml/tube) sterile PBS until use.

Tumor challenge, chemotherapy, and dendritic cell vaccination

CD1 mice (n = 5/group) were inoculated with i.p. injection of 0.25×10^6 EAC cells. On day 14, mice were treated with 4 mg CTX/mouse. On day 15, mice were vaccinated subcutaneous (s.c.) injection of 2×10^6 DCs generated either from naïve or EAC bearing mice and loaded with 1 mg of EAC lysate. DC vaccination was followed by an i.p. injection of 200 μg/mouse poly(I:C) (100 μg i.p. and 100 μg at the site of vaccination) as we previously described.[18-20] On day 21, mice were bled and sacrificed to measure peripheral blood count, the yield of spleen, and BM.

Complete blood count analysis

Mice were anesthetized by inhalation of isoflurane (1-chloro-2, 2, 2-trifluoroethyldifluoromethyl ether; Hospira, Inc., Lake Forest, IL, USA) and bled from the orbital sinus using heparinized microhematocrit tubes into 1.5-ml Eppendorf tubes. Samples were analyzed for the total number of leukocytes using an automated instrument for complete blood counts (VetScan HM2™ Hematology System, Abaxis, Union City, CA, USA) to determine white blood cells (WBCs,) platelets, relative and absolute number of neutrophils, and lymphocytes.

Preparation of spleen cell suspensions

Spleen cell suspensions were prepared according to Salem *et al*.[21] Briefly, the spleen was homogenized by gently pressing between the rough ends of two glass slides and filtered through nylon mesh filters (100 μm; BD Biosciences, CA, USA). The cells were suspended in RPMI (Sigma Chemical Co., St. Louis, USA) and washed twice. Red blood cells were lysed with ammonium chloride-potassium buffer (ACK; Invitrogen, Carlsbad, CA, USA) and the remaining cells were again washed 3 times and counted. Viability was determined by Trypan blue exclusion and consistently exceeded 90%.

Statistics

Numerical data obtained from each experiment were expressed as a mean ± standard error and the statistical differences between experimental and control groups were assessed using one-way analysis of variance. The $P \leq 0.05$ were considered statistically significant.

RESULTS

Characterization of dendritic cells generated from naïve and tumor-bearing mice

The result revealed that the BM cell count from naïve and tumor-bearing mice were 23×10^6 and 29×10^6 per mouse, respectively, indicating that the yield of DCs from naïve or tumor bearing mice is similar. Furthermore, the microscopic investigation showed that the shape of DCs changed greatly throughout the days of the cell culture. On day 0, the majority of the cultured cells were round, oval-shaped, and small in size. On day 4, cells with dendrites appeared in the culture and significantly increased by day 6, indicating the differentiation of monocytes, as well as DC precursors into DCs. On day 7, the majority of DCs were already detached from the plate where cells with typical DC morphology still appear in the culture [Figure 1a]. Interestingly, BM culture from EAC bearing mice showed similar shape of DCs on day 7 [Figure 1b].

When the responses of DCs generated from naïve and EAC bearing mice to stimulation with the TLR3 ligand poly(I:C)

were compared, both DCs responded similarly as measured by the expression of the costimulatory molecules CD40 and CD80 [Figure 2]. As such, poly(I:C) was used in subsequent experiments to stimulate tumor lysate-loaded DCs. The result showed that poly(I:C) induced significance increase of the expression of CD40 (by 1.9-fold) and CD80 (1.3-fold) for DCs generated from naïve mice and activated with poly(I:C) compared to DCs with no poly(I:C). Meanwhile, poly(I:C) induced increases in the expression of CD40 by 1.4-fold and CD80 by (1.1-fold) on DCs generated from EAC bearing mice as compared to DCs with no poly(I:C) [Figure 2a and b].

Impact of different concentrations of tumor lysate on viability and phenotype of dendritic cells

Pulsing of DCs on day 6 with different concentrations of EAC lysate (0.5 mg, 1 mg, 3 mg, and 5 mg), followed by activation with poly(I:C) revealed that 1 mg had no effect on the viability of DCs as compared to the other concentrations which decreased the viability of DCs [Figure 3]. In addition, the numbers of DCs which express total CD11c were higher (20.86%) when loaded with 1 mg EAC lysate as compared to those loaded with 0.5 mg (18.26%), 3 mg (17.99%), and 5 mg (12.41%), respectively, and to unloaded DCs activated with poly(I:C) (36.4%) as control. As such, we selected 1 mg in subsequent experiments to pulse DCs for vaccination of EAC-bearing mice [Figure 4].

The effect of dendritic cells vaccine on the total number of white blood cells, spleen, and bone marrow

Mice were inoculated with an i.p. injection of 0.25×10^6 EAC cells. On day 14, mice were treated with 4 mg CTX followed 1 day later with s.c. injection of 2×10^6 DCs generated either from naïve or EAC bearing mice loaded with or without 1 mg EAC cell lysate. The vaccination was followed immediately by an i.p. injection of 50 μg/mouse poly(I:C). On day 21, mice were bled for peripheral blood count and sacrificed for spleen and BM cellularity.

The results revealed that treatment of EAC-bearing mice with CTX alone has no effect on the number of spleen cells while it decrease the number of BM cells as compared to untreated EAC-bearing mice. Regardless the source of DCs (naïve or EAC mice) or loading with EAC lysate, treatment of EAC-bearing mice with DCs significantly decreased the numbers of spleen cells and BM as compared to CTX-treated EAC bearing. Of note, nDCs ± EAC lysate showed relatively but not significant more decrease in the total number of BM as compared tDC ± EAC lysate [Figure 5a and b].

The result showed that treatment of EAC-bearing mice with CTX alone significantly decreased the total number of WBCs compared to EAC-bearing mice. However, treatment with DC from naïve (nDC) ± EAC lysate, as well as unloaded DCs from EAC bearing mice (tDC) ameliorated the effect of CTX. In contrast, treatment with tDC loaded with EAC lysate induced a higher decrease in the number of WBCs as compared to treatment with CTX for EAC bearing mice [Figure 5c].

Effect of dendritic cell vaccine on differential cell numbers of peripheral blood mononuclear cells

Treatment of EAC-bearing mice with CTX alone has no effect on the relative number of lymphocytes as compared to EAC-untreated mice. However, Vaccination of EAC mice with nDC ± EAC lysate after treatment with CTX induced significant increases in the relative and absolute numbers of lymphocytes and absolute number of monocytes as compared to CTX-treated EAC-bearing mice. In contrast, vaccination with DCs from EAC bearing mice loaded with tumor lysate induced significant decreases in the relative numbers of lymphocytes and absolute number of monocytes. These data suggest that DCs from naïve EAC-bearing mice have opposing effects on the relative and absolute numbers of lymphocytes and monocytes [Figure 6a and b].

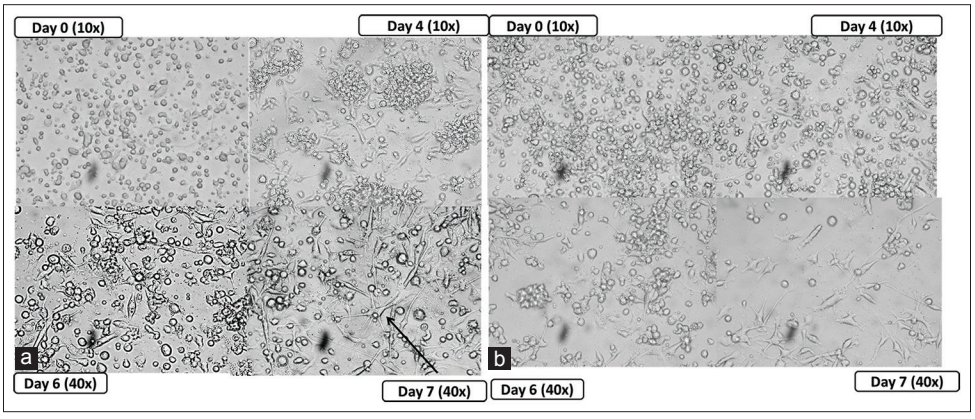

Figure 1: The morphology of dendritic cells: Dendritic cells were induced from mouse bone marrow cells. Bone marrow cells were obtained from murine bone marrow and induced in medium with 20 ng/ml interleukin-4 and granulocyte macrophage colony-stimulating factor for 7 days. On the day (0, 4, 6, and 7) under an inverted microscope. (a) Dendritic cells generated from naïve mice. (b) Dendritic cells generated from tumor-bearing mice

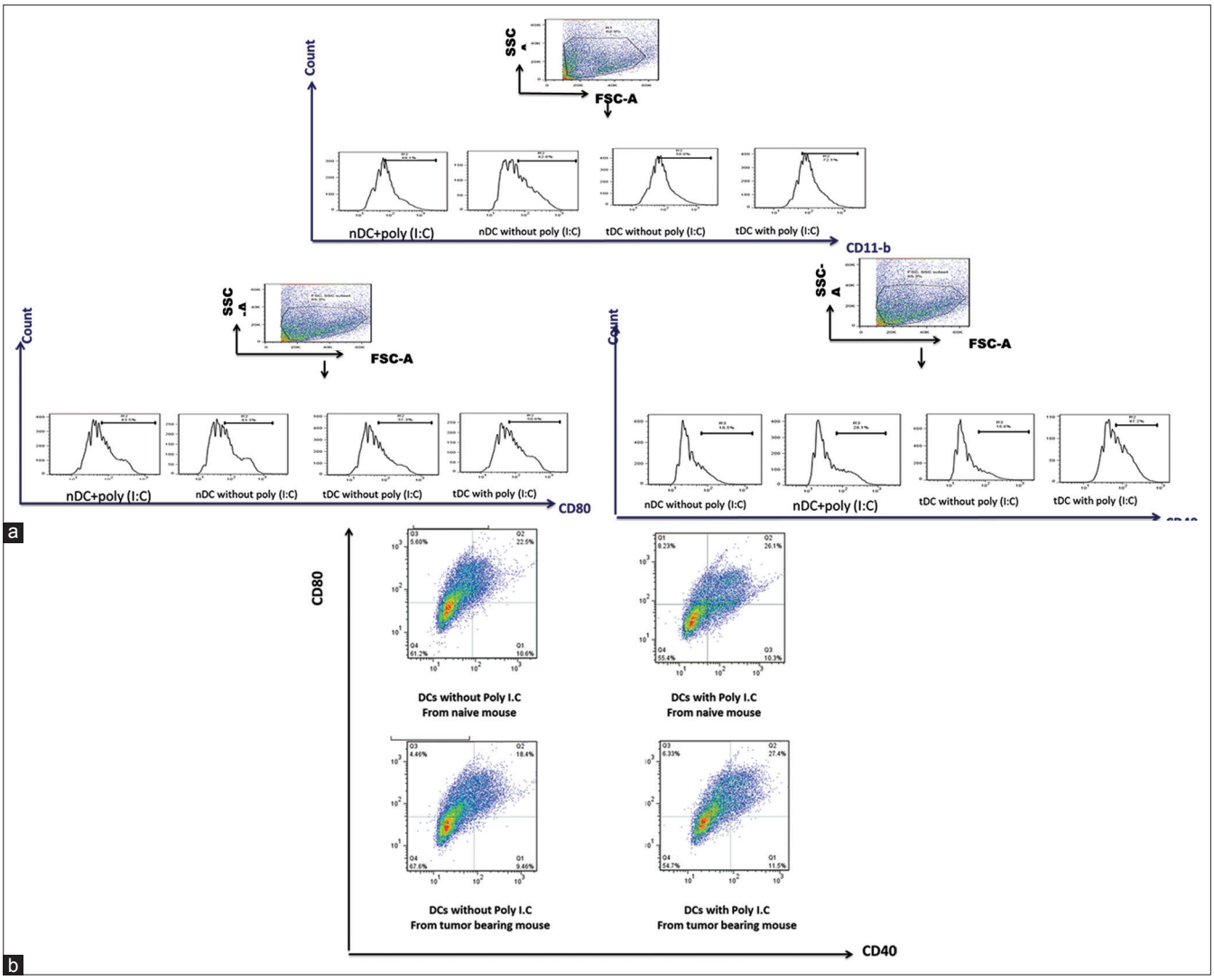

Figure 2: (a) Gating strategy of CD11b, CD40, and CD80 of Dendritic cells population: Bone marrow cells were obtained induced in medium with interleukin-4 and granulocyte macrophage colony-stimulating factor for 7 days. Then stained with anti-mouse CD11c, Ly6-G, CD11b, CD40, and CD80 antibodies. (b) Dot plot flow cytometry analysis of the effect of poly(I:C) on the maturation of *in vitro* generated dendritic cells. Dendritic cells were generated from naïve or Ehrlich ascites carcinoma mice. Bone marrow cells were stimulated *in vitro* with murine granulocyte macrophage colony-stimulating factor (20 ng/ml) and interleukin-4 (20 ng/ml) at 1 × 106 cells/ml and incubated at 37°C with 5% CO_2. On day 6, media were supplemented with or without 25 μg/ml poly(I:C). Generated nonadherent dendritic cell were harvested on day 7. Generated dendritic cells were analyzed by flow cytometry. (b) Data represent the cells were labeled with the cell surface markers CD40, CD80

Interestingly, treatment of EAC-bearing mice with CTX alone decreased the relative and absolute number of neutrophils, where vaccination with tDCs induced significant increases in the absolute number of neutrophils as compared to CTX-treated EAC mice [Figure 6c].

DISCUSSION

In this study, we investigated the microscopic, phenotypic characteristics of DCs generated from naïve or EAC tumor-bearing mice loaded with or without EAC lysate. The effects of vaccination with these cells on the host cellularity of immune cells were compared. The microscopic investigation showed that DCs generated from naïve or tumor bearing mice expressed similar kinetics of changes in morphology in the culture. These data are consistent with previous studies addressing, most of DCs generated from mouse BM,[22,23] as well as to the culture of monocytes from human BM peripheral blood[24] had expanded cytoplasm and small dendrite-like structures. Their responses to stimulation with TLR3 ligand poly(I:C) were also similar as measured by the expression of the costimulatory CD40 and CD80. Importantly, both cells were capable of inducing restoration of the host cellularity of immune cells. Taken together, it does not exclude they are functionally different. Indeed, *in vivo* studies indicate that both DCs functionally different since they had an opposing effect on certain cell subsets and no effect on other subsets.

Few preclinical and clinical studies have investigated the impact of tumor on the quality and quantity of *ex vivo* generated DCs may be because most of DC-based

vaccination depends on autologous DCs from cancer patients. However, vaccination with allogenic DCs has been found to effectively induce favorable antitumor immunity in several preclinical[3,24] and clinical[25-27] studies. The clinical efficacy of allogenic DCs led to the preparation of on-shelf DCs vaccine.[28,29] As such, this study was designed to test whether generated from naïve and tumor-bearing host is similar in phenotype and function. DCs can be generated from BM, spleen, or peripheral blood in particular after mobilization with the growth factor G-CSF, which increase the numbers stem cell and myeloid cell precursors in BM and accelerate their and release into circulation.[30-33] We used the conventional method of generation of DCs from monocytes in BM using GM-CSF and IL-4 to avoid the impact of G-CSF on these cells since it has been reported to skew the phenotype and function of the generated DCs to plasmacytoid rather than myeloid DCs.[34-37]

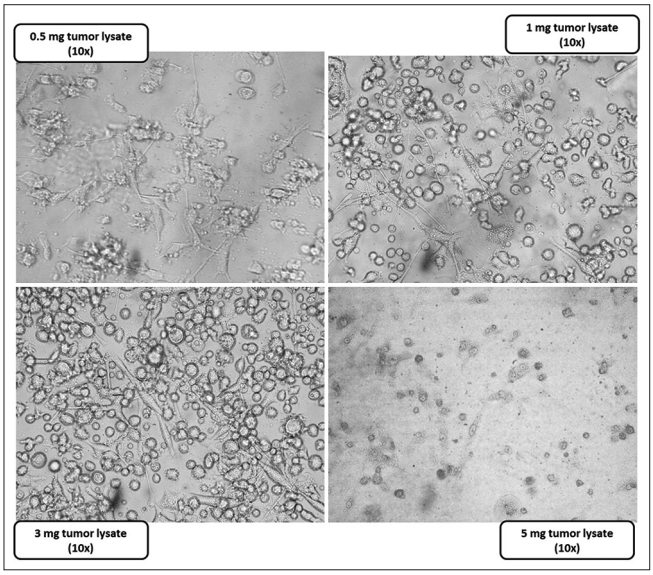

Figure 3: The impact of different concentrations of tumor lysate on the viability of dendritic cells: Bone marrow cells were obtained from murine bone marrow and induced in Roswell Park Memorial Institute medium with interleukin-4 and granulocyte macrophage colony-stimulating factor for 7 days. On day 7, Cells were loaded with 0.5 mg, 1 mg, 3 mg, and 5 mg tumor lysate for 24 h. Followed by activation with poly(I:C) then observed under inverted microscope

To investigate whether the presence has any qualitative or quantitative effects impacts DC generation, mice were inoculated with EAC to form tumor ascites in about 7 days and then generated DCs from the BM of these mice, as well as from control mice with no tumor. Analysis of DCs after 7-day BM cultures from both groups of mice showed similar total numbers, viability, and phenotype indicating that DC precursors (mostly monocytic lineage) in BM from control and tumor-bearing mice have similar capability of the kinetics of differentiation into DCs.[38,39] DCs generated from control BM and from EAC BM showed the typical phenotypes of myeloid DCs ($CD11c^+$ $CD11b^+$). They also responded similarly to the stimulatory effects of the TLR3 agonist poly(I:C) as DCs from both sources expressed high levels of the costimulatory molecules CD40 and CD80 on

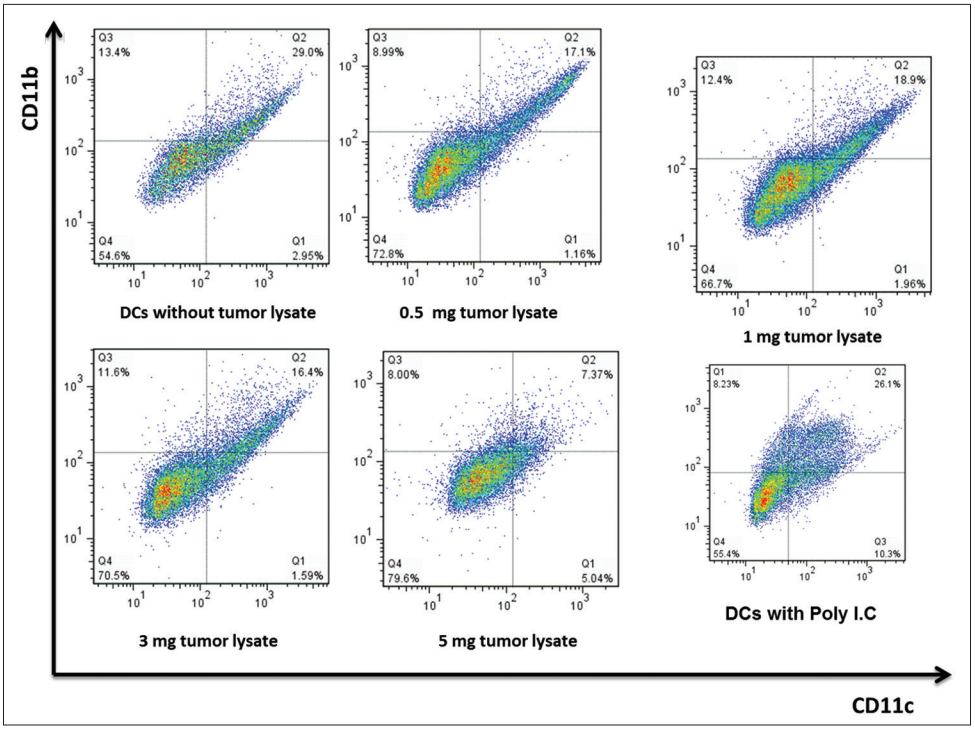

Figure 4: Phenotypic analysis of bone marrow derived dendritic cell (BMDCs) following loading with tumor lysate: Dendritic cells treated with various concentrations of tumor lysate include 0.5 mg, 1 mg, 3 mg, and 5 mg. Cells were labeled with fluorophore-conjugated antibodies against the cell surface markers CD11b, CD11c, and Ly6G fixed in 2% paraformaldehyde solution and analyzed by flow cytometry. Cells were gated on size (forward scatter) and granularity (side scatter) patterns for doublet exclusion, and expression of the cell surface marker CD11c was used for dendritic cell selection

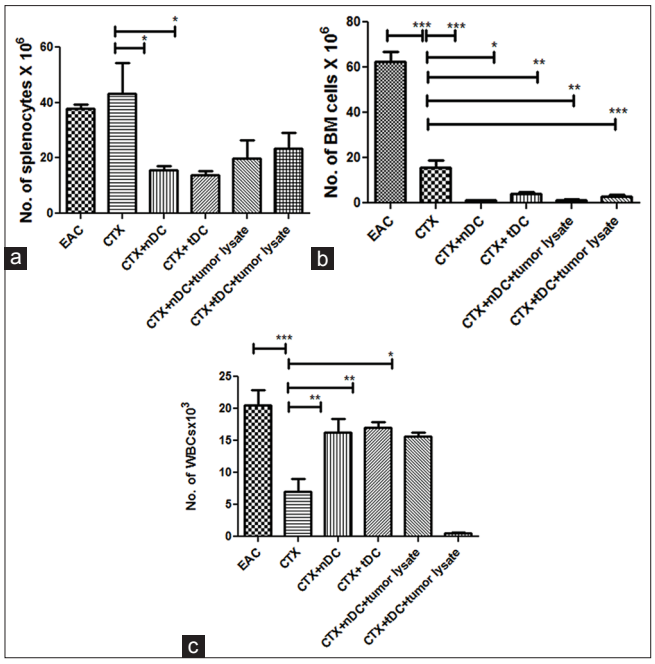

Figure 5: The effect of dendritic cells vaccine on. (a) Total number of spleens cells. (b) Total number of bone marrow cells. (c) White blood cells. Mice were inoculated with an intraperitoneal injection of 0.25 × 106 Ehrlich ascites carcinoma. On day 14, mice were treated with 4 mg cyclophosphamide followed 1 day later with a subcutaneous injection of 2 × 106 dendritic cells generated either from naïve (nDC) or Ehrlich ascites carcinoma-bearing mice (tDC) loaded with or without 1 mg Ehrlich ascites carcinoma lysate. On day 21, mice were bled for peripheral blood count and sacrificed for spleen and bone marrow cellularity. (a) Represent total number of spleen. **P* < 0.05; ***P* < 0.01, as compared with cyclophosphamide (positive control)

their surface. Several studies have established that poly(I:C) *per se* is a potent stimulator of DCs.[22,40] In addition, the *in vitro* studies show nDC and tDC are similar in their number and phenotypic characteristics, it does not exclude they are functionally different. Indeed, *in vivo* studies indicate that both DCs functionally different since they had an opposing effect on certain cell subsets and no effect on other subsets. These data are consistent with previous studies showing that poly(I:C) induces maturation of both mouse and human DCs,[40] inducing them to express stable and high levels of the costimulatory molecules.[41] Taken together, our studies, although pilot, indicate that the presence of tumor, at least in our EAC model system, does not impact the generation, phenotype, and activation of DCs.[42-46]

To explore whether loading of the generated cell with tumor lysate influences their quality, we compared the viability of DCs generated from naïve or EAC bearing mice with different concentrations of EAC tumor lysate. We used tumor lysate as it contains a vast array of immunogenic epitopes to activate both $CD4^+$ and $CD8^+$ tumor-specific T cells to prevent tumor escape, in particular, this approach has been found to be promising to augment DC-whole tumor antigen-based vaccination.[47] Our result revealed that 1 mg EAC lysate had no effect on the viability of DCs as compared to the other concentrations. Moreover, the phenotypic analysis of the

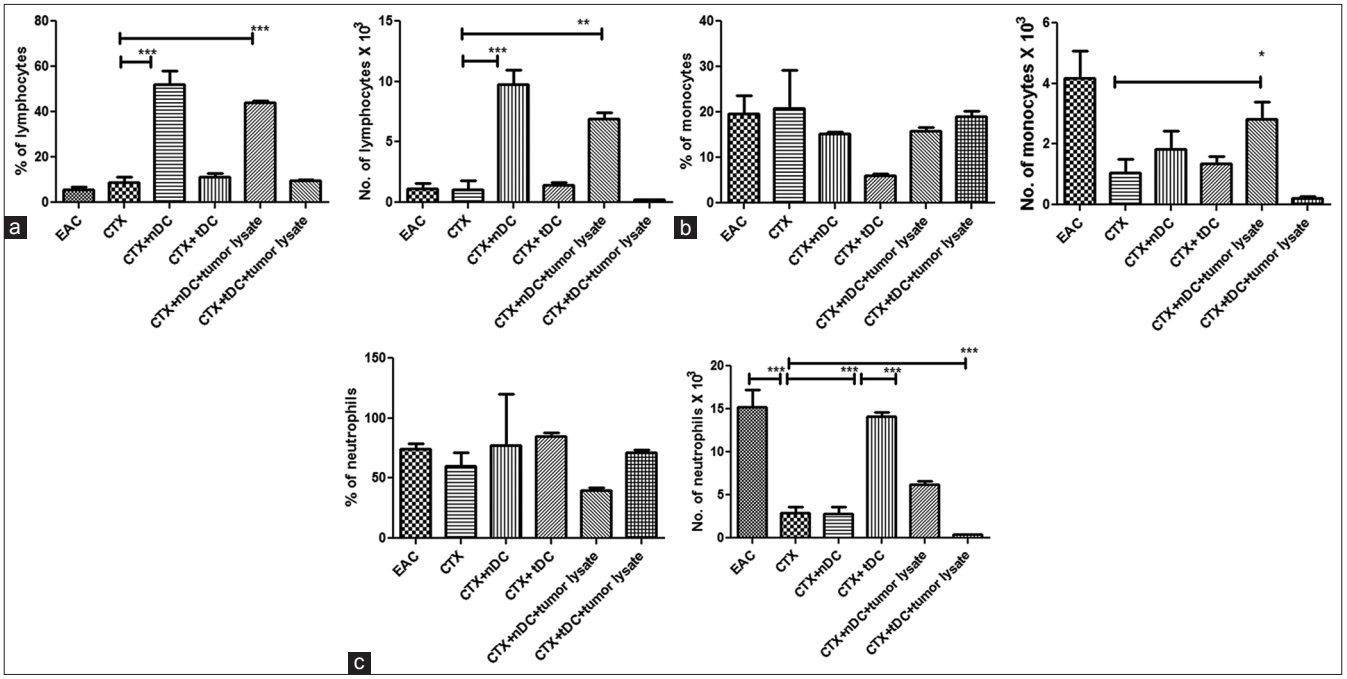

Figure 6: Effects of dendritic cell-based vaccination on the relative and absolute number of (a) Lymphocytes. (b) Monocytes, and (c) Neutrophiles: Mice were inoculated with 0.25 × 106 Ehrlich ascites carcinoma. On day 14, mice were treated with 4 mg cyclophosphamide followed 1 day later with an injection of 2 × 106 dendritic cells generated either from naïve or Ehrlich ascites carcinoma-bearing mice ± 1 mg Ehrlich ascites carcinoma lysate followed by an intraperitoneal injection of poly(I:C). On day 21, mice were bled for PB count. **P* < 0.05; ***P* < 0.01, as compared with cyclophosphamide (positive control)

DCs expressing CD11c was higher on SCs loaded with 1 mg versus other concentration and unloaded DCs. These data in line with the previous finding that 1 mg/ml of ovalbumin have to be fed to DCs before ovalbumin-specific clones are activated.[48,49] Taken together, we selected the concentration of 1 mg to pulse DCs for vaccination of EAC mice. We then compared the effect of the generated DCs on the host cellularity of immune cells. We used CTX, as an anticancer drug, as well as to induce transient leukopenia in peripheral blood [Figure 5b and c].[50-53]

Indeed, we have reported recently the precise kinetics and mechanisms of CTX-induced leukopenia in mice and the detailed alteration in the host cellularity. Using this model system, we found that vaccination with tumor antigen-loaded DCs generated either from control or EC-bearing mice similarly corrected the leukopenia induced by CTX in spleen, BM, and peripheral blood suggesting that both DCs are biologically functional in term of induction of stimulation of leukopenia-induced homeostatic proliferation of the host immune cells.

Our result is in agreement with previous studies showing that DC-based immunotherapy combined with chemotherapy is safe and effective in patients with advanced pancreatic cancer refractory to standard treatment through inducing expansion of leukocytes in peripheral blood, as well as the percentage of lymphocytes that restore the leukopenia associated with chemotherapy.[54] A prior study also showed that very low concentrations of different chemotherapeutic agents increase the ability of DC vaccine to induce T cell proliferation.[55]

Interestingly, vaccination with tDCs induced significant increases in the absolute number of neutrophils as compared to CTX-treated EAC mice [Figure 6c]. It could be suggested that tDC had some suppressor factors such as TGF-β, prostaglandin E2, as well as IL-10 which are known to induce expansion myeloid-derived immunosuppressive cells. Alternatively, these cells might express some co-inhibitory molecules such as PD-1 and CTLA-4, which act as a break for T-Cell responses;[56-58] however, this hypothesis need investigation in our model system.

Although we have not analyzed the mechanisms mediating the modulatory effects of the DC vaccine on the host cellularity of immune cells, it could be mediated, at least in part by stimulation of the endogenous DCs. With this regard, we have reported previously that single treatment of mice with the same dose of CTX (4 mg/mouse) can induce a substantial expansion of DCs in the peripheral blood during the recovery from lymphopenia, peaking on day 12[18] associated with higher numbers of proliferating cells with DC phenotype ($CD11c^+$ $CD11b^+$) in BM peaking on 3 days of CTX treatment.[22] These studies indicate that post-CTX BM is rich in DC precursors with higher tendency to differentiate into DCs which migrate to circulation. In another study, we found that treatment of CTX preconditioned mice with poly(I:C), which we used in this study, in combination with DC vaccine induces a robust activation of the DCs in the blood and their migration to lymph nodes. Accordingly, we suggest that endogenous DCs in CTX-treated mice may mediate the effects of vaccination with exogenous DCs. This hypothesis, however, requires further studies.

CONCLUSION

DCs can be generated *ex vivo* from BM-derived progenitors from naïve or tumor bearing mice with similar microscopic and phenotypic characteristics. In addition, the *in vitro* studies show nDC and tDC are similar in their number and phenotypic characteristics, it does not exclude they are functionally different. Indeed, *in vivo* studies indicate that both DCs functionally different since they had an opposing effect on certain cell subsets and no effect on other subsets. The effect on peripheral blood leukocytes was coincided with the restoration of the relative numbers of lymphocytes, monocytes, and granulocytes. This study opens new avenues for vaccination with allogeneic DC-based vaccines and provides the knowledge and advances to design rational and efficient on shelf DC-based vaccine treatments to achieve long-term clinical response.

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Conflicts of interest

There are no conflicts of interest.

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