Original Article

Efficacy of Combined Administration of Chemoimmunotherapy with Bone Marrow Cells or Granulocyte-colony Stimulating Factor-mobilized Stem Cells on Expansion of Myeloid and Stem Cells

Soha Gomaa Ramadan Abdel Salam[1], Mohamed Labib Salem[1,2], Mohamed Nassef[1], Said Hammad Abdu[2,3], Rania El-Adl[1]

[1]Department of Zoology, Immunology and Biotechnology Division, Faculty of Science, Tanta University, [2]Center of Excellence in Cancer Research, Tanta University, [3]Department of Clinical Pathology, Faculty of Medicine, Tanta University, Tanta, Egypt

Abstract

Background: Integrating immunotherapy and chemotherapy is most likely to be the basis for new optimism in targeting cancer therapies to form local tumor microenvironment and attack tumors early in their development. This regimen has some potential risks such as myelo- and immunosuppression and chemo-resistant tumor cells. **Aim:** The present study aimed to investigate the combination of chemotherapy, immunotherapy, and the prospective of mobilized stem cells for optimization and modulation of the immune system to overcome immunosuppression and kill distant cancer cells. **Materials and Methods:** Ehrlich ascetic carcinoma (EAC) cell line-bearing mice treated with cyclophosphamide (CTX) followed by adoptively transferred with *in vitro*-activated T-cells either harvested from naïve or EAC-bearing host with or without unfractionated bone marrow (BM) cells or granulocyte-colony stimulating factor-mobilized hematopoietic stem cells (HSCs) 1-day post-CTX treatment. All mice were vaccinated with EAC lysate and Hiltonol. **Results:** Cotransfer of activated T-cells obtained from EAC-bearing mice with HSC-progenitors induced the highest antitumor effect through increasing the percentage of apoptosis and decreasing DNA replication in S phase of EAC cells. Besides, marked an increase in the percentage of myeloid cells in spleen and stem cell populations in BM cells. Interestingly, Adoptive T-cell transfer (ACT) derived from EAC-bearing host with or without BM cells induced mobilization of stem cells from BM to circulation increasing their expansion. **Conclusion:** Combination of chemotherapy with ACT plus vaccination may constitute a potent antitumor therapy that provides more efficacious antitumor responses when it is combined with BM cells fostering more effective antitumor immunotherapy strategies.

Keywords: *Adoptive T-cell transfer, cancer, chemotherapy, immunotherapy*

Introduction

Our previous studies and others have been used chemotherapy in conjunction with immunotherapy to generate immune responses against tumors.[1-5] Because the two forms of treatment are considered to be antagonistic,[6,7] and they have the benefit of planned short duration combined therapy with long remission and achieves disease control and survival prolongation, chemoimmunotherapy has been the current standard treatment regimen.[8]

Adoptive T-cell transfer (ACT) is a potential approach which has emerged as a promising advance in cancer immunotherapy after lymphodepletion.[9,10] An immune lymphodepletion can be induced in the recipient host by treatment with anticancer chemotherapeutic drugs, such as cyclophosphamide (CTX), before adoptive transfer of *in vitro*-activated T cells, and this regimen markedly improved the survival, persistence, and antitumor efficacy of the transferred T-cells.[11,12] In addition, the preconditioning of host with lymphodepletion regimens by CTX before adoptively transferred T cells in response to vaccination effectively augment the antitumor efficacy[13-16] and depress the number of regulatory T-cells and myeloid-derived suppressor cells.[17] Furthermore, combinations of adoptive transfer of T cells and specific vaccination against the cognate antigen can be envisaged to more enhancement of the effectiveness of conventional cellular therapies.[18] Combination of CTX with growth factors such as granulocyte-colony stimulating factor (G-CSF) has been

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Address for correspondence:
Dr. Soha Gomaa Ramadan Abdel Salam, Department of Zoology, Immunology and Biotechnology Division, Faculty of Science, Tanta University, Tanta, Egypt. E-mail: sohassd@science.tanta.edu.eg

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widely used for the mobilization of hematopoietic stem cells (HSCs) from bone marrow (BM) to circulation for correction of leukopenia and selective mobilization of dendritic cell (DC) progenitors *in vivo*,[19-21] as it acts on the residual progenitors in BM after CTX treatment.[22] Besides, HSCs can augment the reconstitution of the lymphoid compartment by increasing the expansion and survival of host cells as well as adoptively transferred T cells leading to destruction of large tumor burdens in the absence of cancer vaccines.[23] Moreover, G-CSF administration continuously for 5 consecutive days enhances both the induction of myeloid cell recovery or disease-related myelosuppression and the mobilization of progenitor cells,[24-27] increasing the content of progenitor cells in the BM and change their biological characteristics as well, making them similar to peripheral blood (PB) stem cells.[28] This might modulate the immunological network, activation of lymphocytes and granulocytes and shorten the duration of neutropenia following chemotherapy.[29,30]

Despite chemoimmunotherapy achieves disease control and survival prolongation and becomes the current standard cancer treatment, it has potential risks such as myelo- and immunosuppression and their consequences. Therefore, the present study focused on the evaluation of the efficacy of cotreatment of ACT with BM progenitor cells or G-CSF-mobilized stem cells after chemotherapy on the expansion of both myeloid cells and HSCs in PB and spleen in addition to investigate the changes in the DNA content of tumor cells after chemoimmunotherapy.

Materials and Methods

Mice

Female Swiss albino mice (4–6 weeks old and weighed between 22 and 25 g) were obtained from Company for Biological Products and Vaccines (VACSERA), Cairo, Egypt. Mice were handled and kept in a specific pathogen-free facility at Faculty of Science, Tanta University in accordance with the ethical guidelines of the local Institutional Animals Care and Use Committee. A total of 27 mice were randomly divided into nine groups of three each, including 1 control group.

Reagents and cell lines

CTX (Sigma-Aldrich, USA) was reconstituted in phosphate-buffered saline (PBS) and frozen at −20° C until used. G-CSF was purchased from Biopharmaceutical Co., Ltd., USA. Hiltonol®, received as a gift from Dr. Andres Salazar (Oncovir, Washington, DC, USA), was dissolved in PBS for intraperitoneal (i.p.) injection. Ehrlich ascites carcinoma (EAC) cell line was purchased from National Cancer Institute, Cairo, Egypt and maintained in the ascitic form by sequential passages in female Swiss albino mice by means of biweekly i.p. injection of 2.5×10^5 tumor cells/mouse suspended in 0.1 ml PBS.

Preparation of tumor cell lysate

The ascitic fluid of EAC was collected using a syringe, and the EAC cells were counted using a Neubauer hemocytometer, and the cell viability was determined 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 at −80°C until use. Frozen tumor cells were disrupted by four repetitive freeze–thaw cycles. Lysis was monitored by light microscopy, and larger particles were removed by centrifugation (300 g 10 min; 4°C). Supernatants were then passed through a 0.2 μm filter, protein concentration was determined by Bio-Rad protein assay, and aliquots were frozen at −80°C until use.

In vitro T-cells activation

Splenocytes were harvested from naïve or EAC-bearing mice. Single cell suspension was prepared under sterile conditions. Cells (2.5×10^6 cells/ml) were stimulated *in vitro* with 5 ng/ml Concanavalin A and 10 ng/ml interleukin 2 and incubated at 37°C and 5% CO_2 for 48 h. Activated cells were harvested and processed for the assay of interest.

Flow cytometry

Fresh single cell suspensions of leukocytes from blood and spleen were prepared. PB samples were collected by bleeding each mouse from retro-orbital plexus. 1×10^6 cells were stained with anti-CD11b, anti-Ly6G (Gr-1), anti-Sca-1 and anti-C-kit and incubated for 30 min on ice. The cells were washed twice and resuspended in 0.3 ml 0.5% bovine serum albumin and 0.02% sodium azide solution. Cells were analyzed by flow cytometry using the Cell Quest software package (Becton Dickinson, San Jose, CA, USA).

Tumor challenge, chemotherapy, and adoptive transfer of T-cells

Naïve female mice were i.p. injected with 0.25×10^6 EAC cells/mouse against control group injected with PBS. On day 7, EAC-bearing mice were treated with 4 mg/mouse CTX followed by adoptive transfer of *in vitro*-activated splenocytes generated either from naive or from EAC-bearing mice by intravenous (i.v.) injection in mice tail against negative control injected with PBS.

Adoptive transfer of bone marrow, granulocyte-colony stimulating factor treatment, and vaccination

For adoptive transfer of BM cells, donor unfractionated BM cells were prepared from naïve mice. Fresh BM cells were washed twice, resuspended in PBS, and transferred (5×10^6 cells/mouse) through lateral tail vein into tumor-bearing mice treated 2 days before with CTX and adoptively transferred with activated splenocytes. While in G-CSF treatment, tumor-bearing mice treated 2 days before with CTX and adoptively transferred with activated splenocytes were subcutaneously (s.c.) administered with 5 μg/mouse G-CSF for five successive days. For tumor

lysate vaccination, tumor-bearing mice treated with CTX and adoptively transferred with activated splenocytes followed by coadministration of BM cells or G-CSF were s.c. vaccinated with 100 μg/mouse tumor lysate and 50 μg Hiltonol. All mice were bled and sacrificed on day 15 and prepared for different assays.

Results

Efficacy of chemoimmunotherapy with bone marrow or granulocyte-colony stimulating factor on DNA content and cell cycle of Ehrlich ascetic carcinoma-cells

Our past study[31] showed that adoptive transfer of activated splenocytes from tumor-bearing mice significantly decreased fold change of total number of tumor cells to 0.09×10^6, and this effect was more enhanced by combining this regimen with BM cells that induced the highest inhibitory effect on the fold of tumor cell count (0.08×10^6) when compared to CTX control (0.19×10^6).

In the present study, antitumor efficacy of combinatorial treatment regimen consisting of CTX preconditioning, adoptive transfer of activated splenocytes followed by BM cell injection and vaccination with tumor lysate, and Hiltonol® was assessed. Mice were i.p. injected with PBS or CTX and then left without further treatment or adoptively transferred with activated splenocytes with or without BM injection or s.c. injection of G-CSF daily for 5 consecutive days to mobilize endogenous stem and myeloid cells. Three days later, mice were vaccinated with tumor lysate and Hiltonol®. The current results indicated that all treatments increased the percentage of apoptosis of EAC-cells comparing to control CTX treatment. Interestingly, adoptive transfer of activated splenocytes obtained from tumor-bearing mice (TSp) in combination with or without BM cells induced the highest apoptotic effect on EAC-cells (34.07% and 35.57%, respectively), the lowest decrease in DNA content represented by S phase (19.1% and 22.21%) and (7.75% and 3.84) in the postreplicative plus mitotic (G2/M) phase in comparison to CTX control (9.4%, 12.87% and 5.42%, correspondingly) [Figure 1].

Effect of combinatorial treatment of chemoimmunotherapy with or without bone marrow or granulocyte-colony stimulating factor on hematopoietic stem cells in bone marrow and peripheral blood

To understand the impact of cotransfer of chemoimmunotherapy with or without BM or G-CSF on expansion of HSCs in BM or PB, mice were i.p. injected with CTX (4 mg/mouse) and then left without further treatment or adoptively transferred with activated splenocytes with or without BM cell injection or s.c. injection of G-CSF daily for 5 days after adoptive transfer of splenocytes. Mice were vaccinated with tumor lysate plus Hiltonol® 3 days postadoptive cell transfer. As shown in Figure 2, administration of adoptive transfer of activated splenocytes obtained from naïve or tumor-bearing mice

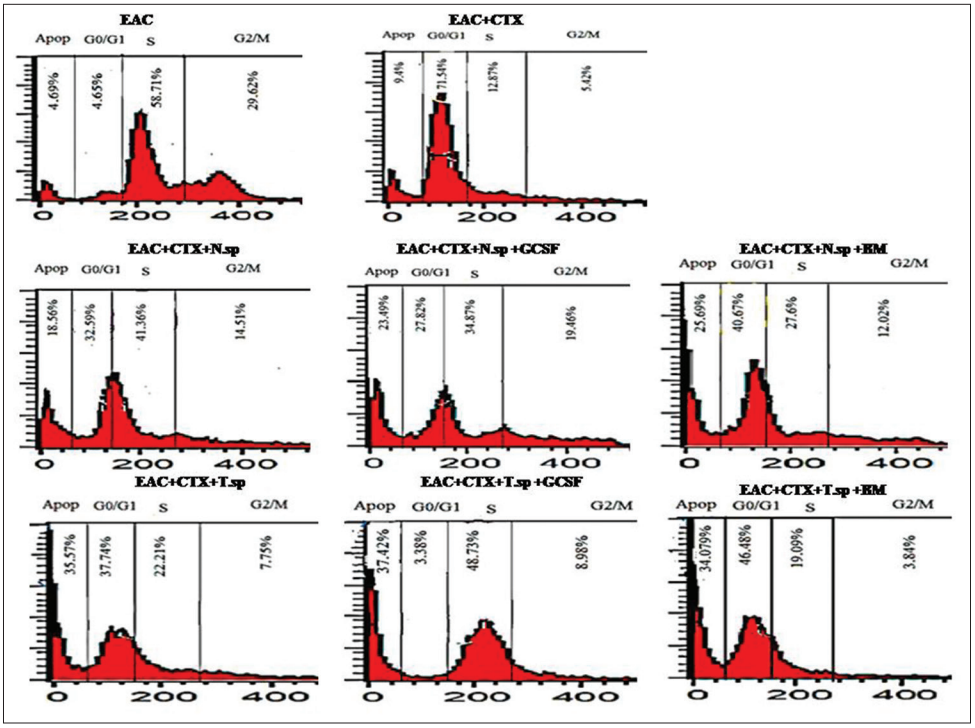

Figure 1: DNA content and cell cycle of Ehrlich ascetic carcinoma cells after combinatorial treatment of chemoimmunotherapy with or without coadministration of bone marrow or granulocyte-colony stimulating factor: Ehrlich ascetic carcinoma cells were harvested from peritoneal cavity, washed twice, and stained with propidium iodide. DNA content and cell cycle of tumor cells were analyzed by flow cytometry for the markers indicated on the representative histograms

with or without BM or G-CSFinduced proliferation and expansion of HSCs (C-Kit+ Sca-1+) in BM. Of interest, ACT of activated splenocytes obtained from naïve mice (NSp) with or without G-CSF administration showed the highest percentage of expansion of HSCs (C-Kit+ Sca-1+) by five- and four-fold, respectively, in comparison to positive CTX control. The results further revealed that activated splenocytes from tumor-bearing mice with or without BM cells markedly enhanced mobilization of HSCs (C-Kit+ population) from BM to circulation by 4.5- and 5-fold correspondingly and 2.5- and 2.7-fold, respectively, for Sca-1+ population [Figures 3 and 4].

To this end, ACT with activated NSp with or without G-CSF administration induced proliferation and expansion of HSCs (C-Kit+ Sca-1+) in BM; however, ACT with activated TSp with or without BM cells injection enhanced mobilization of HSCs (C-Kit+ Sca-1+ populations) from BM to circulation.

The impact of coadministration of bone marrow cells or granulocyte-colony stimulating factor post-chemoimmunotherapy on myeloid cells in spleen

To assess the effect of BM or G-CSF administration of post-chemoimmunotherapy on myeloid cells expansion in spleen, phenotypic analysis of myeloid cells was examined by flow cytometry under the treatment protocol described above. The results revealed that combination of ACT of activated NSp or TSp with BM or G-CSF treatment clearly increased the percentage of myeloid cells (CD11b+ Ly6G+ cells) in spleen as compared with positive CTX control. Interestingly, cotransfer of BM post-ACT of activated TSp induced the highest expansion of CD11b+ Ly6G+ cells by around 2-fold [Figure 5].

Discussion

Albeit active and adoptive immunotherapies are quite effective against small tumor burdens, it seems to be unable to control large tumor masses, as well as, the major limitation for combining antiblastic chemotherapy and immunotherapy is that cytoreductive agents are generally regarded as immunosuppressive because of toxicity to the dividing immune cells in the BM and peripheral lymphoid tissues, so our study evaluated cotransfer of chemoimmunotherapy treatment with BM cells injection or G-CSF administration.

The current study revealed that coadministration of chemoimmunotherapy regimen consisting of activated T-cells obtained from EAC-bearing mice (TSp) plus vaccination post-CTX treatment with or without BM cells provided the highest antitumor effect inducing the percentage of apoptosis and decrease in DNA replication

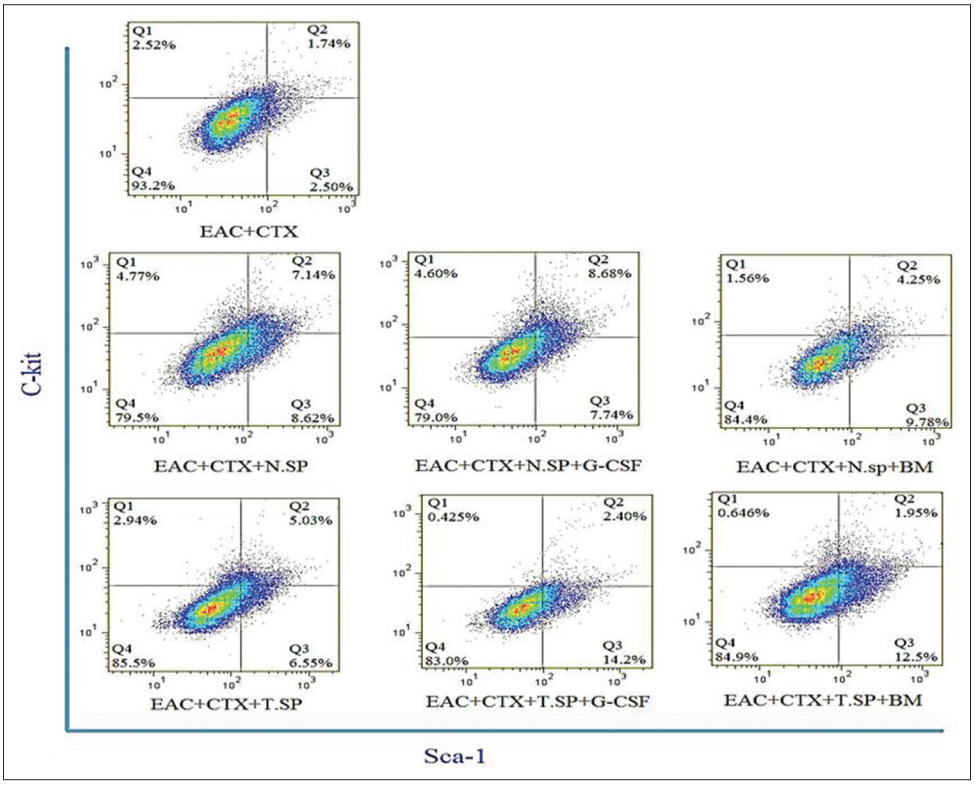

Figure 2: Phenotypic analysis of hematopoietic stem cells markers in bone marrow after combinatorial treatment of chemoimmunotherapy with or without coadministration of bone marrow or granulocyte-colony stimulating factor: Mice were intraperitoneally injected with phosphate-buffered saline or cyclophosphamide and then left without further treatment (phosphate-buffered saline group and cyclophosphamide group) or adoptively transferred with activated splenocytes with or without bone marrow or granulocyte-colony stimulating factor. Mice were sacrificed on day 7, and bone marrow cells were harvested, incubated with Sca-1, c-Kit mAbs then analyzed by flow cytometry for the markers indicated on the representative histograms

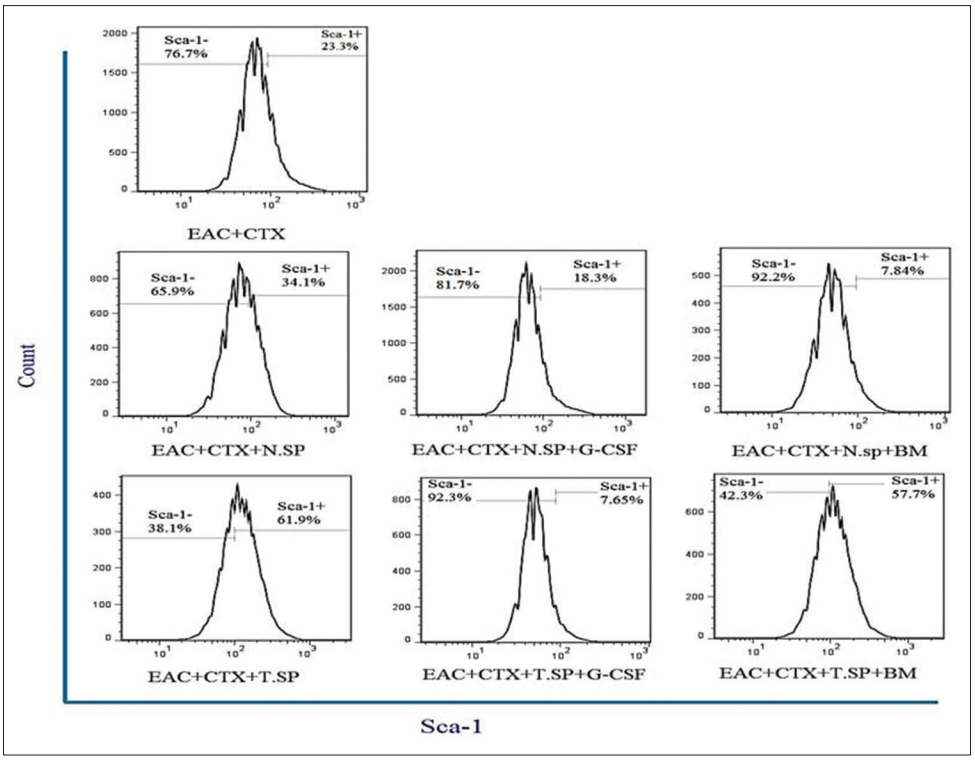

Figure 3: Effect of coadministration of bone marrow or granulocyte-colony stimulating factor post-chemoimmunotherapy on phenotypic analysis of stem cells (Sca-1+) in peripheral blood: cells were incubated with c-Kit+ and Sca-1+ mAbs then analyzed by flow cytometry for the markers indicated on the representative histograms

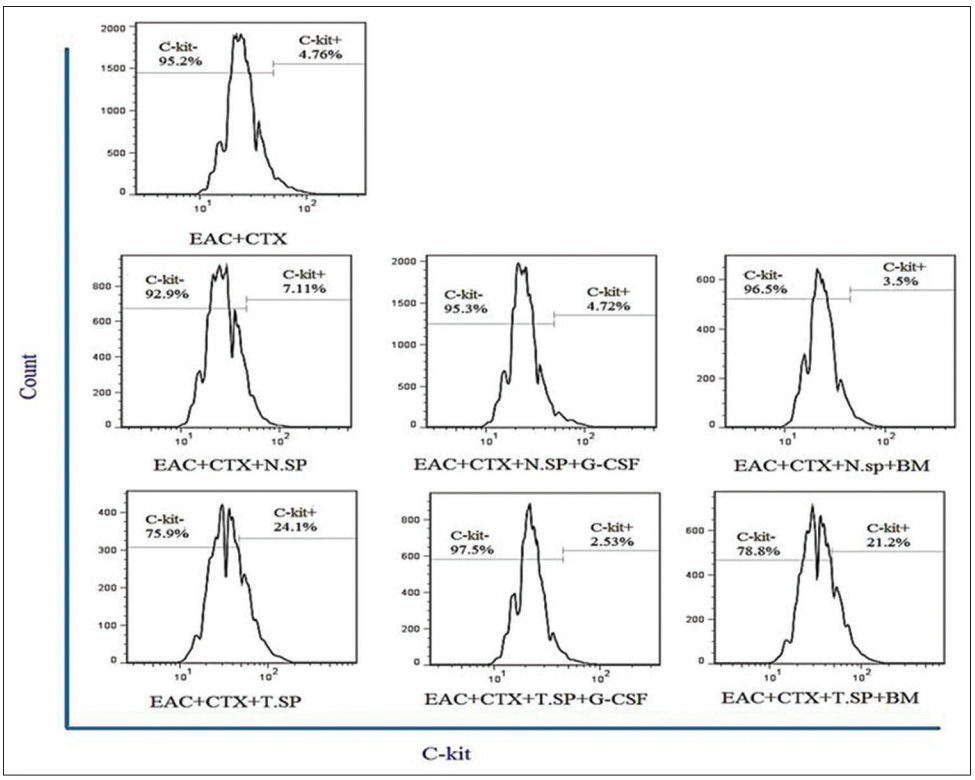

Figure 4: Effect of coadministration of bone marrow or granulocyte-colony stimulating factor post-chemo-immunotherapy on phenotypic analysis of stem cells (c-Kit+) in peripheral blood: Cells were incubated with c-Kit+ mAbs then analyzed by flow cytometry for the markers indicated on the representative histograms

in S phase of EAC cells causing tumor regression and inhibition of its proliferation. These results were in line with this notion,[32] chemotherapeutic agents can kill tumor cells by activating common apoptotic pathways,

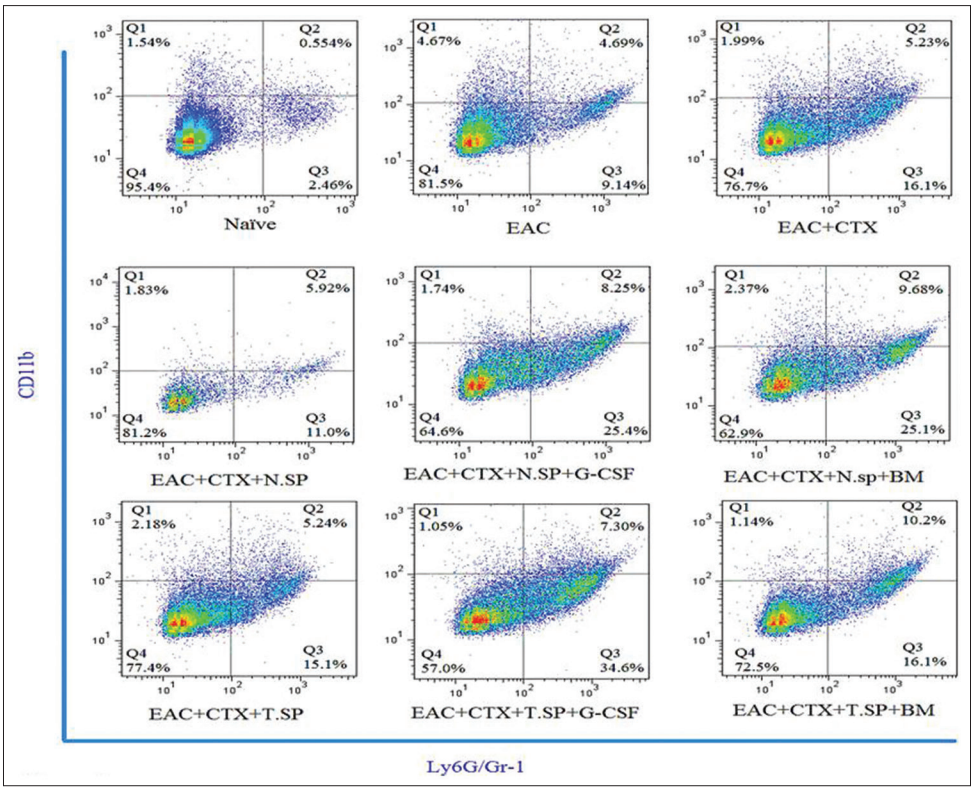

Figure 5: Impact of cotreatment of ACT with bone marrow or granulocyte-colony stimulating factor on expansion of myeloid cells (CD11b+ Ly6G+) in spleen: Mice were intraperitoneally injected with phosphate-buffered saline or cyclophosphamide and then left without further treatment (phosphate-buffered saline group and cyclophosphamide group) or adoptively transferred with activated splenocytes with or without bone marrow or granulocyte-colony stimulating factor. Mice were sacrificed on day 7 and splenocytes were harvested, incubated with CD11b, Ly6G mAbs then analyzed by flow cytometry for the markers indicated on the representative histograms

the extrinsic or death receptor pathway, or through the intrinsic or mitochondrial apoptotic cascades, inducing cellular death by forming DNA adducts which is known to block DNA replication and as a sequence of cytotoxicity.[33] Furthermore, CTX induces an immunogenic apoptosis within the tumor mass that acts as a priming event for the induction of antitumor immunity through the release of large amounts of antigenic material and soluble factors recruiting and activating DCs into the tumor bed.[34] Moreover, cancer immunotherapy aims at induction of an endogenous, long-lasting tumor antigen-specific immune response combining both humoral and cytotoxic T-cell effector mechanisms by the host's immune system following vaccination, as well as induction of an immune response *in vivo* by administration of a tumor antigen as a vaccine to the host's APCs is a method to break the patient's immune tolerance for tumor-associated antigen.[35]

To further clarify changes in HSCs proliferation and mobilization from BM to circulation, we analyzed HSCs in BM and PB in mice with combined treatment of chemoimmunotherapy with or without coadministration of G-CSF or BM cells using flow cytometry based on phenotypic cell surface markers. The results of the current investigation showed that all treatments used in this study increased the proliferation and expansion of HSCs (Sca-1+ c-kit+ cells) in BM. Of interest, ACT of activated NSp with or without G-CSF treatment markedly induced proliferation of stem cell progenitors in BM; however, cotransfer of activated TSp with or without BM cell injection enhanced mobilization of stem cell progenitors into the systemic circulation. Consistent with this notion, coadministration of CTX with G-CSF has been extensively used for the mobilization of HSCs from BM to circulation[36,37] for correction of leukopenia,[38] and HSCs mobilization is significantly enhanced along with the expansion of the marrow c-kitSca-1 cell pool.[39] Moreover, daily stimulation with cytokines for 5–6 days such as G-CSF and/or chemotherapeutic DNA-damaging agents such as CTX cause marked increase in the release of stem cell into the periphery.[40] As well, effect of G-CSF administration on BM progenitor cells collected from normal BM donors showed similar response kinetics in both BM and PB on total nucleated cells and absolute number of CD34+ cells and increase the content of progenitor cells in the BM changing their biological characteristics as well as there were reciprocal changes in the percentage of CD34+ cells in the BM and PB compartments, confirming the concept of their mobilization from BM into PB.

Furthermore, the present study revealed an increase in the percentage of myeloid cells (CD11b+ Ly6G+ cells) in spleen upon treatment with ACT of activated NSp or TSp with or without cotransfer of BM. Remarkably,

coadministration of BM with ACT of activated NSp or TSp increased the proliferation and expansion of CD11b+ Ly6G+ cells by two-fold above baseline (CTX control), and this may be due to activation of BM progenitor cells post-chemo-immunotherapy with G-CSF or BM injection which more enhance the proliferation of BM progenitor cells and their differentiation to different myeloid cells type and this notion is consistent with the results of Jiang *et al.*'s[41] study which showed that a systemic expansion of a population expressing the phenotype of myeloid cells (Gr-1+ CD11b+) post-CTX plus G-CSF treatment and G-CSF supports proliferation and survival of murine BM cells enhancing increase in granulocyte numbers. Moreover, HSCs can augment the reconstitution of the lymphoid compartment by increasing the expansion of survival host cells and adoptively transferred T cells leading to destroy large tumor burdens in the absence of cancer vaccines. More to the point, combining chemotherapy with immunotherapy triggered the enhancement of immune response favoring tumor cell death, inducing tumor antigen cross-presentation *in vivo* and the production of cytokines favoring homeostatic proliferation and/or ablation of immunosuppression mechanisms.[42]

Conclusion

Taken together, combination of chemotherapy with immunotherapy plus vaccination constitutes a potent antitumor therapy and this therapy provides more efficacious antitumor responses when it is combined with BM cells or G-CSF treatment repopulating capacity of cells in the expanded marrow c-kitSca-1 cell pool, homing of the mobilized c-kitSca-1 cells in the systemic circulation, and facilitating the strategic development of chemoimmunotherapy treatment regimens to maximize tumor regression and the antitumor immune response for the long-term clinical benefit of cancer patients. In summary, our data may provide additional information for understanding the significance of the hematopoietic precursor cell response to cancer functions as an important component of the host immune defense response proposing a new standard therapeutic option and application of combinatorial treatments for cancer therapy at earlier stages of tumorigenesis.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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