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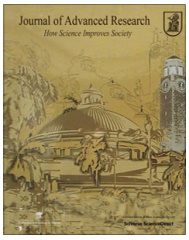

ORIGINAL ARTICLE

# Acute inflammation induces immunomodulatory effects on myeloid cells associated with anti-tumor responses in a tumor mouse model

**Mohamed L. Salem** [a,b,*], **Zeinab I. Attia** [c], **Sohaila M. Galal** [c]

[a] *Immunology and Biotechnology Unit, Zoology Department, Faculty of Science, Tanta University, Tanta, Egypt*
[b] *Center of Excellence in Cancer Research, Zoology Department, Faculty of Science, Tanta University, Tanta, Egypt*
[c] *Physiology Unit, Zoology Department, Faculty of Science, Tanta University, Tanta, Egypt*

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ABSTRACT

Given the self nature of cancer, anti-tumor immune response is weak. As such, acute inflammation induced by microbial products can induce signals that result in initiation of an inflammatory cascade that helps activation of immune cells. We aimed to compare the nature and magnitude of acute inflammation induced by toll-like receptor ligands (TLRLs) on the tumor growth and the associated inflammatory immune responses. To induce acute inflammation in tumor-bearing host, CD1 mice were inoculated with intraperitoneal (i.p.) injection of Ehrlich ascites carcinoma (EAC) ($5 \times 10^5$ cells/mouse), and then treated with i.p. injection on day 1, day 7 or days 1 + 7 with: (1) polyinosinic:polycytidylic (poly(I:C)) (TLR3L); (2) Poly-ICLC (clinical grade of TLR3L); (3) Bacillus Calmette Guerin (BCG) (coding for TLR9L); (4) Complete Freund's adjuvant (CFA) (coding for TLR9L); and (5) Incomplete Freund's Adjuvant (IFA). Treatment with poly(I:C), Poly-ICLC, BCG, CFA, or IFA induced anti-tumor activities as measured by 79.1%, 75.94%, 73.94%, 71.88% and 47.75% decreases, respectively in the total number of tumor cells collected 7 days after tumor challenge. Among the tested TLRLs, both poly(I:C) (TLR3L) and BCG (contain TLR9L) showed the highest anti-tumor effects as reflected by the decrease in the number of EAc cells. These effects were associated with a 2-fold increase in the numbers of inflammatory cells expressing the myeloid markers $CD11b^+$-$Ly6G^+$, $CD11b^+Ly6G^-$, and $CD11b^+Ly6G^-$. We concluded that Provision of the proper inflammatory signal with optimally defined magnitude and duration during tumor growth can induce inflammatory immune cells with potent anti-tumor responses without vaccination.



* Corresponding author. Tel.: +20 1274272624.
E-mail addresses: cecr@unv.tanta.edu.eg, mohamed.labib@science.tanta.edu.eg (M.L. Salem).

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## Introduction

For many years, treatment of cancer was primarily focused on surgery, chemotherapy and radiation, but as researchers learn more about how the body fights cancer on its own, antitumour immunotherapies have been developed. With this regard, recent preclinical and clinical studies have been focusing on designing antitumor treatment strategies based on induction of specific anti-tumor immune responses [1]. Unfortunately, however, these immunotherapeutic approaches have not reached the optimal efficiency against tumor [2]. In addition, they require the identification of certain tumor antigens and tumor-reactive T cells, which are not available in many of cancer settings. As such, immunotherapeutic approaches that depend on induction of non-specific immune responses could be advantageous to the approaches since they do not need requirements. Therefore, exploring and developing non specific immunotherapies is of paramount significance in the clinical application of cancer therapy.

One approach for non specific immunotherapy could be by the induction of inflammation in particular acute inflammation with agents that code for danger signals [3]. Microbial products, which bind to toll like receptors (TLRLs) on immune cells in general and innate immune cells in particular, are the optimal candidate to induce acute inflammation since they code for danger signals that are known to activate immune cells [4]. TLRL are a class of transmembrane signaling proteins that play a critical role in the innate and adaptive immune response against invading pathogen by recognizing various protein, carbohydrates, lipids, and nucleic acids of invading microorganisms [5]. They are expressed by different types of leukocytes or other cell types [6,7]. TLRL expression profiles differ among tissues and cell types. TLRL are predominantly expressed on antigen-presenting cells (APCs), such as macrophages or dendritic cells, and their signaling activates APCs to provoke innate immunity and as a consequence adaptive immunity [8,9]. TLRL are mainly located on the plasma membrane with the exception of TLR3, TLR7 and TLR9 which are localized in the endoplasmic reticulum (ER) [8–10].

Mammalian TLRL include a large family consisting of ten to thirteen different types of toll-like receptors named simply TLR1 to TLR13. To date, ten human and thirteen murine TLR have been identified, TLR1–TLR9 are conserved between the human and mice [11]. However, there are TLRL found in humans and not present in all mammals, for example, TLR10 in humans is present in mice [12]. It has been found that each TLR has been shown to recognize specific microbial component and that TLR have common effects, including inflammatory cytokine or up-regulation of co-stimulatory molecule expression, but also have their specific function such as production of IFN-β [13]. TLR are substances that bind to and activate TLR. The latter constituent in different types of organisms at the cell surface or at the internal cell compartments.

The most common TLRLs that have been used in induction of potent acute inflammation is poly(I:C) which is a synthetic double-stranded RNA that mimics virus and binds to TLR3 [5]. Poly-ICLC (Hiltinol®) is a clinical grade of poly(I:C) which is a synthetic, nuclease-resistant, hydrophilic complex of poly(I:C) and stabilized with poly-L-lysine and carboxymethyl cellulose [14]. BCG is an inflammatory signal to macrophage, lymphocytes, granulocytes, and dendritic cells [15]. It contains cytidine phosphate guanosine (CpG) which is known to bind to TLR9 [16]. BCG can be used alone or integrated into IFA to form CFA.

EAC cells increased via rapid cell division during the proliferating phase and in the load peritoneal cavity. Ascites fluid accumulation occurred in parallelism with the proliferation of tumor cells [17].

In this study, we aimed to determine the impact of the nature, magnitude, and timing of different inflammatory stimuli on the host anti-tumor activity. Our hypothesis is that provision of the proper inflammatory signal with optimally defined magnitude and duration during cancer growth can induce inflammatory cells with potent anti-tumor responses leading to significant decreases in tumor growth even in the absence of vaccination.

## Material and methods

### *Mice*

All experiments were carried out on adult female Swiss albino mice 20 g and aged between 8 and 16 weeks. The mice were purchased from Theodore Bilharz Research Institute, Giza, Egypt. Mice were acclimatized at least two weeks before experimentation and randomly divided into the experimental groups, ten or twelve mice for each. Mice were maintained at regular light and dark cycles, and provided with standard food and water *ad libitum*. *This work was conducted based on the guidelines for the use of experimental animals in research at Department of Zoology, Faculty of Science, Tanta University, Egypt.*

### *Tumor cells*

All experiments in this study were performed using the breast tumor cell line Ehrlich ascites carcinoma (EAC). EAC is a transplantable, poorly differentiated malignant tumor which appeared originally as a spontaneous breast carcinoma in a mouse. It grows in both solid and ascitic forms [18]. The parent cell line was purchased from The National Cancer Institute, Cairo University, Egypt. The tumor cell line was maintained by serial intraperitoneal (i.p.) transplantation of $2.5 \times 10^6$ viable tumor cells in 0.3 ml of saline into female swiss albino mice (8–10 weeks old).

### *Reagents*

Polyinosinic-polycytidylic acid (poly(I:C)), purchased from Sigma Chem. Co., (St. Louis, Mo., USA), was stored at 4 °C in dark until use. Poly(I:C) was dissolved in saline (0. 9%). Poly-ICLC is kindly gifted by Dr. Salazar Andres (Oncovir, Washington, DC, USA). All reagents were obtained in suspension form and stored at 2–8 °C. Poly-ICLC was diluted in saline (0.9%). Complete Freund's adjuvant (CFA) was purchased from Sigma to Aldrich, USA. Incomplete Freund's Adjuvant (IFA) was purchased from Sigma Aldrich, USA. Bacillus Calmette Guerin (Immune BCG-T) was purchased from the vacsera company, Giza, Egypt. It is a suspension of a live attenuated mycobacterium *Bacillus calmette Guerin* is a stabilizing medium. For injection each vial containing 90 mg/3 ml was suspended in 50 ml (0.9%) saline.

*Tumor challenge and treatment*

Seven days after i.p. implantation of $0.5 \times 10^6$ EAC, 3 or 4 mice were killed and EAC cells were collected from the peritoneal cavity, washed for at least twice with 30 ml PBS by centrifugation for 10 min at 1200 rpm, 40C. After making an appropriate dilution, the total number of tumor cells was determined with trypan blue exclusion test. Harvested cells were diluted with saline (0.9%) to the required concentration (usually $0.5 \times 10^6$ cells/ml PBS) used in each experiment, and then 100μL containing $0.5 \times 10^6$ EAC cells were implanted through i.p. injection into the mouse of the experimental groups and treated with PBS or inflammatory stimuli. On day 1 or day 15 post EAC injection, mice were i.p. treated with PBS, a single injection of (100 μg/mouse in 200 μl) BCG ($1 \times 10^6$ c.f.u), the other groups were treated with (100 μg/mouse in 200 μl) poly(I:C), (50 μg/mouse in 200 μl) Poly-ICLC, (100 μg/mouse in 100 μl) CFA, (100 μl/mouse) IFA.

*Assessment of EAC proliferation*

Seven days or fifteen days after i.p implantation ($0.5 \times 10^6$) mice were sacrificed and (EAC) cells were collected. Tumor cells were grown slowly from day 1 to 7 post cell inoculation and then aggressively after day 7 onward. When the mice were sacrificed on day 7 the tumor cells were grow aggressively onward. To insure that all tumor cells were harvested the peritoneal cavity was washed twice by 5 ml PBS and all cells were pooled. Cells were washed for at least twice. After making an appropriate dilution, the total number of tumor cells was determined with trypan blue exclusion assay. Harvested cells were diluted with saline (0.9%) to the required concentration used in each experiment and counted with hemocytometer.

*Flow cytometry*

At the indicated time points, mice were bled from the orbital sinus to harvest peripheral blood and then sacrificed to harvest the spleen and tumor cells. Erythrocytes were then depleted with ACK buffer (Invitrogen, Carlsbad, CA) [19]. Spleen cell suspensions were prepared and counted using a hemocytometer with trypan blue dye exclusion as described previously [20,21]. Table 1 showed different subsets of myeloid cells. Cells were stained with mAbs against CD11b (FITC anti-CD11b), Ly6G (APC anti-Ly6G) for 20 min in dark at room temperature. The cells were then washed twice with PBS and then acquired using Partec flow cytometer and analyzed using flow Jo software (BD Biosciences).

**Table 1** Different subset of myeloid cells.

| Myeloid cells subset | Description |
|---|---|
| $CD11b^+Ly6G^+$ | Immature neutrophil |
| $CD11b^+Ly6G^-$ | Macrophage in case of spleen and monocytes in case of peripheral blood |
| $CD11b^-Ly6G^+$ | Mature neutrophil |

*Statistical analysis*

Statistical analyses were performed using Student's *t*-test [22]. GraphPad Prism (GraphPad Software, Inc., San Diego, CA) was used to analyze the mouse survival data. *P* values less than 0.05 were considered significant. Data were represented as mean ± SD.

## Results

*Comparing the anti-tumor effects of the inflammatory signals on tumor growth*

We compared the effect of the TLR3L agonists poly(I:C) and Poly IC-LC as well as BCG and CFA which contain TLR9L agonists on the anti-tumor response against EAC cells. In addition, we used IFA which is similar to CFA except that it does not contain BCG. All of these agents were injected on days 1 and 15 post EAC challenges. Treatment with these inflammatory stimuli induced decreases in the numbers of EAC harvested from the peritoneal cavity as compared with control tumor-bearing mice (Fig. 1A), where Poly-ICLC, BCG, CFA, poly(I:C) and IFA induced 79.1%, 75.49%, 73.94%, 71.88% and 47.75%, respectively (Fig. 1B). Similar results were obtained when these agents were injected on days 1 + 7 and the analysis was done on day 8 post EAC challenge (data not shown).

*Comparing the immunomodulatory effects of the inflammatory signals on myeloid cells infiltrate in EAC ascites*

To understand whether the anti-tumor effect shown in Fig. 1 was associated with effect on immune cell we analyzed the number of myeloid cells in tumor site. Infiltration of myeloid cells into tumor has been shown to be critical in mediation in the anti-tumor immune response [23]. As such, we analyzed the number of cells expressing the myeloid receptors Ly6G and CD11b in the tumor. Mice were challenged with EAC and then treated with the inflammatory stimuli on both days 1 and 7. Analysis of the expression of CD11b Ly6G in these mice (day 8) after treatment showed that each inflammatory stimulus induced a different effect. As shown in Fig. 2A, BCG resulted in a significant increase in the percentage of $CD11b^+Ly6G^+$ (2-fold) when compared with tumor bearing mice. In contrast, IFA induced decrease (2-fold) in percentage of these cells. Treatment with BCG or IFA did not induce any changes on the percentage of either $CD11b^+$ or $Ly6G^+$ single positive cells. While poly(I:C) did mot induce a marked change in the percentage of $CD11b^+Ly6G^+$, it induced 1.5-fold increases in $CD11b^+$ $Ly6G^-$ or $Ly6G^+$ $CD11b^-$, respectively. Treatment with Poly-ICLC or CFA induced a 2-fold decrease in percentage of $CD11b^+Ly6G^+$ and 5- and 3-fold decreases in $CD11b^+$ $Ly6G^-$ and $Ly6G^+$ $CD11b^-$, respectively Fig. 2B and C.

*Impact of the timing of administration of the inflammatory signals on their anti-tumor effects*

Since poly(I:C) and BCG showed similar effects and they are coding different TLRLs (Figs. 1 and 2), these microbial

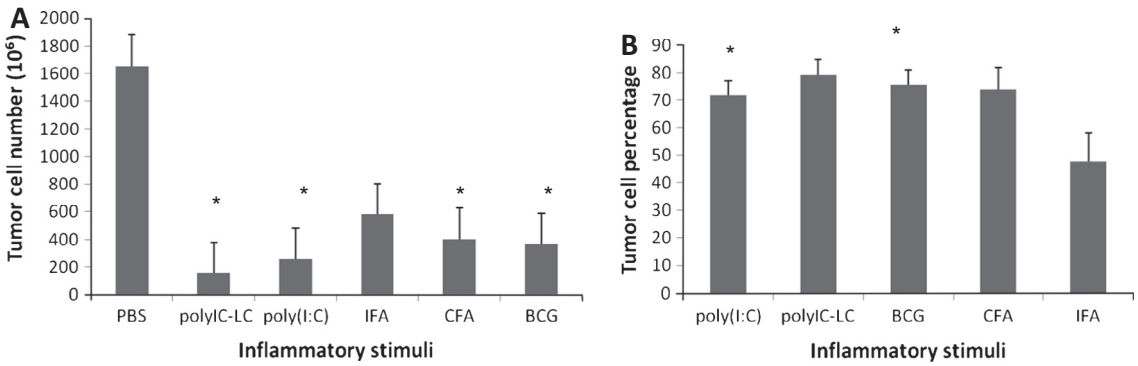
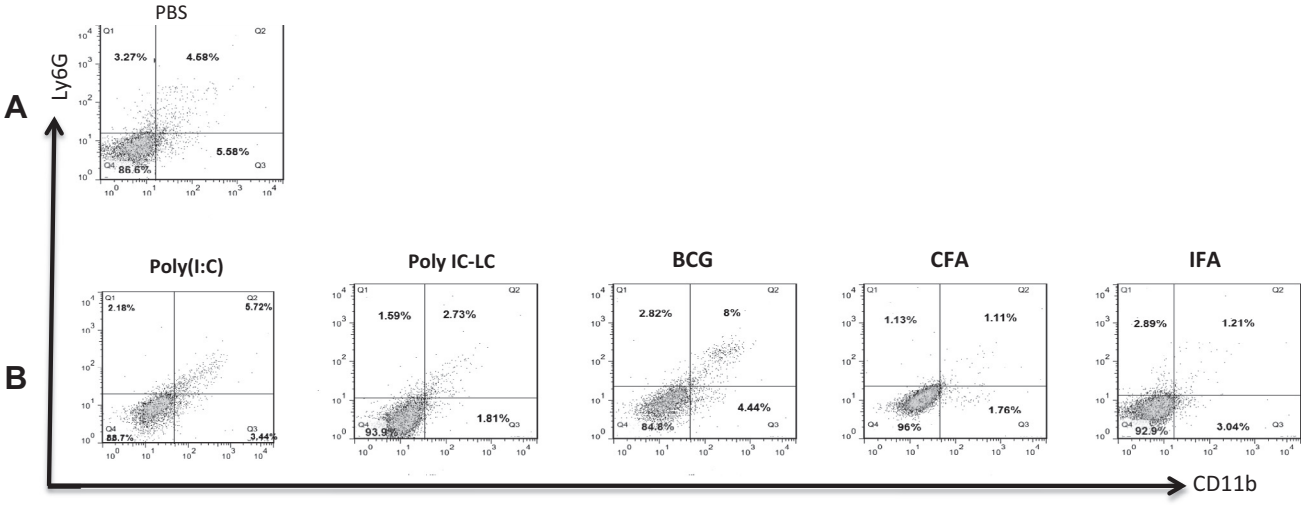



**Fig. 1** The anti-tumor effects of the inflammatory signals on tumor growth. (A) Shows the total number of EAC cells harvested in each group. (B) Shows the percentage of EAC cells. $^{*}P$ value $\leqslant 0.01$ as compared to control.




C

| Myeloid cells | PBS | Poly(I:C) | Poly IC-LC | BCG | CFA | IFA |
|---|---|---|---|---|---|---|
| CD11b $^{+}$Ly6G $^{+}$ | 4.58 | 5.72 | 2.73 | 8 | 1.11 | 1.21 |
| CD11b $^{-}$Ly6G $^{+}$ | 3.27 | 2.18 | 1.59 | 2.82 | 1.13 | 2.89 |
| CD11b $^{+}$Ly6G $^{-}$ | 5.58 | 3.44 | 1.81 | 4.44 | 1.76 | 3.04 |

**Fig. 2** Effects of the inflammatory signals on myeloid cells infiltrate in EAC ascites. (A) Shows a representative control in tumor. (B) Shows the number of cell expressing myeloid (Ly6G$^{+}$ CD11b$^{+}$) or (Ly6G$^{+}$ CD11b$^{-}$) or (Ly6G$^{-}$ CD11b$^{+}$) were estimated after staining with anti-Ly6G and anti-CD11b using flow cytometry. (C) Table shows the percentage of myeloid cells in quadrates.

products were selected in next experiments to test whether the timing of their administration is critical to their anti-tumor effects. To address this issue, EAC-bearing mice were treated with poly(I:C) or BCG either on day 1 or 7 or both and then the mice were sacrificed on day 8 to count EAC number. As shown in (Fig. 3B), when poly(I:C) was administrated both on days 1 + 7 or on day 1 it induced 63.01% and 61.24% decreases in the numbers of EAC (Fig. 3A). However, it induced 33.7% when administrated on day 7 only. When BCG was administrated on days 1 + 7 or on day 1, it induced decrease in the number of EAC by 84.02% and 68.63%, respectively. Interestingly, however, when BCG was administrated only on day 7 it did not induce any change in the numbers of EAC. Taken together, these results indicate that the timing of injection of the inflammatory signals is critical for induction of their anti-tumor effect since injection of BCG in day 1 but not in day 7 increases antitumor effect.

*Comparing the impact of the timing of the inflammatory signals on the frequency of myeloid cells*

Mice were injected with tumor on d0, and treated on day 1 or 7 or both days 1 + 7 with either poly(I:C) (100 μg) or BCG (500 μg). Mice were bled 4 h after each injection of poly(I:C) and BCG and then all mice were sacrificed on day 8 to analyze the numbers of Ly6G$^{+}$ and CD11b$^{+}$ expressed cells in blood, spleen and tumor. Analysis of the frequency of cells expressing Ly6G and CD11b in the tumor site showed that

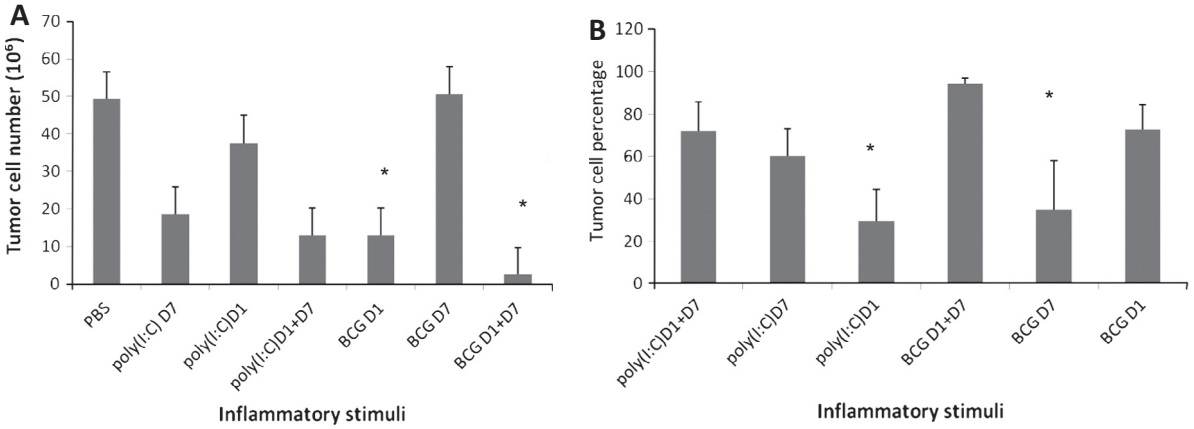



**Fig. 3** Impact of the timing of administration of the inflammatory signals on their anti-tumor effects, (A) shows the total number of EAC cells harvested in each group and (B) shows the percentage of EAC cells. $^{*}P$ value $\leqslant 0.01$ as compared to control.

administration of BCG on day 1 + 7 or day 7 resulted in significant increase in the percentage of $CD11b^{+}Ly6G^{+}$ by 30- and 6-fold, respectively and also 11- and 1.8-fold, respectively, of $Ly6G^{-}CD11b^{+}$ but induced increase of 1.5-fold when administered on day 1 only (Fig. 4A and B). Its administration on days 1 + 7, but not on either of these days alone, resulted in a 4, 5-fold increase in percentage of $Ly6G^{+}CD11b^{-}$ cells (Fig. 4C).

Poly(I:C) administration on days 1 + 7 induced 3-fold increase in the numbers of $CD11b^{+}Ly6G^{+}$ cells and 7-fold increase in their numbers when administered either on day 1 or 7 (Fig. 4A). Interestingly, however, administration of poly(I:C) on day 1 or 7 or both days 1 and 7 induced 2, 7.3 and 12-fold increases, respectively, in the numbers of $Ly6G^{-}CD11b^{+}$ cells (Fig. 4B). Further, its administration on day 1 or days 1 + 7, but not on day 7 alone, induced 2, 2.5-fold increase in the numbers of $Ly6G^{+}CD11b^{-}$ cells (Fig. 4C).

In spleen, BCG, but not poly(I:C), induced a 16-fold decrease in percentage of $CD11b^{+}Ly6G^{+}$ cells and a 4-fold decrease in the number of $Ly6G^{-}CD11b^{+}$ cells. In contrast, however, poly(I:C), but not BCG, induced a 2-fold increase in the numbers of $Ly6G^{+}CD11b^{-}$ (Fig. 5B) as compared with the control group PBS (Fig. 5A).

Administrated of BCG, but not poly(I:C), on days 1 + 7 induced 2-fold decrease in numbers of $CD11b^{+}Ly6G^{+}$. Although administration of BCG or poly(I:C) on days 1 + 7

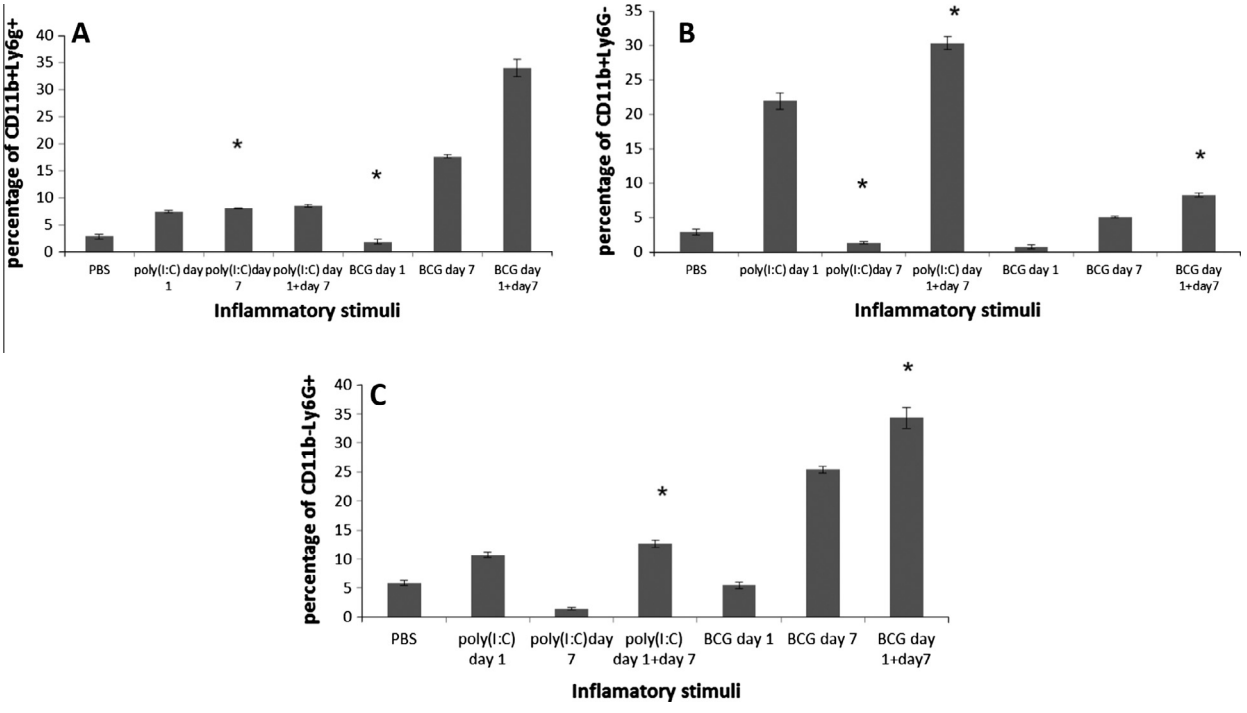
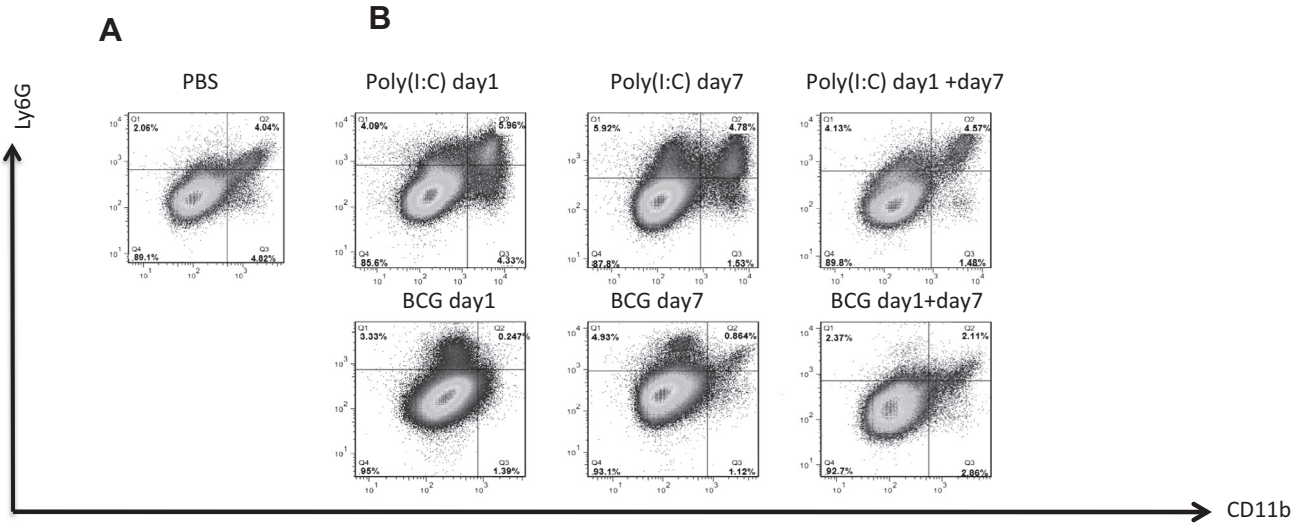



**Fig. 4** Effects of the timing of the inflammatory signals on myeloid cells in tumor site. (A) Shows the number of cell expressing myeloid ($Ly6G^{+}$ $CD11b^{+}$) was estimated after staining with anti-Ly6G and anti-CD11b using flow cytometry. (B) Shows the number of cell expressing myeloid ($Ly6G^{-}$ $CD11b^{+}$) was estimated after staining with anti-Ly6G and anti-CD11b using flow cytometry. (C) Shows the number of cell expressing myeloid ($Ly6G^{+}$ $CD11b^{-}$) was estimated after staining with anti-Ly6G and anti-CD11b using flow cytometry.




C

| Myeloid cells | PBS | Poly(I:C) Day 1 | Poly(I:C) Day7 | Poly(I:C) day1+7 | BCG Day 1 | BCG Day 7 | BCG Day1+7 |
|---|---|---|---|---|---|---|---|
| $CD11b^{+}Ly6G^{+}$ | 4.04 | 5.96 | 4.78 | 4.57 | 0.247 | 0.864 | 2.11 |
| $CD11b^{-}Ly6G^{+}$ | 2.06 | 4.09 | 5.92 | 4.13 | 3.33 | 4.93 | 2.37 |
| $CD11b^{+}Ly6G^{-}$ | 4.82 | 4.33 | 1.53 | 1.48 | 1.39 | 1.12 | 2.86 |

**Fig. 5** Effects of inflammation on myeloid cells in spleen. (A) Shows representative control. (B) Shows analysis of the number of expressing cells of myeloid ($Ly6G^{+}$ $CD11b^{+}$) or ($Ly6G^{+}$ $CD11b^{-}$) or ($Ly6G^{-}$ $CD11b^{+}$) were estimated after staining with anti-Ly6G and anti-CD11b using flow cytometry. (C) Table shows the percentage of myeloid cells in quadrates.

induced 2 and 3-fold decreases in the numbers of $Ly6G^{-}CD11b^{+}$ cells, only poly(I:C) induced 2-fold increase in the number of $Ly6G^{+}CD11b^{-}$ cells. Administration of BCG, but not poly(I:C), on day 7 induced 4-fold decrease in number of $CD11b^{+}Ly6G^{+}$. However, BCG and poly(I:C) induced 4-fold and 3-fold decreases, respectively, in the numbers of $Ly6G^{-}CD11b^{+}$ cells and 2-fold increase in the number of $Ly6G^{+}CD11b^{-}$ cells (Fig. 5B).

Analyses of the frequency of cells expressing Ly6G and CD11b in the blood showed that administration of poly(I:C) or BCG on day 1 had no effect on the number of $CD11b^{+}Ly6G^{+}$ but induced 25 and 16-fold decreases, respectively, in the numbers of $Ly6G^{+}CD11b^{-}$ (Fig. 6A and C). While poly(I:C) induced 2-fold increase in the numbers of $Ly6G^{-}CD11b^{+}$ cells, BCG induced 3.5-fold increase (Fig. 6C) as compared with control group PBS.

Administrated of poly(I:C) on days 1 + 7 induced 2-fold increase in the number of $CD11b^{+}Ly6G^{+}$ while it induced 3.2 and 10-fold decreases in the numbers of $Ly6G^{-}CD11b^{+}$ and $Ly6G^{+}CD11b^{-}$ cells, respectively (Fig. 6A–C). Its administration on day 7 only induced 1.8-fold and 7-fold increases in numbers of $CD11b^{+}Ly6G^{+}$ and $Ly6G^{+}CD11b^{-}$ cells but with no effect on $Ly6G^{-}CD11b^{+}$ cells.

Administration of BCG on days 1 + 7 induced a 2-fold increase in the numbers of $Ly6G^{+}CD11b^{-}$ cells while it induced 5 and 2.5-fold decreases in the numbers of $Ly6G^{-}CD11b^{+}$ and $Ly6G^{-}CD11b^{+}$, respectively (Fig. 6A–C). Its administration on day 7 induced 1.7-fold increase in the numbers of $CD11b^{+}Ly6G^{+}$ cells and 10-fold decrease in the numbers of Ly6G-$CD11b^{+}$ cells but with no effect on $Ly6G^{-}CD11b^{+}$ cells.

*Comparing the anti-tumor effects of inflammation on tumor growth according to magnitude*

To further evaluate whether the antitumor effects of poly(I:C) and BCG depend on their magnitude, they were injected at different doses. They were injected on days 1 + 7 post tumor injection since they showed the optimal effects when they were injected at these 2 time points. Mice were sacrificed on day 8. Consistent with the data in Fig. 1, administration of these two agents at the doses used in the legend of Fig. 1 (100 μg) induced decreases in the numbers of EAC harvested from the peritoneal cavity as compared with control tumor-bearing mice (Fig. 7A). Unexpectedly, however, injection of poly(I:C) at higher (200 μg) dose induced only 69.14% antitumor effect as compared with its effect at 100 μg (89.93%), and its effects disappeared when injected at 50 μg. In contrast to poly(I:C), however, injection of BCG at 1000, 500, and 100 μg induced 89.89%, 76.86% and 81.9% decrease, respectively, in the numbers of EAC as compared to untreated mice (Fig. 7B). Taken together, these results indicate that the dose of TLR is critical for induction of their anti-tumor effect.

*Comparing the impact of magnitude of inflammation on the numbers of myeloid cells*

Administration of 100, 500 and 1000 μg BCG induced 29, 9, and 11-fold increases, respectively, in numbers of $CD11b^{+}Ly6G^{+}$ cells in the tumor site (Fig. 8A). Injection of BCG at 500 μg induced 3-fold increase in percentage of

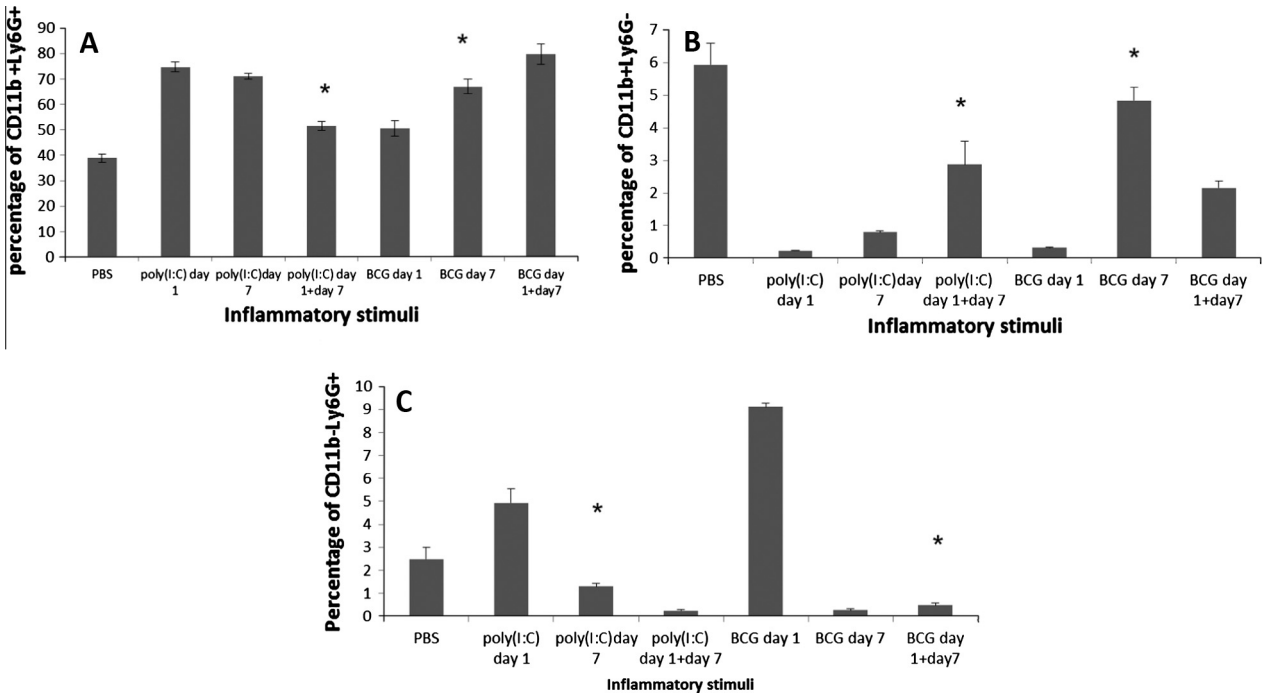



**Fig. 6** Effects of inflammation on myeloid cells in blood. (A) Shows the number of cell expressing myeloid ($Ly6G^+$ $CD11b^+$) was estimated after staining with anti-Ly6G and anti-CD11b using flow cytometry. (B) Shows the number of cell expressing myeloid ($Ly6G^-$ $CD11b^+$) was estimated after staining with anti-Ly6G and anti-CD11b using flow cytometry. (C) Shows the number of cell expressing myeloid ($Ly6G^+$ $CD11b^-$) was estimated after staining with anti-Ly6G and anti-CD11b using flow cytometry.

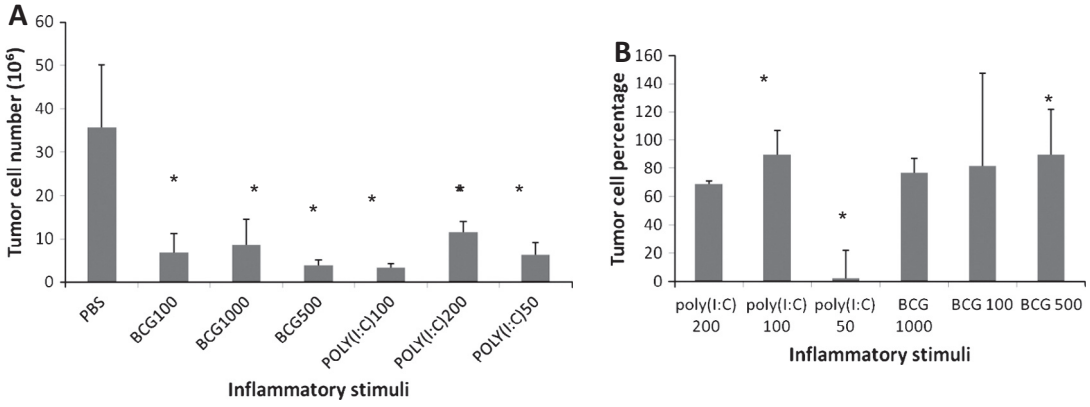



**Fig. 7** The anti-tumor effects on tumor growth according to magnitude. (A) Shows the total number of EAC cells harvested in each group. (B) Shows the percentage of EAC cells. $^*P$ value $\leqslant 0.01$ as compared to control.

$Ly6G^-CD11b^+$ and induced a 2-fold increase in the numbers of $Ly6G^+CD11b^-$ cells (Fig. 8B and C). Its injection at 100 or 1000 μg induced 5 or 90-fold increase in the numbers of $Ly6G^+CD11b^-$ cells, respectively but with no effect on $Ly6G^-CD11b^+$ cells in the tumor site.

Administration of poly(I:C) at 50 or 100 or 200 μg had no effect on the numbers of $CD11b^+Ly6G^+$ cells as compared with untreated EAC bearing mice (Fig. 8A). poly(I:C) at 100 μg, but not at 50 or 200 μg, however, resulted in 3.5-fold decrease and 9-fold increase in the number of $Ly6G^-CD11b^+$ and $Ly6G^-CD11b^+$ cells, respectively, in the tumor site (Fig. 8B and C).

In case of spleen as shown in Fig. 9, we found that BCG(1000 μg) and BCG(100 μg) induced increase of 1.5, 2.5-fold but BCG(500 μg) induced increase (3.5-fold) in percentage of $CD11b^+Ly6G^+$ however all induced decrease (3.8, 6.3 and 2.7-fold) respectively in $CD11b^+Ly6G^-$. In contrast, all induced increase (12.3, 11.3 and 18-fold) respectively in $Ly6G^+CD11b^-$.

Administration of poly(I:C) at 200 μg, but not at 100 μg, induced 1.5-fold increase in the number of $CD11b^+Ly6G^+$ cells, while it induced 2-fold decrease in their number when injected at 50 μg. Treatment with poly(I:C) at 50, 100 and 200 μg induced 3.5-, 1.5 and 2-fold decreases, respectively, in the numbers of $Ly6G^-CD11b^+$ cells and induced 3-, 2 and 2-fold increases, respectively, in the numbers of $Ly6G^+CD11b^-$ cells (Fig. 9).

Fig. 10A shows the numbers of $CD11b^+$ and $Ly6G^+$ cells analyzed in the blood 4 h after administration of poly(I:C) or BCG after 4 h of 1st injection on day 1 of tumor challenge.

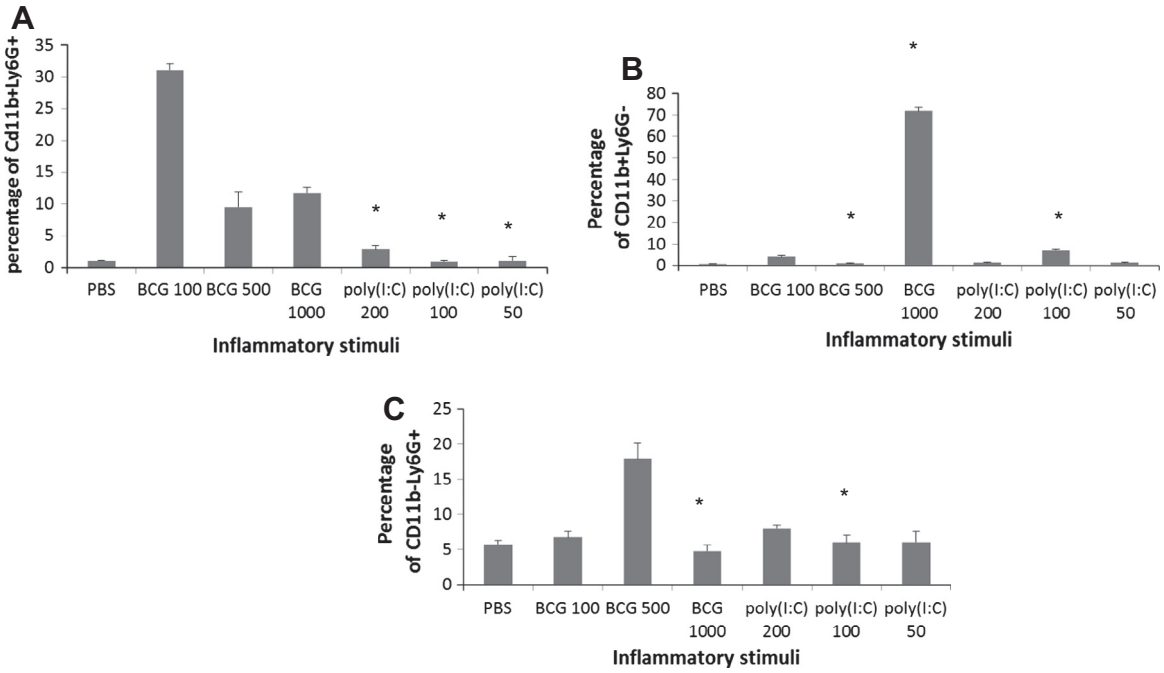
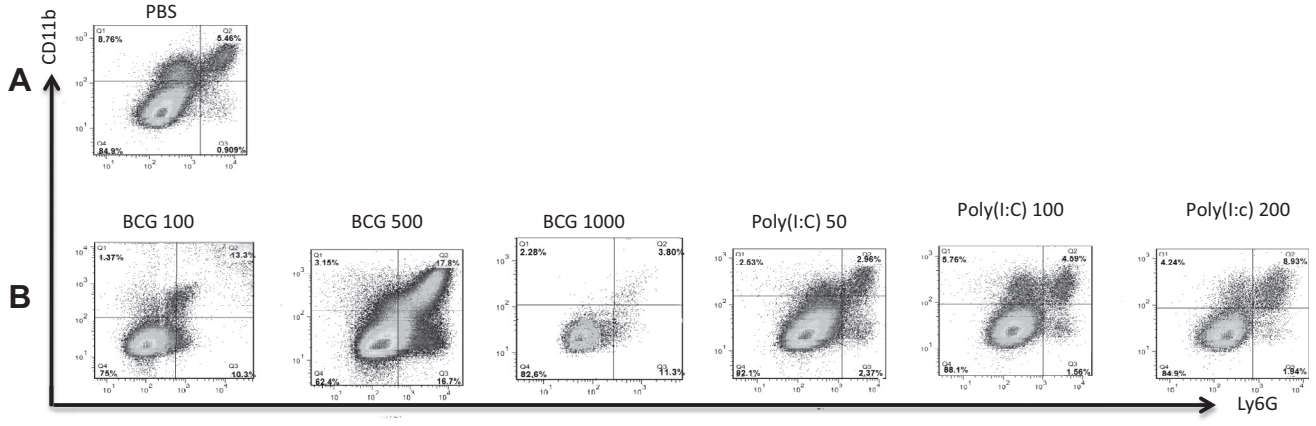



**Fig. 8** Effects of inflammation on myeloid cells in tumor bearing mice. (A) Shows the number of cell expressing myeloid ($Ly6G^+$ $CD11b^+$) was estimated after staining with anti-Ly6G and anti-CD11b using flow cytometry. (B) Shows the number of cell expressing myeloid ($Ly6G^-$ $CD11b^+$) was estimated after staining with anti-Ly6G and anti-CD11b using flow cytometry. (C) Shows the number of cell expressing myeloid ($Ly6G^+$ $CD11b^-$) was estimated after staining with anti-Ly6G and anti-CD11b using flow cytometry.



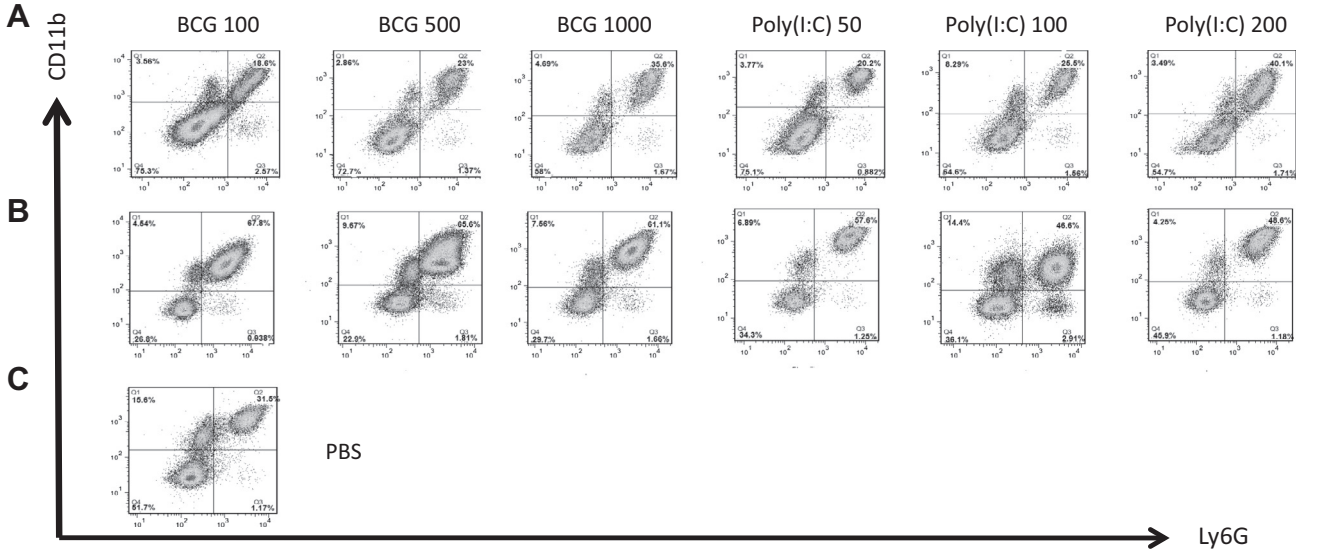

C

| Myeloid cells | PBS | BCG 100 | BCG 500 | BCG 1000 | Poly(I:C) 50 | Poly(I:C) 100 | Poly(I:C) 200 |
|---|---|---|---|---|---|---|---|
| $CD11b^+Ly6G^+$ | 5.46 | 13.3 | 17.8 | 3.80 | 2.96 | 4.59 | 8.93 |
| $CD11b^-Ly6G^+$ | 0.909 | 10,3 | 16.7 | 11.3 | 2.37 | 1.56 | 1.94 |
| $CD11b^+Ly6G^-$ | 8.76 | 1.73 | 3.15 | 2.28 | 2.53 | 5.76 | 4.24 |

**Fig. 9** Effects of inflammation on myeloid cells In spleen. (A) Shows representative control. (B) Shows in the analysis of the number of expressing cells of myeloid ($Ly6G^+$ $CD11b^+$) or ($Ly6G^+$ $CD11b^-$) or ($Ly6G^-$ $CD11b^+$), were estimated after staining with anti-Ly6G and anti-CD11b using flow cytometry. (C) Table shows the percentage of myeloid cells in quadrates.

Administration of BCG at 100 or 500 μg, but not at 1000 μg, induced 1.5-fold decrease in the number of $CD11b^+Ly6G^+$ cells in the blood. At 100 μg, but not at 500 or 1000 μg, BCG induced 2.5-fold increase in the numbers of $Ly6G^-CD11b^+$ cells. In contrast, however, injection of BCG at 100, 500, and 1000 μg induced 4, 5, and 3-fold decreases, respectively, in the numbers of $Ly6G^+CD11b^-$ cells in the blood as compared with control group (Fig. 10C).

Treatment with 200 μg poly(I:C) resulted in 1.5-fold increase in the number of $CD11b^+Ly6G^+$ cells as compared to untreated EAC bearing mice. In contrast, however, its administration at 50 or 100 μg induced 1.5-fold decrease in




D

| Myeloid cells | PBS | BCG 100 | BCG 500 | BCG 1000 | Poly(I:C) 50 | Poly(I:C) 100 | Poly(I:C) 200 |
|---|---|---|---|---|---|---|---|
| $CD11b^{+}Ly6G^{+}$ | 31.5 | 18.6 | 23 | 35.5 | 20.2 | 25.5 | 40.1 |
| $CD11b^{-}Ly6G^{+}$ | 1.17 | 2.57 | 1.73 | 1.67 | 0.882 | 1.56 | 1.71 |
| $CD11b^{+}Ly6G^{-}$ | 15.6 | 3.56 | 2.86 | 4.89 | 3.77 | 8.29 | 3.49 |
| Myeloid cells | PBS | BCG 100 | BCG 500 | BCG 1000 | Poly(I:C) 50 | Poly(I:C) 100 | Poly(I:C) 200 |
| $CD11b^{+}Ly6G^{+}$ | 31.5 | 67.8 | 65.5 | 61.1 | 57.6 | 46.6 | 48.6 |
| $CD11b^{-}Ly6G^{+}$ | 1.17 | 0.938 | 1.81 | 1.66 | 1.25 | 2.91 | 1.18 |
| $CD11b^{+}Ly6G^{-}$ | 15.6 | 4.54 | 9.67 | 7.56 | 6.89 | 14.4 | 4.25 |

**Fig. 10** Effects of inflammation on myeloid cells in blood. The number of cells expressing myeloid ($Ly6G^{+}$ $CD11b^{+}$) or ($Ly6G^{+}$ $CD11b^{-}$) or ($Ly6G^{-}$ $CD11b^{+}$) after staining with anti-Ly6G and anti-CD11b using flow cytometry in blood were analyzed after 4 h of the 1st (A) and the 2nd (B) injection of poly(I:C) and BCG, (C) shows a representative data for control blood, (D) table is shown the percentage of myeloid cells in quadrates.

number of these cells. Interestingly, although administration of 100 or 200 μg/mouse poly(I:C) on day 1 had no effects on $CD11b^{+}Ly6G^{-}$ cells, administration of 50 μg/mouse poly(I:C) induced 4-fold decrease in the number of these cells. In contrast, treatment with poly(I:C) at 50, 100, and 200 μg induced 4.5, 2 and 4-fold decreases, respectively, in the numbers of $Ly6G^{+}CD11b^{-}$ cells.

Interestingly, in Fig. 10B we found that BCG at 100 or 500 or 1000 μg/mouse was analyzed in the blood 4 h after 2nd injection in day 7 induced increase (2.5, 3 and 3-fold), respectively in percentage of $CD11b^{+}Ly6G^{+}$ when. Also BCG at 1000 and 100 μg/mouse induced increase (1.5-fold) in $CD11b^{+}$ $Ly6G^{-}$ while 500 μg did not induce any changes. BCG at 100 or 500 or 1000 μg/mouse induced decrease (8, 14 and 7-fold) in percentage of $CD11b^{+}Ly6G^{-}$, respectively.

Treatment with poly(I:C) at 50, 100, and 200 μg induced 2, 2 and 2.5-fold increases, respectively, in the numbers of $CD11b^{+}Ly6G^{+}$ cells when compared with tumor bearing mice. In case of $Ly6G^{-}CD11b^{+}$ cells, however, only treatment with 200 μg, but not at 50 or 100 μg, poly(I:C) induced 3-fold increase in their numbers in the blood. In case of $Ly6G^{+}CD11b^{-}$ cells, however treatment with poly(I:C) at 50, 100, and 200 μg induced 14, 14.5 and 7-fold decreases, respectively, in their numbers in the blood (Fig. 10C).

## Discussion

In this study we aimed to determine the impact of the nature, magnitude, and timing of different inflammatory stimuli by its agonists poly(I:C) and Poly-ICLC (TLR3) BCG and CFA (which are known to code the TLR9 agonist CpG) on the host anti-tumor activity and the associated response of the immune cells. Administration of these immune stimuli during the tumor progression associated with anti-tumor effects which were dependent on both the magnitude and the timing of induction of the acute inflammation during tumor growth. These antitumor effects also associated with alteration in the numbers of the myeloid cells with $CD11b^{+}Ly6G^{+}$ (immature neutrophils), $CD11b^{-}Ly6G^{+}$ (mature neutrophils) and $CD11b^{+}Ly6G^{-}$ (macrophage in case of spleen and monocytes in case of peripheral blood) phenotypes. Our results indicate that provision of the proper inflammatory signal with optimally defined magnitude and duration during cancer growth can induce inflammatory cells with potent anti-tumor responses leading to significant decreases in tumor growth. The results obtained from this study would led to a simple and effective anti-tumor treatment using the available inflammatory agents even in the absence of vaccination and chemotherapy.

As shown in Fig. 1, BCG, poly(I:C), polyIC-LC and CFA induced similar at anti-tumor effects while IFA showed the lowest effect, indicating that the inflammatory stimuli which code for a TLR ligand are more effective to induce anti-tumor effects than those without danger signals. The nature of the TLR ligand seems not important since BCG and CFA which code for TLR9 showed similar anti-tumor effects to those of poly(I:C) and polyIC-LC which code for TLR3 ligand. These data also suggest that it is possible to induce anti-tumor effects in the absence of antigen-specific immunotherapy if the proper non-specific inflammatory stimuli exist during tumor progression. Taken our results together with those in the literature, it can be suggested that the addition of particular inflammatory stimuli during immunotherapy will significantly enhance the resultant anti-tumor immunity. In line with this hypothesis, we and others have recently reported that the addition of the TLR3 agonist poly(I:C) and other TLR agonists during vaccination against melanoma markedly enhanced the resultant anti-tumor CD8 + T cell responses in terms of the quantity and quality of immune responses [24,25]. In these studies the adjuvant effects of TLRLs were tested in lymphodepleted hosts and with or without adoptive T cell therapy [26]. The studies in which lymphodepletion was applied suggest that combinatorial treatments with chemotherapy/immunotherapy and ACT can markedly improve memory T cell responses [27]. Accordingly, our results indicate that combination of these inflammatory stimuli briefly after anti-cancer chemotherapy can optimally augment the resultant anti-tumor responses even in the absence of vaccination.

Although we did not analyze the exact mechanism underlying anti-tumor effects of these TLR ligands against EAC, the antitumor effects of the tested inflammatory stimuli could be explained by their stimulatory effects on the non specific components of immune system such as macrophag, neutrophils and NK cells. With this regard, we found that poly(I:C) increased the number of neutrophils ($Ly6G^+$) by 1.5-fold and macrophage ($CD11b^+$) by 8-fold. Since the BCG and CFA did not markedly affect these two populations, it could be suggested that the anti-tumor effects of these stimuli are dependent on other cells such as NK and DCs. Recent studies also showed that triggering of TLR signaling pathways induces pro-inflammatory mediators, including cytokines, chemokines, which in turn induces maturation of DCs [28]. These mediators in combination with matured DCs activate cytotoxic T lymphocytes (CTLs) and NK cells, promoting adaptive immunity [15].

Even though we tested the antitumor effects of TLRLs using a non transgenic tumor mouse model and in the absence of vaccination or chemotherapy, the resultant anti-tumor effects could be mediated by antigen-specific T cell response. We challenged the mice with EAC tumor and then treated them with the TLRLs.

Recent studies including ours showed that myeloid derived suppressive cells (MDSC) with the phenotype $Ly6G^+CD11b^+$ expand under the effect of tumor and infection and result in suppression of immune response [29,30]. Interestingly, we found that poly(I:C), polyIC-LC, BCG and CFA induce increases in the number of the cells with this phenotype at tumor site. Recent studies showed that mouse-derived liver MDSC, but not other myeloid cells $CD11b^+$ $Gr1^-$, suppressed T cell proliferation in allogenic MLR in a dose-dependent manner [31], indicating that the presence of proper inflammatory stimuli might interfere with the suppressive function of these cells or induce their activation. Since poly(I:C) and BCG increased the number of these cells, it can be suggested that their adjuvant effects bypassed the suppressive effects of these cells or they induced their maturation or activation. Currently, we are testing these two hypotheses. Alternatively, these cells are not MDSC but mature neutrophils. Studies in our laboratory are ongoing to address this hypothesis.

As shown in Fig. 3, treatment with BCG on day 1 post EAC challenge induced 68.1% reduction in the tumor growth while it had no effect when it was injected on day 7 but retained or even high (84%) anti-tumor effects when injected on days 1 + 7. In contrast, when poly(I:C) was injected on day 1 or day 7 or both, it induced significant anti-tumor effects than when injected only on day 1. These results indicate that BCG need to be injected early during tumor growth but poly(I:C) can be still effective even if administrated at later time points after tumor progression. Although the reason behind the difference between the anti-tumor effects of these two danger signals is not clear, it might be related to the fact that poly(I:C) is specific for TLR3 and BCG contains other TLR ligand other than CpG.

Besides the importance of the timing of the administration of the TLR agonist, our results also indicate the importance of their magnitude. With this regard, we found that increasing the timing of these stimuli had higher effect on the number of $CD11b^+Ly6G^+$ while it decreased the numbers of $CD11b^+$ and $Ly6G^+$ in the blood and spleen. Interestingly, poly(I:C) and BCG induced different patterns on the numbers of these myeloid cells in the tumor site as compared to circulation, indicating that inflammatory stimuli might impact the trafficking of these cells.

The anti-tumor effects of the tested TLR ligands against EAC could be attributed to the direct effects on the tumor cells since recent studies showed that triggering of TLR3 signaling pathway in cancer cells can decrease their proliferation by blocking progression through the cell cycle [32,33]. This would explain in recent studies the clinical interest of TLR3 as indicator of tumor aggressiveness and as a prognostic indicator in gastric cancer [34].

## Conclusions

In sum, our results clearly indicate that provision of certain inflammatory stimuli early or late during tumor progression can effectively induce tumor regression even in the absence of vaccination. This effect is probably mediated by the inflammatory cells such as myeloid cells. Ultimately, our results would open further studies in which we can combine these inflammatory signals with both conventional chemotherapy and immunotherapy such as dendritic cells pulsed with tumor lysate.

## Conflict of Interest

*The authors have declared no conflict of interest.*

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## References


[1] Cassileth BR, Deng G. Complementary and alternative therapies for cancer. Oncologist 2004;9(1):80–9.
[2] Fox BA, Schendel DJ, Butterfield LH, Aamdal S, Allison JP, Ascierto PA, et al. Defining the critical hurdles in cancer immunotherapy. J Transl Med 2011;9(1):214.
[3] Osman MA, Rashid MM, Aziz MA, Habib MR, Karim MR. Inhibition of Ehrlich ascites carcinoma by Manilkara zapota L. stem bark in Swiss albino mice. Asian Pac J Trop Biomed 2011;1(6):448–51.
[4] Wijewardana V, Kristoff J, Xu C, Ma D, Haret-Richter G, Stock JL, et al. Kinetics of myeloid dendritic cell trafficking and activation: impact on progressive, nonprogressive and controlled SIV infections. PLoS Pathog 2013;9(10):e1003600.
[5] Matsumoto M, Seya T. TLR3: interferon induction by double-stranded RNA including poly(I:C). Adv Drug Deliv Rev 2008;60(7):805–12.
[6] Pulendran B. Modulating vaccine responses with dendritic cells and Toll-like receptors. Immunol Rev 2004;199:227–50.
[7] Takeda K, Akira S. Toll-like receptors. Curr Protoc Immunol. Chapter 14: p. Unit 14 12; 2007.
[8] Nishiya T, DeFranco AL. Ligand-regulated chimeric receptor approach reveals distinctive subcellular localization and signaling properties of the Toll-like receptors. J Biol Chem 2004;279(18):19008–17.
[9] He S, Zhang C, Hu Y, Zhou L, He J, Xu K, et al. Some experience of field medical station equipment and materials management. Zhongguo Yi Liao Qi Xie Za Zhi 2013;37(6):454–6.
[10] Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2001;2(8):675–80.
[11] Sivori S, Carlomagno S, Pesce S, Moretta A, Vitale M, Marcenaro E. TLR/NCR/KIR: which one to use and when? Front Immunol 2014;5:105.
[12] Ariffin JK, Sweet MJ. Differences in the repertoire, regulation and function of Toll-like Receptors and inflammasome-forming Nod-like Receptors between human and mouse. Curr Opin Microbiol 2013.
[13] Das S, Rani M, Rabidas V, Pandey K, Sahoo GC, Das P. TLR9 and MyD88 are crucial for the maturation and activation of dendritic cells by paromomycin-miltefosine combination therapy in visceral leishmaniasis. Br J Pharmacol 2014;171(5):1260–74.
[14] Cho HI, Barrios K, Lee YR, Linowski AK, Celis E. BiVax: a peptide/poly-IC subunit vaccine that mimics an acute infection elicits vast and effective anti-tumor CD8 T-cell responses. Cancer Immunol Immunother 2013;62(4):787–99.
[15] Sivori S, Falco M, Della Chiesa M, Carlomagno S, Vitale M, Moretta L, et al. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. Proc Natl Acad Sci USA 2004;101(27):10116–21.
[16] Zuiverloon TC, Nieuweboer AJ, Vékony H, Kirkels WJ, Bangma CH, Zwarthoff EC. Markers predicting response to bacillus Calmette-Guerin immunotherapy in high-risk bladder cancer patients: a systematic review. Eur Urol 2012;61(1):128–45.
[17] Schmidt S, Gorissen S, Eilers H, Fahnenstich H, Dorer A, Krebs D. Animal experiments for the evaluation of laserspectroscopy in the fetus during labor. J Perinat Med 1991;19(1–2):107–13.
[18] Li JJ, Cuthbertson TL, Li SA. Specific androgen binding in the kidney and estrogen-dependent renal carcinoma of the Syrian hamster. Endocrinology 1977;101(4):1006–15.
[19] Lou Y, Wang G, Lizée G, Kim GJ, Finkelstein SE, Feng C, et al. Dendritic cells strongly boost the antitumor activity of adoptively transferred T cells in vivo. Cancer Res 2004;64(18):6783–90.
[20] Lutz MB, Kukutsch N, Ogilvie AL, Rössner S, Koch F, Romani N, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods 1999;223(1):77–92.
[21] Díaz-Montero CM, El Naggar S, Al Khami A, El Naggar R, Montero AJ, Cole DJ, et al. Priming of naive CD8+ T cells in the presence of IL-12 selectively enhances the survival of CD8+CD62Lhi cells and results in superior anti-tumor activity in a tolerogenic murine model. Cancer Immunol Immunother 2008;57(4):563–72.
[22] Glantz S. Primer of biostatistics. United States of America: McGraw-Hill; 2001. p. 489.
[23] Gutkin DW, Shurin MR. Clinical evaluation of systemic and local immune responses in cancer: time for integration. Cancer Immunol Immunother 2014;63(1):45–57.
[24] Davila E, Kang YM, Park YW, Sawai H, He X, Pryshchep S, et al. Cell-based immunotherapy with suppressor CD8+ T cells in rheumatoid arthritis. J Immunol 2005;174(11):7292–301.
[25] Craft N, Bruhn KW, Nguyen BD, Prins R, Lin JW, Liau LM, et al. The TLR7 agonist imiquimod enhances the anti-melanoma effects of a recombinant Listeria monocytogenes vaccine. J Immunol 2005;175(3):1983–90.
[26] Garay RP, Viens P, Bauer J, Normier G, Bardou M, Jeannin JF. Cancer relapse under chemotherapy: why TLR2/4 receptor agonists can help. Eur J Pharmacol 2007;563(1–3):1–17.
[27] Bos R, van Duikeren S, van Hall T, Lauwen MM, Parrington M, Berinstein NL, et al. Characterization of antigen-specific immune responses induced by canarypox virus vaccines. J Immunol 2007;179(9):6115–22.
[28] Matsushima H, Yamada N, Matsue H, Shimada S. TLR3-, TLR7-, and TLR9-mediated production of proinflammatory cytokines and chemokines from murine connective tissue type skin-derived mast cells but not from bone marrow-derived mast cells. J Immunol 2004;173(1):531–41.
[29] Ray A, Chakraborty K, Ray P. Immunosuppressive MDSCs induced by TLR signaling during infection and role in resolution of inflammation. Front Cell Infect Microbiol 2013;3:52.
[30] Källberg E, Stenström M, Liberg D, Ivars F, Leanderson T. CD11b+Ly6C++Ly6G- cells show distinct function in mice with chronic inflammation or tumor burden. BMC Immunol 2012;13:69.
[31] Chen S, Akbar SM, Abe M, Hiasa Y, Onji M. Immunosuppressive functions of hepatic myeloid-derived suppressor cells of normal mice and in a murine model of chronic hepatitis B virus. Clin Exp Immunol 2011;166(1):134–42.
[32] Bhattacharya D, Yusuf N. Expression of toll-like receptors on breast tumors: taking a toll on tumor microenvironment. Int J Breast Cancer 2012;2012:716564.
[33] Sato Y, Goto Y, Narita N, Hoon DS. Cancer cells expressing toll-like receptors and the tumor microenvironment. Cancer Microenviron 2009;2(Suppl. 1):205–14.
[34] Fernandez-Garcia B, Eiró N, González-Reyes S, González L, Aguirre A, González LO, et al. Clinical significance of toll-like receptor 3, 4, and 9 in gastric cancer. J Immunother 2014;37(2):77–83.