Original Article

Enhancing the immunomodulatory effects of the Toll-like receptors 3 agonist poly(I:C) by conjugation with polymers

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ABSTRACT

Background: Poly(I:C) is a Toll-like receptors 3 agonist, which induces potent innate immune responses, and as a consequence adaptive immunity. However, the rapid degradation of poly(I:C) influences its half-life and its adjuvant effects in preclinical and clinical uses. **Aims:** We aimed in this study to conjugate poly(I:C) with polyethylene glycol/poly D, L-actide-co-glycolide (PLGA/PEG) polymers as an approach for better delivery and immunomodulatory effects. **Materials and Methods:** Female CD1 mice were treated once with PEG/PLGA, poly(I:C)/PLGA/PEG (50 μg), poly(I:C)/PLGA/PEG (10 μg) or PEG via intraperitoneal injection and mice were sacrificed 1 day later for complete blood count analysis and analysis of the immune cells by flow cytometry. **Results:** Treatment with PEG/PLGA, poly(I:C)/PLGA/PEG (50 μg), poly(I:C)/PLGA/PEG (10 μg) or PEG administration resulted in significant ($P = 0.0197$) increases (1.89, 1.76, 1.69, and 1.42-fold, respectively) in the absolute number of neutrophils as compared to naïve mice. **Conclusion:** Conjugation of poly(I:C) with polymers does not hamper its immunomodulatory effects, instead it enhances its effects on increasing the number of immune cells opening an avenue for further studies on the beneficial effects of this conjugate.

Key words: Blood, cancer, immune cells, poly(I:C), polymer

INTRODUCTION

Toll-like receptors (TLRs) are a group of receptors that are expressed at high levels by innate immune cells and low levels by adaptive immune cells. TLR3 binds to a number of microbial products called TLR ligands (TLRLs) such as LPS (TLR4 L), viral single and double-stranded ribonucleic acid (TLR7/8 L and TLR3 L, respectively), bacterial and viral deoxyribonucleic acid (TLR9 L).[1] TLR engagement stimulates the innate immune cells, such as dendritic cells (DCs), macrophages, and natural killer (NK) cells, and as a consequence results in activation of T cells. As such, TLRLs have been utilized as adjuvants to potentiate antitumor immune responses in preclinical studies.[2] Of interest, following the Immunotherapy Agent Workshop held recently at the National Cancer Institute, both polyinosinic-polycytidylic acid (poly(I:C)) and CpG were included on a ranked list of 20, out of 124, agents with high potential for use in treating cancer (http://web.ncifcrf.gov/research/brb/workshops.asp). Poly(I:C), a synthetic double-stranded RNA, has been identified as a synthetic TLR3 agonist.[3] We and others have reported that poly(I:C) is a potent inducer of DC and NK cell activation and function due to the rapid release of inflammatory cytokines, including interferon-α, resulting in robust expansion, activation, survival of antigen-specific T cell responses and as a consequence anti-tumor effects against advanced melanoma treatment.[4-9] One limitation of poly(I:C), however, is its rapid degradation by the endogenous endonucleases and as a consequence shortening its half-life and it's biological efficacy.[10,11] Two clinical forms of poly(I:C) have been developed to enhance delivery of poly(I:C) named polyICLC (Hiltonol®) and Ampligen® by chemical stabilization[12,13] and nucleotide mismatching,[14] respectively. Developing further

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DOI: 10.4103/2278-0513.154811

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strategies that can avoid rapid degradation of poly(I:C) is of a great significance to enhance its biological effect.[15]

One of the most biocompatible, popular polymers is polyethylene glycol (PEG) due to its excellent profile.[16] It can reduce the cytotoxicity of the polymer/DNA complex. PEGylation increases the circulation time of nanoparticles and enhances their ability to accumulate in target organs and enhanced permeability and retention effect.[17] Also, conjugation of polyion complex micelles with PEG/poly (L-lysine) (pLL) polymer showed the highest transfection efficiency to human hepatoma HepG2 cells at a 4:1 charge ratio which was higher than that of pLL of the same molecular weight.[18] When microparticle systems contains poly D, L-actide-co-glycolide (PLGA) polymer is preferable, as PLGA has acidic degradation products providing a supportive privilege to proteins and nucleic acids degradation within the acidic microclimate inside PLGA particles.[19] In addition, when PLGA is combined with PEG polymer it results in a potent activity in delivery of cisplatin and targeting the prostate cancer cells *in vitro* using functionalized aptamer.[20] Furthermore, the conjugation of the two efficient polymers PLGA, PEG has been proved to be effective in combination with Ps-341 at CF mice which enhanced pro-inflammatory response in CF lungs disease.[21] Taken together, these studies indicate that modification of biological response by certain polymers can enhance their biological effects. The aim of this study was to increase the immunomodulatory effects of poly(I:C) via its conjugation to PLGA and PEG polymers.

MATERIALS AND METHODS

Mice

Female Swiss Albino (CD1) mice 10-week old and 20–25 g ($n = 6$ per group) were purchased from the National Research Center, Cairo, Egypt. All animals were housed at Animal Facility Unit, Zoology Department, Faculty of Science, Tanta University under the guidelines of the Ethical Committee of this University.

Reagents

Poly(I:C) was purchased from Sigma Chem. Co., (St. Louis, Mo., USA) and prepared under aseptic conditions and dissolved in saline (0.9%) and diluted to the required dose for intraperitoneal (i.p.) injection. Poly(I:C)/PLGA/PEG was prepared at Nanothech Inc., Cairo, Egypt. Anti CD16/CD32, anti-CD11b, anti-Ly6G (Gr1) were purchased from Pharmingen (San Diego, CA, USA).

Preparation of poly (I:C)/poly D, L-actide-co-glycolide/polyethylene glycol

About 13.3 mg of PLGA copolymer, lactide: Glycolide (50:50), mol wt 30,000–60,000, (Sigma-Aldrich Co.), was dissolved in 2 mL chloroform (Elmaadi for Medical Service, Cairo, Egypt). About 250 mg of (PEG) polymer (BioUltra, mol wt 20,000) was dissolved in 5 mL distilled water on the magnetic plate. Then, poly(I:C) (500 μg/100 μl) was added after stirring for 2 min. PLGA solution was added to PEG/poly(I:C) solution drop by drop for 5 h by oil/water emulsion/solvent evaporation method. For preparation of nanoparticles, shaking sonicator was applied into the resultant solution for 1 h to prepare two different concentration poly(I:C)/PLGA/PEG (50 μg), poly(I:C)/PLGA/PEG (10 μg). For preparation of PLGA-PEG, about 13.3 mg of PLGA copolymer, lactide: Glycolide (50:50), mol wt 30,000–60,000 was dissolved in 2 mL chloroform added to 250 mg of PEG polymer (BioUltra, mol wt 20,000) previously dissolved in 5 mL distilled water. PLGA solution was added to PEG solution drop by drop for 5 h by oil/water emulsion/solvent evaporation method. For the preparation of nanoparticles, shaking sonicator was applied into the resultant solution for 1 h. For preparation of PEG (2.5 mg), about 250 mg of PEG polymer (BioUltra 20,000) was dissolved in 5 mL distilled water, shaking sonicator for the preparation of nanoparticles was applied into the resultant solution for 1 h.

Treatment protocol

Naïve mice were treated once with i.p. injection of PBS, treated mice were injected with poly(I:C) (100 μg), poly(I:C)/PLGA/PEG (50 μg/13.3 μg/2.5 mg), poly(I:C)/PLGA/PEG (10 μg/2.66 μg/0.5 mg), PEG/PLGA (2.5 mg/13.3 μg) or PEG (2.5 mg) and dissected 1 day later.

Complete blood count analysis

At the indicated time points, mice were bled from the orbital sinus to harvest peripheral blood and then sacrificed to harvest liver and spleen. The total number of leukocytes in peripheral blood was enumerated using an automated instrument for complete blood count (CBC) (VetScan HM2™ Hematology System, Abaxis®, Union City, CA, USA).

Flow cytometry

About 1×10^6 cells were treated with anti-CD16/CD32 for 5 min on ice. Cells were then stained with the indicated fluorochrome conjugated mAbs, including anti-CD11b, anti-Ly6G (Gr1), and incubated for 30 min on ice in the dark. The cells were washed twice and resuspended in 0.3 mL of 0.5% BSA, 0.02% sodium azide solution. Cells were acquired on a FACS Calibur™ (BD Bio-sciences, San Jose, CA, USA) and analyzed using FlowJo Software (BD Biosciences, San Jose, CA, USA). The absolute number of stained cells = percentage of stained cells × WBCs from CBC/100.

Statistics

Numerical data obtained from each experiment were expressed as mean ± SD and statistical differences between

experimental and control groups were assessed using the Student's *t*-test. $P < 0.05$ were considered statistically significant.

RESULTS

Effects of treatments on total and differential numbers in peripheral blood mononuclear cells

Administration of poly(I:C) at 100 μg, PEG, poly(I:C)/PLGA/PEG at 10 or at 50 μg had no effect on the total number of white blood cells as compared to naïve mice. However, the administration of PEG/PLGA induced significant (P = 0.0434) increase (1.36-fold) in the total number of white blood cells as compared to the naïve mice [Figure 1].

Administration of poly(I:C) at 100 μg, poly(I:C)/PLGA/PEG at 10 μg, at 50 μg, PEG or PEG/PLGA, induced significant (P = 0.0001) increase (2.87, 5.6, 2.68, 4.75 and 2.87-fold, respectively) in the relative numbers of neutrophils [Figure 2b], induced significant (P = 0.0001) increase (2.5, 2, 1.75, 1.25, and 1.5-fold, respectively) in the relative number of monocytes [Figure 2d] as compared to naïve mice.

Administration of poly(I:C) at 100 μg induced significant (P = 0.0197) decrease (0.8-fold) in the absolute number of neutrophils, induced significant (P = 0.0129) decrease (0.78-fold) in the absolute number of monocytes as compared to naïve mice. However, administration of PEG, PEG/PLGA, poly(I:C)/PLGA/PEG at 50 μg or at 10 μg induced significant (P = 0.0197) increases (1.89, 1.76, 1.69 and 1.42-fold, respectively) in the absolute number of neutrophils [Figure 2a], induced significant (P = 0.0129) increases (2.18, 1.82, 1.83, and 1.32-fold, respectively) in the absolute number of monocytes [Figure 2c] as compared to naïve mice.

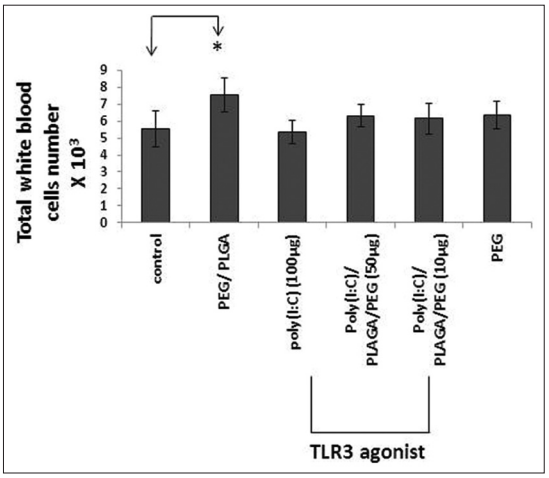

Figure 1: The total number of peripheral blood mononuclear cells after single administration of (polyethylene glycol/poly D,L-actide-co-glycolide [PEG/PLGA]), poly(I:C) (100 μg), poly(I:C)/PLGA/PEG (50 μg), poly(I:C)/PLGA/PEG (10 μg) or PEG into naïve mice. Mice were bled 24 h after injection

Administration of poly(I:C) at 100 μg, PEG/PLGA or poly(I:C)/PLGA/PEG at 10 μg had no significant effect on the relative number of lymphocytes as compared to naïve mice [Figure 3b]. In contrast, administration of PEG or poly(I:C)/PLGA/PEG at 50 μg induced significant (P = 0.0001) decreases (0.81, 0.79-fold, respectively) in the relative number of lymphocyte as compared to naïve mice. Administration of poly(I:C) at 100 μg, PEG, poly(I:C)/PLGA/PEG at 10 μg or at 50 μg had no significant effect on the absolute number of lymphocyte as compared to naïve mice. However, administration of PEG/PLGA induced significant (P = 0.0242) increase (1.27-fold) in the absolute number of lymphocytes as compared to naïve mice [Figure 3a].

Effects of treatments on myeloid cell populations in peripheral blood mononuclear cells

Administration of poly(I:C) at 100 μg, PEG/PLGA, PEG, poly(I:C)/PLGA/PEG at 10 μg or at 50 μg had no significant effect on the relative number of $CD11b^+$ $Ly6G^-$ cells as compared to naïve mice [Figures 4 and 5b). Administration of poly(I:C) at 100 μg induced significant (P = 0.0045) decrease (0.66-fold) in the absolute number of $CD11b^+$ $Ly6G^-$ cells as compared to the naïve group. However, administration of PEG/PLGA, PEG, poly(I: C)/PLGA/PEG at 10 μg or at 50 μg had no significant effect on the absolute number of $CD11b^+$ $Ly6G^-$ cells as compared to naïve mice [Figures 4 and 5a].

Administration of poly (I:C) at 100 μg, PEG/PLGA, poly(I:C)/PLGA/PEG at 10 μg or at 50 μg had no significant effect on the relative number of $CD11b^+$ $Ly6G^+$ cells as compared to naïve mice. However, administration of PEG induced significant (P = 0.0049) increase (1.66-fold) in the relative number of $CD11b^+$ $Ly6G^+$ cells as compared to naïve mice [Figures 4 and 5d].

Administration of poly(I:C) at 100 μg, poly(I:C)/PLGA/PEG at 50 μg or at 10 μg had no significant effect in the absolute number of $CD11b^+Ly6G^+$ cells as compared to naïve mice. However, administration of PEG or PEG/PLGA induced significant (P = 0.0001) increases (1.91, 1.79-fold, respectively) in the absolute number of $CD11b^+$ $Ly6G^+$ cells as compared to naïve mice [Figures 4 and 5c].

DISCUSSION

Poly(I:C) is known to be rapidly degraded by endogenous endonucleases resulting in shortening its half-life, and as a consequence its biological efficacy.[10,11] As such, improving poly(I:C) prosperities by its conjugation with PLGA/PEG copolymers may solve this limitation. We determined that administration of conjugated poly(I:C) resulted in activation of both neutrophil and monocyte indicating the capability of these polymers to enhance their adjuvant

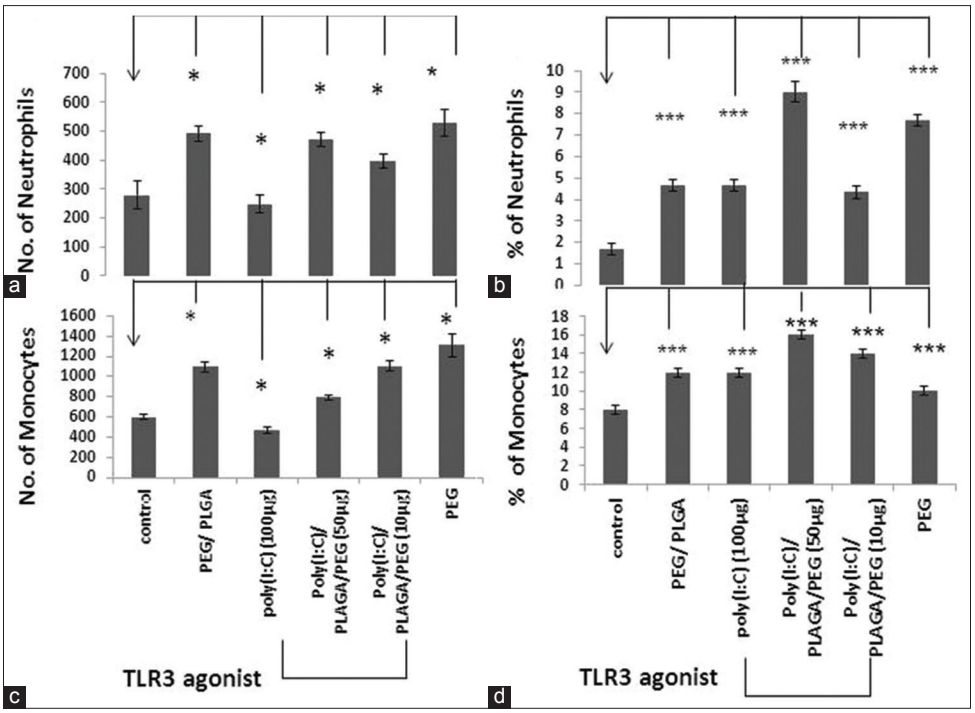

Figure 2: Naïve mice were administered single injection with (polyethylene glycol/poly D,L-actide-co-glycolide [PEG/PLGA]), poly(I:C) (100 µg), poly(I:C)/PLGA/PEG (50 µg), poly(I:C)/PLGA/PEG (10 µg) or PEG. Mice were bled 24 h after injection. (a) The absolute number of neutrophils, (b) the percentage of neutrophils, (c) the absolute number of monocytes and (d) the percentage of monocytes

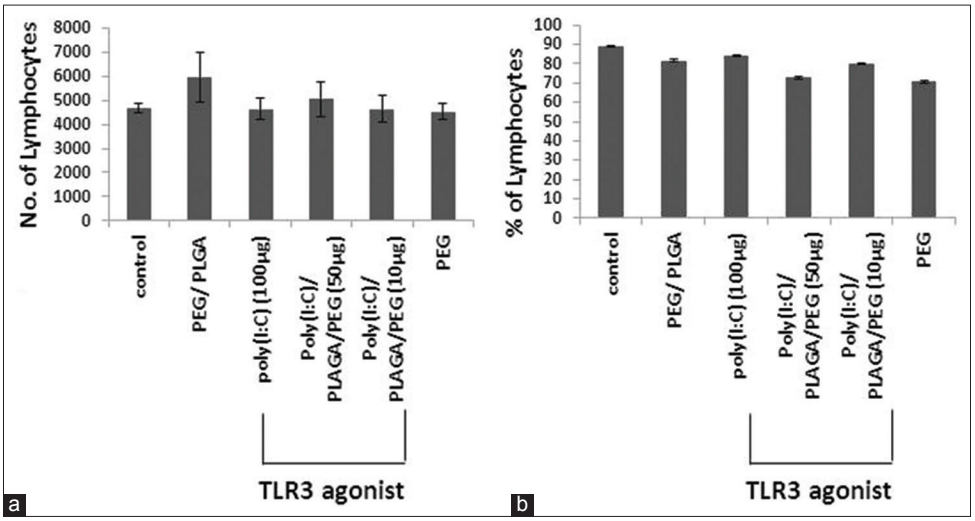

Figure 3: Naïve mice were administered single injection with (polyethylene glycol/poly D,L-actide-co-glycolide [PEG/PLGA]), poly(I:C) (100 µg), poly(I:C)/PLGA/PEG (50 µg), poly(I:C)/PLGA/PEG (10 µg) or PEG. Mice were bled 24 h after injection. (a) The absolute number of lymphocytes and (b) the parentage of lymphocytes

efficacy. Of interest, this effect of conjugated poly(I:C) was dose-dependent, indicating the importance of the quantity of the administered poly(I:C). Previous studies showed that poly(I:C) promotes cross-presentation via expressing specific antigens on DCs, enhancing T lymphocyte response and providing antiviral protection.[22] In the present study, poly(I:C) at 100 µg administration induced decreases in the absolute number of neutrophils and monocytes in the blood. The phenotypic analysis of peripheral blood mononuclear cells verified these results as indicated by the decreases in the number of $CD11b^+ Ly6G^-$ (monocytes). In line with this decreased cell population in blood, we found that poly(I:C) induced alteration in trafficking of these cell population from blood to other organs such as liver, spleen (unpublished data) as suggested by attrition of $CD11b^+ Ly6G^+$ in the blood after 4 h of poly(I:C) administration. Further studies, however, are needed to confirm this observation.

As compared to free poly(I:C), poly(I:C)/PLGA/PEG conjugate induced increase in the number of neutrophils and monocytes in the blood. Taken together the biological effect of free and conjugated poly(I:C), it can be suggested

that the polymers in this conjugate are biologically active. Further, administration of PEG induced increased in the total number of white blood cells, number of neutrophils and monocytes in the blood as well as the phenotypic analysis of $CD11b^+$ $Ly6G^+$ (neutrophils). The biological activities of poly(I:C)/PLGA/PEG could be explained by prolonged blood circulating polymeric vehicles, as the conjugation of drug with diblock hydrophobic PEGylated polymer will result in polymeric vehicle with hydrophobic core with entrapped drug and surrounded with hydrophilic PEG shell will lead to more stability for the particle and prolonged existence within the circulation.[23]

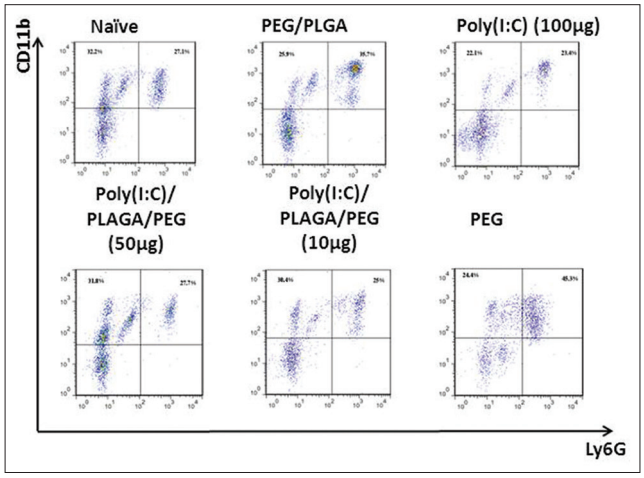

Figure 4: Naïve mice were administered single injection with (polyethylene glycol/poly D,L-actide-co-glycolide [PEG/PLGA]), poly(I:C) (100 µg), poly(I:C)/PLGA/PEG (50 µg), poly(I:C)/PLGA/PEG (10 µg) or PEG. Mice were bled 24 h after injection. The gating of myeloid cell populations expressing CD11b and/or Ly6G molecules were estimated using flow cytometry after staining with anti-CD11b and anti-Ly6G monoclonal antibodies

Although, we did not test the biological activity of PLGA alone previous studies have already described the biological activity of this polymer *per se* Indeed, PLGA proved to be effective microparticle-based vaccine delivery system.[24] Similar to the enhancing effect of PEG/PLGA on the biological effect of poly(I:C) on the innate immune cells, conjugation of poly(I:C) with calcium phosphate nanoparticles has been found to induce DCs and T cell activation.[25] Given that, poly(I:C) proved to improve the immunostimulatory potential of cetuximab against several cancer cell[26] and the conjugation of TLR4 and cancer associated antigen to PLGA resulted in $CD8^+$ T cell induction mediating anti-tumor immunity.[27] It can be suggested that conjugation of poly(I:C) with anti-cancer drugs into PLGA/PEG polymers will lead to robust immunostimulatory effects and anti-cancer effects.

In conclusion, conjugation of poly(I:C) with certain polymers such as PEG and PLGA can enhance its biological activity in immune response in particular innate immune response. These results might be a significant implication in the preclinical application of poly(I:C). However, further studies are needed to evaluate the anti-cancer attribute of this conjugate.

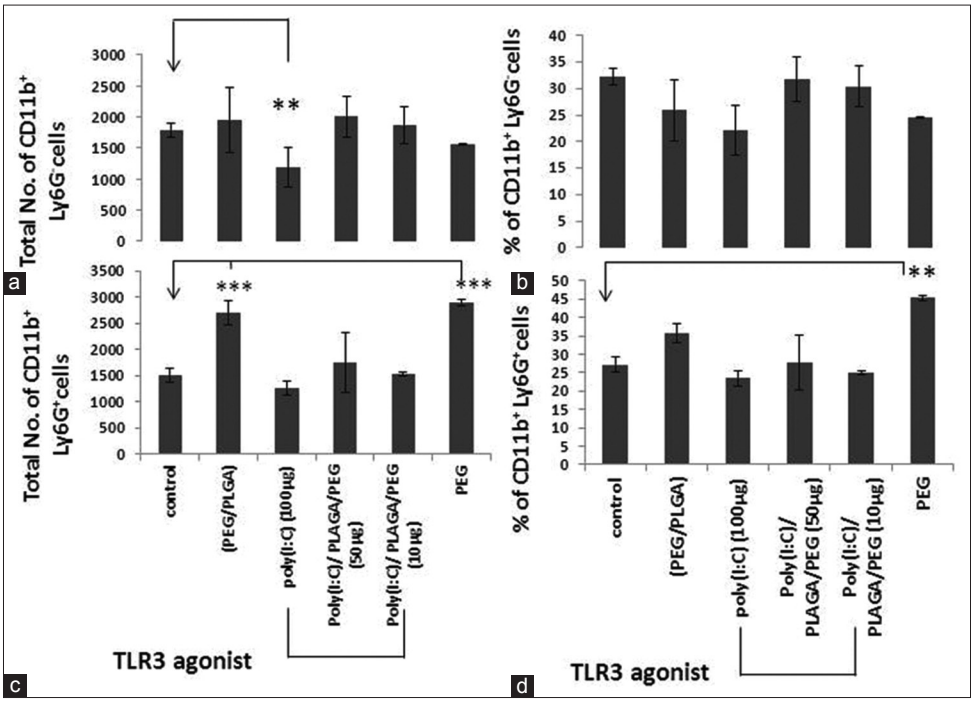

Figure 5: Naïve mice were administered single injection with (polyethylene glycol/poly D,L-actide-co-glycolide [PEG/PLGA]), poly(I:C) (100 µg), poly(I:C)/PLGA/PEG (50 µg), poly(I:C)/PLGA/PEG (10 µg) or PEG. Mice were bled 24 h after injection. (a) The absolute number of $CD11b^+Ly6G^-$ cells, (b) the percentage of $CD11b^+Ly6G^-$ cells, (c) the absolute number of $CD11b^+Ly6G^+$cells and (d) the percentage of $CD11b^+Ly6G^+$cells. The gating of myeloid cell populations expressing CD11b and/or Ly6G molecules were estimated using flow cytometry after staining with anti-CD11b and anti-Ly6G monoclonal antibodies

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Cite this article as: Salem ML, Tosson EM, Mohamed MB, Aboualkheir LM. Enhancing the immunomodulatory effects of the Toll-like receptors 3 agonist poly(I:C) by conjugation with polymers. Clin Cancer Investig J 2015;4:333-8.

Source of Support: Nil, **Conflict of Interest:** None declared.