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Elimination of neutrophils in zymosan-induced ankle inflammation by etoposide

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Article info:

Received: 30 April 2014
Accepted: 28 July 2014

ABSTRACT

Neutrophils play a crucial role in the pathogenesis of joint inflammatory diseases such as rheumatoid arthritis (RA). Therefore their elimination and/or a functional inhibition might have beneficial or even therapeutic effects in these diseases. In the present study we exploited the cytotoxic action of etoposide to deplete neutrophils. We administrated the drug twice (at day -3 and day -1) to SCID mice having intact innate immunity and a fail in T- and B-cell maturation. Ankle inflammation was induced by the injection of zymosan (ZY). Joint damage was evaluated by histology grading system for cell infiltration and proteoglycan loss and degree of cartilage erosion. The frequencies of mature Ly6G⁺CD11b⁺ cells in bone marrow (BM) were monitored at days -4, -2 and 0 by flow cytometry. At day 7 of ankle inflammation the amounts of pro-inflammatory cytokines tumor necrosis factor (TNF)- α and interleukin (IL)-17 were measured by enzyme-linked immunosorbent assay (ELISA). Histological analysis of the joint sections showed decreased scores for cell infiltration and cartilage proteoglycan loss and reduced cartilage erosion in drug-treated zymosan injected mice in comparison to untreated group with ankle inflammation. Etoposide diminished cell numbers in BM, inhibits granulopoiesis triggered by zymosan and decreased the frequencies of mature Ly6G⁺CD11b⁺ cells in BM and eliminated Ly6G⁺ cells from blood and synovial fluid. We observed reduced TNF- α and impaired IL-17 production in etoposide-treated ZY group. Our data provide a proof-of principle that the elimination of neutrophils might be exploited in a design of new therapeutic approaches for joint inflammatory diseases.

Key words: neutrophils, etoposide, zymosan-induced arthritis, IL-17, TNF- α

Introduction

Neutrophils are innate immune cells involved in the initiation and perpetuation of inflammation in RA. The disease affects cartilage, bone and ligaments causing disabilities. RA development is associated with alterations in neutrophils functions such as increased tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-1 β production and activation of nuclear factor κ B (NF- κ B) pathway (Cascão *et al.*, 2010). Neutrophils from arthritic mice or patients secreted spontaneously matrix metalloproteinases (MMPs), osteoprotegerin (OPG), IL-17, interferon (IFN)- γ and expressed Toll-like (TLR)-, complement- and Fc γ receptors, receptor activator of nuclear factor kappa-B (RANK) and its

ligand RANKL, a member of the TNF ligand superfamily (TNFSF11) (Cascão *et al.*, 2010; Milanova *et al.*, 2014). Therefore an inhibition of these neutrophil-related factors by specific drugs, antibodies or via specific mechanisms might have beneficial or even therapeutic effects in arthritis.

Etoposide is a derivative of podophyllotoxin. It inhibits ubiquitously expressed nuclear enzyme topoisomerase II which remains DNA topology during chromosomal recombination, replication and transcription (Wang, 1985; McClendon & Osheroff, 2007; Montecucco & Biamonti, 2007; Hande, 2008). Etoposide induces DNA double-strand breaks increasing the genetic instability and triggers the disassembly of replication factories in intra-S-phase causing the cell cycle arrest in the G2 phase (Montecucco *et al.*,

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2001). The drug increases caspase 3 activity, processing of caspase-2 and 8 and enhances poly ADP ribose polymerase (PARP) cleavage (Day *et al.*, 2009). Etoposide induces p53 target gene (*GADD45*, *BAX* and *MDM2*) expression and stabilization (Cosse *et al.*, 2007). Based on these cytotoxic effects etoposide is used for the treatment of neoplastic diseases in children and adults (Montecucco & Biamonti, 2007). Recently it has been demonstrated that the drug re-localizes and slows the stable long-term nuclear localization of highly mutagenic activation-induced cytidine deaminase that in turn enables DNA demethylation during epigenetic reprogramming (Lambert *et al.*, 2013).

At low doses etoposide has anti-inflammatory potential. It reduces atherosclerotic lesions in rabbits by decreasing the protein expression of lipoprotein receptors (LDL receptor, LDL-related protein-1, cluster of differentiation 36, and scavenger receptor class B member 1), inflammatory cytokines (IL-1 β and TNF- α) and MMP-9 (Tavares *et al.*, 2011). Previously we have observed that the administration of etoposide inhibited the development of zymosan-induced acute inflammation (Remickova *et al.*, 2008). Herein we have exploited etoposide ability to eliminate neutrophils in chronic joint inflammatory conditions. Our aim was to delineate the consequences of the neutrophils loss on joint damage and on the level of joint-erosion related proteins TNF- α and IL-17 in synovial fluid.

Materials and Methods

Mice

SCID (CB17TM) mice were purchased from the Charles River Laboratories (USA), kept under standard conditions of a 12-12 h light-dark cycle and fed with a laboratory diet and water *ad libitum*. Mice (weigh 20–22 g) were anesthetized by intra-peritoneal injection (i.p.) of sodium pentobarbital (50 mg/kg; Sigma-Aldrich, Germany) supplemented with buprenorphine hydrochloride analgetic (0.1 mg/kg; Sigma-Aldrich). All experiments were approved by the Animal Care Committee at the Institute of Microbiology, Sofia in accordance with the National and European Guidelines.

Induction of knee inflammation

SCID mice under anesthesia were injected intra-articularly (i.a.) at ankles with 10 μ l of zymosan suspension (20 mg/ml; Sigma-Aldrich) or 10 μ l of endotoxin-free phosphate-buffered saline (PBS; Lonza®, Basel, Switzerland).

Etoposide administration

In vivo neutrophil elimination was performed in SCID mice by intra-peritoneal injection of 2.5 mg/kg etoposide (Sigma-Aldrich, Munich, Germany) at days -3 and -1 of ankle inflammation. Disease progression for 7 days was compared to untreated PBS- or zymosan-injected groups. To monitor neutrophil elimination Ly6G⁺ cell frequencies were assessed in bone marrow (BM) and blood at days -4, -2 and 0 by flow cytometry.

Histology

At day 7 ankle joints were dissected, fixed in 10% paraformaldehyde/PBS, decalcified in 5% nitric acid for 1 week, dehydrated, embedded in paraffin, cut and stained with hematoxylin and eosin or safranin O (Dimitrova *et al.*, 2010). The degree of injury was graded by a three score system applied for cell infiltration and proteoglycan loss (score 0 - no abnormality; score 3 - severe abnormalities) and determined by two independent observers using light microscopy (Leica Microsystems, Wetzlar, Germany). Cartilage erosion was expressed as the percentage of impaired cartilage from the total cartilage surface and was determined after photo capturing by a DS-Ri1 Nikon camera (Nikon Instruments Europe, Amstelveen, Netherlands) and image analysis by ImageJ 1.42 software (Research Services Branch, NIH, Bethesda, MD, USA).

Cytokine assay

Synovial fluid was harvested from the inter-joint ankle space by lavage with 25 μ l of PBS containing 1 mM ethylenediaminetetraacetic acid (Sigma-Aldrich, Munich, Germany) followed by centrifugation at 350 \times g, 4°C. IL-17 and TNF- α were quantified in synovial fluid by ELISA using commercial kits from Biolegend (London, UK; detection limit < 8 pg/ml) and PeproTech (Cambridge, UK; detection limit < 10 pg/ml). The samples were assayed in triplicate. The concentrations of IL-17 and TNF- α were calculated from a standard curve of the respective recombinant mouse protein using Gen5 Data Analysis Software (BioTek Instruments, Bad Friedrichshall, Germany).

Cell isolation and phenotype

Synovial cells were isolated by centrifugation of synovial fluids at 350 \times g, 4°C. Peripheral cells were obtained from heparinized blood after HistopaqueTM (Sigma-Aldrich, Munich, Germany) density gradient centrifugation. BM cells were collected from the femur. Exclusion dye staining with 0.05% Trypan blue showed more than 95% viable cells in the

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obtained populations. After washing, cells were resuspended at 1×10^5 /ml in 2% fetal calf serum (FCS)/PBS and incubated with antibodies against mouse Ly6G (clone 1A8; Biolegend, London, UK) and CD11b (clone M1-70; Biolegend, London, UK). The samples were analyzed with a flow cytometer (BD LSR II) using BD FACSDiva v6.1.2 Software (Becton Dickinson GmbH, San Jose, CA, USA).

Statistical analysis

Statistical analysis was accomplished by InStat3.0 and GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Data were expressed as mean \pm SEM. Kruskal-Wallis and Mann-Whitney U tests were performed to compare the histological scores and the percentage of cartilage erosion between groups, and to calculate statistical significance of the differences. For other data, the differences in the mean values between groups were analyzed with the two-tailed Student's *t*-test. Differences were considered significant when $p < 0.05$.

Results***Etoposide decreases the severity of cartilage damage induced by ankle zymosan injection***

We evaluated the anti-inflammatory and joint damage-related activity of etoposide in SCID mice lacking mature B lymphocytes and T cells, both sources of secreted pro-inflammatory cytokines and mediators. The dose and the way of etoposide administration have been obtained from our previous investigation (Remichkova *et al.*, 2008). SCID mice were injected with etoposide at day -3 and -1 prior ankle inflammation (day 0). Histological evaluation of the joint sections showed decreased scores of cell infiltration and cartilage proteoglycan loss in drug-treated zymosan injected (ZY)-SCID mice in comparison to control ZY group (Figure 1B). Impaired cartilage was reduced with 20% after etoposide administration in mice with ankle inflammation (Figure 1B). Our data showed the drug ability to decrease the severity of cartilage damage.

Etoposide reduces the frequencies of mature neutrophils in BM and eliminates Ly6G⁺ cells from blood and synovial fluid

Our data showed attenuated ankle inflammation and cartilage erosion in etoposide-treated ZY mice. We next monitored the cell number and frequencies of Ly6G⁺CD11b⁺ cells in BM at days -4, -2 and 0 of zymosan injection. The cell loss in BM was registered after single drug

administration at day -3 (see the data at day -2; Figure 2A). Etoposide diminished twice the neutrophil frequencies in BM and blood at day 0 (Figure 2B). Despite that the injection of zymosan induced granulopoiesis at day 7, mature neutrophils didn't recover completely and were around 20% in BM of etoposide-treated ZY mice as well in PBS-injected group confirming prolonged anti-proliferative and apoptotic effects of the drug (Figure 2C). Decreased generation of Ly6G⁺ cells in BM by etoposide resulted in a loss of neutrophils in blood (Figure 2D) and lack of these cells in synovial fluid (Figure 2E) of ZY group.

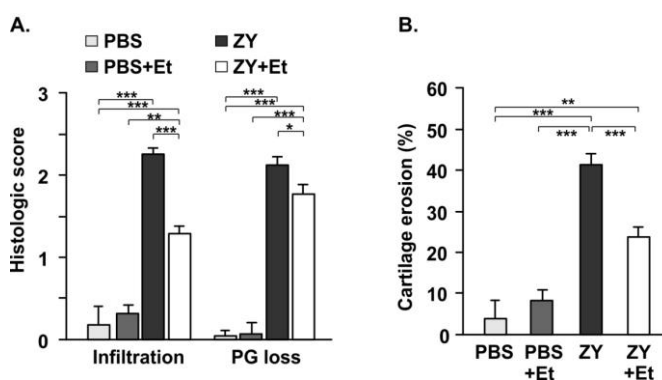


Figure 1. Etoposide decreases the severity of cartilage damage induced by ankle zymosan injection as shown by **A)** histology scores for cell infiltration and cartilage proteoglycan loss and **B)** percentages of impaired cartilage from the total cartilage surface. SCID mice ($n=10$ /group) were injected with etoposide (Et) at day -3 and -1 prior inflammation (day 0). Zymosan (ZY) or saline (PBS) were injected intra-articularly and ankle joints were dissected, fixed, decalcified, embedded in paraffin, cut (20 cuts/sample) and stained with hematoxylin and eosin or safranin O. Results are expressed as mean \pm SEM of 3 individual experiments, Kruskal-Wallis and Mann-Whitney U tests, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Reduced TNF- α and impaired IL-17 production in synovial fluid of etoposide-treated mice with ankle inflammation

We found few Ly6G⁺ cells in blood and in synovial fluid of etoposide-treated mice (Figure 2C-E). TNF- α production increased in etoposide-treated PBS group in comparison to untreated one indicating that the drug might turn compensatory mechanisms for cytokine production as well (Figure 3A). Nevertheless zymosan greatly increased the production of TNF- α in synovial fluid (Figure 3A). The administration of the drug decreased TNF- α synovial level and completely abolished the amount of IL-17 in synovial

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fluid of mice with ankle inflammation (Figure 3B). As both factors are involved directly in cartilage erosion, and drive osteolastogenesis and bone resorption, their inhibition was beneficial in limitation of zymosan-induced joint damage.

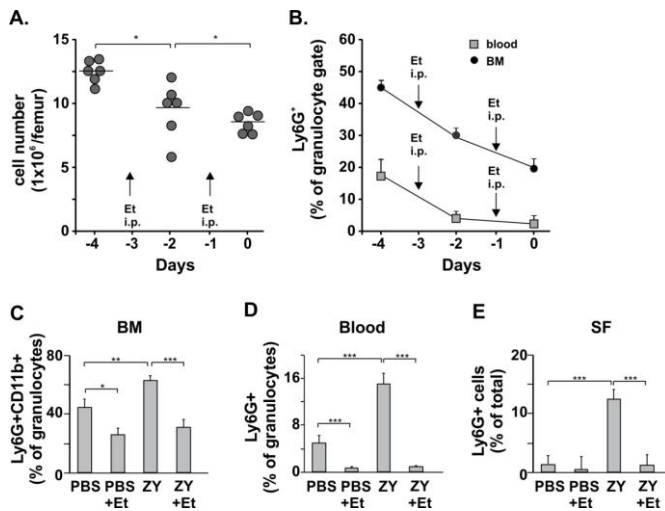


Figure 2. Etoposide reduces the frequencies of mature neutrophils in BM and eliminates $Ly6G^+$ cells from blood and synovial fluid. **A)** Number of BM cells (per femur) and **B)** frequencies of mature $Ly6G^+CD11b^+$ cells in BM and blood were determined at days -4, -2, 0 prior zymosan injection in etoposide (Et)-treated or untreated mice ($n=6$ mice/group). Results in **A)** represents individual data with median value (line), Student's *t*-test, $*p < 0.05$. In **B)** data are expressed as mean \pm SEM. At day 7 of zymosan (ZY) injection **C)** $Ly6G^+CD11b^+$ cells in BM, **D)** $Ly6G^+$ cells in blood and **E)** in synovial fluid of etoposide (Et) treated or untreated mice were determined by flow cytometry. Data are expressed as mean \pm SEM from 3 experiments involving 6 mice per group, Student's *t*-test, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$.

Discussion

In the present study we provide evidences that the elimination of neutrophils prior the induction of inflammation can inhibit $TNF-\alpha$ and IL-17 production and can attenuate consequent cartilage proteoglycan loss and erosion.

The critical role of neutrophils for the initiation of joint inflammation has been described in a model of collagen-antibody induced arthritis after cell depletion by RB6-8C5 antibody (Tanaka *et al.*, 2006). This strategy was also used to investigate the involvement of neutrophils in certain diseases (Mócsai, 2013). Previously we found that a depletion of $Ly6G^+$ neutrophils by specific 1A8 antibody in SCID mice

inhibited the process of proteoglycan loss triggered upon knee zymosan injection (Milanova *et al.*, 2014). Herein our data present the proof-of-principle that the elimination of neutrophils can attenuate joint damage. Specific antibodies are used at concentrations of 100-200 μg /mouse and are administered at least 3 times prior the induction of inflammation. Their effect is estimated not longer than 3 days after their last administration since the granulocyte population recovers faster in BM. By using etoposide we want to prolong the effect of neutrophil elimination. The drug is 95% bound to proteins in serum, with half-life of about 1.5 hours and low intracellular concentrations (Hande, 1998). Nevertheless at low doses etoposide can induce persistent DNA instability, can increase Bax protein levels and apoptosis of myeloid cells (McClendon & Osheroff, 2007). We found decreased numbers of BM cells in etoposide-treated mice. The drug can alter granulocyte differentiation via inhibition of the proliferative promyelocytes and myelocytes stages. As a result few granulocytes can enter at the stage of terminal maturation. Indeed we found decreased frequencies of mature $Ly6G^+CD11b^+$ cells in BM. Etoposide prevented zymosan-induced granulopoiesis. This effect was sustained till day 7 and resulted in the loss of neutrophils in blood and synovial fluid.

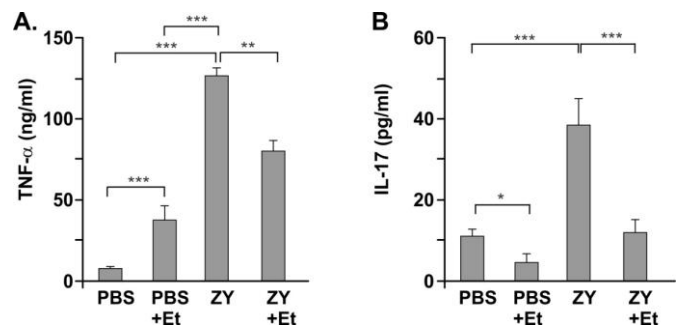


Figure 3. Reduced $TNF-\alpha$ (**A)** and impaired IL-17 production (**B)** in synovial fluid of etoposide-treated mice with ankle inflammation. Mice were treated with etoposide (Et) and injected at ankle as described in section Material and Methods. The cytokine production in synovial fluid was determined by ELISA. Data are expressed as mean \pm SEM from 3 experiments involving 6 mice per group, Student's *t*-test, $***p < 0.001$, $**p < 0.01$.

Consistent lack of neutrophils in circulation prevented cartilage damage and decreased proteoglycans loss. This might be due to a migration of limited number of $Ly6G^+$ cells in synovial fluid. In addition neutrophils depletion restricts

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MMPs secretion that decreases focal angiogenesis required for neutrophil diapedesis and extravasation (Harris *et al.*, 2005). Neutrophils can contribute directly to cartilage erosion by secreted proteases and reactive oxygen species. Previously we found that neutrophils are the source of IL-17, RANKL and OPG (Milanova *et al.*, 2014), all involved in bone resorption, activation of osteoclasts and osteoclastogenesis. Herein we confirmed that the neutrophils can produce IL-17 as their elimination abolished synovial IL-17 production. We think that neutrophils together with other innate immune cells like mast cells and monocytes can initiate IL-17-dependent immune reaction building the inflammatory and bone destructive responses.

The elimination of neutrophils by etoposide altered the level of TNF- α in synovial fluid. In control PBS group the administration of the drug increased the cytokine synovial concentrations. Etoposide can directly interfere with p38 mitogen activated signaling pathway leading to a significant accumulation of TNF- α , IL-1 β and IL-6 mRNAs (Elsea *et al.*, 2008). This drug property is used to block chemotherapy-induced inflammatory cytokine production without inhibiting drug-induced cytotoxicity in cancer. Nevertheless, p38 MAPK inhibitors fail to provide the therapeutic benefit in RA since they act on the anti-inflammatory IL-10 pathway as well (Page *et al.*, 2010). It should be also considered that the elimination of neutrophils can trigger compensatory mechanisms involving other innate immune cells like macrophages, monocytes and NK cells. In a model of LPS-induced shock the administration of RB6-8C5 or 1A8 antibody increased significantly TNF- α production in peritoneal exudates (Elsea *et al.*, 2008; Tanaka *et al.*, 2012). By contrast in our study etoposide was able to suppress zymosan-induced TNF- α production. This effect might be due to a suppression of macrophage/monocyte activation triggered via TLR2. However our previous investigations showed that etoposide did not change the survival rate and had a little influence on organ toxicity when administered in SCID mice in contrast to BALB/c (Remichkova *et al.*, 2008). These data showed that etoposide effects might depend on the genetic background and the severity of inflammatory process studied.

Conclusion

Our data showed that etoposide decreased cartilage erosion via inhibition of granulopoiesis and elimination of circulating neutrophils. These proof-of-principle data are

important for the design of new therapeutic approaches for joint inflammatory diseases.

Acknowledgement

The work was financially supported by Inter-Pasteurien Concerted Actions Grant A05_11, France. PD designed the research and study conception. VM, PD, NI were involved in the acquisition of the data. The authors declare that they have no competing interests.

References

- Cascão R, Rosário HS, Souto-Carneiro MM, Fonseca JE. 2010. Neutrophils in rheumatoid arthritis: More than simple final effectors. *Autoimmunity Reviews*, 9(8): 531-535.
- Cosse JP, Sermeus A, Vannuvel K, Ninane N, Raes M, Michiels C. 2007. Differential effects of hypoxia on etoposide-induced apoptosis according to the cancer cell lines. *Molecular Cancer*, 6(1): 61.
- Day TW, Wu CH, Safa AR. 2009. Etoposide induces protein kinase C δ - and caspase-3-dependent apoptosis in neuroblastoma cancer cells. *Molecular Pharmacology*, 76(3): 632-640.
- Dimitrova P, Ivanovska N, Schwaeble W, Gyurkovska V, Stover C. 2010. The role of properdin in murine zymosan-induced arthritis. *Molecular Immunology*, 47(7-8): 1458-1466.
- Elsea CR, Roberts DA, Druker BJ, Wood LJ. 2008. Inhibition of p38 MAPK suppresses inflammatory cytokine induction by etoposide, 5-fluorouracil, and doxorubicin without affecting tumoricidal activity. *PLoS ONE*, 3(6): e2355.
- Hande KR. 1998. Etoposide: four decades of development of a topoisomerase II inhibitor. *European Journal of Cancer*, 34(10): 1514-1521.
- Hande KR. 2008. Topoisomerase II inhibitors. *Update on Cancer Therapeutics*, 3(1): 13-26.
- Harris A, Ergul A, Kozak A, Machado L, Johnson M, Fagan S. 2005. Effect of neutrophil depletion on gelatinase expression, edema formation and hemorrhagic transformation after focal ischemic stroke. *BMC Neuroscience*, 6(1): 49.
- Lambert LJ, Walker S, Feltham J, Lee HJ, Reik W, Houseley J. 2013. Etoposide induces nuclear re-localisation of AID. *PLoS ONE*, 8(12): e82110.
- McClendon AK, Osheroff N. 2007. DNA topoisomerase II, genotoxicity, and cancer. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 623(1-2): 83-97.
- Milanova V, Ivanovska N, Dimitrova P. 2014. TLR2 elicits IL-17-mediated RANKL expression, IL-17, and OPG production in neutrophils from arthritic mice. *Mediators of Inflammation*, 2014, doi: 10.1155/2014/643406.
- Mócsai A. 2013. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. *The Journal of Experimental Medicine*, 210(7): 1283-1299.
- Montecucco A, Biamonti G. 2007. Cellular response to etoposide treatment. *Cancer Letters*, 252(1): 9-18.

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- Montecucco A, Rossi R, Ferrari G, Scovassi AI, Prosperi E, Biamonti G. 2001. Etoposide induces the dispersal of DNA ligase I from replication factories. *Molecular Biology of the Cell*, 12(7): 2109-2118.
- Page TH, Brown A, Timms EM, Foxwell BMJ, Ray KP. 2010. Inhibitors of p38 suppress cytokine production in rheumatoid arthritis synovial membranes: Does variable inhibition of interleukin-6 production limit effectiveness in vivo? *Arthritis & Rheumatism*, 62(11): 3221-3231.
- Remichkova M, Yordanov M, Dimitrova P. 2008. Etoposide attenuates zymosan-induced shock in mice. *Inflammation*, 31(1): 57-64.
- Tanaka D, Kagari T, Doi H, Shimozato T. 2006. Essential role of neutrophils in anti-type II collagen antibody and lipopolysaccharide-induced arthritis. *Immunology*, 119(2): 195-202.
- Tanaka Y, Nagai Y, Kuroishi T, Endo Y, Sugawara S. 2012. Stimulation of Ly-6G on neutrophils in LPS-primed mice induces platelet-activating factor (PAF)-mediated anaphylaxis-like shock. *Journal of Leukocyte Biology*, 91(3): 485-494.
- Tavares ER, Freitas FR, Diament J, Maranhao RC. 2011. Reduction of atherosclerotic lesions in rabbits treated with etoposide associated with cholesterol-rich nanoemulsions. *International Journal of Nanomedicine*, 6: 2297-2304.
- Wang JC. 1985. DNA Topoisomerases. *Annual Review of Biochemistry*, 54(1): 665-697.