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Rapamycin and Filgrastim effect on Regulation of Peripheral and Splenic Tissue B- and T-lymphocytes in TAA-Induced Splenic Injury in Rats

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ABSTRACT

Background: Splenomegaly; a bigger spleen pool for blood, affecting circulating and its resident blood cells. **Aim:** this study aimed to evaluate the effects of Rapamycin (RAPA) and Filgrastim (FIL) treatment separately and in combination on the blood and splenic lymphocytes in thioacetamide (TAA)-induced splenic injury. **Methods:** Thirty-six adult male albino rats were divided into six groups; negative control, TAA-treated, TAA+RAPA-group,TAA+FIL-group, TAA+RAPA+FIL-group and TAA+RAPA then FIL-group. For each group, complete blood count (CBC), flow cytometry for blood CD3 and CD19 lymphocytes, and immunohistochemical detection of splenic CD3 and CD20 positive lymphocytes were performed. **Results:** the results demonstrated significant increased blood CD3 and CD19 lymphocytes with increased splenic CD3 and CD20 lymphocytes, decreased Red blood cells (RBCs), Hemoglobin (Hb) and increased White blood cells (WBCs) with marked effect on spleen morphology after TAA injection. RAPA treatment showed decreased splenic lymphocytes while increased blood T-cells with increased Hb levels and granulocytes percentage, lowered WBCs count with more decreased lymphocytes percentage and further reduced Platelets (Plts) count. Also, FIL monotherapy showed decreased splenic lymphocytes with decrease in circulating WBCs with increased Hb levels. Both combination treatments caused an increment in blood CD3 lymphocytes, while only decreased CD19 lymphocytes with TAA+RAPA+FIL-group. **Conclusion:** Splenic and blood lymphocytes were affected by RAPA and FIL separately or in combination as combination showed a synergistic effect on spleen lymphocytes.

Introduction:

Splenomegaly is a big problematic outcome of chronic liver disease in Egypt, decreasing life quality (1). Chronic liver diseases, like fibrosis, occasionally associated with splenomegaly due to partial blockade of spleen efflux to liver (2). Splenomegaly; results in problems for patients such as hypersplenism with subsequent thrombocytopenia or even pancytopenia which leave patients with few numbers or even no chances for other treatments (1). Splenomegaly results in increasing pooling of blood, making its elements stay more time inside in contact with spleen macrophage which in turn, diminishes one or more of blood forming elements resulting in cytopenia (3). Nearly 1-6% of splenomegaly causes were due to primary problems in the spleen itself. (4).

Rapamycin (RAPA) is used in reducing spleen size in cases of splenomegaly. It is a 31-membered macrolide immunosuppressant member having some antiproliferation effects on lymphoid and non-lymphoid cells (5). It is classified as xenobiotic immunosuppressant which are produced via endogenic processes and derived from microbes with antiproliferation effect on lymphocytes (6). RAPA was traditionally used to prevent kidney transplantation rejection, also used in treatment of rheumatoid arthritis and some types of cancers (7). RAPA is one of the two clinically used mTOR (mammalian target of rapamycin) inhibitors, rapamycin and everolimus, approved by Food and Drug Administration (FDA) in clinical use (6). RAPA inhibits proliferation of splenic T- and B-cells (8).

mTOR was found to be over expressed in enlarged spleen so, the inhibitory effect of RAPA on mTOR could improve splenomegaly (1). Unfortunately, RAPA monotherapy causes incomplete inhibition of mTORC1 (mammalian target of rapamycin complex-1)-dependent pathways, so it may be compensated by other agents for other feedback loops which make that blockade useless (9).

Filgrastim (FIL) is a recombinant human methionyl granulocyte colony-stimulating factor (G-CSF) responsible for neutrophil progenitor cells activation, proliferation and differentiation into mature functioning neutrophils inside bone marrow and spleen. Hence, it is used to treat patient with neutrophils dysfunctions. Filgrastim was mainly used for patients receiving chemotherapy and developing cytopenia, thus making them under stress of bacterial infection and its complication (10). Filgrastim applied to chemotherapeutic patients with low blood forming elements which give rise to granulocytes as well as platelets (11). It is well tolerated to most patients with few side effects (10). Side effects are well-tolerated, and include mild splenomegaly in about one-fourth of patients in case of long-term applications, increased serum uric acid, lactate dehydrogenase and leucocyte alkaline phosphatase which all are likely due to increased leucocyte production by Filgrastim, beside the medullary bone pain (12).

The present study aimed to induce splenomegaly by chronic intra-peritoneal administration of TAA. Then investigate the possible effects of RAPA and FIL administration on splenomegaly separately or in combination on both peripheral and splenic T- and B-lymphocytes.

Material and Methods:

Chemicals:

Thioacetamide (TAA): (98.5% extra pure) was purchased from Lobachemie for laboratory reagents & fine chemicals, CAS No. 62-55-5, LOT No. L206471610.

Rapamycin: ≥99% was purchased from Alfa Aesar, Thermo Fisher Scientific Chemicals, Inc., Germany, product of USA. CAS No. 53123-88-9, stock number J62473, LOT: Y13D043. Imported by international Co. for scientific and medical supplies, Cairo,

Egypt. Rapamycin was prepared by dissolving in Dimethyl sulphoxide (DMSO) nearly 250 mg/ml DMSO(13) to make stock solution of rapamycin. The stock solution was mixed with a vehicle of 10% polyethylene glycol 6000 (PEG 6000) (14) and 2.5% tween 80 (15)

Filgrastim: Filgrastim Sedico® 300µg/ml/vial, SEDICO pharmaceutical Co., Egypt. Was purchased from local pharmacy. CAS No. AJ05001A10100, LOT No. 0118113.

Dimethyl sulphoxide (DMSO): 99% purity, Batch No. L16A/0116/2511/13, was purchased from El-Gomhoureya Co. for trading medicines, chemicals & Medical appliances, Zagazig. Order No. 1001/120/3635. Manufactured by SDFCL - S D Fine-Chem Limited, India.

Tween 80: 100% purity, Batch No. 2016/3. Was purchased from El-Gomhoureya Co. for trading medicines, chemicals & Medical appliances, Zagazig. Manufactured by El Naser Pharmaceutical Chemical Co. (ADWIC).

Poly ethylene glycol 6000 (PEG-6000): solid, CAS No. 25322-68-3, Batch No. 1212240916, was purchased from El-Gomhoureya Co. for trading medicines, chemicals & Medical appliances, Zagazig. Manufactured by research-lab fine chem. industries, India. Order No. 1001/120/3626/20025.

CD3 immunoexpression: Ready-to-use Polyclonal Rabbit Anti-Human CD3 (Code GA503, Dako Denmark A/S-Produktionsvej 42 -DK-2600 Glostrup – Denmark).

CD20 immunoexpression: CD20 Polyclonal Rabbit IgG Antibody (Catalog # PA5 16701, dilution 1:300, Thermo Fisher Scientific, Rockford, USA).

Animals:

Thirty-six adult male albino rats of mean weight of 180 g were used in this study. Rats were obtained from Faculty of medicine, Zagazig University, Zagazig, Egypt. They were kept at 12 h of light and dark cycles with free access to water and feed. All animals were housed at animal house, faculty of science, Zagazig university according to the guidelines for animal research issued by the National Institute of Health (16), and approved by Animal Ethics Committee, Zagazig University (ZU-IACUC) under number of **ZU-IACUC/1/F/3/2018.**

After one week acclimatization, the animals were divided equally into six groups as follows:

Group I (negative control group): Rats received a vehicle of tween 80 (2.5%) and PEG 6000 (10%) dissolved in saline by intraperitoneal injection for 10 weeks.

Group II (TAA-treated): Received TAA dissolved in sterile normal saline (0.9%) and injected intraperitoneally at a dose of 200 mg/Kg/day, day after day (3 times a week) for 8 weeks (2).

Group III (TAA+RAPA): Received TAA as group II followed by intraperitoneal injection of Rapamycin at a dose of 2 mg/kg/day for 2 successive weeks (1).

Group IV (TAA+FIL): Received TAA as group II followed by Filgrastim administered by subcutaneous injection at a dose of 10 µg/kg every 48 hours for 2 successive weeks (11)

Group V (TAA+RAPA+FIL): Received TAA as group II followed by intraperitoneal injection of Rapamycin with the same dose of group III, and at the same time along with Rapamycin; Filgrastim was administered at the same dose of group IV.

Group VI (TAA+RAPA then FIL): Received TAA as group II followed by Rapamycin before Filgrastim injection at the same doses of group III and IV.

Sampling:

At the end of the experiment, animals of all groups were anaesthetized with ether inhalation. Blood samples were collected from the preiorbital vein. Spleen was dissected for histopathological and immunohistochemistry examination.

Blood sampling:

The whole blood on EDTA was collected and stored refrigerated for complete blood count (CBC), CD3 and CD19.

Tissue sampling:

Specimens of spleen tissues were collected from all groups and prepared for light microscope examination. Spleen was fixed in Bouin's solution, dehydrated and embedded in paraffin wax. 5 µm thick sections were stained with hematoxylin and eosin (H&E), examined and photographed by the light microscope for routine histological examination (17).

Methods:

Flow cytometry using Monoclonal Anti-human CD3 and CD19:

Cell surface expression of CD3 and CD19 is determined by flow cytometer analysis according to Picot, Guerin (18).

Immunohistochemical study:

Splenic CD3 and CD20 were performed using the streptavidin–biotin complex immunoperoxidase technique with the corresponding primary antibodies; used a ready-to-use Polyclonal Rabbit Anti-Human CD3 for CD3 immunoexpression and CD20 Polyclonal Rabbit IgG Antibody for CD20 immunoexpression. Then application of the secondary antibody biotinylated goat anti-rabbit or anti-mouse IgG according to Ramos-Vara, Kiupel (19).

Image analysis and morphometric study:

The area percent (area %) for the immunohistochemical reaction of CD3 and CD20 were morphometrically analyzed using image analyzer computer system. The data were obtained using Leica Qwin 500 image analyzer computer system (Cambridge, UK, Leica Microsystems Imaging Solutions Ltd) in the image analyzing unit at Pathology Department, Faculty of Dentistry, Cairo University, Egypt. The image analyzer consisted of a coloured video camera, coloured monitor, hard disc of IBM personal computer connected to the Olympus microscope (CX 41) and controlled by Leica Qwin 500 software. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units.

CBC:

It was done using Prokan PE-6000 Hematology Analyzer to get Hb, RBCs, WBCs with differential (LYM% and GRAN%)

Statistical analysis:

The data obtained (results of CBC, flow cytometry and morphometric results) for all groups were expressed as means and standard deviations (SD) and subjected to statistical analysis using one-way analysis of variance (ANOVA) test for comparison between the different studied groups (more than two groups) with Posthook: Tukey's test (20).

Results

Levels of circulating Lymphocytes (CD-3 for T-cells and CD-19 for B-cells):

Table (1) described the levels of both T- and B-cells in blood and spleen. The mean values of blood CD-3 and CD-19 lymphocytes of control group were 46.8% and 6.0% respectively. There were a significant increment in TAA group by 22.1% and 25.0% respectively. After treatment with RAPA, CD-3 lymphocytes were significantly increased by 14.6% and CD-19 lymphocytes were insignificantly decreased by 10.0% compared to TAA-treated group. In case of treating with FIL, CD-3 lymphocytes showed insignificant increase only by 11.7%, also CD-19 lymphocytes showed insignificant decrease only by 4.7%. On the other hand, in case of combination therapy, it was found that CD-3 lymphocytes increased by 22.4% and CD-19 lymphocytes decreased by 22.7% for RAPA+FIL group, but CD-3 and CD-19 lymphocytes were both increased by

12.5% and 10.0%; respectively, for RAPA then FIL group. The most increases in CD-3 and decrease in CD-19 lymphocytes percent were found to be in RAPA+FIL group than TAA-treated group.

Levels of splenic Lymphocytes (CD-3 for T-cells and CD-20 for B-cells):

According to table (1), it was found that splenic T- and B-cells were increased after TAA by 259.4% and 5399%; respectively. After treatment with RAPA, T-cells were decreased in splenic tissue by 60.4% but increased in blood. Although, B-cells decreased in spleen by 75.7%, it insignificantly decreased in blood. In case of FIL treatment, both T- and B-cells were reduced by 65.6% and 83.6%; respectively, with no significance effect on blood lymphocytes. The two combination protocols decreased splenic CD-3 and CD-20 cells, with the most decrease was in RAPA+FIL group with 70.0% and 91.6%; respectively. In case of RAPA then FIL protocol, it was 58.3% and 73.5% reduction in both splenic CD-3 and CD-20 cells; respectively. On the other hand, combination therapy increased circulating T-cells in both protocols, but decreased B-cells with the RAPA+FIL and insignificant increase with RAPA then FIL protocol.

Hematological results:

WBCs count:

Significant increase in WBCs count was observed after exposure to TAA (by 30.6% over the control group). After treatment with RAPA, WBCs were decreased by 30.8% compared to FIL which only decreased it by 12.3%. Also, there were further decrease in case of combination therapy, RAPA+FIL or RAPA then FIL group, by 25.0% and 27.4% respectively, with significance ($p<0.001$) to TAA group.

WBCs pattern (GRAN% and LYM%):

Granulocytes percent (GRAN%) and lymphocytes percent (LYM%) are the main WBCs members circulating in the blood. It was found that GRAN% increased in TAA group only by 17.0% and LYM% decrease only by 1.8%, with no significance. After the treatment with RAPA, GRAN% further increased by 106.4% and LYM% further decreased by 23.1%, with significance ($P<0.001$). While treating with FIL affect GRAN% and LYM% with no significance increase, only by 6.1% and 1.4%; respectively. In case of combination therapy, it was found that GRAN% to be increased by 68.4% and LYM% decreased by 16.9% for RAPA+FIL group and by 43.7% and 19.8% for RAPA then FIL group; respectively.

The most increase in GRAN% was found to be in RAPA monotherapy group (106.4%) and the most decrease in LYM% was found to be in the same group (23.1%) than TAA group. These results show that the control group has the lowest GRAN% and the highest LYM%.

Haemoglobin levels:

As shown in Table (2) that described the CBC results obtained for all group, It was found that Hb decreased after treatment with TAA by 3.2 g/dl (19.5%) than found in control group with decreased RBCs count by 20.8% with significance ($P<0.001$). After medication by Rapamycin only, there was significant increment compared to TAA-treated group by 1.5 g/dl (11.5%). Filgrastim treatment after TAA, increased Hb by 0.9 g/dl (7.0%). In case of combination group (RAPA+FIL), it showed a 1.6 g/dl increasing (12.3%) with no significant difference to RAPA monotherapy. In case of RAPA then FIL protocol therapy, Hb was increased by 13.8% that was significant than FIL monotherapy. The most increase in Hb level was found in RAPA then FIL group than TAA-treated group.

RBCs count:

RBCs count was decreased in TAA group by 20.8%. After treatment of splenomegaly with RAPA or FIL alone, there was no significant increase in RBCs, only by 9.0% and 7.4% more than TAA group; respectively. Also, in case of combination therapy, RAPA+FIL group and RAPA then FIL group, there was no significant increase in RBCs noticed (only by 11.9% and 9.1% respectively). The most increase in RBCs count was found to be in RAPA+FIL group than TAA group.

Platelets (Plts) count:

TAA group showed insignificant decreased number of Plts by 8.0% which also not increased after RAPA treatment but unfortunately further decreased by 36.0%. Treatment of TAA group with FIL showed the lower drop in Plts count, with only about 4% reduction, compared to RAPA-treated group. No group showed any increase in Plts count. The two combination protocol therapies, showed only 19% decrease with a significant difference ($P<0.001$) between the combination therapy and either RAPA or FIL monotherapy.

Histological Results:

Light microscope examination of spleen sections:

H&E-stained sections of the control adult male albino rats' spleen revealed normal spleen architecture with its two major components; white pulp and red pulp. Thin connective tissue capsule and trabeculae can be seen **(Fig. 1A)**. TAA-treated group showed hyperplastic lymphatic follicles and some of them were disorganized, congested red pulp and thickened trabeculum with dilated and congested trabecular veins **(Fig. 1B)**. Rapamycin-treated group showed lymphatic follicles with thickened and dilated central arteriole. Disorganized connective tissue trabeculae with congested trabecular vein were also seen **(Fig. 1C)**. Filgrastim-treated group showed lymphatic follicle with peripheral arteriole, congested red pulp and thin trabeculum could be seen **(Fig. 1D)**. Combined Filgrastim and Rapamycin group showed normal shaped lymphatic follicles with peripherally located central arteriole. The red pulp showed normal appearance with thin trabeculae and covered by thin connective tissue capsule **(Fig. 1E)**. Rapamycin then Filgrastim group showed normal appearance of lymphatic follicles, slightly congested red pulp, thin capsule, disorganized trabeculum and dilated venous sinus **(Fig. 1F)**.

Immunohistochemistry results:

Examination of CD3 immunostained sections in the spleen of control group showed CD3+ T cells appearing mainly in periarterial lymphatic sheath (PALS) of the white pulp. **(Fig. 2A)**. TAA group showed marked increase in the number of CD3+ T cells in the PALS of the white pulp in comparison to control group **(Fig. 2B)**. Rapamycin-treated group showed an apparent increase in the number of CD3+ T cells in the PALS compared to control group **(Fig. 2C)**. Filgrastim-treated group showed normal appearance of CD3+ T cells in the PALS **(Fig. 2D)**. Combined Rapamycin and Filgrastim treatment group **(Fig. 2E),** showed normal appearance of CD3+ T cells in the PALS while, Rapamycin then Filgrastim group showed an apparent increase in the number of CD3+ T cells in the PALS **(Fig. 2F)**

Examination of CD20 immunostained sections in the spleen of control group showed CD20+ B cells appearing mainly in lymphatic follicles of white pulp **(Fig. 3A)** while, TAA-treated group showed marked increase in expression of CD20+ B cells both in lymphatic follicles of white pulp and in red pulp when compared to control group **(Fig. 3B)**. Rapamycin- treated group **(Fig. 3C)** showed expression of CD20+ B cells lymphatic follicles of white pulp and few cells appeared in red pulp. On the other hand, nearly normal appearance of CD20+ B cells localized in lymphatic

follicles of white pulp were seen in spleen sections of Filgrastim-treated group **(Fig. 3D)**, and combined Filgrastim and Rapamycin group **(Fig. 3E).** Rapamycin then Filgrastim group showed CD20+ B cells in lymphatic follicles of white pulp and few cells appeared in red pulp **(Fig. 3F).**

Discussion

Spleen is an important immune system organ in blood homeostasis. It also acts as a reservoir for platelets; nearly 33% of platelets are stored. Hence maintaining or restoring a proper spleen function is of great importance on circulating blood cells count and functions (21). One of the most common causes of splenomegaly is liver disease, especially portal hypertension and infectious HCV (22).

Thioacetamide (TAA) induces splenomegaly by its direct effect on spleen and also on liver tissue, inducing portal hypertension with subsequent splenomegaly (23). The overall increased splenic lymphoid tissue proliferation and neovascularization in the splenic red pulp resulted in splenomegaly (24). This splenomegaly was confirmed by histopathological examination as explained in figure 1.

Lymphocytes were associated with alterations in spleen structure and blood distribution according to the critical role of spleen on storing and dealing with lymphocytes. Both CD3 and CD19 surface markers that refer to T- and B-lymphocytes; respectively, were increased in TAA-group. T-cells results matches that found in hepatocellular carcinoma-bearing mouse model stated in Fang, Zhu (25) both in blood and splenic tissue after splenomegaly induction. Another study of Fujii, Kimura (26) showed that TAA leads to increased amount of CD3 lymphocytes inside hepatocyte, indicating that TAA may increase CD3 proliferation that reach blood and also reside inside splenic tissues. Also, blood B-cells were increased according to Fujii, Kimura (26). In case of splenic B-cells, our results showed vast increasing as reported in Fang, Zhu (25) results. This massive increased splenic B-cells but only the small extent increasing in the blood, because nearly all B-cells reside lymphoid tissues, like spleen, with only 2% in the blood (27).

T-cells proliferation has a link with mTOR activation and inhibition (28). This explains the increased expression of $CD3^+$ T cells in TAA group and decrease with RAPA groups in the present study; due to inhibition of mTOR signaling as mentioned in Zheng, Collins (29) as described as T-cells anergy (loss of its function and detection). This reduction in T-cells was confirmed by Luo, Duguid (30) on mice that caused thymus atrophy with a strong negative effect on T-cell proliferation. Another approach explaining the increased circulating CD3 cells is by the protective role of RAPA as mentioned by Shu, Chen (31) giving it more chances to be increased in the blood. Also, decreased amount of T- and B-cells in spleen is due to anti-inflammatory effect of RAPA (32).

The present study revealed that RAPA treatment reduces the number of blood or spleen B-cells. Decreased blood B-lymphocytes in case of concomitant RAPA and FIL treatment but not separately. It was found that, RAPA treatment makes these cells of lower survival rates via reduction of the germinal center B-cells proliferation, hence lower amount available in the blood and spleen via RAPA direct effect on mTOR inhibition (28). Also, blocking of B-cells resulted in activation of some types of T-cells that matches our results concerning this impact (28).

Filgrastim alone showed insignificant effect on circulating T- and B-cells; its effect on lymphocyte subtypes is less in case of presence of illness like splenic pathology. In case of concomitant RAPA and FIL treatment, the effect on blood

CD3 cells was found to be additive value. But separately RAPA and FIL treatment was considered as the same as using FIL alone; end of RAPA effect then the FIL appears to take its role with less effect with the persistent splenic pathology.

WBCs count was increased with TAA-group, likely to participate in fighting the xenobiotic (23). FIL treatment alone showed lower WBCs count than TAA-group, due to the little effect of FIL on spleen pathology; large pooling capacity of the splenic tissue (33). On the other hand, all groups received RAPA showed lower WBCs count, due to reduced proinflammatory cytokines after RAPA according to in vivo experiment done by Kim, Min (32). This RAPA effect on decreasing WBCs count matched the effect of RAPA on decreasing the synthetic capacity to vast number of components in the body.

Treating rats with TAA nearly doesn't affect peripheral LYM% (34). All groups received RAPA were found to have decreased blood LYM% due to effect of RAPA (35). Filgrastim effect on blood LYM% may be considered as neglected effect. These results are reversed in case of talking about GRAN% for RAPA-group, where GRAN% was increased. TAA-group showed slight GRAN% increasing that further increased when treated with RAPA, but with no significant effect when treated with FIL (36). Our results showed significant increase in blood GRAN% with RAPA, due to ameliorated spleen that liberates more neutrophils to the blood stream and not infiltrate other tissues rather blood. This increase in GRAN% is less prominent in case of concomitant RAPA and FIL treatment and lesser in separated RAPA and FIL treatment, showing that RAPA is doing well alone on GRAN% increasing. It is proved that FIL has the ability to liberate granulocytes from bone marrow into circulation (37). Hence the concomitant RAPA and FIL treatment showed a higher percent than the separated one. Because the increased formed neutrophils in case of injected RAPA tend to disappear when RAPA is stopped in case of the separated strategy, giving the chance for those previously formed granulocytes to be destroyed, with fewer chances from FIL to keep it elevated for longer time.

Levels of Hb and RBCs were significantly decreased with insignificant decreased Plts count in TAA-group due to splenomegaly that increased blood sequestration and reduced production of new platelets (38), also the reduced thrombopoietin production by the liver. Reduced blood RBCs count, in turn, reduced Hb level. The decreased RBCs and Hb were reversed by RAPA alone; reducing reservoir capacity of the spleen in case of groups received RAPA. These results agreed with the findings in Mejias, Garcia-Pras (1) with increased RBCs and Hb.

Platelets count was decreased in all groups of our study. The marked decrease with RAPA treatment could be explained by the lowered synthetic capacity of RAPA. Splenomegaly rats treated with FIL showed nearly no further decrease in Plts count. In the concomitant RAPA and FIL treatment, more splenomegaly reduction resulted in more Plts available in the blood, better than RAPA or FIL alone. Chen, Burke (39) reported that, treatment with Peg-G-CSF (a modified form of G-CSF), improved platelet counts in radiation injury (RI) mice, suggesting that G-CSF can stimulate megakaryocytes in the bone marrow.

Conclusion:

A more prominent effect on lowering splenic lymphocytes expression evolved with combined Rapamycin and Filgrastim at the same time accompanied to better splenic status. Splenic injury is characterized with a vast increasing in splenic B-cells. It is recommended to

find a way that improves platelets count with Rapamycin administration.

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Table 1 mmunological measurements among the studied groups

Variable	Group	Mean ±SD
Blood CD3(T-cells)	Control -ve	46.83 ±5.19
	Control +ve (TAA)	57.17 [a] ±3.43
	TAA + RAPA	65.50 [a,b] ±3.94
	TAA + FIL	63.83 [a] ±2.64
	TAA+RAPA+FIL	70.00 [a,b] ±6.45
	TAA+RAPA then FIL	64.33 [a,b] ±1.63
Blood CD19(B-cells)	Control -ve	6.00 ±0.71
	Control +ve (TAA)	7.50 [a] ±1.18
	TAA + RAPA	6.75 ±0.76
	TAA + FIL	7.15 ±0.80
	TAA+RAPA+FIL	5.80 [b] ±0.86
	TAA+RAPA then FIL	8.25 [a,c,e] ±0.94
Splenic CD3 (T-Cells)	Control -ve	7.99 ±0.85
	Control +ve (TAA)	28.71 [a] ±1.60
	TAA + RAPA	11.37 [a,b] ±2.19
	TAA + FIL	9.85 [b] ±1.53
	TAA+RAPA+FIL	8.61 [b] ±1.75
	TAA+RAPA then FIL	11.95 [a,b,e] ±1.31
Splenic CD20 (B-Cells)	Control -ve	0.35 ±0.12
	Control +ve (TAA)	18.97 [a] ±4.00
	TAA + RAPA	4.60 [a,b] ±1.24
	TAA + FIL	3.10 [b] ±1.55
	TAA+RAPA+FIL	1.58 [b] ±0.41
	TAA+RAPA then FIL	5.02 [a,b,e] ±0.41

SD: Stander deviation, all are of highly significant ($p<0.001$), a: significant with control –ve, b: significant with TAA-treated, c: significant with TAA + RAPA, d: significant with TAA + FIL, e: significant with TAA+RAPA+FIL.

Table 2 CBC results among the studied groups

Variable	Group	Mean ±SD
Hb	Control -ve	16.52 ±0.48
	Control +ve (TAA)	13.30 [a] ±0.28
	TAA + RAPA	14.83 [a,b] ±0.34
	TAA + FIL	14.23 [a,b] ±0.42
	TAA+RAPA+FIL	14.93 [a,b] ±0.22
	TAA+RAPA then FIL	15.13 [a,b,d] ±0.50
WBCs	Control -ve	9.65 ±0.52
	Control +ve (TAA)	12.60 [a] ±0.75
	TAA + RAPA	8.72 [b] ±0.73
	TAA + FIL	11.05 [a,b,c] ±0.72
	TAA+RAPA+FIL	9.45 [b,d] ±0.31
	TAA+RAPA then FIL	9.15 [b,d] ±0.40
LYM%	Control -ve	67.07 ±1.63
	Control +ve (TAA)	65.83 ±5.97
	TAA + RAPA	50.60 [a,b] ±6.34
	TAA + FIL	66.73 [c] ±3.16
	TAA+RAPA+FIL	54.72 [a,b,d] ±3.32
	TAA+RAPA then FIL	52.80 [a,b,d] ±2.94
GRAN%	Control -ve	15.37 ±0.73
	Control +ve (TAA)	17.98 ±0.72
	TAA + RAPA	37.12 [a,b] ±4.84
	TAA + FIL	19.08 [c] ±0.75

	TAA+RAPA+FIL	30.28 [a,b,c,d] ±3.23
	TAA+RAPA then FIL	25.83 [a,b,c,d,e] ±2.32
RBCs	Control -ve	7.67 ±0.36
	Control +ve (TAA)	6.07 [a] ±0.32
	TAA + RAPA	6.62 [a] ±0.36
	TAA + FIL	6.52 [a] ±0.23
	TAA+RAPA+FIL	6.79 [a] ±0.21
	TAA+RAPA then FIL	6.63 [a] ±0.39
Plts	Control -ve	891.67 ±58.45
	Control +ve (TAA)	819.33 ±76.07
	TAA + RAPA	526.83 [a,b] ±76.97
	TAA + FIL	783.00 [a,c] ±64.13
	TAA+RAPA+FIL	663.00 [a,b,c,d] ±47.68
	TAA+RAPA then FIL	664.50 [a,b,c,d] ±51.11

SD: Stander deviation, all are of highly significant ($p<0.001$), a: significant with control –ve, b: significant with TAA-treated, c: significant with TAA + RAPA, d: significant with TAA + FIL, e: significant with TAA+RAPA+FIL.

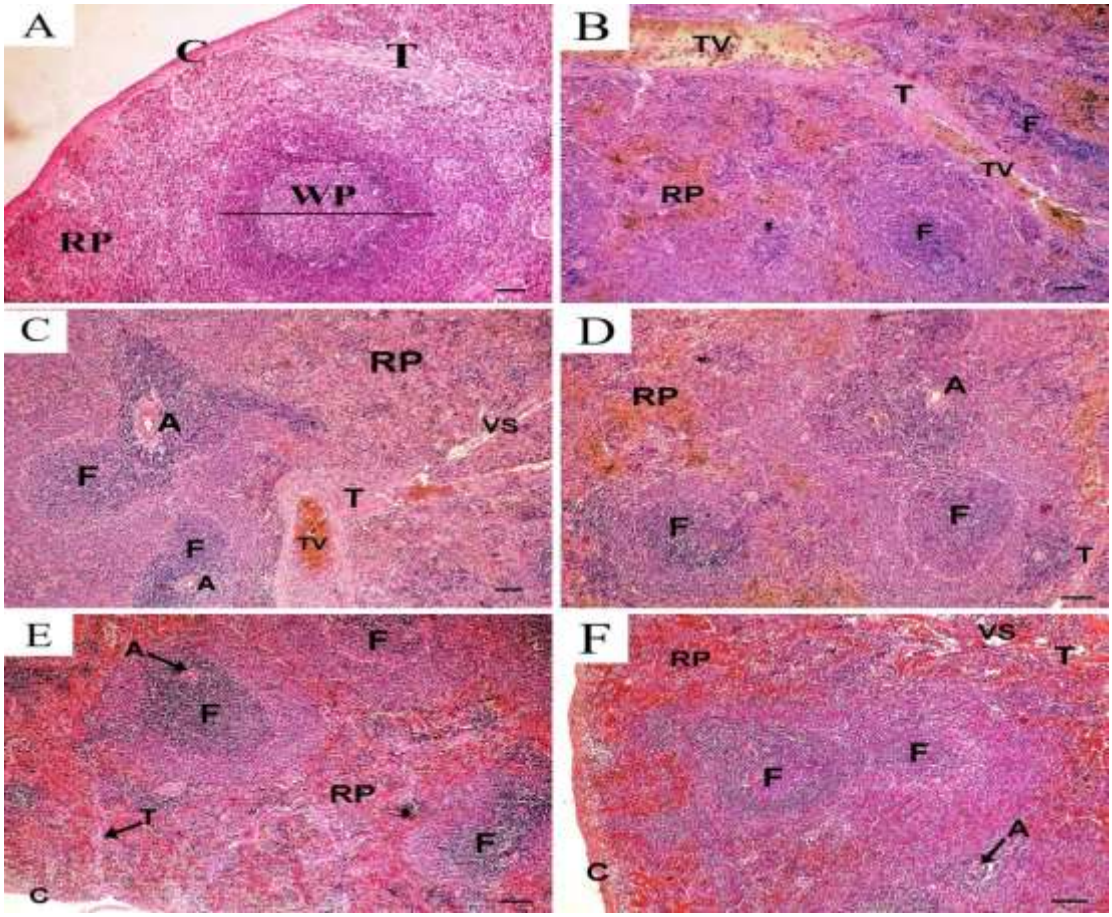

Figure (1): H & E-stained sections of adult male albino rats' spleen. **A**: the control group shows normal spleen architecture with its two major components; white pulp (WP), and red pulp (RP). Thin connective tissue capsule (C) and trabeculae (T) can be seen. **B**: TAA-treated group shows hyperplastic lymphatic follicles and some of them are disorganized (F), congested red pulp (RP) and thickened trabeculum (T) with dilated and congested trabecular vein (TV). **C**: Rapamycin-treated group shows lymphatic follicle (F) with thickened and dilated central arteriole (A). Disorganized connective tissue trabeculae (T) with congested trabecular vein (TV) are also seen. **D**: Filgrastim-treated group shows lymphatic follicles (F) with peripheral arteriole (A), congested red pulp (RP) and thin trabeculum (T). **E**: Combined Filgrastim and Rapamycin group shows normal shaped lymphatic follicles (F) with peripherally located central arteriole (A). The red pulp (RP) shows normal appearance with thin trabeculae (T) and covered by thin connective tissue capsule (C). **F**: Rapamycin then Filgrastim group shows normal appearance of lymphatic follicles (F), slightly congested red pulp (RP), thin capsule (C), disorganized trabeculum (T) and dilated venous sinus (VS). **(H & E stain x 10, scale bar 50 µm)**

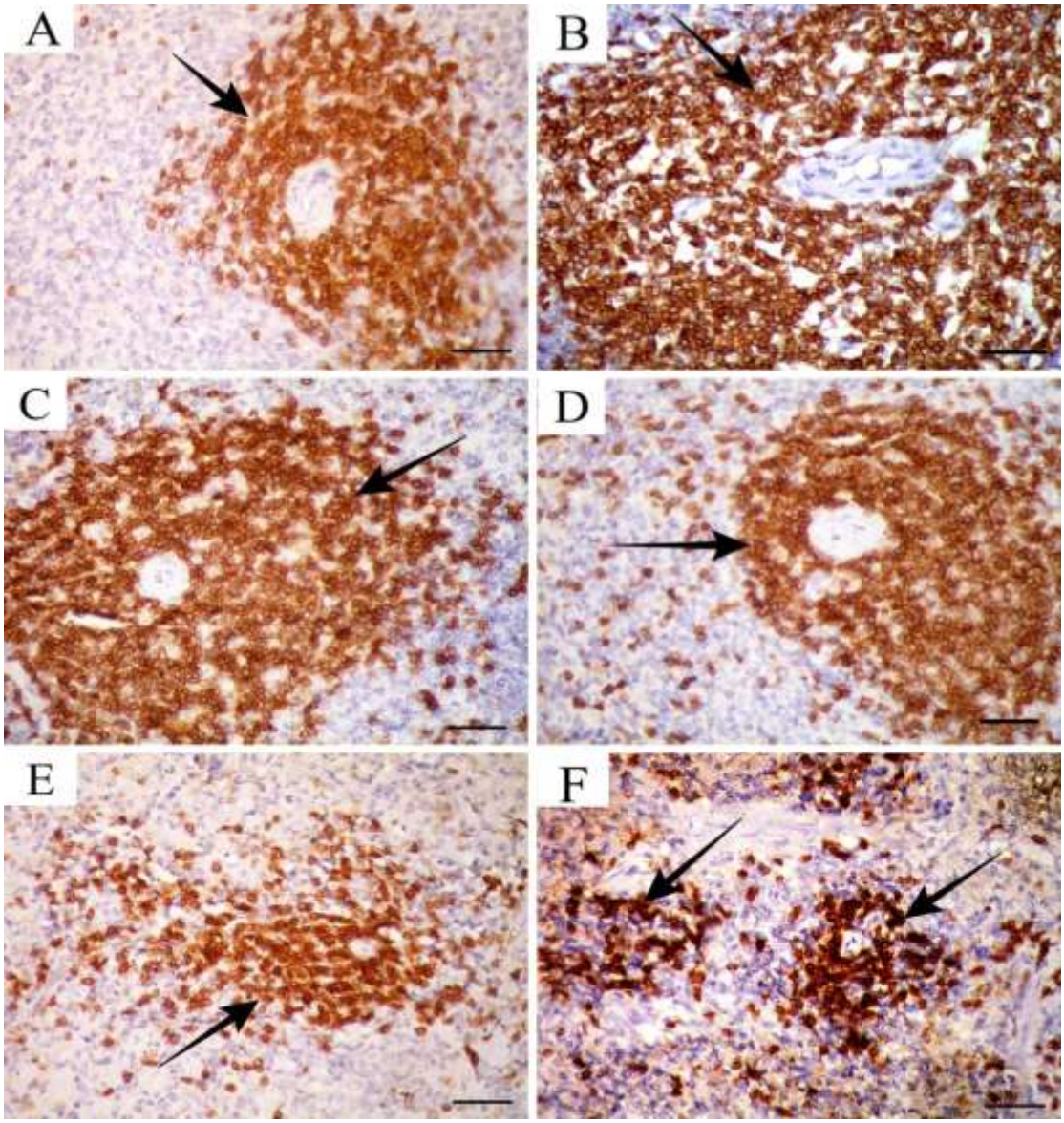

Figure (2): CD3 immunostained sections in the spleen showing **A:** control group shows CD3+ T cells appearing mainly in periarterial lymphatic sheath (PALS) of the white pulp. **B:** TAA group shows marked increase in the number of CD3+ T cells in the PALS of the white pulp. **C:** Rapamycin-treated group shows an apparent increase in the number of CD3+ T cells in the PALS. **D:** Filgrastim-treated group shows normal appearance of CD3+ T cells. **E:** Combined Rapamycin and Filgrastim treatment shows normal appearance of CD3+ T cells in the PALS while, **F:** Rapamycin then Filgrastim group shows an apparent increase in the number of CD3+ T cells in the PALS. **(Immunoperoxidase technique for CD3 x 40, scale bar 30 µm)**

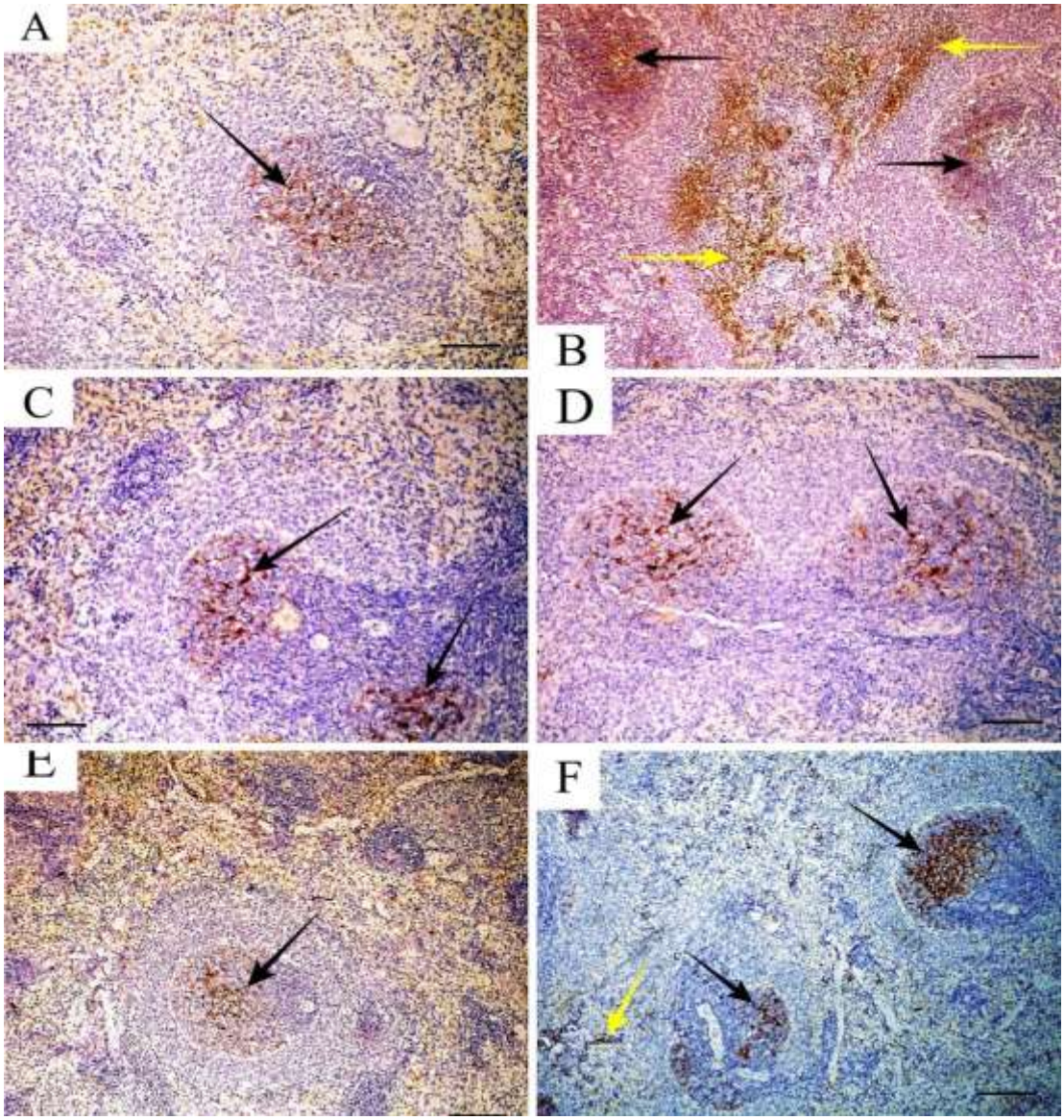

Figure (3): CD20 immunostained sections in the spleen showing **A**: control group shows CD20+ B cells appearing mainly in lymphatic follicles of white pulp (arrow). **B**: TAA-treated group shows marked increase in expression of CD20+ B cells both in lymphatic follicles of white pulp (black arrow) and in red pulp (yellow arrow). **C**: Rapamycin- treated group shows CD20+ B cells lymphatic follicles of white pulp (arrow) and few cells appear in red pulp. **D &E**: nearly normal appearance of CD20+ B cells localized in lymphatic follicles of white pulp are seen in sections of Filgrastim-treated group, and combined Filgrastim and Rapamycin group. **F**: Rapamycin then Filgrastim group shows CD20+ B cells in lymphatic follicles of white pulp (black arrow) and few cells appear in red pulp (yellow arrow). **(Immunoperoxidase technique for CD20 x 20, scale bar 50 µm)**