

How to Cite:

Nichita, A., Anoor, P. K., & Burgula, S. (2022). Retinol rescues immune cells from inflammation in bacterial infection. *International Journal of Health Sciences*, 6(S1), 2712–2721.
<https://doi.org/10.53730/ijhs.v6nS1.5328>

Retinol Rescues Immune Cells from Inflammation in Bacterial Infection

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Abstract---Retinol (Vitamin A), is a well-known supplement for the repair of the wound, cell growth, and good vision. Through its implication in immune function is well established, further its recommendation as a supplement during the prognosis from infection along with antibacterial substances is still uncertain. Our study aims at understanding its anti-inflammatory effect on some known inflammatory markers, blood from a Healthy donor was collected for isolation of PBMCs and further induced with or without LPS (*E. coli* and *Klebsiella*). After 18 h of incubation, mRNA was isolated for qRT PCR quantification and studied for its effect. Retinol had good inhibitory action on genes under study such as IL6, TNF α , NF κ B, SAA, TLR4, PNOC which are known to be pro-inflammatory in presence of LPS. Whereas its action on IL10 a well-known anti-inflammatory cytokine is contradictory to its suppressive action. Indicating its role as an anti-inflammatory supplement that can hold the expression of many mediators participating in inflammation and aid in controlling inflammation-led cytokine Strom.

Keywords---Peripheral blood mononuclear cells (PBMCs), LPS, Proinflammatory markers.

Introduction

Retinol is available as a supplement, as it has many health benefits. It is necessary for good eyesight, improves cell growth, and reduces skin wrinkles. More importantly, its application is very well studied in preventing infection [1] reducing morbidity in children suffering from diarrhea [2], and also as an immune modulator [3]. Retinol is been used as an anti-inflammatory agent for therapeutic

International Journal of Health Sciences ISSN 2550-6978 E-ISSN 2550-696X © 2022.

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Manuscript submitted: 27 Jan 2022, Manuscript revised: 18 Feb 2022, Accepted for publication: 9 March 2022
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supplement in treating infectious diseases [4]. Its availability in the market is also very frequent and rational. Its multifaceted function leads to an investigation of its role as an anti-inflammatory supplement to prevent inflammation under uncontrollable circumstances [5]

During bacterial infection, most of the Gram-negative bacteria trigger inflammation due to lipopolysaccharide LPS binding to TLR4 receptor [6], a sequence of cascade begins which initiates the production of different cytokines IL6, TNF α , IL10 [7] inflammatory peptides SAA, PNO, NGAL, and other transcription factors NF κ B, STAT3 and other mediators which are very essential for establishing defense against bacterial infection and also stimulate immune cell proliferation and differentiation, this stage is described as acute phase response [8] Exaggerated LPS signal or failure of neutralization of LPS leads to a different condition which leads to cytokine Storm resulting in deleterious effects and organ failure [9].

The mechanism of regulating the inflammation signals by acute-phase proteins APP follows proinflammatory cytokines exaggerating inflammatory signals and antiinflammation cytokines decline inflammatory signals [10]. Proinflammatory signals are very crucial for proper immune function and establishing a defense against infection. Whereas anti-inflammatory signals are very critical for controlled production of inflammation which prime to cell damage [11]

Our study is designed to understand the insight of Retinol as an anti-inflammatory supplement. Its action on transcripts of prominent inflammatory markers after induction of PBMC with LPS is studied. Retinol prevails as a powerful anti-inflammatory mediator [12] which can be used as a supplement along with antibacterial substances extended during treating an infected person. This study can establish retinol as an important supplement obtained universally and at a very low cost to include in treating a person suffering irrepressible inflammation due to bacterial infection.

Materials and Methodology

The study was conducted in the Department of Microbiology, Osmania University where Blood was drawn from a healthy donor collected, and processed further for isolation of PBMCs.

Isolation of PBMCs

PBMCs were isolated from 5ml of blood added with anticoagulant, to which 2.5ml of Histopaque (Sigma -Aldrich, India) was added and gently mixed to create two different layers and centrifuged for 30 minutes at 100 X g at 4 °C. After centrifugation, a whitish buffy coat formed between the interface is aspirated into a separate tube. The PBMCs obtained thus are washed twice with 5ml PBS and centrifuged for 10minutes, later the pellets were resuspended in 1ml of PBS. Controls were maintained simultaneously.

Induction of LPS and costimulation with Retinol

PBMCs were suspended in RPMI medium supplemented with Glutamine (Gibco™ RPMI 1600) in a 2:1 ratio. 1 ml of PBMCs suspended in RPMI were subjected to induction to *E. coli* and *k. pneumonia* (Sigma-Aldrich USA) LPS in 10ng/ml and stimulated with or without 5µg/ml retinol (R7632 Sigma-Aldrich, USA) and incubate for 18 hours at 37 °C and adequate CO₂. The treated PBMCs were subjected to RNA isolation.

RNA Isolation by TRI reagent

Treated PBMCs were subjected to RNA isolation by TRI reagent, to 1ml of treated PBMCs 1ml TRI reagent (Sigma- Aldrich, India) was added, mixed, and further incubated at RT for 5 minutes. To this 0.2ml of cold chloroform was added, tubes were inverted and incubated for another 3 minutes at RT. Then tubes were subjected to centrifugation for 15 minutes at 12,000 X g at 4 ° C, resulting in the upper aqueous phase of RNA, which was collected into a fresh tube, and 0.5ml of cold isopropanol was added and incubated for 10 minutes at RT, immediately centrifuged at 12000 X g for 10 minutes to obtain RNA in form of a pellet, which is washed with 0.5ml Of 75% Ethanol, air-dried and reconstituted in 50 µl of RNAase- free water.

cDNA Synthesis

Isolated RNA was subjected to agarose gel electrophoresis for visualizing the quality of RNA, later subjected to the synthesis of cDNA by iScript cDNA synthesis kit (Bio-Rad).

Real-Time PCR (qRT PCR)

After cDNA synthesis, the reaction mix was subjected to qRT PCR with SYBER Green premix (Applied Biosystem, USA). The genes under study were IL6, TNFα, IL10, NFκB, SAA, PNOC, STAT3, TLR4, primer sequence is listed in Table 1. The gene amplification was analyzed using StepOnePlus RT PCR System (Thermo Fisher Scientific), for each gene separate reaction mixes were maintained in triplicates. The set of primers was designed using Primer 3 software.

Statistical analysis

All the results were statistically analyzed and a significant difference of (P<0.05) was considered statistically significant

Results

Inhibition of inflammatory Markers by Retinol

The treated PBMCs subjected to qRT PCR and $\Delta\Delta C_t$ normalized against GAPDH, showed inhibition of genes under study by Retinol. It exerted inhibition on genes involved in inflammation such as IL6, TNFα, NFκB, TLR4, SAA, PNOC, whereas STAT3 and IL10 showed increased expression. When LPS has added alone all

genes showed upregulation, Retinol in costimulation with LPS could mask the effect of LPS and suppress their expression (Figure 1 and Figure 2).

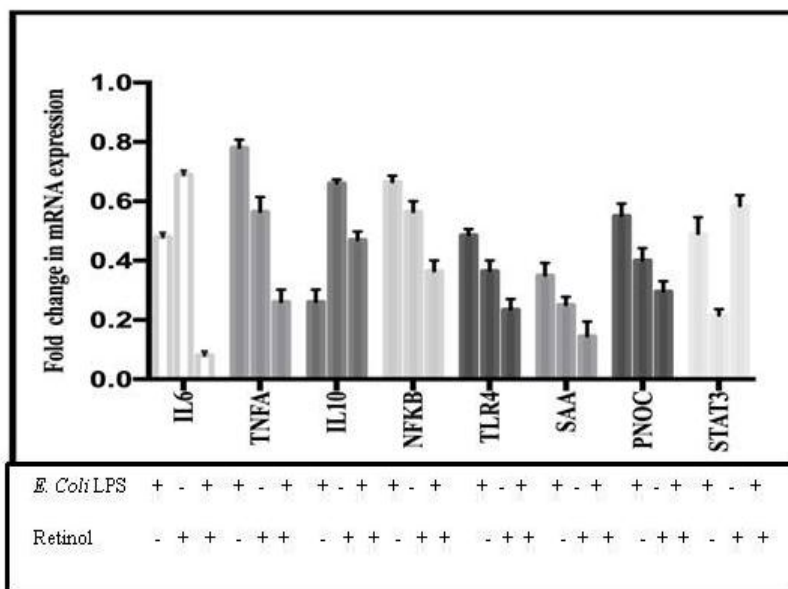


Figure 1 shows the effect of retinol in presence of *E. coli* LPS on inflammatory markers
(+ indicates its presence and - indicates its absence)

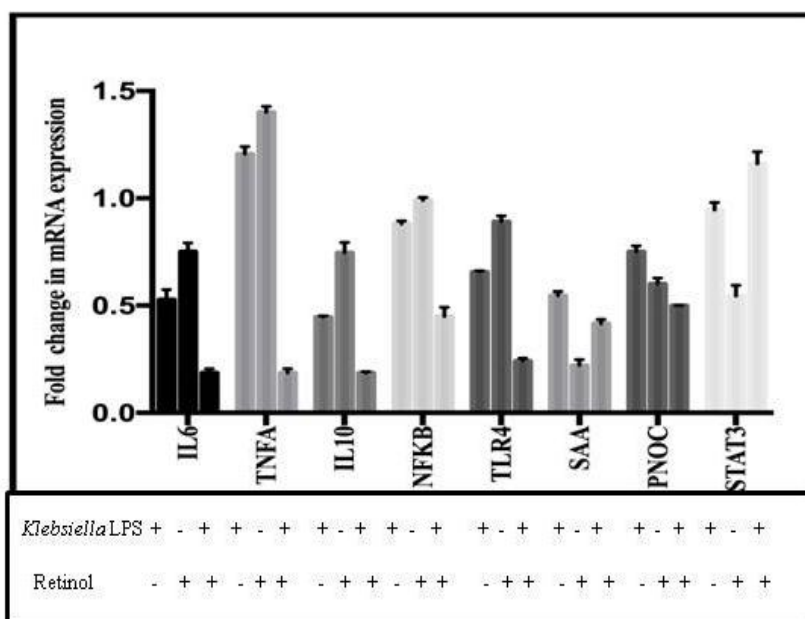


Figure 2 shows the effect of retinol in presence of *Klebsiella* LPS on inflammatory markers. (+ indicates its presence and - indicates its absence)

Effect of Retinol on IL6 and TNF α

Prominent Inflammatory markers IL6 and TNF α were upregulated in presence of both LPS, *Klebsiella* LPS was able to induce the makers more profoundly. A comparative analysis between IL6 and TNF α (Figure 3), shows a 0.4- and 0.53-fold decrease in IL6 and TNF α transcripts respectively in presence of *E. coli* LPS and Retinol. Similarly, a 0.3- and 1-fold decrease was observed for IL6 and TNF α co-stimulated with retinol and *Klebsiella* LPS.

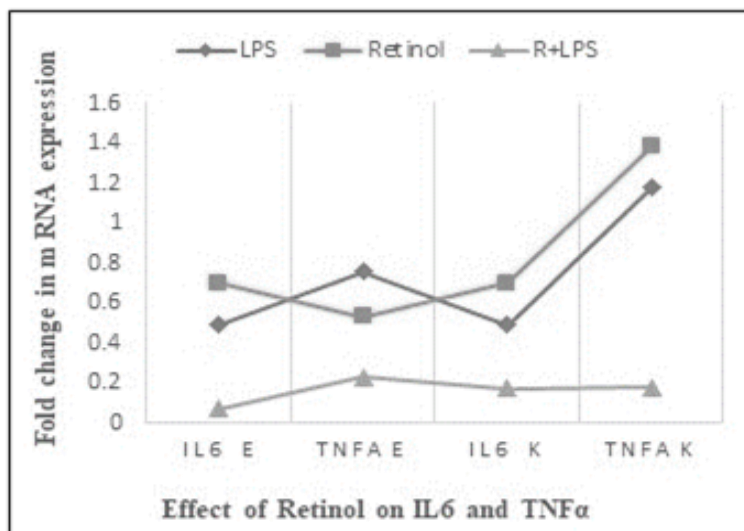


Figure 3 shows a comparative study on the effect of Retinol on inflammatory markers in presence of LPS
(Here E is *E. coli* LPS and K is *Klebsiella* LPS)

Fold Change in mRNA expression of Inflammatory markers

Marked inhibition was observed for individual genes, the fold change was significant (Table 4). IL6 showed 0.42 and 0.32, TNF α 0.53 and 1, NF κ B fold change was 0.31 and 0.45, TLR4 showed 0.26- and 0.43-fold change respectively in presence of *E. coli* and *Klebsiella* LPS and retinol respectively. IL10 showed upregulation along with STAT3, the fold increases were 0.22 and 0.46 for IL10 and 0.11 and 0.2 for STAT3 in presence of *E. coli* and *Klebsiella* LPS and retinol respectively. A significant difference of $P < 0.05$ was considered.

Table 1 Shows Fold change in mRNA expression of genes understudy

S.No	Gene	Fold change in presence of LPS	
		<i>E. coli</i>	<i>Klebsiella</i>
1	IL6	0.42	0.32
2	TNFα	0.53	1
3	NFκB	0.31	0.45
4	TLR4	0.26	0.43
5	SAA	0.21	0.12
6	PNOC	0.25	0.24
7	IL10	+0.22	+0.46
8	STAT3	+0.11	+0.2

Effect of Retinol on STAT3

STAT3 mRNA transcript showed a varied regulation when compared to other markers. The inhibitory effect of retinol was not shown on it in the presence of LPS. Further retinol alone showed suppression of STAT3.

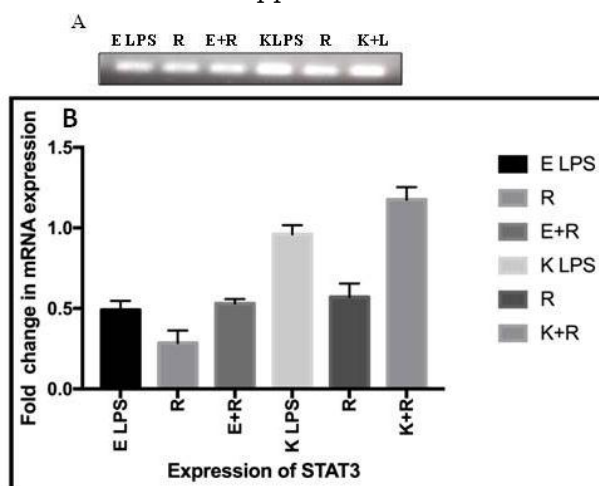


Figure 4 shows the effect of retinol on STAT3 in presence of with and without LPS.

Discussion

During infection, to initiate inflammation, many kinds of cytokines, inflammatory peptides, and inflammatory mediators from a network of the cascade generate a regulated immune response against invading infection. It is a controlled process, due to lack of neutralization of signal or overwhelmed reaction of the immune system towards the infection, it can lead to a condition known as cytokine storm [13]. It can lead to devastating conditions in the cell, which can end up in the failure of the organ. Inflammatory signals thus are sent to the cell by proinflammatory molecules which can exaggerate the condition to the worst. To

regulate the function of proinflammatory molecules, anti-inflammatory molecules counteract them [14]. Our study aims at exploring the anti-inflammatory effect of Retinol in form of a supplement on prominent inflammation biomarkers. To this end LPS of *E. coli* and *Klebsiella* at a concentration of 10ng/ml was sufficient to create an inflammatory condition in PBMCs. Further co-stimulation with retinol was able to reverse the inflammatory condition by exerting an anti-inflammatory effect on genes involved in inflammation Figures 1 and 2. Its inhibition or suppression was very profoundly detected. All known inflammatory markers IL6, TNF α , NF κ B, TLR4, SAA, PNOC were involved in our study were able to be down-regulated by the addition of retinol in presence of LPS. And also, it was found that the IL10 anti-inflammatory cytokine was up-regulated and STAT3, a transcription factor independent of NF κ B was also shown up-regulation in figure 4, further investigation needs to confirm this outcome.

Antimicrobial agents are generally used as a source to stop the multiplication of bacteria, as well as they, act as anti-inflammatory agents especially macrolides with fewer side effects [15] but others may have major disadvantages which prove to be possessing many side effects that in turn may impair the immune system [16]. During the surge of COVID 19, many different anti-inflammatory drugs were underused [17]. Our study is aiming at the importance of anti-inflammatory agents which can be used in combination with antimicrobial substances and have very minute side effects on the host under observation. In this quest retinol as an anti-inflammatory supplement was observed due to its significance during LPS induction and its attenuation of LPS induced inflammation [18]. Its suppressive role is due to the diminution of major signal transducers participating in inflammation pathways such as NF κ B/ JNK pathways, which play a central dogma in initiating the inflammation circuit.

Indicating the role of Retinol as a therapeutic substance that could be supplemented along with antimicrobial substances which could be prescribed to a patient undergoing treatment.

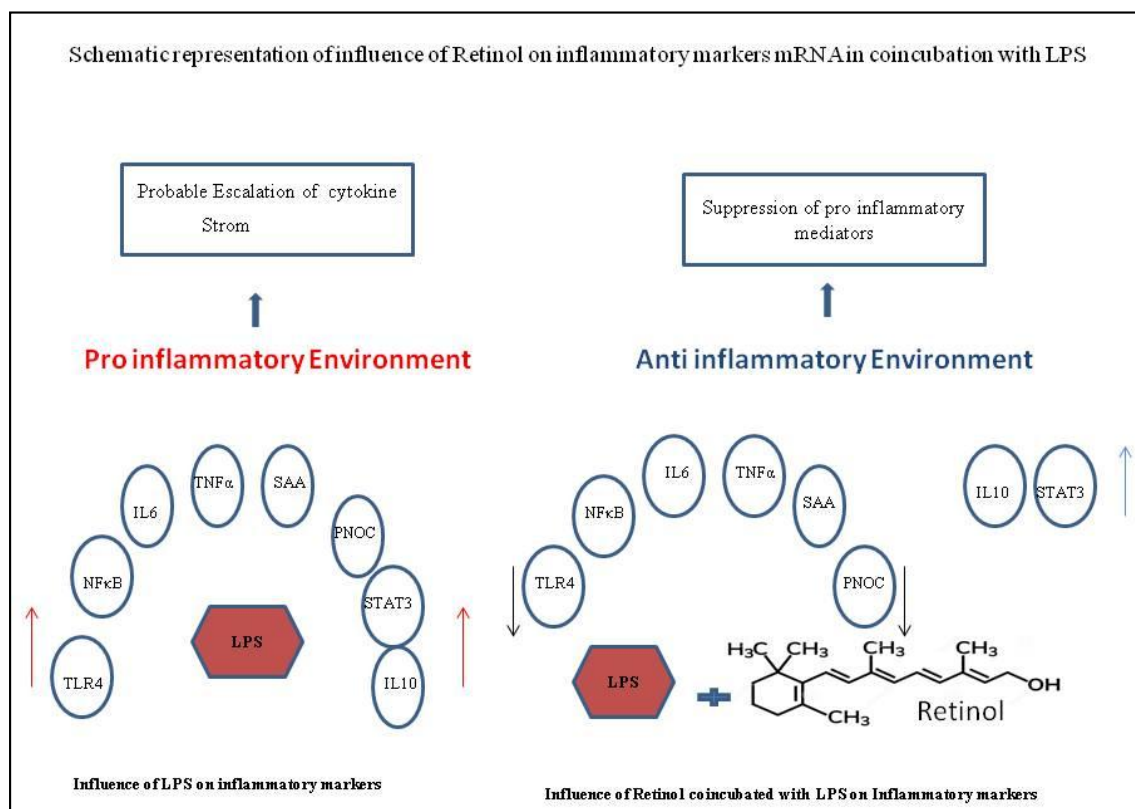


Figure 5 shows schematic representation of Retinol on inflammatory markers mRNA in coinubation with LPS

Conclusion

Our study supports the anti-inflammatory role of retinol during the prevailed inflammatory condition in a host. Our study concludes that as retinol is safe to be administered and has many health benefits, its inclusion as a prescription for a patient suffering from infection could have a therapeutic approach towards the infection leading to inflammation. Our study adds to the existing information on the anti-inflammatory role of retinol. Further investigations can be evaluated to better understand its profound application in treatment and therapeutics.

Conflict of interest: None

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