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Neutrophil Transmigration into the Joint of RA-Induced Mouse Is Markedly Blocked by PLAG, a monoacetyl diglyceride, via STAT3 Signaling

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Abstract

Inappropriate regulation of leukocyte trafficking can lead to impaired neutrophil clearance, which increases tissue damage by the accumulation of neutrophil-secreted proteases and reactive oxygen species at the sites of inflammation. In this study, we have discovered that 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) modulates neutrophil migration by regulating the activity of signal transducer and activator of transcription 3 (STAT3), which is a master regulator of IL-6 expression. PLAG has been isolated from the antlers of Sika deer (Cervus nippon Temminck), which were known to have immunosuppressive and anti-arthritic activities. PLAG caused the decrease of IL-6 production in a macrophage cell line, RAW264.7, and RA-fibroblast-like synoviocytes (RA-FLS) via the regulation of STAT3 signaling without affecting NF-κB signaling, which is also a well-known regulator of IL-6 expression. In collagen-induced arthritis (CIA) mouse model, arthritic symptoms were recapitulated with an increase of IL-6 level in the synovium, which was recuperated by the treatment of PLAG to the level comparable with commercial therapeutics (such as Remicade or Methotrexate). When the joint tissues were stained with neutrophil-specific antibodies, PLAG significantly reduced the infiltration of neutrophils into the joint synovium of CIA mouse. The inhibitory effect of PLAG on IL-6-STAT3 signaling also reduced the invasion of differentiated neutrophils in vitro. Therefore, PLAG inhibits the infiltration of destructive neutrophils into inflammatory sites, and can be utilized as a potent therapeutic agent for the treatment of sustained inflammation and joint destruction.

Introduction

Arthritis means joint inflammation which categorized into osteoarthritis, rheumatoid arthritis, and gout. Rheumatoid arthritis (RA) is a chronic inflammatory disease that causes progressive destruction of the extracellular matrices of bone and cartilage resulting in irreversible joint damage, deformity, and significant disability. As a soft layer of connective tissues lining the joint cavity, the synovium is the major target of inflammatory processes in RA. A heterogeneous group of inflammatory cells as lymphocytes, activated macrophages, and plasma cells infiltrate into the synovium during joint inflammation. RA synovial fluid is primarily characterized by the abundance of major pro-inflammatory cytokines, such as IL-1β and tumor necrosis factor alpha (TNF-α) mainly produced by MLS, and IL-6 by FLS [1]. Especially, IL-6 is known to recruit neutrophils into the inflammatory sites [2].

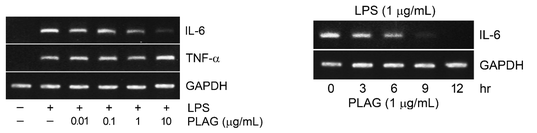
Neutrophils contribute to the pathogenesis of a number of inflammatory diseases. One of the earliest clinical signs of inflammation in an inflammatory arthritis model is the presence of neutrophils in the synovial regions of the ankle joint [3]. Ultrastructural studies of cartilage revealed immune complexes embedded in the superficial layers [4], thereby providing a solid surface to facilitate neutrophil adherence and activation. Neutrophils exert critical roles in initiating and maintaining inflammatory processes in the joint where they accumulate, engulf immune complexes and release proteolytic enzymes causing rheumatic tissue destruction [5, 6].

A monoacetyl-diglyceride (1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol; PLAG) has been isolated from the antlers of sika deer (Cervus nippon Temminck), and chemically synthesized as a single compound with immune-modulatory functions [7, 8]. In this study, we showed that PLAG administration inhibited the progression of RA phenotypes in collagen-induced arthritis (CIA) mouse model. PLAG regulated the activation mechanism of signal transducer and activator of transcription 3 (STAT3), which is the key mediator of both chronic inflammation and joint destruction in RA, and the consequent blocking of the cytokine amplification loop by IL-6–STAT3 signaling that promotes sustained inflammation and joint destruction.

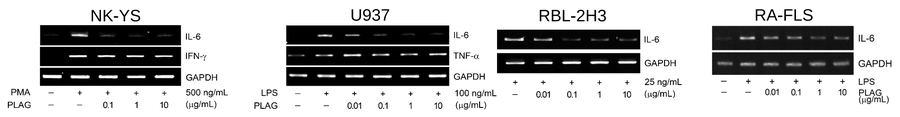
Results

1. PLAG reduces IL-6 expression from stimulated immune cells

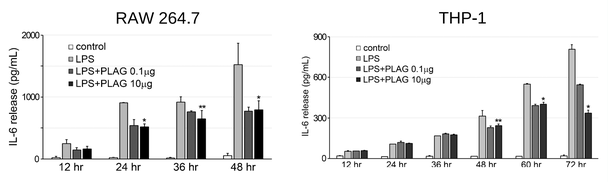
LPS (1 μg/mL) induced IL-6 expression in RAW264.7 cells, which was specifically inhibited by PLAG in concentration- and time-dependent manners.



The effect of PLAG on the transcription of IL-6 was confirmed in various immune cell lines (below). NK-YS was stimulated with PMA, U937 and RA-fibroblast-like synoviocytes (RA-FLS) with LPS, and RBL-2H3 with an antigen, respectively. The expression of other pro-inflammatory cytokines such as IFN-γ and TNF-α was not affected by PLAG.

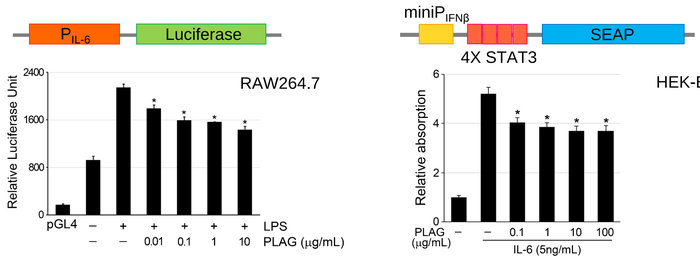


IL-6 secretion from the stimulated RAW 264.7 and THP-1 cells was decreased by PLAG treatment. The cells were stimulated with 100 ng/mL of LPS and/or various concentration of PLAG, and IL-6 protein level was analyzed by ELISA at different time points. *p<0.005, **p<0.01.

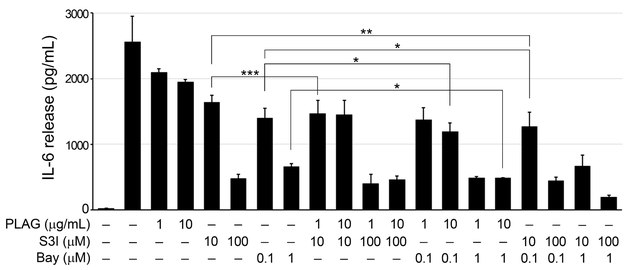


2. PLAG regulates STAT3 activity specifically

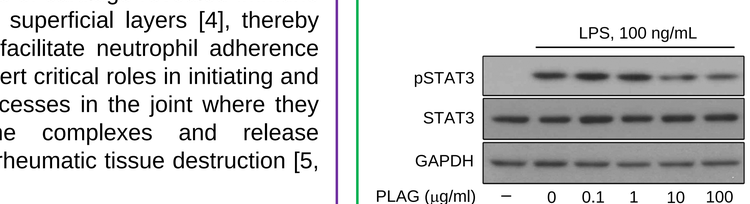
LPS (100 ng/mL) activated reporter gene expression, which was blocked by PLAG demonstrating transcriptional regulation for IL-6 expression by PLAG. *p<0.005.

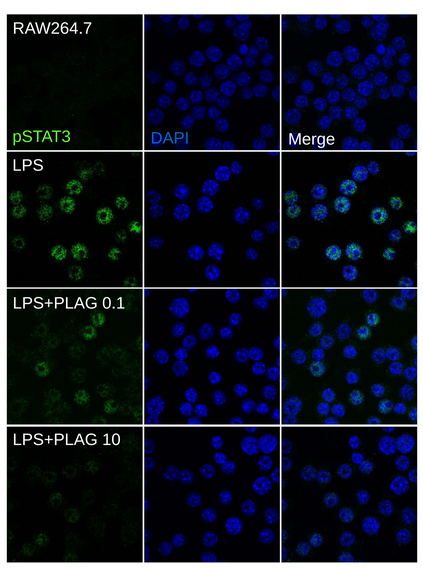


Both NF-κB and STAT3 inhibitors exhibited synergistic effects on the repressive activity of PLAG in RAW264.7. Co-treatment of Bay 11-7082 (for NF-κB) or S3I-201 (for STAT3) with PLAG induced more efficient inhibition of IL-6 production from 100 ng/mL of LPS-treated cells compared with individual treatment. *p<0.005, **p<0.01, ***p<0.05.

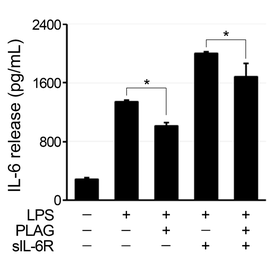


As a major transcription factor for regulating IL-6 expression, STAT3 was activated by phosphorylation in LPS-stimulated RAW 264.7 cells, which was decreased by PLAG treatment in a concentration-dependent manner (below). Nuclear translocalization of the activated STAT3 was inhibited by PLAG (right). An NF-κB subunit, p65, was also localized into the nucleus by LPS treatment. However, nuclear-localized p65 was not affected by PLAG, demonstrating the specificity of PLAG effect on STAT3 regulation (data not shown).



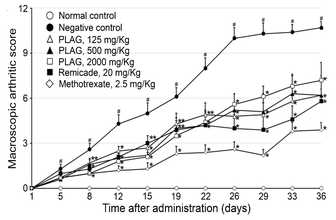


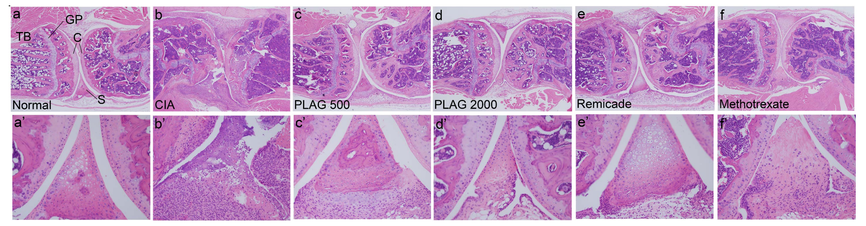
Co-treatment of soluble IL-6 receptor (sIL-6R) enhanced IL-6 production from LPS-only treated RA-FLS, which does not express IL-6 receptor. IL-6 produced by LPS stimulation could function as a self-stimulatory signal to boost IL-6 expression. This autocrine functions of IL-6 was inhibited by the treatment of PLAG. *p<0.005



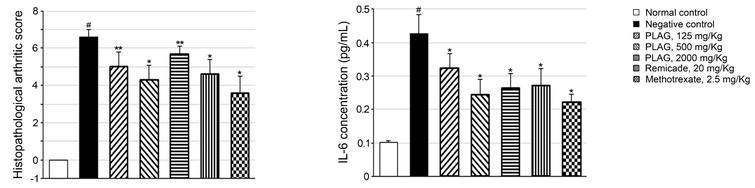
3. PLAG alleviated RA phenotypes in mouse CIA model

PLAG administration alleviated the joint destruction in collagen-induced arthritis (CIA) mice to the levels similar to the commercial therapeutics. Macroscopic arthritic scores and the dosage and duration of therapeutics administration were summarized in the graph (right). Pathologic phenotypes were analyzed by H&E staining, and representative images were displayed below. a-f : 40X images showing the joint region, a'-f': 200X images showing the synovium. #p<0.01 (Normal vs CIA), *p<0.01, **p<0.05 (CIA vs Therapy).

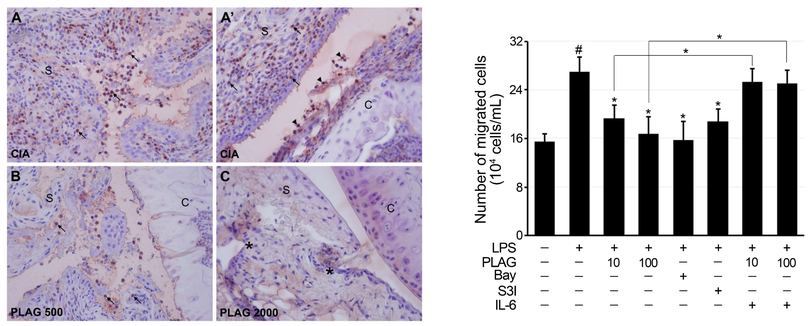




Pathological phenotypes of the joint tissues were statistically analyzed (left), and IL-6 concentration in the synovial fluids collected from the CIA mice were analyzed by ELISA (right). #p<0.005 (Normal vs CIA), *p<0.005, **p<0.01 (CIA vs Therapy).

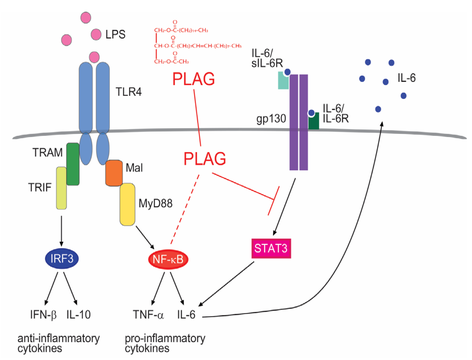


A large number of neutrophils infiltrated into the articular joints. The synovial region (A) and the area between the synovium and cartilage (A') were shown at 400X magnification. Some of the infiltrated neutrophils were marked by arrow (in the synovium) or arrowhead (at the surface of cartilage). Neutrophil infiltration was significantly decreased by the administration of PLAG with dose dependency (B, C). Invasion of differentiated HL-60 cells into the culture media of LPS-treated RA-FLS was significantly inhibited by PLAG, which was recovered by the addition of recombinant IL-6. #p<0.005 (control vs LPS), *p<0.005 (LPS vs co-treatment).



Conclusion

- PLAG regulated the expression of IL-6, which is a pro-inflammatory cytokine and induces chemotaxis of immune cells in the inflammatory sites.
- PLAG controls the transcriptional activity of STAT3 to regulated IL-6 expression.
- PLAG alleviates arthritic phenotypes in a collagen-induced arthritis model.
- PLAG administration reduced the infiltration of neutrophils into the arthritic joints.



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