Neutrophil Transmigration into the Joint of RA-Induced Mouse Is Markedly Blocked by EC-18, a monoacetyl diglyceride, via STAT3 Signaling

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Abstract

Inappropriate regulation of leukocyte trafficking can lead to impaired neutrophil clearance and increased tissue damage from the accumulation of neutrophil-secreted proteases and reactive oxygen species at the site of inflammation. In collagen-induced arthritis (CIA) mouse model, arthritic symptoms were recapitulated with an increase of interleukin (IL)-6 level in the synovium, which was recuperated by the treatment of 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) to the level comparable with commercial therapeutics such as Remicade or Methotrexate. EC-18, a monoacetyl-diglyceride, has been isolated from the antlers of Sika deer (*Cervus nippon* Temminck) which are known to have immunosuppressive and anti-arthritic activities. We have discovered that EC-18 regulates the activity of signal transducer and activator of transcription 3 (STAT3), which is a master regulator of IL-6 expression. EC-18 caused the selective inhibition of IL-6 production in a macrophage cell line, RAW264.7, and RA-fibroblast-like synoviocyte (RA-FLS) via the regulation of STAT3 signaling without affecting NF-κB signaling, which is also a well-known regulator of IL-6 expression. When the joint tissues from CIA mice were stained with neutrophil-specific antibodies, EC-18 significantly reduced neutrophil infiltration into the synovium correlated with tissue recovery . IL-6, a multifunctional pro-inflammatory cytokine, plays a critical role in the pathogenesis of the joint and systemic inflammation in RA. Therefore, EC-18 could be utilized as a potential therapeutic agent for the treatment of sustained inflammation and joint destruction.

Introduction

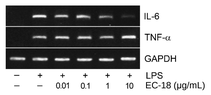
Arthritis is a disorder that involves inflammation in joint(s), and it is categorized into osteoarthritis, rheumatoid arthritis, and gout. Rheumatoid arthritis (RA) is a chronic inflammatory disease that causes progressive destruction of the extracellular matrices of bone and cartilage resulting in irreversible 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].

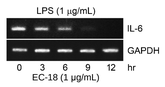
Neutrophils contribute to the pathogenesis of a number of inflammatory diseases. One of the earliest clinical signs of inflammation in an inflammatory arthritis model is the presence of neutrophils in the synovial regions of the ankle joint [3]. Ultrastructural studies of cartilage revealed immune complexes 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; EC-18) was originally isolated as a component of an extract from the antlers of sika deer (Cervus nippon Temminck); it is now manufactured by chemical synthesis as a single compound with immune-modulatory functions [7, 8]. In this study, we showed that EC-18 inhibited the progression of RA phenotypes in collagen-induced arthritis (CIA) mouse model. EC-18 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

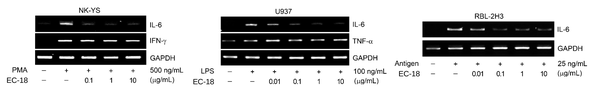
1. EC-18 reduced IL-6 expression from activated immune cells



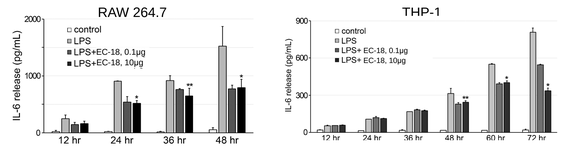


LPS (1 μg/mL) induced IL-6 expression in RAW264.7 cells, which was specifically inhibited by EC-18 in concentration- and time-dependent manners (upper).

The effect of EC-18 on the transcription of IL-6 was confirmed in various immune cell lines (below). NK-YS was stimulated with PMA, U937 with LPS, and RBL-2H3 with a specific antigen. The expression of other pro-inflammatory cytokines such as IFN-γ and TNF-α was not affected by EC-18.

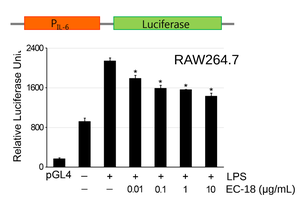


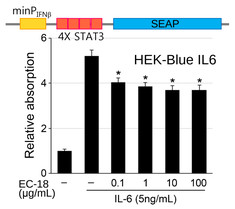
Secreted IL-6 level in the supernatant of stimulated RAW 264.7 macrophage and THP-1 cells was decreased by EC-18 treatment. The cells were stimulated with 100 ng/mL of LPS and/or various concentration of EC-18, and IL-6 protein level was analyzed by ELISA at different time points. **p*<0.005, ***p*<0.01.



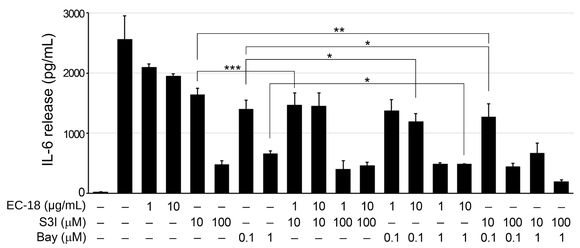
2. EC-18 regulated STAT3 activity specifically

LPS (100 ng/mL) activated reporter gene expression, which was blocked by EC-18 demonstrating transcriptional regulation for IL-6 expression by EC-18. **p*<0.005.

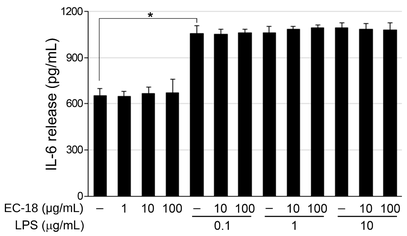




Both NF-κB and STAT3 inhibitors exhibited synergistic effects on the repressive activity of EC-18 in RAW264.7. Co-treatment of Bay 11-7082 (for NF-κB) or S3I (for STAT3) with EC-18 induced more efficient inhibition of IL-6 production from 100 ng/mL of LPS-treated cells compared with individual treatment. **p*<0.005, ***p*<0.01, ****p*<0.05.

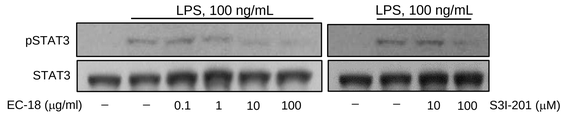


However, EC-18 did not appear to have direct effect on NF-κB signaling pathway. IL-6 production in RA-FLS is solely dependent on NF-κB pathway because the amplification loop of IL-6/STAT3 is not working in RA-FLS which does not express IL-6 receptor (IL-6R). EC-18 did not block LPS-induced IL-6 expression in RA-FLS. **p*<0.005.

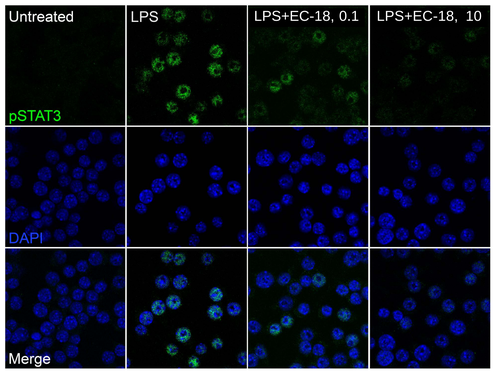


3. EC-18 regulated STAT3 phosphorylation

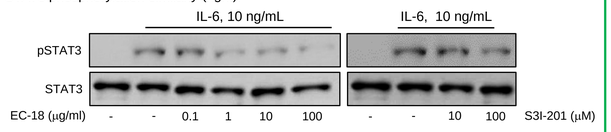
STAT3 is a major transcription factor regulating IL-6 expression. In LPS-stimulated RAW 264.7 cells, the phosphorylation of STAT3 was detected, which was decreased by EC-18 treatment in a concentration-dependent manner (left). The inactivating de-phosphorylation was similarly induced by the treatment of S3I-201, a STAT3 inhibitor (right).



LPS (100 ng/mL) induced the phosphorylation and nuclear translocalization of STAT3, which was blocked by EC-18 in a concentration dependent manner.

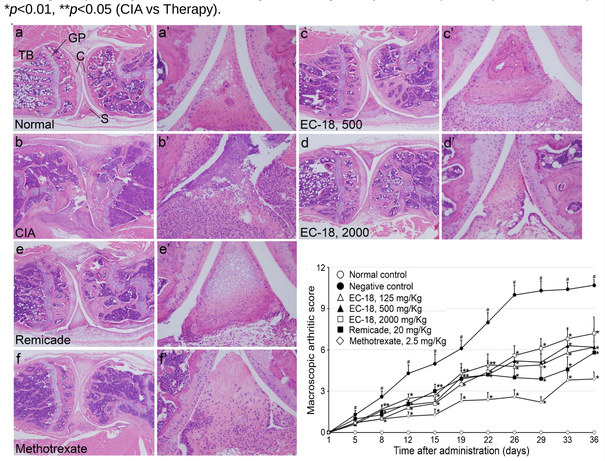


IL-6 treatment on RAW 264.7 cells induced STAT3 phosphorylation which could function as an self-stimulatory signal to express IL-6. This autocrine functions of IL-6 was inhibited by the treatment of EC-18 in a concentration-dependent manner (left). STAT3 inhibitor was also blocked STAT3 phosphorylation similarly (right).

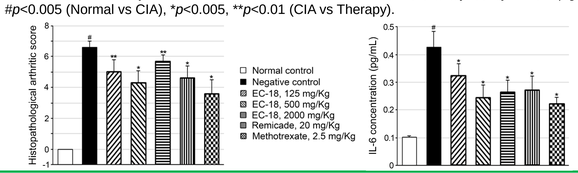


4. EC-18 alleviated RA phenotypes in mouse CIA model

EC-18 reduced the joint destruction in collagen-induced arthritis (CIA) mice to the levels similar to the commercial therapeutics. The macroscopic arthritic scores and the dosage and duration of therapeutics administration were summarized in the graph (right bottom). 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).

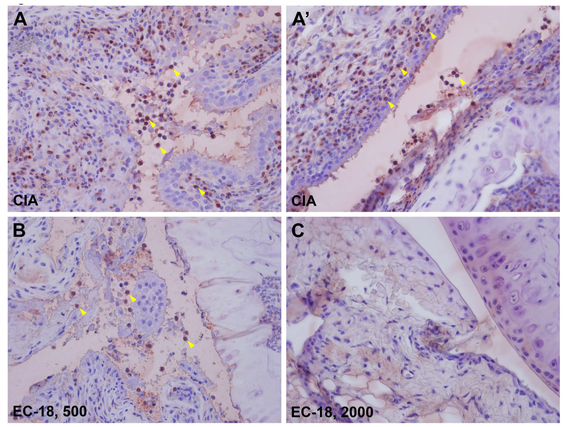


Pathological phenotypes of the joint tissues were statistically analyzed (left), and IL-6 concentration in the synovial fluids collected from the CIA mice were analyzed by ELISA (right). #*p*<0.005 (Normal vs CIA), **p*<0.005, ***p*<0.01 (CIA vs Therapy).



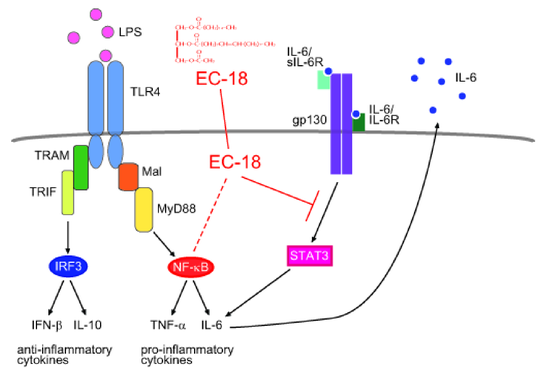
5. EC-18 inhibited neutrophil infiltration into RA synovium

The joints from CIA mice were stained with a neutrophil-specific antibody (NIMP-R14). Neutrophils were not detected in the joint region of normal mice (data not shown). However, a large number of neutrophils infiltrated into the CIA joint (A and A'), which were inhibited by EC-18 with dose dependent manner (B and C). Representative images for the joint synovium were displayed showing the infiltrated neutrophils (arrowheads).



Conclusion

- EC-18 regulated the expression of IL-6, which is a pro-inflammatory cytokine and induces chemotaxis of immune cells in the inflammatory sites.
- EC-18 controled the transcriptional activity of STAT3 to regulated IL-6 expression.
- EC-18 alleviated arthritic phenotypes in a collagen-induced arthritis model.
- EC-18 reduced the infiltration of neutrophils into the arthritic joints.



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