International Immunopharmacology 30 (2016) 74–84

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

The pentacyclic triterpene Lupeol switches M1 macrophages to M2 and ameliorates experimental inflammatory bowel disease

Yeshan Zhu [a,*], Xueqing Li [b], Jianquan Chen [c], Tongjun Chen [a], Zhimin Shi [a], Miaona Lei [a], Yanjun Zhang [a], Pengfei Bai [a], Yifang Li [a], Xuan Fei [a]

[a] Department of Digestive Internal Medicine, Tangshan Hospital of Traditional Chinese Medicine, Tangshan, Hebei Province, China
[b] Department of Acupuncture, North China University of Science and Technology School of Traditional Chinese Medicine, Tangshan, Hebei Province, China
[c] Department of Traditional Chinese Medicine, Yutian Hospital, Tangshan, Hebei Province, China

ARTICLE INFO

Article history:
Received 20 August 2015
Received in revised form 5 November 2015
Accepted 24 November 2015
Available online 3 December 2015

Keywords:
Lupeol
Inflammatory bowel disease
Macrophages
Phenotypes
Cytokines
Epithelial cells

ABSTRACT

Background: Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, is a chronic inflammatory disease in the lower gastrointestinal tract. Mounting evidence suggests that the predominance of the classically activated (M1) macrophages versus the alternatively activated (M2) macrophages plays a role in the progression of IBD. Thus, agents able to shift pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages may be beneficial to IBD. The pentacyclic triterpene Lup-20(29)-en-3β-ol (Lupeol), a potent anti-inflammatory natural product, has been shown to inhibit pro-inflammatory cytokine production, suggesting it is potentially able to modulate macrophage polarization, thereby beneficial to IBD.
Methods: $CD4^+$ monocytes were differentiated to M1 or M2 macrophages, which were cocultured with epithelial cell lines, T84 and Caco-2, in the absence or presence of Lupeol (10 μM). Experimental colitis was induced with dextran sodium sulfate (DSS), with or without oral administration of Lupeol (50 mg/kg, q.d.). Cytokines were measured with Luminex kits. M1/M2 genes were measured with real-time polymerase chain reaction. Macrophage phenotypes were defined by measuring M1 and M2 markers with confocal microscopy. Proteins were measured with Western blotting, while cell surface markers were measured with confocal microscopy or flow cytometry. Histology was evaluated with H&E staining.
Results: Treatment of M1 macrophages with Lupeol resulted in a marked decrease in the production of pro-inflammatory cytokines, including IL-12, IL6, IL-1β and TNFα, and a marked increase in the production of IL-10, an anti-inflammatory cytokine. This was associated with a down-regulation of CD86, a typical marker of M1 macrophages, and an up-regulation of CD206, a typical M2 macrophage marker. IRF5, a transcription factor that is critically involved in M1 polarization, was down-regulated in M1 macrophages after being incubated with Lupeol, associated with a marked decrease in the phosphorylation of p38 mitogen activated protein kinase. Coculture of epithelial cells with M1 macrophages resulted in down-regulation of the tight junction protein ZO-1 and disruption of epithelial integrity, which were blocked by Lupeol treatment of the M1 macrophages. Moreover, oral administration of Lupeol to dextran sulfate sodium (DSS)-induced colitis mice resulted in mitigated intestinal inflammation and increased survival from lethal colitis, associated with decreased expression of M1-related genes and increased expression of M2-related genes.
Conclusion: Lupeol ameliorates experimental inflammatory bowel disease through, at least in part, inhibiting M1 and promoting M2 macrophages.

1. Introduction

Inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease, is a chronic inflammatory disease with recurring relapses and remissions in the lower gastrointestinal tract [11,27,42]. IBD often result in morbidity due to a high incidence of diarrhea, abdominal pain, rectal bleeding and malnutrition [27]. Despite significant progresses in the past decades, our understanding of the inflammatory regulators that contribute to the pathogenesis of IBD is still limited.

Recent evidence suggests that defects in mucosal innate immune functions may be involved in the etiology of IBD. Macrophages are central mediators of innate immune system. The "classically activated" macrophages (or M1 macrophages) produce pro-inflammatory cytokines such as TNF-α, interleukin (IL)-1β, and IL-6, and effector

* Corresponding author.
E-mail addresses: zhuyeshan1965@163.com (Y. Zhu), 15531558238@163.com (X. Li), 741094776@qq.com (J. Chen), tongjunchen@sina.com (T. Chen), shizhimin0613@163.com (Z. Shi), eimiaona001@sina.com (M. Lei), zhangyj1312@126.com (Y. Zhang), 854951455@qq.com (P. Bai), liyifang02@163.com (Y. Li), 1tsfuyou@126.com (X. Fei).

http://dx.doi.org/10.1016/j.intimp.2015.11.031
1567-5769/

molecules like reactive oxygen species or nitric oxide, thereby regulating T-helper (Th) 1-cell and mediating acute inflammation [15,44,54]. In contrast, the "alternatively activated" macrophages (or M2 macrophages), which express large amounts of IL-10, scavenger-, mannose- and galactose-type receptors, are involved in Th2-cell activation and regulate extracellular matrix molecule synthesis that is required for wound repair [15,44,54]. Previous studies have demonstrated that M1 macrophages are increased and M2 macrophages are decreased in colitis, accompanied by inflammatory cytokine induction and anti-inflammatory cytokine suppression [24,54]. It is therefore hypothesized that disequilibrium of macrophage subsets promotes colitis development. Supporting this hypothesis, transfer of M2 macrophages reduce dextran sodium sulfate (DSS)-induced colitis by inducing IL-10 production and promoting regulatory T-cell generation [54], and a shift from the M1 to M2 phenotype reduces colitis by inducing IL-10 [5]. Thus, mobilization of M2 macrophages could be a novel approach to colitis therapy.

The pentacyclic triterpene Lup-20(29)-en-3β-ol (Lupeol) is found in vegetables such as white cabbage, pepper, cucumber, tomato, in fruits such as olive, fig, mango, strawberry, red grapes and in medicinal plants such as American ginseng, Shea butter plant, *Tamarindus indica*, and *Sebastiania adenophora* used by native people in North America, Latin America, Japan, China, Africa and Caribbean islands [34,39]. Lupeol and its derivatives (linoleate, acetate and palmitate) have been shown to exhibit potent anti-inflammatory activity in rat and mouse models of inflammation [13,38,47–49]. Several studies have been carried out to understand the molecular mechanism through which Lupeol inhibits or abrogates the inflammatory processes under in vitro and in vivo situations. For instance, Lupeol treatment is shown to decrease the generation of pro-inflammatory cytokines such as TNF-α and IL-β in lipopolysaccharide (LPS)-treated macrophages [11]. Lupeol has also been shown to exhibit significantly high wound healing potential in a dead space wound healing mouse model through decreasing the level of monocytes and docking with GSK3β protein [16]. Together, these compelling evidences suggest that the therapeutic usefulness of Lupeol for inflammatory conditions is attractive. However, whether Lupeol is beneficial for IBD has not been investigated thus far.

We hypothesize that Lupeol may play an anti-inflammatory role through modulating macrophage phenotypes. To this end, we treated M1 or M2 macrophages with Lupeol and assessed cytokine production and cell surface marker expression. Moreover, we administered Lupeol to an experimental colitis mouse model and evaluated its therapeutic effects and underlying mechanisms. We demonstrated that Lupeol treatment resulted in a switch of macrophages from M1 to M2 phenotype, and that administration of Lupeol to DSS-induced colitis mouse model mitigated intestinal inflammation, leading to increased mouse survival from lethal colitis.

2. Materials and methods

2.1. Animal colitis model and Lupeol treatment

All animal experiments were approved by hospital's Institutional Animal Care and Use Committee (IACUC 2013-0051 1E03030). Male C57BL/6 mice, (aged 8–10 weeks; 21–30 g, average ≈ 25 g), were purchased from Model Animal Research Center of Nanjing University and housed in a temperature-controlled environment with a 12-hour day/night light cycle. Individual body weights were assessed daily over an initial acclimation period of 7 days. All mice were non-fasting and had access to food and autoclaved tap water for drinking ad libitum during experiment. The following 4 groups each consisting of 12 mice were randomly assigned: negative control, Lupeol, DSS/vehicle, and DSS/Lupeol. Colitis was induced by feeding mice with 4% w/v dextran sulfate sodium (DSS, MW = 40,000–50,000, USB, Affymetrix Inc., Ohio, USA) dissolved in drinking water from day 1 to day 7. Control mice received water without DSS from day 1 to day 7. To observe the therapeutic effect of Lupeol on DSS-induced colitis, animals were fed with a normal diet and dosed daily by oral gavage of Lupeol (50 mg/kg, purity ≥ 94%, Sigma-Aldrich) dissolved in olive oil, or an equal amount of olive oil once daily from day 3 for 10 days [36,41]. Animal weights and stool scores (0, normal; 1, soft; 2, diarrhea/minimal anorectal bleeding; 3, diarrhea/severe anorectal bleeding) were recorded daily [6]. The Experimental design needed for the present in vivo study has been summarized in Fig. 1.

2.2. Histological inflammatory scores of the mouse colon

Histology scoring of H&E-stained sections was performed in a blinded fashion according to a previously published scoring system [46]. Scoring based on cell infiltration: 0, occasional inflammatory cells in the LP; 1, increased infiltration in the LP, predominantly at the base of the crypts; 2, inflammatory infiltrate extending into the mucosa; 3, transmural extension of the infiltrate. Scoring based on tissue damage: 0, no mucosal damage; 1, partial (up to 50%) loss of crypts in large areas; 2, partial to total (50%–100%) loss of crypts in large areas with an intact epithelium; 3, total loss of crypts in large areas and of the epithelium. Finally, a combined score of inflammatory cell infiltration and tissue damage was calculated.

2.3. Generation of M1 or M2 macrophages

Monocytes were isolated and polarized into macrophages as described previously [29]. In brief, $CD14^+$ cells were prepared from peripheral blood mononuclear cells by Ficoll density gradient centrifugation (GE Healthcare, Freiburg, Germany) and subsequent magnetic cell sorting using CD14 MACS MicroBeads (Miltenyi Biotech, Bergisch-Gladbach, Germany). To generate M1 and M2 macrophages, $CD14^+$ cells (95% monocytes) were cultured for 6 days in the presence of GM-CSF (50 ng/ml) before being stimulated with LPS (1 ng/ml) and IFN-γ (20 ng/ml) for 24 h or in the presence of M-CSF (100 ng/ml) for 6 days before being stimulated with IL-4 (20 ng/ml) for 24 h, respectively.

2.4. Preparation of Lupeol and cell treatment

A stock solution of Lupeol (30 mM) which was purchased from Sigma was prepared by dissolving it in warm alcohol and diluted in DMSO in a 1:1 ratio. For dose-dependent studies, cells (50% confluent) were treated with Lupeol (0.01–10 μM) for 24 h, or otherwise indicated, in complete cell medium. The final concentrations of DMSO and alcohol were 0.25% and 0.075%, respectively, in all treatment protocols. After 24 h of treatment with Lupeol, the cells were harvested for different experimental purposes.

2.5. Cytokines analysis

Luminex multiplex panel technology was used for simultaneous measurement of IL-1β, IL-12, and IL-10. Briefly, 50 μL of cell culture medium and reaction standards were added, in duplicate, to a 96 multi-wells plate containing analyte beads followed by incubation for 30 min at room temperature. After washing, the antibody-biotin reporter was added and incubated for 10 min with streptavidin–phycoerythrin. The levels of the cytokines were determined using the Bio-Plex array reader (Luminex, Austin, TX). The Bio-Plex Manager software optimized the standard curves automatically and returned the reading data as Median Fluorescence Intensity (MFI) and concentration (pg/mL).

2.6. Cell viability analysis

Analysis of cell viability was performed with MTT (3-[4, 5-methylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide) assay. Briefly,