Immunology Letters xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Immunology Letters

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

Piperlongumine alleviates lupus nephritis in MRL-Fas(lpr) mice by regulating the frequency of Th17 and regulatory T cells

Lan Yao, Hai-ping Chen*, Qing Ma

Department of Nephrology, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China

ARTICLE INFO

Article history:
Received 26 March 2014
Received in revised form 5 May 2014
Accepted 6 May 2014
Available online xxx

Keywords:
Piperlongumine
Lupus
MRL-Fas(lpr) mice
Th17
Regulatory T cells

ABSTRACT

Recent data have shown that piperlongumine (PL), an important component of *Piper longum* fruits, is known to possess anti-inflammatory and vascular-protective activities. This study aimed to examine the therapeutic effects and underlying mechanisms of PL on lupus-prone MRL-Fas(lpr) mice. Female MRL-Fas(lpr) mice were intraperitoneally treated with PL (2.4 mg kg^{-1} d^{-1}) for 10 weeks, and the proteinuria level was biweekly monitored. After the mice were euthanized, serum biochemical parameters and renal damage were determined. Splenocytes of MRL-Fas(lpr) mice were isolated for *in vitro* study. Treatment of the mice with PL significantly attenuated the progression of proteinuria and glomerulonephritis. The improvement was accompanied by decreased serum levels of nephritogenic anti-dsDNA antibodies, IL-6, IL-17, IL-23 and TNF-α. Treatment of the mice with PL suppressed the frequency of Th17 cells and increased the regulatory T cells (Tregs). *In vitro*, the levels of IL-6, IL-17, IL-23 and TNF-α were significantly decreased in the cultures of splenocytes from PL-treated mice compared with those from vehicle-treated mice. In addition, PL treatment impeded activation of the JAK/STAT3 signaling in splenocytes. Of great important, the survival of MRL-Fas(lpr) mice were improved by PL treatment. In summary, PL effectively ameliorates lupus syndrome in MRL-Fas(lpr) mice by suppressing the pathogenic Th17 cells and increasing the Tregs as well as inhibiting activation of the JAK/STAT3 signaling pathway. This study sheds new light on the immune-modulatory role of PL.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder characterized by the aberrant function of the immune system and the production of numerous autoantibodies [1]. Lupus nephritis (LN) depends on autoantibody deposition and activation of multiple immune cell types that promote kidney inflammation, including lymphocytes and monocyte/macrophages [2]. Patients at risk for progressive tissue damage are treated with unselective immunosuppressive drugs such as steroids, cyclophosphamide, mycophenolate mofetil and calcineurin inhibitors, but drug toxicity and lack of efficacy are often associated with poor outcomes [3]. Novel drugs that interfere with the disease pathomechanisms more specifically are needed to improve lupus management.

Piper longum L. (Piperaceae) is a slender aromatic climber with perennial woody roots, and notably, in traditional medicines, *P. longum* fruits have been attributed with the treatment of bronchial diseases, menstrual pain, tuberculosis, muscular pain, sleeping disorders, and certain cancers [4]. The primary constituents isolated from various parts of *P. longum* L. are piperine, piperlongumine, sylvatin, sesamin and diaeudesmin piperlonguminine [4]. Among them, piperlonguminine shows various biological properties including anti-hyperlipidemic, anti-platelet and anti-melanogenesis activities [5–9]. And recently, piperlonguminine has been demonstrated to have the vascular inflammatory protective and anti-inflammatory functions. Since the inflammatory responses are an important component in the pathogenesis of SLE, we explored the effects of piperlonguminine on murine lupus and the molecular mechanisms in this study *in vitro* and *in vivo*.

2. Materials and methods

2.1. Animals

Female MRL-Fas(lpr) mice were obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. All mice were housed under specific pathogen-free conditions. All experiments were performed according to the institutional ethical

* Corresponding author at: Department of Nephrology, Beijing Friendship Hospital, Capital Medical University, No. 95 Yong-An Road, Xicheng District, Beijing 100050, China. Tel.: +86 1063014411.
E-mail address: chenhai_ping@yeah.net (H.-p. Chen).

http://dx.doi.org/10.1016/j.imlet.2014.05.001
0165-2478/

guidelines on animal care and approved by the Institute Animal Care and Use Committee at the Capital Medical University, Beijing, China.

2.2. Treatment regimens

In this study, 11-week-old female MRL-Fas(lpr) mice were intraperitoneally treated with PL for daily for 10 weeks. At the initiation of treatment, the mice were randomly divided into two groups ($n = 8$ per group from two independent experiments): vehicle (1% dimethyl sulfoxide, DMSO)-treated control group and a PL-treated group (Tocris Bioscience, 2.4 mg kg^{-1} d^{-1}) as described before [9]. The proteinuria levels were monitored biweekly.

2.3. Biochemical parameters, renal histopathology and immunofluorescence examination

Urinary protein concentrations were determined with a Coomassie blue G dye-binding assay. Blood urea nitrogen (BUN) and creatinine (CRE) concentrations were determined using a HITACHI-7080 automatic biochemical analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan).

At the time of euthanization, the kidneys were removed and cut in half. One half of the kidney from each mouse was fixed in formalin, embedded in paraffin, sectioned, and stained with Periodic acid-Schiff (PAS). The sections were then graded by two independent renal pathologists for renal damage according to a grading scheme as described [10]. Briefly, glomerulonephritis and lymphocyte infiltration in the kidneys were examined graded separately in each animal, using a scale of 1–4, where 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked, as reported previously [11].

Snap-frozen kidneys were used for immunofluorescence examination of IgG and C3 deposits, and staining was analyzed and scored as 0–4 as above.

2.4. Cell purification

Mouse CD4+ T cells from 10-week-old non-treated mice were isolated from splenocytes using a mouse CD4-negative selection kit (Dynal Biotech). For isolation of CD4+ CD2 – T cells, the purified CD4+ T cell populations were incubated with PE-labeled anti-CD25 antibody and anti-PE magnetic beads and isolated using a MACS separation column (Miltenyi Biotec). Naive CD4+ CD2 – CD62L+ T cells were prepared after a further purification using the CD62L microbeads component from a CD4+ CD62L+ T cell isolation kit (Miltenyi Biotec).

2.5. Cell culture

For mouse T cell differentiation, the above purified naive CD4+ T cells were cultured in RPMI supplemented with 10% fetal bovine serum in the presence of anti-CD3/28 antibodies, followed by treatment with or without PL(5 μM). 2 ng/ml TGF-β, 10 μg/ml anti-IL-4, and 10 μg/ml anti-IFN-γ (for Treg); or 10 ng/ml IL-6, 2 ng/ml TGF-β, 10 μg/ml anti-IL-4, and 10 μg/ml anti-IFN-γ (for Th17) were added to drive T cell polarization. After 4 days, cells were collected for flow cytometry analysis.

2.6. Flow cytometry

For surface staining of CD4, T cells were resuspended in PBS containing 1% BSA and were incubated on ice for 30 min with fluorochrome-conjugated antibody (eBioscience). For intracellular staining, cells were restimulated with 50 ng/ml PMA (Sigma–Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences, USA) for 5 h before intracellular staining. Cells were fixed, permeabilized, and stained with fluorochrome-conjugated antibodies (BD). The stained cells were then analyzed using a FACSCalibur or BD FACSAria instrument. Supernatants were collected, and the cytokine concentration was determined by ELISA.

2.7. ELISA

Serum anti-dsDNA Abs was detected using previously reported methods [9]. Briefly, 96-well high-binding microplates were coated with calf thymus dsDNA (Sigma–Aldrich, St. Louis, MO, USA) and incubated at 4 °C overnight. Each diluted serum sample was added and incubated. HRP-conjugated goat anti-mouse IgG Abs (Invitrogen, San diego, CA, USA) were then added and incubated. Serum from female MRL-Fas(lpr) mice at 5 months of age was used as the standard.

Cytokines in sera or culture supernatants were assayed using mouse IL-6, IL-17, IL-23 and TNF-α ELISA kits (all from BD Pharmingen) according to the users' instructions.

2.8. Western blot analysis

Splenocytes from different groups of MRL-Fas(lpr) mice or BMDC cultures were directly lysed in sodium dodecyl sulfate (SDS) sample buffer and boiled for 10 min at 100 °C. Proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech) for immunoblotting. Anti-JAK1, anti-phospho-JAK1, anti-STAT3, anti-phospho-STAT3 and anti-GAPDH were from Cell Signaling Technology (Beverly, MA, USA). Proteins were visualized using HRP-conjugated anti-rabbit IgG and the ECL system (Amersham Biosciences). Western blot analysis was determined by densitometric analyses (Image J) and corrected by the internal control GAPDH.

2.9. Statistical analysis

Proteinuria data were analyzed using one-way analysis of variance (ANOVA) for repeated measures, corrected with the Bonferroni posttest. Nonparametric data (histological scores) were analyzed using a Mann–Whitney U test. Additionally, data were analyzed using Student's t-test and one-way ANOVA. $P < 0.05$ was considered statistically significant. For statistical analyses, GraphPad Prism 5.0 software was used.

3. Results

3.1. Effects of PL on proteinuria and renal function in female MRL-Fas(lpr) mice

PL treatment significantly inhibited the progression and aggravation of proteinuria of lupus-prone mice (Fig. 1A). Additionally, PL prevented the worsening of renal function, represented by a significant decrease in blood urea nitrogen (BUN) and creatinine (Cr) levels compared with the vehicle-treated group (Fig. 1B and C).

3.2. Effects of PL on the kidney pathology and IgG and C3 deposition of MRL-Fas(lpr) mice

To evaluate the effects of PL on renal injury and immune-complex deposition, kidneys were examined by histopathology and an immunofluorescence assay. We found that kidney sections from vehicle-treated mice exhibited severe renal injury, characterized by glomerulonephtitis, tubular cast/cyst formation, diffuse perivascular and interstitial mononuclear cell infiltration. Treatment with

Please cite this article in press as: Yao L, et al. Piperlongumine alleviates lupus nephritis in MRL-Fas(lpr) mice by regulating the frequency of Th17 and regulatory T cells. Immunol Lett (2014), http://dx.doi.org/10.1016/j.imlet.2014.05.001