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A77 1726, the active metabolite of leflunomide, attenuates lupus nephritis by promoting the development of regulatory T cells and inhibiting IL-17-producing double negative T cells

Guilin Qiao[a], Lifen Yang[a], Zhenping Li[a], James W. Williams[b,*], Jian Zhang[a,c,**]

[a] Section of Nephrology, Department of Medicine, The University of Chicago, 5841 S. Maryland Ave., Chicago, IL 60637, USA
[b] Cinkate Pharmaceutical Corporation, Chicago, IL 60605, USA
[c] Department of Microbial Infection and Immunity, The Ohio State University, Columbus, OH 43210, USA

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Abstract Lupus nephritis (LN) is a challenging problem that affects 50% of patients with systemic lupus erythematosus (SLE) without effective therapy. Here, we report that A77 1726, the active metabolite of leflunomide, effectively inhibits development of LN and attenuates the generalized autoimmune features. A77 1726 suppresses the expansion of double negative (DN) T cells, and inhibits T and B cell activation. Intriguingly, A77 1726 treatment significantly increases $CD4^+Foxp3^+$ regulatory T cells but suppresses potential "pathogenic" IL-17-producing DN T cells in lymph nodes. In vitro experiment shows that A77 1726 potentiates the conversion of naive $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ inducible regulatory T cells (iTregs) by inhibiting Akt. Taken together, our data indicate that the therapeutic effects of A77 1726 in murine LN are mediated, at least in part, by augmenting iTregs which suppress pathogenic IL-17-producing DN T cells through an Akt-dependent mechanism.

1. Introduction

Systemic lupus erythematosus (SLE) is a potentially fatal non-organ-specific autoimmune disease that predominantly affects young women. Lupus nephritis (LN) affects more than 50% of patients with SLE and is a major cause of morbidity

* Correspondence to: J. Williams, Cinkate Pharmaceutical Corp, Chicago, IL 60605, USA.
** Correspondence to: J. Zhang, Department of Microbial Infection & Immunity, The Ohio State University, Columbus, OH 43210, USA.
E-mail addresses: jwilliams@cinkate.com (J.W. Williams), Jian.zhang@osumc.edu (J. Zhang).

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[1]. The clinical features of LN generally manifest in 75% of SLE patients within five years of initial diagnosis, however, pathological lesions can be found in the biopsy of almost 100% of symptom-free patients five years or more after the diagnosis of SLE. Despite intervention, the rates of end-stage renal disease due to LN are increasing worldwide [1,2]. T cells are central to SLE pathogenesis, as they provide help to autoreactive B cells, infiltrate target tissues, and fail to perform appropriate regulatory functions [3,4]. The precise effector mechanisms used by T cells to promote disease pathology are still unclear. It was recently reported that IL-17, a pro-inflammatory cytokine produced by a subset of T cells, may play a role in the pathogenesis of LN [5]. Specifically, DN T cells from patients with SLE produce significant amounts of IL-17 and IFN-γ and expand when stimulated in vitro with an anti-CD3 antibody in the presence of accessory cells [6]. More importantly, IL-17 and DN T cells are found in kidney biopsies of patients with active LN [7]. These data add to previous studies which have shown that DN T cells provide help to B cells and contribute to the abnormal autoantibody profile of lupus patients [8,9]. Furthermore, it has been shown that serum of patients with SLE contains high levels not only of IL-17 and but also of IL-23 [5,10], a cytokine important for the development, expansion, and proliferation of Th17 cells [11]. Lupus-prone mice have also increased numbers of Th17 cells, which, after being conditioned with IL-23 in vitro, can instigate disease when transferred into *Rag1*$^{-/-}$ mice [10]. Taken together, these findings suggest that an abnormally activated IL-23/IL-17 axis may be contributing to the pathogenesis of LN [10]. In support of this notion, IL-23 receptor deficiency prevents LN in C57BL/6-*lpr* mice [5].

Although corticosteroids and immunosuppressive drugs have improved the outcome of LN, they are burdened with significant adverse effects. The search for alternative, less toxic therapeutic strategies has prompted a number of clinical studies. Several recent studies have shown that the small molecule drug, leflunomide, is a safe and probably efficacious treatment in patients with LN who do not respond to or cannot tolerate conventional treatments [12–14]. Leflunomide is an immunosuppressive disease-modifying anti-rheumatic drug (DMARD), used in active moderate to severe rheumatoid arthritis and psoriatic arthritis [15,16]. It is a pyrimidine synthesis inhibitor but its immune suppression activity has been shown to be independent of pyrimidine synthesis inhibition [17–19]. As T cells play a central role in the orchestration of both physiological and pathological immune responses, it has been proposed that the ability of leflunomide to suppress inappropriate and undesirable immunity is related to a functional inhibition of T cells [18–21]. However, the mechanism by which the therapeutic effect of leflunomide on LN is achieved is currently unknown. Moreover, the active metabolite of leflunomide, A77 1726, may be more effective and better tolerated by the intestinal tract than pro-drug leflunomide but has not been tested in either human or mouse lupus models.

In this study, we show that a reliably absorbed A77 1726 attenuates LN in a mouse model of lupus. A77 1726 treatment significantly suppresses the activation of potentially harmful T cells, the expansion of pathogenic T cells that produce inflammatory factor IL-17, and the generation of auto-antibodies. Therefore, A77 1726 inhibits LN, and may provide a novel potent yet safe therapeutic approach to the disease.

2. Materials and methods

2.1. Mice and A77 1726 treatment

MRL-*lpr* mice (female) and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). A771726 was suspended in 1.5% carboxymethyl cellulose (CMC) and dosed at 15 mg/kg, 30 mg/kg, 45 mg/kg, and 60 mg/kg. MRL-*lpr* mice (10 mice/group) at 10 weeks of age were treated with A77 1726 at the above doses or carboxymethyl cellulose (CMC) daily by gavage for 10 weeks. Pyrimidine supplementation (Orotate) was simultaneously given at 200 mg/kg by gavage because A77 1726 inhibits pyrimidine biosynthesis [21]. General health status was monitored daily, and body weights were measured weekly and recorded during the entire experimental period. All the mice were sacrificed on day 70 after treatment. Four hours after the last treatment, blood and urine samples were collected. Lymph nodes, spleens, and kidneys were also collected. The experimental protocols followed NIH guidelines, and were approved by the institutional animal care and use committees of the University of Chicago.

2.2. Detection of serum autoantibody titers and proteinuria

Serum anti-dsDNA and anti-ANA antibody titers were detected by ELISA as described previously [22]. Urinary albumin concentrations were measured with a mouse albumin ELISA kit (Bethyl Laboratories, Montgomery, TX) and normalized to creatinine concentrations in the same urine (measured with Stanbio Creatinine Procedure No. 0400; Stanbio Laboratory, Boerne, TX).

2.3. Kidney histology

To evaluate renal pathologic changes, kidney tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) and examined by a renal pathologist in a blinded manner. For each slide, the severity of GN, glomerulosclerosis, interstitial nephritis, and arteritis was graded in a semiquantitative (0 to 4) manner as described previously [23]. The number of glomeruli with sclerosis and/or hyalinosis and crescent formation was determined and expressed as a percentage of total glomeruli observed in the entire cortical field.

2.4. Flow cytometry

The following Abs (BD Biosciences; San Diego, CA) were used: anti-CD4 (clone GK1.5) and anti-CD8 (53–6.7) for lineage markers; anti-CD69 (H1.2F3), anti-CD44 (IM7), anti-CD62L (MEL-14), and anti-CD25 (PC81) for activation markers. Fluorescence-conjugated anti-Foxp3 (FJK-16s), anti-IL-17 (17B7), and the respective isotypic control mAbs were purchased from eBioscience (San Diego, CA). Acquisition of samples was performed on a FACScan flow cytometer, and