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Laquinimod ameliorates spontaneous colitis in interleukin-10-gene-deficient mice with improved barrier function

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ABSTRACT

Background and aims: Crohn's disease is an autoimmune disease associated with imbalanced mucosal immunity, mediated with increased Th1 and Th17 cells. Laquinimod, an immunomodulatory drug, has shown efficacy in regulating the differentiation of T cells. The aim of the study was to investigate the therapeutic effect of laquinimod on spontaneous colitis in interleukin-10-gene-deficient mice, an animal model of Crohn's disease.
Methods: Male *Il10*$^{-/-}$ mice aged 16 weeks in the laquinimod group were treated with laquinimod with distilled water at a dose of 25 mg/kg by oral gavage, 3 times a week. *Il10*$^{-/-}$ mice in the IL-10-KO group and wild type mice received equal volume of phosphate buffered saline by oral gavage, 3 times a week. After 4 weeks, mice were sacrificed for analysis. Severity of colitis, epithelial expression of T-cell-associated cytokines, expression and distribution of tight junction proteins in the lamina propria and NF-κB signaling pathway associated mRNA expression were measured at the end of the experiment.
Results: Laquinimod treatment ameliorated spontaneous colitis in *Il10*$^{-/-}$ mice, which was associated with decreased T-cell-associated pro-inflammatory cytokines. Increased expression and correct distribution of tight junction proteins (occludin and ZO-1) were found in *Il10*$^{-/-}$ mice treated with laquinimod. In addition, in mice treated with laquinimod, NF-κB signaling pathway associated mRNA in the colon was also downregulated.
Conclusions: Our results indicated that laquinimod treatment ameliorates colitis in *Il10*$^{-/-}$ mice and improves intestinal barrier function, which may support a new therapeutic approach to Crohn's disease.

1. Introduction

Crohn's disease (CD) is a chronic relapsing inflammatory disorder characterized by chronic inflammation and mucosal tissue damage of the gastrointestinal tract. Although the precise etiology of Crohn's disease remains unclear, research has thus far demonstrated that CD is caused by a combination of genetic, environmental and immunoregulatory factors [1–3]. Increasing evidence suggests that an imbalanced mucosal barrier may play an important role in the pathogenesis and disease progression of CD. Massive numbers of microorganisms reside in the gut lumen, and the intestinal mucosa constitutes an immunologic organ, which tolerates and defends against harmful organisms [4]. Epithelial cells and tight junctions (TJ) comprise the largest number of intestinal barriers [5,6]. When the epithelium is intact, intestinal barrier function is largely defined by the construction and expression of TJ as well as intestinal barrier permeability characteristics [7].

In Crohn's disease, the intestinal barrier at the interface between the intestinal microbiome and the lymphoid tissue plays a critical role in shaping the mucosal immune response. In this situation, the intestinal barrier is impaired, and bacterial products and dietary antigens cross the epithelium and enter the lamina propria. Antigen-presenting cells (APCs) take up foreign materials and regulate the differentiation of T cells [8]. For many years, it has been assumed that Crohn's disease is a T helper 1 (Th1)-cell-mediated disease [9], and a novel subset of IL-17-producing CD4 + Th cells, Th17 cells, have more recently been implicated in the pathogenesis of CD [10]. In the most appropriate model of human CD, interleukin-10-gene-deficient (*Il10*$^{-/-}$) mice [11] exhibit increases in CD4 + Th1 and Th17 cells, which secrete a large number of pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), IL-1β, interferon-γ (IFN-γ), and IL-17A [12,13].

Laquinimod (C19H17ClN2O3, 5-chloro-N-ethyl-4-hydroxy-1-methyl-2-oxo-N-phenyl-1, 2-dihydroquinoline-3-carboxamide) is an immunomodulatory drug that has extensively shown its efficacy in inflammatory and autoimmune disorders, especially multiple sclerosis (MS) [14]. Laquinimod is a small molecule that concentrates in the peripheral immune system as well as in the central nervous system (CNS) [15]. In MS model mice, laquinimod has shown immunomodulatory effects by downregulating Th1 and Th17 cells. Preclinical data suggest that laquinimod has a direct inhibitory effect on antigen-presenting cells and results in anti-inflammatory T cell polarization manifested by a

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1567-5769/

Table 1
Primer for polymerase chain reaction.

TNF-α	Forward:	5′-TGGGAGTAGACAAGGTACAACCC-3′
	Reverse:	5′-CATCTTCTCAAAATTCGAGTGACAA-3′
IFN-γ	Forward:	5′-AACGCTACACACTGCATCTTGG-3′
	Reverse:	5′-GCCGTGGCAGTAACAGCC-3′
IL-1β	Forward:	5′-CAACCAACAAGTGATATTCTCCATG-3′
	Reverse:	5′-GATCCACACTCTCCAGCTGCA-3′
IL-17A	Forward:	5′-GCTCCAGAAGGCCCTCAGA-3′
	Reverse:	5′-AGCTTTCCCTCCGCATTGA-3′
GAPDH	Forward:	5′-AGGCCGGTGCTGAGTATGTC-3′
	Reverse:	5′-TGCCTGCTTCACCACCTTCT-3′

reduction in the frequencies of pro-inflammatory Th1 (IFN-γ + CD4 +) cells and Th17 (IL-17A + CD4 +) cells [16]. Thus, the proposed mechanism of laquinimod may make it ideally suited to reduce gastrointestinal inflammation. However, the effects of laquinimod on colitis have been heretofore unstudied. In this investigation, we determined whether laquinimod ameliorates established spontaneous colitis in *Il10*$^{-/-}$ mice, and we attempted to explain the potential mechanisms that account for these effects.

2. Materials and methods

2.1. Animals

Both wild type (WT) and *Il10*$^{-/-}$ mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The mice were bred and maintained under specific pathogen-free (SPF) conditions at the Model Animal Research Center of Nanjing University (Nanjing, China). Previous experiment has demonstrated that most *Il10*$^{-/-}$ mice on the C57BL/6 strain under SPF conditions develop spontaneous colitis at 12 weeks of age [17]. The mice used in our study were 16 weeks of age. The experimental procedures were performed in accordance with the Guidelines for Animal Experiments at Jinling Hospital and were approved by the Ethics Committee of Jinling Hospital (Jiangsu, China).

2.2. Drug treatment of mice

Wild-type and *Il10*$^{-/-}$ mice were divided into wild-type group (WT, wild-type mice), control group (IL-10-KO, *Il10*$^{-/-}$ mice) and treatment group (laquinimod, *Il10*$^{-/-}$ mice), and each group contains 6 mice. To investigate the effects of laquinimod on wild type mice, 6 wild type mice (control group) also received laquinimod treatment. Laquinimod (TEVA Pharmaceuticals Industries, Ltd (Israel), purity > 98%) was dissolved in distilled water and administered 25 mg/kg by oral gavage, 32 times a week. As reported in a study by Lourenço EV et al. [16], we found that this dose was well tolerated in mice, and no obvious macroscopic adverse effects were observed. Mice in the IL-10-KO group and the WT group received an equal volume of phosphate buffered saline (PBS) by oral gavage. Four weeks after administration, the mice were sacrificed by an overdose of anesthesia with pentobarbital sodium, and proximal colons were collected for experiments.

2.3. Weight and disease activity index (DAI)

Animals were weighed on a balance, and measurements were recorded to the nearest 0.1 g. The Disease Activity Index (DAI) was recorded as a composite score of stool consistency (0–2) and fecal blood (0–1). Animals were given a score of 0 for hard stools, 1 for soft-formed stools, and 2 for frank diarrhea. The absence (no points) or presence (+1 point) of fecal blood was determined using the Hemoccult® Sensa® card (Beckman Coulter, Miami, FL, USA) [18].

2.4. Histological evaluation

After the mice were sacrificed, samples were obtained from the proximal colon. The samples were fixed in 4% paraformaldehyde for 24 h and paraffin-embedded. Fixed tissues were then sectioned at a thickness of 5 μm and stained with hematoxylin and eosin (H&E). A histological score of H&E-stained samples of the proximal colon was determined by two independent pathologists in a blind manner according to the method described by Singh et al. [19]. Briefly, a score (0–4) was given: grade 0 indicated no change from normal tissue; grade 1 indicated one or a few multifocal mononuclear cell infiltrates in the lamina propria, minimal hyperplasia, and no mucus depletion; grade 2 indicated intestinal lesions involving several multifocal, mild, inflammatory cell infiltrates in the lamina propria composed of mononuclear cells with no inflammation in the submucosa; grade 3 indicated lesions involving moderate inflammation and epithelial hyperplasia; grade 4 indicated inflammation involving most of the intestinal sections. Each mouse (6 in every group) got a histological score from 0 to 4, and all scores from the mice were included in the statistics of the corresponding group. Normally, the intestinal epithelial caves in and forms glandular architecture called gland in the lamina propria. The normal architecture of gland is a single straight tubular gland opening to the mucosal surface, and is comprised with columnar cells, goblet cells, endocrine cells and a few of undifferentiated cells. When the score is higher than grade 2, the architecture of gland began destroyed.

2.5. Intestinal permeability in vitro

The permeability of mouse intestine was measured with the Ussing Chamber Analyses. Segments of proximal colon were immediately removed for assessment of intestinal permeability. We used the method previously reported by Arrieta et al. [20]. Briefly, the mucosa was mounted in Lucite chambers exposing mucosal and serosal surfaces to 10 ml of oxygenated Krebs buffer (115 mmol/l NaCl, 8 mmol/l KCl, 1.25 mmol/l $CaCl_2$, 1.2 mmol/l $MgCl_2$, 2 mmol/l KH_2PO_4 and 225

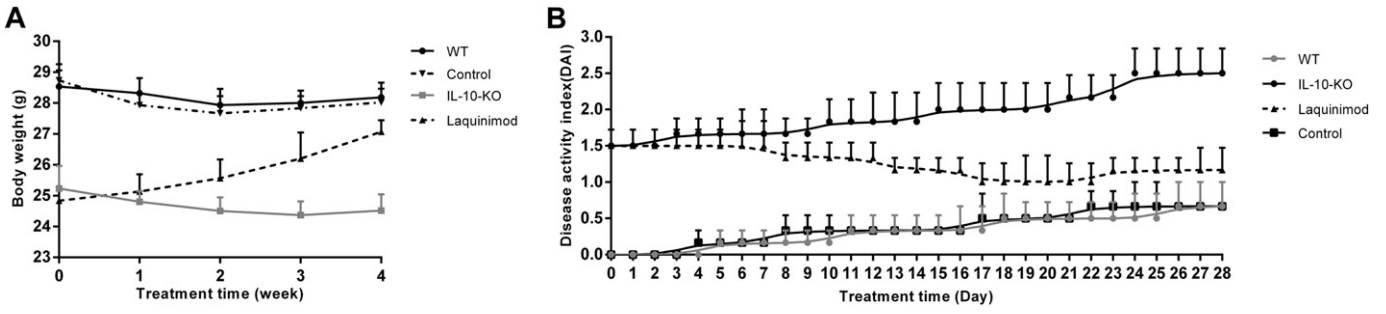

Fig. 1. Clinical manifestation of mice. (A) Body weight weighed every week. (B) Disease Activity Index recorded every day according to stool consistency and fecal blood (0 for absence and 1 for presence of fecal blood). The result of each group was distinguished with different line types. Data are presented as means ± SEM (n = 6 for each group).