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Research Paper

Induction and Amelioration of Methotrexate-Induced Gastrointestinal Toxicity are Related to Immune Response and Gut Microbiota

Bailing Zhou [a,1], Xuyang Xia [a,b,1], Peiqi Wang [c,1], Shuang Chen [a], Chaoheng Yu [a], Rong Huang [a], Rui Zhang [a], Yantai Wang [a], Lian Lu [a], Fengjiao Yuan [a], Yaomei Tian [a], Yingzi Fan [a], Xueyan Zhang [a], Yang Shu [a,b], Shouyue Zhang [a,b], Ding Bai [c], Lei Wu [a], Heng Xu [a,b,*], Li Yang [a,*]

[a] State Key Laboratory of Biotherapy, West China Hospital, Sichuan University and Collaborative Innovation Center, Chengdu, Sichuan, China
[b] Department of Laboratory Medicine, Research Center of Clinical Laboratory Medicine and Precision Medicine Center, Precision Medicine Key Laboratory of Sichuan Province, West China Hospital, Sichuan University, Sichuan, China
[c] State Key laboratory of Oral diseases, National Clinical Research Center for Oral Diseases, West China Hospital of Stomatology, Sichuan University and Collaborative Innovation Center, Chengdu, China

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ABSTRACT

As a widely used anticancer and immunosuppressive agent, methotrexate (MTX) can induce multiple adverse drug reactions (ADRs), such as gastrointestinal toxicity, the mechanisms are poorly understood. Gut microbiota has been widely reported to be associated with the onset of multiple diseases as well as treatment outcomes of different drugs. In this study, mucosal injury was observed in MTX-treated mice, leading to significant changes in macrophages (i.e., M1/M2 ratio, $P < 0.05$) but not in dendritic cells. Moreover, the population, diversity and principal components of the gut microbiota in mice were dramatically altered after MTX treatment in a time-dependent manner, and *Bacteroidales* exhibited the most distinct variation among all the taxa ($P < 0.05$). *Bacteroides fragilis* was significantly decreased with MTX treatment ($P < 0.01$) and tended to decrease proportionately with increasing macrophage density. Gavage of mice with *B. fragilis* ameliorated MTX-induced inflammatory reactions and modulate macrophage polarization. In conclusion, our results delineate a strong impact of the gut microbiota on MTX-induced intestinal mucositis and provide a potential method for the prevention of such ADRs.

1. Introduction

Methotrexate (MTX), a structural analog of folic acid, blocks folate metabolism via competitive inhibition of dihydrofolate reductase (DHFR), thus leading to the suppression of de novo synthesis of purines and pyrimidines [1–3]. Over the past decades, MTX has been successfully used to treat various cancers and autoimmune diseases either alone or in combination with other agents [4]. Unfortunately, the curative potential of MTX is sometimes reduced due to its morbid multi-organ toxicity, including gastrointestinal toxicity, bone marrow toxicity, cardiotoxicity, nephrotoxicity and hepatotoxicity [5–7]. Currently, intestinal toxicity is the major dose-limiting factor for MTX administration, and MTX-induced intestinal mucositis represents a significant burden to patients. The condition may affect the entire gastrointestinal tract and is typically accompanied with nausea, bloating, abdominal pain and diarrhea, which often result in malabsorption, weight loss and disrupted chemotherapy [8–10]. Despite all the efforts being made to ameliorate MTX-induced intestinal damage [11–14], there is no satisfactory therapeutic intervention so far that prevents or treats all the symptoms [15]. This is due, at least in part, to the lack of understanding of the mechanisms by which MTX induces intestinal impairment. A limited number of studies suggest that the administration of MTX induces DNA strand breaks in rapidly proliferating intestinal epithelial cells [16] and causes significant oxidative stress [17, 18]. More importantly, MTX may exert deleterious effects through a dynamic sequence of complex inflammatory events initiated by direct cellular injury in the intestinal epithelium and submucosal tissues [16, 19]. According to various reports, the mononuclear phagocyte system (MPS) plays a key role in the maintenance of gut homeostasis and exhibits multiple functions during immune responses in the intestine. The MPS mainly comprises dendritic cells (DCs) and macrophages [20], which initiate adaptive immune responses [21] and act as innate effector cells [22], respectively. When tissues are damaged following infection or injury, inflammatory monocytes are recruited from the circulation or the intestinal reservoir for homeostatic adaptation. Therefore,

* Corresponding authors at: State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, No. 17, South Renmin Road, Chengdu, Sichuan 610041, China.
E-mail addresses: xuheng81916@scu.edu.cn, (H. Xu), yl.tracy73@gmail.com (L. Yang).
[1] These authors contributed equally to this work.

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it is possible that the MTX-induced intestinal damage may cause dysregulation of the intestinal MPS, thus leading to a vicious inflammatory cycle.

On the other hand, the activated immune system in the intestine can also impact the gut microbiota, a diverse microbial community mainly composed of bacteria that colonize the gastrointestinal tract. Despite the complexity of the gut microbiota, there is a mutualistic relationship between the host and microorganisms in which the microbiota contributes to many physiological processes of the host, and in turn the host provides niches and nutrients for microbial survival [23, 24]. As the homeostasis of the gut microbiota is important to the intestinal epithelium and immune system and may modulate the intestinal metabolism of drugs, aberrant changes of the gut microbiota can result in altered drug response, including treatment inefficiency as well as adverse drug reactions (ADRs) [25]. For instance, the antitumor efficacy of CTLA-4 blockade-based immunotherapy is dependent on distinct *Bacteroides* species in the intestine, and response to CTLA-4 inhibitors may be lost in antibiotic-treated or germ-free mice [26]. Therefore, it is worthwhile to investigate the relationship among MTX-induced intestinal toxicity, immune responses and disruption of the gut microbiota.

In this study, we determined the impact of MTX on the intestinal mucosal damage, alteration in the MPS properties, and consequent imbalance in the host gut microbiota. We also observed that via a dynamic sequence of detrimental intestinal inflammatory reactions, MTX profoundly aggravates intestinal toxicity, which can be ameliorated by gavage with specific *Bacteroides* species. The current study may fuel subsequent studies investigating the prevention of treatment of MTX-induced intestinal damage.

2. Methods

2.1. Mice and Treatment

Eight-week-old male Balb/c mice were purchased from Beijing Vital River Laboratory Animal Co. Ltd. (Beijing, China). All mice were maintained in a pathogen-free animal facility. All experimental procedures and animal care were carried out in compliance with the regulations of the Animal Care Committee of Sichuan University. The mice were intraperitoneally (i.p.) injected with 1 mg/kg of MTX (Sigma-Aldrich, USA) or PBS every 3 days. The mice were treated with or without metronidazole (Sigma-Aldrich, USA) for 2 weeks before MTX injection and given antibiotics until the beginning of the experiment. Metronidazole (1 mg/ml) was added to sterile drinking water. The solutions and bottles were changed 2 times a week.

2.2. Cell Culture and Reagents

Colon adenocarcinoma Caco2 cells and intestinal epithelial IEC6 cells were cultured at 37 °C in 5% CO_2 in DMEM (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA), 100 units/ml penicillin (Beyotime Biotechnology, China) and 100 μg/ml streptomycin (Beyotime Biotechnology, China). Macrophage RAW264.7 cells were cultured at 37 °C in 5% CO_2 in RPMI-1640 (Gibco, USA) medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA), 100 units/ml penicillin (Beyotime Biotechnology, China) and 100 μg/ml streptomycin (Beyotime Biotechnology, China).

2.3. Microbial DNA Extraction, 16S rDNA Amplicon Sequencing

The fecal samples used in this study were collected before or after injection of MTX (or PBS) from mice under metronidazole regimen or water and kept at −80 °C until further analysis. Frozen fecal samples were processed for DNA isolation using the Stool DNA Isolation Kit according to manufacturer's instructions. One nanogram of purified fecal DNA was used for PCR amplification. Amplicons spanning the variable region 4 (V4) of the 16S rRNA gene were generated by using the following primers: forward, 5′-GTGCCAGCMGCCGCGGTAA-3′; reverse, 5′-GGACTACHVGGGTWTCTAAT-3′. The PCR products were then sequenced on an Illumina Hi-seq sequencer at Novogene (Novogene, Beijing, China).

2.4. Taxonomic Microbiota Analysis

FLASH (V 1.2.7, http://ccb.jhu.edu/software/FLASH/) [27] was used to obtain assembly reads, i.e., the raw tags, for each sample, from which the sequences of barcodes and primers were truncated. The raw tags were then processed using the QIIME (Quantitative Insights Into Microbial Ecology, http://www.qiime.org) analysis pipeline to obtain clean tags [28–30]. Using Uparse (V 7.0.1001, http://drive5.com/uparse/) [31], sequences sharing 97% nucleotide sequence identity in the 16S region were binned into operational taxonomic units (OTUs). Taxonomical classification was performed using the RDP-classifier (V 2.2, http://sourceforge.net/projects/rdp-classifier/) [32], and OTU mapping was employed using the SILVA database (http://www.arb-silva.de/) [33]. For alpha diversity, QIIME and R (V 2.15.3) were used to construct the Chao1 curve. For beta diversity, the Unifrac distance was calculated using QIIME, and PCA was performed using R.

2.5. Co-Culture of B. fragilis with RAW264.7 Macrophages

B. fragilis (ATCC 25285) were plated at 1×10^6 per well with 100 μl BHI and cultured with or without 1×10^6/ml RAW264.7 cells. After 16 h, the *B. fragilis* was collected, diluted and cultured on sheep blood agar plates for 24 h.

2.6. Gut Colonization with Dedicated Bacterial Species

For inoculation of mice untreated or treated with metronidazole, colonization was performed on the day following the first MTX injection by oral gavage with 100 μl PBS containing 1×10^9 bacterial cells. *B. fragilis* was grown on sheep blood agar plates for 48 h at 37 °C under anaerobic conditions. *B. fragilis* was harvested from the sheep blood agar plates, suspended in sterile PBS, centrifuged and washed with PBS and then resuspended in sterile PBS at an optical density (600 nm) of 1, which corresponds to approximately 1×10^9 colony-forming units (CFUs) per ml.

2.7. Flow Cytometry

Mesenteric lymph nodes (MLNs) and spleens were harvested from mice on day 7 or day 14 after the first injection of MTX. The tissues were cut into small pieces and digested with type IV collagenase (Sigma, USA) at 37 °C for 30 min with shaking. The mixture was subsequently filtered through a 70 μm cell strainer. The cells were stained with antibodies against the following surface markers for flow cytometry: CD11b, F4/80, CD206 and CD11c. The antibodies were purchased from BD Biosciences and BioLegend. Cell populations were gated as follows: M1 macrophage ($CD11b^+F4/80^+$), M2 macrophage ($CD11b^+CD206^+$) and DC ($CD11c^+$). Flow cytometry was performed on a FACSCalibur (BD, USA) flow cytometer, and the data were analyzed with the software FlowJo 6.0.

2.8. Histology of Gut Tissue and Immunofluorescence Staining of the Gut Leukocytes

The whole tissue of the small intestine (duodenum, jejunum and ileum) and colon was harvested, cleaned from feces, fixed in 4% paraformaldehyde for 24 h and then embedded longitudinally in paraffin. Small intestine or colon tissues were cut into 4 μm longitudinal sections and stained with hematoxylin and eosin for histological analyses. To

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