The Role and Mechanism of M2c Macrophages in Lipopolysaccharide/D-Galactosamine Induced Acute Hepatitis

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The study was based on the evaluation of the positive effect of M2c macrophages on liver tissue inflammation, pathological changes and tissue damage in lipopolysaccharide/D-galactosamine induced acute hepatitis. Meanwhile, whether M2c macrophages induced anti-inflammatory effects through Janus kinase/signal transducer and activator of transcription signaling pathway was also investigated. Forty institute of cancer research female mice in the same batch with no significant difference in body weight were distributed randomly into 5 different groups namely the control group, solvent control group, lipopolysaccharide/D-galactosamine, lipopolysaccharide/D-galactosamine+M2c and lipopolysaccharide/D-galactosamine+M2c+ruxolitinib, 8 in each group. 6 h after the last treatment of mice in each group, serum was collected to detect the content of alanine aminotransferase and aspartate transferase. The pathology of liver tissue was determined using hematoxylineosin staining. The determination of the levels of expression of anti-inflammatory factors, pro-inflammatory factors, related apoptosis proteins and Janus kinase/signal transducer and activator of transcription signaling pathway related proteins in liver tissue were carried out by Western blot. After 6 h of induction with lipopolysaccharide/D-galactosamine, the liver function (alanine aminotransferase, aspartate aminotransferase), pathological score of liver slices, liver pro-inflammatory factors and anti-inflammatory factors (interleukin-6, tumor necrosis factor-alpha, interleukin-1beta and interleukin-10) were significantly improved in the mice blood. Comparative to the normal control group, apoptosis-related proteins (B-cell lymphoma-extra-large, B-cell lymphoma 2, Bel-2-associated X protein) and other indicators were higher with a significant difference (p<0.05) under the acute hepatitis model induced lipopolysaccharide/D-galactosamine, after infusion of M2c macrophages from the tail vein of mice. The above indexes of mice were improved and exhibited a statistically significant difference (p<0.05). After adding ruxolitinib to intervene Janus kinase/signal transducer and activator of transcription signaling pathway on the basis of lipopolysaccharide/D-galactosamine+M2c group, the liver function, liver slice pathological score, pro and anti-inflammatory factor and apoptosis-related protein expressions were significantly higher as compared to the lipopolysaccharide/D-galactosamine+M2c group with significant difference (p<0.05), while the observed differences were not significant (p>0.05) in the lipopolysaccharide/D-galactosamine group. Lipopolysaccharide/D-galactosamine induces the occurrence of acute hepatitis, which can well mimic the virus the pathogenic process of hepatitis. M2c macrophages exhibited a critical anti-inflammatory role in acute hepatitis via activating the Janus kinase/signal transducer and activator of transcription signaling pathway.

Key words: Lipopolysaccharide/D-galactosamine hydrochloride, alanine aminotransferase, interleukin-6, tumor necrosis factor-alpha

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reduce and eliminate the damage caused by harmful factors to the human body, but also promote tissue repair in the later phase, serious adverse reactions, even life-threatening.

As the most important immune defense cells in inflammatory diseases, macrophages can release pro-inflammatory mediators to promote the formation of inflammatory factors. The plasticity and functional polarization of macrophages exhibited a critical role in the inflammatory response. In a specific microenvironment, macrophages can undergo the transformation of M1 and M2 phenotypes. M1 macrophages are capable to secrete various pro-inflammatory factors in the early stages of an inflammatory response, M1 macrophages can secrete a variety of pro-inflammatory factors including Interleukin-6 (IL-6), and Tumor Necrosis Factor-Alpha (TNF-α), etc. It has the ability to activate type I immune response, causing tissue cell damage and preventing the process of inflammatory response. In contrast, M2 macrophages are capable to secrete various inflammatory inhibitory factors. M2 macrophages are divided into different subtypes such as alternate activation (M2a), type II alternate activation (M2b) or acquired inactivation (M2c). The three have special surface markers and are capable to secrete various anti-inflammatory factors and chemokine’s including IL-10, IL-13 and CC Chemokine Ligand 17 (CCL-17). The results of multiple polarizations of macrophages can affect the outcome of many diseases. M2c macrophages are considered to be the main subtype that determines the initiation of inflammation and its anti-inflammatory factor IL-10 can inhibit inflammation through signaling pathways. The development of the response further promotes the self-healing process of tissue damage. Various researches has described the possibility of the Janus Kinase (JAK)/Signal Transducer and Activator of Transcription 3 (STAT3) signaling pathway participating in certain physiological processes including cell differentiation, proliferation and apoptosis after liver injury by mediating the signal transduction of various anti-inflammatory and pro-inflammatory factors. Ruxolitinib is a novel targeted drug that selectively inhibits JAK. It has been clinically used for targeted therapy of hematological diseases such as myelofibrosis. Activation of the JAK2/STAT3 pathway has a significant inhibitory effect.

The animal model of Lipopolysaccharide/D-Galactosamine (LPS/D-gal) induced acute hepatitis is a representative model of viral acute hepatitis and its pathogenic mechanism is related to oxidative stress, stimulation, hepatocyte necrosis and inflammatory response. LPS is an endotoxin that activates inflammatory cytokines and can cause liver tissue damage. D-gal is a hepatotoxic substance that mainly induces liver toxicity by inhibiting the hepatocytes and protein synthesis. D-gal increases the sensitization effect of LPS more than 1000 times. Therefore, the liver injury model induced LPS/D-gal has been used excessively to better understand the underlying acute liver injury mechanism and discover new hepatoprotective agents. The combined application of LPS and D-gal increased the Reactive Oxygen Species (ROS) production. Excessive ROS can stimulate macrophages to produce pro-inflammatory cytokines, cause hepatocyte apoptosis and aggravate hepatic inflammatory damage.

This study intends to investigate whether the increase of M2c macrophages has a positive effect on the inflammation, pathological changes and tissue damage of acute hepatitis liver tissue in a mouse acute hepatitis model induced by LPS/D-gal combination. In the meanwhile, via inhibit JAK/STAT3 by ruxolitinib pathway; this study further verified whether M2c macrophages exert anti-inflammatory effects via the JAK/STAT3 signaling pathway.

**MATERIALS AND METHODS**

Research objects:
The study was conducted on the Specific Pathogen-Free (SPF), Institute of Cancer Research (ICR) female mice. The mice aged 6-8 w, weighing 18-25 g and randomly divided into 8 mice in each group. The approval of this experiment was obtained by the hospital ethics committee.

**Grouping, model preparation and drug intervention:**
Forty ICR female mice with no significant difference in body weight in the same batch were divided into 6 different groups (8 mice/group).

**Normal control group:** Without any treatment, injected with exactly the same proportion of normal saline as the LPS/D-gal group.

**Solvent control group:** Injected with the same proportion of solvent in the LPS/D-gal group without adding solute.

**LPS/D-gal:** Model group received intraperitoneal injection of LPS/D-gal (LPS 40 μg/kg+D-gal 800 mg/kg).
LPS/D-gal+M2c: In the model group intravenous infusion of 1×10⁶ M2c macrophages.

LPS/D-gal+M2c+ruxolitinib: After the model group was established, 1×10⁶ M2c macrophages were infused from the tail vein and ruxolitinib was administered orally after modeling (INCB018424) 180 mg/kg.

M2c macrophage preparation in this study: Macrophage-Colony Stimulating Factor (M-CSF) (50 ng/ml)+IL-10 (50 ng/ml) was added to macrophages (RAW 264.7).

Liver histopathological section preparation:
6 h after the last treatment of the mice in each group, the experimental mice were dissected, and about 30 mg of liver tissue (including the middle and edge parts) was taken, and then transferred to the embedding box with its the original structure not damaged as much as possible, and fixed in 10 % formaldehyde solution. Paraffin embedding, sectioning, Hematoxylin and Eosin (HE) staining, observation under the microscope, photographing and recording were performed. Tissue sections were scoring according to the severity of the pathological. A score range of 0-4 points is set and 3 fields of view are selected for each pathological section. The average value was calculated and the degree of inflammatory cell infiltration was the standard. More than 80 % were scored as 4 points; 60 % as 3 points; 40 % as 2 points; 20 % as 1 point; if there is no inflammatory cell infiltration it is counted as 0 points. The specific scores are calculated according to the actual test results.

Serological index detection:
6 h after the last treatment, the serum of each mouse in each group were collected by centrifuging blood sample of each mouse at 4°, 3000 r/min and 15°. The separated serum was tested in strict accordance with the instructions of Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) detection kits.

Western blot detection of protein expression levels:
After 6 h of the last treatment, the experimental mice were dissected in each group and 30 mg of mouse liver tissue was taken in duplicate and labeled. Then, the tissue protein was extracted followed by the determination of the protein concentration via the Bicinchoninic Acid (BCA) method using the BCA protein quantitative analysis kit. Finally, the protein expression was detected by Western blot. For specific operation steps, please refer to the corresponding testing manual.

Statistical analysis:
For statistical analysis of the data, Statistical Package for the Social Sciences (SPSS) 20.0 software was used and the results were represented as mean±standard deviation (x±s). In different treatment groups, independent samples t test was used, while the comparison of the differences among multiple groups was carried out using a one-way analysis of variance, p<0.05 suggested a statistically significant difference.

RESULTS AND DISCUSSION
Each group was investigated for the serum levels of AST and ALT in mice. The study found that under the combined effect of LPS/D-gal, a significantly higher serum level of ALT and AST was observed in the LPS/D-gal group (354.2±36.7) U/l and (652.5±53.2) U/l, than that of normal control (52.3±9.2) U/l and (132.5±19.3) U/l, respectively (p<0.001). ALT and AST between the normal control and the solvent control groups did not exhibit a significant difference (p>0.05). As compared to the LPS/D-gal group, the ALT and AST levels of mice serum in the LPS/D-gal+M2c group were lower with a statistically significant difference (p<0.001). Further analysis revealed the increased level of ALT and AST of the mice after oral administration of ruxolitinib (LPS/D-gal+M2c+ruxolitinib group) upon comparison with the LPS/D-gal+M2c group, with a statistically significant difference (p<0.001) as shown in fig.1.

The pathological sections were performed on the mice liver tissues of each group, and the results exhibited no abnormal changes in the normal control group and the solvent control group. After 6 h of LPS/D-gal induction, the pathological sections of the liver in the LPS/D-gal model group showed, massive hemorrhage, partial liquefaction and necrosis of hepatocytes, partial vacuolar degeneration, infiltration of a huge extent of inflammatory cells in the local liver parenchyma,
increased phagocytic cells in the hepatic sinus and histopathological changes such as individual vascular embolism. Upon comparison with the LPS/D-gal group, the mice in the LPS/D-gal+M2c group showed partial hemorrhage in the liver, decreased hepatocyte necrosis, decreased phagocytic cells in the hepatic sinus, and some histopathological changes such as vascular embolism. The histopathological sections of the liver in the LPS/D-gal+M2c+ruxolitinib group were similar to the LPS/D-gal group. Each group’s pathological sections of liver tissue were scored and then those scores were compared across groups. The observed average score of the LPS/D-gal group was (2.6±0.5) points, while the scores of the normal control and the solvent control groups were both 0 points. LPS/D-gal group exhibited a significant difference upon comparison with control groups (normal control group and solvent control group) (p<0.001). The observed findings exhibited that the treatment of LPS/D-gal would cause severe case damage to liver tissue. As compared to the LPS/D-gal group, the scores of the liver histopathological sections of the mice in the LPS/D-gal+M2c group were reduced. Statistical analysis showed that only the LPS/D-gal+M2c and the LPS/D-gal groups had significant differences in the scores of liver pathological sections (p<0.001). As compared to the LPS/D-gal+M2c group, the pathological section score of the LPS/D-gal+M2c+ruxolitinib group increased to (2.65±0.62) points with a significant difference (p<0.001), as illustrated in fig. 2. However, in the scores of liver pathological sections between the LPS/D-gal and LPS/D-gal+M2c+ruxolitinib groups, no significant difference was observed (p>0.05), indicating that the addition of M2c improved the liver damage of the mice, but the addition of ruxolitinib will inhibit JAK/STAT3 pathway. Thus, it was speculated that M2c no longer played an anti-inflammatory role after JAK/STAT3 pathway was inhibited. The results of liver pathological damage in the LPS/D-gal+M2c+ruxolitinib group were consistent with the LPS/D-gal group.

IL-6, IL-1β and TNF-α are the main inflammatory factors, and IL-10 is an anti-inflammatory factor. The results of the Western blot exhibited the increased expression of IL-1β, IL-6, TNF-α and IL10 in the LPS/D-gal group upon comparison with the normal control group and the solvent control group with significant difference (p<0.001). Upon comparison with the LPS/D-gal group, the decreased protein expressions of IL-1β, TNF-α and IL-6 were observed in the LPS/D-gal+M2c group (p<0.05), with a significant decrease (p<0.05) in IL-10 expression (anti-inflammatory factor). The result also showed the increased IL-1β, IL-6 and TNF-α expressions in the LPS/D-gal+M2c+ruxolitinib group as compared to the LPS/D-gal+M2c group, while the expression of IL10 was decreased with statistically significant difference (p<0.05). The results were described in fig. 3.

BCL-2, BAX and BCL-xL are the main proteins associated with apoptosis, among which BCL-xL and BAX are anti-apoptotic proteins and can promote cancer cell proliferation, while BAX is a pro-apoptotic protein and can promote cancer cell apoptosis. The results of this study showed the increased Bcl-2 and BCL-xL protein expressions in the LPS/D-gal group upon comparison with the normal control group, while BAX expression was decreased with a statistically significant difference (p<0.001). As compared to the LPS/D-gal group, the BCL-2 and BCL-xL expressions in the LPS/D-gal+M2c group were both decreased (p<0.001) with increased protein expression of BAX (p<0.05). Upon comparison with the LPS/D-gal+M2c group, the protein expressions of BCL-2 and BCL-xL in the LPS/D-gal+M2c+ruxolitinib group increased with decreased BAX expression and exhibited statistically significant differences in the expression of the apoptosis-related proteins between the two groups (p<0.05) as described in fig. 4.

To further investigate whether the anti-inflammatory effect of M2c macrophages in mice with acute hepatitis is through the intervention of JAK/STAT3 signaling pathway, ruxolitinib (INCB018424) was added to inhibit the JAK/STAT3 signaling pathway in the acute hepatitis model in mice. The study found that compared with the LPS/D-gal group, the expression levels of JAK and STAT3 proteins in the LPS/D-gal+M2c group were increased with a statistically significant difference (p<0.001). After the addition of ruxolitinib (INCB018424) inhibition, the protein expression levels of JAK and STAT3 proteins in the LPS/D-gal+M2c group were increased with a statistically significant difference (p<0.001). Therefore, the anti-inflammatory effect of M2c macrophages was through the inhibition of JAK/STAT3 signaling pathway.

In response to environmental signals, macrophages are capable of switching between functional phenotypes. Classically activated macrophages (M1) have pro-
inflammatory effects and are important cellular components in anti-inflammatory, antibacterial and antitumor immunity. While alternative activated macrophages (M2) are present in the presence of IL-13 or IL-4 cytokines, especially its subtype M2c macrophages perform a series of physiological processes by producing anti-inflammatory factors such as IL-10, including anti-inflammatory, wound repair and tissue remodeling\(^6\). Some studies have found that\(^{15}\), LPS/D-gal induces the synthesis and secretion of early pro-inflammatory factors including IL-1\(\beta\), IL-6 and TNF-\(\alpha\), thereby inducing the incidence and progress of inflammatory responses. While M2c inhibits the occurrence of immune response by activating the expression of anti-inflammatory factor IL-10 in later phase of inflammation, hinders the excessive activation of inflammatory response and promotes the body to maintain homeostasis.

The JAK-STAT and activator of transcription signal transduction pathway is an important pathway that mediates the signal transduction of a variety of cytokines, and is involved in many aspects of the body’s inflammatory response, immune response and immune cell differentiation. The STAT3 signaling pathway has been shown in recent studies to play a vital role in the recovery from liver injury, which can promote hepatocyte proliferation, regulate carbohydrate and lipid metabolism, and activate the expression of hepatoprotective acute-phase proteins\(^{16}\).

The JAK2/STAT3 signaling pathway can widely participate in the pathological processes including cell differentiation, proliferation and apoptosis after liver injury by mediating the signal transduction of various inflammatory factors\(^9\). Under the stimulation of inflammatory factors, JAK2 receptors are prone to phosphorylation, which activates JAK2 and further activates STAT3 and then mediates inflammation such as Interferon Gamma (IFN-\(\gamma\)), IL-1\(\beta\), TNF-\(\alpha\) and IL-6 by regulating gene transcription. At the same time, the overexpressed inflammatory factors can accelerate the phosphorylation of JAK/STAT3, thereby causing the occurrence and expansion of the inflammatory cascade\(^{17}\).

The mechanism of action between M2c macrophages and the JAK/STAT3 signaling pathway is still unclear. Komohara et al.\(^{18}\) elucidated that M2c macrophages inhibited the proliferation of glioma cells by activating the transcription factor STAT3. There are studies indicate\(^{19}\) that in glioblastoma patients, inhibiting STAT3 not only inhibits the growth of tumor cells but also reverses tumor immune tolerance by weakening the immunosuppressive influence of M2c macrophages. Some studies\(^{20}\) have also shown that the inhibition of the JAK/STAT3 signaling pathway may further inhibit the M2-type polarization of macrophages, that is, inhibit the activation of M2-type macrophages, so that anti-inflammatory factors cannot be expressed and functioned. In acute hepatitis induced by LPS/D-gal, the relationship between the JAK/STAT3 signaling pathway and M2c macrophages and the mechanism of action are still unclear. Therefore, in this study, ruxolitinib was added to inhibit the JAK/STAT3 signaling pathway and the mechanism of action between the two was explored by comparing the changes of related indicators in mice with acute hepatitis before and after adding ruxolitinib.

The finding of this study revealed that after 6 h of induction with LPS/D-gal, the blood liver function (ALT, AST), liver slice pathological score, liver pro-inflammatory factors and anti-inflammatory factors (IL-1\(\beta\), TNF-\(\alpha\), IL-6 and IL10) and its apoptosis-related proteins (BCL-2, BCL-xL, BAX) etc., were higher as compared to the normal control group, indicating that LPS/D-gal induces the occurrence of acute hepatitis, which can effectively mimics the pathogenic process of viral hepatitis. Under the LPS/D-gal-induced acute hepatitis model, after infusion of M2c macrophages from the tail vein of mice, all the above indicators in mice were improved, indicating that M2c macrophages have a positive impact on the inflammatory response of mice.

After adding ruxolitinib (INCB018424) on the basis of LPS/D-gal+M2c group to inhibit JAK/STAT3 signaling pathway, the liver function, liver slice pathological score, pro and anti-inflammatory factors and expression of apoptosis-related proteins were significantly different from those in LPS/D-gal+Mac group (\(p<0.05\)). However, there was no statistically significant difference compare to LPS/D-gal group (\(p>0.05\)), indicating that after inhibiting the activity of the JAK/STAT3 signaling pathway, even adding M2c macrophages did not improve the inflammatory response of the mice. Therefore, this study speculates that M2c macrophages play an anti-inflammatory role in acute hepatitis through the JAK/STAT3 signaling pathway. Once this pathway is inhibited, M2c macrophages cannot be activated.

In conclusion, this study established an acute hepatitis model by injecting LPS/D-gal into the tail vein of mice.
**Fig. 1:** Levels of ALT and AST in mice serum of each group
Note: **p<0.001 indicates a statistically significant difference

**Fig. 2:** Analysis of histopathological changes in the liver of mice in each group
Note: **p<0.001 indicates a statistically significant difference

**Fig. 3:** Levels of IL-1β, TNF-α, IL-6 and IL10 protein expression
Note: *p<0.05 and **p<0.001 both indicates a statistically significant difference
and successfully simulated the pathological process of clinical immune liver disease. To further explore the anti-inflammatory influence of M2c macrophages on mice with acute liver injury by regulating the JAK/STAT3 signaling pathway, thereby inhibiting the inflammatory cascade.

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