Rapamycin alleviates experimental autoimmune hepatitis by immunoregulation of Th1 and Th17 cells

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Research article

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Abstract

Background

Increasing evidence in recent years has suggested that Th1, Th17 cells act as a crucial regulatorin autoimmune hepatitis (AIH). Rapamycin, as a inhibitor of mammalian target of rapamycin (mTOR), which modulates T cell activation, differentiation and migration, is known to be able to selectively inhibit the activation of T helper cells including Th1 and Th17 subsets. However, little is known about the underlying regulatory mechanisms.

Results

In this study, we demonstrated that rapamycin ameliorated AIH in mice model by inhibiting Th1 and Th17 cells. Further analysis revealed that the suppressive function of rapamycin was associated with the downregulation of p70s6k-STAT3 signaling, which is downstream of mTOR, in AIH mice.

Conclusions

These findings suggest a previously unrecognized role for rapamycin immunoregulatory function and its protective effect on AIH, providing additional insight for the selection of alternative clinical protocols for autoimmune diseases.

Background

Autoimmune hepatitis (AIH) is a chronic liver disease putatively caused by loss of tolerance to hepatocyte-specific autoantigens. The immunological mechanism of AIH is considered to be an abnormal T lymphocyte, particularly Th1 and Th17, reaction to hepatocytes [1]. Histological features of AIH include mild to severe interface hepatitis with inflammatorycell infiltration, bridging necrosis, increased hepatocyte apoptosis, multilobular collapse, and bile duct injury or loss [2]. AIH patients require lifelong immunosuppression, which does not always prevent progression to end-stage liver damage [3]. Consequently, the development of high effective and well tolerated novel therapies is an urgent need.

Rapamycin is an inhibitor of the mTOR (mammalian target of rapamycin) kinase that selectively inhibits the function of T helper cell subsets [4, 5], for example, the differentiation and proliferation of effector Th17 cells [6]. The potential role of rapamycin in autoimmune diseases application have demonstrated in the development of experimental autoimmune encephalomyelitis (EAE), type 2 experimental autoimmune hepatitis[7], and post-transplant autoimmune hepatitis [8] by inhibiting Th1 and Th17 cells [9, 10]. However, the concrete mechanism of drug action in AIH is unclear and needs further exploration.

The p70s6k-STAT3 signaling pathway is downstream of mTOR, which activation is considered a pathogenesis for autoimmune diseases [11, 12]. Inhibition of the p70s6k-STAT3 pathway was associated

with suppression of Th17cell differentiation [13, 14]. Therefore, we designed this study to determine the relationship between the dynamic proportions of Th1 and Th17 cells and of the activity of p70s6k-STAT3 pathway in S-100 induced AIH mice.

Results

Rapamycin attenuates liver injury in AIH mice

To explore whether rapamycin could attenuate AIH mice liver injury, liver histopathology and biochemical indexes were measured. AIH mice had varying degrees of pathological changes of grade 2 to grade 3 with portal area and its surroundings showing obvious infiltration of inflammatory cells and hepatic cell necrosis. However, we observed signs of mild inflammation with remission of infiltration of inflammatory cells, and less hepatic cell necrosis in samples with rapamycin treatment as compared with NS group (Fig. 1a, 1c). In the meanwhile, the function indexes ALT and AST were significantly decreased in rapamycin treated liver as compared with those from NS group (Fig. 1d). We also verified that rapamycin could remarkably decreased hepatocyte apoptosisin AIH mice model by TUNEL assay (Fig. 1b, 1e). Combined with the above results, we concluded rapamycin could alleviate liver injury in AIH mice.

Rapamycin inhibit inflammation by downregulating Th1 and Th17 cells in the liver

Next, we tested whether the liver injury protection effect of rapamycin is due to its inhibitory effect of inflammatory cytokine release into the liver. We found that rapamycin treatment could significantly reduce liver IFN-γand IL-17 levels and therefore the inflammation severity (Fig. 2a, 2b). It indicated that the interrelated cytokines IFN-γand IL-17 from Th1 and Th17 cells might play significant role in AIH, and rapamycin could at least partially reverse this inflammatory state. Since the Th1 and Th17 are major sources of IFN-γ and IL-17, we further directly measured the ratios of Th1 and Th17 cells in liver lymphocytes by Flow cytometry. The results showed they were significantly increased in AIH mice as compared with control. After intervention with rapamycin, the ratios were significantly decreased as compared with NS group (Fig. 2c, 2d). Our works indicated rapamycin might inhibit inflammatory Th1 and Th17 cells infiltration in AIH mice.

Rapamycin suppresses the hepatic p70s6k-STAT3 signaling pathway

Because of the important role of p70s6k-STAT3 pathway in modulating immune inflammation, we further asked if rapamycin treatment could suppresses the phosphorylation of both p70s6k and STAT3 which indicates activation of the p70s6k-STAT3 pathway. Rapamycin treatment normalized the levels of p-p70s6k/p70s6k and p-STAT3/STAT3 comparable to healthy control mice in AIH mice (Fig.3). Collectively, these results suggest that rapamycin treatment could reverse the autoimmune inflammatory p70s6k-STAT3 signaling activation in AIH mice.

Discussion

Hepatic necrosis, apoptosis, ALT and AST had been proved to be significantly increased in autoimmune hepatitis, and had been demonstrated to be associated with disease severity [15, 16]. We found rapamycin could significantly alleviate this pathological process.

Immune reaction to autoantigens is not evoked under normal conditions, particularly in liver which is believed to be a immuntolerant organ. However in abnormal scenario, once a liver autoantigen is recognized via costimulatory signals, naive T cells are activated and the immune reaction is initiated [17]. Naive T cells can be activated and differentiated into different types of inflammatory CD4 + T cells, these T cells then begin to trigger immune cascades and participate in the destruction of the liver [18, 19]. In order to achieve pathogenicity, these inflammatory CD4 + T cells are primed in the spleen and migrate to target organs. Emerging evidence reveals that various cytokines contribute to liver inflammation and autoimmunity, in which IFN-γand IL-17 family are highly acknowledged. We confirmed in mouse models of AIH that the expressions of IFN-γand IL-17 in the liver were significantly upregulated compared to the control mice, and rapamycin could markedly decreased Th1 and Th17 cells levels and their produced cytokines, IFN-γ and IL-17. It was reported that Th1 cells play an important role in promoting AIH pathology, and high levels of IFN-γ may be a key factor [20, 21]. Th17 cells have been found to be associated with the pathogenesis of MS, SLE, and autoimmune hepatitis [22–24]. Many autoimmune diseases progression of could be weakened by suppressing Th1 and Th17 cells responses. Thus, we examined the proportions of Th1, Th17 cells in liver lymphocytes in different pathological situation and we found rapamycin could effectively inhibit effector Th1 and Th17 cells.

STAT3 is a critical nuclear transcription factor that mediates the activation of T helper cell subsets and expression of inflammatory cytokines, the differentiation of Th17 cell is largely regulated by the activation of STAT3 [25, 26]. Actually, increasing evidence implies that inhibition of p70s6k-STAT3 pathway alleviate the degree of inflammatory diseases. We observed in this study that phosphorylation of mTOR downstream proteins p70s6k and STAT3 induced by S-100 could be effectively inhibited by rapamycin treatment. Unfortunatuely, we could not tell in this study whether cell type specific p70s6k-STAT3 pathway ultimately modulate liver Th1 and Th17 cells in the liver and further investigation was needed.

Conclusion

We demonstrated that rapamycin treatment, probably by regulating the p70s6k-STAT3 activity and further affected Th1 and Th17differentiation and function, reduced inflammatory infiltration into the liver and reversed AIH development. This finding may imply a new therapeutic option for autoimmune hepatitis via targeting the specific T cell subsets, mainly Th1 and Th17 cell.

Methods

Animals

Twenty-four male C57/BL6 mice (aged 6 weeks), which were housed in a specific-pathogen-free (SPF) facility, were obtained from experimental animal center of Shanxi Medical University (license number: SCXK (Jin) 2015-0001, Taiyuan, China). All animal experiments were approved by the Institutional Animal Care and Treatment Committee of Shanxi Medical University in China.

Animal experiment

All mice were randomly divided into three groups after 1 week of adaptive feeding as follows: NS (normal saline) group (n=6), AIH+NS group (n=6) and AIH+RAP group (n=6). The AIH mouse model was established as previously reported [1-3] by intraperitoneal administration of 0.5 ml fresh S-100 antigen emulsified in an equal volume of complete Freund's adjuvant (CFA, Sigma, Cat No.:F5881, USA) (the concentration of S-100 antigen is 1 mg/mL) on 1st and 7th day. On 4th day, the AIH+NS group and AIH+RAP group were given 0.2mL intraperitoneal injection with normal saline (NS) and rapamycin (Sangon, Cat No.:A606203, China, 1 mg/kg), respectively, once a day for 20 days. All experimental animals were anesthetized by intraperitoneal injection with sodium pentobarbital (60 mg/kg). After deep anesthesia, cervical dislocation was performed and euthanasia was performed. Animal studies were approved by the Animal Use and Care Committee of Shanxi medical university, and were conducted in accordance with the National Research Council Guide for Care and Use of Laboratory Animals.

Biochemical indexes

On 24th day, blood was drawn from retro-orbital plexus and sera were collected by centrifugation (4 °C, 3000rpm, 15 min) and stored at −80 °C. The serum samples were analyzed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by automatic biochemical analyzer.

Histopathology, TUNEL and Immunohistochemistry assay

Liver tissues were fixed with 10% neutral formalin and embedded in paraffin wax and sliced. The TUNEL Apoptosis Assay Kit (Beyotime, Cat No.:C1086, China) was used to detect the apoptosis of liver, according to the manufacturer's instruction. The slides were stained with hematoxylin-eosin (HE). The degree of inflammation of the liver was graded as follows: grade 0 = normal liver tissue; grade 1 = mild infiltration of inflammatory cells with rare hepatic cells necrosis; grade 2 = medium damage of hepatic cells accompanied by infiltration of inflammatory cells and regional hepatic cell necrosis; grade 3 = wide range inflammatory cells infiltration in portal area and hepatic lobules accompanied with wide range hepatic cells necrosis. Three randomly selected fields were scored by three individuals in a blinded manner. Liver Sections deparaffinization, rehydration, blockade of endogenous peroxidase, and antigen retrieval were sequentially performed, and then incubated with primary antibody anti-IFN-γ(1:100; Bioss, Cat No.:bs-0481R, China), or anti-IL-17(1:100; Bioss, Cat No.:bs-1183R, China) at 4°C overnight. After being incubated in secondary antibody (1:500, Boster, Cat No.:BA1041, China), the sections were then colorized by diaminobenzidine reaction.

Liver tissue lymphocytes isolation

Liver tissues were dissected into small pieces, ground and filtered through stainless steel meshes. Liver lymphocytes were isolated from the cell suspensions by gradient centrifugation at 1,400×g for 25 min with Percoll (Solarbio, Cat No.:P8370, China) and lymphocytes were suspended in RPMI 1640 media. The proportion of living cells is verified by trypan blue dye, which is greater than 95%.

Intracellular cytokine staining and Flow cytometry

For measuring intracellular amount of IFN-γ and IL-17 positive cells, liver lymphocytes (5×10^6 cells) were added to 6-well plate. Firstly, cells were incubated and stimulated with phorbolmyristate acetate (PMA, Sigma, Cat No.:P1585, USA, 50 ng/ml), ionomycin (Beyotime, Cat No.:S1672, China, 5 μg/ml) and brefeldin A (BD, Cat No.:555029, USA, 20 μg/ml) at 37 °C for 6 h. Following fixation and permeabilization, the cultured cells were stained with anti-CD4 antibody (1:5000, Cat BD, No.:553654, USA) anti-IFN-γ (1:5000, BD, Cat No.:560660, USA), and anti-IL-17 (1:5000, BD, Cat No.:560821, USA) for 30 min. Flow cytometry was used to detect the frequency of Th1 and Th17 cells.

Western blotting

Briefly, liver tissues were homogenized in RIPA lysis buffer (Boster, Cat No.:AR0102, China) containing a protease inhibitor cocktail (Boster Cat No.:AR1183, China). Then, the protein concentrations were detected by BCA protein assay kit (Boster Cat No.:AR1189, China) according to the manufacturer's instruction. Protein extracts were separated on SDS-PAGE gels and further electro-transferred to NC membranes (Boster Cat No.:AR0135-04, China). After being blocked with 5% nonfat skim milk, the membranes were incubated overnight at 4 °C with appropriate primary antibodies against p70s6k (1:1000, CST, Cat No.:2708, USA), phospho-p70s6k (Thr421/Ser424, 1:1000, CST, Cat No.:9204, USA), STAT3 (1:2000; CST, Cat No.:12640, USA), phospho-STAT3 (Tyr705, 1:2000, CST, Cat No.:9145, USA), respectively. Membranes were then washed with TBST and incubated with peroxidase-conjugated secondary antibody (1:5000, Beyotime, Cat No.:A0208, China) for 1 h at room temperature. Finally, the bands on the membranes were visualized and analyzed by electrochemiluminescence detection. The β-actin staining (1:2000, Bioworld, Cat No.:AP0714, China) was used as internal control.

Statistical analysis

All data were analyzed using SPSS17.0 software (SPAA Inc., USA). Results were expressed as mean ±standard deviation and one-way analysis of variance was used. Comparison among groups used LSD analysis (homogeneity of variance) or Dunnett's T3 (heterogeneous variance). $P<0.05$ was statistically significant.

Declarations

Acknowledgements

Not applicable.

Competing Interest

All authors of the article declare that they have no conflict of interest.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yan Tian, Shuting Zhang, Qingqing Liu, Haixia Tian, Jianjun Zhou and Weiping Fan. The first draft of the manuscript was written by Yan Tian and all authors commented on previous versions of the manuscript. All authors have read and approved the manuscript.

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Ethics approval and consent to participate

The experimental protocols were established according to the Declaration of Helsinki, and the experiments were approved by the Experimental Animal Ethics Committee of the Animal Use and Care Committee of Shanxi medical university, (Approval NO.2018010).

Consent for publication

Not applicable.

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Figures

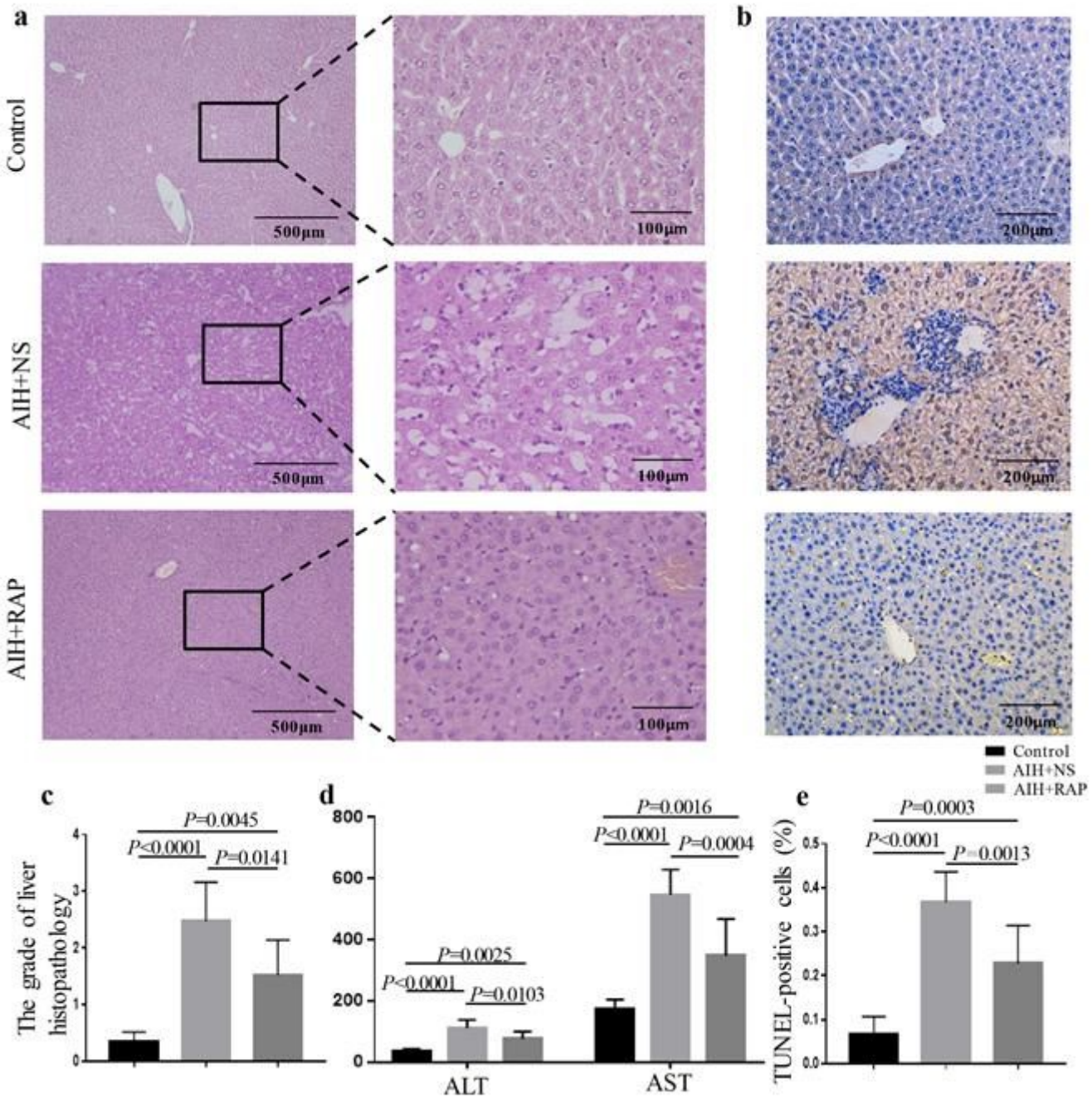

Figure 1

Rapamycin alleviates liver injury in AIH mice. After 3 days of AIH model preparation, the AIH+NS and AIH+RAP group received NS and RAP (1 mg/kg/d) treatments. Representative histological analyses for the Control group, AIH+NS group, and AIH+RAP group (HE-staining) are shown at 100× and 400× magnification (a). TUNEL results for the Control group, AIH+NS group, and AIH+RAP group are shown at 200×magnification (b). The average histological inflammation grade score (c), results of the ALT and AST (d), biochemical assays and TUNEL-positive cells (%) (e) are also shown.

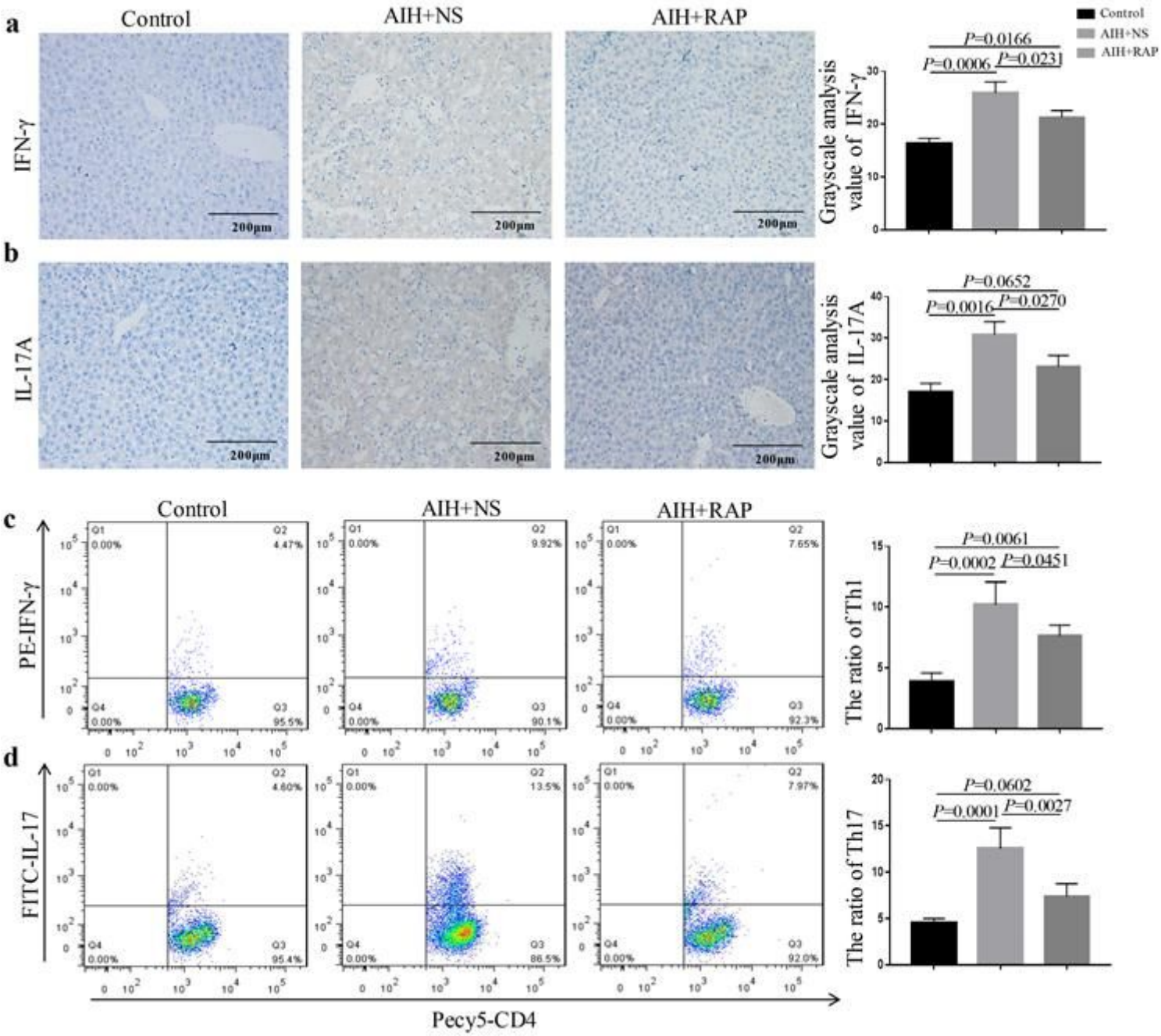

Figure 2

Rapamycin inhibit inflammation in the liver. After 3 days of AIH model preparation, the AIH+NS and AIH+RAP group received NS and RAP (1 mg/kg/d) treatments. Immunohistochemistry indicated the IFN-γ (a) and IL-17 (b) in liver tissue in Control group, AIH+NS group and AIH+RAP group, they are shown at 100×magnification. Flow cytometry analysis of Th1 (c) and Th17 (d) cells in the CD4+ T cell proportion of each group in liver lymphocytes.

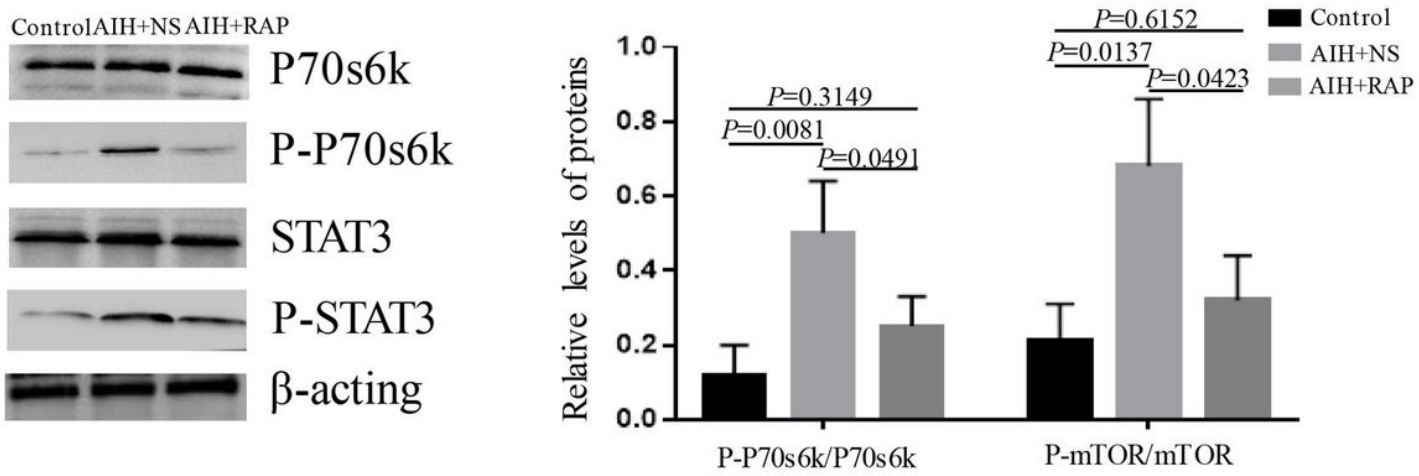

Figure 3

Rapamycinsuppresses p70s6k-STAT3 signaling pathway. After 3 days of AIH model preparation, the AIH+NS and AIH+RAP group received NS and RAP (1 mg/kg/d) treatments.Western bloting showed p-p70s6k/ p70s6k and p-STAT3/STAT3 in control group, AIH+NS group and AIH+RAP group.

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