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Mice lacking adenosine 2A receptor reveal increased severity of MCD-induced NASH

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Abstract

Adenosine 2A receptor (A2A-R) exerts a protective role in obesity-related non-alcoholic fatty liver disease. Here, we examined whether A2A-R protects against non-alcoholic steatohepatitis (NASH). In C57BL/6j mice, feeding a methionine- and choline-deficient diet (MCD) resulted in significant weight loss, overt hepatic steatosis, and massive aggregation of macrophages in the liver compared with mice fed a chow diet. MCD feeding also significantly increased the numbers of A2A-R-positive macrophages/Kupffer cells in liver sections although decreasing A2A-R amount in liver lysates compared with chow diet feeding. Next, MCD-induced NASH phenotype was examined in A2A-R-disrupted mice and control mice. Upon MCD feeding, A2A-R-disrupted mice and control mice displayed comparable decreases in body weight and fat mass. However, MCD-fed A2A-R-disrupted mice revealed greater liver weight and increased severity of hepatic steatosis compared with MCD-fed control mice. Moreover, A2A-R-disrupted mice displayed increased severity of MCD-induced liver inflammation, indicated by massive aggregation of macrophages and increased phosphorylation states of JNK p46 and nuclear factor kappa B (NFκB) p65 and mRNA levels of tumor necrosis factor alpha, interleukin-1 beta, and interleukin-6. In vitro, incubation with MCD-mimicking media increased lipopolysaccharide (LPS)-induced phosphorylation states of JNK p46 and/or NFκB p65 and cytokine mRNAs in control macrophages and RAW264.7 cells, but not primary hepatocytes. Additionally, MCD-mimicking media significantly increased lipopolysaccharide-induced phosphorylation states of p38 and NFκB p65 in A2A-R-deficient macrophages, but insignificantly decreased lipopolysaccharide-induced phosphorylation states of JNK p46 and NFκB p65 in A2A-R-deficient hepatocytes. Collectively, these results suggest that A2A-R disruption exacerbates MCD-induced NASH, which is attributable to, in large part, increased inflammatory responses in macrophages.

Key Words

- adenosine 2A receptor
- non-alcoholic steatohepatitis
- lipodystrophy
- macrophage

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by excessive fat deposition in hepatocytes (steatosis) (Browning et al. 2004, Sanyal 2005). When the liver displays overt inflammatory damage due to fat deposition and inflammatory mediators from extrahepatic tissues, simple steatosis progresses to non-alcoholic steatohepatitis (NASH) as the advanced form of NAFLD (Cohen et al. 2011, Chalasani et al. 2018). Epidemiological data indicate that NASH affects 1.5–6.45% of the general populations (Younossi et al. 2016, Estes et al. 2018). Alarmingly, the incidence of NASH in both adults and children is rising continuously due to ongoing epidemics of obesity (Younossi et al. 2016, Estes et al. 2018). NASH is one of the most common causes of liver cirrhosis and hepatocellular carcinoma (Bugianesi et al. 2002, Marrero et al. 2002, Powell et al. 2005, Starley et al. 2010). To date, there is no effective treatment for NASH (Neuschwander-Tetri 2010, Chalasani et al. 2018).

Because NAFLD is highly prevalent in obese populations (Younossi et al. 2016, Estes et al. 2018), obesity-associated inflammation is accepted as a critical factor that initiates or exacerbates NAFLD. As supported by evidence from both human and animal studies, inflammation can impair hepatic insulin resistance and dysregulate hepatic fat metabolism, which in turn brings about pathological increases in hepatocyte fat deposition (Kang et al. 2008, Odegaard et al. 2008, Menghini et al. 2009). In addition, obesity-associated adipose tissue dysfunction has also been implicated to play a critical role in development of NAFLD. Indeed, this role of dysfunctional adipose tissue is highlighted by the ‘second-hit’ hypothesis. In support of this, adipocyte-specific overexpression of monocyte chemoattractant protein-1 (MCP1), an inflammatory molecule upregulated in adipose tissue of obese mice and human subjects, mediates the effect of adipose tissue inflammation to bring about an increase in hepatic triglyceride content (Kamei et al. 2006). These results and many others suggest that dysfunctional adipose tissue contributes to hepatic steatosis by increasing the delivery of fatty acid flux to the liver (Tilg & Moschen 2010) and by impairing liver insulin signaling through adipose tissue-driven inflammation (Kelley et al. 2003, Schaffer et al. 2005). During obesity, however, inflammation exists in both the liver and adipose tissue (Xu et al. 2014, Guo et al. 2016) and complicates with fat deposition, for example, adiposity and hepatic steatosis. This makes it difficult to separate the effects of inflammation from those of fat deposition. Furthermore, increased adiposity, when displaying fat composition characterized by an elevation of palmitoleate, can promote hepatic steatosis, but decrease liver inflammation (Huo et al. 2012). Given this, there is a critical need to better understand the role of inflammation in regulating NAFLD/NASH in the absence of obesity.

Adenosine 2A receptor (A2AR) is one of the four adenosine receptors that belong to the superfamily of G-protein-coupled receptors and displays powerful anti-inflammatory effects in immune cells such as macrophages and neutrophils (Gessi et al. 2000, Haskó et al. 2008). Previous studies have validated a critical role for A2AR in the pathophysiology of NAFLD/NASH. Specifically, A2AR activation is shown to ameliorate NASH phenotype in both rats and mice (Imarisio et al. 2012, Alchera et al. 2017). In contrast, using A2AR-disrupted mice, Cai et al. provide complementary evidence to support a protective role for A2AR in NAFLD (Cai et al. 2018), although the study by Csoka et al. suggests a contradictory role for A2AR in obesity and NAFLD (Csóka et al. 2017). Of importance, the protective role for A2AR is largely attributed to the effect of A2AR on suppressing inflammation derived from lipotoxicity (Imarisio et al. 2012, Alchera et al. 2017). However, it is not clear in the liver how the A2AR is altered by inflammation in the absence of obesity as it relates to development and progression of NAFLD/NASH. In the present study, we examined the expression of A2AR in livers of mice fed a methionine- and choline-deficient diet (MCD). We also examined the effect of A2AR disruption on MCD-induced NASH phenotype and examined the effects of MCD-mimicking media on the proinflammatory responses in both hepatocytes and macrophages.

Materials and methods

Animal experiments

A2AR-disrupted (A2AR−/− or A2AR+/−) mice, in which A2AR was disrupted in all cells, and their wild-type (WT) littermates (A2AR+/+ mice) were generated as previously described (Cai et al. 2018). Additional C57BL/6J mice were obtained from the Jackson Laboratory. All mice were maintained on a 12:12-h light–darkness cycle (lights on at 06:00). Study 1: male C57BL/6J mice, at 9–10 weeks of age (when body weight was greater than 20 g), were fed an MCD for 5 weeks or maintained on a Chow diet (CD) to examine A2AR abundance in relation to diet-induced NASH. Study 2: both male and female A2AR-disrupted mice and A2AR+/+ mice, at 9–10 weeks of age, were fed an MCD for 5 weeks to induce NASH (Rinella et al. 2008, Luo et al. 2018).
All diets are products of Research Diets, Inc. At 1 day prior to harvest, all mice were subjected to EchoMRI™ analyzer (EchoMRI LLC, Houston, TX, USA) to measure body composition. After the feeding period, all mice were fasted for 4 h before killing for collection of blood and tissue samples as previously described (Guo et al. 2010, Huo et al. 2010, Cai et al. 2018). The levels of plasma alanine aminotransferase (ALT) were measured using an assay kit (BioVision, Inc. Milpitas, CA, USA). These protocols were approved by the Institutional Animal Care and Use Committee of Texas A&M University.

**Histological and immunohistochemical analyses**

The sections of paraffin-embedded liver blocks were stained with H&E and/or stained for F4/80 expression with rabbit anti-F4/80 antibodies (1:100) (AbD Serotec, Raleigh, NC, USA). Also, co-staining of F4/80 (rat anti-mouse, MCA497, Bio-Rad) and A2AR (7F6-G5-A2, Cat# sc-32261, Santa Cruz Biotechnology, Inc.) in liver sections was evaluated by double immunofluorescent labeling according to the manufacturer’s instructions (Vector Laboratories, Inc. Burlingame, CA, USA) as previously described (Pei et al. 2018). Following staining, images were obtained using Leica TCS SPE Confocal Microscope System. The sections of frozen livers were stained with Oil Red O as previously described (Guo et al. 2016).

**Cell culture and treatment**

Primary mouse hepatocytes were isolated from male A2AR−/− mice and A2AR+/+ mice, at 11–12 weeks of age, using a collagenase digestion method as previously described (Cai et al. 2018, Luo et al. 2018). After attachment, hepatocytes were further incubated in M199 supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 μg/mL streptomycin for 24 h. Thereafter, hepatocytes were incubated in MCD-mimicking media (US Biological Life Sciences, Salem, MA, USA) for an additional 24 h. Prior to harvest, hepatocytes were treated with or without lipopolysaccharide (LPS, 100 ng/mL) for 30 min. Cell lysates were prepared for Western blot analysis. Also, bone marrow cells were isolated from male A2AR−/− mice and A2AR+/+ mice, at 11–12 weeks of age, and differentiated into macrophages (BMDM) as previously described (Xu et al. 2014). After differentiation, BMDM were incubated with MCD-mimicking media for 24 h and treated with or without LPS (100 ng/mL) for the last 30 min. Cell lysates were measured for proinflammatory signaling using Western blot analysis. For a confirmatory study, RAW264.7 cells were treated with MCD-mimicking media and/or control media, and assayed for proinflammatory signaling and cytokine expression same as BMDM.

**Western blot analysis**

Frozen liver tissues and cultured cells were prepared in a lysis buffer containing 50 mM HEPES (pH 7.4), 10 mM EDTA, 50 mM sodium pyrophosphate, 0.1 M sodium fluoride, 10 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 2 mM benzamidine, and 1% Triton X-100. After protein electrophoresis and transfer, immunoblots were performed using rabbit anti-serum as primary antibody at a 1:1000 dilution. This dilution was used for each of the primary antibodies used for the present study. After washing, the blot was incubated with a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and followed by a chemiluminescent kit (Immobilon™ Western; EMD Millipore) as described (Qi et al. 2017). GAPDH was used as a loading control. The maximum intensity of each band was quantified using ImageJ software. Protein amount of αSMA and/or A2AR was normalized to GAPDH and adjusted relative to the average of CD-fed mice. Similarly, ratios of Pp46/p46 and/or Pp65/65 were normalized to GAPDH and adjusted relative to the average of CD-fed WT mice, MCD-fed A2AR−/− mice, or control medium-treated primary hepatocytes or BMDM from A2AR−/− mice, which was arbitrarily set as 1 (AU). Antibodies against Pp46, p46, Pp65, p65, αSMA, and A2AR were products of Cell Signaling.

**RNA isolation, reverse transcription, and real-time PCR**

Total RNA was isolated from liver tissues. Reverse transcription was performed using the GoScript™ Reverse Transcription System (Promega) and real-time PCR analysis was performed using SYBR Green (LightCycler® 480 system; Roche) (Guo et al. 2012, 2013). The mRNA levels were analyzed for tumor necrosis factor alpha (TNfa), interleukin 1 beta (Il1β), Il6, fatty acid synthase (Fas), carnitine palmitoyltransferase 1a (Cpt1a), and sterol regulatory element-binding protein 1c (Srebp1c). A total of 0.1 μg RNA was used for the determination. Results were normalized to 18s ribosomal RNA and plotted as relative expression to the average of MCD-fed A2AR−/− mice, which was set as 1. Primer sequences are available upon request.
Statistical methods

Numeric data are presented as means±s.e.m. (standard error). Statistical significance was assessed by unpaired, two-way ANOVA (for comparisons including three or more groups) and/or two-tailed Student’s t tests (for variables only involving two groups). Differences were considered significant at the two-tailed P<0.05. Tukey’s range test was used as a post hoc test.

Results

MCD feeding induces lipoatrophy and severe NASH in mice

Feeding a high-fat diet (HFD) to C57BL/6J mice induces hepatic steatosis and inflammation that are associated with obesity and increased adiposity (Cai et al. 2018, Luo et al. 2018). In this type of mouse model, dysfunctional adipose tissue is thought to also contribute to NAFLD phenotype. Considering the existence of steatohepatitis in human subjects without obesity, we sought to feed male C57BL/6J mice an MCD and examined hepatic steatosis and inflammation in the presence of lipoatrophy. Compared with CD-fed male mice, MCD-fed male C57BL/6J mice revealed significant decreases in food intake and body weight (Fig. 1A and B). Additionally, MCD-fed mice displayed nearly no fat mass (Fig. 1C). When NASH phenotype was analyzed, the levels of plasma alanine aminotransferase (ALT) in MCD-fed mice were significantly increased compared with those in CD-fed mice (Fig. 1D). In contrast, MCD-fed mice revealed significantly decreased liver weight relative to CD-fed mice (Fig. 1E). Consistent with NASH phenotype, liver sections of MCD-fed mice displayed overt hepatic steatosis and revealed mass aggregation of F4/80+ macrophages (Fig. 1F). In addition, liver lysates of MCD-fed mice showed significant increases in the phosphorylation states of JNK 1 p46 and the amount of αSMA, a marker of liver fibrosis (Fig. 1G). These results validate MCD-fed mice as a model of NASH, which reveals hepatic steatosis and severe inflammation in the presence of lipoatrophy.

Figure 1

MCD feeding induces severe hepatic steatosis and inflammation while causing lipoatrophy. Male C57BL/6J mice, at 9–10 weeks of age, were fed a methionine- and choline-deficient (MCD) for 5 weeks or maintained on a chow diet (CD). (A and B) Body weight was measured during the feeding period (A). Also, foods consumed by the mice were recorded during the feeding period and used to calculate food intake (B). (C) At 1 day prior to the end of feeding period, body composition of the mice was analyzed using an EchoMRI analyzer. (D) Plasma levels of alanine aminotransferase (ALT). (E) Liver weight was measured after harvest of mice. (F) Liver sections were stained with H&E or for F4/80 expression. (G) Liver lysates were examined for the phosphorylation states of JNK 1 p46 and the amount of αSMA. Bar graphs, quantification of blots. For A, B, C, D, E and G, numeric data are means±s.e.m. (standard error), n=8–10. AU, arbitrary unit. *P<0.05 and **P<0.01 MCD vs CD (in D, E, and G) for the same time point (in A and B) or for the same type of mass (in C). A full colour version of this figure is available at https://doi.org/10.1530/JOE-19-0198.
MCD feeding induces NASH and alters hepatic A<sub>2A</sub>R expression in mice

We showed before that hepatic A<sub>2A</sub>R expression was increased in mice with obesity-associated NAFLD and validated the increase in A<sub>2A</sub>R expression as a defensive response (Cai et al. 2018). Here we examined hepatic A<sub>2A</sub>R expression in MCD-fed mice. Unlike the changes in HFD-fed mice, the amount of A<sub>2A</sub>R in liver lysates of MCD-fed C57BL/6 mice was decreased compared with that in CD-fed mice (Fig. 2A). Next, we used immunofluorescent staining to examine the expression pattern of A<sub>2A</sub>R in liver macrophages/Kupffer cells; given that liver sections of MCD-fed C57BL/6j mice displayed marked aggregation of F4/80<sup>+</sup> cells. Compared with those of CD-fed mice, liver sections of MCD-fed mice contained much more A<sub>2A</sub>R-positive cells (Fig. 2B), most of which were F4/80<sup>+</sup> cells (macrophages/Kupffer cells). Together, these results suggest that during MCD-induced NASH the liver revealed increased A<sub>2A</sub>R expression in macrophages although A<sub>2A</sub>R expression was decreased in liver lysates.

Validation of A<sub>2A</sub>R disruption in livers of MCD-fed mice

MCD feeding altered liver A<sub>2A</sub>R expression. Next, we sought to use A<sub>2A</sub>R-disrupted mice to examine whether A<sub>2A</sub>R exerts a protective role in the pathogenesis of MCD-induced NASH. Initially, we analyzed A<sub>2A</sub>R expression in liver sections of MCD-fed male A<sub>2A</sub>R<sup>−/−</sup> mice and their wild-type (WT, A<sub>2A</sub>R<sup>+/+</sup>) littermates. Upon immunofluorescent staining, liver sections of MCD-fed male A<sub>2A</sub>R<sup>+/+</sup> mice displayed significant amount of A<sub>2A</sub>R-positive cells, many of which were F4/80<sup>+</sup> macrophages/Kupffer cells. In contrast, liver sections of MCD-fed male A<sub>2A</sub>R<sup>−/−</sup> mice contained almost no A<sub>2A</sub>R-positive cells, but revealed significant increases in the numbers of F4/80-positive cells compared with those of MCD-fed male A<sub>2A</sub>R<sup>+/+</sup> mice (Fig. 3). These results validated A<sub>2A</sub>R deficiency and confirmed that MCD feeding induced mass macrophage aggregation.

A<sub>2A</sub>R disruption does not alter the effect of MCD feeding on decreasing body weight and abdominal fat mass

A<sub>2A</sub>R disruption exacerbates HFD-induced weight gain (Cai et al. 2018). However, it is not clear whether A<sub>2A</sub>R disruption influences MCD-induced weight loss. We measured body weight and analyzed body composition of MCD-fed male and female A<sub>2A</sub>R-disrupted mice and their WT littermates. During and after MCD feeding period, all mice revealed significant decreases in body weight regardless of A<sub>2A</sub>R disruption and sex of the mice. Also, all mice started to consume fewer foods after MCD feeding.
for 2 weeks. However, these changes in A2AR-disrupted mice were comparable with those in WT littermates (Fig. 4A and B, data from male mice only). In addition, abdominal fat mass and adiposity in A2AR-disrupted mice did not differ significantly from those in WT littermates (Fig. 4C and D). Specifically, epididymal fat mass was 0.079 ± 0.009 g for MCD-A2AR−/− mice, 0.078 ± 0.0064 g for MCD-A2AR+/− mice, and 0.061 ± 0.006 g for MCD-A2AR+/+ mice (data from male mice only). These results suggest that A2AR disruption did not significantly alter MCD-induced decreases in body weight and abdominal fat mass.

**A2AR disruption exacerbates MCD-induced hepatic steatosis and inflammation**

MCD feeding induces NASH while decreasing liver weight. In the present study, the levels of plasma ALT in MCD-fed male A2AR−/− mice were significantly higher than those in MCD-fed male A2AR+/+ mice (Fig. 5A). Also, liver weight of A2AR-disrupted male mice was greater than that in WT male mice (Fig. 5B), although all mice revealed significant decreases in liver weight relative to CD-fed mice. Compared with male A2AR+/+ mice, male A2AR−/− mice also revealed a significant increase in the severity of MCD-induced hepatic steatosis, indicated by the results from liver sections stained with H&E and/or Oil Red O (Fig. 5C). Furthermore, the severity of MCD-induced hepatic steatosis in male A2AR−/− mice was greater than in male A2AR+/+ mice. Male A2AR−/− mice also displayed a significant increase in accumulation of liver macrophages/Kupffer cells. This increase in male A2AR−/− mice was much greater than in male A2AR+/+ mice. Of note, macrophages/Kupffer cells were aggregated in large groups in livers of MCD-fed male A2AR+/− mice. When inflammatory signaling was analyzed, the phosphorylation states of JNK p46 and NFκB p65 were increased in MCD-fed male A2AR−/− or A2AR+/− mice compared with MCD-fed male A2AR+/+ mice (Fig. 5D). Additionally, MCD-fed male A2AR−/− or A2AR+/− mice displayed significant increases in Tnfα, Il1β, and Il6 mRNAs (Fig. 5E). Next, we analyzed the expression of lipogenic genes/enzymes and observed that hepatic Acc1, Fas, and Srebp1c mRNAs in MCD-fed male A2AR−/− mice were significantly higher than their respective levels in MCD-fed male A2AR+/+ mice, whereas Cpt1a mRNAs in MCD-fed male A2AR−/− or A2AR+/+ mice did not differ from those in MCD-fed male A2AR+/+ mice (Fig. 5E). These results suggest that A2AR disruption exacerbates NASH phenotype in MCD-fed male mice. In female mice, we observed similar phenotype upon MCD feeding (data not shown).

**A2AR-disrupted macrophages, but not hepatocytes, reveal increased proinflammatory responses upon incubation with MCD-mimicking media**

A2AR deficiency exacerbated MCD-induced liver inflammation. Considering that A2AR-deficient mice lacked A2AR in both hepatocytes and macrophages, we sought to determine the direct effects of MCD-mimicking media on the proinflammatory responses of A2AR-deficient hepatocytes and/or macrophages. Upon incubation with MCD-mimicking media, primary hepatocytes from either A2AR−/− mice or A2AR+/+ mice did not reveal significant changes in LPS-induced phosphorylation states of JNK1 p46 and NFκB p65 compared with hepatocytes incubated with control media (Fig. 6A), suggesting that MCD-mimicking media have limited effects on altering hepatocyte proinflammatory responses regardless of the presence or absence of A2AR. In contrast, incubation with MCD-mimicking media caused a significant increase in LPS-induced phosphorylation states of NFκB p65 in A2AR−/−-BMDM (Fig. 6B) relative to control media. Moreover, upon incubation with MCD-mimicking media, A2AR−/−-BMDM revealed significant increases in...
LPS-induced phosphorylation states of p38 and NFκB p65 compared with A2AR+/−-BMDM (Fig. 6B). Since MCD increased NFκB p65 phosphorylation in WT BMDM but not A2AR−/− BMDM, we next measured cytokine expression in WT BMDM incubated with MCD-mimicking media or control media. Compared with control, MCD-mimicking media significantly increased Tnfα, Il1b, and Il6 mRNAs (Fig. 6C). In a confirmatory study involving RAW264.7 cells, incubation with MCD-mimicking media caused significant increases in the phosphorylation states of JNK p46 and/or NFκB p65 relative to control media under both basal and LPS-stimulated conditions (Fig. 6D). Additionally, Tnfα, Il1b, and Il6 mRNAs in MCD-mimicking media-treated RAW264.7 cells were significantly higher than their respective levels in control media-treated RAW264.7 cells (Fig. 6E). These results suggest that MCD-mimicking media enhance the proinflammatory responses in macrophages, but not hepatocytes.

**Discussion**

The prevalence of NAFLD is markedly increased among obese subjects due to, in large part, adiposity-associated overflow of fats and adipokines/cytokines to the liver to trigger or exacerbate hepatic steatosis and inflammation (Nugent & Younossi 2007, van der Poorten et al. 2008, Estes et al. 2018). Interestingly, there also is a good amount of evidence suggesting the presence of NAFLD/NASH in lean subjects (Feldman et al. 2017, Kim et al. 2019), in which genetic factors, but not obesity-related factors, are thought to contribute to the pathogenesis of
NAFLD/NASH. Similarly, the prevalence of NAFLD/NASH or even liver fibrosis is also increased among HIV-infected patients who have normal BMI or lipodystrophy (Pérez-Matute et al. 2013, van Welzen et al. 2019). In this case, HIV infection-related inflammation is considered as a key factor to drive the pathogenesis of NAFLD/NASH or liver fibrosis, although the precise underlying mechanisms remain to be elucidated. To date, there is a lack of a perfect animal model for studying NAFLD pathophysiology in the absence of obesity or in the presence of lipodystrophy. Because feeding an MCD to C57BL/6 mice causes severe hepatic steatosis and inflammation while causing lipoatrophy, we analyzed changes in hepatic expression of A2aR in MCD-fed mice and examined the effect of A2aR disruption on MCD-induced NASH phenotype.

Similar to HFD-fed C57BL/6j mice (Cai et al. 2018), MCD-fed C57BL/6j mice displayed hepatic steatosis relative to chow diet-fed mice. However, the severity of liver inflammation in MCD-fed C57BL/6j mice was much greater than that in HFD-fed C57BL/6j mice, which is consistent with our previous report (Luo et al. 2018). In particular, liver sections of MCD-fed C57BL/6j mice contained significantly more F4/80-positive cells, many of which were aggregated. In addition, MCD-fed C57BL/6j mice contained almost no fat mass, in particular visceral fat mass. These characteristics not only validated MCD-fed C57BL/6j mice as a model for studying NASH in the presence of lipoatrophy, but also indicated that MCD-fed mice developed liver inflammation via mechanism(s) different from those for HFD-fed mice. As supporting evidence, hepatic expression of A2aR, an ant-inflammatory molecule, was differentially regulated in MCD-fed mice compared with HFD-fed mice. Specifically, A2aR abundance in liver lysates, unlike that in liver lysates of HFD-C57BL/6j fed mice (Cai et al. 2018), was decreased in MCD-fed C57BL/6j mice. Upon examining A2aR expression using immunofluorescence staining, we showed that liver sections of MCD-fed mice, indeed, contained significant more A2aR-positive cells than those of CD-fed mice. Because most A2aR-positive cells were F4/80-positive cells, we argued that MCD feeding increased A2aR expression in macrophages, although it decreased whole liver A2aR abundance. The latter may be attributable to decreased A2aR expression in hepatocytes, which is opposite to that in HFD-fed mice where A2aR expression was increased in hepatocytes (Cai et al. 2018). Considering that A2aR exerts powerful anti-inflammatory effects, we speculated that the A2aR in macrophages could play a more important role than the A2aR in hepatocyte in terms of regulating liver inflammation in MCD-fed mice.
This concept was substantiated by the differences in the responses of hepatocytes versus macrophages to culture media mimicking MCD (see below).

Activation of A2AR by an agonist has been shown to ameliorate MCD-induced NASH phenotype in both rats and mice (Imarisio et al. 2012, Alchera et al. 2017). Considering this, we speculated that the decrease in A2AR amount in liver lysates of MCD-fed mice was indicative of reduced protection of NASH phenotype, although A2AR expression was increased in macrophages/Kupffer cells of liver sections from MCD-fed mice. In agreement with our speculation, A2AR-deficient mice revealed significant increases in the severity of MCD-induced hepatic steatosis and inflammation compared with WT control mice. Of note, the severity of hepatic steatosis in MCD-fed homozygous A2AR-deficient mice, indicated by the results of liver histology, was significantly greater than that in MCD-fed heterozygous A2AR-disrupted mice. Moreover, the degrees of liver inflammation in MCD-fed homozygous A2AR-deficient mice, indicated by the numbers of F4/80-positive macrophages/Kupffer cells, the phosphorylation states of NF-kB p65, and the mRNA levels of Il1b and Il6, in livers from MCD-fed homozygous A2AR-deficient mice were also significantly higher than their respective levels in MCD-fed heterozygous A2AR-disrupted mice. These findings, along with the results indicative of increased severity of NASH phenotype in heterozygous A2AR-deficient mice relative to that in WT mice, strongly suggest that A2AR exerts a gene-dose-dependent effect on protecting against MCD-induced NASH phenotype. What should be noted is that A2AR exerts a similarly gene-dose-dependent effect on protecting against NAFLD phenotype in obese mice (Cai et al. 2018), where inflammation, but not adiposity, is the characteristic shared by MCD-fed mice. Therefore, the anti-inflammatory effect of A2AR likely accounts for, to a large extent, protection of NASH in MCD-fed mice.

In MCD-fed mice, A2AR expression was increased in liver macrophages/Kupffer cells. This increase appeared to a defensive response and was different from the A2AR in non-macrophages/Kupffer cells where decreased A2AR expression might be a consequence or adaptive response to MCD feeding. In other words, it is possible that upon MCD feeding macrophage A2AR expression was increased to counter against massive inflammation whereas the A2AR in other cells, mainly, hepatocytes, was decreased and contributed to increases in hepatocyte fat deposition and proinflammatory responses. At this point, the mechanisms by which MCD feeding differentially regulates A2AR expression in macrophages/Kupffer cells and other cells, for example, hepatocytes, are not clear. However, MCD feeding appears to primarily account for decreasing hepatocyte A2AR expression; considering that HFD feeding increases hepatocyte A2AR expression (Cai et al. 2018). To be noted, methionine and choline are key nutrients involved in methionine cycle and methylation reactions. Given this, alterations of hepatocyte methylation reactions in response to deficiency of methionine and choline in diet likely are MCD-induced upstream events of hepatocyte A2AR expression. This view, however, is in need of validation by future study. Nonetheless, the differential expression of A2AR in different liver cells led us to examine the effect of MCD-mimicking media on the direct responses of macrophages versus hepatocytes. Indeed, we validated that MCD-mimicking media generated different effects on proinflammatory responses in the two types of cells. Notably, MCD-mimicking media increased LPS-induced phosphorylation states of Nfkb p65 in WT macrophages and insignificant increases in the phosphorylation states of p38 and Nfkb p65 in A2AR-deficient macrophages. In contrast, MCD-mimicking media did not significantly alter or even tended to decrease LPS-induced phosphorylation states of Jnk p46 and/or Nfkb p65 in both WT and A2AR-deficient hepatocytes. Because of this, we speculated that the A2AR in macrophages directly responded to MCD feeding in a defensive manner to exert an anti-inflammatory effect. However, future study is needed to examine the extent to which A2AR disruption only in myeloid cells also protects against MCD-induced NASH phenotype.

In summary, we validated that A2AR expression was increased in liver macrophages/Kupffer cells, but was decreased in liver lysates of MCD-fed WT mice. We then demonstrated a protective role for A2AR in MCD-induced NASH phenotype. At the cellular level, the A2AR in macrophages appears to be more important than that in hepatocytes in terms of suppressing the effect of MCD or MCD-mimicking media on stimulating the proinflammatory responses. Therefore, we provide additional evidence supporting the potential of targeting A2AR to suppress liver inflammation as a therapeutic strategy for the treatment of NASH.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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Author contribution statement
J Z carried out most of animal experiments. H L carried out all histological and immunohchemical assays. Y Cai, L M, D M, B L, B Z, and L Z collected tissue and cell samples, performed molecular and biochemical assays, and analyzed the data. C W came up the concept of the study. Y Chen, X Q, X X, Qifu L, S G, Y H, L Z, Y T, and Qingsheng L contributed to scientific discussion. C W supervised all experiments and wrote the manuscript.

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