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## Regulation of Adipose Tissue Inflammation by Adenosine 2A Receptor in Obese Mice

Ya Pei<sup>#1</sup>, Honggui Li<sup>#1</sup>, Yuli Cai<sup>#1,2</sup>, Jing Zhou<sup>1</sup>, Xianjun Luo<sup>1</sup>, Linqiang Ma<sup>1</sup>, Kelly McDaniel<sup>3</sup>, Tianshu Zeng<sup>4</sup>, Yanming Chen<sup>5</sup>, Xiaoxian Qian<sup>6</sup>, Yuqing Huo<sup>7,8</sup>, Shannon Glaser<sup>3</sup>, Fanyin Meng<sup>3</sup>, Gianfranco Alpini<sup>3</sup>, Lulu Chen<sup>4,†</sup>, and Chaodong Wu<sup>1,†</sup>

<sup>1</sup>Department of Nutrition and Food Science, Texas A&M University, College Station, TX 77843, USA

<sup>2</sup>Department of Endocrinology, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, China

<sup>3</sup>Research, Central Texas Veterans Health Care System, Baylor Scott & White Digestive Disease Research Center, Baylor Scott & White Health, Department of Medical Physiology, Texas A&M University College of Medicine, Temple, TX 76504

<sup>4</sup>Department of Endocrinology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430022, China

<sup>5</sup>Department of Endocrinology, the Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong 510630, China

<sup>6</sup>Department of Cardiology, the Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong 510630, China

<sup>7</sup>Vascular Biology Center, Department of Cellular Biology and Anatomy, Medical College of Georgia, Augusta University, Augusta, GA 30912, USA

<sup>8</sup>Drug Discovery Center, Key Laboratory of Chemical Genomics, Peking University Shenzhen Graduate School, Shenzhen 518055, China

# These authors contributed equally to this work.

### Abstract

Adenosine 2A receptor (A<sub>2A</sub>R) exerts anti-inflammatory effects. However, the role for A<sub>2A</sub>R in obesity-associated adipose tissue inflammation remains to be elucidated. The present study examined the expression of A<sub>2A</sub>R in adipose tissue of mice with diet-induced obesity, and determined the effect of A<sub>2A</sub>R disruption on the status of obesity-associated adipose tissue

<sup>†</sup>Correspondence: Chaodong Wu, MD, PhD, College Station, TX 77843, cdwu@tamu.edu; or Lulu Chen, MD, PhD, Wuhan, Hubei 430022, China, cheria\_chen@126.com.

#### AUTHORS' CONTRIBUTION

Y.P., Y. Cai, and C.W. designed the research; Y.P., Y. Cai, H.L., J.Z., X.L. L.M., and K.M. conducted both animal and cell experiments; Y.P., Y. Cai, H.L., and C.W. analyzed the data and wrote the manuscript. T.Z., Y. Chen, X.Q., Y.H., S.G., F.M., G.A., and L.C. were involved in scientific discussion. S.G., F.M., and G.A. critically reviewed and edited the manuscript. C.W. and L.C. had primary responsibility for the final content. All authors read and approved the final manuscript.

#### DECLARATION of INTEREST

The authors declare that there is no competing financial interest.

inflammation. Wild-type C57BL/6J mice and A<sub>2A</sub>R-disrupted mice were fed a high-fat diet (HFD) for 12 weeks to induce obesity and adipose tissue inflammation. *In vitro*, bone marrow-derived macrophages from A<sub>2A</sub>R-disrupted mice and wild-type control mice were treated with palmitate and examined for macrophage proinflammatory activation. Compared with that of low-fat diet (LFD)-fed wild-type mice, A<sub>2A</sub>R expression in adipose tissue of HFD-fed wild-type mice was increased significantly and was present predominantly in adipose tissue macrophages. The increase in adipose tissue A<sub>2A</sub>R expression in HFD-fed mice was accompanied with increased phosphorylation states of c-Jun N-terminal kinase 1 p46 and nuclear factor kappa B p65 and mRNA levels of interleukin (*Il*)-1beta, *Il6*, and tumor necrosis factor alpha. In A<sub>2A</sub>R-disrupted mice, HFD feeding induced significant increases in adipose tissue inflammation, indicated by enhanced proinflammatory signaling and increased proinflammatory cytokine expression, and adipose tissue insulin resistance, indicated by a decrease in insulin-stimulated Akt phosphorylation relative to those in wild-type mice. Lastly, A<sub>2A</sub>R disruption enhanced palmitate-induced macrophage proinflammatory activation. Taken together, these results suggest that A<sub>2A</sub>R plays a protective role in obesity-associated adipose tissue inflammation, which is attributable to, in large part, A<sub>2A</sub>R suppression of macrophage proinflammatory activation.

### Keywords

adenosine 2A receptor; obesity; adipose tissue inflammation; macrophage

## INTRODUCTION

Obesity is an ongoing pandemic and critically contributes to the development and progression of a wide variety of metabolic diseases such as type 2 diabetes, non-alcoholic fatty liver disease, and cardiovascular disease. Over the past decade, much evidence has demonstrated the persistence of low-grade inflammation in adipose tissue during obesity (Lumeng, et al. 2007; Weisberg, et al. 2003; Xu, et al. 2003), and validated adipose tissue inflammation as a critical factor that causes insulin resistance and glucose and fat metabolic dysregulation locally in adipose tissue and distally in key metabolic tissues such as the liver and skeletal muscle (Berg, et al. 2001; Boyle, et al. 2011; Cheung, et al. 2000; Hotamisligil, et al. 1996; Huo, et al. 2010; Huo, et al. 2012; Kabir, et al. 2005; Kamei, et al. 2006; Trujillo and Scherer 2006; Ye, et al. 2007). Given this, a better understanding of adipose tissue inflammation is essential for the development of new and effective approaches for treatment and/or prevention of obesity-associated metabolic diseases.

Since the discovery of macrophage infiltration in adipose tissue of mice with diet-induced obesity (DIO) (Weisberg et al. 2003; Xu et al. 2003), a significant number of studies have investigated how adipose tissue cells, mainly adipocytes and macrophages, regulate the development of adipose tissue inflammation. Using genetically modified mouse models, many researchers have shown that genes/proteins varying from signaling molecules to metabolic and regulatory enzymes (Choe, et al. 2014; Han, et al. 2013; Huo et al. 2010; Huo et al. 2012; Jiao, et al. 2012; Kamei et al. 2006; Kumari, et al. 2016; Menghini, et al. 2009; Nomiya, et al. 2007; Odegaard, et al. 2007; Saberi, et al. 2009; Solinas, et al. 2007), and even the players of circadian core loop (Xu, et al. 2014) function to either promote or inhibit

the development and progression of adipose tissue inflammation. Of note, signaling molecules have drawn particular attention. For instance, signaling molecules such as c-Jun N-terminal kinase 1 (JNK1) and interferon regulatory transcription factor 3 (IRF3) have been validated to play detrimental roles in adipose tissue inflammation (Kumari et al. 2016; Solinas et al. 2007). There is also evidence suggesting the importance of cell surface receptors such as Toll-like receptor 4 (TLR4) and G-protein coupled receptor 120 (GPR120) in the regulation of the pathogenesis of adipose tissue inflammation (Oh, et al. 2010; Saberi et al. 2009). However, it remains to be elucidated how nutrition stress regulates cell surface receptors in the context of altering the development and progression of adipose tissue inflammation.

Adenosine receptors (AR) belong to superfamily of G-protein-coupled receptors. To date,  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$  and  $A_3$  are the four AR that have been validated to mediate various physiological functions of adenosine (Haskó, et al. 2008). Among the four AR,  $A_{2A}R$  exerts powerful anti-inflammatory effects in immune cells such as macrophages and neutrophils (Gessi, et al. 2000; Haskó et al. 2008). Recent studies have also demonstrated that  $A_{2A}R$  activation exhibits anti-inflammatory effects in cultured hepatocytes, and in rodent models of inflammatory liver diseases (Alchera, et al. 2017; Imarisio, et al. 2012). In contrast, using  $A_{2A}R$ -disrupted mice, Cai et al. provide complementary evidence to support a protective role for  $A_{2A}R$  in inflammatory liver disease (Cai, et al. 2018). Considering that  $A_{2A}R$  critically regulates the proinflammatory activation of macrophages that determine adipose tissue inflammation, investigators have postulated  $A_{2A}R$  as an essential regulator of adipose tissue inflammation. Indeed, a recent study has suggested a role for  $A_{2A}R$  activation in decreasing macrophage infiltration in adipose tissue of DIO mice (DeOliveira, et al. 2017). However, it remains unknown about the effect of nutrition stress, i.e., high-fat diet (HFD) feeding, on altering adipose tissue  $A_{2A}R$  expression as it relates to the regulation of adipose tissue inflammation. The present study provides primary evidence to support a protective role for  $A_{2A}R$  in obesity-associated adipose tissue inflammation. In addition,  $A_{2A}R$  regulates how macrophages respond to palmitate, a major macronutrient accounting for obesity and related diseases.

## MATERIALS and METHODS

### Animal experiments

Wild-type (WT) C57BL/6J were obtained from Jackson Laboratory (Bar Harbor, ME).  $A_{2A}R$ -disrupted ( $A_{2A}R^{-/-}$  or  $A_{2A}R^{+/-}$ ) mice and their WT littermates ( $A_{2A}R^{+/+}$  mice) were generated by breeding male  $A_{2A}R^{+/-}$  mice with female  $A_{2A}R^{+/-}$  mice (all in C57BL/6J background) as described (Chen, et al. 1999). After weaning, offspring were ear-tagged, and subjected to collection of tail samples for genotyping. Genomic DNA was prepared following manufacturer's instruction (Extract-N-Amp™ Tissue PCR Kit, Sigma-Aldrich, St. Louis, MO). PCR was performed according to a standard procedure. PCR products were loaded in a 2% agarose gel for visualization. Primers are forward: 5'-GGGCTCCTCGGTGTACA, and reverse: 5'-CCCACAGATCTAGCCTTA. All mice were housed on 12:12-h light-dark cycles (light on at 06:00). **Study 1:** male WT C57BL/6J mice, at 5 - 6 weeks of age, were fed an HFD (60% fat calories, 20% protein calories, and 20%

carbohydrate calories) or low-fat diet (LFD, 10% fat calories, 20% protein calories, and 70% carbohydrate calories) for 12 weeks as described previously (Cai et al. 2018; Huo et al. 2010; Xu et al. 2014) to examine A<sub>2A</sub>R abundance in relation to diet-induced adipose tissue inflammation. Additional age-matched male WT mice were maintained on a standard chow diet (CD) and served as an additional control. All diets (D12492, D12450J, and D10001) were products from Research Diets, Inc. (New Brunswick, NJ, USA). **Study 2:** male A<sub>2A</sub>R<sup>-/-</sup>, A<sub>2A</sub>R<sup>+/-</sup>, and A<sub>2A</sub>R<sup>+/+</sup> mice, at 5 - 6 weeks of age, were fed an HFD for 12 weeks to analyze A<sub>2A</sub>R regulation of adipose tissue inflammation. Some mice in Study 2 were subjected to the Promethion™ system (Sable Systems International, North Las Vegas, NV) to measure energy metabolism. All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

### Measurement of plasma glucose levels

After the feeding period, the mice of both studies were fasted for 4 hr prior to collection of blood samples. Briefly, the fasted-mice were anesthetized by ketamine/xylazine. Under anesthesia, mice were subjected to heart puncture to collect blood, which was transferred to a heparinized tube. After centrifugation, plasma was collected and assayed for the levels of glucose using a metabolic assay kit (Sigma, St. Louis, MO).

### Histological and immunohistochemical analyses

Paraffin-embedded adipose tissue (epididymal fat) blocks were cut into sections of 5 μm thickness. The sections of adipose tissue from Study 1 were stained for A<sub>2A</sub>R expression using mouse monoclonal antibodies against A<sub>2A</sub>R (7F6-G5-A2, Cat# sc-32261, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Additional sections of adipose tissue from study 1, along with those from Study 2, were stained with H&E and for F4/80 expression with rabbit anti-F4/80 antibodies (1:100) (AbD Serotec, Raleigh, NC). Also, the co-staining of F4/80 (rat anti-mouse, MCA497, Bio-Rad, Hercules, CA) and A<sub>2A</sub>R in adipose tissue sections was evaluated by double immunofluorescent labeling according to the manufacturer's instructions (Vector Laboratories, Inc. Burlingame, CA). Following staining, images were obtained using Leica TCS SPE Confocal Microscope System (Buffalo Grove, IL).

### Adipose tissue stromal vascular cells and flow cytometry analysis

Adipose tissue stromal vascular cells (SVC) were isolated from epididymal fat depots using the collagenase digestion method (Lumeng et al. 2007; Stienstra, et al. 2008). The isolated SVC were subjected to FACS analyses (Wentworth, et al. 2010; Xu et al. 2014). Briefly, adipose tissue SVC were stained with fluorescence-tagged antibodies: anti-F4/80, anti-CD11b for macrophages, and anti-CD11c and anti-CD206 for macrophage activation, and analyzed using BD Accuri™ C6 Plus flow cytometer (BD Biosciences, San Jose, California, USA). Initially, SVC were analyzed based on FSC-A and SSC-A. Living cells were then examined for F4/80 (FITC) and CD11b (APC) expression. Mature macrophages (F4/80<sup>+</sup> CD11b<sup>+</sup> cells) were then gated for CD11c (PE/Cy7) and CD206 (PE) expression (macrophage polarization). Mature macrophages that were positive for CD11c but negative for CD206 were considered as proinflammatory (M1) macrophages (F4/80<sup>+</sup> CD11b<sup>+</sup> CD11c

<sup>+</sup> CD206<sup>-</sup> cells) whereas CD11c<sup>-</sup> CD206<sup>+</sup> mature macrophages were considered as alternatively activated (M2) macrophages (F4/80<sup>+</sup> CD11b<sup>+</sup> CD11c<sup>-</sup> CD206<sup>+</sup> cells).

### Cell culture and treatment

Bone marrow cells were isolated from male A<sub>2A</sub>R<sup>-/-</sup> and A<sub>2A</sub>R<sup>+/+</sup> mice and differentiated into macrophages (BMDM) as described (Xu et al. 2014). To examine the extent to which A<sub>2A</sub>R disruption alters the effect of palmitate on macrophage proinflammatory activation, both WT and A<sub>2A</sub>R<sup>-/-</sup> BMDM were incubated with media in the presence or absence of palmitate (250 μM, conjugated in bovine serum albumin (BSA)) or BSA for 24 hr. Cell lysates were examined for proinflammatory signaling using Western blot analysis.

### Western blot analysis

Frozen adipose tissue (epididymal fat) 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 1:1,000 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, Billerica, MA, USA) as described (Qi, et al. 2017). GAPDH was used as a loading control. The maximum intensity of each band was quantified using ImageJ software. Ratios of Pp46/p46, Pp65/65, or P-Akt/Akt were normalized to GAPDH and adjusted relative to the average of LFD- or CD-fed mice, HFD-fed A<sub>2A</sub>R<sup>+/+</sup> mice, phosphate-buffered saline (PBS)-treated A<sub>2A</sub>R<sup>+/+</sup> mice, or BSA-treated A<sub>2A</sub>R<sup>+/+</sup> cells, which was arbitrarily set as 1 (AU). Antibodies against Pp46, p46, Pp65, p65, and hormone-sensitive lipase (HSL), P-HSL were products of Cell Signaling (Danvers, MA, USA). Antibodies against P-Akt and Akt and anti-rabbit IgG antisera were products of Santa Cruz Biotechnology, Inc.

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

Total RNA was isolated from adipose tissue (epididymal fat) and cultured macrophages. 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. 2013; Guo, et al. 2012). The mRNA levels were analyzed for tumor necrosis factor alpha (*Tnfα*), interleukin 1 beta (*Ilb*), *Il6*, *Il10*, arginase 1, A<sub>2A</sub>R (*Adora2a*), peroxisome proliferator-activated receptor gamma (*Pparg*), macrophage chemoattractant protein 1 (*Mcp1*), adiponectin, and *Hsl*. 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 LFD-fed mice or HFD-A<sub>2A</sub>R<sup>+/+</sup> mice, which was set as 1. Primer sequences are provided in Supplementary Table 1.

## Statistical Methods

Numeric data are presented as means  $\pm$  SEM (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 for post-hoc test.

## RESULTS

### HFD feeding increases adipose tissue A<sub>2A</sub>R expression and proinflammatory responses

HFD-fed WT mice, a model of DIO, displayed a significant increase in adipose tissue amount of A<sub>2A</sub>R compared with LFD-fed WT mice (Fig. 1A). When histological and immunohistochemical assays were performed, HFD-fed WT mice revealed significant increases in adipocyte size and in adipose tissue macrophage infiltrations as indicated by immunostaining of F4/80 cells (macrophages) in adipose tissue sections (Fig. 1B). When stained for A<sub>2A</sub>R expression, adipose tissue sections of HFD-fed WT mice contained markedly more A<sub>2A</sub>R-positive cells than those of LFD-fed mice. Also, nearly all A<sub>2A</sub>R-positive cells belong to stromal cells including macrophages (Fig. 1B). The staining intensity of A<sub>2A</sub>R in adipose tissue sections of HFD-fed WT mice was much stronger when compared with that of LFD-fed WT mice. Further analysis using immunofluorescent staining not only confirmed that HFD feeding stimulated adipose tissue A<sub>2A</sub>R expression compared with LFD or CD feeding, but also validated that most A<sub>2A</sub>R-positive cells were macrophages (Fig. 1C). When adipose tissue inflammatory responses were analyzed, HFD-fed WT mice displayed significant increases in adipose tissue proinflammatory signaling through JNK p46 and NF $\kappa$ B p65 and in adipose tissue mRNA levels of proinflammatory mediators such as *Tnfa*, *Iib*, *Il6*, and *Mcp1* (Fig. 1D,E) compared with LFD- or CD-fed mice. In terms of regulating adipose tissue proinflammatory signaling, LFD did not differ significantly from CD (Supplemental Fig. S1). Taken together, these results suggest that increased adipose tissue A<sub>2A</sub>R expression is associated with adipose tissue inflammation.

### A<sub>2A</sub>R disruption exacerbates the effects of HFD on inducing weight gain, adiposity, and hyperglycemia

Next, male homozygous A<sub>2A</sub>R-disrupted (A<sub>2A</sub>R<sup>-/-</sup>) mice, heterozygous A<sub>2A</sub>R-disrupted (A<sub>2A</sub>R<sup>+/-</sup>) mice, and their WT (A<sub>2A</sub>R<sup>+/+</sup>) littermates (Fig. 2A) were fed an HFD. After the feeding period, adipose tissue A<sub>2A</sub>R disruption was verified using real-time RT-PCR (Fig. 2B) and immunofluorescent staining (Fig. 2C). Initially, body weight and food intake of the mice were analyzed. Prior to HFD feeding, there was no difference in body weight among the mice (Fig. 3B). After HFD feeding, A<sub>2A</sub>R<sup>-/-</sup> or A<sub>2A</sub>R<sup>+/-</sup> mice gained much more body weight than A<sub>2A</sub>R<sup>+/+</sup> mice (Fig. 3B); although the mice consumed comparable amount of foods (Fig. 3A). When energy metabolism was analyzed, HFD-fed A<sub>2A</sub>R<sup>-/-</sup> mice and HFD-fed A<sub>2A</sub>R<sup>+/+</sup> mice displayed comparable amount of energy expenditure; although HFD-fed A<sub>2A</sub>R<sup>-/-</sup> mice, interestingly, revealed a shift in time of peak energy expenditure during night time period (~ 3 hr ahead of HFD-fed A<sub>2A</sub>R<sup>+/+</sup> mice) (Supplemental Fig. S2). Consistently, HFD-fed A<sub>2A</sub>R<sup>-/-</sup> or A<sub>2A</sub>R<sup>+/-</sup> mice displayed significant increases in abdominal fat mass and adiposity compared with HFD-fed A<sub>2A</sub>R<sup>+/+</sup> mice (Fig. 3C). Also, adipocyte size of

HFD-fed  $A_{2A}R^{-/-}$  or  $A_{2A}R^{+/-}$  mice was significantly increased compared with that of HFD-fed  $A_{2A}R^{+/+}$  mice (see below in Fig. 4A, top panels). At whole animal level, male  $A_{2A}R$ -disrupted mice revealed increased severity of HFD-induced systemic insulin resistance and glucose intolerance (supplemental data of the published study by Cai et al. (Cai et al. 2018)). Similar analyses were also performed in female mice. However, female  $A_{2A}R$ -disrupted mice did not gain more body weight compared with WT mice. Also, female homozygous, but not heterozygous  $A_{2A}R$ -disrupted mice revealed an increase in the severity of HFD-induced systemic insulin resistance (Cai et al. 2018). When plasma levels of glucose were examined,  $A_{2A}R^{-/-}$  or  $A_{2A}R^{+/-}$  mice displayed a greater increase in the severity of HFD-induced hyperglycemia than  $A_{2A}R^{+/+}$  mice (Fig. 3D). Also, HFD-induced hyperglycemia in  $A_{2A}R^{-/-}$  mice was severer than that in  $A_{2A}R^{+/-}$  mice.

### **$A_{2A}R$ disruption exacerbates HFD-induced adipose tissue inflammation**

When adipose tissue inflammation was analyzed, HFD-fed  $A_{2A}R$ -disrupted mice accumulated significantly more macrophages in adipose tissue compared with HFD-fed  $A_{2A}R^{+/+}$  mice (Fig. 4A, bottom panels). Among adipose tissue macrophages, there were more proinflammatory (M1) macrophages and fewer alternative or anti-inflammatory (M2) macrophages in HFD-fed  $A_{2A}R$ -disrupted mice than in HFD-fed  $A_{2A}R^{+/+}$  mice (Fig. 4B). Consistently, the phosphorylation states of adipose tissue JNK p46 and NF $\kappa$ B p65 and the mRNA levels of proinflammatory cytokines in HFD-fed  $A_{2A}R$ -disrupted mice were significantly higher than their respective levels in HFD-fed  $A_{2A}R^{+/+}$  mice (Fig. 4C,D). In contrast, the mRNA levels of *Il10*, *Arginase*, and *Pparg* in HFD-fed  $A_{2A}R$ -disrupted mice were significantly lower than their respective levels in HFD-fed  $A_{2A}R^{+/+}$  mice (Fig. 4D). Taken together, these results suggest that  $A_{2A}R$  disruption exacerbates diet-induced adipose tissue inflammation.

### **$A_{2A}R$ disruption exacerbates HFD-induced adipose tissue insulin resistance**

We examined  $A_{2A}R$  regulation of adipose tissue insulin sensitivity, and observed that insulin-stimulated Akt phosphorylation was significantly decreased in HFD-fed  $A_{2A}R$ -disrupted mice in a gene-dose-dependent manner (Fig. 5A). Also, we examined the expression of several genes or enzymes related to adipose tissue metabolic responses. The mRNA levels of adiponectin and *Hsl* in HFD-fed  $A_{2A}R^{-/-}$  mice were significantly decreased compared with their respective levels in HFD-fed  $A_{2A}R^{+/+}$  mice (Fig. 5B). Consistently, the amount and phosphorylation states of HSL in HFD-fed  $A_{2A}R$ -disrupted mice were significantly lower than those in HFD-fed  $A_{2A}R^{+/+}$  mice (Fig. 5C). However, *Mcp1* mRNAs in HFD-fed  $A_{2A}R$ -disrupted mice did not differ significantly from those in control mice. These results suggest that  $A_{2A}R$  disruption exacerbates diet-induced adipose tissue insulin resistance.

### **$A_{2A}R$ disruption enhances the effect of palmitate on stimulating macrophage proinflammatory signaling**

The present study examined the extent to which  $A_{2A}R$  disruption alters the effect of palmitate on regulating macrophage proinflammatory signaling. In WT BMDM, treatment with palmitate caused marginal or insignificant increases in the phosphorylation states of JNK p46 and NF $\kappa$ B p65 (Fig. 6). However, in  $A_{2A}R^{-/-}$  BMDM, treatment with palmitate

caused a significant increase in the phosphorylation states of JNK p46; although palmitate did not significantly alter the phosphorylation states of NF $\kappa$ B p65 (Fig. 6). These results suggest that A<sub>2A</sub>R disruption enhances the effect of palmitate on stimulating macrophage proinflammatory signaling.

## DISCUSSION

In the present study, we validated an association between increased A<sub>2A</sub>R expression and adipose tissue inflammation in WT mice upon feeding an HFD. Furthermore, using A<sub>2A</sub>R-disrupted mice, we demonstrated an increase in the severity of HFD-induced adipose tissue inflammation in relative to that in WT control mice. This finding is complementary to the finding by DeOliveira et al, which indicates a suppressive effect of A<sub>2A</sub>R activation on adipose tissue inflammation (DeOliveira et al. 2017). At the cellular level, the present study validated that A<sub>2A</sub>R deficiency exacerbated the stimulatory effect of palmitate on macrophage proinflammatory activation, suggesting that A<sub>2A</sub>R suppression of adipose tissue inflammation is attributable to, at least in part, the effect of A<sub>2A</sub>R on inhibiting macrophage activation.

While A<sub>2A</sub>R is expressed in adipose tissue, the response of A<sub>2A</sub>R expression to obesity in the context of adipose tissue inflammation is not defined. To address this, the present study examined adipose tissue A<sub>2A</sub>R amount in WT C57BL/6J mice fed an HFD or LFD, and demonstrated a stimulatory effect of HFD feeding on adipose tissue A<sub>2A</sub>R expression. Of note, adipose tissue of HFD-fed WT mice contained many more A<sub>2A</sub>R-positive cells than that of LFD-fed WT mice, and nearly all of the A<sub>2A</sub>R-positive cells were located in crown structure areas that were mainly composed of macrophages. Upon co-staining A<sub>2A</sub>R and F4/80 using immunofluorescent assay, it was confirmed that most A<sub>2A</sub>R-positive cells were macrophages. Furthermore, the staining intensity of A<sub>2A</sub>R-positive cells in adipose tissue of HFD-fed WT mice was much stronger than that of LFD-fed WT mice. Because of this, it is conceivable that obesity increases adipose tissue A<sub>2A</sub>R abundance through increasing macrophage infiltration into adipose tissue and stimulating A<sub>2A</sub>R expression in the infiltrated macrophages. To be noted, increased adipose tissue macrophage A<sub>2A</sub>R expression in HFD-fed WT mice was accompanied with enhanced adipose tissue inflammatory responses, as indicated by increases in the phosphorylation states of JNK p46 and NF $\kappa$ B p65 and in the mRNA levels of proinflammatory cytokines including *Tnfa*, *Il1b*, and *Il6*. This correlation suggested a defensive response of adipose tissue macrophage A<sub>2A</sub>R to obesity or HFD feeding rather than a causal role for A<sub>2A</sub>R in adipose tissue inflammation. This is because the A<sub>2A</sub>R in macrophage, *per se*, is anti-inflammatory (Cai et al. 2018; Lukashev, et al. 2004). As substantial evidence, A<sub>2A</sub>R deficiency exacerbated the effect of HFD feeding on inducing adipose tissue inflammation *in vivo* and enhanced the effect of palmitate on stimulating macrophage proinflammatory activation *in vitro*. However, we do not rule out the possibility that prolonged increase in A<sub>2A</sub>R expression may cause harmful effects, which warrants future investigation.

The regulatory role for A<sub>2A</sub>R in adipose tissue inflammation is supported by recent evidence obtained from obese mice upon treatment with an A<sub>2A</sub>R agonist (CGS-21680) (DeOliveira et al. 2017). Specifically, treatment of obese mice with CGS-21680 caused a significant



decrease in adipose tissue macrophage infiltration; although treatment with CGS-21680 did not significantly alter the phosphorylation states of adipose tissue JNK p46. As complementary evidence, the present study revealed that the severity of HFD-induced adipose tissue inflammation in  $A_{2A}R$ -disrupted mice was significantly greater than that in  $A_{2A}R^{+/+}$  mice. Notably, HFD-fed  $A_{2A}R$ -disrupted mice displayed significant increases in adipose tissue macrophage infiltration, as well as percentages of proinflammatory macrophages among adipose tissue macrophages. In addition, HFD-fed  $A_{2A}R$ -disrupted mice displayed significant increases in the phosphorylation states of adipose tissue JNK p46 and NF $\kappa$ B p65 and in the mRNA levels of *Tnfa*, *Il1b*, and *Il6*. Taken together, these results argued in favor that  $A_{2A}R$  plays a suppressive role in obesity-associated adipose tissue inflammation.

Additional to inflammation, adipose tissue insulin resistance is another feature commonly associated with obesity. In the present study, increased adipose tissue inflammation in HFD-fed  $A_{2A}R$ -deficient mice was accompanied with increased severity of HFD-induced adipose tissue insulin resistance. In particular, the states of insulin-induced Akt phosphorylation in HFD-fed  $A_{2A}R$ -disrupted mice were significantly lower than those in HFD-fed control mice. To this point, however, it remains to be explored whether decreased adipose tissue insulin sensitivity in HFD-fed  $A_{2A}R$ -deficient mice was due to increased adipose inflammation, increased adipose tissue fat mass, or both. With regard to changes in body weight of HFD-fed mice, our findings are contradictory to those published by Csoka et al. (Csóka, et al. 2017). The exactly reasons for the discrepancy are not clear, but may be attributable to the differences in the responses of WT mice to HFD feeding. In the study by Csoka et al., WT mice displayed a marked increase in body weight upon HFD feeding, and appeared to be hyperphagic compared with the WT mice used by us and others (DeOliveira et al. 2017; Gnad, et al. 2014). Given this, interpretation of the phenotype of  $A_{2A}R$  disruption appeared to be influenced by what control was used for comparisons. In the present study,  $A_{2A}R$ -deficient mice gained more body weight upon HFD feeding compared with WT mice; although consuming comparable amount of foods and revealing similar energy expenditure. A possible explanation is that HFD-fed  $A_{2A}R$ -deficient mice had increased efficiency in energy absorption. This postulation was based on two reasons. First, in rodent models of DIO, intestine inflammation is evident and likely enhances energy absorption. Second,  $A_{2A}R$  exerts a suppressive effect on intestine inflammation (Odashima, et al. 2005). However, future study is needed to examine  $A_{2A}R$  regulation of intestine inflammation in HFD-fed mice to validate our postulation. In HFD-fed  $A_{2A}R$ -disrupted mice, the phosphorylation states of HSL were significantly decreased compared with those in control mice. This appeared to also account for increased adiposity in  $A_{2A}R$ -disrupted mice. What should be pointed out is that decreased HSL phosphorylation in HFD-fed  $A_{2A}R$ -disrupted mice likely indicates a decrease in adipose tissue release of fatty acids. The latter, when undergoing oxidation, contributes largely to energy expenditure. In other words, decreased adipose tissue HSL phosphorylation was expected to associate with an increase in respiratory quotient in HFD-fed  $A_{2A}R$ -disrupted mice relative to control mice. However, this was not the case. We speculate that increased energy absorption could provide more fatty acids to offset a decrease in the availability of fatty acids due to decreased adipose tissue

lipolysis. Nonetheless, it is conceivable that A<sub>2A</sub>R has a role in protecting against HFD-induced adipose tissue inflammation and insulin resistance.

While A<sub>2A</sub>R exerted anti-inflammatory effects on adipose tissue inflammation, an interesting question raised was to what extent the A<sub>2A</sub>R in macrophages *versus* adipocytes contributes to the regulation of adipose tissue inflammation. To address this question may rely on analyzing diet-induced adipose tissue inflammation in mice whose A<sub>2A</sub>R is disrupted only in adipocytes or myeloid cells (macrophages). However, the results generated by DeOliveira et al. and by the present study appear to indicate a more important role for the A<sub>2A</sub>R in macrophages in determining overall adipose tissue inflammation; although the data from adipocyte- or macrophage-specific A<sub>2A</sub>R knockout is not available. This view is supported by three lines of evidence. Firstly, in adipose tissue of HFD-fed WT mice, A<sub>2A</sub>R was expressed predominantly in macrophages, and barely in adipocytes. Secondly, when adipose tissue macrophage infiltration was increased in HFD-fed A<sub>2A</sub>R-disrupted mice, adipose tissue amount of *Mcp1*, whose secretion by adipocytes regulates adipose tissue macrophage infiltration, was not significantly altered. This indicates a role for the A<sub>2A</sub>R in macrophages in determining adipose tissue macrophage infiltration, which was consistent with the finding that A<sub>2A</sub>R-disrupted leukocytes (monocytes) likely are easier to infiltrate into inflammatory tissues when compared with WT cells (Wang, et al. 2010). Thirdly, A<sub>2A</sub>R activation has been shown to decrease macrophage infiltration, which may be attributable to A<sub>2A</sub>R activation in macrophages (Garcia, et al. 2008). Taken together, it appears that the A<sub>2A</sub>R in macrophages determines obesity-associated adipose tissue inflammation. At the cellular level, A<sub>2A</sub>R has a direct role in regulating macrophage inflammatory activation, which was validated by the finding that A<sub>2A</sub>R deficiency enhanced the effect of palmitate, a major component of HFD, on stimulating macrophage proinflammatory activation.

In summary, the present study demonstrated a critical role for A<sub>2A</sub>R in regulating obesity-associated adipose tissue inflammation. Specifically, A<sub>2A</sub>R expression was increased in adipose tissue of obese mice, which appeared to be a defensive response. Also, in the absence of A<sub>2A</sub>R, macrophage proinflammatory status was increased and likely accounted for exacerbation of adipose tissue inflammation, adiposity, and adipose tissue insulin resistance under obese conditions. Accordingly, targeting A<sub>2A</sub>R to suppress adipose tissue inflammation would be a beneficial approach for management of obesity-associated inflammatory and metabolic diseases. The present study also has several limitations. First, it remains to be determined what signals are responsible for stimulating macrophage A<sub>2A</sub>R expression *in vivo*. Second, it is not clear whether increased adiposity in A<sub>2A</sub>R-disrupted mice was attributable to, in part, increased hepatic production of endogenous fat. Third, it remains to be determined the extent to which A<sub>2A</sub>R-disruption-related adipose tissue inflammation also acts through causing hepatic and muscle insulin resistance to bring about systemic insulin resistance. We would like to address these questions in our future studies.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGEMENTS

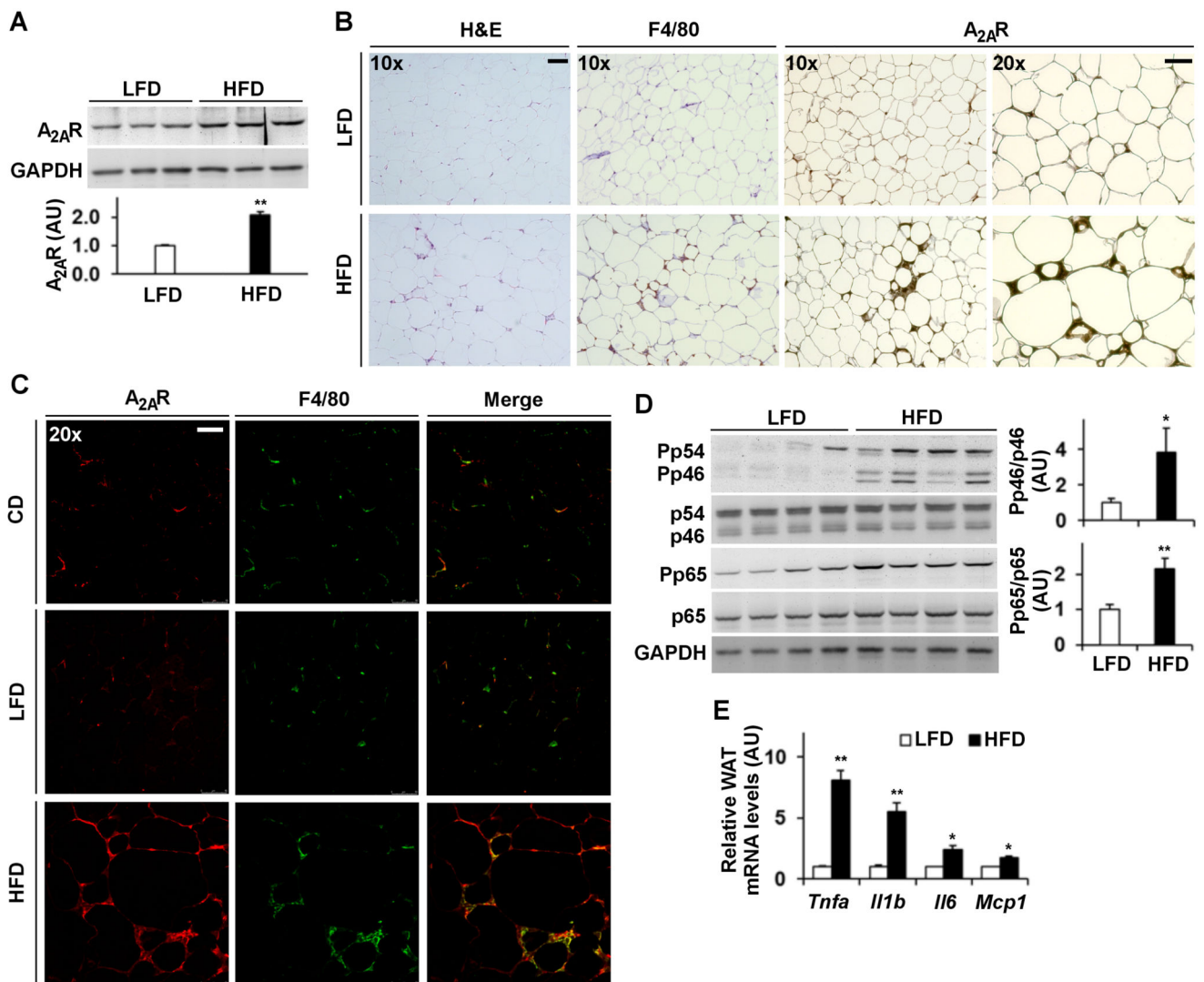
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**Figure 1. HFD feeding increases adipose tissue adenosine 2A receptor (A<sub>2A</sub>R) expression and proinflammatory responses.**

Male C57BL/6J mice, at 5 – 6 weeks of age, were fed a high-fat diet (HFD) or low-fat diet (LFD) for 12 weeks or maintained on a standard chow diet (CD). (A) Adipose tissue (epididymal fat) lysates were examined for A<sub>2A</sub>R amount using Western blot analysis. (B) Adipose tissue (epididymal fat) sections were stained with H&E (panels in the first row from top) or stained for F4/80 expression (a macrophage marker) (panels of the second row from top) or A<sub>2A</sub>R expression (panels of bottom two rows) using immunohistochemistry. Representative images were from HFD-fed mice (right column) and LFD-fed mice (left column). (C) Adipose tissue (epididymal fat) sections were subjected to immunofluorescent staining of A<sub>2A</sub>R (left column) and/or F4/80 (middle column) expression. (D) Adipose tissue (epididymal fat) lysates were examined for proinflammatory signaling using Western blot analysis. (E) Adipose tissue (epididymal fat) mRNA levels of cytokines were quantified using real-time RT-PCR. For A and D, blots were quantified using densitometry. For A, D, and E, numeric data are means ± SEM. n = 4 - 6. Statistical difference between HFD and

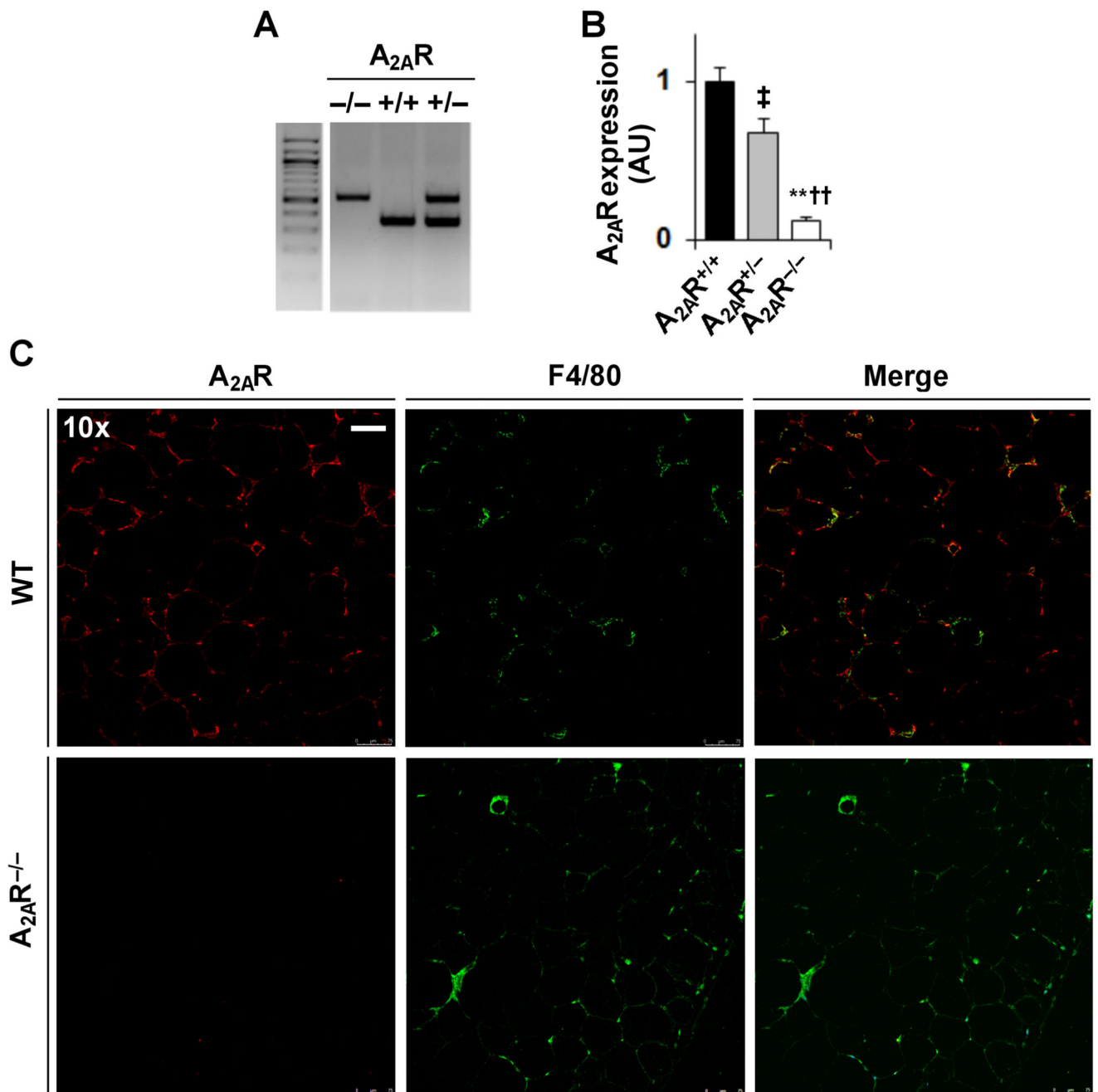
LFD: \*,  $P < 0.05$  and \*\*,  $P < 0.01$  in bar graphs of A and D or in E for the same gene. For B and C, the scale bar is 75  $\mu\text{m}$  for 10 $\times$  images or 50  $\mu\text{m}$  for 20 $\times$  images.

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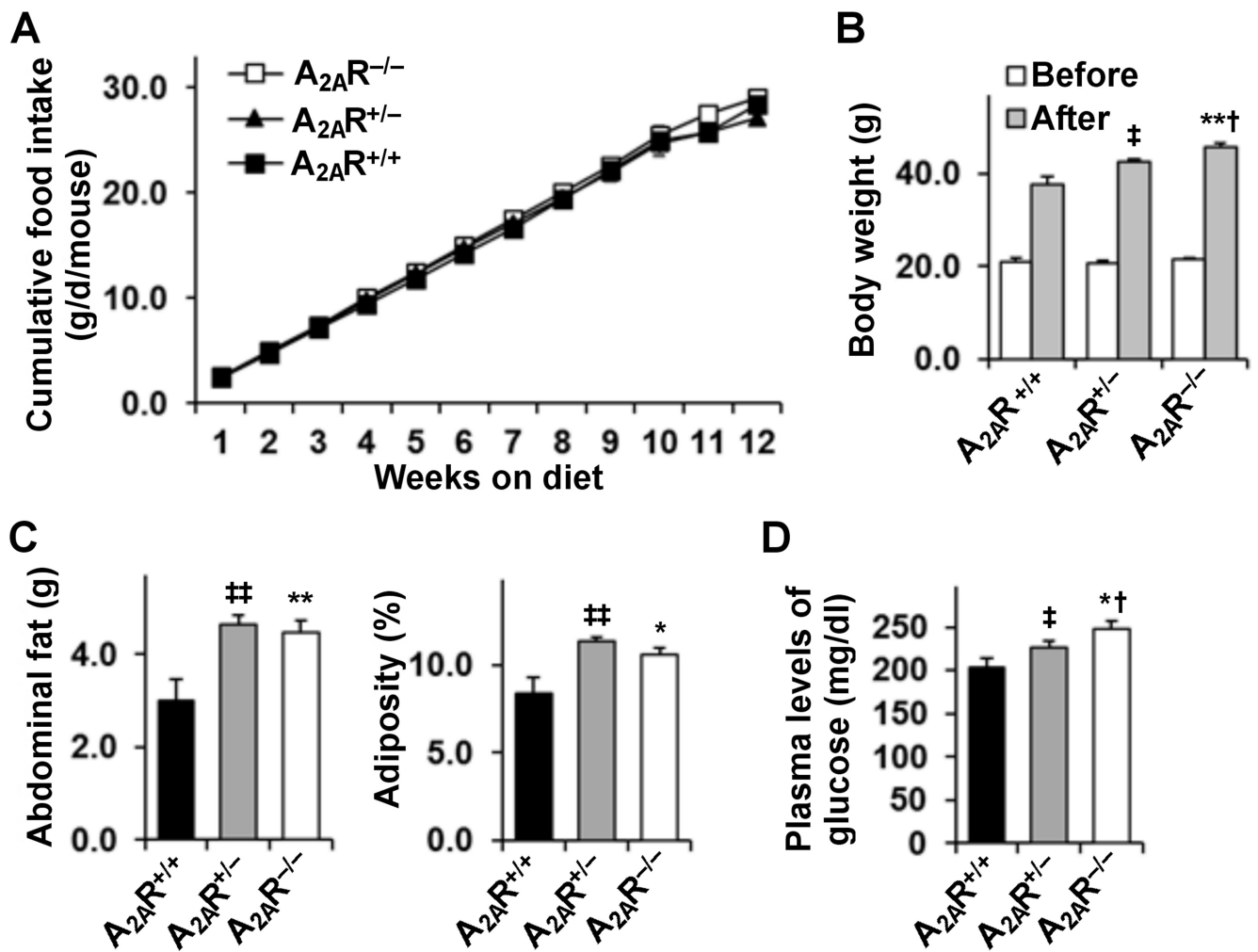


### Figure 2. Validation of adipose tissue A<sub>2</sub>AR disruption

(A) Genomic DNA was subjected to PCR analysis for genotyping of homozygous A<sub>2</sub>AR-disrupted (A<sub>2</sub>AR<sup>-/-</sup>) mice, heterozygous A<sub>2</sub>AR-disrupted (A<sub>2</sub>AR<sup>+/-</sup>) mice and their wild-type (WT, A<sub>2</sub>AR<sup>+/+</sup>) littermates. (B,C) Adipose tissue A<sub>2</sub>AR disruption. Male A<sub>2</sub>AR-disrupted (A<sub>2</sub>AR<sup>-/-</sup> and A<sub>2</sub>AR<sup>+/-</sup>) mice and A<sub>2</sub>AR<sup>+/+</sup> mice, at 5 - 6 weeks of age, were fed an HFD for 12 weeks. For B, adipose tissue (epididymal fat) mRNA levels of A<sub>2</sub>AR were quantified using real-time RT-PCR. Data are means ± SEM. n = 8 - 10. Statistical difference between A<sub>2</sub>AR<sup>-/-</sup> and A<sub>2</sub>AR<sup>+/+</sup>: \*\*, *P* < 0.01; statistical difference between A<sub>2</sub>AR<sup>-/-</sup> and

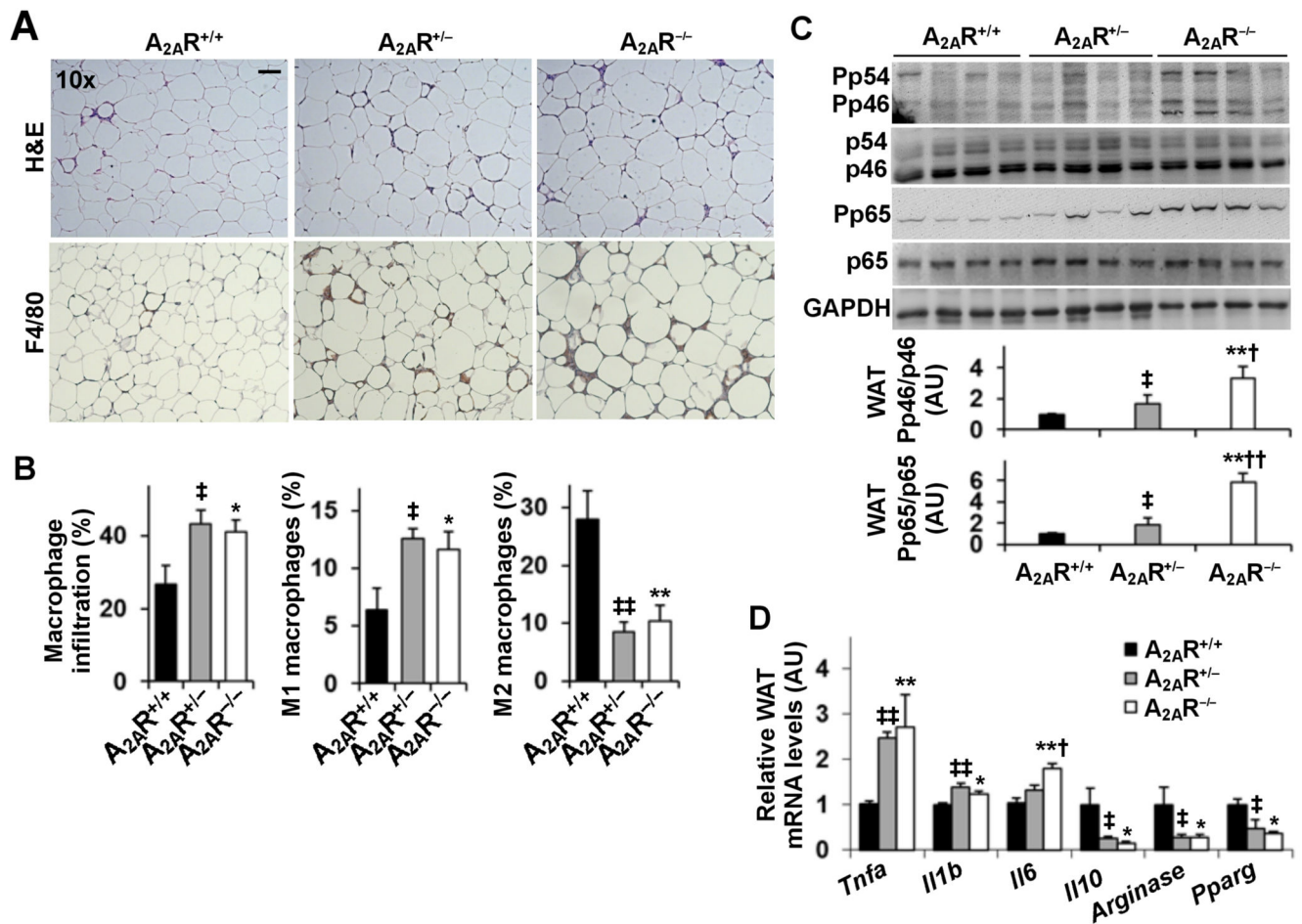


$A_{2A}R^{+/-}$ :  $\dagger\dagger$ ,  $P < 0.01$ ; statistical difference between  $A_{2A}R^{+/-}$  and  $A_{2A}R^{+/+}$ :  $\ddagger$ ,  $P < 0.05$ . For C, adipose tissue (epididymal fat) sections were subjected to immunofluorescent staining of  $A_{2A}R$  and/or F4/80 expression. The scale bar is 75  $\mu\text{m}$  for 10 $\times$  images.



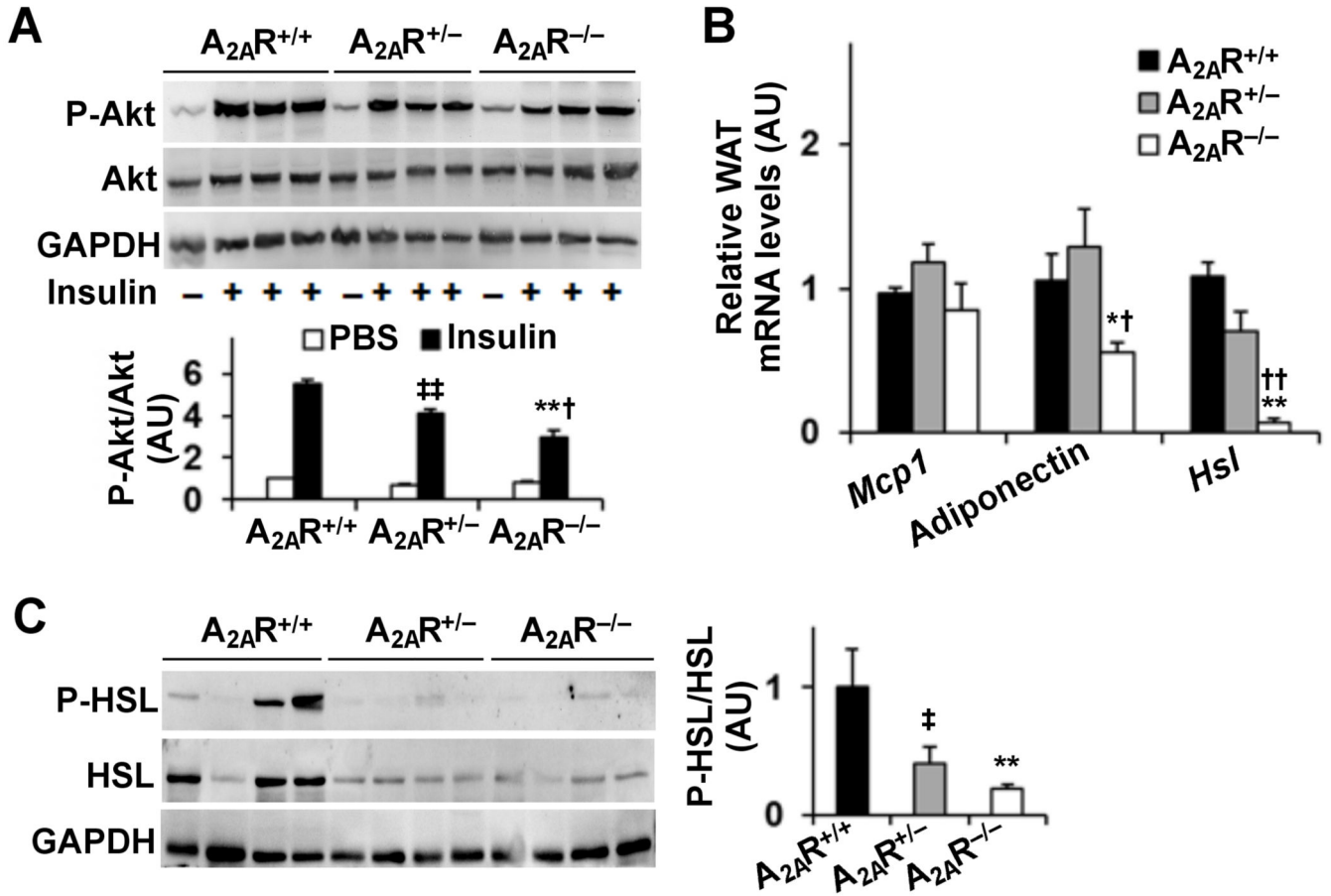
**Figure 3.  $A_{2A}R$  disruption exacerbates the effects of HFD feeding on inducing weight gain, adiposity, and hyperglycemia**

Male  $A_{2A}R^{-/-}$ ,  $A_{2A}R^{+/-}$ , and  $A_{2A}R^{+/+}$  mice, at 5 - 6 weeks of age, were fed an HFD for 12 weeks. (A) Cumulative food intake during feeding period. (B) Body weight was measured before and after the feeding period. (C) Abdominal fat mass was calculated as the sum of epididymal fat, mesenteric fat, and perinephric fat. Adiposity was calculated as the ratio of abdominal fat mass to body weight. (D) After the feeding period, mice were fasted for 4 hr prior to collection of blood samples. Plasma levels of glucose were quantified using a metabolic kit. For A - D, data are means  $\pm$  SEM.  $n = 4 - 6$  (A) or  $10 - 12$  (C - D). Statistical difference between  $A_{2A}R^{-/-}$  and  $A_{2A}R^{+/+}$ : \*,  $P < 0.05$  and \*\*,  $P < 0.01$  in B after feeding period or in C and D; statistical difference between  $A_{2A}R^{-/-}$  and  $A_{2A}R^{+/-}$ : †,  $P < 0.05$  in B after feeding period or in D; statistical difference between  $A_{2A}R^{+/-}$  and  $A_{2A}R^{+/+}$ : ‡,  $P < 0.05$  ‡‡,  $P < 0.01$  in B after feeding period or in C and D.

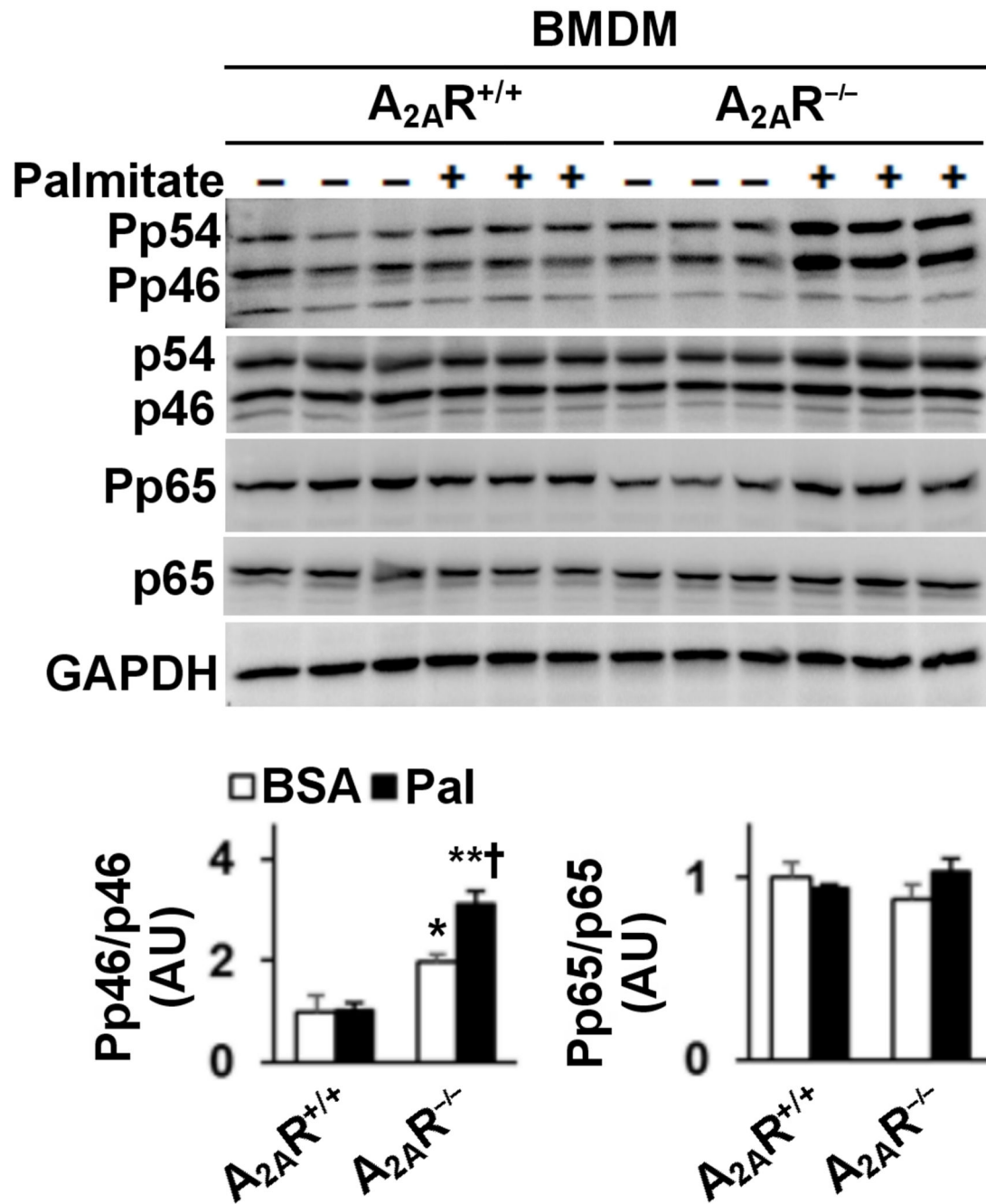


**Figure 4.  $A_{2A}R$  disruption exacerbates HFD-induced adipose tissue inflammation**

Male  $A_{2A}R^{-/-}$ ,  $A_{2A}R^{+/-}$ , and  $A_{2A}R^{+/+}$  mice, at 5 - 6 weeks of age, were fed an HFD for 12 weeks. (A) Sections of epididymal fat were stained with H&E (top three panels) or stained for F4/80 expression (bottom three panels) using immunohistochemistry. Representative images were presented. (B) Adipose tissue macrophage infiltration and polarization. Stromal vascular cells (SVC) were isolated from epididymal fat depots and analyzed for CD11b and F4/80 expression. Mature macrophages (CD11b<sup>+</sup> and F4/80<sup>+</sup> cells) were further analyzed for CD11c and CD206 expression. Left panel, percentages of mature macrophages; middle panel, percentages of M1 macrophages (F4/80<sup>+</sup> CD11b<sup>+</sup> CD11c<sup>+</sup> CD206<sup>-</sup> cells); right panel, percentages of M2 macrophages (F4/80<sup>+</sup> CD11b<sup>+</sup> CD11c<sup>-</sup> CD206<sup>+</sup> cells). (C) Lysates of epididymal fat were examined for proinflammatory signaling using Western blot analysis. Bar graphs, quantifications of blots. (D) Adipose tissue (epididymal fat) mRNA levels of cytokines and markers related to macrophage polarization were quantified using real-time RT-PCR. For B - D, numeric data are means  $\pm$  SEM. n = 8 - 10. Statistical difference between  $A_{2A}R^{-/-}$  and  $A_{2A}R^{+/+}$ : \*,  $P < 0.05$  and \*\*,  $P < 0.01$  in B and C or in D for the same gene; statistical difference between  $A_{2A}R^{-/-}$  and  $A_{2A}R^{+/-}$ : †,  $P < 0.05$  and ††,  $P < 0.01$  in C or in D for the same gene; statistical difference between  $A_{2A}R^{+/-}$  and  $A_{2A}R^{+/+}$ : ‡,  $P < 0.05$  and ‡‡,  $P < 0.01$  in B and C or in D for the same gene.



**Figure 5. A<sub>2A</sub>R disruption exacerbates HFD-induced adipose tissue insulin resistance**  
 Male A<sub>2A</sub>R<sup>-/-</sup>, A<sub>2A</sub>R<sup>+/-</sup>, and A<sub>2A</sub>R<sup>+/+</sup> mice, at 5 – 6 weeks of age, were fed an HFD for 12 weeks. **(A)** Adipose tissue insulin signaling. Prior to harvest, mice were given an injection of insulin (1 U/kg body weight) into the portal vein for 5 min. Lysates of epididymal fat were subjected to Western blot analysis. Bar graphs, quantifications of blots. **(B)** The mRNA levels of genes for adipose tissue metabolic responses were quantified using real-time RT-PCR. **(C)** Lysates of epididymal fat were examined for HSL amount and phosphorylation states using Western blot analysis. For A and C, numeric data are means ± SEM. n = 6 - 8. Statistical difference between A<sub>2A</sub>R<sup>-/-</sup> and A<sub>2A</sub>R<sup>+/+</sup>: \*, P < 0.05 and \*\*, P < 0.01 in A under the same condition, in B for the same gene, or in C; statistical difference between A<sub>2A</sub>R<sup>-/-</sup> and A<sub>2A</sub>R<sup>+/-</sup>: †, P < 0.05 and ††, P < 0.01 in A under the same condition, in B for the same gene, or in C; statistical difference between A<sub>2A</sub>R<sup>+/-</sup> and A<sub>2A</sub>R<sup>+/+</sup>: ‡, P < 0.05 and ‡‡, P < 0.01 in A under the same condition or in C.



**Figure 6.  $A_{2A}R$  disruption enhances macrophage proinflammatory signaling**

Bone marrow cells were isolated from male  $A_{2A}R^{-/-}$  and  $A_{2A}R^{+/+}$  mice, and differentiated into macrophages (BMDM). Cells were incubated in growth media in the presence of palmitate (250  $\mu$ M, conjugated in BSA) or BSA for 24 hr. Cell lysates were subjected to Western blot analysis. Bar graphs, quantifications of blots. Numeric data are means  $\pm$  SEM.  $n = 6 - 8$ . Statistical difference between  $A_{2A}R^{-/-}$  and  $A_{2A}R^{+/+}$  with the same treatment: \*\*,  $P < 0.01$ .