Glucocorticoid induces hepatic steatosis by inhibiting ATF3/S100A9 signaling in granulocytic MDSCs

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Running titer: G-MDSCs contributes to drug-induced hepatic steatosis

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ABSTRACT

Glucocorticoids (GCs) used as inflammation suppressors have harmful side effects, including induction of hepatic steatosis. The underlying mechanisms of GCs-promoted dysregulation of lipid metabolism, however, are not fully understood. GCs could facilitate the accumulation of myeloid-derived suppressor cell (MDSC) in the liver of animals, the potential role of MDSCs in GC-induced hepatic steatosis was therefore investigated in this study. We demonstrated that granulocytic (G)-MDSC accumulation mediated the effects of GCs on the fatty liver, in which activating transcription factor 3 (ATF3)/S100A9 signaling plays an important role. ATF3-deficient mice developed hepatic steatosis and displayed expansion of G-MDSCs in the liver and multiple immune organs, which shared highly similarity with the phenotype observed in GC treated wild-type littermates. Adoptive transfer of GC-induced or ATF3-deficient G-MDSCs promoted lipid accumulation in the liver, while depletion of G-MDSCs alleviated these effects. Mechanistic studies showed that in MDSCs, ATF3 was transrepressed by GC receptor GR through direct binding to the negative GR response element (nGRE). S100A9 is the major transcriptional target of ATF3 in G-MDSCs. Silencing S100A9 clearly alleviated G-MDSCs expansion and hepatitis steatosis caused by ATF3 deficiency or GC treatment. Conclusion: Our study uncovers an important role of G-MDSCs in GC-induced hepatic steatosis, in which ATF3 may have potential therapeutic implications.

Hepatic steatosis is characterized by abnormal lipid accumulation in hepatocytes, which was caused by the imbalance between lipogenesis, lipid catabolism, and free fatty acid uptake (1). Clinical administration of exogenous glucocorticoids (GCs) to patients, subjected to organ transplantation or suffering from severe inflammation-related diseases, is known to cause side effects, including deregulation of lipid metabolism and hepatic steatosis (2,3). The mechanisms underlying GC-induced hepatic steatosis are still not fully understood. Recent studies have shown that administration of a synthetic GC dexamethasone (Dex) could cause the expansion of distinctive proinflammatory
monocytes, they were CD11b\(^+\)Gr-1\(^+\) and expressed signature molecule of tumor-induced myeloid-derived suppressor cells (MDSCs), both in human and mouse (4). Accumulation of immature myeloid (CD11b\(^+\)Ly6C\(^+\)Ly6G\(^-\)) cells was also observed in mice fed with high-fat diet (5). These observations indicate a possible relationship between GC-induced lipid metabolism and myeloid cell differentiation.

MDSCs represent a heterogeneous population consisting of immature myeloid cells and different stages of myeloid progenitors in which their full differentiation into mature myeloid cells has been prevented (6,7). MDSCs expansion has been observed under various pathological conditions, including but not limited to, cancer, inflammation, infections and trauma (8-10). Functioning through suppression of other immune cells, MDSCs are believed to be one of the key brakes in the immune system (7). The clinical beneficial effect of GCs on the prevention of graft-versus-host reaction or excessive inflammation is attributed, at least in part, to immune suppressive function of MDSCs (11,12).

To determine a potential association between MDSCs and GC-induced hepatic steatosis, we have previously performed gene expression analysis of MDSCs purified from liver of Dex-treated and vehicle control mice. We found that the expression of activating transcription factor 3 (ATF3) was dramatically downregulated in MDSCs upon Dex treatment. ATF3, a basic leucine zipper transcription factor belonging to the ATF/cyclic AMP response element-binding (ATF/CREB) family is encoded by an adaptive-response gene, i.e., its expression is induced by extracellular or intracellular stress signaling (13,14). Importantly, ATF3 has been demonstrated as an integrative factor in bridging metabolism, inflammation, and immunity (15).

In this study, we investigated the role of ATF3 in linking MDSCs and GC-induced hepatic steatosis. We demonstrate that MDSCs contribute to GC-induced hepatic steatosis through ATF3/S100A9 axis. Targeting ATF3 may be promising in therapeutic application of lipid metabolic disorders.

**RESULTS**

**GC-induced hepatic steatosis is dependent on ATF3 deficiency**- To gain insights into the potential role of ATF3 in GC-induced hepatic steatosis, female wild type (WT) and ATF3-deficient (ATF3\(^{-/-}\)) mice were intraperitoneally injected with synthetic GC, dexamethasone (Dex), or DMSO (Veh). Mice were analyzed for liver weight, as well as lipid contents in the liver and peripheral blood. As expected, in WT mice, Dex induced hepatomegaly and clear elevation of triglyceride, cholesterol, and free fatty acid contents in both liver and circulation. Interestingly, in the absence of Dex, ATF3\(^{-/-}\) mice displayed significantly higher levels of liver weight and lipids than those of WT littermates, reaching comparable levels of Dex-treated WT controls. No further increases were observed in ATF3\(^{-/-}\) mice after Dex administration (Fig.1A). The development of liver steatosis was further confirmed by staining with lipid-specific Oil-Red O and hematoxylin/eosin, which revealed small fat droplets in liver sections from ATF3\(^{-/-}\) and Dex-treated WT animals (Fig.1B). The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum, indicative of liver damage, displayed similar changes with those of lipids (Data not shown). These observations indicate that mice lacking ATF3 developed spontaneous hepatosteatosis and were refractory to Dex treatment, suggesting ATF3 deficiency may be important for Dex-induced hepatic steatosis.

**GC-induced MDSCs accumulation is dependent on ATF3 deficiency**- To investigate whether MDSCs contributed to the...
development of hepatic steatosis, flow cytometric analysis was performed in WT and ATF3−/− mice before and after Dex treatment. The CD11b+Gr1high and CD11b+Gr1inter populations were corresponded to granulocytic (G)-MDSCs (CD11b+Ly6G−Ly6C+) and monocytic (M)-MDSCs (CD11b+Ly6G+Ly6C+) subsets, respectively (Data not shown). Consistent with previous report (16), Dex treatment caused significant increase in the frequency of MDSCs in WT mice, including liver and mutiple immune organs. Further analysis showed that this increase was due to G-MDSCs, but not M-MDSCs (Fig. 2A). ATF3 deficiency caused 2-3-fold increase in the proportion of G-MDSCs in multiple tissues, reaching the levels of Dex-treated WT animals; whereas no further increase was observed upon Dex administration in ATF3−/− mice. The levels of M-MDSCs did not display any noticeable difference among distinct groups (Fig. 2A). In line with the changes in MDSCs, the proportions of mature myeloid cells, including conventional dendritic cells (DCs, CD11c+MHCII+) and macrophages (CD11b+F4/80+), were lower in Dex-treated WT and Veh-ATF3−/− mice, compared to those in Veh-treated WT control (Fig. 2B). In line with these observations, the levels of mature myeloid cells upon Dex treatment was observed only in WT BM cells, not in ATF3−/− mice (Fig. 2B). As further confirmation, BM cells lacking ATF3 displayed stronger capability in the generation of MDSCs than WT control in vitro assay. Dex promoted MDSCs accumulation from WT BM as expected, while no effect was observed for cells from ATF3−/− mice (Fig. 2C), which was accompanied with a reduction in the mature CD11c−MHCII− DC cells upon Dex treatment or ATF3 deletion (Fig. 2C). These results indicate that ATF3 deficiency may be required for GC-induced MDSC accumulation.

ATF3 mediates the effect of G-MDSCs on GC-induced hepatic steatosis—To determine whether a causal relationship exists between MDSC and hepatic lipid accumulation in response to GC administration, adoptive transfer of G-MDSCs was performed. Results showed that G-MDSCs from Dex-treated WT or Veh-treated ATF3−/− mice caused a significant increase in liver weight, as well as lipid contents in WT recipient mice. The corresponding control from Veh-treated WT mice, however, did not elicit any effects (Fig. 3A). Purified M-MDSCs from these groups failed to display any effects (Data not shown). The hepatic lipid accumulation was further confirmed by Oil-Red O and hematoxylin/eosin staining (Fig. 3B). We also tested the survival of MDSCs in the adoptive transfer assay. Results showed that G-MDSCs from donor mice (CD45.2+) proliferate at the same rate with those from recipient mice (CD45.1+), indicating that they survived normally as in vivo (data not shown). The role of G-MDSCs in the development of hepatic steatosis was further corroborated when G-MDSCs were depleted using anti-Ly6G antibody. The 2-3-fold reduction in G-MDSCs population in liver clearly alleviated steatosis symptoms in both Dex-treated WT and ATF3−/− mice (Fig. 3C-E). Therefore, these results further determined the important role of G-MDSCs in development of GC-induced hepatic steatosis, and represents a novel mechanism of GC-induced fatty liver, in which ATF3 deficiency may be required.

ATF3 is transrepressed by GR in MDSCs—Based on the observations that ATF3 deficiency may be prerequisite for GC-induced MDSCs expansion and fatty liver, we hypothesized that GC might transrepress ATF3 in myeloid cells. It was found that Dex inhibited ATF3 expression in cultured BM cells in a concentration-dependent manner, both at mRNA and protein levels (Fig. 4A). This observation was confirmed in purified MDSCs from BM, spleen, and liver (Fig. 4B), while no effects were detected in T cells from these tissues (Data not shown). These indicate that suppression of ATF3 by GC was limited to
myeloid cells. Pretreatment with GR antagonist RU486 completely abrogated this effect (Fig. 4C), supporting that the suppression is GR-dependent. Binding with negative GR response elements (nGREs, with core sequence CTCCnGGAG) mediates the transrepression of targets by GR (17). Detailed analysis of ATF3 regulatory region revealed a potential nGRE located between -2300 to -2310 bp from the transcription start site (Fig. 4D). Transfection with ATF3 luciferase reporter plasmid containing the predicted nGRE showed that GC downregulated the activity of ATF3 promoter in 32D myeloid cells, whereas site-directed mutation of nGRE completely abolished this repression (Fig. 4D). As expectedly, RU486 abrogated the effect of Dex on ATF3 reporter (Data not shown). Further chromatin immunoprecipitation (ChIP) assay confirmed the binding of GR protein to nGRE located within ATF3 regulatory region, no signals were detected in BM cells from ATF3 -/- mice, as expected (Fig. 4E). Overall, these results revealed that GR transrepresses ATF3 in myeloid cells.

**ATF3 regulates the development of G-MDSCs** - The participation of ATF3 in MDSC-mediated hepatic steatosis in response to GC suggests a potential role of ATF3 in the differentiation of myeloid cells. Flow cytometric analysis showed that the level of CD11b*Gr1* immature myeloid cells (iMCs) was elevated in the tissues from ATF3-/- mice, with more than 2-fold increase in the liver and spleen; The increase was mostly provided by CD11b*Ly6G*Ly6C- granulocytes, not CD11b*Ly6G*Ly6C+ monocytes (Fig. 5A). In line with the increase in iMCs, the proportion of mature myeloid cells, including CD11c*MHCII* dendritic cells and CD11b*F4/80* macrophages, was clearly lower in ATF3-/- mice than in their WT littermates. In contrast, the composition of lymphocyte population, including total T and B cells, as well as regulatory T cells, was not impacted by ATF3 deficiency (Fig. 5B). The major characteristic of MDSCs is their suppressive activity (18). Aim to know whether the elevated CD11b*Ly6G*Ly6C- were granulocytic MDSCs (G-MDSC), co-culturing with autologous T cells experiments was performed. Results showed that splenic CD11b*Ly6G*Ly6C- from ATF3-/- mice were suppressive towards T cells, indicating that they were G-MDSCs. Consistent with previous report (19), the corresponding control iMCs from WT mice had no effect (Fig. 5C). The reactive oxygen species (ROS) content, as well as the expression of p47phox (component of the NADPH oxidase complex responsible for ROS production), was significantly higher in G-MDSCs from ATF3-/- mice than that from WT control (Data not shown). Administration of a ROS inhibitor NAC completely abrogated the suppressive activity of MDSCs in ATF3-/- mice (Data not shown). These observations indicate that ATF3 deficiency facilitated the activation of MDSC in a ROS-dependent manner.

The apoptosis and proliferation of MDSC, revealed by Annexin-V or Ki67 staining, remained unchanged in the presence or absence of ATF3 (Data not shown), indicating that the elevation of G-MDSCs was not caused by cell survival. We next evaluated whether ATF3 regulates the development of G-MDSCs. Lentiviral over-expression of ATF3 caused a reduction in percentage of MDSCs generated from cultured bone marrow cells (Fig. 5D). MDSCs could be induced to differentiating into mature myeloid cells under proper conditions (20). Splenic CD11b*Gr1* with ATF3 deletion had clearly lower ability to differentiating into mature myeloid cells, when cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (Fig. 5E). These results collectively indicate that ATF3 represent a novel regulator in the development and activation of MDSCs.

**S100A9 is a novel target of ATF3 in G-MDSCs** - To investigate the mechanism
underlying the role of ATF3 in MDSCs, gene expression analysis was performed to screen the potential targets of ATF3 by qRT-PCR. S100A8 and S100A9, known to be involved in myeloid cell differentiation (21), were significantly upregulated in ATF3-deficient MDSCs, both at the mRNA and protein levels (Fig. 6A). Other MDSC-related genes, including GM-CSF and IL-6, did not show noticeable differences in the presence or absence of ATF3 (data not shown). The effect was further confirmed when lentiviral overexpression of ATF3 led to downregulation of S100A8 and S100A9 in MDSCs (Fig. 6B).

The amounts of S100A8/9 in serum from Dex treated-WT and ATF3−/− mice were elevated, as compared with WT controls (Fig. 6C). Heterodimer formed by S100A8 and S100A9, functioning as transcriptional targets of STAT3, has been demonstrated to be important in MDSCs accumulation and migration in cancer (22). The phosphorylation of STAT3 and other STAT proteins, however, remained largely unchanged in MDSCs, regardless of ATF3 expression (Fig. 6D), indicating that the regulation of S100A8 and S100A9 by ATF3 is independent of STAT3.

Further sequence analysis revealed two potential ATF/CRE consensus sites within the promoters of S100A8 and S100A9 (Fig. 6E). Chromatin immunoprecipitation (ChIP) assay showed that ATF3 bound to canonical ATF3 binding sites located in the promoters of S100A8 (-416 to -425 bp) and S100A9 (-1324 to -1331 bp) (Fig. 6E). Further luciferase reporter assays using 32D cell line confirmed that the regulation of S100A8/A9 by ATF3 is independent of STAT3.

S100A8 and S100A9 are transcriptional targets of ATF3, S100A9 play a major role in mediating the effect of ATF3 on MDSCs.

**S100A9 silencing alleviates GC-induced hepatosteatosis** - We next determined whether silencing S100A9 could alleviate hepatic steatosis under either Dex treatment or ATF3 deficiency conditions. For this purpose, lentiviral particles carrying S100A9-specific short hairpin (sh) RNA or scrambled shRNA (Ctrl), were intravenously injected into ATF3−/− or Dex-treated WT mice. Knockdown of S100A9 in MDSCs was confirmed by western-blot (Fig.7A). A clear reduction of G-MDSCs, in both the liver and immune organs was observed upon S100A9 silencing in ATF3−/− mice (Fig.7B) and Dex-treated WT mice (Data not shown). The lipid accumulation in the liver and serum was alleviated as expected (Fig.7C-D). Meanwhile, Dex failed to display any effect on hepatic steatosis after silencing S100A9 in WT animals (Data not shown). These results demonstrate that ATF3/S100A9 mediates the effect of MDSCs on hepatic steatosis caused by GC administration.

**TLR4−/− mice is sensitive to GC-induced hepatosteatosis** - Toll-like receptors (TLRs) signaling, especially TLR4, plays important role in mediating the anti-inflammatory and immunosuppressive effects of glucocorticoids (23). Activation of TLRs is associated with liver diseases including alcoholic liver injury, liver fibrosis, and liver cancer (24,25). We therefore investigated the potential role of TLRs in GC-induced hepatosteatosis. TLR4 was chosen due to its well-recognition in inflammation and liver diseases (26). Flow cytometric analysis showed that G-MDSCs (CD11b+Gr1high) populations were significant increased in TLR4−/− mice, upon Dex treatment (Fig. 8A), which is consistent with the results from in vitro culture (Fig. 8B). No difference was observed between TLR4−/− and WT controls mice (Fig. 8A-B). The development
of steatosis liver in Dex-treated TLR4−/− mice was further confirmed by staining with lipid-specific Oil-Red O and hematoxylin/eosin (Fig. 8C). These observation showed that deficiency of TLR4 failed to impair the effects of Dex on G-MDSCs and fatty liver, indicating that TLR4 is dispensable for the GC-induced hepatosteatosis. However, we have to point out that the possibility of other TLRs in GC-induced hepatosteatosis could not be excluded at this time.

In summary, we uncovered the mechanism underlying the role of MDSCs in GC-induced hepatic steatosis. Upon activation by GC, GR represses ATF3 transcription by binding to nGRE (Negative Glucocorticoid Responses Element), which leads to transactivation of S100A9, the important pro-inflammatory molecule and regulator of myeloid cell differentiation. The induction of S100A9 causes expansion and activation of G-MDSCs and hepatic lipid accumulation. Therefore, our study uncovers an important role of G-MDSCs in GC-induced hepatic steatosis, in which ATF3/S100A9 is the dominant signaling event. 

**DISCUSSION**

GC is widely prescribed anti-inflammatory and immunosuppressive medicine in clinic. The side effects of GC, mostly dysregulated lipid metabolism and fatty liver, remains the major concern in patients (27). Understanding the mechanism of GC-induced hepatic steatosis is expetely to provide intervention target to avoid this side effect. In this study, we demonstrated that G-MDSCs represent a novel player in promoting GC-induced hepatic steatosis, in which ATF3/S100A9 is the dominant signaling event.

MDSCs, characterized by their immunosuppressive capability, has been well-studied in a variety of diseases, especially in cancer (7). Studies have shown that liver is one of the major sites of MDSCs, in addition to the immune organs (28). Accumulation of MDSC in liver has been observed in tumor-bearing hosts, HBV-infected individuals, and high-fat-diet-fed animals (4)(29,30). Also, hepatic stellate cells were reported to support the expansion of MDSCs in vitro (31). Our study reveals the importance of MDSCs in the modulation of lipid metabolism in liver. These studies collectively support that MDSCs are active player in hepatic immunity.

The origin of hepatic MDSCs remains to be elucidated. It is possible that MDSCs could migrate to the liver from bone marrow in response to chemokines released by hepatocytes or liver stroma cells; Alternatively, MDSCs could differentiate from hematopoietic stem or progenitor cells residing in the liver. In this study, we found that adoptive transfer of MDSCs resulted in their homing to the liver and spleen, which supports the first possibility. However, MDSC differentiation within the liver can not be excluded.

The exact role of MDSCs in liver is context dependent. MDSC could facilitate tumor escape by down-regulation the anti-tumor immunity in both liver and the circulation (32). In other aspect, MDSCs in liver could protect against Con A-induced T cell-mediated hepatitis (33). Accumulated immature myeloid cells in mouse liver caused by high-fat diet could induce the apoptosis of natural killer cells by releasing some pro-inflammatory cytokines (5). In this study, we found that G-MDSCs in liver could promote lipid accumulation and fatty liver, in the GC-treatment. The mechanism underlying MDSCs-promoted lipid dysregulation deserves further investigation. It is plausible that MDSCs could secrete some pro-inflammatory cytokines, such as S100A9, and cause inflammatory responses and the dysfunction of hepatocytes, which finally leads to lipid accumulation in liver. Cumulatively, these findings indicate that the beneficial or
G-MDSCs contributes to drug-induced hepatic steatosis
detrimental activities of MDSCs in the liver may be context-dependent.
S100A9 belongs to the S100 family of calcium-binding proteins and functions as a pro-inflammatory danger signal transducer in the form of a homodimer or heterodimer with S100A8. The S100A9 protein is released by myeloid cells in response to cell damage, infection, or inflammation (34). Functioning as transcriptional target of STAT3, S100A9 could facilitate MDSC accumulation and migration in cancer (21). Our study show that S100A9 can be transrepressed by ATF3, a well-known stress responsive protein. This reveals a novel mechanism of S100A9 regulation in myeloid cells, suggesting that expression of S100A9 may be responsive to stress.

ATF3 has been demonstrated as key intersection point in linking lipid metabolism, inflammation and immune responses. ATF3 functions as a negative regulator of the inflammatory responses in macrophages by antagonizing Toll-like receptor (TLR)-induced NF-κB signaling (35), and ATF3 deletion increases lipid accumulation and foam cell formation through epigenetic repression of the cholesterol 25-hydroxylase gene Ch25h (36). ATF3 has been demonstrated to mediate the anti-inflammatory and metabolic activities of high-density lipoprotein (HDL) by downregulating TLR-induced proinflammatory cytokines (37). Here we provide novel evidence that ATF3 play an important role in linking lipid metabolism and immunity through regulation of MDSC development and activation.

The role of ATF3 in neutrophils biology has recently been reported. ATF3 was shown to be important for neutrophil migration in the lungs via transrepression of CXCL1 chemokine (38). Another study reported that ATF3 functions as a novel negative regulator of neutrophil differentiation by modulating Ly6G expression (39). Consistently, we found that ATF3 deletion promoted the increase in CD11b’Ly6G’ cells and their acquisition of immunosuppressive functions, which characterizes this cell population as G-MDSCs, not neutrophils. The accumulation of G-MDSCs in ATF3-deficient mice was also confirmed in tumor-bearing mouse model (data not shown). Our study provides further insights into the role of ATF3 in the differentiation of myeloid cells.

The clinical significance of our data remains to be investigated. The evaluation of the MDSC population in liver biopsies of patients with GC-induced hepatic steatosis would be helpful; however, given that GC-treated patients usually suffer from complicated disorders and may take other medications, the results of clinical studies should be carefully analyzed in view of confounding factors.

EXPERIMENTAL PROCEDURES

Mouse strain- All experiments performed on mice were approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University. ATF3−/− mice (background: C57BL/6) were kindly provided by Dr. Tsonwin Hai (Ohio State University, Columbus, Ohio, USA). Female TLR4−/− mice (background: C57BL/6) mice and the congenic female C57BL/6SJL strain (C57BL/6, CD45.1 alloantigen) were obtained from Nanjing Bionedical Research Institute of Nanjing University and The Jackson Labs (Bar Harbor, ME), respectively. All mice were housed in a specific pathogen-free facility, and age matched wild type littermates (6-8 weeks) were used as controls. All mice were given normal feed diet.

Reagents- Dexamethasone, mifepristone (RU486), N-Acetyl-L-cysteine (NAC), L-Arginine, ConA and dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO). GM-CSF was obtained from Peprotech (Rocky Hill, NJ). The antibodies against ATF3, S100A8, S100A9, P47phox, Arg1, STAT6(Tyr641,#9361), p-STAT6, GR(CST:#12041), β-actin and
HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) or Cell Signaling Technology. NW-hydroxy-nor-Arginine (nor-NOHA) and L-NG-monomethyl-Arginine (L-NMMA) were obtained from BioVision (Milpitas, CA). The following fluorescein-conjugated anti-mouse antibodies: CD11c-PE-Cy5, MHC-II-PE, Gr-1-PE-Cy7, Gr-1-PE, Ly-6C-PerCP-Cy5.5, Ly6G-PE, B220-PE, CD11b-FITC, CD11b-PE-Cy7, F4/80-PerCP-Cyanine5.5, CD3e-FITC, CD4-PE, CD25-PE-Cy7, CD8a-PE-Cy5, CD8a-PE-Cy7, FITC-p-STAT3 (Tyr705(D3A7), #9145), PE-cy7-p-STAT5(Tyr694(D47E7), #4322) and the corresponding IgG isotype antibodies were obtained from eBioscience (San Diego, CA). BrdU (5-bromo-2-deoxyuridine; BD). S100A8 and S100A9 neutralizing antibodies were purchased from R&D Systems (Minneapolis, Minn). The shRNAs for S100A9 and the control shRNA were purchased from Cell Signaling Technology (Beverly, MA). In vivo-jetPEITM, PolyPlus-Transfection, Illkirch, France. Lipofectamine 2000, 5,6 carboxyfluorescein diacetate succinimidyl ester (CFSE) and the reagents for cell culture were from Invitrogen (Carlsbad, CA). Anti-Ly6G antibody (IA8; BD,Biosciences), or anti-IgG antibody (BD,Biosciences).

Flow cytometric analysis and sorting- Flow cytometric analysis and sorting was performed as described previously (40). Briefly, single-cell suspensions were prepared and were stained with fluorochrome-conjugated antibodies. Data were collected on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR). Data were acquired as the fraction of labeled cells within a live-cell gate set for 50000 events. For flow cytometric sorting, cells were stained with specific antibodies and isolated on a BD FACS Aria cell sorter (BD Bioscience). The purity after sorting was greater than 95%.

In vivo Dex treatment- Female 8-week-old mice were intraperitoneally injected with Dex (1mg/kg body weight/daily, 5 days) (41). The symptoms of hepatic steatosis and the levels of immune cells were analyzed on day 6. For blocking of T cell apoptosis, IL-7 (5 µg/kg body weight) were intraperitoneally injected with or without Dex (42).

MDSC adoptive transfer- Adoptive transfer of G-MDSCs or M-MDSCs was performed as described previously (43). Briefly, MDSC subsets were isolated from spleen of ATF3-KO or Dex-treated WT mice by flow cytometric sorting, followed by tail vein injection (3×10⁶) into WT recipients on days 1 and 3. The symptoms of hepatic steatosis were evaluated on day 6. G-MDSCs (3×10⁶) from Dex-treated WT (WT-Dex), or Veh-treated ATF3-KO mice were adoptively transferred into WT CD45.1 receipts mice, and receipts mice were injected with Brdu at days 5. The levels of CD45.2⁺ cell, CD11b⁺Gr1⁺ cell and Brdu⁺ cell were evaluated by flow cytometric.

MDSC depletion- G-MDSC depletion was performed as described previously (44). Briefly, anti-Ly6G antibody (1A8) or anti-IgG antibody (rat IgG2b) were injected (120 µg per injection) through tail vein on days 0 and 3 at post-Dex injection. The levels of immune cells and hepatic steatosis were evaluated on day 6.

In vivo S100A9 shRNA infection- Lentiviral particles carrying S100A9 specific short hairpin (sh) RNA (scrambled control shRNA as control) were prepared. A commercially available cationic polymer transfection reagent (in vivo-jetPEITM) was used to deliver lentivirus via intravenous injection. Briefly, 150 µg of lentiviral particles diluted in 200µL 5% glucose solution was mixed with vivo-jetPEI solution. The mixture was incubated for 15 min at room temperature, and then was injected into mice (45).
knockdown efficiency was determined by RT-PCR.

Quantitative reverse transcription (qRT)-PCR: Total RNA was extracted with TRIzol (Invitrogen), and was subjected to reverse transcription using reverse transcriptase (TAKARA) at 42 °C-15 min, and the resulting cDNA was amplified by PCR using gene-specific primers (40). The primers were designed based on genome-wide using the Primer 3 program and cross-checked by a BLAST search of the NCBI data base. The qRT-PCR primers are following as:

- S100A8-for (NM_009114.3):
  5' -ATGCCGTCTACAGGGATGAC-3'
- S100A8-rev:
  5' -ACTGAGGACACTCGGTCTTA-3';
- S100A9-for (NM_009114.3):
  5' -GGACCTGGACACAAATGCAG-3'
- S100A9-rev:
  5' -ACCCTCGTGCA TCTTCTCG-3';
- AT5F-for (NM_007498):
  5' -TTTGCTAACCTGACACCCTTG-3'
- AT5F-rev:
  5' -AGAGGACATCCGATGGCAGA-3';
- β-actin-for (NM_007393.5):
  5' -TACCACAGGCATTGTGATGG-3'
- β-actin-rev:
  5' -TTTGATGTCACGCACGATTT-3'.

Luciferase activity analysis: Rat ATF3 promoter reporter pATF3/Luc vector, pS100A8/Luc and pS100A9/Luc vector were constructed by pGL3/Luc plasmid. Corresponding site-mutated plasmids were constructed by the two-step PCR method. Lipofectamine 2000 (Invitrogen) was used to transfect 32D cells according to the technical manual. Twenty-four hours post-transfection, cells were treated with Veh (DMSO) or Dex in DMEM only for 48 h. Cells were then harvested, and their luciferase activities were measured with the Dual-Luciferase Reporter Assay kit (Promega) according to the technical manual. Cloning primers used were:

- ATF3-reporter-for: 5' -TCTGAGGGAGTGAGACTGAGGC-3';
- S100A8-reporter-for:
  5' -CGCTGAGTGACACTGTGGC-3' and S100A8-reporter-rev:
  5' -CACCTGCAACAAACTGAGGACAC-3';
- S100A9-reporter-for:
  5' -GGCAGGTTAGCTGTCTTG-3' and S100A9-reporter-rev:
  5' -GGCAGGTTAGCTGTCTTG-3';

Chromatin immunoprecipitation (ChIP) assay: In order to test whether activated GR could bind to nGRE sequence within ATF3 promoter, WT or ATF3−/− BM cells were treated with Dex for 3 d. For proved that ATF3 could bind to S100A8/A9 promoter area, WT BM cells were infected lentivirus carrying over-expressed ATF3 for 3 d. After treatment, cells were fixed in 1% paraformaldehyde for 10 min at room temperature and harvested in cell lysis buffer containing protease inhibitors (Millipore). Nuclei were collected and resuspended in shearing buffer [10 mM Tris-HCl (pH 8), 1 mM EDTA, 140 mM NaCl, 1% SDS, 0.1% Nadeoxycholate, 1% Triton X-100, protease inhibitor mixture]. DNA was sheared by sonication using a Branson Sonifier. Protein-DNA complexes were immunoprecipitated overnight with equivalent amounts of specific antibody or anti-IgG. Immunoprecipitated protein-DNA complexes were recovered with protein A magnetic beads (Millipore), washed, and eluted. After the cross-links were reversed, the DNA was purified using a QIAquick DNA purification kit (Qiagen). ChIP DNA samples were then subject for real-time qPCR analysis with the following primers:

- ChIP-ATF3-for: 5' -CTGGGATAGGCGTTTCTC-3' and ChIP-ATF3-rev5' -CCAAGTCGTACCTGGATAGA-3';
- ChIP-A8-site1-for: 5' -TGGCAACTCTGGAAAGGAA-3' and ChIP-A8-site1-rev5' -CGCTAAGGAGAGGCTT-3';
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ACACTG-3';
ChIP-A8-site2-for:5'-TACCCATCTAAATAG
AACCACA-3' and
ChIP-A8-site2-rev:5'-CGCTAAGGAG
AGCCTACACT-3';
ChIP-A9-site1-for:5'-GCTGGGAATTTCAC
AAAACA-3' and
ChIP-A9-site1-rev:5'-GTTAGATACATGGTT
ATCTCG-3';
ChIP-A9-site2-for:5'-GGAACCCTTGA TGC
CCAGCCC-3' and
ChIP-A9-site2-rev:5'-TTCTCCTTGTACCT
TCTGA-3';

Statistical analysis- Statistical tests were performed using GraphPad Prism version 5.0a software and SPSS 17.0. Unpaired Student t tests and analysis of variance (ANOVA) were used to confirm most comparisons. Correlations between different parameters were analyzed using the Spearman rank test. *:p<0.05, **:p<0.01, was considered significant.

Acknowledgement
We thanks Dr. Tsonwin Hai from Department of Molecular and Cellular Biochemistry, Ohio State University (USA), for kindly providing the ATF3-KO mouse strain. We thanks Dr. Hongliang Li from Collaborative Innovation Center of Model Animal, Wuhan University (China), for providing assistance in ATF3-KO mouse breeding.

Declaration of Interest statement:
The authors have no conflicts of interest to declare.

Author Contributions:
Jie Zhou supervised the study, design the experiments and wrote the manuscript. Yu-feng Liu performed the experiments, participated in the experiments design and manuscript writing; Jian-yang Wei, Mao-hua Shi, and Hua Jiang participated in the flow cytometric analysis and animal experiments. All the authors approved the submission of the manuscript

REFERENCES:


**FOOTNOTES**
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The abbreviations used are: MDSCs, myeloid-derived suppressor cells; Dex, dexamethasone; GC, glucocorticoid; ATF3, activating transcription factor 3; RLU: relative luciferase unit; nGRE, negative glucocorticoid response elements.

**FIGURE LEGENDS**

**FIGURE 1: GC-induced hepatic steatosis is dependent on ATF3 deficiency**
WT and ATF3⁻/⁻ mice (n=6) were injected with dexamethasone (Dex, 1 mg/kg of weight/day) or DMSO (Veh) for 5 days. (A) The ratio of liver to body weight, and lipid contents in the serum and liver were measured. TG, triglyceride; Chol, cholesterol; FFA, free fatty acids. Data represent the mean ± SEM of all mice analyzed. *: p<0.05, **: p<0.01, unpaired t tests. (B) Representative results of Oil-Red O staining and hematoxylin and eosin (HE) staining of liver section. Original magnification, ×200.

**FIGURE 2: GC-induced MDSC accumulation is dependent on ATF3 deficiency**
(A-B) WT and ATF3⁻/⁻ mice were injected with dexamethasone (Dex) or DMSO (Veh) for 5 days (n=6). (A) The levels of MDSC subsets in bone marrow (BM), spleen (SP), peripheral blood (PB) and liver were evaluated by flow cytometric analysis. (B) The population of DC (CD11c⁺MHCII⁺) and macrophage (CD11b⁺F4/80⁺) subsets in BM were evaluated by flow cytometric analysis. (A, B) The left flow panels are representative results, the right graphs were mean ± SEMs from all mice analyzed. (C) BM cells were cultured in medium containing GM-CSF, in the presence of Dex or Veh. The frequencies of MDSCs (CD11b⁺Gr1⁺) and DCs (CD11c⁺MHCII⁺) were analyzed by flow cytometry on day 5. Both representative results (left) and mean ± SEMs from 3 independent experiments (right) were shown. *: p<0.05, unpaired t tests.

**FIGURE 3: ATF3 mediates the effect of G-MDSCs on GC-induced hepatic steatosis**
(A-B) G-MDSC adoptive transfer: G-MDSCs (3×10⁶) from WT, Dex-treated WT (WT-Dex), or ATF3⁻/⁻ mice were adoptively transferred into WT receipts (n=6), mice were sacrificed at day 6. PBS was used as negative control. (A) The ratio of liver to body weight and the lipid contents; (B)
Representative Oil-Red O and hematoxylin/eosin (HE) staining of liver sections were shown; (C-E) G-MDSCs depletion assay: anti-Ly6G or anti-IgG antibodies were injected intravenously into Dex-treated WT or Veh-treated ATF3\(^{-/-}\) mice, as described in methods. (C) The proportions of MDSCs were evaluated by flow cytometric analysis; (D) The ratio of liver to the body weight, and lipid contents in the serum and liver tissue; (E) Representative Oil-Red O staining and hematoxylin and eosin (HE) staining of liver tissue. (B, E) Original magnification, ×200. (A, C, D) Data represent the mean ± SEMs of 6 mice per group of four independent experiments. *: p<0.05, unpaired t tests.

**FIGURE 4: ATF3 is transrepressed by GR in MDSCs**

(A) BM cells were treated with Dex at indicated concentrations, ATF3 expression was measured by qRT-PCR (upper) and WB (lower). (B) ATF3 expression in sorted MDSCs from BM, spleen (SP) and liver was determined by qRT-PCR (upper) and WB (lower). (C) BM cells were treated as indicated, ATF3 expression was measured by qRT-PCR (left) and WB (right). RU486: GR antagonist. (D) Left: Scheme of ATF3 promoter sequence with potential nGRE indicated; Right: As well as its site-directed mutant (Mut): ATF3-Luc activity: 32D cells transfected with pATF3/Luc plasmid were treated with Dex, and luciferase activity was measured using the Dual Luciferase Reporter Assay (Promega). RLU: relative luciferase unit. (E) ChIP assay was performed on BM cells from Dex treated WT or ATF3\(^{-/-}\) mice, using anti-GR or anti-IgG antibody, the presence of ATF3 promoter harbouring the nGRE was determined by qRT-PCR. Data were normalized against input and presented as fold increase over IgG control. (A-E) Results were graphed as mean ± SEM from 3 independent experiments; WB in (A-C) were representative data from 3 independent experiments, each sample was pooled from 3 mice. *: p<0.05, **: p<0.01, unpaired t tests were used.

**FIGURE 5: ATF3 regulates the development of G-MDSCs**

(A) The proportions of the indicated myeloid cell populations in WT and ATF3-KO mice were analyzed by flow cytometry. IMC (immature myeloid cells, CD11b\(^+\)Gr1\(^+\)), Granulo (granulocytes, CD11b\(^+\)Ly6G\(^+\)Ly6C\(^-\)), and Mono (monocytes, CD11b\(^+\)Ly6G\(^-\)Ly6C\(^+\)). (B) The proportions of indicated immune cells in WT and ATF3-KO mice were determined by flow cytometric analysis: DC (CD11c\(^+\)MHCII\(^+\)), macrophage (CD11b\(^+\)Gr1\(^-\)F4/80\(^+\)), Treg (CD4\(^+\)CD25\(^+\)), B cell (B220\(^+\)), CD4 T (CD3\(^+\)CD4\(^+\)), and CD8 T (CD3\(^+\)CD8\(^+\)). (C) Allogeneic mixed lymphocytes reaction (MLR). CD3\(^+\)T cells were stimulated with concanavalin A (ConA) and cocultured with allogeneic splenic CD11b\(^+\)Ly6G\(^+\) at different ratios for 3 days. T-cell proliferation was evaluated by carboxyfluorescein diacetate, succinimyl ester (CFSE) dilution, unstimulated T cells were used as negative control. (D) BM cells were infected with lentivirus expressing ATF3 or vector (Vec), and cultured in medium containing GM-CSF and IL6. The frequencies of MDSCs (Gr1\(^+\)CD11b\(^+\)), DCs (CD11c\(^+\)MHCII\(^+\)) and macrophages (CD11b\(^+\)F4/80\(^+\)) among GFP\(^+\) cells were analyzed by flow cytometry at day 6. Medium alone was used as control. (E) Splenic MDSCs were cultured with GM-CSF and IL-4 for 3 and 5 days, and the proportions of DCs (CD11c\(^+\)CD11b\(^+\)) and macrophages (CD11b\(^+\)F4/80\(^+\)) were determined. (A-D) Both representative data and mean ± SEM from 6 mice (A) or 3 independent experiments (B-D) were shown. *: p<0.05, **: p<0.01, unpaired t tests were used.

**FIGURE 6: S100A9 mediates the effect of ATF3 on G-MDSCs**

(A) Expression of S100A8/A9 in splenic MDSCs was determined by qRT-PCR (upper) and WB (lower). (B) BM cells were infected with lentivirus expressing ATF3 or vector (with GFP tag), gene expression in GFP\(^+\) cells was evaluated by qRT-PCR (upper) and WB (lower). (C) The levels of
S100A8/A9 protein in the serum from the indicated animals were measured by ELISA. (D) The phosphorylation of STAT3 and STAT5 in splenic MDSCs was measured by flow cytometry (left), the STAT6 phosphorylation was evaluated by WB (right). (E) Scheme of S100A8/A9 promoters with potential ATF3 binding sites as indicated. S100A8: site 1: -416 to -425bp, site 2: -954 to -961bp; S100A9: site 1: -506 to -515bp, site 2: -1324 to -1331bp. Over-expressed ATF3 lentivirus were infected with WT BM cells for 3 days, and then cells were fixed in 1% paraformaldehyde for ChIP assay. The presence of S100A8/A9 promoters harbouring the ATF3-binding sites was determined by qRT-PCR. Data were normalized against input and presented as fold increase over IgG control. (F) S100A8/A9-Luc activity: 32D cell transfected with pS100A8/Luc or pS100A9/Luc plasmid and then were infected with over-expressed ATF3 lentivirus. Luciferase activity was measured using the Dual Luciferase Reporter Assay (Promega). (G) BM cells were cultured with GM-CSF and IL6 in presence or absence of neutralizing antibodies against S100A8 or S100A9, and anti-IgG was used as control. The percentages of the indicated populations were analyzed by flow cytometry. (A,B,C,E,F,G) Results were mean ± SEM from 3 independent experiments; WB in (A, B, D) were representative data from 3 independent experiments, each sample was pooled from 3 mice. *: p< 0.05, **: p< 0.01, unpaired t tests were used.

**FIGURE 7: S100A9 silencing alleviates GC-induced hepatosteatosis**

Lentiviral particles carrying S100A9-shRNA or scrambled control were intravenously injected into ATF3−/− mice, and then mice were injected with dexamethasone (Dex) or DMSO (Veh). (A) Knockdown of S100A9 expression in sorted MDSCs was determined by WB. (B) The proportions of MDSC subsets were determined by flow cytometric analysis, both representative data (left) and mean ± SEM from 6 mice (right) were included. (C) Ratio of liver to the body weight, and the lipid contents in the serum and liver tissue were measured. Data were presented as mean ± SEM from 6 mice. (D) Representative Oil-Red O and hematoxylin and eosin (HE) staining of liver sections. Original magnification, ×200. *: p< 0.05, unpaired t tests were used.

**FIGURE 8: TLR4−/− mice is sensitive to GC-induced hepatosteatosis**

(A) WT, TLR4−/− mice were injected with dexamethasone (Dex) or DMSO (Veh) for 5 days (n=6). The levels of MDSCs subsets in bone marrow (BM) and liver were evaluated by flow cytometric analysis. The left flow panels are representative results, the right graphs were mean ± SEMs from all mice analyzed. (B) BM cells were cultured in medium containing GM-CSF, in the presence of Dex or Veh. The frequencies of MDSCs were analyzed by flow cytometry on day 5. (C) Representative results of Oil-Red O staining and hematoxylin and eosin (HE) staining of liver section. Original magnification, ×200. *: p< 0.05, unpaired t tests were used. (D) Scheme depicting ATF3 mediated GC-induced hepatic steatosis: The mechanism underlying the role of MDSCs in GC-induced hepatic steatosis. Activation of GR by GC represses ATF3 transcription by binding to nGRE, which leads to transactivation of S100A9. The induction of S100A9 causes the expansion and activation of G-MDSCs and hepatic lipid accumulation.
Figure 1

A

![Graphs showing liver/body weight and serum TG, Chol, and FFA levels for WT and ATF3−/− mice with Veh and Dex treatments.]

B

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G-MDSCs contributes to drug-induced hepatic steatosis.
G-MDSCs contributes to drug-induced hepatic steatosis
Figure 3

A

Liver/body

Serum triglycerides

Liver triglycerides

B

PBS  WT-Veh  WT-Dex  ATF3

Oil red O

HE

C

WT-Dex

ATF3

IgG  Ly6G

BM

2.54  0.5

SP

Liver

G1

CD11b

D

Liver/body

Serum triglycerides

Liver triglycerides

E

WT-Dex

ATF3

IgG  Ly6G

Oil red O

HE
G-MDSCs contributes to drug-induced hepatic steatosis
G-MDSCs contributes to drug-induced hepatic steatosis

Figure 5

A

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% of cells
G-MDSCs contributes to drug-induced hepatic steatosis

![Figure 6](image-url)

- **A**: Relative mRNA level of S100A8 and S100A9 in WT and ATF3-/-.
- **B**: Relative mRNA level of S100A8, S100A9, and ATF3 in Vector and ATF3.
- **C**: Serum levels of S100A8 and S100A9 in Veh and Dex.
- **D**: Western blot analysis of p-STAT3 and p-STAT5 in WT and ATF3-/-.
- **E**: S100A8 promoter binding sites and enrichment analysis.
- **F**: Fold enrichment and RLU of S100A8 in WT and ATF3.

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Figure 7

A

G-MDSCs contributes to drug-induced hepatic steatosis

B

C

D

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G-MDSCs contributes to drug-induced hepatic steatosis

Figure 8

A

B

C

D


G-MDSCs

S100A8

GR

ATF3

GR bind on ATF3 promoter

GCs

nGRE

Veh

Dex

WT

TLR4−/−

Veh

Dex

WT

TLR4−/−

Veh

Dex

WT

TLR4−/−

Oil Red O

HE

Fatty liver

G-MDSCs

S100A8

ATF3

nGRE

GR

GCs

Veh

Dex

WT

TLR4−/−

Veh

Dex

WT

TLR4−/−

Veh

Dex

WT

TLR4−/−

Veh

Dex

WT

TLR4−/−

Veh

Dex

WT

TLR4−/−
Glucocorticoid induces hepatic steatosis by inhibiting ATF3/S100A9 signaling in granulocytic MDSCs

Yu-Feng Liu, Jian-Yang Wei, Mao-Hua Shi, Hua Jiang and Jie Zhou

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