Immunology and Inflammation

Leucine alleviates cytokine storm syndrome by regulating macrophage polarization via the mTORC1/LXRα signaling pathway

Hui Yan, **Yao Liu, Xipeng Li, Bing Yu, Jun He, Xiangbing Mao, Jie Yu, Zhiqing Huang, Yuheng Luo, Junqiu Luo, Aimin Wu, Daiwen Chen**

Key Laboratory of Animal Disease-Resistance Nutrition of China Ministry of Education, Key laboratory of Animal Disease-resistant Nutrition and Feed of China Ministry of Agriculture and Rural Affairs, Key laboratory of Animal Disease-resistant Nutrition of Sichuan Province, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

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Abstract

Cytokine storms are associated with severe pathological damage and death in some diseases. Excessive activation of M1 macrophages and the subsequent secretion of pro-inflammatory cytokines are a major cause of cytokine storms. Therefore, promoting the polarization of M2 macrophages to restore immune balance is a promising therapeutic strategy for treating cytokine storm syndrome (CSS). This study was aimed at investigating the potential protective effects of leucine on lipopolysaccharide (LPS)-induced CSS in mice and exploring the underlying mechanisms. CSS was induced by LPS administration in mice, which were concurrently administered leucine orally. In vitro, BMDMs were polarized to M1 and M2 phenotypes with LPS and IL-4, respectively, and treated with leucine. Leucine decreased mortality in mice treated with lethal doses of LPS. Specifically, leucine decreased M1 polarization and promoted M2 polarization, thus diminishing pro-inflammatory cytokine levels and ameliorating CSS in mice. Further studies revealed that leucine induced macrophage polarization through the mechanistic target of rapamycin complex 1 (mTORC1)/liver X receptor α (LXRα) pathway, which synergistically enhanced the expression of the IL-4-induced M2 marker Arg1 and subsequent M2 polarization. In summary, this study revealed that leucine ameliorates CSS in LPS mice by promoting M2 polarization through the mTORC1/LXRα/Arg1 signaling pathway. Our findings indicate that a fundamental link between metabolism and immunity contributes to the resolution of inflammation and the repair of damaged tissues.

eLife assessment

The study has added value to what we have already known in the potential pharmacological immunomodulatory therapies in LPS-induced sepsis, and especially the use of oral leucine might be of great interest to the readers engaged in this field. We believe this study is **important** and provides **solid** evidence on the potential use of leucine in sepsis.

Introduction

Cytokine storm syndrome (CSS), characterized by severe systemic inflammation, is the leading cause of death in certain infectious diseases, such as Coronavirus disease 2019 and acute respiratory distress syndrome (Kim et al., 2021; Ramasamy and Subbian, 2021; Wang et al., 2020). Acute overwhelming inflammation caused by CSS is characterized by elevated levels of circulating cytokines and hyperactivation of immune cells, particularly macrophages (Grom et al., 2016). Macrophage hyperactivation markedly increases the levels of many proinflammatory cytokines, such as IL-1β, IL-6, TNFα and IFNγ, thereby leading to a hyperinflammatory state associated with high mortality (Schulert and Grom, 2015). Macrophages are broadly classified into two categories according to their function: M1 (classical) and M2 (alternative). M1 triggers a rapid proinflammatory response to infection and tissue damage, whereas M2 exhibits anti-inflammatory and reparative activity, participating in inflammation remission and tissue repair (Covarrubias et al., 2015; Ginhoux et al., 2016; Murray et al., 2014). Therefore, targeting macrophages to achieve a precise balance of anti-inflammatory and pro-inflammatory activity is a major avenue for the treatment of CSS. Currently, the main approach to treating CSS involves mitigating excessive immune responses through broad (e.g., glucocorticoid) and targeted (e.g., anti-cytokine) approaches (Schulert and Grom, 2014). However, those approaches elicit numerous adverse effects, such as hypertension and liver damage. Nutritional intervention is a relatively safe approach to alleviate CSS.

Macrophage polarization to M1 or M2 phenotypes has distinct metabolic requirements, in which the mechanistic target of rapamycin complex 1 (mTORC1) pathway plays a key role (Kang et al., 2018). mTORC1 is an important metabolic regulator that controls cell proliferation, differentiation, and immunity by sensing cellular energy status, nutrients, and external stimuli (Dibble et al., 2012). Amino acid restriction reprograms macrophage function through an mTOR-centric cascade (Orillion et al., 2018). Leucine, an important essential amino acid, is also a critical mTORC1 regulator (Jewell et al., 2015). The mTORC1 pathway plays a key role in controlling macrophage polarization (Byles et al., 2013). Recent studies have indicated that leucine blocks macrophage infiltration in obese animals, thus decreasing levels of the proinflammatory cytokine TNF-α and the macrophage marker $F4/80^{+}$ in adipocytes(Macotela et al., 2011). However, the mechanism through which leucine alleviates inflammation is unclear.

In our study, we explored the effects of leucine on LPS-induced CSS and elucidated the essential roles of leucine in CSS pathogenesis. We observed that leucine decreased mortality in mice treated with lethal doses of LPS. In addition, leucine decreased the expression and secretion of inflammatory factors in the serum and tissues of mice. *In vitro* data further confirmed that leucine inhibited LPS-driven M1 polarization and promoted M2 polarization through the mTORC1 signaling pathway, and leucine promoted M2 macrophage polarization through enhancing the expression of LXRα induced by IL-4. Thus, our findings revealed a basic link between metabolism and immunity, in which leucine, via mTORC1/LXRα/Arg1, regulates the polarization of macrophages to M2 and alleviates inflammation.

Results

Leucine improves survival and inhibits cytokine storm in LPS-induced endotoxemic mice

Firstly, we determined whether leucine prevents LPS-induced acute endotoxemia by i.p. injection of 25 mg/kg LPS. The injection of a lethal dose of LPS caused the death of all mice within 72 hours. Providing 2% leucine from feed or 5% leucine from water improved survival rate of LPS-injected mice to 34% and 60%, respectively (**Figure 1A**). Thus, leucine appeared to protect against LPS-induced acute endotoxemia in mice.

LPS-induced endotoxemia and death are partially due to systemic and local inflammation. We next investigated the effect of leucine on acute inflammation caused by non-lethal doses of LPS in mice. Mice fed leucine in the feed, drinking water, or a combination of both exhibited higher body weight gain than control mice before LPS stimulation (**Figure 1B**). In addition, LPS, compared with the control, significantly increased levels of the proinflammatory cytokines IL-6, IFN-γ and TNF-α in both the serum and liver after 6-hour i.p. injection. Mice administered 2% leucine in the feed, 5% leucine in the drinking water, or a combination of both, compared with the LPS group, showed significantly lower cytokine levels in the serum and liver (**Figure 1C-D**). We also investigated expression of inflammation related markers in various tissues. Compared with that in the LPS group, the expression of IL-6, IL-1β, NLRP3, MCP1, and iNOS in liver and spleen was significantly lower in mice receiving 2% leucine from the feed, 5% leucine from the drinking water, or a combination of both (**Figure 1E-G**). In addition, mice receiving 2% leucine from the feed, 5% leucine from the drinking water, or a combination of both, compared with the LPS group, showed lower expression of IL-6, IL-1β and MCP1, and higher expression of the anti-inflammation related markers Arg1, Mgl1 and Mgl2 in the bone marrow (**Figure 1E-G**).

Collectively, our results indicated that 2% leucine from feed, 5% leucine from drinking water, or a combination of both significantly inhibits proinflammatory cytokine production in mice with LPS-induced endotoxemia.

Leucine regulates macrophage polarization in endotoxemic mice

Inflammatory cytokines are derived primarily from a variety of immune cells, such as macrophages and neutrophils. Briefly, LPS i.p. injection resulted in significantly more white blood cells, neutrophils, monocytes, eosinophils, and basophils in the blood than observed in the control group. Providing leucine in the drinking water, feed, or both significantly decreased white blood cells, neutrophils, monocytes, eosinophils, and basophils (**Figure 2A**). Thus, the alleviation of inflammation and death in CSS might have been due to changes in immune cell populations and the regulation of immune cell differentiation by leucine.

Macrophages are derived from monocytes and are the main cells producing cytokines. Typically, macrophages polarize to pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes, depending on their microenvironment (Locati et al., 2020). Therefore, we determined how leucine modulates macrophage polarization by performing flow cytometry sorting in endotoxemic mice. The immune cell population was labeled by $CD45^+$, and $CD11b^+$ and $F4/80^+$ double-positive labeled macrophages were used. To determine the proportions of macrophage subsets, we used $CD86^+$ and $CD206^+$ as molecular markers for M1 and M2 macrophages, respectively (**Figure 2B**). Briefly, LPS i.p. injection significantly increased the percentage of $CD86^+$ and decreased the percentage of $CD206^+$ in both the bone marrow and spleen, thus suggesting that LPS i.p. injection led to a transition toward M1 polarization in mice (**Figure 2C-D**). In contrast, providing leucine in the drinking water, feed or both decreased the percentage of $CD86^+$ and increased the percentage of $CD206^+$, thus indicating that leucine ameliorated inflammation in mice and decreased macrophage M1 polarization, but markedly promoted M2 polarization (**Figure 2C-D**).

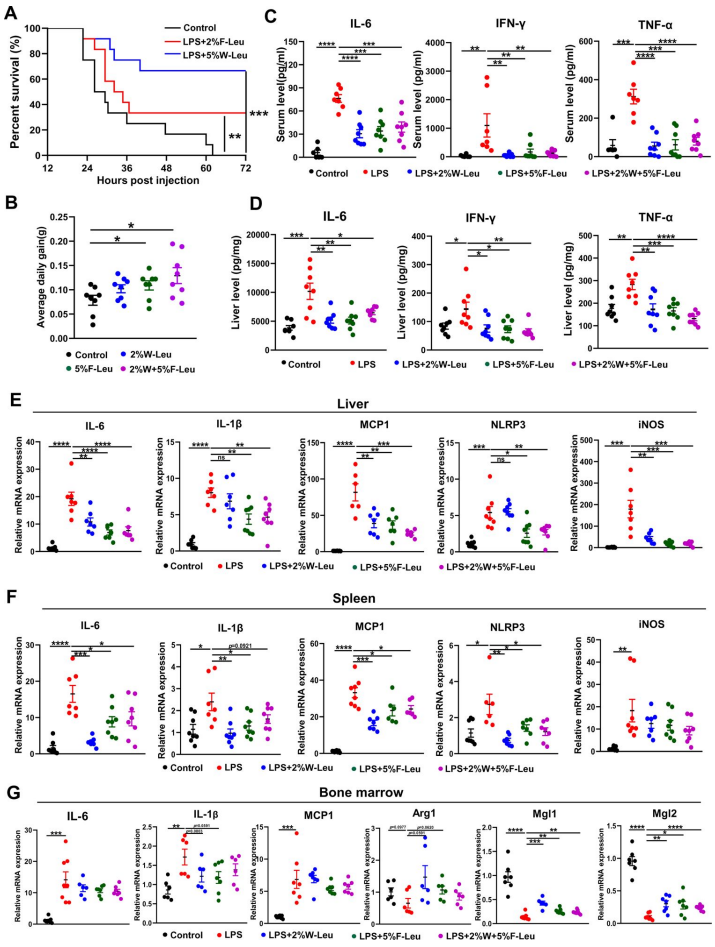

Figure 1.

Leucine ameliorates LPS-induced inflammation. (A) Kaplan-Meier curve showing survival of the mice (n=12). (B) Average daily weight gain of mice (n=8). (C-D) Measurement of IL-6, IFN-γ, and TNF-α secretion in mouse serum and liver by ELISA after treatment with LPS for 6 h. (E-F) mRNA expression of IL-6, IL-1β, NLRP3, MCP1, and iNOS, measured by RT-PCR in the liver and spleen. (G) mRNA expression of IL-6, IL-1β, MCP1, Arg1, Mgl1, and Mgl2, measured by RT-PCR in the bone marrow. Student's *t-test* was used to determine statistical significance, defined as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, and $****P < 0.0001$.

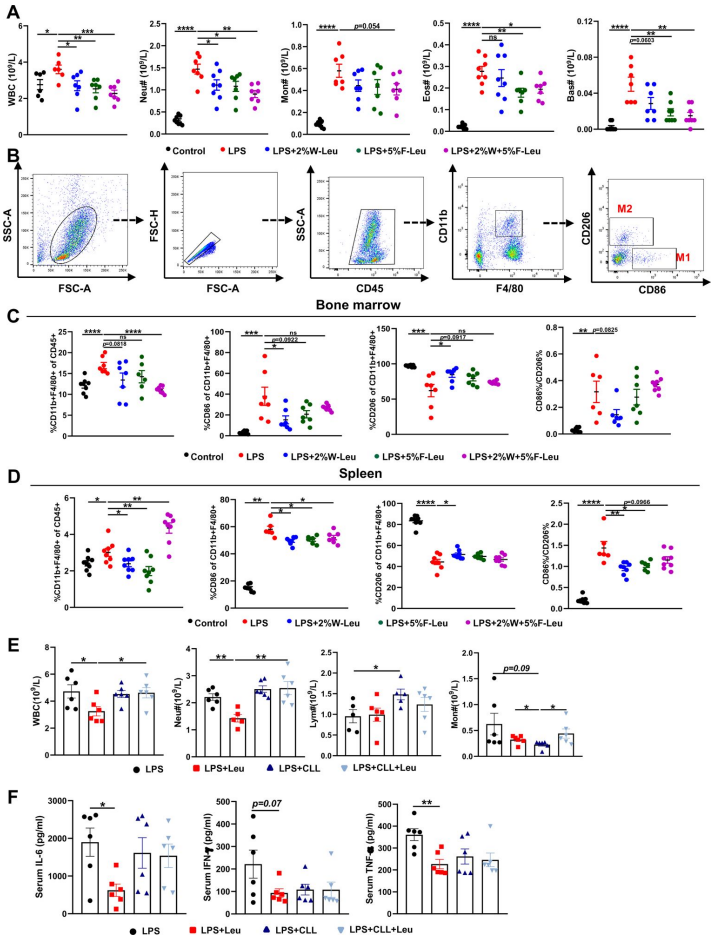

Figure 2.

Leucine inhibits M1 polarization and promotes M2 polarization in mice. (A) White blood cell composition and proportion in mice (n=8). (B) Gating strategy for macrophage flow cytometry in the bone marrow. (C) Percentages of $CD45^+$, $CD86^+$, $CD206^+$, and $CD86^+/CD206^+$, detected by flow cytometry in the bone marrow (n=8). (D) Percentages of $CD45^+$, $CD86^+$, $CD206^+$, and $CD86^+/CD206^+$, detected by flow cytometry in the spleen (n = 8). (E) White blood cell composition and proportion in mice (n=5-6). (F) Measurement of IL-6, IFN-γ and TNF-α secretion in mouse serum by ELISA after treatment with LPS for 6 h (n=6). Student's *t-test* was used to determine statistical significance, defined as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

Together, these results indicated that the anti-inflammatory effects of leucine were probably mediated by modulating macrophage polarization, through suppressing M1 polarization but promoting M2 polarization.

To further evaluated the role of macrophage in leucine-mediated alleviation of CSS, macrophages were depleted in mice by vein injection of clodronate-containing liposomes before administration of LPS. Macrophage depletion significantly eliminated the mitigating effect of leucine in endotoxemic mice by blood immune cells (**Figure 2E**) and serum inflammatory factors IL-1β, TNFα and IFN-γ (**Figure 2F**).

Leucine promotes M2 polarization in BMDMs

To further determine the effects of leucine on macrophage polarization, we induced BMDMs to differentiate into M1 or M2 macrophages through stimulation with LPS or IL-4, respectively (**Figure 3A**). LPS promoted M1 macrophage polarization, as indicated by increased release of the pro-inflammatory cytokines IL-6 and TNF-α, and induction of mRNA expression of IL-1β, TNFα, IL-6 and NLRP3 in BMDMs (**Figure 3B-C**). In LPS-stimulated cells, leucine decreased the secretion of IL-6 and TNF-α in culture supernatant and suppressed the expression of IL-1β and TNF-α, particularly at 10 mM concentration (**Figure 3B-C**). However, the mRNA expression of IL-6 and NLRP3, which is regulated by NFκB, was uninfluenced by leucine treatment (**Figure 3C**). To rule out that leucine treatment was toxic to cells and thus indirectly regulated the inflammatory response, we examined the effects of 2 mM and 10 mM leucine on cell viability. The results revealed that cell viability was increased after 6h and 24h of 2mM and 10mM leucine treatment, further suggesting that leucine was a direct inhibitor of inflammatory cytokine production (**Supplement Figure 2A**).

Next, we investigated the effects of leucine on M2 macrophage polarization. IL-4 promoted M2 macrophage polarization, as indicated by increased activity of arginase-1, and induction of mRNA expression of Arg1, Ym1, Fizz1, and Mgl2 in BMDMs. Arg1, a hallmark feature of M2 macrophages, competes with inducible nitric oxide synthase for L-arginine, and decreases nitric oxide synthesis, thereby preventing local inflammation and tissue repair (Arlauckas et al., 2018). In IL-4-stimulated cells, leucine increased the activity of Arg1 in the culture supernatant and promoted the protein expression of Arg1, particularly when administered at 10 mM concentration (**Figure 3D-F**). Additionally, leucine promoted the mRNA expression of Arg1, Ym1, Fizz1 and Mgl2, thus further validating that leucine promotes M2 polarization (**Figure 3G**). Together, these findings suggested that leucine promotes M2 polarization in BMDMs.

mTORC1 mediates leucine induced M2 polarization

M2 polarization involves activation of signal transducer and activator of transcription 6 (STAT6), which directly mediates the transcriptional activation of M2 macrophage-specific genes such as Arg1 (Goenka and Kaplan, 2011). In general, IL-4 activates the STAT6 signaling pathway and consequently promotes Arg1 expression, thus contributing to M2 polarization (Yang et al., 2021). However, our results indicated that leucine did not further activate STAT6; therefore, leucine did not promote M2 polarization through the STAT6 pathway (**Figure 4A**). A key effector of leucine is believed to activate the mTORC1 protein kinase (Cangelosi et al., 2022). In our study, inhibiting mTORC1 was found to suppress Arg1 expression and inhibit leucine-mediated M2 polarization (**Figure 4A** and **Supplement Figure 3A**). Notably, Torin1 inhibited M2 polarization more significantly compared to rapamycin. The above findings underscored the pivotal role of leucine in driving M2 polarization through the mTORC1 pathway.

To further confirm the role of leucine in regulating M2 polarization via mTORC1, we next directly detected the activity of arginase 1 in culture supernatants and cells. Inhibition of mTORC1 was followed by arginase 1 activity inhibition (**Figure 4B-C**). Moreover, the expression of the M2

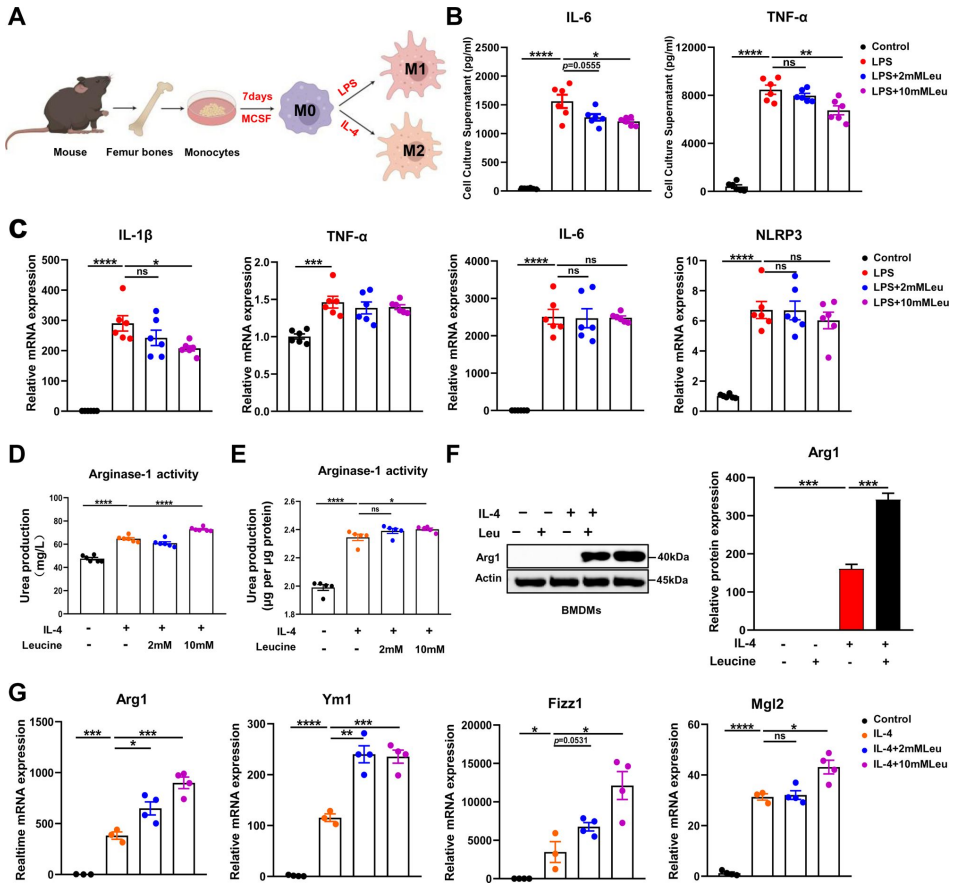

Figure 3.

Leucine promotes M2 polarization in BMDMs. (A) Schematic diagram of macrophage polarization. (B) Measurement of IL-6 and TNF-α secretion in cell culture supernatants by ELISA (n=6). (C) mRNA expression of IL-1β, TNF-α, IL-6, and NLRP3, measured by RT-PCR in BMDMs (n=6). (D-E) Detection of arginase-1 activity in the medium and BMDMs (n =5-6). (F) BMDMs isolated from mice were stimulated with leucine, IL-4, or both, and the protein expression of Arg1 was determined. (G) mRNA expression of Arg1, Ym1, Fizz1, and Mgl2, measured by RT-PCR in BMDMs (n=3-4). Student's *t-test* was used to determine statistical significance, defined as **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

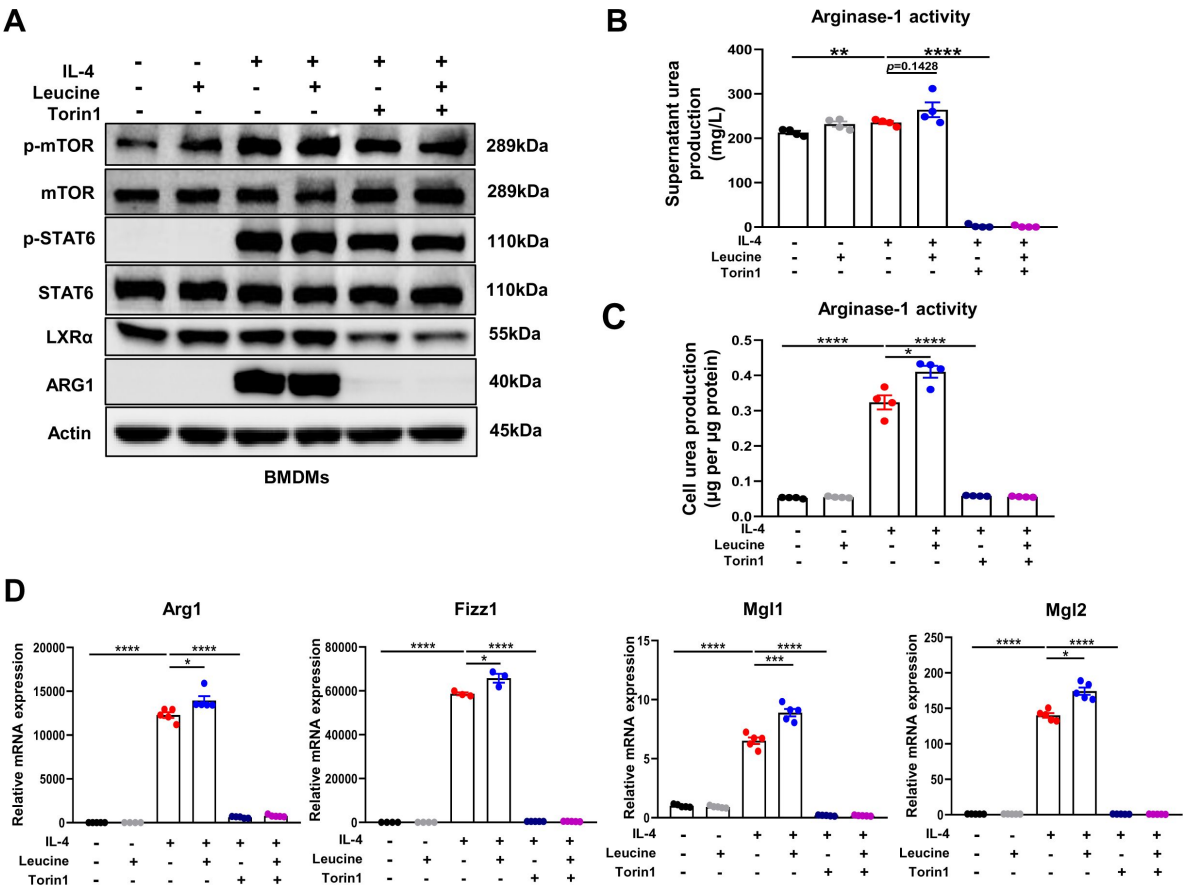

Figure 4.

mTORC1 signaling is necessary for M2 polarization. (A) Protein levels of ARG1, p-STAT6, STAT6, p-mTOR, and mTOR, determined by western blotting. (B-C) Detection of arginase-1 activity in the medium and BMDMs (n=4). (D) mRNA expression of Arg1, Fizz1, Mgl1, and Mgl2, measured by RT-PCR in BMDMs (n=3-5). Student's *t-test* was used to determine statistical significance, defined as **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

marker genes Arg1, Fizz1, Mgl1, and Mgl2 was also completely inhibited (**Figure 4D**). The above results again confirmed that the effect of leucine on M2 polarization occurs through mTORC1 signaling.

In addition, although inhibition of mTORC1 slightly inhibited p-STAT6 activation, it was insufficient to explain the complete inhibition of Arg1 caused by inhibition of mTORC1, thus indicating that mTORC1 regulates M2 polarization by mediating other pathways. Together, our findings suggested that leucine mediates M2 polarization via the mTORC1 pathway.

LXRα is essential for leucine-induced macrophage polarization

LXRα is a transcription factor for Arg1 in macrophages, and its activation enhances the expression of Arg1 (Pourcet et al., 2011). In our study, leucine promoted the protein expression of LXRα upon IL-4 activation of M2 macrophage (**Figure 5A**). The transcriptional regulation of Arg1 requires the entry of LXRα into the nucleus. Leucine increased the abundance of LXRα in the nucleus (**Figure 5B**) and also increased Arg1 expression (**Figure 5E**) in IL-4 stimulated cells. Moreover, inhibition of LXRα activity by GSK2033 muted the effect of leucine on Arg1 activity and mRNA expression of M2 macrohphage markers Fizz1, Mgl1 and Mgl2demonstrating that LXRα mediates the effect of leucine on M2 polarization (**Figure 5C-D**). Next, we inhibited LXRα in LPS-stimulated mice by i.v. injection of GSK2033, and found that the reduction of cytokine in serum and peritoneal fluid (**Supplementary Figure 4A**). These results showed that LXRα is essential for leucine-induced macrophage polarization.

Discussion

CSS is an uncontrolled and immune dysregulated immune response involving the sustained activation and expansion of macrophages, which secrete large amounts of cytokines, these cytokines in turn lead to overwhelming systemic inflammation and multi-organ failure with high mortality (Chen et al., 2020; Copaescu et al., 2020). An imbalance between pro-inflammatory and anti-inflammatory systems is the main cause of CSS, and macrophage polarization is important for maintaining immune homeostasis. M1 macrophages arise in inflammatory settings dominated by the interferon signaling associated with immunity to bacteria and intracellular pathogens, whereas M2 macrophages relieve inflammation and play important roles in fighting against and recovering from infection (Murray, 2017). Thus, therapeutic strategies that target macrophage polarization may be an approach to alleviate CSS.

In our study, leucine decreased LPS-induced inflammation and mortality. The uptake and metabolism of leucine regulated immune cell activation through the mTORC1 signaling pathway. Targeting leucine to manipulate immune responses have been suggested to be useful in the treatment of infections and autoimmunity (Ananieva Elitsa A et al., 2016). A previous study has found that leucine inhibits the expression of inflammatory factors in LPS-stimulated RAW 264.7 cells, but the specific mechanism has not been clarified (Lee et al., 2017). Our research further verified that leucine alleviates inflammation by inhibiting LPS-stimulated M1 polarization and promoting M2 polarization in both animals and BMDMs.

Macrophage polarization is critical for immune homeostasis. In our study, leucine not only decreased the LPS-mediated production of pro-inflammatory factors such as IL-6, IFN-γ, and TNF-α, but also promoted the expression of the M2 macrophage markers Mgl1 and Mgl2. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are sensitive indicators of liver damage. LPS led to significant increase in AST and ALT in both serum and liver, whereas leucine decreased AST and ALT levels, indicating that leucine mitigated tissue damage (**Figure supplement 1A**). Importantly, leucine significantly inhibited the high mortality caused by LPS. Therefore, we hypothesized that leucine might regulate macrophage polarization in LPS-induced

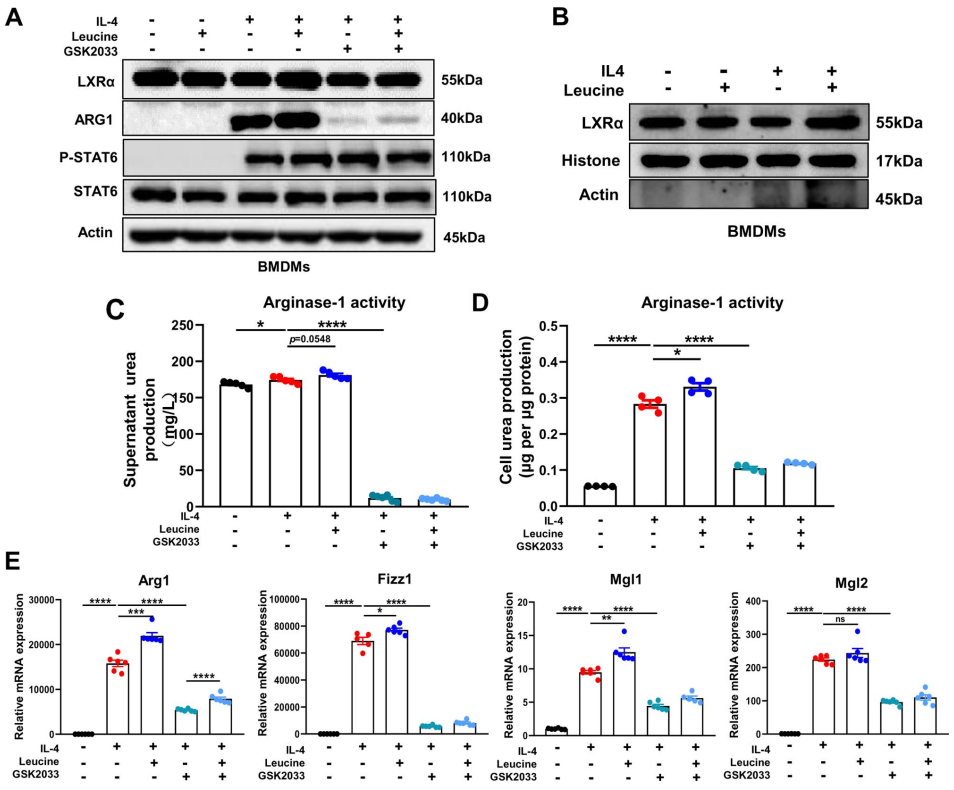

Figure 5.

Leucine promotes M2 polarization via mTORC1/LXRα signaling. (A) Protein levels of LXRα, ARG1, p-STAT6 and STAT6, determined by western blotting. (B) The nuclear proteins of BMDMs were extracted, and the protein levels of histones and LXRα were determined by western blotting. (C-D) Detection of arginase-1 activity in the medium and BMDMs (n=4). (E) mRNA expression of Arg1, Fizz1, Mgl1, and Mgl2, measured by RT-PCR in BMDMs (n=5-6). Student's *t-test* was used to determine statistical significance, defined as **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

CSS. Indeed, leucine has been found to directly promote M2 macrophage polarization *in vitro*, because the addition of leucine markedly increases the expression of M2-associated genes in IL-4-stimulated BMDMs, as well as arginase-1 activity, which is crucial for M2 macrophage polarization (Y. Zhang et al., 2020). Moreover, leucine alone did not increase the expression of M2-associated genes in BMDMs, thus further indicating that leucine synergistically enhances IL-4-induced gene expression and subsequent M2 polarization. CSS is caused by a severe pro-inflammatory/anti-inflammatory imbalance of macrophages, in which M2, which plays important roles in inflammation relief and tissue repair, is diminished (Cosín-Roger et al., 2016). Herein, we demonstrated that leucine alleviates inflammation in LPS-induced CSS, possibly through its ability to promote M2 polarization.

mTORC1 is known to integrate information about cellular nutritional status, including amino acid levels, thus altering cell signaling through a wide range of downstream targets (Wolfson and Sabatini, 2017). Recent studies have revealed the crucial importance of the mTORC1 pathway in controlling macrophage polarization, and the strong connections among the regulation of macrophage activity, nutrition sensing, and metabolic status (Papathanassiu et al., 2017) Byles et al., 2013b; Kobayashi et al., 2021). Nutrient status, particularly that of amino acids, regulates macrophage polarization via mTORC1 remains unclear. Among amino acids, leucine is a potent activator of mTORC1 in macrophages (X. Zhang et al., 2020). Leucine activates mTORC1 primarily by blocking the inhibitory effect of the protein setrin2 on the GATOR2, a complex that activates mTORC1 (Wolfson et al., 2016). Recently, leucine has been found induce mTORC1 activation in macrophages, thus further regulating macrophage polarization (Li et al., 2019).

However, the specific mechanism through which leucine regulates macrophage polarization has not been reported. Akt-mTORC1 signaling integrates metabolic inputs to control macrophage activation. Wortamannin inhibition of AKT was followed by inhibition of M2 polarization, suggesting that AKT signaling is involved in M2 polarization (**Supplement Figure 3B**). Studies reported that mTORC1 activation inhibits pAkt (T308), inhibition of mTORC1 in turn activate Akt (Byles et al., 2013a), promoting M2 polarization as a feed back to compensate the inhibition of mTORC1 induced suppression of M2 polarization. mTORC2, directly phosphrlate Akt at S473, and inhibition of mTORC2 inhibits p-Akt (S473) (Leontieva et al., 2014), further inhibiting M2 porlarization. Torin1 is the inhibitor for both, while rapamycin is specially for mTORC1 (Zhao et al., 2015). In this study, torin significantly decreased p-Akt (S473), and thus additionally inhibits mTORC2 showing a better inhibition of M2 than rapamycin (**Supplement Figure 3C**).

In M2 macrophages, Arg1 plays a pivotal role in a hallmark characteristic by catalyzing the hydrolysis of L-arginine into L-proline and polyamines, which subsequently downregulate the transcription of pro-inflammatory cytokine TNF-α in macrophages, thereby attenuating local inflammation and promoting tissue repair. Our research findings demonstrate that inhibiting mTORC1 significantly reduces Arg1 expression. Macrophage polarization is regulated by various transcription factors, among which STAT6 is indispensable for M2 polarization. Stimulation of macrophages with IL-4 leads to IL-4R signaling and phosphorylation of the transcription factor STAT6 at tyrosine residues, facilitating its nuclear translocation and induction of target genes. Studies have shown that STAT6/Arg1 is an important signaling mechanism in macrophage phenotypic regulation (Cai et al., 2019). Furthermore, emerging evidence suggests that ketone bodies promote M2 macrophage polarization through a STAT6-dependent signaling pathway(Huang et al., 2022). Addition of 2% leucine to drinking water increased blood ketone levels and decreases blood glucose levels (**Figure supplement 1C**). Therefore, we hypothesized that leucine might regulate M2 polarization through the mTORC1/STAT6/Arg1 pathway. Indeed, after IL-4 stimulation, STAT6 was tyrosine phosphorylated, but inhibition of mTORC1 did not alter the expression of STAT6. Moreover, leucine increased STAT6 phosphorylation in IL-4-stimulated BMDMs, but leucine alone did not activate STAT6. These findings indicated that leucine promotes macrophage M2 polarization independently of the mTORC1/STAT6/Arg1 pathway.

Beyond STAT6, a prior study has identified that LXRα regulates Arg1 expression in macrophages by promoting binding of the hematopoietic transcription factors IRF8 and PU.1 to the transcription start site in the Arg1 gene (Pourcet et al., 2011). The importance of LXRα in inhibiting the expression of inflammatory mediators in macrophages and macrophage differentiation has been reported (A-Gonzalez et al., 2013; Joseph et al., 2003). LXRα knockout decreases Arg1 expression, thus enhancing inflammation signatures of macrophages and ultimately inhibiting recovery after injury (Mao et al., 2021). However, whether leucine regulates M2 polarization through mTORC1/LXRα must be verified. After inhibition of mTORC1, the protein expression of LXRα decreased, and Arg1 expression was also significantly inhibited. Moreover, after LXRα inhibition, the expression of Arg1 decreased significantly, thereby confirming that active LXRα is necessary for M2 polarization. These findings indicated that leucine-mediated M2 polarization may occur via the mTORC1/LXRα/Arg1 pathway. Indeed, we extracted macrophage nuclear proteins and found that leucine effectively promoted LXRα entry into the nucleus. Thus, our results showed that mTORC1 integrates intracellular leucine signaling and external IL-4 signaling, thus activating its downstream transduction factor LXRα, and promoting LXRα entry into the nucleus and the induction of the target gene Arg1, and ultimately leading to M2 polarization.

Our results suggested that leucine regulates M2 via the mTORC1/LXRα/Arg1 pathway, thus alleviating LPS-mediated CSS. Leucine also has regulatory effects on M1. In present study, we report the first evidence that leucine decreases mortality in mice after a lethal dose of LPS, and attenuates secretion of the proinflammatory factors IL-6, IFN-γ, and TNF-α in the serum. The anti-inflammatory effects of leucine were probably mediated by modulation of macrophage polarization, because leucine suppressed CD86 expression (M1 macrophage marker) but increased CD206 (M1 macrophage marker) expression in both the bone marrow and spleen. This possibility was corroborated by *in vitro* data in BMDMs. Leucine inhibited LPS-driven M1 polarization, and decreased the secretion of IL-6, IL-1β, and TNF-α in BMDMs. Together, our in vivo and in vitro results suggest that leucine may also inhibit inflammation driven by M1 macrophages. Therefore, subsequent studies of the specific mode of action of leucine on the polarization of M1 macrophages will be essential.

Conclusions

In summary, the present study revealed that leucine ameliorates CSS in mice exposed to LPS by inhibiting macrophage M1 polarization and promoting M2 polarization. On the basis of our results, a role of leucine in macrophage inflammatory responses via the mTORC1/LXRα/Arg1 axis is proposed (**Figure 6**), in which leucine promotes M2 macrophage polarization through the mTORC1/LXRα/Arg1 signaling pathway, thereby contributing to the resolution of inflammation and the repair of damaged tissues.

Materials and methods

Animals

The experimental procedures and animal care were performed in accordance with the regulations of the Animal Care Committee of Sichuan Agricultural University (No. 20180701). Forty male C57BL/6J mice (8 weeks) were purchased from Dashuo Laboratory Animal Co., Ltd. (Chengdu, China). Mice with similar body weights (n = 8) were randomly divided into five treatment groups: 1) control group; 2) LPS group; 3) LPS + 2% leucine drinking water group (LPS + 2% W Leu); 4) LPS + 5% leucine food group (LPS + 5% F Leu); and 5) LPS + 2% leucine drinking water + 5% leucine food group (LPS + 2% W + 5%F Leu). All mice were housed in cages with constant humidity (40–70%) and temperature (20–25 °C) under a 12 h light/dark cycle, and were given free access to

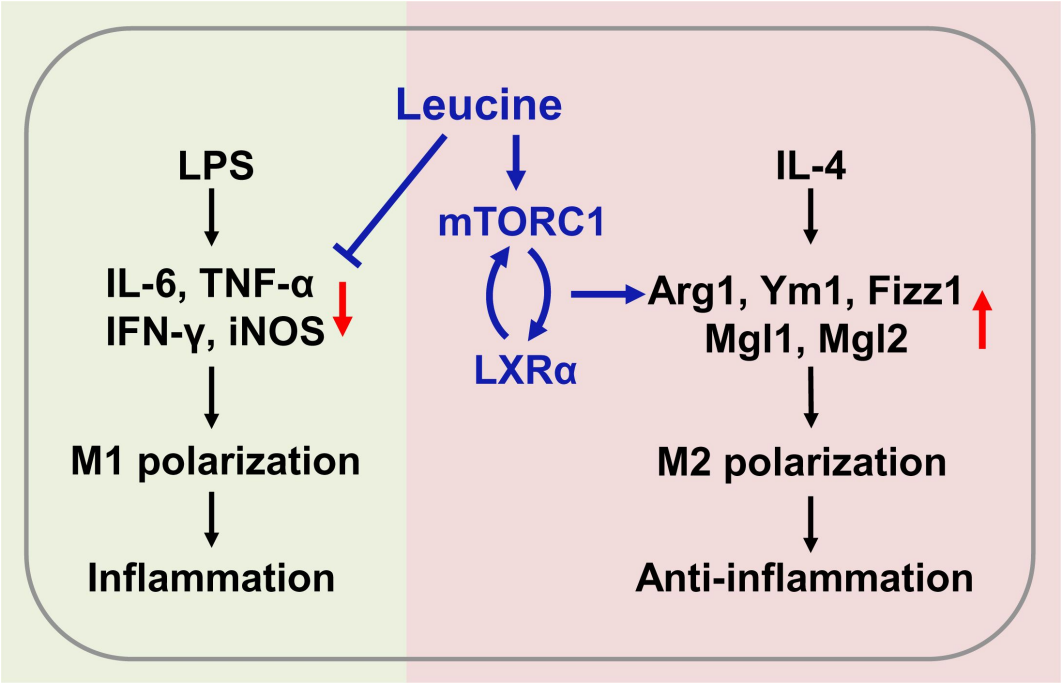

Figure 6.

Mechanism of leucine alleviating LPS-induced CSS by modulating mTORC1/LXRα signaling. In macrophages, LPS promotes M1 polarization to promote the secretion of inflammatory factors leading to inflammation, and IL-4 promotes M2 polarization to alleviate inflammation. Leucine further promotes IL-4-induced M2 polarization by activating mTORC1/LXRα to alleviate inflammation and repair damaged tissues, while leucine also inhibits LPS-mediated M1 polarization and reduces the expression and secretion of inflammatory factors in the organism.

drinking water and food for 21 days. On day 21, the mice were challenged with LPS (intraperitoneal injection); 6 hours after the challenge, the mice were anesthetized by 20 s exposure to carbon dioxide, and blood samples were collected through cardiac puncture. Collected samples were snap frozen in liquid nitrogen and stored at −80 °C until analysis. Blood samples were centrifuged at 3000 × g for 15 min at 4 °C, and then the serum was separated and stored at -20 °C until further analysis.

Macrophage isolation and stimulation

BMDMs were prepared as previously described (Byles et al., 2013). Briefly, after 6–8-week-old C57BL/6J mice were euthanized with CO_2, the femurs and tibias were removed and centrifuged to obtain cells. For macrophage differentiation, bone marrow-derived cells were plated in Petri dishes and cultured for 7 days in αMEM (containing 10% FBS and 1% penicillin/streptomycin) supplemented with 10 ng/mL M-CSF. Adherent cells were collected and seeded into new dishes for subsequent experiments. For M1-like activation, 25×10^4 BMDMs were placed in 12-well plates and treated with 100 ng/mL LPS (Sigma–Aldrich) for 6 h. For M2 polarization, cells were treated with 20 ng/mL IL-4 (Peprotech) for 24 hours. Leucine treatment was usually performed 1 hour before LPS/IL-4 stimulation.

Real-time quantitative PCR

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The reaction solution was prepared according to the instructions of the reverse transcription kit (Takara) to reverse-transcribe RNA to cDNA. QPCR was then performed with a reaction mixture consisting of 5 μL SYBR Green (Takara), 0.2 μL Rox, 3 μL dH_2O, 0.4 μl primers (F+R) for each gene used in the study, and 1 μl cDNA. Relative gene expression was calculated with the ΔΔCT method, and results were normalized to values for the housekeeping gene Cyclophilin. Primer sequences are listed in **Table S1**.

Western blot analysis

Cells were washed with ice-cold PBS, and proteins were extracted with RIPA lysis buffer (containing PMSF and phosphatase inhibitors). After a 30 min incubation at 4°C, samples were sonicated and centrifuged at 12,000 g for 15 min at 4°C, and clear supernatant was collected. Concentrations were determined, and samples were assayed with a BCA protein assay kit (Thermo Scientific, MA, USA). Equal amounts of protein were then separated through 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Merck Millipore Ltd., Tullagreen, Ireland). Membranes were blocked in 5% nonfat dry milk in 1× TBST for 1 hour at room temperature, then incubated with specific primary antibodies overnight at 4°C. Membranes were washed three times with TBST and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Finally, protein bands were visualized with an ECL chemiluminescence kit (Beyotime Biotechnology, Shanghai, China). Protein band density was quantified in Image Lab software (Bio-Rad). The ratio of the densitometric values of the target protein to the reference protein was calculated and expressed relative to the control value. Antibody information is listed in **Table S2**.

Flow cytometry analysis

Fluorescently labeled antibodies (purified anti-mouse CD16/32, FITC anti-mouse CD45, APC anti-mouse F4/80, PerCP/Cy5.5 anti-mouse CD11b, APC/Cy7 anti-mouse CD86, and PE/Cy7 anti-mouse CD206) were used according to the manufacturer's instructions. Cells were collected on a BD FACSVerse^{TM} instrument (BD Biosciences) and analyzed in FlowJo10 software. Antibody information is listed in **Table S2**.

Arginase assays

Arginase was measured as described above (Corraliza et al., 1994). In brief, cells were lysed with 0.1% Triton X100 and incubated at 37°C for 30 min to release enzymes by cell rupture. Subsequently, $MnCl_2$ and Tris-HCl (pH = 7.5) were added and heated at 56°C for 10 minutes to activate arginase 1. Subsequently, 500 mM L-arginine (pH = 9.7) was added and incubated at 37°C for 30 min to hydrolyze L-arginine. Hydrolysis was stopped with acid stop solution (H_2SO_4: H_3PO_4: H_2O = 1:3:7 v/v). Finally, 9% α-isonitrosopropiophenone (dissolved in 100% ethanol) was added and heated at 100°C for 15 min. Urea was measured at 540 nm, and all samples were read in triplicate.

Enzyme-linked immunosorbent assays

The cytokines TNF-a, IL-6, and INF-y (Beijing Sizhengbai Biotechnology, China) were determined with commercial enzyme-linked immunosorbent assays. Briefly, serum or cell culture supernatants were collected and analyzed according to the manufacturer's recommendations.

AST and ALT assays

For liver function tests, glutamic oxaloacetic transaminase (also known as aspartate transaminase, AST) and alanine aminotransferase (ALT) levels in mouse serum and liver homogenate supernatants were detected with kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Routine blood examination

Analysis of white blood cell composition and proportion (WBC, Neu#, Mon#, Lym#, Bas#, Eos#) was performed with an automatic biochemical analyzer (Hitachi 3100).

Statistical analysis

The results are presented as mean ± standard error (SEM) of the mean. Groups were compared with unpaired two-tailed Student's t-test and/or one-way analysis of variance. The *P* values are indicated in the figures as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ and ns, not significant ($P > 0.05$). All results were plotted in GraphPad Prism 8 software.

Acknowledgements

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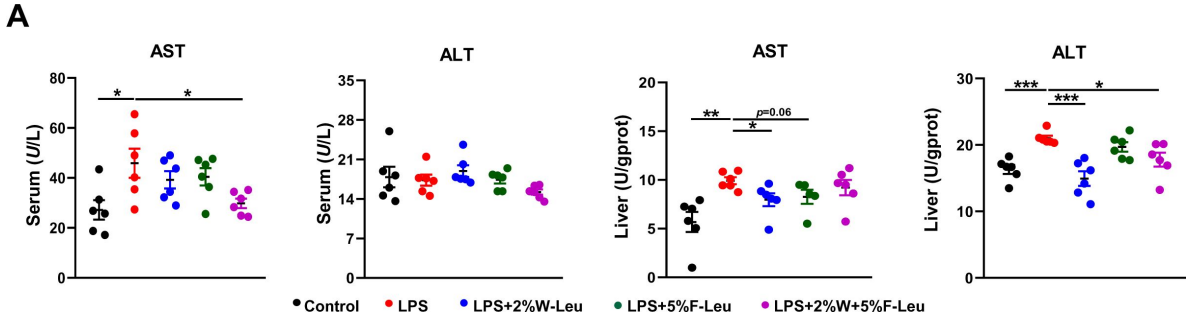
Conflicts of Interest

There are no conflicts of interest to declare.

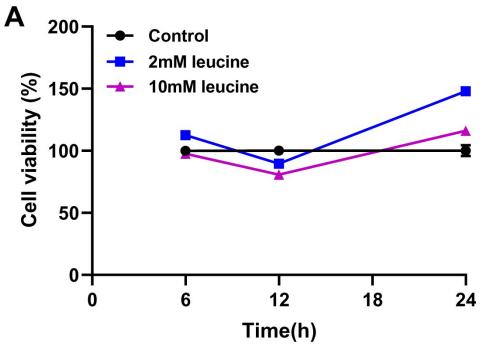
Figure Legends

Supplementary material



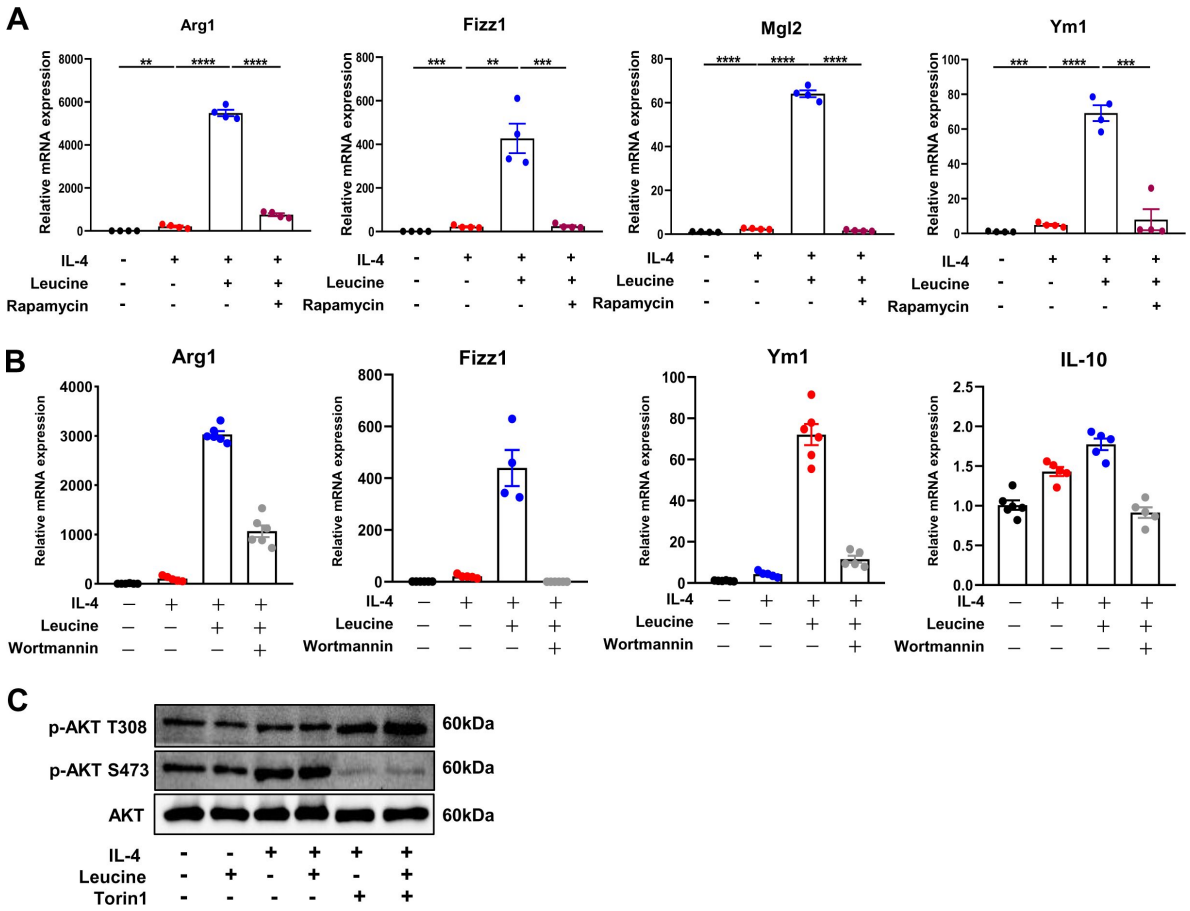
Supplementary Figure 1.

(A) AST and ALT levels in serum and liver (n=6). Student's *t-test* was used to determine statistical significance, defined as **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.



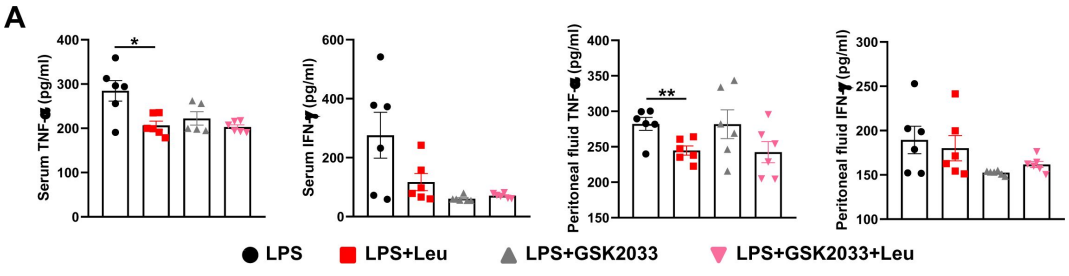
Supplementary Figure 2.

(A) Cell viability of 2 mM and 10 mM leucine treatments detected by CCK8 (n=5). Student's *t-test* was used to determine statistical significance, defined as **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.



Supplementary Figure 3.

(A) mRNA expression of Arg1, Fizz1, Mgl1 and Mgl2, measured by RT-PCR in BMDMs (n=4). (B) mRNA expression of Arg1, Fizz1, Mgl1 and Mgl2, measured by RT-PCR in BMDMs (n=4-6) (C) Protein levels of p-AKT (T308) and p-AKT (S473), determined by western blotting. Student's *t-test* was used to determine statistical significance, defined as **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.



Supplementary Figure 4.

(A) Measurement of IFN-γ and TNF-α secretion in mouse serum and peritoneal fluid by ELISA after treatment with LPS for 6 h (n=6). Student's *t-test* was used to determine statistical significance, defined as **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Gene	Forward 5′-3′	Reverse 5’-3’
Arg-1	AGCACTGAGGAAAGCTGGTC	CAGACCGTGGGTTCTTCACA
Mgl-1	TGCAACAGCTGAGGAAGGACTTGA	AACCAATAGCAGCTGCCTTCATGC
Mgl-2	GCATGAAGGCAGCTGCTATTGGTT	TAGGCCCATCCAGCTAAGCACATT
Fizz-1	TCCAGCTGATGGTCCCAGTGAATA	ACAAGCACACCCAGTAGCAGTCAT
Ym1	AGAAGGGAGTTTCAAACCT	GTCTTGCTCATGTGTGTAAGTGA
IL-6	AAAATTTCCTCTGGTCTTCTGGAGT	TTCTGTGACTCCAGCTTATCTCTTG
IL-1β	GCTTCCTTGTGCAAGTGTCTGA	TCAAAAGGTGGCATTTCACAGT
MCP1	CCACTCACCTGCTGCTACTCA	TGGTGATCCTCTTGTAGCTCTCC
iNOS	CAGGTCTTTGACGCTCGGAA	GCCTGAAGTCATGTTTGCCG
NLRP3	ATTACCCGCCCGAGAAAGG	TCGCAGCAAAGATCCACACAG
TNF-α	TCTCATGCACCACCATCAAGGACT	ACCACTCTCCCTTTGCAGAACTCA
Cyclophilin	TGCCCGCAAGTCAAAAGAAAT	ACTGAATGGCTGGATGGCAAG

Supplementary Table S1.

qPCR primer sequences.

Antibodies	Source	Identifier
Purified Rat Anti-Mouse CD16/CD32	BD Pharmingen™	Cat # 553141
FITC Rat Anti-Mouse CD45	BD Pharmingen™	Cat # 553080
Alexa Fluor® 647 Rat Anti-Mouse F4/80	BD Pharmingen™	Cat # 565853
PerCP-Cy™5.5 Rat Anti-CD11b (M1/70)	BD Pharmingen™	Cat # 550993
Alexa Fluor®647 Rat Anti-Mouse CD206	BD Pharmingen™	Cat # 565250
APC/Cyanine7 anti-mouse CD86	Biolegend	Cat # 105030
β-Actin Rabbit mAb	CST	Cat # 4970S
LXR alpha Antibody	NOVUS	Cat # NBP2-66938
Arginase-1 XP® Rabbit mAb	CST	Cat # 93668
Stat6 Rabbit mAb	CST	Cat # 5397S
Phospho-Stat6 (Tyr641) Rabbit mAb	CST	Cat # 56554S
4E-BP1 Rabbit mAb	CST	Cat # 9644S
Phospho-4E-BP1 Rabbit mAb	CST	Cat # 13443S
Akt (pan) (11E7) Rabbit mAb	CST	Cat # 4691S
Phospho-Akt (Ser473) XP® Rabbit mAb	CST	Cat # 4060S
Phospho-Akt (Thr308) XP® Rabbit mAb	CST	Cat # 13038S
p70 S6 Kinase Rabbit mAb	CST	Cat # 2708S
Phospho-Drosophila p70 S6 Kinase (Thr398) Antibody	CST	Cat # 9209S

Supplementary Table S2.

Antibody information.

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Article and author information

Hui Yan
Key Laboratory of Animal Disease-Resistance Nutrition of China Ministry of Education, Key laboratory of Animal Disease-resistant Nutrition and Feed of China Ministry of Agriculture and Rural Affairs, Key laboratory of Animal Disease-resistant Nutrition of Sichuan Province, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China
For correspondence: yan.hui@sicau.edu.cn

Yao Liu
Key Laboratory of Animal Disease-Resistance Nutrition of China Ministry of Education, Key laboratory of Animal Disease-resistant Nutrition and Feed of China Ministry of Agriculture and Rural Affairs, Key laboratory of Animal Disease-resistant Nutrition of Sichuan Province, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

Xipeng Li
Key Laboratory of Animal Disease-Resistance Nutrition of China Ministry of Education, Key laboratory of Animal Disease-resistant Nutrition and Feed of China Ministry of Agriculture and Rural Affairs, Key laboratory of Animal Disease-resistant Nutrition of Sichuan Province, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

Bing Yu
Key Laboratory of Animal Disease-Resistance Nutrition of China Ministry of Education, Key laboratory of Animal Disease-resistant Nutrition and Feed of China Ministry of Agriculture and Rural Affairs, Key laboratory of Animal Disease-resistant Nutrition of Sichuan Province, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

Jun He
Key Laboratory of Animal Disease-Resistance Nutrition of China Ministry of Education, Key laboratory of Animal Disease-resistant Nutrition and Feed of China Ministry of Agriculture and Rural Affairs, Key laboratory of Animal Disease-resistant Nutrition of Sichuan Province, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

Xiangbing Mao
Key Laboratory of Animal Disease-Resistance Nutrition of China Ministry of Education, Key laboratory of Animal Disease-resistant Nutrition and Feed of China Ministry of Agriculture and Rural Affairs, Key laboratory of Animal Disease-resistant Nutrition of Sichuan Province, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

Jie Yu
Key Laboratory of Animal Disease-Resistance Nutrition of China Ministry of Education, Key laboratory of Animal Disease-resistant Nutrition and Feed of China Ministry of Agriculture and Rural Affairs, Key laboratory of Animal Disease-resistant Nutrition of Sichuan Province, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

Zhiqing Huang
Key Laboratory of Animal Disease-Resistance Nutrition of China Ministry of Education, Key laboratory of Animal Disease-resistant Nutrition and Feed of China Ministry of Agriculture and Rural Affairs, Key laboratory of Animal Disease-resistant Nutrition of Sichuan Province, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

Yuheng Luo
Key Laboratory of Animal Disease-Resistance Nutrition of China Ministry of Education, Key laboratory of Animal Disease-resistant Nutrition and Feed of China Ministry of Agriculture and Rural Affairs, Key laboratory of Animal Disease-resistant Nutrition of Sichuan Province, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

Junqiu Luo
Key Laboratory of Animal Disease-Resistance Nutrition of China Ministry of Education, Key laboratory of Animal Disease-resistant Nutrition and Feed of China Ministry of Agriculture and Rural Affairs, Key laboratory of Animal Disease-resistant Nutrition of Sichuan Province, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

Aimin Wu
Key Laboratory of Animal Disease-Resistance Nutrition of China Ministry of Education, Key laboratory of Animal Disease-resistant Nutrition and Feed of China Ministry of Agriculture and Rural Affairs, Key laboratory of Animal Disease-resistant Nutrition of Sichuan Province, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China

Daiwen Chen
Key Laboratory of Animal Disease-Resistance Nutrition of China Ministry of Education, Key laboratory of Animal Disease-resistant Nutrition and Feed of China Ministry of Agriculture and Rural Affairs, Key laboratory of Animal Disease-resistant Nutrition of Sichuan Province, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China
For correspondence: dwchen@sicau.edu.cn

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Joint Public Review:

Summary:

The major purpose of this manuscript is to examine whether leucine treatment would be a potential strategy to treat cytokine storm syndrome (CSS). CSS is a common symptom in multiple infectious diseases in clinic, gradually leads to multiple organ failure and high mortality. Strategies to treat CSS including pulse steroid therapy normally leads to severe side effects. Therefore, it is still required to develop safe strategy with high efficacy to treat CSS. In clinic, sepsis is well characterized to exhibit CSS and therefore multiple studies utilized LPS-induced sepsis model to evaluate CSS symptom. In this study, the authors examined whether leucine, an essential amino acid that has been absorbed daily in our body, could ameliorate CSS symptom in the LPS-induced sepsis mouse model. They found a potential protective effect of leucine in terms of the survival rate and inflammatory responses.

Strengths:

The study is overall well designed and the results are well analyzed with only minor issues. The methods they utilized is appropriate.

Weaknesses:

The mechanistical insights are not sufficient and could not fully explain the phenotype they found. Considering the importance of this study is to identify the potential protective role of leucine in CSS, the authors could also consider investigator-initiated clinical trials to further expand the significance of this study.

- https://doi.org/10.7554/eLife.89750.2.sa0

Author Response

The following is the authors' response to the original reviews.

I greatly appreciate the time you and the reviewers have taken to review my paper and provide detailed feedback and suggestions. I have carefully considered the reviewers' comments and made thorough modifications to the paper. Below are my responses to each comment and the revisions I have made.

> ***Reviewer #1 (Recommendations for The Authors):***
>
> *Although the paper has strengths in understanding better the pathway of activation leading to polarization, the mechanisms contributing to cytokine storm are weak. In the context of cellular in vitro changes, it would be very interesting to map these molecular changes to strengthen the pathways affected in this model. In vivo, stronger evidence is required to bridge the gap between the in vitro model and mechanisms regulating in*

> *vivo disease development. Reporting of experiments needs to be considerably strengthened. Individual data points are shown, however, it is unclear whether these represent biological or technical, or how many experiments have been undertaken. The addition of this information is essential for uzndersta nding the robustness and repeatability of findings. Currently, these cannot be assessed from the information provided. Furthermore, it is unclear whether the error bars represent s.e.m or s.d. which greatly impacts data interpretation.*

Answer: thank you for the valuable comments! We have added some in vivo experiments to strengthen the bridge between the in vitro and in vivo model. 1) The depletion of macrophage by clodronate-liposomes (CLL) i.v. injection was performed in endotoxemic mice with leucine. The alleviation of LPS-induced cytokine production by leucine was muted with macrophage depletion (Figure 2E, F), suggesting the anti-inflammatory effect of leucine was exerted via the regulation of macrophage. 2) The LXRα inhibitor, GSK2033, was applied to mice via i.v. injection prior to LPS-challenge. In GSK2033 treated mice, the effects of leucine on the serum levels of inflammatory cytokines were neutralized (Supplementary Figure 4), partially indicating the importance of LXRα in the regulation of cytokine release. We acknowledge the limitation of LXRα inhibition by GSK2033 in this study. In our future study, we plan to use monocyte specific LXRα knockout mice by LysM-cre to elucidate the importance of LXRα in the progression of CSS, and specifically focuse on the molecular mechanism how mTORC1 interacts with LXRα to modulate M2 macrophage polarization. Additionally, we made modifications in the manuscript to clarify that the error bars represented as the standard error of the mean (SEM) (line 416).

> ***Reviewer #2 (Recommendations for The Authors):***
>
> 1. *The whole manuscript is based on the 2% leucine from feed and 5% leucine from water. Is there any rationale for using these two types of different concentrations in this study? Often, a dose-dependent treatment is utilized in vivo in pharmacological study. Therefore, the authors should at least test two different concentrations in each type to confirm the conclusion.*

Answer: thank you for your comment and suggestion. The 2% leucine in feed and 5% leucine in water in this study were based on the literatures. In those studies, leucine was reported to activate mTORC1 and regulate metabolism at such types of different concentration as shown below, although there is lack of leucine in the regulation of macrophage activation. In this study, we found leucine supplementation in such types significantly increased the average body weight gain of mice, suggesting growth promoting and no toxicity of leucine on mice.

(1) Jiang X, Zhang Y, Hu W, Liang Y, Zheng L, Zheng J, Wang B, Guo X. 2021. Different Effects of Leucine Supplementation and/or Exercise on Systemic Insulin Sensitivity in Mice. Front Endocrinol (Lausanne) 12:651303. doi:10.3389/fendo.2021.651303

(2) Holler M, Grottke A, Mueck K, Manes J, Jücker M, Rodemann HP, Toulany M. 2016. Dual Targeting of Akt and mTORC1 Impairs Repair of DNA Double-Strand Breaks and Increases Radiation Sensitivity of Human Tumor Cells. PLoS One 11: e0154745. doi:10.1371/ journal. pone.0154745

> *1. The authors focus on macrophage polarization as the major cellular event affected by leucine treatment; however, they also report that the proportion of multiple immune cell types has been suppressed by leucine treatment. As some of these immune cells can also produce inflammatory cytokines, the authors should confirm the anti-inflammatory effects of leucine were mainly mediated by modulating macrophage polarization as they suggested in the manuscript. For example, the authors could utilize Anti-CSF1 or clodronate to deplete macrophage and observed whether leucine-reduced inflammatory cytokines production was largely diminished.*

Answer: thank you for your valuable suggestion! We used clodronate-liposome (CLL) i.v. injection to deplete macrophages to further validate the specific contribution of macrophage polarization to the anti-inflammatory effects of leucine. The results revealed that clodronate treatment decreased blood monocyte counts and eliminated the effect of leucine in lowering serum inflammatory factors IL-6, IFN-γ and TNF-α (Figure 2E-F), suggesting the importance of leucine-mediacted macrophage activation on the anti-inflammation.

> *1. It would be important to examine whether 10 mM leucine would exhibit cytotoxicity to bone marrow derived monocytes/macrophages. This would confirm that leucine treatment directly suppresses inflammatory cytokines production or reduces cell viability to indirectly modulates inflammatory responses.*

Answer: thank you for your valuable suggestion! We performed cell viability assays after treating BMDM with 2 mM and 10 mM leucine for 6h or 24h (consistent with the timing of leucine treatment in article). The results showed that at 6h, 2 mM leucine significantly increased cell viability, while 10 mM leucine had no significant effect on cell viability. At 24h, both 2 mM and 10 mM leucine significantly increased cell viability. In conclusion, 2 mM and 10 mM leucine were not cytotoxic to BMDM, and the anti-inflammatory effect of leucine was not derived from the reduction in cell viability (Supplementary Figure 2).

> *1. The authors found that leucine promotes mTORC1-LXRα for arginase-1 transcription and M2 polarization. The pathway the authors elucidated is not surprising, which has already been reported in other studies. What about the other M2 markers? The authors could examine whether arginiase-1 deficiency would deplete leucine-increased other M2 marker genes expression. Moreover, what about the molecular mechanism for leucine-reduced M1 polarization?*

Answer: Thank you for the valuable comments! To clarify that Arginase-1 activity, mRNA expression of Fizz1, Mgl1, Mgl2, and Ym1 were well established markers for M2 macrophage. Specifically, Arginase-1 activity is important to define M2 functionality. These markers were used to define the level of M2 macrophage polarization. Only a few studies indicated the involvement of mTORC1 in the M2 polarization as shown below; however, there is no molecular mechanism about how mTORC1 modulates this process. In this study, we provide the evidence that LXRα mediated the mTORC1 associated M2 polarization, and leucine regulated mTORC1-LXRα to promote M2 polarization, which was in dependent of IL-4-induced STAT6 signaling. In our future study, we are focusing on the molecular mechanism how mTORC1 interacts with LXRα to modulate M2 macrophage polarization.

(1) Byles V, Covarrubias AJ, Ben-Sahra I, Lamming DW, Sabatini DM, Manning BD, Horng T. 2013. The TSC-mTOR pathway regulates macrophage polarization. Nat Commun 4:2834. doi:10.1038/ncomms3834

(2) Kimura T, Nada S, Takegahara N, Okuno T, Nojima S, Kang S, Ito D, Morimoto K, Hosokawa T, Hayama Y, Mitsui Y, Sakurai N, Sarashina-Kida H, Nishide M, Maeda Y, Takamatsu H,

Okuzaki D, Yamada M, Okada M, Kumanogoh A. 2016. Polarization of M2 macrophages requires Lamtor1 that integrates cytokine and amino-acid signals. Nat Commun 7:13130. doi:10.1038/ncomms13130

> *1. In Fig. 1A, what's the P-value among these two groups? Moreover, what about the result with combination treatment as the authors performed in other panels?*

Answer: thank you for the valuable comments from the reviewer! In Figure 1A, the P-value between the LPS and LPS+2% Leucine groups is 0.0031, and the P-value between the LPS and LPS+5% Leucine groups is 0.0009. I have marked the significance in Figure 1A accordingly. Due to the limited number of mice, we only treated mice in two different ways respectively. Initially, we performed survival experiment and observed that the addition of leucine prolonged survive of mice at lethal dose. Based on these findings, we further investigated whether a combination of the two methods would yield better results on the regulation of inflammation, but the combination exhibited the similar effect on cytokines production, and it is not necessary to repeat the survival experiment with the combination.

> *1. It seems not much difference could be observed between 2% leucine from feed and 5% leucine from water in the expression of inflammatory genes and anti-inflammation-related markers. However, it seems that 5% leucine from water would exhibit a better survival rate than 2% leucine from feed. The authors should explain potential reasons and at least examine it in vitro.*

Answer: we appreciate the valuable comments from the reviewer! There are two possible reasons: 1) When lethal dose of LPS applied, mice were too weak to eat but still drank a small amount of water; 2) the absorption of leucine from the water were much easier than from the feed, thus leucine from the water exhibited much better efficiency in a short period of survival experiment. On the other hand, the cytokine levels and expressions were measure in non-lethal experiments, in which mice were in much better condition for lecine absorption.

> *1. In Fig. 4A, the authors examined the expression of p-mTOR. The authors should further examine the expression of p-AKT (S473, T308) and p-S6 to clarify whether mTORC1 or mTORC2 has been modulated. As reported, leucine should act on GATOR2 for mTORC1 activation. However, the authors reported that Torin, a mTORC1/mTORC2 inhibitor, inhibited M2 polarization more significantly compared to rapamycin, a mTORC1 inhibitor. These observations seem to indicate that leucine has other targets except mTORC1, such as mTORC2, which might raise novel mechanisms that have never been reported before.*

Answer: thank you for the valuable comments! Akt-mTORC1 signaling integrates metabolic inputs to control macrophage activation. Wortamannin inhibition of AKT was followed by inhibition of M2 polarization, suggesting that AKT signaling is involved in M2 polarization. Studies reported that mTORC1 activation inhibits pAkt (T308), inhibition of mTORC1 in turn activate Akt (1), promoting M2 polarization as a feed back to compensate the inhibition of mTORC1 induced suppression of M2 polarization. mTORC2, directly phosphrlate Akt at S473, and inhibition of mTORC2 inhibits p-Akt (S473) (2), further inhibiting M2 porlarization. Torin1 is the inhibitor for both, while rapamycin is specially for mTORC1 (3). The explanation was included in Line 252-262

(1) Leontieva OV, Demidenko ZN, Blagosklonny MV. 2014. Rapamycin reverses insulin resistance (IR) in high-glucose medium without causing IR in normoglycemic medium. Cell Death Dis 5: e1214. doi:10.1038/cddis.2014. 178Byles.

(2) Holler M, Grottke A, Mueck K, Manes J, Jücker M, Rodemann HP, Toulany M. 2016. Dual Targeting of Akt and mTORC1 Impairs Repair of DNA Double-Strand Breaks and Increases Radiation Sensitivity of Human Tumor Cells. PLoS One 11: e0154745. doi:10.1371/journal.pone .0154745

(3) V, Covarrubias AJ, Ben-Sahra I, Lamming DW, Sabatini DM, Manning BD, Horng T. 2013. The TSC-mTOR pathway regulates macrophage polarization. Nat Commun 4:2834. doi:10.1038/ncomms3834.

> *1. In Fig.5B, frankly speaking, I do not observe much difference in LXRa expression. Also, the actin band is too poor to get any conclusion.*

Answer: thank you for the valuable comments from the reviewer! In Fig. 5B, the extracted protein is specifically mentioned as nuclear protein in the text. It is stated that actin is expressed in the cytoplasm, while histone is expressed in the nucleus. The figure shows that actin expression is almost absent, which is mentioned to demonstrate the purity of the extracted nuclear protein.

> *1. In Fig. 5C and 5D, it is amazing that GSK2033 would reduce urea production even largely greater than the basal condition (lane 1). As GSK2033 normalized IL-4 or IL-4 combination with Leucine raised urea production in cells, how GSK2033 could reduce urea in medium. The authors should explain this discrepancy.*

Answer: thank you for the valuable comments from the reviewer! In Fig. 5C, urea production was measured directly in the culture medium using a commercial assay kit, and GSK2033 indeed led to a significant decrease in urea production. In Fig. 5D, on the other hand, we assessed the activity of arginase-1 by lysing the cells, activating arginase-1, providing the substrate arginine, and then measuring urea production. In response to your question, the explanation is that in the assay measuring arginase-1 activity, we supplied a sufficient amount of substrate arginine, which may better reflect the enzyme's activity and the results were consistent with our expectations. Additionally, when GSK2033 was used in combination with IL-4 or IL-4 plus leucine, it might interact with the IL-4 signaling pathway or leucine metabolism pathway, leading to an increase in urea production. This is just our preliminary explanation for the contradictory results, and we acknowledge that further research is needed to explore the mechanism of action of GSK2033 and its interactions with IL-4 or leucine.

> *1. Line 98, "INF-gamma" should be IFN-gamma.*

Answer: We appreciate your attention to detail. We apologize for the error in line 98, where "INF-gamma" should indeed be corrected to "IFN-gamma (IFN-γ)." We will make the necessary correction in the revised version of the manuscript.