

Different doses of systemic LPS induce different degrees of polarization of microglia and astrocytes

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Research

Keywords: Systemic lipopolysaccharides, polarization, microglia, astrocytes, C3aR

Posted Date: April 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-362185/v1>

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Abstract

Background

Microglia and astrocytes are activated in different phenotypes to exert opposite effects. The recently reported intraperitoneal injection of 5 mg/kg lipopolysaccharides (LPS) to promote A1 astrocytes by activating M1 microglia was found to cause high mortality. Furthermore, reported doses of systemic LPS used to induce M1 microglia vary widely (0.1 ~ 5 mg/kg). We aimed to study microglia and astrocytes polarization induced by various LPS doses in the central nervous system, and assess whether downregulation of C3a receptor (C3aR) in astrocytes contributes to an increased A2/A1 ratio.

Methods

Rats were randomly divided into six LPS dosage groups (0, 0.1, 0.33, 1, 3, and 5 mg/kg, intraperitoneally). Seventy-two genes for A1, A2, A-pan, M1, and M2 markers were detected by real-time polymerase chain reaction 24 hours after LPS treatment in the cerebral cortex, hippocampus, and spinal cord. C3aR in astrocytes was knocked down by intrathecal injection of AAV-C3aR-GFAP 21 days before LPS administration. Co-immunofluorescence of C3aR with microglia, astrocytes, and neuron markers were performed to verify the specificity of C3aR knockdown in astrocytes. Changes in the 72 genes in the spinal cord were detected again 24 hours after LPS injection.

Results

Systemic LPS activated not only A1 and M1, but also A2, M2, and A-pan in the cerebral cortex, hippocampus, and spinal cord. The same LPS dose induced a similar activation level of M1 and M2, both of which were upregulated with increasing LPS. A1 and A-pan were polarized more than A2 at all LPS doses in the cortex and spinal cord. Microglia were more activated at 5 mg/kg than at 3 mg/kg LPS, but astrocytes presented no activation advantage at 5 mg/kg LPS. Marco, Ym1, and C3 showed a significant dose-dependent increase in LPS concentration. Specific knockdown of C3aR in astrocytes upregulated more markers of A2 than A1 and A-pan.

Conclusions

A larger systemic LPS dose contributes to greater polarization of M1 and M2 microglia, but no dominant phenotype. More A1 and A-pan astrocytes were activated than A2, even at low LPS doses. Downregulation of C3aR in astrocytes contributes to the polarization of anti-inflammatory phenotypes induced by LPS.

Background

Activated microglia and astrocytes play a crucial role in neuroinflammation, which is known to be involved in neurodegenerative diseases, neuropsychiatric disorders, brain trauma, and pain^[1-3]. Microglia are sensitive to pathogens or damage; they can be stimulated by lipopolysaccharides (LPS) or interferon (IFN)- γ to an M1 phenotype for the expression of pro-inflammatory cytokines or by interleukin (IL-4) or IL-13 to an M2 phenotype to resolve inflammation and repair tissue^[4-5]. Upon injury, astrocytes are also activated and play dual roles. Neuroinflammation induced by LPS promotes neurotoxic astrocytic polarization, termed the A1 phenotype, while ischemia induced by middle cerebral artery occlusion (MCAO) leads to a neuroprotective astrocyte polarization, termed the A2 phenotype^[6]. Systemic LPS-induced neuroinflammation has been evidenced by increased microglia and astrocytes activation^[7,8]. Numerous scientific studies have demonstrated that systemic LPS can activate microglia to an M1 phenotype and astrocytes to the A1 phenotype. Furthermore^[9,10], A1 astrocytes induced by LPS were found to be activated through microglial secretion of the neuroinflammatory cytokines tumor necrosis factor (TNF)- α , IL-1 α , and the complement component C1q^[11]. However, unlike the commonly used low dose of LPS used to induce M1 activation^[12-14], the dosage used to activate A1 astrocytes has been reported to be as high as 5 mg/kg^[11,15], a similar dose to induce liver injury or sepsis^[16,17]. In another ongoing study on the mechanism of A1 astrocytes in a rat chronic postoperative pain model, we employed 5 mg/kg systemic LPS to induce A1 astrocytes. However, a high mortality rate was observed 24 hours after LPS injection, and the survival rate could not be improved by increasing the weight of rats from 250 g to 350 g. Since this LPS dose was also used to create a liver injury and sepsis model, which could lead to death^[16,17], we wondered whether decreasing the LPS dose could still successfully induce A1 polarization. LPS has been found to induce A1 polarization not directly but through M1 activation, and the reported LPS dose-activating M1 phenotype was much lower than 5 mg/kg, although it varied^[11-15].

Each phenotype has multiple markers, and the selected markers often vary^[7,18,19]. In a study performed by Xu et al., TNF- α and IL-1 β in the cerebral cortex were used as M1 markers, and CD206, Ym1/2, and Arg1 were used as M2 markers^[18]. In Wang's research, CD16, TNF- α , inducible nitric oxide synthase (iNOS), and monocyte chemoattractant protein (MCP)-1 in the hippocampus were used as M1 markers and CD206, transforming growth factor (TGF)- β , Arg1, and Ym1 as M2 characteristic markers^[19]. It remains unclear whether any of these phenotype markers are appropriate classical markers of LPS in any central nervous tissue. Zamanian et al. showed that reactive astrocytes induced by LPS or ischemic stroke upregulated over 1,000 genes^[7]. Some of the upregulated genes are unique to the LPS subtype (A1) or the MCAO subtype (A2); for example, H2-D1 and Serping1 are markers for A1 astrocytes. It remains unclear whether the upregulated genes induced by LPS present a dose-dependent response to LPS.

Targeted inhibition of pro-inflammatory phenotypes or promotion of anti-inflammatory phenotypes is a promising technique for alleviation of neuroinflammation. Downregulation of A1 astrocytes has been shown to protect against white matter injury in the chronic hypoperfusion model mice^[20]. Furthermore, promoting astrocytes polarized into A2 astrocytes by silencing miR-21 was shown to improve the

formation of synapses and nerve growth after acute ischemic spinal cord injury^[21]. Complement C3 was recently found to be a classical marker of A1 astrocytes^[11,15], and its interaction with the C3a receptor (C3aR) plays a crucial role in neuroinflammation regulation^[22,23]. C3 and C3aR expression have been reported to be positively correlated with cognitive decline. Deletion of C3aR in PS19 mice reportedly attenuates neuroinflammation, synaptic deficits, and neurodegeneration^[22]. Interestingly, in *in vitro* astrocytes, C3 was found to interact with itself C3aR, and downregulation of C3aR in astrocytes not only decreased C3 expression but also many transcription factors of A1 astrocytes^[24]. To test whether downregulation of C3aR in astrocytes could protect astrocytes from polarization into the detrimental A1 phenotype induced by LPS *in vivo*, we knocked down C3aR in astrocytes by intrathecal injection with AAV2/9-r-C3ar1 shRNA-glia fibrillary acidic protein (GFAP) before LPS administration.

Although targeting of the anti-inflammatory phenotypes shows much promise, the doses of systemic LPS adopted to induce microglia and astrocytes polarization vary considerably. Therefore, we conducted this study to explore the differences in various LPS dosages by covering multiple phenotype markers and tissue variances. By comparing the differences, we expect to provide more specific information about a proper marker. Furthermore, the involvement of C3aR in astrocytes in LPS-induced pro-inflammatory phenotypes was also studied.

Methods

Animals

Adult male Sprague-Dawley rats weighting 120~150 g and 250~300 g used in this study were provided by the Experimental Animal Center of the Chinese Academy of Medical Sciences (Beijing, China). Rats were housed at 23 ± 1 °C under a 12 hours light-dark cycle with food and water ad libitum. Experimental protocols were approved by the Institutional Animal Care and Use Committee in the Chinese Academy of Medical Sciences (Beijing, China), and were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. For the LPS dose groups, rats weighting 250~300 g were randomly divided into 6 groups (0, 0.1, 0.33, 1, 3, and 5 mg/kg LPS), $n = 8$. To verify the efficiency and specificity of AAV, rats weighting 120~150 g were randomly divided into 2 groups (AAV2/9-r-C3ar1 shRNA-GFAP, AAV2/9-GFAP), $n = 3$. For the involvement of C3aR in LPS induced neuroinflammation, rats weighting 120~150 g were randomly divided into 2 groups (AAV2/9-r-C3ar1 shRNA-GFAP + LPS, AAV2/9-GFAP + LPS), $n = 4$.

LPS treatment

LPS from *Escherichia coli* 055:B5 (dissolved in endotoxin-free PBS) was purified by phenol extraction (L2880, Sigma-Aldrich, St. Louis, MO). The animals were injected with 0.1, 0.33, 1, 3 and 5 mg/kg LPS intraperitoneally (i.p.), respectively. The injection volume was diluted to 500 μ l with endotoxin-free PBS for each injection. In the control group, 500 μ l endotoxin-free PBS were injected i.p.. Tissues including the cerebral cortex, hippocampus and spinal cord were extracted 24 hours after LPS treatment.

Intrathecal administration

Under anesthesia, rats were put in a prone position with a manual bolster under the lower abdomen to fully expose the L4-L5 intervertebral space. Incisions were made in the back skin of the rats, and the lumbar fascia was exposed. The L5 acantha was held with a clamp by the left hand, and a 25 μ l microsyringe needle was vertically inserted into the L4-L5 intervertebral space through the ligamenta flava with the right hand. The microsyringe was stopped when a tail flick or a paw retraction was observed, as these responses demonstrated correct intrathecal needle placement. Then, 20 μ l AAV2/9-r-C3ar1 shRNA-GFAP or AAV2/9-GFAP was injected slowly through the needle and held a few seconds before removal. A tail flick could be observed when the needle was removed, and then the wound was sutured. Rats that showed any movement disorder on the next day were excluded from the experiments. Twenty-one days after AAV administration, rats received a single dose of LPS injection determined by the above LPS dosage response experiment. Chemogenetical AAV (titer, 2.5×10^{12} vg/mL) was purchased from Hanbio Technology (Shanghai, China).

Real-time polymerase chain reaction (RT-PCR)

Rats were sacrificed with overdose anesthetics 24 hours after LPS treatment. The cerebral cortex, hippocampus and L4-L5 spinal cord from the same group were mixed respectively, and total RNA was extracted with Trizol reagent (15596026, Invitrogen, Carlsbad, CA) by using ultrasonic cracking, and reverse transcribed with Prime Script RT Master Mix (RR036, Takara, Otsu, Japan) according to the manufacturer's instructions. Quantitative reverse transcriptase (RT-PCR) was performed using SYBR Premix Ex Taq (RR820, Takara) and a Step One Real Time PCR System (Applied Biosystems, Foster City, CA). Three repeated spottings were set, and if necessary, another three more spottings were repeated when the three spottings showed inconsistent result. The sequences of primers used were presented in Supplementary Table 1.

Immunohistochemistry

Rats ($n = 3$) from the 3 mg/kg LPS group and the control group were sacrificed with excessive anesthetics 24 hours after LPS treatment, and sacrificed 21 days after AAV intervention. Rats were then transcardially perfused with PBS followed by fresh 4% paraformaldehyde. The whole brain and L4-L5 spinal cord were extracted and postfixed in 4% paraformaldehyde for 4 hours, and then dehydrated in 30% sucrose overnight until the tissues settled to the bottom of the EP pipe at 4 °C. Tissues were embedded in OCT (4583, Tissue-Tek, SAKURA, USA) and frozen at -80 °C, and then sectioned at 12 μ m thick using a cryostat (Leica 2000, Germany). The sections were permeated in 0.3% Triton-X-100 in PBS for 30 min and incubated with 10% bovine serum albumin in PBS for 60 mins, following an overnight incubation with primary antibodies at 4 °C. Then sections were incubated with corresponding secondary antibodies for 1 hour at room temperature (the primary and secondary antibodies used for immunohistochemistry were listed in Supplementary Table 2), and visualized using a CCD spot camera (Olympus DP71).

Statistical analysis

Results were presented as means \pm SEM, and statistical analyses were performed using GraphPad Prism 8.0 software package (version 8 for Windows, San Diego, CA).

Results

Systemic LPS induced polarization of microglia and astrocytes

As a classical reagent to induce M1 and A1 activation, systemic LPS is expected to predominantly polarize the pro-inflammatory M1 microglia and/or A1 astrocytes. However, in addition to the expected reactive M1 and A1, we observed obvious M2 and A2, especially A-pan activation in the cerebral cortex, hippocampus, and spinal cord (Figure 1). The majority of markers of A1 and A-pan astrocytes showed an increase when exposed to LPS, even at a low dose in the cerebral cortex, and a large LPS dose induced more activation. Markers of M1 and A2 showed an inconsistent trend with increasing LPS, with a fair number of markers showing no change, such as IL-12a, IL-6, Ptgs2 of M1 genes, and Clcf1, Emp1, and Sphk1 of A2 genes, while some others showed an obvious increase with LPS. Many M2 markers, such as IL-10, Jak3, IL-1ra, Ym1, and Clec7a, were also activated by LPS (Figure 1A). Reactive microglia induced by LPS in the hippocampus were similar to those in the cortex, while much fewer markers of A2 and A-pan were activated in the hippocampus compared to the cortex (Figure 1B). The majority of markers in A1 and A-pan, except Ugt1a1 and Amigo2, showed an increase with LPS in the spinal cord, and the increased consistency of A2 markers was better than that in the cortex and hippocampus. Changes in the M1 and M2 markers in the spinal cord were similar to those in the cortex (Figure 1C). To further confirm the mRNA data, we performed co-immunofluorescence of reactive astrocytes.

RT-PCR results showed a 7.9-fold in upregulation of C3, which marks A1, a 2.3-fold increase in S100a10, which marks A2, and a 2.2-fold increase in GFAP, which marks A-pan in the 3 mg/kg LPS group in the spinal cord. Consistent with these results, our immunofluorescence data showed an obvious increase in the expression of C3, S100a10, and GFAP. The co-immunofluorescence of C3 with GFAP (Figure 2A) and S100a10 with GFAP (Figure 2B) were also upregulated in the 3 mg/kg LPS group compared to the control group.

Number of phenotypic markers upregulated more than 2-fold by LPS

Interestingly, in the LPS-induced activation of M1 and A1, some classical markers did not show a large increase, but most showed a 2- to 10-fold upregulation, such as CD86 (2- to 4-fold), TNF- α (2- to 9-fold), and C1qa (2- to 4-fold). For further quantitative analysis of the complex heat map results, we compared the number of markers with an upregulation of more than 2-fold under LPS stimulation. When exposed to a low LPS dose, such as 0.1 or 0.33 mg/kg, most markers of A1 and A-pan in the cortex and spinal cord were activated, while fewer markers of M1, M2, and A2 were reactive. With increasing LPS dose, markers of M1 and M2 presented a similar upregulation in both the brain and spinal cord. The majority of A1 and A-pan markers were activated, especially in the cortex and spinal cord, and those of A2 markers presented

a relatively lower activation at large LPS doses. Although largely increased, there seems to be no difference in the activated numbers between 3 and 5 mg/kg LPS doses (Figure 3).

Large LPS dose induced more microglia activation, but not astrocytes

Considering that 5 mg/kg LPS led to higher mortality, and similar numbers (≥ 2 -fold) of reactive gliocytes induced by 3 and 5 mg/kg LPS (Figure 3), we further analyzed whether 3 mg/kg LPS could replace 5 mg/kg LPS to induce microglial and astrocytes polarization. We compared the number of markers reaching the maximum in all LPS doses at 3 mg/kg or 5 mg/kg LPS.

Results showed that all the phenotypes except the A1 subtype in the cortex reached a maximum at 5 mg/kg LPS. More markers of M1, M2 and A-pan in the hippocampus peaked at 5 mg/kg compared to 3 mg/kg LPS. However, only more markers of M2 in the spinal cord peaked at 5 mg/kg compared to 3 mg/kg LPS. From the phenotype perspective, more M1, M2, and A-pan in the cranial tissues were activated at 5 mg/kg LPS than at 3 mg/kg. For A1 and A2 astrocytes, there was no obvious difference between the two LPS doses (Figure 4).

Phenotypic markers presenting a good dose response with LPS

More than ten markers were classified as special genes of each phenotype, but only a few of the 72 markers showed a good dose response with LPS in any nervous tissue; they were Marco marked M1, Ym1 marked M2, and C3 marked A1 (Figure 5).

Downregulation of C3aR in astrocytes reversed A1/A2 ratio induced by LPS

Since C3aR was reported expressed in neurons, microglia, and astrocytes, to prove the specificity knockdown of AAV2/9-r-C3ar1 shRNA-GFAP in astrocytes, we conducted co-immunofluorescence of C3aR with astrocytes (GFAP), microglia (IBA1), and neuron (Neun) (Figure 6).

Our results showed that C3aR was widely co-expressed with GFAP in the spinal white matter (Figure 6A) and Neun in the spinal gray matter (Figure 6B), and decreased in the AAV2/9-r-C3ar1 shRNA-GFAP group (AAV). Compared with the AAV2/9-GFAP group (Control), C3aR was mostly downregulated in astrocytes in the AAV group (Figure 6A), with almost no changes in microglia (Figure 6C) and neurons (Figure 6B). In addition, the expression of C3aR mRNA in the spinal cord of the AAV group was significantly downregulated (Supplementary Fig. 1), indicating the efficiency of AAV2/9-r-C3ar1 shRNA-GFAP. Rats were then injected with 3 mg/kg LPS (i.p.). The dose was selected because no activation advantage of A1 and A2 astrocytes was found at 5 mg/kg LPS compared with 3 mg/kg LPS (Figure 5), while the latter contributed to a higher survival rate (Supplementary Fig. 2).

Data showed that C3aR downregulation in astrocytes significantly upregulated more markers of anti-inflammatory A2 (7/11, CD14, Clcf1, Emp1, Ptx3, Sphk1, Slc10a6, and S100a10) than pro-inflammatory A1 (2/13, Ggta1, Srgn) and A-pan (3/12, Cp, Steap4, and Osmr). In addition, C3aR downregulation also decreased the expression of Fkbp5 and C3, which was marked A1. Unexpectedly, C3aR knockdown in

astrocytes also affected microglial polarization. It decreased several M1 markers (IL-1 β , Ptgs2, Stat1, and TNF- α)—for example, TNF- α by 0.28-fold—and increased markers of M2 (5/15, Retnla, IL-1ra, CD206, Clec7a, and Msx3) —for example, CD206 by 39.3-fold. It also increased some markers of M1 (6/21, Marco, Mr1, Il17, IFN- γ , Ccl5, C1qa) similar to that of IFN- γ , which was upregulated 193.6-fold (Figure 7).

Discussion

LPS-induced M1 polarization has been broadly validated in rodents, but doses of LPS vary between studies^[10,12-14,25]. Yang injected mice i.p. with 1 mg/kg LPS for 4 hours to induce M1 polarization, and observed an upregulation of CD16 and CD86, which marks M1, and downregulation of M2 markers such as Arg1, Ym1, and TGF- β 1 in the brain^[10]. Fenn^[13] and Liao's^[14] data showed that a 0.33 mg/kg LPS injection could increase the expression of M1 markers such as IL-1 β , IL-6, TNF- α , and iNOS. Liu et al. treated mice with an even lower LPS dose (0.1 mg/kg) for 24 hours to induce M1 activation, and they detected upregulation of TNF- α , IL-1 β , IL-6, CD86, CD16, and CD32 in the brain^[12]. In our experiments, we showed that systemic LPS with doses ranging from 0.1 to 5 mg/kg induced M1 activation in both the brain and spinal cord. As the LPS dose increased, the number of activated M1 markers (\geq 2-fold) increased slowly, with 13 of 22 activated at 5 mg/kg LPS. In particular, M2 markers presented a similar increasing trend and degree as M1. Thus, we infer that systemic LPS lasting 24 hours with a dosage ranging from 0.1 to 5 mg/kg may not be a proper choice to induce M1 polarization. On the one hand, it did not make microglia polarize into M1 more than M2; on the other hand, only half of the M1 genes were activated, with less than 25% upregulated at the most reported dose of 0.1 mg/kg. In a study comparing 4 hours and 24 hours of LPS treatment, a weaker activation of M1 markers CD86, iNOS, and IL-1 β was detected at 24 hours compared to the 4-hour group^[13]. Whether a shortened stimulation of LPS contributes more to M1 polarization than M2 polarization is not clear. Moreover, in Wang and Zhao's study^[19,26], small LPS doses (0.75 and 0.25 mg/kg, respectively) were administered i.p. for 7 days to induce cognitive deficits by activating M1 microglia.

LPS has been recently reported to induce A1 activation. Studies by Liddelow^[11], Zamanian^[7], and Kano^[27] all used systemic LPS 5 mg/kg to induce A1 polarization, and Zhang^[28] selected 0.83 mg/kg LPS to activate A1 astrocytes to create murine depression-like behavior. Our results showed that even a small intraperitoneal LPS dose (0.1 mg/kg) could activate more than half of the A1 markers. As LPS increased, more than 80% of A1 markers were activated in both the brain and spinal cord. Furthermore, fewer A2 markers were activated, especially in the brain, indicating that LPS contributes to superior A1 polarization, thus supporting it as a good inducer to activate A1 astrocytes.

Besides the upregulated M1 and A1 genes induced by LPS, we also found that M2 and A2 markers were significantly increased, as demonstrated by our co-immunofluorescence of elevated S100a10 with GFAP certificates. The dichotomy of M1 and M2, or A1 and A2, is an oversimplified conceptual framework. M2, which is often induced by IL-4 can be further characterized into several main states^[29]: M2a with alternate activation and involvement in repair and regeneration; M2b with an immunoregulatory

phenotype; and M2c with an acquired-deactivating phenotype. Chhor et al. found that the majority of M1 markers (10/13) and M2b markers (8/13) were maximally expressed after 12 hours of exposure to LPS. Moreover, M2c genes (IL-10, IL-4R α , and SOCS3) were also increased in enriched microglia 4 hours after LPS injection^[30]. Similarly, we found that more than half of M2 genes, especially M2a genes, were activated when the dose of LPS injection was larger than 0.33 mg/kg, and most reached a maximum at LPS 5 mg/kg. Identically, although less sensitive to LPS compared with A1, A2 was also activated, particularly in the spinal cord. A2 is often induced by MCAO and upregulates many beneficial inflammatory factors, such as hypoxia-induced factors, IL-6 and IL-10^[11,31]. Markers of A2 could also be activated by LPS, although they were more sensitive to MCAO. For example, as A1 markers, H2-D1 was induced 30-fold by LPS, but only 3-fold by MCAO, and Serping1 was induced 6.5-fold after MCAO and 34-fold after LPS^[11]. Our results showed that Serping1 was induced 34-fold in the cortex, 111-fold in the hippocampus, and 55-fold in the spinal cord under LPS 5 mg/kg, indicating a difference in tissues induced by LPS. Overall, LPS exposure could induce not only a pro-inflammatory response, but also anti-inflammatory action. However, the question remains whether the transient M2 or A2 polarization is a self-limiting reaction that is beneficial for regeneration or just a recovery failure.

Genes that are induced by both neuroinflammation and ischemia were classified as pan-reactive (A-pan) genes^[15], different from the specific genes (A1) induced by neuroinflammation. Our research showed that, with increasing LPS dose, A-pan markers presented a similar trend to A1, especially in the cerebral cortex and spinal cord. It should be noted that the dichotomy of A1, A2, and A-pan is an oversimplified conceptual framework, and the status of astrocytes may include a battery of different, but overlapping, functional phenotypes. Although LPS is often used as a classical reagent to induce A1 astrocytes, it could activate other astrocyte phenotypes like A-pan, which may show an overlapping function with A1. We infer that selective blocking of A1 formation could be discounted by the function-similar A-pan, and this requires further study.

In addition to the various LPS doses, gene changes in different tissues were also detected. The cortex and hippocampus were preferred when studying LPS-induced impairments in spatial learning and memory. In Wang et al.'s study, mice were administered i.p. with LPS (250 μ g/kg) once daily for 7 days, a 2-fold increase in TNF- α was found in the cortex and hippocampus by ELISA, and increases were nearly 3-fold for IL-1 β in the cortex and 2.5-fold in the hippocampus^[19]. Consistent with this, our results showed that when exposed to 0.33 mg/kg LPS, TNF- α mRNA was increased 2-fold both in the cortex and hippocampus, while IL-1 β was raised by 19-fold in the hippocampus and 36-fold in the cortex, which may correlate with the increased LPS dose-induced pro-inflammatory state. In particular, more markers of microglia and fewer astrocytes were activated in the hippocampus than in the cortex and spinal cord. These data remind us that activated genes induced by LPS vary not only with LPS dose, but also tissue type and stimulation time. The complex signals in the lesion microenvironment determine the polarization of glial cells.

Our results showed that several markers, such as Marco, Ym1, and C3, presented a significant dose-response relationship with LPS increase in any tissue. TLR4 is an important receptor of LPS, and the interaction of TLR4 with adaptor MyD88 leads to the activation of downstream NF- κ B and subsequent production of pro-inflammatory cytokines^[32]. With increasing LPS, more pro-inflammatory cytokines were released. Whether these dose-dependent markers are involved in inflammation production or could reflect the degree of inflammation or glial cell polarization remains to be elucidated.

Lectin Ym1, a well-established marker of murine M2, is a secretory protein strongly induced by IL-4 and IL-13. The protective action of Ym1 expression after stroke has been demonstrated in several studies. Fumagalli et al. found increased Ym1 levels with reduced infarct sizes 24 h after transient MCAO in CX3CR1-deficient mice^[33]. Barbera-Cremades showed the expression of Ym1 was associated with the inhibition of macrophage proliferation and is induced both by anti-inflammatory signal adenosine and ATP, and pro-inflammatory signal LPS^[34]. This is consistent with Lee's study, which showed that LPS significantly increased Ym1 in the anterior cortex, hippocampus, and entorhinal cortex^[35]. This raises questions regarding how the upregulation of Ym1 and other M2 or A2 markers induced by LPS is regulated by pro-inflammatory stimuli, and why mRNA levels of Ym1 are increased in AD patients and animal models of amyloid deposition, which are typically considered to be associated with a pro-inflammatory (M1 or A1) cytokine environment. These results also suggest a more complex set of microglia and astrocyte phenotypes than the bipolar M1/M2 or A1/A2 characterization, and indicate that LPS-induced neuroinflammation may be mediated by distinct activation subtypes. Meanwhile, the classical markers of M1, such as CD86 and M2 marker CD206, increased with LPS in the cortex and hippocampus, but not in the spinal cord, suggesting that the particular markers of phenotypes should vary with tissues.

Downregulation of C3aR in astrocytes affected the status of activated markers A1 and A2 induced by LPS. Only Ggta1 (4-fold) and Srgn (2-fold) of A1 were upregulated, while more markers of A2, including S100a10, Clcf1, Emp1, Sphk1, Slc10a6, and especially Ptx3 (10-fold) and CD14 (7.4-fold), were elevated, indicating that A2 polarization dominated the reactive astrocytes when C3aR in astrocytes was knocked down. Interestingly, C3aR downregulation in astrocytes under LPS stimulation also affected microglial activation, such as decreasing IL-1 β , Stat1, and TNF- α in M1 and increasing CD206, IL-1ra, and Clec7a in M2. The significant upregulation of IFN- γ and downregulation of TNF- α reflect a dominant anti-inflammatory state, consistent with the superior activation of M2 and A2. We infer that the superior M2 activation may be caused by the A2 polarization. During development, astrocytes can sense subtle changes in neurons to induce the production of C1q in neuronal synapses, which interacts with the microglial C3aR to prune the neuronal synapses through the classic cascade complement pathway^[23]. In the context of Alzheimer's disease pathology, overproduction of C3 from astrocytes can simultaneously communicate with microglial C3aR and neuronal C3aR to dynamically regulate microglial phagocytosis and impair dendritic morphology, as well as synaptic function, subsequently resulting in deterioration of cognitive function^[22]. Meanwhile, reactive A2 astrocytes could also stimulate more active M2 microglia to produce an anti-inflammatory microenvironment.

Limitations

There were several limitations to our study. Our focus was on the dose relationship between LPS and gliocyte phenotypes, so we mainly used RT-PCR to quantify this. Morphology data on gliocyte polarization were partly shown, which could provide more information about polarization. We employed three variables—dose, phenotypes, and tissues—in our study, and did not include time variants. Considering that astrocytes are often activated following microglia, polarization changes with time need to be detected in future studies. Though we did not show the classical one-way ANOVA or Student's *t* test statistical results which we have done, the presented figures could help better interpretate the multiple-dimensional RT-PCR results.

In conclusion, our results showed that systemic LPS activates not only M1 and A1, but also M2, A2, and A-pan. Only a few markers of the 72 genes showed a significant dose response to LPS, which may correlate with the degree of inflammation caused by LPS. No more A1 astrocytes were polarized at 5 mg/kg LPS than at 3 mg/kg LPS in the brain and spinal cord. In addition, the downregulation of C3aR in astrocytes contributed to more A2 and M2 polarization in the spinal cord when exposed to LPS.

Declarations

Ethics approval: All animal procedures performed in this study were reviewed and approved by the Institutional Animal Care and Use Committee in the Chinese Academy of Medical Sciences (Beijing, China), and were performed in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

Consent for publication: Not applicable.

Availability of data and materials: There are no data, software, databases, and application/tool available apart from the reported in the present study. All data are provided in the manuscript and supplementary data.

Competing interests: The authors declare that they have no any competing interests.

Funding: This work was supported by the National Natural Science Foundation of China #81901148 (Afang Zhu), #82071252 (Yuguang Huang), and the Postdoctoral Research Foundation of China #2019M650566 (Afang Zhu).

Authors' contributions: Afang Zhu drafted the manuscript, and performed the RT-PCR procedures. Huan Cui performed the immunofluorescence staining. Wenliang Su performed the intrathecal and intraperitoneal injection. Chaoqun Liu performed the tissues extraction. Le Shen helped to draft the manuscript. Xuerong Yu performed the data analysis and study design. Yuguang Huang conceived of the study and participated in its design. All authors have read and approved the final manuscript.

Acknowledgements: We thank Prof. Chao Ma in the Department of Anatomy, Histology and Embryology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences for his kindness help in immunohistochemistry.

Abbreviations

C3aR, C3a receptor; GFAP, glial fibrillary acidic protein; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; i.p., intraperitoneally; LPS, lipopolysaccharides; MCAO, middle cerebral artery occlusion; MCP, monocyte chemoattractant protein; TGF, transforming growth factor; TNF, tumor necrosis factor.

References

1. Kwon HS, Koh SH. Neuroinflammation in neurodegenerative disorders: the roles of microglia and astrocytes. *Transl Neurodegener.* 2020;91(1):42.
2. Najjar S, Pearlman DM, Alper K, Najjar A, Devinsky O. Neuroinflammation and psychiatric illness. *J Neuroinflammation.* 2013;10:43.
3. Ji RR, Nackley A, Huh Y, Terrando N, Maixner W. Neuroinflammation and central sensitization in chronic and widespread pain. *Anesthesiology.* 2018;129(2):343-66.
4. Ji J, Xue TF, Guo XD, Yang J, Guo RB, Wang J, et al. Antagonizing peroxisome proliferator-activated receptor γ facilitates M1-to-M2 shift of microglia by enhancing autophagy via the LKB1-AMPK signaling pathway. *Aging Cell.* 2018;174(4):e12774.
5. Yan C, Ma Z, Ma H, Li Q, Zhai Q, Jiang T, et al. Mitochondrial transplantation attenuates brain dysfunction in sepsis by driving microglial M2 polarization. *Mol Neurobiol.* 2020;57(9):3875-90.
6. Escartin C, Galea E, Lakatos A, O'Callaghan JP, Petzold GC, Serrano-Pozo A, et al. Reactive astrocyte nomenclature, definitions, and future directions. *Nat Neurosci.* 2021;243(3):312-25.
7. Zamanian JL, Xu L, Foo LC, Nouri N, Zhou L, Giffard RG, et al. Genomic analysis of reactive astrogliosis. *J Neurosci.* 2012;3218(18):6391-410.
8. Li N, Stewart T, Sheng L, Shi M, Cilento EM, Wu Y, et al. Immunoregulation of microglial polarization: an unrecognized physiological function of α -synuclein. *J Neurosci.* 2020;171(1):272.
9. Zhang HY, Wang Y, He Y, Wang T, Huang XH, Zhao CM, et al. A1 astrocytes contribute to murine depression-like behavior and cognitive dysfunction, which can be alleviated by IL-10 or fluorocitrate treatment. *J Neuroinflammation.* 2020;17(1):200.
10. Yang X, Xu S, Qian Y, Xiao Q. Resveratrol regulates microglia M1/M2 polarization via PGC-1 α in conditions of neuroinflammatory injury. *Brain Behav Immun.* 2017;64:162-72.
11. Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, et al. Neurotoxic reactive astrocytes are induced by activated microglia. *Nature.* 2017;541(7638):481-7.
12. Liu Q, Zhang Y, Liu S, Liu Y, Yang X, Liu G, et al. Cathepsin C promotes microglia M1 polarization and aggravates neuroinflammation via activation of Ca-dependent PKC/p38MAPK/NF- κ B pathway. *J Neuroinflammation.* 2019;16(1):10.

13. Fenn AM, Henry CJ, Huang Y, Dugan A, Godbout JP. Lipopolysaccharide-induced interleukin (IL)-4 receptor- α expression and corresponding sensitivity to the M2 promoting effects of IL-4 are impaired in microglia of aged mice. *Brain Behav Immun*. 2012;26(5):766-77.
14. Liao S, Wu J, Liu R, Wang S, Luo J, Yang Y, et al. A novel compound DBZ ameliorates neuroinflammation in LPS-stimulated microglia and ischemic stroke rats: Role of Akt(Ser473)/GSK3 β (Ser9)-mediated Nrf2 activation. *Redox Biol*. 2020;36:101644.
15. Clarke LE, Liddel SA, Chakraborty C, Münch AE, Heiman M, Barres BA. Normal aging induces A1-like astrocyte reactivity. *Proc Natl Acad Sci U S A*. 2018;115(8):E1896-905.
16. La Mura V, Pasarín M, Rodríguez-Vilarrupla A, García-Pagán JC, Bosch J, Abalde JG. Liver sinusoidal endothelial dysfunction after LPS administration: a role for inducible-nitric oxide synthase. *J Hepatol*. 2014;616(6):1321-7.
17. Napier BA, Andres-Terre M, Massis LM, Hryckowian AJ, Higginbottom SK, Cumnock K, et al. Western diet regulates immune status and the response to LPS-driven sepsis independent of diet-associated microbiome. *Proc Natl Acad Sci U S A*. 2019;116(9):3688-94.
18. Xu Y, Xu Y, Wang Y, Wang Y, He L, Jiang Z, et al. Telmisartan prevention of LPS-induced microglia activation involves M2 microglia polarization via CaMKK β -dependent AMPK activation. *Brain Behav Immun*. 2015;50:298-313.
19. Wang D, Dong X, Wang B, Liu Y, Li S. Geraniin attenuates lipopolysaccharide-induced cognitive impairment in mice by inhibiting TLR4 activation. *J Agric Food Chem*. 2019;67(36):10079-88.
20. Miyamoto N, Magami S, Inaba T, Ueno Y, Hira K, Kijima C, et al. The effects of A1/A2 astrocytes on oligodendrocyte lineage cells against white matter injury under prolonged cerebral hypoperfusion. *Glia*. 2020;68(9):1910-24.
21. Su Y, Chen Z, Du H, Liu R, Wang W, Li H, et al. Silencing miR-21 induces polarization of astrocytes to the A2 phenotype and improves the formation of synapses by targeting glypican 6 the signal transducer and activator of transcription-3 pathway after acute ischemic spinal cord injury. *FASEB J*. 2019;33(10):10859-71.
22. Litvinchuk A, Wan YW, Swartzlander DB, Chen F, Cole A, Propson NE, et al. Complement C3aR inactivation attenuates pathology and reverses an immune network deregulated in tauopathy models and Alzheimer's disease. 2018;1006(6):1337-1353.e5.
23. Zhang LY, Pan J, Mamtilahun M, Zhu Y, Wang L, Venkatesh A, et al. Microglia exacerbate white matter injury via complement C3/C3aR pathway after hypoperfusion. *Theranostics*. 2020;10(1):74-90.
24. Mogilenko DA, Kudriavtsev IV, Trulioff AS, Shavva VS, Dizhe EB, Missyul BV, et al. Modified low density lipoprotein stimulates complement C3 expression and secretion via liver X receptor and Toll-like receptor 4 activation in human macrophages. *J Biol Chem*, 2012;287(8):5954-68.
25. Li N, Stewart T, Sheng L, Shi M, Cilento EM, Wu Y, et al. Immunoregulation of microglial polarization: an unrecognized physiological function of α -synuclein. *J Neuroinflammation*. 2020;171(1):272.

26. Zhao J, Bi W, Zhang J, Xiao S, Zhou R, Tsang CK, et al. USP8 protects against lipopolysaccharide-induced cognitive and motor deficits by modulating microglia phenotypes through TLR4/MyD88/NF- κ B signaling pathway in mice. *Brain Behav Immun*. 2020;88:582-96.
27. Kano SI, Choi EY, Dohi E, Agarwal S, Chang DJ, Wilson AM, et al. S-Glutathione-transferases promote proinflammatory astrocyte-microglia communication during brain inflammation. *Sci Signal*. 2019;12(569):eaar2124.
28. Zhang HY, Wang Y, He Y, Wang T, Huang XH, Zhao CM, et al. A1 astrocytes contribute to murine depression-like behavior and cognitive dysfunction, which can be alleviated by IL-10 or fluorocitrate treatment. *J Neuroinflammation*. 2020;17(1):200.
29. Colin S, Chinetti-Gbaguidi G, Staels B. Macrophage phenotypes in atherosclerosis. *Immunological Reviews*. 2014;262(1),153-66.
30. Chhor V, Le Charpentier T, Lebon S, Oré MV, Celador IL, Josserand J, et al. Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. *Brain Behav Immun*. 2013;32:70-85.
31. Hong Y, Liu Q, Peng M, Bai M, Li J, Sun R, et al. High-frequency repetitive transcranial magnetic stimulation improves functional recovery by inhibiting neurotoxic polarization of astrocytes in ischemic rats. *J Neuroinflammation*. 2020;171(1):150.
32. Pålsson-McDermott EM, O'Neill LA. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. 2004;1132(2):153-62.
33. Fumagalli S, Perego C, Ortolano F, De Simoni MG. CX3CR1 deficiency induces an early protective inflammatory environment in ischemic mice. 2013;61(6):827-42.
34. Barberà-Cremades M, Baroja-Mazo A, Pelegrín P. Purinergic signaling during macrophage differentiation results in M2 alternative activated macrophages. *J Leukoc Biol*. 2016;99(2):289-99.
35. Lee DC, Rizer J, Selenica ML, Reid P, Kraft C, et al. LPS-induced inflammation exacerbates phospho-tau pathology in rTg4510 mice. *J Neuroinflammation*. 2010;7:56.

Figures

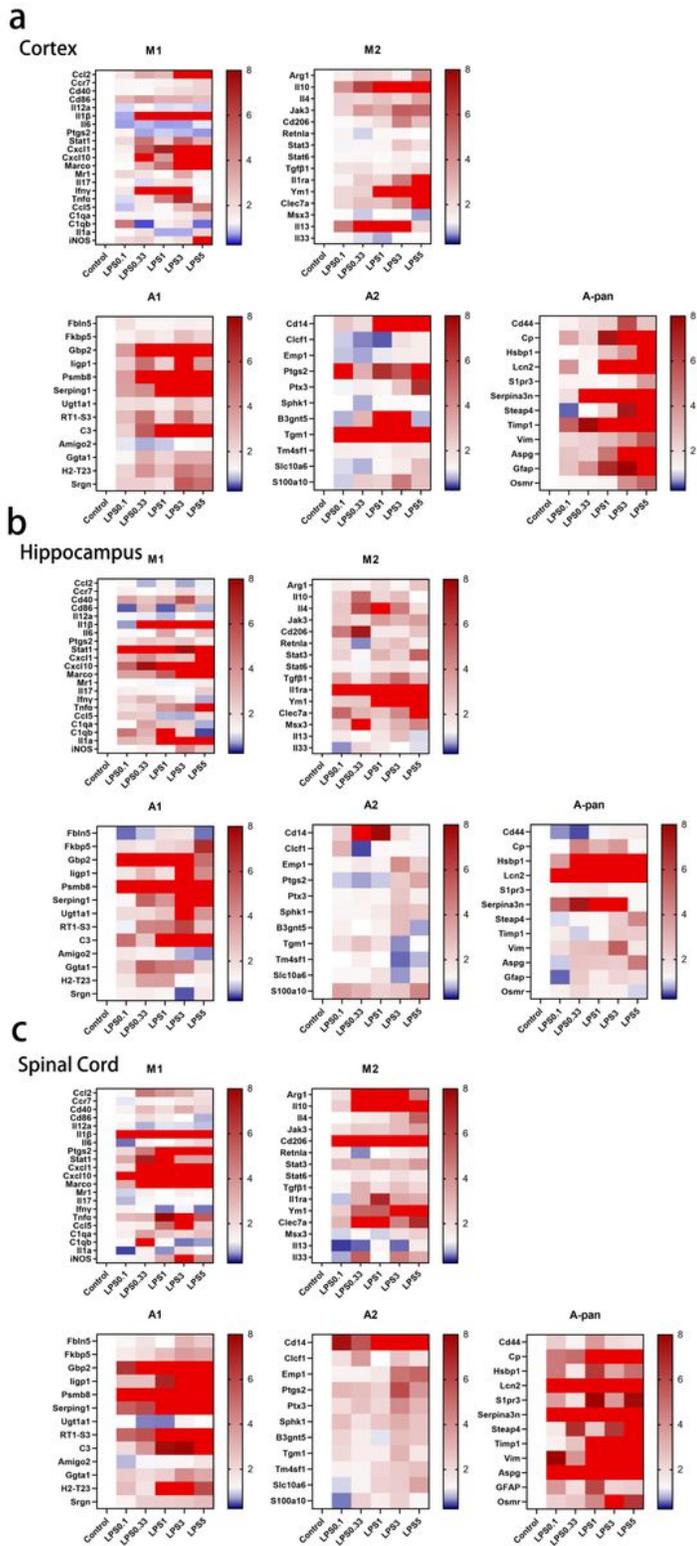


Figure 1

Different doses of systemic LPS induced diverse polarization of microglia and astrocytes. a-c Changes of the selected 72 transcripts associated with markers of M1, M2, A1, A2 and A-pan phenotypes in different groups (control, 0.1, 0.33, 1, 3, 5 mg/kg LPS) detected by RT-PCR 24 hours after LPS treatment in the cerebral cortex, hippocampus and spinal cord. n = 5 in the control group, n = 8 in the 0.1 mg/kg LPS

group, n = 8 in the 0.33 mg/kg LPS group, n = 7 in the 1 mg/kg LPS group, n = 3 in the 3 mg/kg LPS group, n = 3 in the 5 mg/kg LPS group.

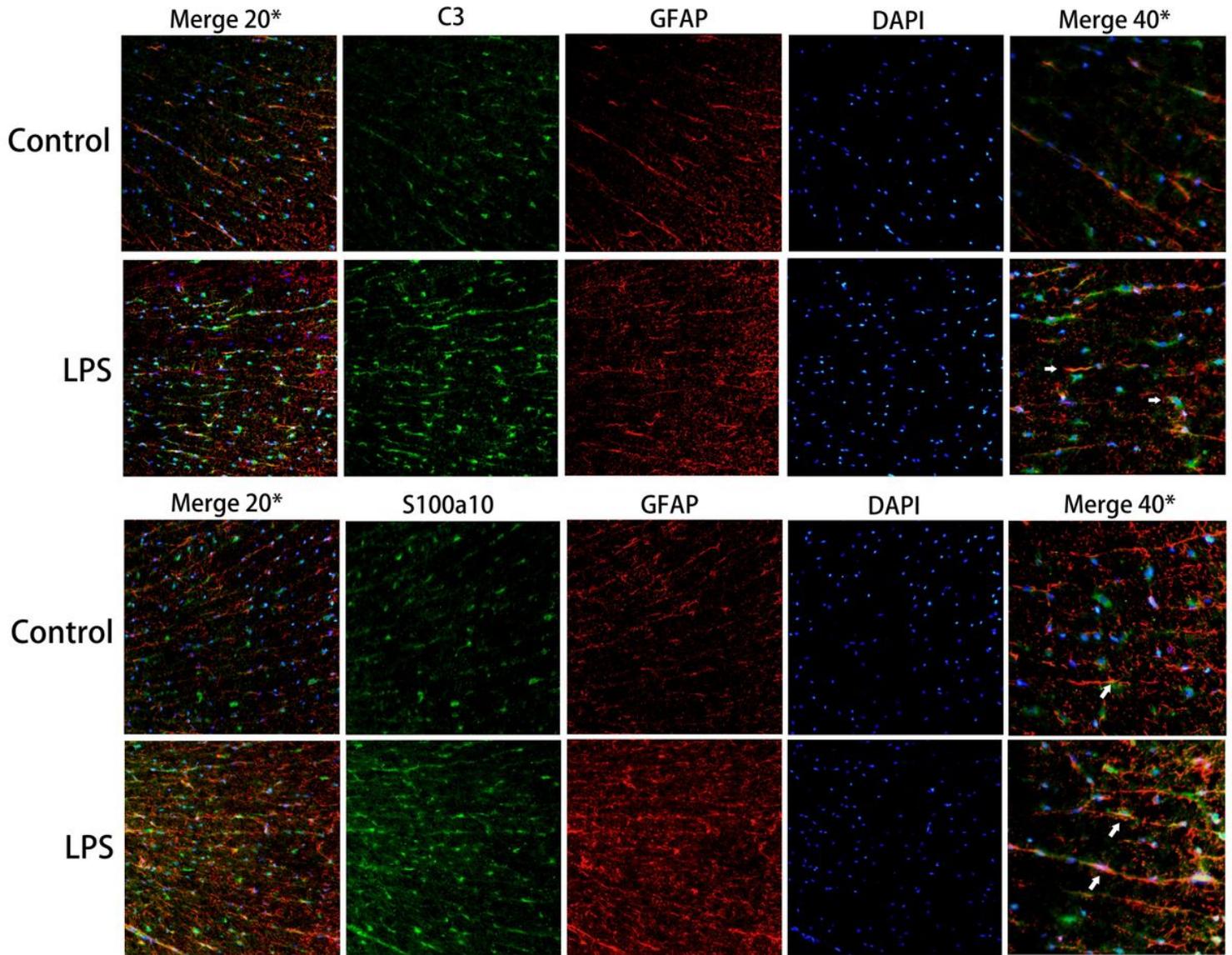


Figure 2

Systemic LPS increased expressions of both A1 marker C3 and A2 marker S100a10. Compared with the control group (equal dose of PBS), injection of 3 mg/kg LPS intraperitoneally increased the colocalization of A1 marker C3 with astrocytic marker GFAP (a), and also A2 marker S100a10 with GFAP (b) in the spinal cord. 20*, 40* presenting magnification. n = 3 per group.

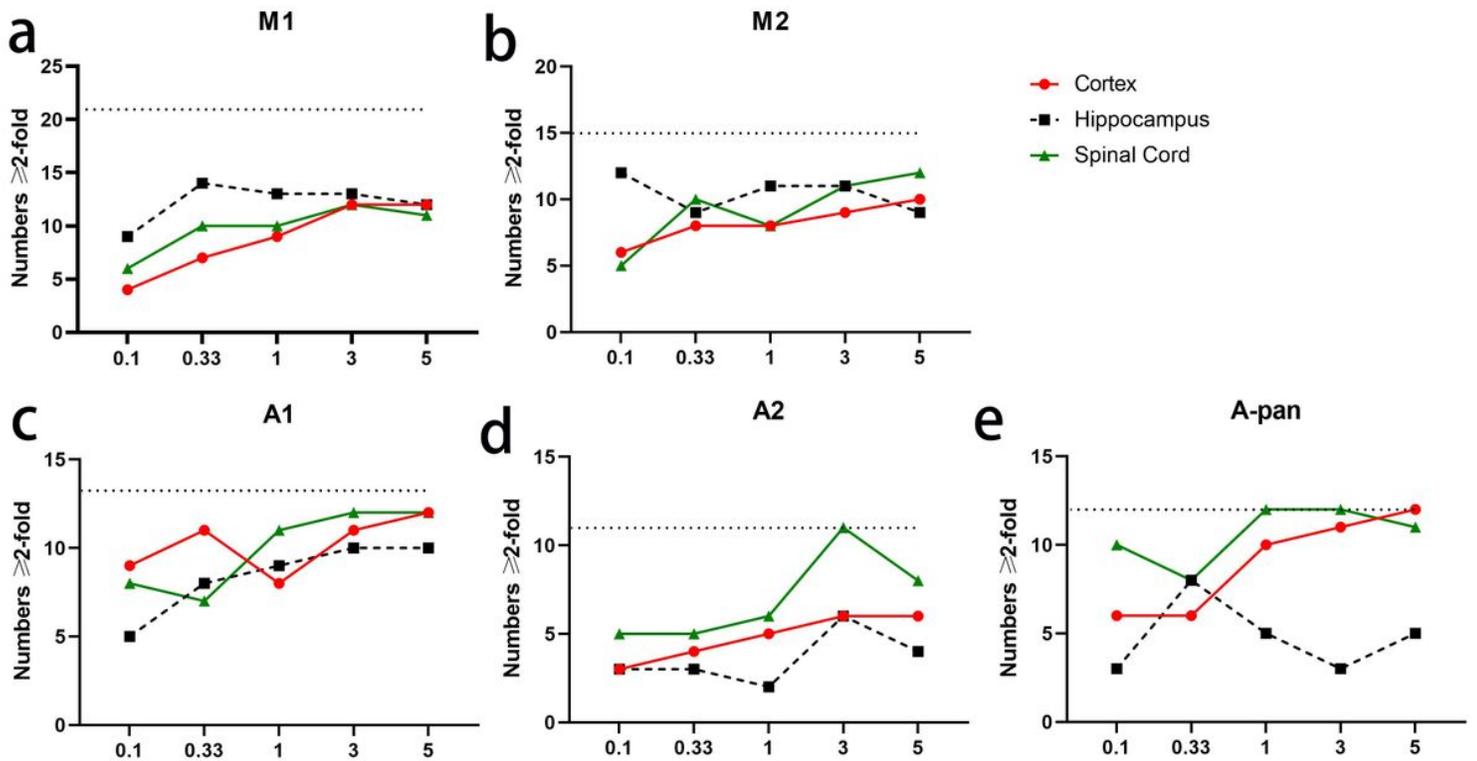


Figure 3

Number of activated phenotypic markers ≥ 2 -fold varied with tissues and LPS doses. a-e Characteristic genes of M1, M2, A1, A2 and A-pan phenotypes upregulated ≥ 2 -fold at various tissues and LPS doses were calculated. The above horizontal dotted line presented the total tested phenotypic markers. n = 3~8 per group.

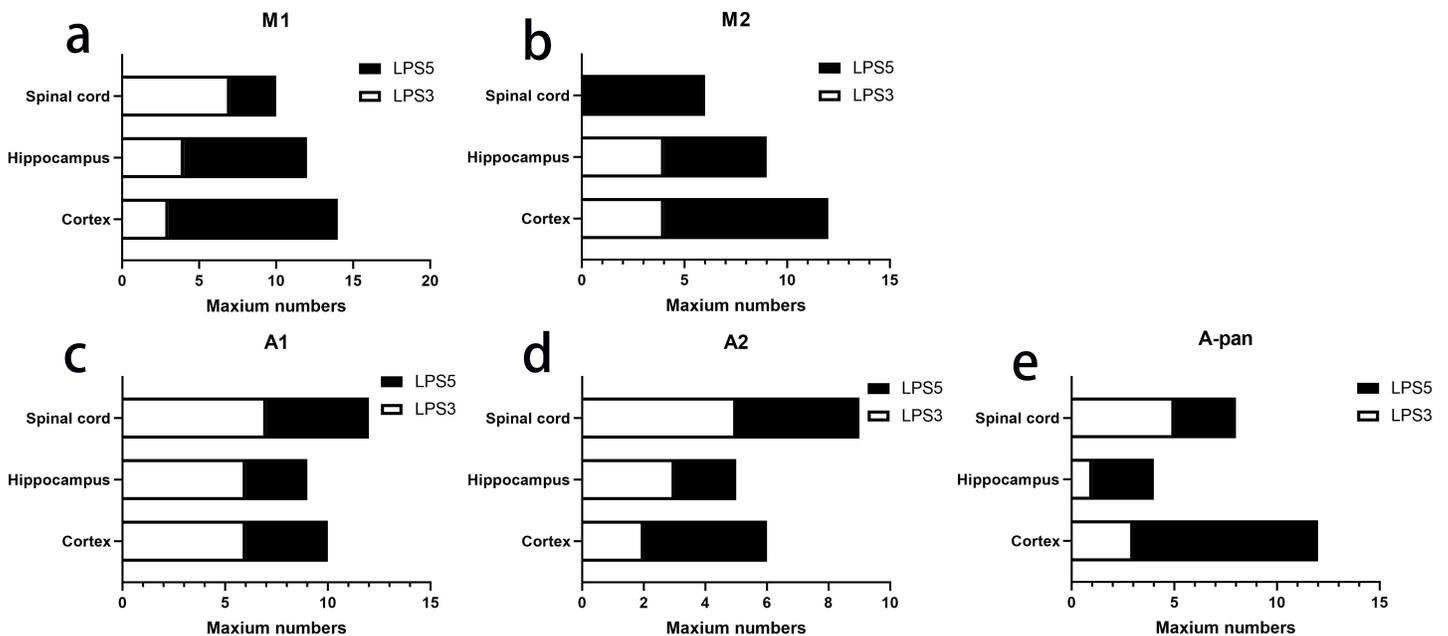


Figure 4

Number of markers reaching maximum of all the LPS doses are compared between 3 and 5 mg/kg LPS. a-b More microglia markers reaching maximum at 5 than 3 mg/kg LPS. c More A1 astrocytes were reactive at 3 than 5 mg/kg LPS. d-e 5 mg/kg LPS activated more A2 and A-pan markers in the brain, while no advantage in the spinal cord compared with 3 mg/kg LPS.

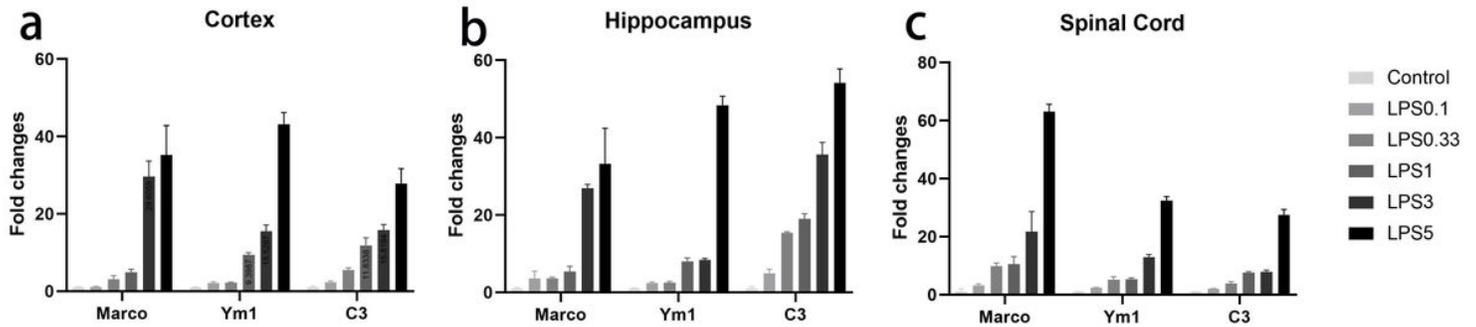


Figure 5

Phenotypic markers presenting a good dose response with LPS. a-c In all the tested 72 genes, Marco marking M1, Ym1 marking M2, C3 marking A1 presented a good dose response with LPS in the cerebral cortex, hippocampus and spinal cord.

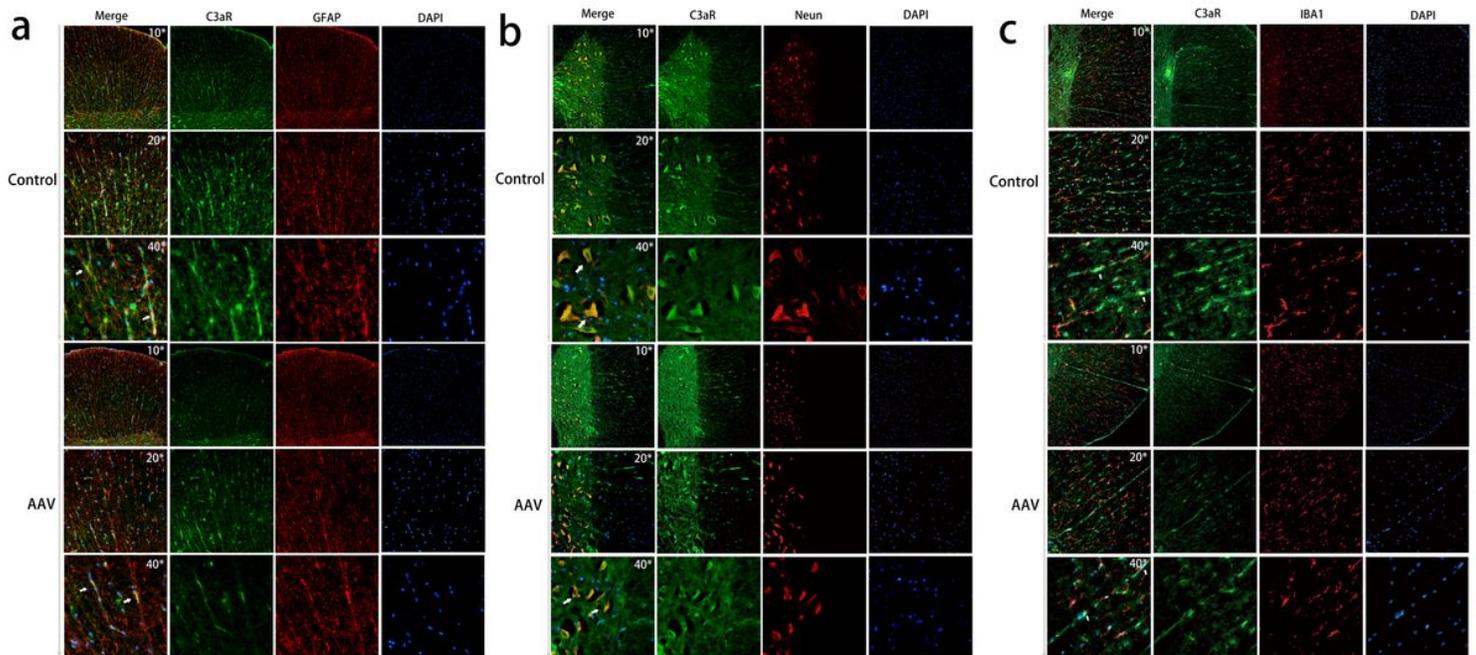


Figure 6

AAV2/9-r-C3ar1 shRNA-GFAP knocked down C3aR expression in astrocytes specifically. a Compared with the AAV2/9-GFAP group (control), downregulation of C3aR in astrocytes by intrathecal injection of AAV2/9-r-C3ar1 shRNA-GFAP decreased the colocalization of C3aR with astrocytic marker GFAP, but not with neuronal marker Neun (b) and microglial marker IBA1 (c) in the spinal cord. 10*, 20*, 40* presenting magnification.

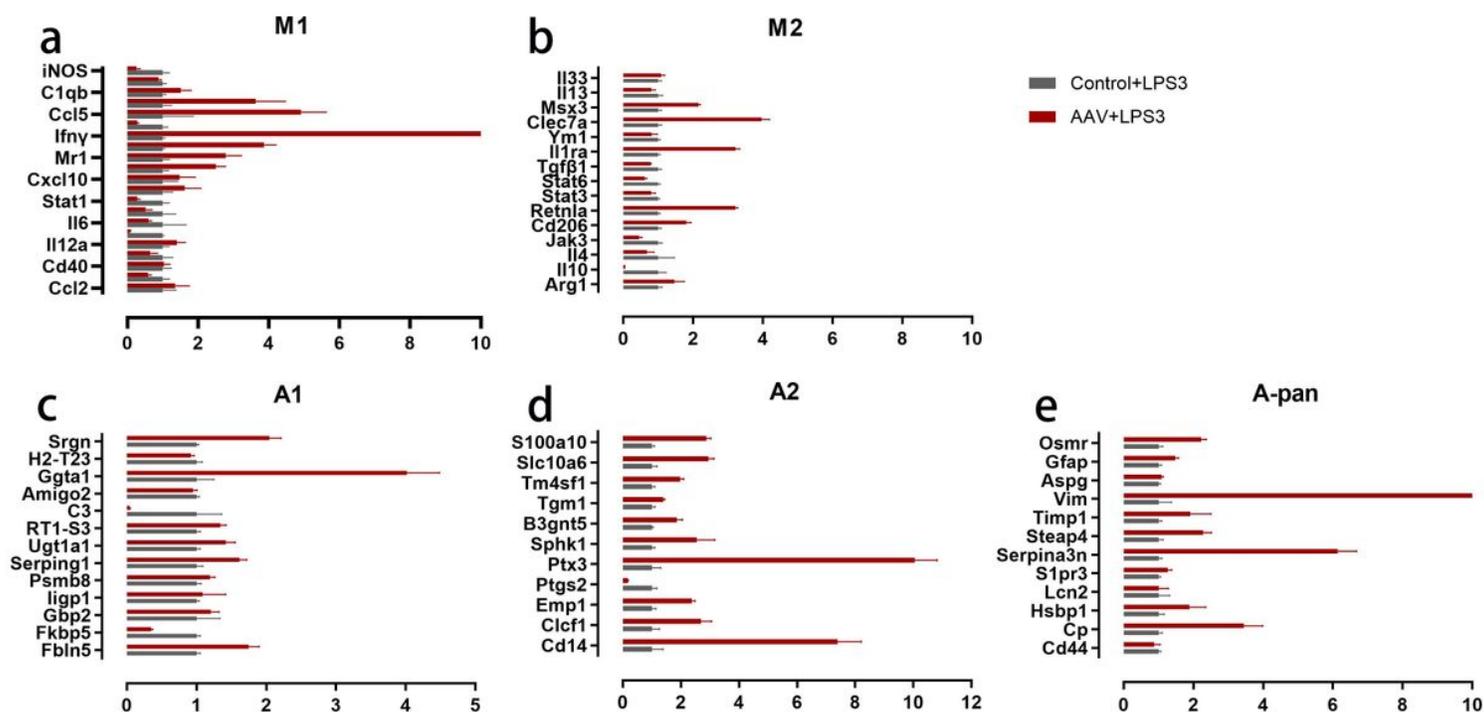


Figure 7

Downregulation of C3aR in astrocytes by AAV promotes an anti-inflammatory state under 3 mg/kg LPS in the spinal cord. a-e The changes of 72 genes marking M1, M2, A1, A2, A-pan phenotypes between the AAV2/9-r-C3ar1 shRNA-GFAP + 3 mg/kg LPS group (AAV + LPS3) and the AAV2/9-GFAP + 3 mg/kg LPS group (Control + LPS3) were detected in the spinal cord. n = 3 per group. Data are presented as mean \pm SEM.

Supplementary Files

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