Cellular Distribution of C-C Motif Chemokine Ligand 2 Like Immunoreactivities in Frontal Cortex and Corpus Callosum of Normal and Lipopolysaccharide Treated Animal

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Abstract

Background: C-C motif chemokine ligand 2 (CCL2) is reported to be involved in the pathogenesis of various neurological and/or psychiatric diseases. Tissue or cellular expression of CCL2, in normal or pathological condition, may play an essential role in recruiting of monocytes or macrophages into the targeted organs, and be involved in a certain pathogenic mechanism. However, only a few studies focused on tissue and cellular distribution of the CCL2 peptide in the brain's grey and white matters (GM, WM), and the changes of the GM and WM cellular CCL2 level in septic or endotoxic encephalopathy was not explored. Hence, the CCL2 cellular distribution in the front brain cortex and the corpus callosum (CC) WM was investigated in the present work by using immunofluorescent staining.

Results: 1) Normally, CCL2 like immunoreactivity (CCL2-ir) in the CC is significantly higher than the cortex, especially when the measurement includes ependymal layer attached to the CC. 2) Structures surrounding the vasculatures contribute major CCL2-ir positive profiles in both GM and WM, but significantly more in the CC WM, in which they are bilaterally distributed and predominantly located in the lateral CC between the cingulate cortex and the lateral ventricles. 3) Following systemic lipopolysaccharide (LPS), the number of neuron-like CCL2-ir positive cells are increased significantly in the cortex, but not in the CC. 4) More CCL2-ir positive elements are accumulated inside microvasculature like structures in the CC WM, compared to those found in the cortex following systemic LPS. 5) Few macrophage/microglia marker-Iba-1 labeled structures exhibit CCL2-ir in normal cortex and CC, but the co-localization is significantly increased following systemic LPS. 6) Following saline or LPS injection, CCL2-ir and GFAP or Iba-1 double labeled structures are observed within the ependymal layer between the lateral ventricles and the CC. No accumulation of neutrophils was detected.

Conclusion: there exist differences in the cellular distribution of the CCL2 peptide in the front brain cortex GM and the subcortical WM - the CC, in both the physiological condition and experimental endotoxemia. Which might cause different pathological change in the GM and WM.

Background

Chemokine C-C motif ligand 2 (CCL2), also termed monocyte chemoattractant protein 1, is known as a pivotal chemoattractant for immune-cells, especially monocytes and T-cells, and is involved in pathogenesis of a number of diseases [1–4]. Some previous studies have shown that the CCL2 synthesized in tissues or cells induced by inflammatory stimulations played an essential role in recruiting monocytes or macrophages into the targeted organs or tissues [2–4]. For example, intervertebral disc herniation induced nociception has been recognized to be resulted not only from nerve compressing but also by local inflammation [2]. The disc cells produce CCL2 when injured, which recruits excessive macrophages with pro-inflammatory phenotype [2]. Hypertension could also induce aortic vascular smooth muscle to produce CCL2 [3], which recruits lipid-filled foam macrophages to accumulate *in situ* and to cause atherosclerosis [4]. More recently, it was shown that one of pathogenic factors for coronavirus induced cardiac injury is related to CCL2 over-expression in myocardial and/or interstitial

cells evoked by virus. The over-expression of CCL2 recruits excessive macrophages that engulf the virus, and the cardiac cells as well [5]. Therefore, illustrating the cellular distribution of CCL2 is significant for understanding the mechanism of pathological changes in certain disease, and beneficial for developing tissue or cell targeted therapeutic approaches against the pathogenic CCL2 [6, 7].

However, the data about CCL2 tissue distribution in the brain and the relationship of this distributive pattern with observed pathological change are still sparse. A set of previous studies indicated that the CCL2 level is higher in the brain white matter (WM) than in the grey matter (GM) [8–10]. For instance, a study in healthy C57BL/6J mice displayed the basic CCL2 mRNA expression in the corpus callosum (CC) WM was about 300-times higher compared to that found in the neighboring cortex [8]. Utilizing Western blot and ELISA, we have shown that the concentration of CCL2 peptide was significantly higher in the brain WM than in the neighboring GM in naïve rats and neurologically health humans [9]. In cases of multiple sclerosis, robust T-cells and macrophages infiltration were observed in the WM, but less frequently in the GM [10, 11]. We also observed in simian immunodeficiency virus (SIV) infected brain that the number of perivascular macrophage cuffing (PC) in the WM was significantly higher than in the GM [12]. Significantly, an autopsy study on an iatrogenic human immunodeficiency virus infected case who deceased shortly after accidentally inoculation showed that the PCs formed fairly early, in a short period after infection [13]. Hence, the higher number of early formed PCs in the WM is possibly related to the higher physiological CCL2 concentration therein [8, 9], rather than the direct effect of the virus [12, 14].

As aforementioned, illustration of *in situ* cellular distribution of the CCL2 would be beneficial for understanding the mechanism of certain pathological changes. As we have known, the CCL2 plays significant role in sepsis and/or endotoxemia related organs injuries [15, 16]; however, there were few studies that compare the cellular CCL2 distribution in the brain GM and the WM of normal subjects and the subjects suffering from sepsis or endotoxemia. Meanwhile, systemic lipopolysaccharide (LPS) is a well-established animal model for the investigation of sepsis and/or endotoxemia related organ-injuries [17]. Therefore, in the present work, we attempted to explore cellular CCL2 distribution in the brain GM and the WM of both normal and LPS treated animals. We carried out CCL2 and astrocyte marker double immunostaining at first to detect the peptide levels in the brain of naïve, saline and LPS injected animals, since expression of CCL2 mRNA or peptide have been frequently identified in cultured astrocytes [18, 19]. Meanwhile, we examined CCL2 labeling in microglia/macrophage and/or neuron, and the potential changes following the systemic LPS. In addition, we traced migration states of neutrophils in the GM and the WM, especially around brain vasculature, using a couple of neutrophil markers, considering they equip Toll-like receptors and upgrade expression of CCL2 receptors after relevant ligands binding to the Toll-like receptors [20, 21].

Materials And Methods

Animals and drugs

The animals used for immunostaining were Wistar rats and C57BL/6J mice, the former has been used by previous authors to show the CCL2 immunostained neurons in normal rat brains [22, 23], and the latter is a background strain for many transgenic preparations including CCR2^{-/-} mice [24].

Six Wistar rats (150 ~ 200g, 4 male and 2 female) and 12 C57BL/6J mice (8 ~ 10 weeks, 8 male and 4 female) were used and divided into 3 groups. For the rats, one rat was used as a normal control, two were intraperitoneally injected (*i.p.*) with sterilized saline equal to the volume of LPS injected, and the *i.p.* delivery of LPS were conducted on the three remaining rats. For the mice, two male mice were used to determine the best approach of CCL2 immunofluorescent staining, and 5 mice (3 male, 2 female) received saline and LPS injections as mentioned above. All experimental protocols and animal care were carried out in line with the *European Union guideline for Laboratory Animal Care and Use* and approved by the Institutional Research and Ethic Committee of the Tongji medical College, Huazhong University of Science and Technology. All efforts were made to minimize animal suffering and the number of animals used in the study.

The LPS (*Escherichia coli*, L2880, Sigma, St Louise, MO) was prepared with a saline solution at 1 mg/ml for rats and 2 mg/ml for mice, and stored at -20°C before using. Both rats and mice would receive a saline or a LPS injection once per day for 5 consecutive days. The rats took LPS at a dose of 1.0 mg/kg, and the mice received LPS at a dose of 1.5 ± 0.3 mg/kg. The animal was euthanized one day after the final injection of the saline or LPS.

Immunofluorescent staining

Immunostaining All rats and mice were euthanized with an overdose of sodium pentobarbital and immediately perfused with saline and followed by a 10% formalin phosphate (Fisher Chemicals) transcardially. The brains were removed, post-fixed overnight, and were cryo-protected by gradient (10% to 30%) sucrose in phosphate-buffered saline (PBS, pH 7.2 ~ 7.4). Coronal frozen sections (14 µm) from the normal, the saline and the LPS injected mice were mounted on the same Plus⁺ slides (see Supplementary Fig. 1) those were immediately saved in a -80°C freezer. Once stained, one section in each slide was isolated by Super PAP Pen as a control section (see Supplementary Fig. 1), on which all procedures were the same except the primary antibody was omitted. After routine immune-blocking, sections were incubated in mouse or rabbit anti-CCL2 antibody (see a list of antibodies in Table 1) overnight. For double staining, either mouse or goat anti-glial fibrillary acidic protein (GFAP) antibody were combined with rabbit anti-CCL2 to detect double labeling. Similarly, rabbit or goat anti-ionizing calcium binding adaptor protein-1 (Iba-1) was used to reveal the macrophages and the microglia. Mouse anti-neuron specific nuclear protein (NeuN) was assumed to find CCL2 expressed neurons. In addition, either monoclonal rat anti-Ly6g or mouse anti-MPO was used and combined with rabbit anti-CCL2 to explore whether there is a neutrophil accumulation and if any neutrophil is CCL2 positive. Alexa Flour 488 or 594 conjugated anti-primary antibody were applied to visualize the immunofluorescent staining. The sections were mounted with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI, Vector Labs).

Quantification of CCL2-like immunoreactivity The CCL2-like immunoreactivity (CCL2-ir) was measured by using the same method we used to quantify the intensity variations of immunofluorescent staining in a previous work [25]. SlideBook 6 (Intelligent Imaging Innovations Inc, Denver, CO) was used to measure and analyze the original CCL2-ir intensity and the change in CCL2-ir intensity following systemic LPS, which was then normalized by the whole measured area. Firstly, we focused on the labeling in the CC WM tract without the ependymal layer and the cortex above the CC. Secondly, the labeling that included the ependymal layer attached to the CC was also selected and measured.

Counting double-labeling of CCL2 with GFAP, Iba-1 or NeuN Then, Image J was applied to count double labeled structures through a “multi-point” recording function. Similarly, the number was normalized by the whole area of either CC or cortex within the photographed scope field, which is finally converted into the number of cells per square millimeter.

Imaging acquisition and processing

Digital images of fluorescent labeling were collected with Keyence BZ-X800E microscope (Keyence Corp. America, Itasca, IL USA) equipped with SlideBook, and the images were analyzed through a Dell computer installed with SlideBook 6 processor. All images for analysis and measurement were observed and captured under 20 X objective lens, except for the verification of double labeling, which was done through 40 X lens. The original pictures were taken through a box size of 960 X 720 pixels and the area was calculated based on scale bar and pixel data. Then, the images were analyzed by Image J for statistical process or organized with Adobe Photoshop CS5 (Adobe Inc, San Jose, CA) for preparing the manuscript.

Statistical Analyses

Group *t*-test was used to compare the intensities of the CCL2-ir labeling in the cortex and CC (also CC + ependymal layer), between normal and saline as well as between saline and LPS groups. Similarly, all of the double labeled cells in the GM and the WM of the different treatment groups were compared with a group *t*-test. Statistical analysis was processed with Graph Pad Prism 5 (Graph Pad Software Inc. La Jolla, CA) and the significant difference of $p < 0.05$, $p < 0.01$ and $p < 0.001$, were represented as “*”, “**” and “***”, respectively.

Results

CCL2-ir levels in the cortex and CC of normal, saline and LPS injected mice

CCL2-ir density in the cortex and CC of normal and saline injected mice The CCL2-ir labeling was distinctively visualized in the immunostained sections (Fig. 1), while no visible positive structure was observed in the control stain (Fig. 1A, B). Most of CCL2-ir positive structures were observed surrounding the vasculature like profiles in both the cortex (Fig. 1C, D) and the CC (E, F arrows). In both the cortex and the CC, some neuron-like cells were encountered, as characterized by the clear DAPI marked nuclei that are centered in CCL2-ir positive ovoid or fusiform profiles (C~F insets). It appears that more neuron-like

cells are scattered in I – III layers of the cortex (Fig. 1C, D) or in the cingulate cortex (Fig. 6). In the CC, CCL2-ir labeling is predominantly situated in bilateral parts of the CC, the areas between cingulate cortex and lateral ventricles (Fig. 1E, F aligned arrows). The staining was resulted from the combination of two CCL2 antibodies (*e.g.* PA5-34505 and AB25124, see Table 1), as illustrated in supplementary figure 1.

Statistical comparison of CCL2-ir intensities in normal and saline GM/WM Firstly, the comparison of CCL2-ir intensity found in the cortex GM and the CC WM showed a significant difference (Fig. 2A, $p < 0.05$). Secondly, the intensity of CCL2-ir staining in the CC including an ependymal layer (CC+Epend) was significantly higher than that in the CC without an ependymal layer (Fig. 2B, $p < 0.05$). The CCL2-ir intensity in the CC+Epend was significantly higher than the CCL2-ir intensity in the cortex (Fig. 2C, $p < 0.01$). Meanwhile, CCL2-ir intensity in the cortex or CC of the normal *vs* saline injected mice showed no obvious difference (Fig. 2D), which indicates the latter can represent the normal cases.

CCL2-ir intensities in cortex and CC of LPS injected mice It seemed that more CCL2-ir labeling overlapped with neuron-like structures (Fig. 1D insets) in the cortex of LPS injected mice. In all rostral, middle and caudal parts of the CC in the LPS injected mice, more CCL2-ir labeling appeared to be confined in the blood vessels (Fig. 1G-J, opened arrows). This kind of labeling was seen less often in the cortex compared to the CC.

*Statistical comparison of CCL2-ir intensities in the saline *vs* LPS injected mice* The intensity of CCL2-ir labeling in the cortex (Fig. 2E) of the LPS injected mice was significantly higher than that in the saline injected cases (Fig. 2E, $p < 0.05$). While in the CC, the difference of CCL2-ir intensity between saline and LPS treated mice was evident, and nearly reached to a statistically significant level (Fig. 2E, $p = 0.054$). Similarly, CCL2-ir intensity in the CC+Epend of the LPS group was obviously higher than that in the saline group (Fig. 2E, $p = 0.074$).

Cellular CCL2-ir distribution in brain of saline and LPS injected mice

Co-localization of CCL2-ir and GFAP-like labeling in the cortex and CC The CCL2-ir and GFAP-like double labeled structures were stained in the cortex and the CC of the normal or saline injected mice (Fig. 3), and the double labeling was usually in areas surrounding vasculature-like (arrows) structures (Fig. 3A, C, arrow-arrowheads, inset). However, the CCL2-ir and GFAP labeling appeared to not always overlap (Fig. 3A GFAP Merged). Co-localization of CCL2 and GFAP immunoreactivity was also observed in the ependymal layers attached to the CC (Fig. 5A, arrow-arrowhead).

Following systemic LPS, co-localization of CCL2 (Fig. 3 arrows) and GFAP-like immunoreactivity was also mainly observed in areas surrounding the vasculature-like structures in both the cortex (Fig. 3B) and the CC (D, insets, arrow-arrowheads). And most of the double labeling in the CC was scattered in regions between the cingulate cortex and the lateral ventricle (Fig. 3C, D). Similarly, the CCL2-ir and GFAP double labeling was also seen abutting or inside the ependymal layer attached to the CC (Fig. 5B, arrow-arrowheads).

Co-localization of CCL2-ir and Iba-1-like labeling in the cortex and CC The co-localization of CCL2-ir (arrows) and Iba-1 (arrowheads) labeling was seldomly visualized in the cortex and CC of the normal or saline injected animals (Fig. 4), likewise, the double labeling was occasionally seen in areas surrounding the vasculature-like (arrows) structures (Fig. 4C inset, arrow-arrowheads). Sporadically, CCL2-ir and Iba-1 double labeled cell was seen in choroid plexus tissue that seems be invaginated from the lateral ventricle (Fig. 5C, arrow-arrowhead).

Following systemic LPS, Iba-1 positive cells with hypertrophic soma and/or hyper-ramified pseudopodia were seen in the cortex and the CC (Fig. 4B, D, opened arrowheads), and more amoeba like Iba-1 positive cells were observed in the CC (Fig. D, framed areas and insets, opened arrowheads). Some hyper-ramified pseudopodia (Fig. 4D, arrow-arrowhead) or amoeba like Iba-1 labeled cells were also CCL2-ir positive (Fig. 4D, framed areas and inset, arrow-arrowheads, Fig. 5E, D opened arrowheads). Meanwhile, co-localization of CCL2-ir (Fig. 5, arrows) and Iba-1 (arrowheads) were identified in or adjacent to the ependymal layers (arrow-arrowheads).

Co-localization of CCL2-ir and NeuN-like labeling in rat cortex and CC In the normal rat brain, most of the CCL2-ir positive (Fig. 6A, arrows) and NeuN labeled cells (arrowheads) were located at I ~ III layer of the cortex (Fig. 6A, insets and arrow-arrowheads). Following systemic LPS, more CCL2-ir (Fig. 6B, arrows) and NeuN (arrowheads) co-localized cells were observed in layer II and III of the cortex (arrow-arrowheads). The CCL2-ir positive neuron like cells (Fig. 6C, D arrows) were also seen in the cingulate cortex above the CC (framed areas and insets, arrow-arrowheads). In addition, the CCL2-ir positive neuron like cells (Fig. 6C, D framed areas and insets, arrow-arrowheads) were encountered in the CC of both normal (C) and LPS treated (D) rats. The result of NeuN immunostaining was from rats, since the data collected from the rats were more consistent.

Co-localization of CCL2-ir and Ly6g or MPO immunostaining in the cortex and CC Clusters of MPO like immunoreactivity were only visualized in the meninges area, or occasionally seen in the choroid plexus in-between the hippocampus of saline or LPS injected mice. Even following systemic LPS, neither MPO nor Ly6g positive cells were observed in the brain parenchyma or the CC+Epend.

Statistical comparison of double labeling between saline and LPS injected mice The double labeled structures (arrow-arrowheads) in the cortex, the CC and the CC+Epend were counted and recorded (Fig. 3-5). The number of double labeled cells from normal, saline and LPS injected mice (Fig. 7A - E) or rats (F), were summarized, statistically analyzed and shown in Fig. 7.

Discussion

We have observed that the CCL2 peptide level in the normal rat CC and the healthy human WM was significantly higher than that in the normal GM [9]. In the present work, we also detected that the CCL2-ir intensities in the CC and the CC+ Epend were significantly higher than that intensity identified in the neighbor cortex. Furthermore, we found that CCL2 labeling in the ependymal layer attached to the CC contributes a significant portion of the CCL2-ir intensities. Several previous studies have reported either

higher basic level of CCL2 mRNA in choroid plexus stromal cells [26] or measurable levels of both CCL2 mRNA and peptide in choroid plexus epithelial cells [27, 28]. Meanwhile, some previous studies implied the possibility of the transmission of the CCL2 from cerebrospinal fluid (CSF) into the brain parenchyma through ependymal cells [29, 30]. It was documented that a basic level of CCL2 peptide (71~200 pg/ml) in CSF was detected either in healthy human [31, 32] or in sham treated animal cases [33]. That concentration in both the choroid plexus and CSF was dramatically increased following pathogen or mechanical stimulations [31–33]. In the current work, CCL2-ir density in the ependymal layer attached to the CC was also enhanced after systemic LPS. While, it is not clear whether the ependymal epithelial cells could actively express CCL2, or the CCL2 peptides in these cells were transferred from the CSF to the CC tissue, or *vice versa*. A previous study substantiates the current finding that the ependymal layers harbor a relatively high density of the CCL2 peptide. In this study on the simian immunodeficiency virus infected brain, more numerous nodular lesions – microglia nodules and/or perivascular macrophage cuff - in the CC were found distributed along the CC border close to the ependymal layers [12].

The other difference of CCL2 cellular distribution between the cortex and CC is that significantly more neurons in the cortex are CCL2-ir positive than in the CC. Further, the number of CCL2-ir and neuron marker co-localization in the cortex was significantly augmented by systemic LPS; whereas, neuron-like CCL2-ir positive cells in the CC did not increase after systemic LPS. This observation might be explained by the fact that the cortex contains more neurons than the CC WM [34]. The fact mentioned above implies that the CCL2 cellular origination in the GM is different from the WM in both physiological and pathological condition, though we do not know what the functional significance for this discrepancy is. The majority of CCL2-ir positive structures in the CC are surrounding the vasculature like profiles, and these CCL2-ir positive rings are predominantly situated bilaterally in lateral parts of the CC between the cingulate cortex and the lateral ventricles. The expression of CCL2 by astrocyte and/or other vascular mural cells is suggested by the fact that the double labeling of CCL2-ir and astrocyte marker was sometimes co-localized around vasculature like structures. Consequently, the number of CCL2-ir and GFAP double labeled structures was significantly higher in the CC than in the cortex, reflecting astrocytes and/or other vascular mural cells contribute more CCL2-ir in the CC. It has been documented that, basic level of CCL2 was identified in murine and human vascular endothelial and mural cells [35–37], and vascular smooth muscle also actively express CCL2 once prompted by plasma cholesterol or by a few unknown soluble proteins [38, 39]. Notably, an immune-electronic microscopy study suggested layers of vascular mural cells may transmit circulating CCL2 from plasma to brain parenchyma, which forms a CCL2 density gradient from plasma to brain tissues [37]. However, which vascular mural cells actively express CCL2, and which of them underlie a transmission mechanism is still an enigma and needs further exploration.

The CCL2-ir positive astrocyte somata are occasionally encountered in both the cortex and CC, but more of them are present in the ependymal layers attached to the CC. It was documented that there exists a specific type of ependymal cells termed as tanycytes, which was originally considered to be a radial glial cell inlaid in the ependymal layer but stretches their long processes far beyond the layer to play a role of molecule transferring between CSF and parenchyma or vascular system therein [29, 30]. It is possible that

the CCL2-ir and GFAP double labeled cells in the ependymal layers that are observed in the current work are tanycytes, since tanycytes were revealed to be GFAP positive by previous immunohistochemistry staining [30, 40]. While, in recent year, the tanycyte has been classified into a few subtypes, such as phenotype of neural stem cell or radial glial cell, or $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ subtypes [40], depending on what kind of specific protein would they synthesize and release. Therefore, it is natural to inquire that what subtype of tanycyte we have encountered could be. It is unknown from the current work whether these tanycyte-like cells could actively synthesize the CCL2 or they just transfer the peptide between the CSF and parenchyma. Further studies are necessary to clarify these questions.

Our result showed that CCL2-ir and Iba-1 double labeled cells are fewer in the cortex, the CC WM and the ependymal layers of both normal and saline injected mice; occasionally, the CCL2-ir positive and Iba-1 labeled pseudopodia was visualized in the area surrounding the vasculature like structures. Reasonably, co-localization of CCL2-ir and Iba-1 labeling was significantly increased following systemic LPS, which is consistent with the previous report on remarkable increase of CCL2 mRNA in macrophages or microglia located in the choroid plexus, the cortex and the CC after systemic LPS [41]. On the other hand, the major cellular components in CSF of the normal brain are T-cells and monocytes [42], and migration of Iba-1 positive cells from the CSF to the parenchyma was observed in the other *in vivo* neuroinflammation model than systemic LPS [43]. Therefore, it is rational for us to consider that the CCL2-ir and Iba-1 double labeled cells in the ependymal layers might be the monocyte derived macrophages that are activated by systemic LPS and entered the CC from the CSF. Of note, neutrophil marker staining showed no neutrophil accumulated in area adjacent to the ependymal layers.

Another interesting observation in this work is that following systemic LPS, CCL2-ir profile appeared to be gathered in the small blood vessels, which occurred more prevalently in the CC. Initially, we thought there might be some blood cells expressing CCL2 and were congested there, because neutrophils could produce CCL2 after LPS stimulation [44]. Then, we attempted to maximize the removal of the blood cells by prolonging the time of saline perfusion, but the situation did not change. On the other hand, no clusters of unclear (marked by DAPI), nor accumulation of neutrophil (labeled by MPO or Ly6g) or monocyte (stained by Iba-1) were observed to co-localize with the CCL2-ir profiles within the blood vessels. These results suggest it is not the blood cells that express CCL2 and congest within the small blood vessels. As we known, structural feature of the vascular system in subcortical and deep WM is substantially different from that in the cortex GM, and the vasculature density and the blood volume in the GM are significantly higher than in the WM [45–47]. Generally, arteries enter and irrigate the superficial GM at first and continue to bifurcate deeply to deep and/or subcortical WM until reaching the ependymal layers; therefore, blood fluid in the WM is a kind of terminal irrigation [45–47]. It was further revealed that when the arteries travel from the cortex to the subcortical WM like the CC, they become tapered and coiled [46]. In addition, following systemic LPS, not only plasma CCL2 elevated but also a serial of related adhesion molecules increased in the circulation, which might build up a molecular network with the CCL2 [48, 49], especially when the vasculatures are tapering and coiling through the CC. Accordingly, we assumed that there would be some accumulation of circulating CCL2 in the terminal vasculatures after systemic LPS, which causes higher CCL2-ir intensity within these vasculatures.

In summary, we indeed observed several differences in the cellular distribution of the CCL2 peptide in the cortex and CC in the current work. 1) CCL2-ir density in the CC is significantly higher than adjacent cortex, especially when the measurement included the attached ependymal layer, which is consistent to our previous report [9] and implies that the ependymal layer contributes to a significant amount of CCL2-ir intensity. 2) Structures surrounding the brain vasculatures contribute to a major amount of CCL2-ir positive profiles in both the GM and the WM, especially in the CC WM. These CCL2-ir positive rings in the CC WM are distributed predominantly in lateral part of the CC between cingulate cortex and lateral ventricles. 3) Significantly more neuron-like CCL2 positive cells in the cortex contributes to CCL2-ir intensity than in the CC WM. Following systemic LPS, the number of them increased significantly in the cortex, but not in the CC. 4) After systematic LPS, more CCL2-ir positive elements appear to be accumulated within microvasculature like structures in the CC WM, comparing to the cortex. 5) Fewer CCL2-ir and Iba-1 double labeled structure is encountered in normal cortex and CC, but the co-localization is significantly increased following systemic LPS in both the cortex and the CC. 6) Following saline or LPS injection, CCL2-ir + GFAP, or + Iba-1 double labeled structures, but neither Ly6g nor MPO positive cells, are observed in the ependymal layer attached to the CC.

Declarations

Ethics approval and consent to participate

All experiments involving animals are approved by Institutional Research and Ethic Committee of Tongji Medical College, Huazhong University of Science and Technology. And the protocol is in accordance with the ARRIVE guidelines (<https://arriveguidelines.org>) to use both sex of the animals equally during statistical analysis, minimize the number of experimental animals and treat the animals carefully in line with the *Laboratory Animal Care and Use Guidelines, etc.*

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

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Author Contributions

Experimental design: Gong XR and Zhang JD.

Saline and LPS injections in mice and rats: Shi X.

Immunofluorescent staining: Shi X, Gong XR and Zhang JD.

Data collection, analysis and photograph: Gong XR and Zhang JD.

Writing and reviewing manuscript: Zhang JD and Xiong HG.

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Conflict of Interest Statement

The authors declare no conflict of interest.

Author Contributions

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Cellular distribution of CCL2-ir in cortex and CC of saline and LPS injected mice. **A** and **B**, control sections stained without CCL2 primary antibody. **C** and **D**, CCL2-ir positive structures (arrows) scatter in the cortex of saline and LPS injected mice, respectively. The insets display neuron-like cells and I ~ IV indicate the cortex layers. The blood vessel walls are also CCL2-ir positive (arrows). **E** and **F**, the CC in saline injected mice, showing majority of CCL2-ir labeled profiles are located in lateral CC between Cg and LV,

meanwhile, neuron-like CCL2-ir positive cells are shown in the insets. **G ~ J**, rostral (**G, H**), middle (**I**) and caudal (**J**) plane of the CC in LPS treated mice, in which a large number of microvasculature like profiles exhibit CCL2-ir positive (opened arrows pointing to typical ones). Cg, cingulate cortex, Ctx, cortex, LV, lateral ventricle, SF, sagittal fissure. Scare bar = 100 μm in all micro-images.

Figure 2

Statistical comparison of CCL2-ir intensities among cortex and CC of normal, saline and LPS injected mice. **A**, intensity of CCL2-ir labeling in the CC WM is significantly higher ($p < 0.05$) than that in the cortex GM, in the naïve animals. **B** and **C**, CCL2-ir intensity in CC+Epend is significantly higher than that in both the CC (**B**, $p < 0.05$) and the cortex (**C**, $p < 0.01$). **D**, CCL2-ir intensity in naïve and saline treated cortex and

CC shows no obvious difference. **E**, following system LPS, the cortex CCL2-ir intensity is significantly increased comparing to the saline injection cases. While, following system LPS, the CCL2-ir intensity in the CC shows a trend toward to a significantly higher than that in the CC of saline injected cases ($p = 0.054$). Similarly, the CCL2-ir intensity in the CC+Epend of saline injected mice is remarkably higher than that in LPS treated animals ($p = 0.074$).

Figure 3

Co-localization of CCL2-ir and GFAP labeling in cortex and CC of saline and LPS injected mice. **A** and **B**, in the cortex of both saline and LPS injected mice, CCL2-ir and GFAP double labeled structures (arrow-arrowheads) are mainly located in area surrounding vasculature like profiles (arrows). While, in some cases, the CCL2-ir positive structure appears to be adjacent to the GFAP labeling (**A**, GFAP Merged, arrowhead). **C** and **D**, similarly, co-localizations of CCL2-ir and GFAP labeling (insets, arrow-arrowheads) are predominantly surrounding the microvasculature like structures (arrows) in the CC of both saline and LPS injected mice. Scale bar = 100 μ m in all micro-images.

Figure 4

Co-localization of CCL2-ir and Iba-1 labeling in cortex and CC of saline and LPS injected mice. **A** and **B**, likewise, CCL2-ir positive structures are mainly encircling the vasculature like profiles (CCL2 panel, arrows). Delicate ramified Iba-1 labeled cells (**A**, Iba-1 panel, arrowheads) are viewed in saline injected mice, while, in LPS injected mice, hyper-ramified and/or hypertrophic soma of Iba-1 labeled cells are encountered (**B**, Iba-1 panel, opened arrowheads). Double labeled structure (**B**, Merged, arrow-arrowheads)

is usually adjacent to the wall of vessels either, but few. **C**, co-localization of CCL2-ir and Iba-1 labeling is occasionally visualized in the CC of saline injected mice (Iba-1 Merged, inset, arrow-arrowhead), which is also adjacent to a microvasculature like profile (framed area). **D**, in the CC of LPS injected mice, more amoeba like Iba-1 positive cells are presented (insets, opened arrowheads), reflecting their activated states. Some of amoeba like Iba-1 labeled cells are also CCL2-ir positive (inset, arrow-arrowhead). Some pseudopodia like structures (arrow) are also CCL2-ir and Iba-1 double positive (Iba-1 Merged, arrow-arrowhead). Scare bar = 100 μ m in all micro-images.

Figure 5

CCL2-ir cellular distribution in ependymal layers attached to the CC. **A** and **B**, CCL2-ir (arrows) and GFAP (arrowheads) double immunostained structures (Merged, arrow-arrowheads) are seen inlaid in the ependymal layers attached to the CC in both saline (**A**) and LPS (**B**) injected mice. **C**, a CCL2-ir (arrow)

and Iba-1 (arrowhead) double labeled cell (Merged, arrow-arrowhead) is seen in choroid plexus tissue that seems be invaginated from lateral ventricle. **D** and **E**, hyper-ramified and amoeba like Iba-1 labeled cells (Iba-1 panel, opened arrowheads) are observed in the CC of LPS injected mice, indicating their activated states. The CCL2-ir and Iba-1 double labeled structures are also regarded in the ependymal layers (Merged, arrow-arrowheads). Scare bar = 100 μ m in all micro-images.

Figure 6

Co-localization of CCL2-ir and neuron marker in cortex and CC of naïve and LPS injected rats. **A**, CCL2-ir positive (arrows) neuron like (arrowheads) cells are mainly observed in cortical layer I ~ II of the naïve cortexes (Merged, insets, arrow-arrowheads). **B**, following system LPS, the number of CCL2-ir positive neuron like cells (arrow-arrowheads) appears to be increased and spread broader in the cortex including cortical layers I ~ III. **C** and **D**, the CCL2-ir positive (arrows) neuron like (arrowheads) cells are also encountered in the CC and the cingulate cortex above the CC, in both naïve and LPS injected rats (Merged, insets, arrow-arrowheads). Scare bar = 100 μ m in all micro-images.

Figure 7

Comparison of co-localization of the CCL2-ir and the other cellular markers in cortex and CC of saline and LPS injected animals. **A**, the number of CCL2-ir and GFAP double labeled structures in normal CC is

significantly higher than that in normal cortex ($p < 0.05$). **B**, this number in the cortex is significantly increased following systematic LPS, comparing to that after saline injection ($p < 0.05$). **C**, this number in the CC+Epend is also significantly augmented by LPS injection, comparing to that in saline injected cases ($p < 0.01$). **D** and **E**, the numbers of co-localization of CCL2-ir and Iba-1 labeling are significantly higher in both the cortex (**D** $p < 0.05$) and the CC+Epend (**E** $p < 0.05$) of LPS injected mice than that of saline injected ones. **F**, Firstly, the ratio of CCL2-ir and NeuN double labeling shows no statistical difference between the brain of naïve and saline injected rats. Secondly, this ratio in the cortex is significantly higher than that in the CC, in both the naïve and saline injected rats ($p < 0.001$ for both). Thirdly, this ratio is significantly raised ($p < 0.05$) in the cortex, but not obviously changed in the CC after the systemic LPS.

Supplementary Files

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