Persistent IL-6 expression is induced in the olfactory bulb of arthritis model mice before the onset of arthritis

Kazuhiro Otani (✉ md11-otani@jikei.ac.jp)  
The Jikei University School of Medicine

Masayuki Yoshiga  
The Jikei University School of Medicine

Masashi Hirano  
The Jikei University School of Medicine

Takayuki Matsushita  
The Jikei University School of Medicine

Kentaro Noda  
The Jikei University School of Medicine

Daitaro Kurosaka  
The Jikei University School of Medicine

Research Article

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Abstract

Background

Rheumatoid arthritis (RA) is complicated by psychiatric symptoms. There are many reports of abnormalities in the brains of RA patients and models of arthritis. However, it is unclear when these abnormalities appear and where they are distributed. In this study, we analyzed the spatiotemporal gene expression changes in the brains of mice with collagen-induced arthritis.

Methods

Mice were divided into three groups: i) collagen-induced arthritis (all mice developed arthritis on day 35): complete Freund’s adjuvant (CFA) and type II collagen at initial immunization, and incomplete Freund’s adjuvant (IFA) and type II collagen at booster immunization; ii) C(+/-) (50% mice developed arthritis on day 35): only IFA at booster immunization; and iii) C(-/-) (no arthritis): only CFA at initial immunization and only IFA at booster immunization. Whole brains were collected at 10 stages of arthritis and divided into six sections. RT-PCR was performed using RNA extracted from the divided brains, and the expressions of proinflammatory cytokines and glial markers were semi-quantified. At the same time, the arthritis score, body weight, and food and water intake were recorded and analyzed for correlation with brain gene expression.

Results

After booster immunization, a transient increase in ITGAM and IL-1β was observed in multiple areas. Interestingly, IL-6 was persistently expressed before the onset of arthritis in the olfactory bulb (OB), which correlated with body weight loss and decreased food intake. This characteristic change in the OB was similarly observed in the C(+/-), but not in the C(-/-). Furthermore, in the C(+/-), non-arthritis mice showed the same changes in the OB as the arthritis mice. This elevation of IL-6 persisted throughout the chronic phase to day 84.

Conclusion

Persistent elevation of IL-6 in the OB from the early stage of arthritis may be an important finding that might explain the neuropsychiatric pathophysiology of RA, which is present in the early stages of disease, and presents as a variety of symptoms over time. These findings also support the idea that the OB may be affected in early disease and persistently under particular peripheral immunoinflammatory conditions, as has been reported in a variety of neurodegenerative diseases.

Background
Rheumatoid arthritis (RA) is a systemic chronic inflammatory disease with synovitis in which an autoimmune response is involved (1). Neuropsychiatric symptoms such as depression, fatigue, cognitive disorder, insomnia, and decreased pain threshold were observed in RA patients (2)-(6). These neuropsychiatric symptoms may decrease the response to treatment for arthritis, reduce the quality of life of patients, and increase long-term mortality (6) (7). Some of these neuropsychiatric symptoms respond to conventional RA treatments and show improvement (6). However, the severity of arthritis assessed by a physician and the degree of neuropsychiatric symptoms can diverge in some cases (8). Therefore, despite a physician's low evaluation of disease activity, neuropsychiatric symptoms may persist, and these patients do not receive adequate treatment. Therefore, one of the clinical challenges for the treatment of RA is to clarify the pathophysiology of arthritis as well as concomitant neuropsychiatric symptoms, and to identify a target site and the appropriate timing for treatment.

Several studies have reported abnormalities of the central nervous system (CNS) in RA (7)(9)(10). Schrepf et al. reported that the inferior parietal lobule (IPL) and medial prefrontal cortex in RA patients have connections to multiple brain networks and that the gray matter in the IPL was reduced and associated with fatigue and cognitive decline (9). Abe et al. reported that RA patients have abnormal functional connections between the anterior cingulate cortex and left insula, which were associated with treatment responsiveness to biological agents using functional magnetic resonance imaging (7). Furthermore, Süß et al. showed that microglial markers were upregulated in the cerebral cortex and behaved differently from the cerebellum in the postmortem brain of RA patients, indicating microglia may be involved in abnormalities of the brain of RA patients and distributed in a site-specific manner (10). However, it is unclear when the CNS network and microglial abnormalities in RA occur and how they spread spatially within the CNS.

Abnormalities of the CNS have been reported in several mouse models of RA (10)(11)(12). Nishioku et al. reported the disruption of the blood–brain barrier in a mouse model of collagen-induced arthritis (CIA) (11). Andersson et al. reported the activation of microglia in the hippocampus, suppression of neurogenesis, and reduction of hippocampal volume in CIA mice (12). Süß et al. reported the activation of microglia in the cortex, striatum, and thalamus in tumor necrosis factor-alpha (TNF-α) transgenic mice, and showed that TNF inhibitors suppressed the activation of microglia (10). Recently, we reported that microglia were activated in the area postrema of CIA mice and that Janus kinase signal transducers and activators of transcription inhibitors suppressed their activation (13)(14). However, it is still unclear when the inflammatory changes in the CNS occur and how they spread spatially in these mouse models.

In the present study, we collected the brains of CIA mice at different stages of arthritis and divided them spatially into six sections. We investigated the temporal and spatial transition of inflammatory changes in the brain, and examined whether there are characteristic changes in the pathology of arthritis.

**Materials And Methods**

**Animals and arthritis model**
Five-week-old DBA/1 J mice were purchased from Sankyo Labo Service (Tokyo, Japan) and immunized intradermally at the dorsal root of the tail with bovine type II collagen (200 μg/mouse; Collagen Research Center, Tokyo, Japan) emulsified in complete Freund’s adjuvant (CFA, Becton Dickinson and Company, Franklin Lakes NJ, USA) (day 0). On day 21, a booster injection of bovine type II collagen emulsified in incomplete Freund’s adjuvant (IFA, Becton Dickinson and Company) was administered in the same manner. The C(+/-) group received the same initial immunization as the CIA group but only IFA as the booster immunization. The C(-/-) group received only CFA at the initial immunization and only IFA at the booster immunization (Fig. 1A).

The severity of arthritis was expressed as the sum of the scores for all four limbs assessed using the following scale: 0, normal; 1, swelling of digits alone or mild swelling of wrist and ankle joints; 2, clear swelling of wrist and ankle joints; and 3, severe swelling of wrist and ankle joints. Fig 1B shows the arthritis score of each group. Each group contained four to six mice.

**Measuring body weight and amount of food and drinking water consumed**

For acclimation, mice (CIA day 35 group) were moved to single-housed cages on day 21 and maintained on a light/dark cycle of 12:12 h (light on at 7:00 and off at 19:00) with food and water available ad libitum. Body weight loss and voluntary feeding were assessed from 12:00 on day 28 until 12:00 on day 35. The body weight of mice and weights of leftover food and water were measured every 24 h to calculate the amount of food and drinking water consumed.

**Brain sampling and segmentation methods**

Mice were euthanized and whole brains were harvested (days 0, 2, 7, 14, 21, 23, 28, 35, 42, and 84) at 12:00. The whole brain was divided into four sections at +3.56, +0.02, and −4.04 mm anteriorly and posteriorly from the bregma (R1, R2, the third sample, R3). The third sample from the anterior was divided further into three sections at ±1.5 mm lateral to the bregma (R4, the middle sample). The middle sample was divided into two sections vertically with reference to the third ventricle (R5, R6). Figure 1C shows the different segments: R1: olfactory bulb (OB); R2: forebrain; R3: midbrain, medulla oblongata, cerebellum; R4: lateral brain including part of the hippocampus and amygdala; R5: parietal brain including the thalamus and hippocampus; and R6: hypothalamus.

**RNA extraction and real-time polymerase chain reaction**

Total RNA was extracted from brain samples using an RNeasy Lipid Tissue Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer’s protocol. Real-time PCR was performed using an Applied Biosystem StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) with TaqMan probes (Thermo Fisher Scientific) and the following primers: integrin subunit alpha M (ITGAM) (Mm00434455_m1), Glial fibrillary acidic protein (GFAP) (Mm01253033_m1), Interleukin (IL)-1β (Mm01336189_m1), IL-6 (Mm00446190_m1), TNF-α (Mm0443258_m1), and Glyceraldehyde 3-phosphate dehydrogenase (Mm9999915_g1).
Statistical analysis

All statistical analyses were performed using GraphPad Prism 4 (GraphPad, Boston, MA, USA). Differences between the means of two groups were analyzed by the unpaired t-test. Correlations were analyzed using the Pearson correlation coefficient. Changes in mRNA expression were analyzed using the one-way analysis of variance and Bonferroni’s multiple comparison test as a post hoc test. P-values <0.05 were considered statistically significant.

Results

Spatiotemporal changes in glial cell marker expression in mice with CIA

To analyze inflammatory changes in the brain during arthritis, we analyzed how ITGAM, a microglial marker, and GFAP, an astrocytic marker, changed after booster immunization in CIA mice. ITGAM expression was upregulated in the R1, R3, and R6 on day 23 compared with day 21 (Fig. 2A), and the upregulation in R3 and R6 was transient and not significantly different from day 21 after day 28. The other sites did not show a significant increase at any time point compared with day 21. GFAP was not upregulated at any site or time phase compared with day 21 (Fig. 2B).

Spatiotemporal changes in proinflammatory cytokine expression in mice with CIA

Next, we analyzed the expressions of proinflammatory cytokines, and found that IL-6 was upregulated in the R1, R4, and R5 (Fig. 3A). Among them, R1 was upregulated from day 23, before the onset of arthritis, and remained elevated until day 42, after the peak of arthritis. IL-1β was significantly elevated in the R1 and R4 on day 28 (Fig. 3B). Other sites also showed a tendency of increased IL-1β on day 28, although this did not reach significance. The same analysis was performed for TNF-α, but no significant increase in expression was observed compared with day 21 (Additional file1: Fig. S1).

Correlation between IL-6 expression in the OB and arthritis-related findings in CIA mice

Because characteristic inflammatory changes were observed in the OB of CIA mice, we analyzed whether these changes correlated with arthritis severity, body weight loss, food intake, and water consumption. The results showed that IL-6 expression on day 35 was negatively correlated with body weight change and food intake (Fig. 4), and IL-6 was not correlated with arthritis severity or water intake.

Changes in ITGAM, IL-6, and IL-1β expression in the OB of C(+/−) mice after booster immunization

To investigate whether the changes in the OB of CIA were characteristic of arthritis pathology, we generated a C(+/−) group that received only IFA without collagen as the booster immunization. These mice unexpectedly developed arthritis (Fig. 1B). However, the severity of the arthritis was milder than that of the CIA group. The expressions of ITGAM, IL-6, and IL-1β in the OB of these mice were analyzed, and the same trends were observed as in CIA mice (Fig. 5A). The C(+/−) group included mice that did not develop arthritis by day 42. The incidence ratios on days 23, 28, 35, and 42 were 0/6, 1/5, 3/6, and 4/5,
respectively. We analyzed the differences in IL-6 expression between the arthritis and non-arthritis mice, and found no significant difference in IL-6 expression at day 35 (Fig. 5B). Because we observed changes in the OB gene expression in non-arthritis mice on day 35, we observed arthritis progression until day 84 to confirm whether these non-arthritis mice were likely to develop arthritis in the future. All mice eventually developed arthritis, although the severity of arthritis was milder than that of the CIA group (Additional file2: Fig. S2). IL-6 expression in the OB of these mice was higher than that in day 21 mice (Fig. 5C).

**Changes in ITGAM, IL-6, and IL-1β expression in the OB of the C-/ - group after booster immunization**

To analyze whether the OB changes in the CIA and C+/ - groups were specific to the arthritis pathology, we generated a C-/ - group that received only CFA without collagen as the initial immunization and only IFA without collagen as the booster immunization. No arthritis was observed in these mice (Fig. 1B) and no upregulation of ITGAM, IL-6, or IL-1β was observed in their OB (Fig. 6).

**Spatiotemporal inflammatory changes in the brains of CIA and C+/- mice after the initial immunization**

Because the CIA and C+/ - groups, which received collagen as the initial immunization, showed characteristic changes in gene expression in the OB before the onset of arthritis, and the C-/ - group, which did not receive collagen as the initial immunization, showed no changes in gene expression, we considered that the response to CFA and collagen at the initial immunization may be important for the inflammatory changes in the OB during the pathogenesis of arthritis. Therefore, we analyzed inflammatory changes in the brain after the initial immunization. ITGAM and IL-1β were transiently upregulated in all regions analyzed (Figs. 7 and 8). GFAP was significantly upregulated in the R1, R2, R4, R5, and R6 (Fig. 7). However, IL6 was upregulated in R4, but no characteristic changes were observed in the OB (Fig. 8).

**Discussion**

The spatiotemporal analysis of inflammatory changes in the brain during arthritis development is important for the early detection of abnormalities in the CNS and for identifying therapeutic targets. Previously, we reported that microglia in the CIA group were activated in the area postrema, a periventricular organ in which the blood–brain barrier does not exist, and that this activation persisted until day 84 (13). Consistent with this, we found an increase in ITGAM expression in the R3, which contains the area postrema, during the course of arthritis (Fig. 2). The most interesting finding of this study is that IL-6 expression in the OB was present before the onset of arthritis and persisted into the chronic phase of arthritis. We hypothesized that IL-6 expression in the OB in the CIA and C+/- groups after booster immunization was related to the particular immune-inflammatory state induced by the booster immunization that was arthritis-inducing, and to the neurological and/or immunological pre-arthritic abnormalities that occurred after the initial immunization.

The OB may be more likely to receive blood-derived information than other brain regions (15)(16). Ueno et al. reported that endogenous albumin leaked more easily into the OB than into the cerebral cortex and
that the blood–brain barrier may not be tight (15). Hasegawa-Ishii et al. reported that peripheral bone marrow-derived cells may be more likely to invade the OB than other brain regions, based on a study using bone marrow transplantation (16). Furthermore, the disruption of the blood–brain barrier was reported to be a characteristic feature of CIA (11). During CIA, activation of the immunoinflammatory system, including elevated serum cytokines and anti-collagen antibody titers, was observed before the onset of arthritis (17) (18). These reports suggest that the early inflammatory changes in the OB observed in this study may have been the result of peripheral immunoinflammatory conditions being transmitted via the blood to the OB. Alternatively, inflammatory changes in the OB may occur via neurological inputs other than blood-derived inputs (19) (20). The presence of inflammation in the nasal cavity was reported to produce microglial activation in the OB via the olfactory nerve (19). Furthermore, gamma-aminobutyric acid (GABA)-ergic neurons project to the OB from the basal forebrain (20), indicating abnormalities other than those of the OB may exist prior to those in the OB and cause inflammatory changes via neurons projecting to the OB. Previously, we reported neuroinflammation in the area postrema, where inflammation might be initiated (13). Thus, there are many possible pathways by which inflammatory changes may occur in the OB after the booster immunization, and this is a subject for future investigation. However, blood-derived and neurological input pathways both originate from a specific peripheral and systemic immunoinflammatory state that can induce arthritis after booster immunization. However, it is unlikely that local abnormalities caused by the intradermal administration of IFA are transmitted via the peripheral nervous system to the CNS without systemic inflammation, resulting in characteristic changes in the OB, as suggested by the lack of OB changes in the C(-/-) group.

Inflammation in peripheral tissues causes widespread inflammatory changes in the CNS (21) (22) (23). Freund’s Adjuvant, which was used to induce arthritis in this study, promotes antigen-specific and -nonspecific immune responses (24). Additionally, granulomatous lesions can occur locally where Freund’s Adjuvant is administered as well as throughout the body (25) (26). Furthermore, the administration of CFA to peripheral tissues was reported to cause inflammatory changes in the CNS (27). However, local and systemic inflammatory changes caused by CFA and IFA alone cannot explain the characteristic changes in the OB in the present study, because we observed changes in the OB in the CIA and C(+/-) groups, but not in the C(-/-) group. In particular, the difference in OB changes after booster immunization in the C(+/-) and C(-/-) groups suggested that abnormalities that occur after the initial immunization with collagen and the arthritogenic response after IFA administration were necessary for the inflammatory change in the OB. Peripheral immunological abnormalities, including anti-collagen immunity in the CIA model, are present at the time of booster immunization (28). The C(+/-) group developed arthritis after IFA administration, indicating a different peripheral immunoinflammatory state was induced from that of the C(-/-) group. Furthermore, inflammatory changes occur throughout the CNS after the initial immunization, suggesting that neurological abnormalities may already be present at the time of booster immunization. These findings suggest that inflammatory changes in the OB are not a phenomenon that occurs in all mice after IFA administration, but rather occurs when IFA is administered to mice with particular neurological and/or immunological abnormalities in the pre-arthritic phase that occur after the initial immunization. However, inflammatory changes in the CNS after the initial
immunization were also seen with CFA alone (Additional file3: Fig. S3), and it is not clear whether CFA + collagen treatment causes neurological abnormalities that are different from those seen with CFA alone. It was reported that antigen-specific Th17 cells in the blood invaded the CNS after an initial immunization with CFA in experimental autoimmune encephalomyelitis, a model of multiple sclerosis, and were involved in the neuroinflammatory process after pathogenesis-inducing stimuli were provided (29). The CIA model can also be used to determine whether arthritis pathogenesis-specific neurological abnormalities occur after the initial immunization in the future.

The most characteristic OB change observed in this study was an increase in IL-6. Several studies of IL-6 knockout mice reported the short-term effects of IL-6 on the CNS under inflammatory pathological conditions (30)(31). Chai et al. reported that IL-6 expression in the CNS was required for fever using IL-6 knockout mice (30). Bluthé et al. reported that lipopolysaccharide and IL-1β administration into the ventricles of the brain reduced sickness behavior in IL-6 knockout mice (31). In the present study, IL-6 expression in the OB correlated with body weight loss and decreased food intake, supporting the previously reported function of IL-6 in the brain under inflammatory conditions. IL-6-expressing cells in the CNS include neurons, microglia, astrocytes, oligodendrocytes, and vascular endothelial cells, as well as infiltrating macrophages and T lymphocytes during inflammatory pathological conditions (32)(33)(34). Histological analysis was not performed in this study; thus, the IL-6-expressing cells are not known. However, because IL-6 expression in the OB correlated with body weight loss and food intake, its expression in the OB may be associated with changes in the brain parenchyma. Although IL-6 expression in the serum and joint tissues was reported in RA and its mouse models (36)(37), our results suggest that the OB may also be a source of IL-6 that can act on the brain parenchyma early in the pathology of arthritis. In RA patients, appetite loss is a common symptom from the early stage of the disease (38)(39), so we are interested in relationship between IL-6 expression in OB and the appetite loss in RA patients.

IL-6 expression persisted even during the chronic phase of arthritis. The prolonged exposure of the CNS to IL-6 was reported to contribute to the depletion of the neural stem cell pool (40). In adult mice, persistent neurogenesis occurs in the subventricular zone (SVZ) and neural stem cells reach the OB through the rostral migratory stream (RMS) (41). IL-6 exposure enhances neurogenesis over the short term, but depletes the neural stem cell pool over the long term (40). However, a decrease in the OB volume was reported in RA (42). Therefore, it will be of interest to investigate whether the persistently elevated expression of IL-6 in the OB under arthritic conditions affects neurogenesis in the SVZ-RMS-OB, and this is a topic for future investigation. In CIA rats, IL-6 inhibitor therapy for 8 weeks after the completion of arthritis was reported to improve the olfactory social memory test better than TNF inhibitor therapy or methotrexate suggesting that IL-6 is involved in long-term brain dysfunction via the OB and may be a therapeutic target (43). Therefore, we are interested in whether the inhibition of IL-6 in the OB improves long-term brain dysfunction.

The OB is a site of abnormalities in Alzheimer’s disease, Parkinson’s disease, and depression during the early pathological stages of disease (44)(45). In addition, reports have suggested that immunological abnormalities, including peripheral acquired immune system and inflammatory conditions, are involved in
these diseases (46)(47)(48). The presence of OB changes early in the pathology of arthritis suggests that it is a site prone to early abnormalities in response to particular immunoinflammatory conditions. Inflammatory changes in the OB were reported to be induced by inflammation in the nose (19), but there are no reports of early and persistent elevations of IL-6 in the OB under other peripheral tissue inflammatory conditions. The early and persistent elevation of IL-6 in the OB under arthritic conditions that do not primarily target the nasal cavity suggests that the OB undergoes early inflammatory changes under certain peripheral immunoinflammatory conditions and that IL-6 may be involved in subsequent brain changes.

**Conclusion**

In this study, we showed that IL-6 was persistently expressed in the OB of arthritis model mice even before the onset of arthritis. The persistent elevation of IL-6 in the OB at the early stage of arthritis is an important finding that might explain the neuropsychiatric pathophysiology of RA, which is present from the early stages of the disease and which presents as a variety of symptoms over time. Our findings also support the idea that the OB may be affected early and persistently, under particular peripheral immunoinflammatory conditions, as has been reported in a variety of neurodegenerative diseases. This is the first report of early and persistent inflammatory changes in the OB of mice under arthritic conditions.

**Abbreviations**

OB Olfactory bulb
RA Rheumatoid arthritis
IPL Inferior parietal lobule
CNS Central nervous system
CIA Collagen-induced arthritis
CFA Complete Freund’s adjuvant
IFA Incomplete Freund’s adjuvant
TNF Tumor necrosis factor
JAK Janus kinase
STAT Signal transducers and activators of transcription
ITGAM Integrin subunit alpha M
GFAP Glial fibrillary acidic protein
IL Interleukin

GAPDH Glyceraldehyde 3-phosphate dehydrogenase

SVZ Subventricular zone

RMS Rostral migratory stream

**Declarations**

**Ethics approval and consent to participate**

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Jikei University, Tokyo, Japan (2017-035, 2018-076, 2018-077).

**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 upon reasonable request.

**Competing interests**

D.K. received grants from Eisai Co., Ltd., Chugai Pharmaceutical Co., Ltd., Ayumi Pharmaceutical Co., Ltd., Daichi Sankyo Co., Ltd., Eli Lilly Japan K.K., AbbVie GK, Asahi Kasei Pharma Co., and Taisho Pharmaceutical Holdings Co., Ltd. None of these pharmaceutical industries had any role in the design of the study, the collection, analysis, and interpretation of data, or in the writing of the manuscript. The other authors declare that they have no competing interests.

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**Authors’ contributions**

K.O. and M.Y. contributed equally to the research. The research project was designed by all authors. M.Y. acquired the data and K.O. analyzed the data. All authors contributed to the interpretation of the data. K.O. wrote the manuscript. All authors approved the submitted version of the manuscript. All authors agreed to be personally accountable for the author’s own contributions and to answer questions related
to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved.

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Figures
Figure 1

Generation of mouse models of arthritis and brain segmentation methods. A. Experimental scheme of the generation of mouse models of arthritis. B. Arthritis scores of the three models. Each symbol represents the average arthritis score at each time point. Bars show the standard deviation (n=four to six per group). C. Graphical image of the brain segments.
Figure 2

Spatiotemporal changes in glial cell marker expression during the course of arthritis. A. Spatiotemporal changes in ITGAM expression. B. Spatiotemporal changes in GFAP expression. Each symbol represents the gene expression of one mouse. Horizontal bars represent the average gene expression. *p<0.05 compared with the day 21 group.
Figure 3

Spatiotemporal changes in proinflammatory cytokine expressions during the course of arthritis. A. Spatiotemporal changes in IL-6 expression. B. Spatiotemporal changes in IL-1β expression. Each symbol represents the gene expression of one mouse. Horizontal bars represent the average gene expression. *p<0.05 compared with the day 21 group.
Figure 4

Correlation between IL-6 expression in the OB and arthritis score, body weight change, and food and drinking water consumed. Body weight change was calculated by the body weight on day 35 divided by the body weight on day 28. Each symbol represents one mouse. The correlation coefficient is $r$. *$p<0.05$. 

![Graphs showing correlation between IL-6 expression and various outcomes.](image)
Figure 5

ITGAM, IL-6, and IL-1β expressions in the R1 of the C(+/−) group. A. Gene expression changes during the course of arthritis. B. IL-6 expression in arthritis and non-arthritis mice on day 35. C. IL-6 expression in mice at day 21 and day 84. Each symbol represents the gene expression of one mouse. Horizontal bars represent the average gene expression. *p<0.05 compared with the day 21 group.
Figure 6

ITGAM, IL-6, and IL-1β expressions in the R1 of the C(-/-) group. Each symbol represents the gene expression of one mouse. Horizontal bars represent the average gene expression. *p<0.05 compared with the day 21 group.
Figure 7

Spatiotemporal changes in glial cell marker expression after the initial immunization. A. Spatiotemporal changes in ITGAM expression. B. Spatiotemporal changes in GFAP expression. Each symbol represents the gene expression of one mouse. Horizontal bars represent the average gene expression. *p<0.05 compared with the day 21 group.
Figure 8

Spatiotemporal changes in proinflammatory cytokine expression after the initial immunization. A. Spatiotemporal changes in IL-6 expression. B. Spatiotemporal changes in IL-1β expression. Each symbol represents the gene expression of one mouse. Horizontal bars represent the average gene expression. *p<0.05 compared with the day 21 group.

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