ARTICLE

Induction of tumor necrosis factor alpha (TNFα) expression in microglia by the accumulation of a superoxide anion donor in rat cerebral cortex

Authors
Hiroshi Kobayashi,[1] Takashi Ishijima,[1] Kazuyuki Nakajima,[1] *

Affiliation
[1]Department of Science and Engineering for Sustainable Innovation, Faculty of Science and Engineering, Soka University, Tokyo 192-8577, Japan

***Corresponding author:**
Kazuyuki Nakajima, Ph.D.
Department of Science and Engineering for Sustainable Innovation, Faculty of Science and Engineering, Soka University,
1-236 Tangi-machi, Hachioji, Tokyo 192-8577, Japan
Phone: +81 426 91 9370
Fax: +81 426 91 9312
E-mail: nakajima@soka.ac.jp

Abstract

Previously we reported that endotoxin-dependent induction of tumor necrosis factor alpha (TNFα expression in microglia was significantly suppressed by the superoxide anion scavenger N-acetyl cysteine (NAC), and that microglia induced TNFα in response to a superoxide anion donor 3-(4-morpholinyl)sydnonimine (SIN-1) *in vitro*. Those findings strongly suggested that superoxide anion is associated with the induction of TNFα in microglia. However, whether TNFα is actually induced in microglia *in vivo* remains to be determined. In the present study, we confirmed the ability of microglia to induce TNFα *in vitro* and examined the effects of SIN-1 on microglial induction of TNFα *in vivo*. The accumulation of SIN-1 solution in rat cerebral cortex led to the induction of TNFα on the ipsilateral, but not the contralateral side. The levels of TNFα in the ipsilateral cortex peaked at 6-12 h post-accumulation. Immunohistochemical study revealed that anti-TNFα antibody-positive cells in the SIN-1-injected region were mainly anti-ionized Ca^{2+} binding adapter molecule-1 (Iba-1) antibody-positive, suggesting that microglia are a major cell type for inducing TNFα. On the other hand, interleukin 1beta (IL-1β) and IL-6 were not detected in the SIN-1-injected cortex. Together, these results indicate that microglia induced TNFα *in vivo* in response to superoxide anion.

Keywords: tumor necrosis factor alpha (TNFα); microglia; superoxide anion; rat brain

1. Introduction

Tumor necrosis factor alpha (TNFα is an inflammatory cytokine that exerts cytocidal action in the nervous system.[1-6] Thus, this cytokine is a target for ameliorating or improving various pathological conditions. From a clinical point of view, it would be helpful to identify the biomolecules critical for inducing TNFα *in vivo*. For this purpose, we need to know the detailed mechanism by which TNFα is induced in specific neural cell types.

We previously reported that microglia *in vitro* induce TNFα in response to endotoxin lipopolysaccharide (LPS),[7] and that such induction was significantly reduced in the presence of superoxide anion scavenger N-acetyl cysteine (NAC).[8] In the same study, we found that superoxide anion donor 3-(4-morpholinyl)sydnonimine (SIN-1) induced TNFα to some extent in microglia *in vitro*.[8] These results strongly suggested that oxygen radicals are somehow associated with the induction of TNFα in microglia. At the same time, we raised the question of whether microglia induce cytokine production *in vivo* in the presence of superoxide anion. However, we had no information regarding the effects of oxygen radicals on the induction of TNFα *in vivo*, and thus the analysis was left for a future investigation. In the present study, we examined the effects of oxygen radicals on the induction of TNFα in rat cerebral cortex.

2. Materials and methods

2.1. Reagents and antibodies

Lipopolysaccharide (LPS) from Escherichia coli was supplied by Difco Laboratories (Detroit, MI). Superoxide anion donor 3-(4-morpholinyl)sydnonimine, hydrochloride (SIN-1)[9] was obtained from Dojindo Laboratories (Kumamoto, Japan). The superoxide anion assay kit and superoxide dismutase (SOD) were obtained from Sigma-Aldrich Japan (Tokyo). The enzyme-linked immunosorbent assay (ELISA) kit for rat TNFα was purchased from R&D Systems Inc. (Minneapolis, MN).

Antibodies against TNFα (L-19) (sc-1351), interleukin 1beta (IL-1β) (M-20) (sc-1251) and actin (C-11) (sc-1615) were supplied by Santa Cruz Biotechnology (Dallas, TX). Anti-IL-6 antibodies were supplied by Santa Cruz Biotechnology (M-19; sc-1265) and Thermo Fisher (ARC0062) (Waltham, MA). Anti-ionized Ca^{2+}-binding adapter molecule 1 (Iba1) antibody (016-20001) was obtained from Fuji film Wako (Osaka, Japan). Anti-glial fibrillary acidic protein (GFAP) antibody (MAB5628) was obtained from Millipore (Temecula, CA). Horseradish peroxidase (HRP)-conjugated anti-goat IgG (sc-2058), HRP-conjugated anti-rabbit IgG (sc-2374) and HRP-conjugated anti-mouse IgG (sc-2055) were purchased from Santa Cruz Biotechnology.

Alexa Fluor 488-conjugated anti-goat IgG (A11055) and Alexa Fluor 568-conjugated anti-rabbit IgG (A11036) were obtained from Invitrogen (Carlsbad, CA).

2.2. Preparation of glial cells and stimulation with LPS

For the preparation of microglia and astrocytes *in vitro*, pregnant Wistar rats were purchased from Clea Japan Inc. (Tokyo) and kept under a 12-h light/dark cycle with food and water *ad libitum*. Animal care and experiments were carried out in accordance with the guidelines of the U.S. National Institutes of Health (NIH) regarding the care and use of animals and were approved by the ethics committee of Soka University (approval number 18005).

Rat microglia were prepared from primary mother cultures derived from newborn rat brains, as described previously.[10] Briefly, microglia were floated by the gentle shaking of a primary mother culture maintained for 10 to 20 days, then seeded on 60-mm dishes (Nunclon^{TM}) at a density of 1.5×10^6. The purity was over 99.9% based on the assessment of Iba1 staining. The adhered microglia were rinsed three times with serum-free Dulbecco's modified Eagle's medium (DMEM) and were then maintained with the same medium for 14–16 h.

Astrocytes were prepared from a primary mother culture maintained for 3 weeks, essentially as described.[11] The astrocytes were subcultured onto 60-mm Nunclon dishes at a density of 1.5×10^6 cells/dish. The cell purity was estimated as 98% based on the staining with anti-GFAP antibody. The astrocyte cultures were rinsed three times with serum-free DMEM and were then maintained with the same medium for 14–16 h.

The ability of microglia/astrocytes to induce TNFα *in vitro* was examined by stimulation with LPS. After LPS stimulation (0.5 μg/mL) for 12 h, microglial conditioned medium (MCM) and astrocytic conditioned medium (ACM) were recovered, respectively, and concentrated by using a Centricut ultrafiltration device (v-10) (Kurabo, Osaka). Each CM was freeze-dried and immunoblotted for TNFα.[8]

2.3. Immunoblotting

The vehicle (methanol)-injected site (control site) and SIN-1-injected site were carefully sliced out at a thickness of 1 mm from the frozen brain. The cut tissue was solubilized with nonreducing sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, and 5% glycerol] and centrifuged at 100,000 g for 30 min. The supernatant of each sample was recovered as tissue extract. The protein in the tissue extract was quantified by the method of Lowry et al.[12] The resultant tissue extract was prepared to contain 10% 2-mercaptoethanol, then was subjected to immunoblotting for TNFα (1:400), IL-1β (1:400), IL-6 (1:100-200) and actin (1:4000).

2.4. Immunoassay for rat TNFα

The amounts of TNFα secreted into the MCM were determined by ELISA (R&D Systems). Each MCM sample (50 μl) diluted three times with diluent buffer and each standard rat TNFα (0-800 pg) were concomitantly poured into the wells of a microplate. After 2 h, the wells were washed 5 times with washing buffer and incubated with TNFα conjugate. Finally, TNFα in each well was colored by applying substrate solution and the absorbance was measured at 450 nm after addition of stop solution (according to the manufacture protocol). Uing this ELISA kit, it was possible to measure TNFα in amounts from 6.5 pg to 800 pg with the standard curve.

2.5. Production of superoxide anion from SIN-1

To confirm that SIN-1 generates superoxide anion, we measured the production by using a superoxide anion assay kit (Sigma-Aldrich) as follows. Briefly, SIN-1 was added to a total volume of 200 μL of a superoxide anion assay medium including 100 μM luminol and 250 μM enhancer at 37. The luminescence of the reaction mixture was determined by a luminometer (Turner Biosystems Instruments, Sunnyvale, CA) at suitable time points. The detected light was expressed in relative light unit (RLU).

2.6. Accumulation of SIN-1 in animals

Wistar rats (8 weeks old, male) were kept on a 12-h daylight cycle *ad libitum* and subjected to the SIN-1 accumulation experiments.

Rats were anesthetized with isoflurane and fixed to a corkboard. In each hemisphere, a hole was made in the skull at the following coordinates: in the left hemisphere, 5 mm posterior, 3 mm lateral to the bregma; in the right hemisphere, 5 mm posterior, 3 mm lateral to the bregma. In the left hole, 5 μL of methanol (vehicle) was injected at 5 mm depth. In the right hole, 5 μL of 200 mM SIN-1/methanol was injected at 5 mm depth. The rats were reared for 3 h, 6 h, 12 h, 24 h, or 3 days before decapitation under anesthesia. The whole brains were removed, frozen on dry ice and stored at –80°C until the specific sites were cut out.

2.7. Immunohistochemistry

Each brain was cut into 20-μm-thick sections with a cryostat (Leica CM1510; Leica Biosystems, Nussloch, Germany) at the level of the SIN-1-injected region, and the sections were frozen at –80°C until staining.

Dual fluorescence staining was carried out essentially as described previously.[13] Briefly, the cryosections were fixed with 3.7% paraformaldehyde and treated with acetone. The sections were then blocked with blocking solution containing 0.2% bovine serum albumin/phosphate-buffered saline. The cryosections were incubated with anti-TNFα antibody (1:200) for 16 h and then with anti-Iba1 antibody (1:200) for 16 h at 4°C. These sections were then incubated with Alexa Fluor 488-conjugated anti-goat IgG (1:200) and Alexa Fluor 568-conjugated anti-rabbit IgG (1:200) for 3 h at room temperature. After washing, the sections were dehydrated, mounted, and observed by a fluorescence microscope (Eclipse TS100; Nikon, Tokyo).

2.8. Statistical analysis

The densities of protein bands (TNFα) in immunoblotting were measured by densitometry using ImageJ software (NIH, Bethesda, MD). These densities were expressed as the means ± SDs of three separate experiments. Differences between the control and SIN-1-injected sites were assessed via Student's *t*-test. In all cases, P values less than 0.05 were considered significant (*P < 0.05, **P < 0.01).

3. Results

3.1. Ability of astrocytes and microglia to induce TNFα

First we examined the ability of astrocytes and microglia to induce TNFα *in vitro*. Two dishes of astrocytes and microglia were prepared, and each dish was stimulated with LPS (0.5 μg/mL) for 12 h. At the end of the culture, each conditioned medium was recovered and immunoblotted for TNFα as described in the Materials and methods section.

TNFα was hardly detected in non-stimulated astrocytic CM (ACM, −) or in non-stimulated microglial CM (MCM, −) (Fig. 1A). A slight amount of TNFα was detected in LPS-stimulated ACM (ACM, +L), and significant amounts of TNFα were induced in LPS-stimulated MCM (MCM, +L) (Fig. 1A). The intensity of the TNFα band suggested that the amounts in ACM (+L) were appropriately 0.5% of those in MCM (+L). These results suggested that microglia are a main cell type that induces TNFα *in vivo*.

The production of TNFα in LPS-stimulated microglia was examined over a time course.

TNFα was first detected at 3 h after stimulation, and the amounts increased with the culture time (Fig. 1B). The amounts appeared to peak at 12 h. We found that microglia begin to produce or secrete TNFα from a relatively early time (3 h) after stimulation.

The amounts of TNFα released in MCM were also measured by an ELISA kit. Microglia were stimulated with LPS (0.5 μg/mL), and the MCM of each dish were recovered at 0, 3, 6, 12, or 24 h after stimulation. A portion of the MCM were used to determine TNFα We found that LPS-stimulated microglia (2 x 10^6 cells) released 0 ± 0.3 ng, 1.7 ± 0.4 ng, 10.6 ± 1.7 ng, 17.7 ± 0.4 ng, 23.4 ± 0.7 ng, and 23.6 ± 1.1 ng TNFα at 0, 1.5, 3, 6, 12 and 24 h, respectively (Fig. 1C, open circle). On the other hand, nonstimulated microglia secreted 0.3 ± 0.1 ng and 0.5 ± 0.2 ng TNFα at 12 h and 24 h, respectively (Fig. 1C, closed circle).

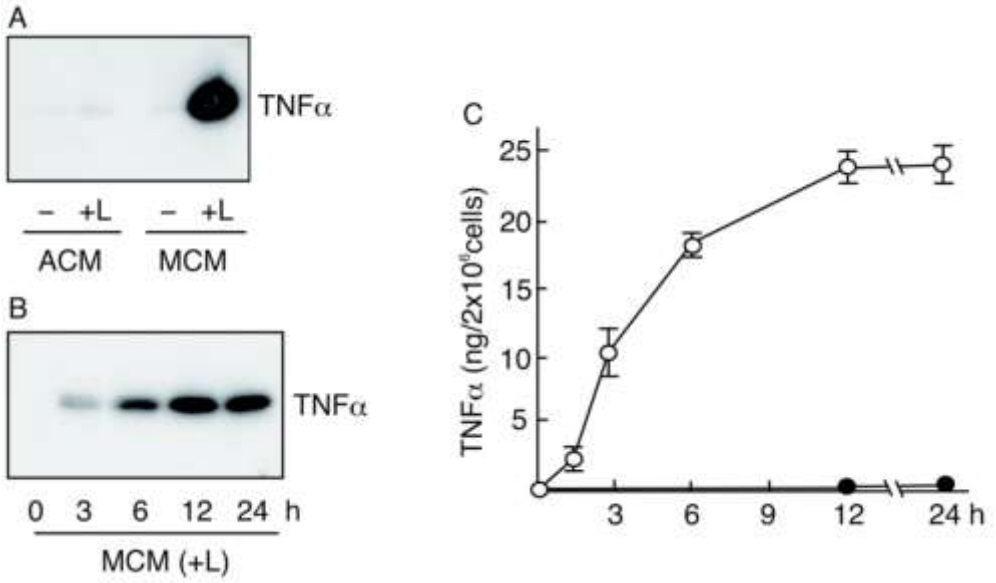

Figure 1 Ability of astrocytes and microglia to induce TNFα

A. Comparison of the abilities between astrocytes and microglia

Two dishes of astrocytic culture and two dishes of microglial culture were prepared as described in the Materials and methods section. The cultures were maintained for 16 h with serum-free DMEM, and then one dish of each type was stimulated with 0.5 μg LPS/mL (+L), while the other was left as a nonstimulated control (–). These dishes were maintained for 12 h, after which each medium was recovered. Astrocytic CM (ACM) and microglial CM (MCM) were concentrated, freeze-dried and immunoblotted for TNFα as described in the Materials and methods section.

B. Time course of TNFα production

Each of five microglial dishes was stimulated with 0.5 μg LPS/mL, and the conditioned medium (MCM) was collected at 0, 3, 6, 12, or 24 h following stimulation. The MCMs were concentrated, freeze-dried, and immunoblotted for TNFα as described in the Materials and methods section.

C. Quantification of TNFα released from microglia

Five microglial cultures were treated with 0.5 μg LPS/mL as described in B (open circle), and two dishes were left without stimulation (closed circle). At 0, 1.5, 3, 6, 12, and 24 h, each medium was collected and the amounts of TNFα were determined by ELISA.

3.2. Production of superoxide anion from SIN-1

In this study, we used SIN-1 to produce superoxide anion *in vivo*. We checked whether or not superoxide anion is actually produced from SIN-1.[9] Figure 2 indicated that SIN-1 generated superoxide anion with a peak at around 2 min, after which production decreased (Fig. 2, open circle, open triangle). When superoxide dismutase (SOD) was added to the assay system, most of superoxide anion was eliminated (Fig. 2, gray square). Thus, SIN-1 was expected to generate significant amounts of superoxide anion if administered *in vivo*.

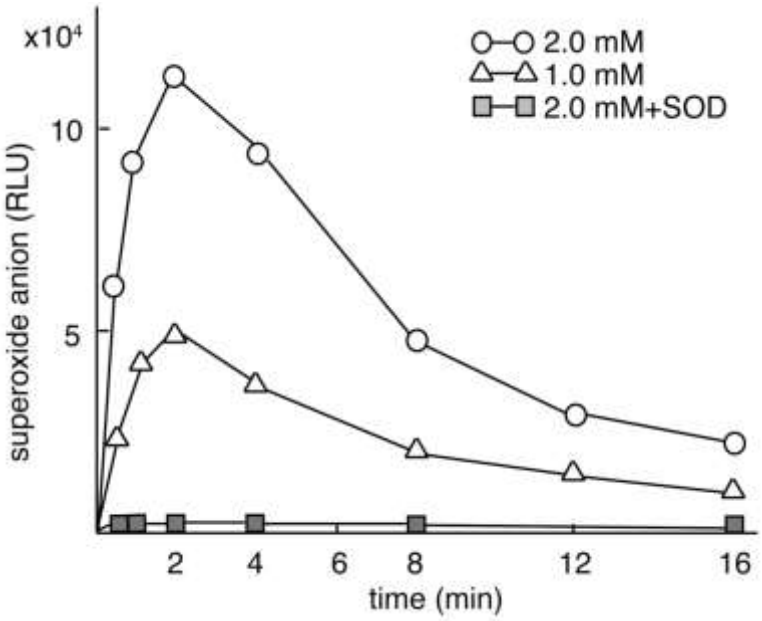

Figure 2 Production of superoxide anion from SIN-1

SIN-1 was added at 1.0 mM (open triangle) and 2.0 mM (open circle) to superoxide anion assay solution (See Methods). In one case, SOD (10 Units) was added to the superoxide anion assay solution containing 2.0 mM SIN-1 (gray square). The generated superoxide anion in these reaction tubes was determined at 0.5, 1, 2, 4, 8, 12, and 16 min. RLU: relative light unit.

3.3. SIN-1-dependent induction of TNFα in vivo

To investigate the effects of SIN-1 *in vivo*, the SIN-1-injected cerebral cortex and vehicle (methanol)-injected cortex were cut from the brains at 3 h, 6 h, 12 h, 24 h, or 3 days after SIN-1 injection. Each tissue lysate of control (C) and SIN-1-injected sites (+S) was immunoblotted for TNFα. The bands for TNFα were observed only in the SIN-1-injected sites (Fig. 3A). TNFα was induced from 3 h after SIN-1 injection and the levels appeared to peak at around 6-12 h (Fig. 3A). The relative intensities of the TNFα band in the SIN-1-injected site at 3 h, 6 h, 12 h, 24 h, and 3 days were 1.0 ± 0.0, 2.18 ± 0.47, 3.38 ± 0.69, 1.10 ± 0.15, and 0.06 ± 0.05, respectively (Fig. 3B), indicating that the greatest induction of TNFα occurred at 12 h post-injection.

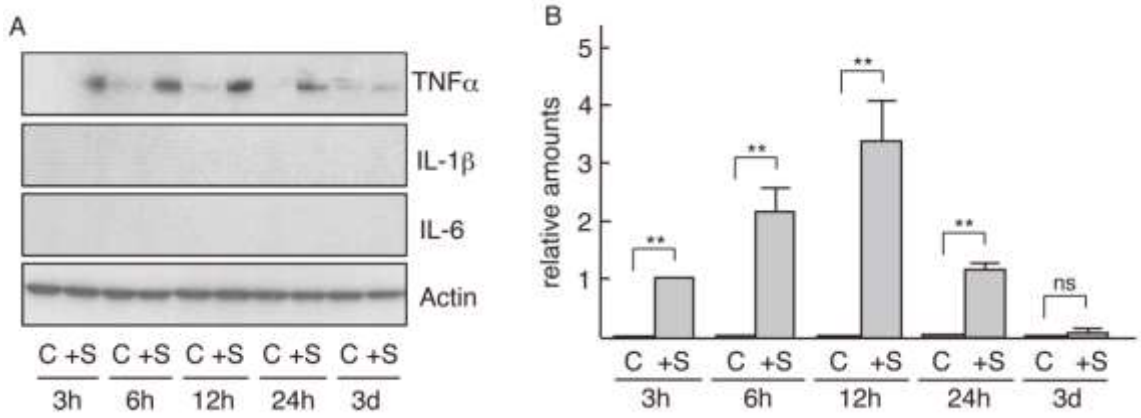

Figure 3 Induction of TNFα in the SIN-1-accumulated cerebral cortex

A. Time course of induction of TNFα

The control site (C; vehicle injected site) and SIN-1-injected site (+S) were recovered at 3 h, 6 h, 12 h, 24 h, and 3 days post-injection, and their tissue extracts were prepared. Each sample set (C, +S) was subjected to immunoblotting for TNFα, IL-1β, IL-6, and actin (Actin).

B. Quantification of TNFα induction

The intensities of the TNFα bands in panel (A) were determined by a densitometer, and each value was expressed relative to that for the SIN-1-injected site at 3 h post-injection (defined as 1.0). The data shown are means ± SDs from three separate experiments (ns: not significant; *P < 0.05, **P < 0.01).

On the other hand, other inflammatory cytokines, IL-1β and IL-6 were not significantly detected at any time point (Fig. 3A). The levels of actin in the tissue extracts of the control (C) and SIN-1-injected (+S) sites were almost constant across the measurement time points, i.e., 3 h, 6 h, 12 h, 24 h, and 3 days (Fig. 3A).

These results indicated that SIN-1 induced TNFα but not IL-1β/IL-6 *in vivo* and that the induced TNFα level peaked at 12 h following SIN-1 injection.

3.4. Immunohistochemistry for TNFα in the SIN-1-injected cerebral cortex

Whether TNFα is induced in microglia in the SIN-1-injected cerebral cortex was immunohistochemically examined at 12 h post-injection. Control and SIN-1-injected cerebral cortices were dually stained by anti-TNFα antibody and anti-Iba1 antibody.

In the vehicle-injected site (ct), we observed hardly anti-TNFα antibody-stained cells (Fig. 4A, ct. TNFα), although microglia were present (Fig. 4A, ct. Iba1).

On the other hand, in the SIN-1 injected site (+SIN) we observed many anti-TNFα antibody-stained cells (Fig. 4B, +SIN. TNFα). In the same field, many anti-Iba1 antibody-positive cells (microglia) were also observed (Fig. 4B, +SIN, Iba1). The merged image indicates that most TNFα-expressing cells were anti-Iba-1 antibody-positive cells (Fig. 4B, +SIN, merged). This immunohistochemical method demonstrated that TNFα is induced in the SIN-1-injected cortex, mainly in microglia.

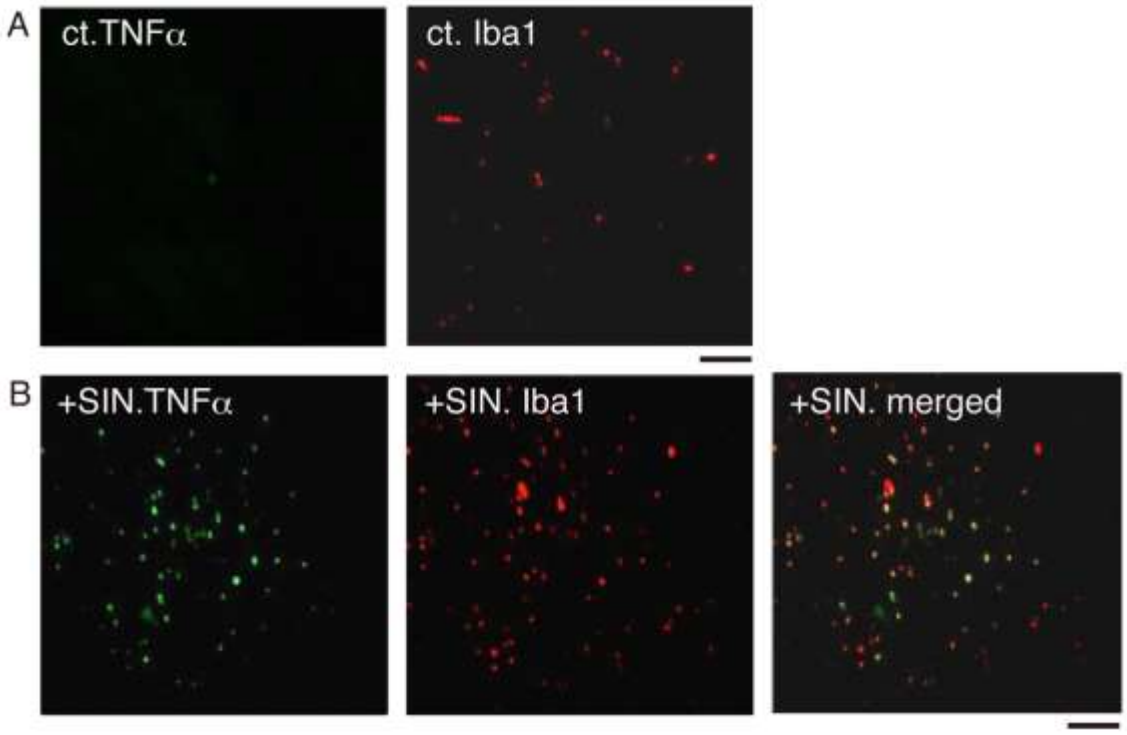

Figure 4 Immunohistochemical detection of TNFα

A. Control side

The brain recovered at 12 h following SIN-1 injection was coronally cut by a cryostat. The vehicle (methanol)-injected side (ct) was stained with anti-TNFα antibody (TNFα) and anti-Iba1 antibody (Iba1). In this control side, anti-TNFα antibody-positive cells were not detected.

B. SIN-1-injected side

The SIN-1-injected side (+SIN) was dually stained by using anti-TNFα antibody (TNFα) and anti-Iba1 antibody (Iba1). Since anti-TNFα antibody-positive cells were detected on this side, anti-TNFα antibody-positive cells (+SIN, TNFα) and anti-Iba1 antibody-positive cells (+SIN, Iba1) were merged in the right hand panel (+SIN, merged). The scale bar is 100 μm.

4. Discussion

TNFα has been generally described as an inflammatory and cytotoxic cytokine, and it has been shown to kill oligodendrocytes[1] and some neurons.[2-6] TNFα has also been shown to exert various actions, including proliferative effects on oligodendrocyte progenitor cells,[14] angiogenic function,[15] activation of glial cells,[16-19] regenerative effects,[20] inhibitory effects on neurite outgrowth[21] and regulatory effects on microglial phagocytosis,[22] suggesting that this cytokine modulates inflammation, degeneration, cell death, reorganization and regeneration in the nervous system. On the basis of these abilities, TNFα is called a pleiotropic factor.

TNFα has often been detected in pathological states of the brain, including in cases of injury,[23-26] Alzheimer's disease,[27] Parkinson's disease,[28] multiple sclerosis,[29] and acquired immunodeficiency syndrome dementia.[30] In these situations, activated microglia are likely candidates for the cell type inducing TNFα. It is thus considered that TNFα is produced from activated microglia in the diseased/injured

nervous system and induces and/or exacerbates the inflammatory condition.[31-34] From a therapeutic point of view, it is desirable to suppress the induction of TNFα in the brain. We thus have to know the detailed mechanism by which TNFα is induced *in vivo*.

Thus far, microglia have been implicated in the production of TNFα in various pathological states.[23, 24, 33, 35] In the present study, therefore, we first investigated the ability of microglia to induce TNFα in an LPS-stimulation system *in vitro*. The results showed that microglia significantly induced TNFα in response to LPS, but astrocytes as the other candidate did so only slightly (Fig. 1A). Although the purity of the astrocytic preparation was suspected, we confirmed that the astrocytes contained GFAP, and not Iba1, indicating that our astrocytes were not contaminated by microglia. Thus we can say that rat astrocytes have a very weak ability to induce TNFα. Accordingly, it is most likely that microglia are a major cell type inducing TNFα *in vivo*.

As shown in Fig. 2, SIN-1 was confirmed to produce a large amount superoxide anion relatively quickly (Fig. 2), and it was expected that the produced superoxide anion immediately stimulated the cells around the SIN-1-injected region.

In fact, TNFα was clearly induced in the SIN-1-injected cerebral cortex (Fig. 3A). Since SIN-1 can produce superoxide anion (Fig. 2), we conjectured that TNFα was induced in microglia by stimulation with superoxide anion. Based on the results, we also anticipated that other inflammatory cytokines, such as IL-1β and IL-6, were similarly induced at the SIN-1-injected sites. However, these cytokines were not significantly detected in any the SIN-1-injected sites (Fig. 3A). It was thus clear that TNFα was specifically induced by the effect of superoxide anion.

Immunohistochemical results indicated that the most TNFα-expressing cells were Iba1-positive microglia in the SIN-1-injected cortex (Fig. 4B). This result was supported by an *in vitro* experiment (Fig. 1A). On the other hand, a small number of TNFα-expressing cells were not stained with anti-Iba1 antibody (Fig. 4B), suggesting that these cells were astrocytes. This result might be explained by reports in which astrocytes were shown to induce TNFα.[36, 37] We also detected low levels of TNFα in the cell homogenate of highly purified astrocytes (Fig. 1A), suggesting that astrocytes have a weak ability to produce TNFα.

Thus far, we have not noticed any effects of reactive oxygen radicals on the induction of TNFα *in vivo*. At present, we know that microglia induce TNFα *in vivo* through stimulation by superoxide anion. This knowledge provides a clue to the possible prevention of TNFα production. It might be possible to reduce TNFα levels in the pathological brain by eliminating reactive oxygen radicals.

Conclusion

The accumulation of superoxide anion generator SIN-1 in the rat cerebral cortex led to the induction of TNFα in the region. Microglia were suggested to be a major cell type to induce TNFα *in vivo*.

Contributors

K. Nakajima designed this study and performed cell culture experiments. H. Kobayashi and T. Ishijima carried out the animal experiments, immunoblotting, and immunohistochemistry.

Competing Interests

The authors have declared that no competing interest exists.

Funding

This research was not supported by any grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgments

We thank Yoko Tohyama for her expert and careful care of the animals.

References

1. Selmaj KW, Raine CS. Tumor necrosis factor mediates myelin and oligodendrocyte damage in vitro. *Ann Neurol*. 1988;23:339-346

2. Venters HD, Dantzer R, Kelley KW. A new concept in neurodegeneration: TNFα is a silencer of survival signals. *Trends Neurosci.* 2000;23:175-180

3. Zassler B, Weis C, Humpel C. Tumor necrosis factor-α triggers cell death of sensitized potassium chloride-stimulated cholinergic neurons. *Brain Res Mol Brain Res.* 2003;113:78-85

4. Sedel F, Béchade C, Vyas S, Triller A. Macrophage-derived tumor necrosis factor α, an early developmental signal for motoneuron death. *J Neurosci.* 2004;24:2236-2246

5. Cueva Vargas JL, Osswald IK, Unsain N, Aurousseau MR, Barker PA, Bowie D, Di Polo A. Soluble tumor necrosis factor α promotes retinal ganglion cell death in glaucoma via calcium-permeable AMPA receptor activation. *J Neurosci.* 2015;35:12088-12102

6. Prajapati P, Sripada L, Singh K, Bhatelia K, Singh R, Singh R. TNF-α regulates miRNA targeting mitochondrial complex-I and induces cell death in dopaminergic cells. *Biochim Biophys Acta.* 2015;1852:451-461

7. Nakajima K, Tohyama Y, Kohsaka S, Kurihara T. Protein kinase Cα requirement in the activation of p38 mitogen-activated protein kinase, which is linked to the induction of tumor necrosis factor α in lipopolysaccharide-stimulated microglia. *Neurochem Int.* 2004;44:205-214

8. Yoshino Y, Yamamoto S, Kohsaka S, Oshiro S, Nakajima K. Superoxide anion contributes to the induction of tumor necrosis factor alpha (TNFα) through activation of the MKK3/6-p38 MAPK cascade in rat microglia. *Brain Res.* 2011;1422:1-12

9. Hogg N, Darley-Usmar VM, Wilson MT, Moncada S. Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. *Biochem J.* 1992;281:419-424

10. Nakajima K, Graeber MB, Sonoda M, Tohyama Y, Kohsaka S, Kurihara T. In vitro proliferation of axotomized rat facial nucleus-derived activated microglia in an autocrine fashion. *J Neurosci Res.* 2006;84:348-359

11. Maeda S, Nakajima K, Tohyama Y, Kohsaka S. Characteristic response of astrocytes to plasminogen/plasmin to upregulate transforming growth factor beta 3 (TGFβ3) production/secretion through proteinase-activated receptor-1 (PAR-1) and the downstream phosphatidylinositol 3-kinase (PI3K)-Akt/PKB signaling cascade. *Brain Res*. 2009;1305:1-13

12. Lowry OJ, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951;193:265-275

13. Ichimiya T, Yamamoto S, Honda Y, Kikuchi R, Kohsaka S, Nakajima K. Functional down-regulation of axotomized rat facial motoneurons. *Brain Res.* 2013;1507:35-44

14. Arnett HA, Mason J, Marino M, Suzuki K, Matsushima GK, Ting JP. TNFα promotes proliferation of oligodendrocyte progenitors

and remyelination. *Nat Neurosci.* 2001;4:1116-1122

15. Leibovich SJ, Polverini PJ, Shepard HM, Wiseman DM, Shively V, Nuseir N. Macrophage-induced angiogenesis is mediated by tumour necrosis factor-α. *Nature.* 1987;329:630-632

16. Barna BP, Estes ML, Jacobs BS, Hudson S, Ransohoff RM. Human astrocytes proliferate in response to tumor necrosis factor α. *J Neuroimmunol.* 1990;30:239-343

17. Aloisi F, Carè A, Borsellino G, Gallo P, Rosa S, Bassani A, Cabibbo A, Testa U, Levi G, Peschle C. Production of hemolymphopoietic cytokines (IL-6, IL-8, colony-stimulating factors) by normal human astrocytes in response to IL-1β and tumor necrosis factor-α. *J Immunol.* 1992;149:2358-2366

18. Panek RB, Lee YJ, Itoh-Lindstrom Y, Ting JP, Benveniste EN. Characterization of astrocyte nuclear proteins involved in IFN-γ- and TNF-α-mediated class II MHC gene expression. *J Immunol.* 1994;153:4555-4564

19. Romero LI, Tatro JB, Field JA, Reichlin S. Roles of IL-1 and TNF-α in endotoxin-induced activation of nitric oxide synthase in cultured rat brain cells. *Am J Physiol.* 1996;270:R326-332

20. Schwartz M, Solomon A, Lavie V, Ben-Bassat S, Belkin M, Cohen A. Tumor necrosis factor facilitates regeneration of injured central nervous system axons. *Brain Res.* 1991;545:334-338

21. Neumann H, Schweigreiter R, Yamashita T, Rosenkranz K, Wekerle H, Barde YA. Tumor necrosis factor inhibits neurite outgrowth and branching of hippocampal neurons by a rho-dependent mechanism. *J Neurosci.* 2002;22:854-862

22. von Zahn J, Möller T, Kettenmann H, Nolte C. Microglial phagocytosis is modulated by pro- and anti-inflammatory cytokines. *Neuroreport.* 1997;8:3851-3856

23. Buttini M, Appel K, Sauter A, Gebicke-Haerter PJ, Boddeke HW. Expression of tumor necrosis factor α after focal cerebral ischaemia in the rat. *Neurosci.* 1996;71:1-16

24. Medana IM, Hunt NH, Chaudhri G. Tumor necrosis factor-α expression in the brain during fatal murine cerebral malaria: evidence for production by microglia and astrocytes. *Am J Pathol.* 1997;150:1473-1486

25. Uno H, Matsuyama T, Akita H, Nishimura H, Sugita M. Induction of tumor necrosis factor-α in the mouse hippocampus following transient forebrain ischemia. *J Cereb Blood Flow Metab.* 1997;17:491-499

26. Barone FC, Arvin B, White RF, Miller A, Webb CL, Willette RN, Lysko PG, Feuerstein GZ. Tumor necrosis factor-α. A mediator of focal ischemic brain injury. *Stroke* 1997;28:1233-1244

27. Maccioni RB, Rojo LE, Fernández JA, Kuljis RO. The role of neuroimmunomodulation in Alzheimer's disease. *Ann NY Acad Sci.* 2009;1153:240-246

28. Reale M, Iarlori C, Thomas A, Gambi D, Perfetti B, Di Nicola M, Onofrj M. Peripheral cytokines profile in Parkinson's disease. *Brain Behav Immun.* 2009;23:55-63

29. Cannella B, Raine CS. The adhesion molecule and cytokine profile of multiple sclerosis lesions. *Ann Neurol.* 1995;37:424-435

30. Yeung MC, Pulliam L, Lau AS. The HIV envelope protein gp120 is toxic to human brain-cell cultures through the induction of interleukin-6 and tumor necrosis factor-α. *AIDS.* 1995;9:137-143

31. Banati RB, Gehrmann J, Schubert P, Kreutzberg GW. Cytotoxicity of microglia. *Glia.* 1993;7:111-118

32. Benveniste EN. Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis. *J Mol Med (Berl).* 1997;75:165-173

33. Gregersen R, Lambertsen K, Finsen B. Microglia and macrophages are the major source of tumor necrosis factor in permanent middle cerebral artery occlusion in mice. *J Cereb Blood Flow Metab.* 2000;20:53-65

34. Kempuraj D, Thangavel R, Natteru PA, Selvakumar GP, Saeed D, Zahoor H, Zaheer S, Iyer SS, Zaheer A. Neuroinflammation induces neurodegeneration. *J Neurol Neurosurg Spine.* 2016;1(1) 1-15

35. Cacci E, Claasen JH, Kokaia Z. Microglia-derived tumor necrosis factor-α exaggerates death of newborn hippocampal progenitor cells in vitro. *J Neurosci Res.* 2005;80:789-797

36. Chung IY, Benveniste EN. Tumor necrosis factor-α production by astrocytes. Induction by lipopolysaccharide, IFN-γ, and IL-1β. *J Immunol.* 1990;144:2999-3007

37. Kim HM, Shin HY, Lim KH, Ryu ST, Shin TY, Chae HJ, Kim HR, Lyu YS, An NH, Lim KS. Taraxacum officinale inhibits tumor necrosis factor-α production from rat astrocytes. *Immunopharmacol Immunotoxicol.* 2000;22:519-530

