BMDCs (**Fig.1f,g**).

Next, to confirm that Prxs are major IL-23 inducers in the brain lysate, we performed an antibody-depletion assay. An apparent reduction of IL-23p19-inducing activity was observed in the No. 2 and No. 3 sucrose gradient fractions after depletion of Prx proteins by adding a mixture of polyclonal antibodies (**Fig.1h**), while antibody-depletion of HMGB1, on the other hand, had little effect (**Supplementary Fig.6**). These results suggest that Prx family proteins in the brain lysate are major IL-23 inducers from BMDC.

Originally, Prx family proteins were reported to function as anti-oxidative enzymes that played a neuroprotective role by catalyzing reactive oxygen species (ROS) [26-28]. Prxs are thiol-dependent antioxidants each containing one (1-cysteine (1-Cys): Prx6) or two (2-Cys: Prx1-5) conserved cysteine residues. 2-Cys Prxs scavenge ROS in cooperation with thioredoxin[26,29,30]. However, once released from the cells, Prx is considered to lose its anti-oxidant capacity, because its cysteine residue is rapidly oxidized in the extracellular space and the redox cycle of Prx is abolished by necrotic change[26,31]. Consistent with this notion, the disruption of cysteine residues by point mutations or iodoacetamide treatment did not affect the IL-23-inducing activity of Prx5 (**Supplementary Fig.7**). Thus, extracellular Prx proteins could function as inducers of IL-23 from BMDCs, being independent of anti-oxidant activity.

Extracellular peroxiredoxin is co-localized with infiltrating macrophages in the ischemic brain

Recent reports have demonstrated that Prx family proteins were released in the extracellular fluid following brain tissue death in rats as well as in acute ischemic stroke patients; however, the function of this extracellular Prx remains unknown[32,33]. Thus, we hypothesized that Prx is released from necrotic brain

cells into the extracellular compartment, where it functions as a danger signal. The expression patterns of Prxs during ischemic brain injury were consistent with this hypothesis. The mRNA expression levels of Prx1, 2, 5, and 6 were very high in normal brain tissue, comparable to those of the housekeeping gene, hypoxanthine phosphoribosyltransferase 1 (HPRT1), and were increased further as a result of ischemic brain injury (**Fig.2a**). These data are consistent with previous reports describing the abundance of Prx family proteins in the brain[30].

We then investigated the expression of Prx in ischemic brain tissue through immunohistostaining. Anti-Prx1/2 and Anti-Prx6 antibodies were successfully utilized for immunostaining. Prx6 expression was also observed in normal brain tissues by means of highly sensitive immunohistochemical staining, as previously reported[34] (**Supplementary Fig.8a**). Cerebral ischemic stress induced much stronger Prx6 expression in the infarct region than in the normal tissues (**Fig.2b**). This increase in Prx6 expression was barely observable until 4 hours after the reperfusion, and Prx6 expression was very evident on day 1 but had diminished by day 4 (**Fig.2b**). Specific staining of Prx6 was confirmed by control IgG experiment, which did not exhibit any such patterns (**Fig.2b**, control IgG). A similar increase in Prx1/2 protein staining upon ischemic stress was observed in WT mice, as shown by staining with anti-Prx1/2 antibody. In contrast, no detectable signals were obtained in $Prx1^{-/-}$ mice (**Supplementary Fig.8b,c**). These data strongly exclude the possibility of a nonspecific reaction of the antibodies to necrotic debris.

In the ischemic brain, strong Prx6 expression was observed in the TUNEL-positive infarct region (**Fig.2c**). By using confocal laser microscopy, we found that Prx6-positive debris-like granules were abundant around TUNEL-positive cells in the infarct core (**Fig.2d**). These observations indicate that Prx6 was induced in injured ischemic cells, then released into the

extracellular compartment from dying cells. Importantly, Prx6-positive debris were often observed to be co-localized with the cell membranes of F4/80-positive macrophages (**Fig.2e,f**). Therefore, it is possible that the extracellular release of Prx6 stimulates infiltrating macrophages.

Peroxiredoxins induce IL-23 via TLR2/4 signaling pathway

TLR2 and TLR4 have been implicated in ischemic brain injury[35]. Thus we tried to determine the relationship between the IL-23-inducing activity of Prx and TLR2/4. As shown in **Fig.3a**, the recombinant Prxs-mediated induction of IL-23p19 was decreased in TLR2- and TLR4-deficient BMDC, and completely absent in TLR2/4 double-deficient BMDC. In addition, we confirmed that Prx5 protein activated NF-κB through TLR4 in 293 cells expressing TLR4 (**Supplementary Fig.9**). Therefore, Prx1, 2, 5, and 6 induced IL-23 by activating both TLR2 and TLR4.

Next, we clarified the relationship between TLR2/4 and the IL-23/IL-17 pathway in the ischemic brain injury. We collected infiltrating immune cells in the ischemic brains of TLR2- and/or TLR4-deficient mice using the Percoll gradient centrifugation method and examined the expression of inflammatory cytokines in the infiltrating immune cells on day 1. The expression levels of IL-23p19, TNF-α, and IL-1β in the infiltrating immune cells were largely dependent on both TLR2 and TLR4, but that of IL-12p35 was not (**Fig.3b**) (physiological data and cerebral blood flow (CBF) are shown in **Supplementary Table 2,3**). Inflammatory cytokine production was more severely reduced in TLR2/4-DKO mice than in TLR2 or TLR4 single KO mice (**Fig.3b**), suggesting a functional redundancy between TLR2 and TLR4. In peripheral blood monocytes, minimal expression of IL-23p19, TNF-α, and IL-1β was observed (**Supplementary Fig.10**), indicating that the activation of macrophages via TLR2/4 occurred mainly in the ischemic

brain, rather than in the periphery.

The infiltration of IL-17-expressing T cells was also dependent on TLR2/4. The absolute number and the fraction of IL-17^{+} T cells were decreased in TLR2 or TLR4 single KO mice, and were further decreased in TLR2/4-DKO mice (**Fig.3c**). However, TLR2/4 deficiency did not significantly decrease the absolute number of IFN-γ^{+} T cells (**Supplementary Fig.11**), this observation is consistent with the minimal effect of TLR2/4 deficiency on IL-12 expression. Thus, both the TLR2 and the TLR4 signaling pathways regulate the IL-23/IL-17 inflammatory axis, but not the IL-12/IFN-γ axis. Bone marrow (BM) chimera experiments indicated that the infiltrating macrophages, but not residential microglia, were responsible for TLR2/4-mediated promotion of ischemic brain injury (**Fig.3d**) and inflammatory cytokine expression (**Supplementary Fig.12**) (CBF is shown in **Supplementary Table 4**). Therefore, both the TLR2 and the TLR4 signaling pathways play essential roles in the induction of ischemic brain damage by infiltrating immune cells.

Extracellular release of peroxiredoxins contributes to the initiation of post-ischemic inflammation

Next, we investigated the therapeutic effects of neutralizing Prxs and/or HMGB1. First, anti-Prx1, 2, 5, and 6 antibodies mixture was administered immediately after stroke onset. The administration of anti-Prx1, 2, 5, and 6 antibodies, but not control IgG, had a neuroprotective effect, associated with a significant reduction in infarct volume (**Fig.4a,b**) (CBF and survival rate are shown in **Supplementary Table 5**). This reduction could still be observed on day 7 (**Supplementary Fig.13**). We also confirmed that these anti-Prx antibodies were delivered to the ischemic brain tissue (**Supplementary Fig.14**). Suppression of infarct volume growth by the administration of anti-Prx antibodies

was dependent on TLR2/4 and IL-23 (**Fig.4c,d** and **Supplementary Fig.15**), further supporting our hypothesis that extracellular Prx induces IL-23 from infiltrating macrophages through TLR2/4 and promotes post-ischemic inflammation and infarct volume growth.

Then we examined the effect of anti-Prx antibodies on inflammation. The administration of anti-Prx antibody mixture immediately after stroke onset decreased the mRNA expression of inflammatory cytokines in the infiltrating immune cells on day 1 (**Fig.4e**). Moreover, the absolute number and the fraction of IL-17-producing T cells on day 3 were significantly decreased in the ischemic brains of mice treated with the anti-Prx antibody mixture (**Fig.4f** and **Supplementary Fig.16**). A significant reduction in the number of TUNEL-positive cells on day 4 was observed in the peri-infarct region of mice treated with the anti-Prx antibody mixture (**Fig.4g**). These results were consistent with our hypothesis that extracellular Prxs function as DAMPs to infiltrating immune cells via the TLR2/4-IL-23-IL-17 pathway.

HMGB1 has been implicated in ischemic brain injury[20]. We confirmed that anti-HMGB1 antibody administered immediately after stroke onset reduced infarct volume (**Fig.4a**). We observed additive therapeutic effects to result from the administration of both anti-Prx and anti-HMGB1 antibodies (**Fig.4a,b**). However, because the extracellular release of HMGB1 is mostly diminished in the ischemic brain within 6 hours after stroke onset[20], and the increase of blood cells infiltration mostly occurs one day after stroke onset[11], we hypothesized that the expression of inflammatory cytokines is more dependent on Prx than on HMGB1. As we expected, the admistration of anti-Prx antibody mixture decreased the mRNA expression of inflammatory cytokines on day 1 more profoundly than one of anti-HMGB1 antibody did (**Fig.4e**).

Then we examined the effect of antibodies administered several hours after

stroke onset, which is important to extend the therapeutic time window. We observed a significant reduction in infarct volume and severity of neurological deficits to result from the administration of the anti-Prx antibody mixture even 6 hours after stroke onset, while the administration of the anti-HMGB1 antibody was much less effective at this time point (**Fig.4h,i**). These data suggest that HMGB1 is a hyperacute DAMP, while extracellular Prx is the secondary one in the acute phase for post-ischemic inflammation. Anti-Prx antibody mixture was therapeutic up to 12 hours after stroke onset (**Fig.4j,k**), however, therapeutic effect was not observed when it was administered 24 hours after stroke onset (**Fig.4l,m**). These results suggest that the neutralization of extracellular Prxs has potential as a novel anti-inflammatory and neuroprotective strategy even at 12 hours after ischemic stroke onset.

The conserved region of peroxiredoxins was essential for IL-23-inducing activity

As already shown in **Fig.1f**, Prx family proteins potently activate BMDCs. We thus suspected that a particular structure common to Prx family proteins could be a TLR2/4 stimulator. Blocking this common structure would be preferable to blocking each entire molecule for preventing post-ischemic inflammation. Thus, we first tried to identify the active region on the Prx that was responsible for the induction of IL-23 by generating deletion mutants of Prx5. We found that IL-23-inducing activity was present between amino acid residues 70 and 90 of Prx5 (**Fig.5a**). This region contained β4 sheet and α3 helix regions that were well conserved among the Prx family as well as among various species[36-38] (**Supplementary Fig.17a,b**). GST-fusion of this region, but not other regions, from Prx1, Prx5, and Prx6 resulted in similar IL-23-inducing activity (**Fig.5b** and **Supplementary Fig.17c,d,e**). These regions locate in the similar surface of

Prx1, Prx5, and Prx6 (**Supplementary Fig.17a)**. These data suggest that this particular conserved region of Prxs commonly activates macrophages.

Next, we generated specific antibodies to these conserved β4 sheet and α3 helix regions of Prx5 and Prx6 ($Prx5_{68\text{-}90}$ and $Prx6_{66\text{-}93}$). Although these two antibodies did not cross-react with each other (**Supplementary Fig.18**), the administration of an anti-$Prx5_{68\text{-}90}$ and $Prx6_{66\text{-}93}$ antibody mixture immediately after stroke onset significantly reduced the infarct volume on day 4 and reduced the severity of neurological deficits compared with those seen in control IgG-administered mice (**Fig.5c,d**). These data suggest that this conserved region of Prx is responsible for the induction of post-ischemic inflammation in the brain, and could be a therapeutic target in ischemic brain injury.

Discussion

ROS and its inactivators have been considered to be implicated in endogenous danger signals[39]. Surprisingly, our study demonstrated that the antioxidant proteins themselves can function as danger signals under conditions of sterile inflammation, such as in ischemic brain injury. A recent study revealed that DNA and the formyl peptide complex derived from mitochondria activated neutrophils through TLR9[18]. This may be related to the fact that mitochondria are evolutionary endosymbionts that are derived from bacteria. Similarly, because Prx family proteins are highly conserved among various species from bacteria to mammals, it is possible that these conserved molecules function as DAMPs recognized by TLRs. As the conserved regions of Prx proteins were sufficient for IL-23 induction, even partially degraded Prxs could function as DAMPs.

We showed that Prx-mediated inflammation after ischemic brain injury was

dependent on TLR2 and TLR4, which seem to be functionally redundant. Although it is rare that one TLR ligand activates multiple TLRs, HMGB1 has been shown to function as a danger signal through TLR2, TLR4, and TLR9[15,40]. Our data suggest that post-ischemic inflammation in the brain is strongly dependent on TLR2 and TLR4 (**Fig.3**). The molecular mechanism underlying TLR2/4 activation by Prx as well as by other endogenous DAMPs remains to be clarified. It is also possible that the other pattern recognition receptors (CD36, RAGE, SRA) in addition to TLRs are implicated in the recognition of endogenous DAMPs and the modification of TLR signaling[41-43].

The expression of Prx family proteins, in particular, Prx5 and Prx6, has been reported to be especially high in brain tissue compared to other organs[30]. This is why Prx family proteins function as potential DAMPs in ischemic brain injury. It has been reported that Prx family proteins are released into the extracellular fluid following brain tissue death in both rats and acute ischemic stroke patients[32,33]. Thus, it is highly likely that extracellular Prxs also function as DAMPs in the ischemic human brain.

Prx family proteins have been implicated as possible anti-oxidative stress proteins, which promote cell survival in brain and other organ injury models[27,28,44]. Indeed, we observed an increase in neural cell death in *Prx1*$^{-/-}$ mice after brain ischemia (unpublished data). Thus, intracellular Prx are probably essential for neuroprotection against ischemic injury. Yet the present study also revealed that extracellular Prx functions as a danger signal that promotes post-ischemic inflammation in the brain. Thus, we propose the two opposing functions of Prx, one inside and one outside the brain cells. Intracellular Prx could be neuroprotective due to anti-oxidative reactions. However, once Prx is released from necrotic brain cells into the extracellular compartment, this extracellular Prx acts as a neurotoxic danger signal via TLR2/4 (**Supplementary**

Fig.19a). These two opposing functions performed by a single molecule may be apposite for self-protection against injury and infection.

HMGB1 has been shown to be an important DAMP in various kinds of tissue damage, including cerebral ischemic injury[20-23]. Although the administration of anti-HMGB1 antibody has been reported to be neuroprotective[45], extracellular HMGB1 may not be a good therapeutic target for reducing inflammation, given that the extracellular release of HMGB1 has been reported to decrease rapidly in the ischemic core, that is, within 6 hours after stroke onset[20,46], while the infiltration of macrophages mainly occurs on day 1 after ischemic injury[11]. It has also been reported that the extracellular release of HMGB1 contributes to BBB breakdown[20]. Our research demonstrated that the extracellular release of Prx was relatively slow and that HMGB1 did not affect the inflammatory cytokine expression in the infiltrating immune cells. In summary, HMGB1 is a hyperacute DAMP for BBB breakdown, while extracellular Prx is the secondary DAMP in the acute phase which initiates post-ischemic inflammation (**Supplementary Fig.19b**). Because there is currently no successful therapy that can be initiated more than 6 hours after the ischemic stroke onset, one of the main benefits of extracellular Prx as a novel therapeutic target would be the fact that its therapeutic time window would be longer than that of HMGB1.

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Author contributions

T.S. designed and performed experiments, analyzed data, and wrote the manuscript; E.H. performed experiments analyzing TLR-deficient mice analysis; A.K. performed TLR signal analysis; R.M., R.S, and T.S. are involved in paper preparation and data analysis; I.T. provided specific input on protein and mass spectrometry analysis; H.O. and T.K. provided technical advice and contributed to experimental design; T.Y. and T.I. provided KO mice and critical input on Prx1's functions; H.T., S.M., and M.N. provided anti-HMGB1 antibody; K.K. provided specific input regarding LC/MS analysis; K.M. and S.A. provided KO mice and critical input on the TLR's functions; A.Y. initiated and directed the entire study, designed experiments, and wrote the manuscript.

Figure legends

Figure 1 Peroxiredoxins are potent IL-23 inducers in the brain lysate. (**a**) The time course of mRNA levels of inflammatory cytokines in WT or *Myd88*$^{-/-}$ BMDC after the addition of sham-operated or ischemic brain lysate (n = 6 for each). (**b**) The IL-23p19 mRNA levels in BMDC 1 hour after stimulation with brain lysate that had been treated with heat (98°C, 10 min), pronase or DNase I (37°C, 1 hour) (n = 3 for each). (**c**) IL-23p19 mRNA levels in BMDC 1 hour after stimulation with each sucrose gradient fraction of the brain lysate (representative data from five independent experiments). (**d**) IL-23p19 mRNA levels in BMDC 1 hour after stimulation with the recombinant proteins identified through LC/MS analysis in the No. 2 and No. 3 sucrose gradient fractions. GST was used as a negative control (n = 3 for each). (**e**) Western blot analysis of indicated proteins in the sucrose gradient fraction of the brain lysate. (**f,g**) The mRNA levels of inflammatory cytokines (**f**) and IL-23 protein levels (**g**) induced in BMDC by the addition of recombinant proteins or GST protein (negative control) or LPS (100 ng ml^{-1}) (n = 3 for each). The protein levels were detected by ELISA in culture supernatant 6 hours after stimulation with 1 μM recombinant proteins. LPS (lipopolysaccharide) was used as a TLR4 ligand (positive control). (**h**) IL-23p19-inducing activity of each sucrose gradient fraction after immunoprecipitation using control IgG or anti-Prx1, 2, 5, and 6 antibody mixture. The depletion of Prx1, 2, 5, and 6 was confirmed by Western blot analysis (left column; representative data from two independent experiments). ***$p < 0.001$ vs. brain lysate (one-way ANOVA with Dunnett's correction; the error bars represent s.e.).

Figure 2 Extracellular peroxiredoxin is co-localized with infiltrating macrophages in the ischemic brain. (**a**) The time course of mRNA levels of Prx family proteins

in the ischemic brain (n = 3 for each). Each value indicates relative expression level compared to *Hprt1* mRNA. (**b**) The time course of immunohistochemical staining of Prx6 in the ischemic brain tissue at indicated time points (bar: 50 μm). (**c**) The immunohistochemical staining of day 1 ischemic brain tissue. (bar: 50 μm). (**d,e,f**) The immunohistochemical staining of the infarct core on day 1 (bar: 10 μm). The images were captured by using conventional fluorescence microscopy (BZ-8000, Keyence) (**b,c**) or confocal laser microscopy (LSM510 META, Carl Zeiss) (**d,e,f**).

Figure 3 Peroxiredoxins induce IL-23 via TLR2/4 signaling pathway. (**a**) IL-23p19 mRNA levels induced in WT, TLR2-, TLR4-, or TLR2/4-deficient BMDC by the addition of 1 μM recombinant Prx proteins (n = 4 for each). (**b**) The mRNA levels of inflammatory cytokines in the infiltrating immune cells on day 1 (n = 7 for each). Each value indicates relative expression compared to that in sham-operated mice. (**c**) The absolute number and the ratio of IL-17^{+} T cells on day 3 (n = 8 for WT and n = 6 for other samples). (**d**) Infarct volume measured by MAP2 immunostaining of brain sections on day 4 in chimeric mice (bar: 1 mm). WT←*Tlr2*$^{-/-}$ BM means transfer of *Tlr2*$^{-/-}$ BM cells into WT mice. The number of mice is shown on each bar. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. WT mice (**b,c**) and WT BM-transferred WT mice (**d**) (one-way ANOVA with Dunnett's correction; the error bars represent s.e.).

Figure 4 Extracellular release of peroxiredoxins contributes to the initiation of post-ischemic inflammation. (**a**) Infarct volume on day 4 and (**b**) neurological scores on days 1 and 3 of mice treated with control IgG (500 μg/mouse), a mixture of anti-Prx1, 2, 5, and 6 antibodies (500 μg/mouse), anti-HMGB1 antibody (200 μg/mouse), or both anti-Prx antibody mixture and anti-HMGB1

antibody immediately after stroke onset (bar: 1 mm). (**c,d**) Infarct volume on day 4 (**c**) and neurological scores (**d**) on days 1 and 3 of TLR2/4 double-deficient mice treated with control IgG or the anti-Prx antibody mixture immediately after stroke onset (n = 7 for each) (bar: 1 mm). (**e**) mRNA levels of inflammatory cytokines in infiltrating immune cells on day 1, (**f**) the absolute number and the ratio of IL-17^{+} T cells on day 3, and (**g**) the absolute number of TUNEL-positive cells in the peri-infarct area on day 4 (bar: 50 μm) in the mice treated with control IgG, anti-Prx antibody mixture, or anti-HMGB1 antibody immediately after stroke onset. (**h**) Infarct volume on day 4 and (**i**) neurological scores on days 1 and 3 of mice treated with control IgG, anti-Prx antibody mixture, or anti-HMGB1 antibody 6 hours after stroke onset (bar: 1 mm). (**j**) Infarct volume on day 4 and (**k**) neurological scores on days 1 and 3 of mice treated with control IgG, anti-Prx antibody mixture 12 hours after stroke onset (bar: 1 mm). (**l**) Infarct volume on day 4 and (**m**) neurological scores on days 1 and 3 of mice treated with control IgG, anti-Prx antibody mixture 24 hours after stroke onset (bar: 1 mm). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control IgG-administered mice (one-way ANOVA with Dunnett's correction (**a,b,e,h,i**) and two-sided Student's t-test (**c,d,f,g,j,k,l,m**); the error bars represent s.e.).

Figure 5 The conserved region of peroxiredoxins was essential for IL-23-inducing activity and infarct increase. (**a**) IL-23p19-inducing activities of the C-terminal deletion mutants of PRX5 (n = 3 for each). The number of amino acid residues contained in each GST fusion PRX5 peptide is shown on the X-axis. (**b**) IL-23p19-inducing activities of the GST fusion PRX1, PRX5, and PRX6 peptides. The number of amino acid residues contained in each PRX1, PRX5, and PRX6 peptide is shown on the X-axis (GST-PRX1$_{47\text{-}70}$, GST-PRX5$_{42\text{-}67}$, GST-PRX6$_{41\text{-}65}$: peptides contained α2 helix region; GST-PRX1$_{71\text{-}94}$,

GST-PRX5$_{68\text{-}90}$, GST-PRX6$_{66\text{-}93}$: peptides contained β4 sheet and α3 helix regions). IL-23p19 mRNA levels were detected by means of quantitative RT-PCR in BMDC 1 hour after stimulation with 1 μM recombinant proteins. (**c,d**) Infarct volume on day 4 (bar: 1 mm) (**c**) and neurological scores (**d**) on days 1 and 3 of mice treated with control IgG or anti-Prx5, Prx6 antibody mixture or anti-Prx5 $_{68\text{-}90}$, Prx6 $_{66\text{-}93}$ (common α3 helix and β4 sheet region) antibody mixture immediately after the induction of brain ischemia (300 μg/mouse). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. GST-PRX5 $_{1\text{-}162}$ (**a**) and control IgG-treated mice (**c,d**) (one-way ANOVA with Dunnett's correction; the error bars represent s.e.).

Methods

Mice. TLR2-KO, TLR4-KO, TLR2/4-DKO, MyD88-KO, and TRIF-KO mice were kindly provided by Dr. K Miyake and Dr. S Akira. Prx1-KO mice were kindly provided by Dr. T Ishii. All mice were from a C57BL/6 background. All experiments were approved by the Institutional Animal Research Committee of Keio University (approval number: 08004).

Murine focal brain ischemia model. Male mice, aged 9 to 12 weeks and weighing 20–30 g, were used for focal brain ischemia experiments. There was no significant difference in weight and age between WT mice and any of the KO groups. We used a transient middle cerebral artery occlusion (MCAO) model induced by means of an intraluminal suture. The method of inducing this transient suture MCAO model has been described previously[11,47]. A greater-than-60% reduction in cerebral blood flow was confirmed by laser Doppler flowmetry, and head temperature was kept at 36°C using a heat lamp.

Sixty minutes after MCAO, the brain was reperfused by the withdrawal of the intraluminal suture.

At the indicated time points after MCAO, neurological function was evaluated using a previously described 4-point-scale neurological score method[11]. For measurement of the infarct volume, the mice were fixed by transcardial perfusion with cold PBS followed by 4% paraformaldehyde/PBS. We embedded 1-mm-thick serial coronal slices from the brains in paraffin sections and immunostained them with MAP2-specific antibody. To determine the border of infarcted and non-infarcted regions at early time points after stroke onset, we examined the nuclear morphological change of neuronal cells stained with DAPI and also performed TUNEL-staining.

Preparation and LC/MS analysis of brain lysate. The mice were perfused with PBS transcardially. The forebrain was removed, homogenized with RPMI-1640, and centrifuged at 15,000 rpm for 5 min. The supernatant was made up to 1 ml with RPMI-1640 and used as the brain lysate. For the pronase and DNaseI digestion assay, the brain lysate was incubated with pronase (1-10 U ml^{-1}, Roche) or DNase I (50 $\mu g\ ml^{-1}$, Roche) at 37°C for 1 hour.

For the sucrose density gradient centrifugation and LC/MS analysis, the brain lysate was ultracentrifuged at 47,000 rpm for 1 hour. The supernatant was applied to DEAE Sepharose Fast Flow (GE Healthcare), and the flow through was condensed by ultrafiltration with an Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-10 membrane (Millipore). Four hundred microliters of condensed solution was layered on a 1-ml 10–40% (w/w) linear sucrose gradient in PBS, and centrifuged at 40,000 rpm for 12 hours. Sucrose was depleted by ultrafiltration from each of the sucrose gradient fractions. We added each of the sucrose gradient fractions to a culture of BMDC to examine its capacity to induce

inflammatory cytokines and analyzed sucrose gradient fractions No. 1–4 by means of LC/MS. LC/MS analysis was performed after trypsin treatment using a Qstar-XL mass spectrometer (Applied Biosystems).

For the antibody-depletion assay, HMGB1 or Prx proteins were depleted by immunoprecipitation with antibody cross-linked Protein G beads using dimethyl pimelimidate (DMP). Depletion of HMGB1 or Prx proteins from the brain lysate was confirmed by Western blot analysis.

Generation of recombinant protein. cDNA clones encoding candidate proteins identified by LC/MS analysis were cloned from a mouse brain cDNA library. All mutations were made with complementary mutagenic oligonucleotides. Final cDNA constructs were inserted into the pGEX6P-3 plasmid (GE Healthcare) and expressed as GST fusion proteins in BL21 competent cells (Stratagene). Following fusion protein purification using Glutathione-Sepharose 4B (GE Healthcare), 100 μl of protein-bound glutathione beads were extensively washed with 10 ml of cold PBS five times. Washed protein-bound glutathione beads were either eluted with 20 mM reduced glutathione (pH 8.0) to elute GST-fusion proteins or incubated with PreScission Protease (GE Healthcare) overnight at 4°C to remove the GST tag. Finally, these recombinant proteins were incubated with Affi-Prep Polymyxin Support (Bio-Rad) for 12 hours at 4°C to remove endotoxins and endotoxin-bound proteins. We confirmed through SDS-PAGE and CBB staining that the same amounts of purified recombinant proteins were applied to BMDC stimulation experiments. Recombinant GST protein was always used as a negative control for cytokine induction in BMDC.

To generate alkylated Prx proteins, recombinant Prx protein with 1 mM dithiothreitol (DTT) was incubated with 55 mM iodoacetamide (Wako Pure

Chemical Industries Ltd.) in a dark chamber at room temperature for 1 hour. Sufficient alkylation of -SH residues in Prx protein was confirmed using dithionitrobenzoic acid (Dojindo Laboratories).

Generation of rabbit polyclonal antibody. Rabbits were immunized with recombinant Prx proteins by Japan Lamb. To affinity-purify antibodies, recombinant Prx proteins were crosslinked to NHS-Sepharose beads. The Protein A-purified antibodies from immunized rabbit serum were applied to Prx protein-bound NHS-Sepharose beads. These affinity-purified antibodies were used for all experiments in this study.

Anti-HMGB1 antibody. Anti-HMGB1 antibody (rat, monoclonal) was kindly provided by Dr. H Takahashi, Dr. S Mori, and Dr. M Nishibori. As previously reported, 200 μg of anti-HMGB1 antibody was administered immediately or 6 hours after the induction of brain ischemia[20,45].

Statistical analysis. Data are expressed as means ± the standard error (s.e.). We performed a one-way analysis of variance (ANOVA) followed by post hoc multiple comparison tests (Dunnett's correction) to analyze differences among three or more groups of mice. Between two groups of mice, an unpaired Student's *t*-test was performed to determine statistical significance. $p < 0.05$ was considered a significant difference.

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