Original Article

Age-related differences in interferon regulatory factor-4 and -5 signaling in ischemic brains of mice

Shou-cai ZHAO^{1,2,#}, Chun WANG^{3,#}, Heng XU², Wen-qian WU², Zhao-hu CHU², Ling-song MA², Ying-dong ZHANG^{1,*}, Fudong LIU^{4,*}

¹Department of Neurology, Nanjing First Hospital, Nanjing Medical University, Nanjing 210006, China; ²Department of Neurology, Wannan Medical College Yijishan Hospital, Wannan 241001, China; ³Department of Stomatology, Bengbu Medical College, Bengbu 233030, China; ⁴Department of Neurology, McGovern Medical School at The University of Texas Health Science Center at Houston, Houston, TX 77030, USA

Abstract

Stroke is a disease that mainly affects the elderly and modeling stroke in aged animals is clinically more relevant. The inflammatory responses to stroke are a fundamental pathological procedure where microglial activation plays an important role. Interferon regulatory factor-5 (IRF5) and IRF4 regulate M1 and M2 activation of macrophages, respectively, in peripheral inflammation; but it is unknown whether IRF5/IRF4 are also involved in cerebral inflammatory responses to stroke and whether age-related differences of the IRF5/IRF4 signaling exist in the ischemic brain. Here, we investigated the impacts of aging on IRF5/IRF4 signaling and post-stroke inflammation in mice. Both young (9–12 weeks) and aged (18 months) male mice were subject to middle cerebral artery occlusion (MCAO). Morphological and biochemical changes in the ischemic brains and behavior deficits were assessed on 1, 3, and 7 d post-stroke. After MCAO, the aged mice showed smaller infarct sizes but worse behavior deficits than young mice. Young mice had significantly more IRF4/CD206 double positive microglia in the ischemic brains, whereas the aged mice had more IRF5⁺ and MHCII⁺ microglia after stroke than their counterparts. In addition, serum pro-inflammatory cytokines (TNF- α , iNOS, IL-6) were more robustly up-regulated in aged mice, whereas serum anti-inflammatory cytokine (TGF- β , IL-4, IL-10) levels were higher in young mice compared to their counterparts after MCAO. Our results demonstrate that aging has a significant effect on stroke outcomes in mice, which is most likely mediated by age-specific inflammatory responses.

Keywords: stroke; MCAO; aging; microglia; IRF4; IRF5; cytokines; neuroinflammation

Acta Pharmacologica Sinica (2017) 38: 1425–1434; doi: 10.1038/aps.2017.122; published online 14 Sep 2017

Introduction

Ischemic brain injury results from a cascade of events initiated by energy depletion including glutamate excitotoxicity, oxidative stress, and inflammation. Cerebral ischemia induces inflammatory responses in both the brain parenchyma and systemic circulation. Within hours after the ischemic insult, cytokines are produced in large amounts, and leukocytes are activated and consequently migrate toward the injured brain tissue^[1]. Concomitantly, microglia undergo a phenotypic transformation from a resting state to an “activated” phenotype and develop macrophage-like abilities^[2]. The activated microglia produce pathological molecules such as reactive

oxygen species, cytokines, and proteases that kill neighboring cells, disrupt the blood-brain barrier, and perpetuate the inflammatory response^[3]. In addition, the activated microglia are also highly plastic cells that can exhibit either a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype depending on the progression of the disease^[4], to aggravate the ischemic injury or attenuate the inflammation respectively.

Age-related differences exist in microglia, and the microglial response to stroke differs with aging. Aging is associated with low-grade neuroinflammation (termed “inflammaging”^[5]) demonstrated by basal increases in levels of circulating pro-inflammatory cytokines, increased expression of pro-inflammatory mediators and decreased anti-inflammatory markers in microglia^[6]. Clinically aged stroke patients have worse outcomes and higher morbidity/mortality than do young patients^[7]. This phenomenon has been recapitulated in experimental studies. For example, our previous data have shown

[#]These authors contributed equally to this work.

^{*}To whom correspondence should be addressed.

E-mail zhangyingdong@aliyun.com (Ying-dong ZHANG);

Fudong.Liu@uth.tmc.edu (Fudong LIU)

Received 2017-04-20 Accepted 2017-06-30

more severe behavioral deficits and higher mortality in aged *vs* young animals after stroke^[8]. Whether the worsened functional outcomes in the aged population are caused by “inflammaging” is not known.

Accumulating data have shown that interferon regulatory factors play an important role in regulating inflammation^[9–11]. IRF4 is a negative regulator of inflammation and mediates macrophage M2 polarization^[10]. IL-4 induces the expression of IRF4 in macrophages and consequently increases the expression of M2 marker genes such as Arg1, Ym1, and Fizz1^[12]. IRF5 is a critical component in the Toll-like receptor 4 (TLR4)-MyD88-IRF5 signaling pathway, which regulates the expression of inflammatory cytokines^[11]. After being activated by TLR4 signal transduction, IRF5 induces the expression of pro-inflammatory cytokines including TNF- α , IL-6, and IL-12 and promotes the M1 polarization of macrophages^[13]. To date, no studies have reported the role of IRF4 or IRF5 signaling in the activation of microglia, the resident macrophages in the brain.

In this study, we used a well-established murine model of focal cerebral ischemia/reperfusion to investigate the temporal kinetics of microglial polarization in mice of different ages after stroke. We found that microglia were differentially activated in young *vs* aged ischemic brains and that microglial IRF4/5 expression corresponded to the age-related difference.

Materials and methods

Animals

C57BL/6 mice were purchased from the Nanjing Qinglongshan Animal Breeding Center. All animal protocols were approved by the Institutional Animal Care and Use Committee of Wannan Medical College. The mice were group-housed and maintained on a 12:12 h light/dark cycle and given *ad libitum* access to water and rodent chow. Both young male mice (9–12 weeks; 21–25 g) and aged male mice (18 months; 30–40 g) were utilized.

Focal cerebral ischemia model

Focal cerebral ischemia was induced by intraluminal occlusion of the right middle cerebral artery (MCA) for 90 min under isoflurane anesthesia, as described previously^[14]. To occlude the MCA, 6-0 nylon suture filaments with different sizes of silicone-coated tips were utilized in young (0.21 mm) and aged (0.23 mm) mice. To assess physiological parameters in mice of both ages, cerebral blood flow (CBF) was monitored by laser Doppler flowmetry (LDF, Moor Instruments Ltd, UK), and blood pH, pO₂, pCO₂, and glucose were examined through femoral artery tubing in a separate non-survival cohort. For all MCAO mice, the rectal temperature was maintained at 37.0 $\pm$ 0.5 °C during surgery and MCA occlusion (MCAO) via a temperature-regulated heating pad. Sham-operated animals underwent the same anesthesia and surgical procedures but were not subjected to MCAO. The animals were randomly assigned to sham and MCAO groups with different reperfusion durations through the use of a lottery-drawing box. All biochemical and histological (immunostaining and cell counting) assessments were performed by investigators who were

blinded to the experimental group assignments. A total of 198 mice (32 sham-operated and 166 ischemic mice) were used in this study, including 22 mice that were excluded from further assessments because of either death after ischemia or failure in ischemia induction.

Neurological behavior tests

At 1, 3 and 7 d of stroke, neurological deficit scores (NDS) were assessed using a nine-point scale method^[15]. The scale was based on the following observations: (1) absence of neurological deficits (0 point), (2) left forelimb flexion upon suspension by the tail or failure to fully extend the right forepaw (1 point), (3) left shoulder adduction upon suspension by the tail (2 points), (4) decreased resistance to a lateral push toward the left (3 points), (5) spontaneous movement in all directions with circling to the left only if pulled by the tail (4 points), (6) circling or walking spontaneously only to the left (5 points), (7) walking only when stimulated (6 points), (8) no response to stimulation (7 points), and (9) stroke-related death (8 points).

The corner test^[16] was performed at 7 d post-stroke. The mouse was placed between two cardboard pieces (each 30 cm $\times$ 20 cm $\times$ 1 cm), and the boards were gradually moved closer to the mouse from both sides to encourage the mouse to enter into a corner of 30° with a small opening along the joint between the two boards. When each mouse entered the deepest part of the corner, both sides of the vibrissae were stimulated by the two boards. Then, the mouse reared forward and upward and turned back to face the open end. Twenty trials were performed for each mouse, and the percentage of right turns was calculated. Only turns involving full rearing along either board were recorded.

Infarct volume measurement

At the indicated time points, the mice were euthanized, and the brains were removed and cut into five 2 mm slices. The slices were stained with 1.5% of 2,3,4-triphenyltetrazolium chloride (TTC) solution and incubated in a 37 °C water bath for 8–10 min; the solution was changed to 4% formalin under a hood, and the slices were fixed overnight. The slice photos were taken within 24 h after TTC staining, and the infarct volumes were quantified with Swanson's method^[17].

Immunofluorescence

Mouse brains were perfused intracardially with 1 $\times$ PBS and 4% formalin; next, the brains were removed and post-fixed in 4% formalin overnight. After post-fixation, the brains were transferred to 30% sucrose solution until equilibration and then preserved in a -80 °C freezer. After all samples were collected, the brains were cut into 30 μ m coronal sections on a freezing microtome, and the slices were then preserved in 96-well plates filled with Antifreeze solution. After being washed and blocked, the brain slices were incubated with primary antibodies overnight, then stained with secondary antibodies. The primary antibodies were mouse anti-CD206 (1:10; Abcam), mouse anti-MCII (1:50; Abcam), rabbit anti-IRF4 (1:62.5; Abcam), rabbit anti-IRF5 (1:500; Abcam), and rat

anti-Iba-1 (1:500; Abcam). The secondary antibodies (Santa Cruz, USA) were either goat anti-rabbit (1:100) or goat anti-rat (1:100)/mouse (1:100) depending on the primary antibodies. The slides were then dipped in DAPI (Abcam) solution for 5 min. Quantification of fluorescence-positive cells was performed with ImageJ. Three randomly selected microscopic fields in the cortex and striatum of each section were examined, and three consecutive sections were analyzed for each brain. Data are expressed as the mean numbers of cells per square millimeter.

Western blotting

At the endpoints, mouse brain samples were obtained by rapid removal of the brain from the skull, resection of the cerebellum, and immediate dissection into right (ipsilateral) and left (contralateral) hemispheres. The ipsilateral hemisphere was homogenized in 1 mL of RIPA buffer containing 1 mmol/L PMSF. The extracts were immediately centrifuged at 12 000 revolutions per minute at 4 °C for 15 min, and the supernatant was collected for measurement of the protein concentration. Samples containing 30 µg of protein per well were loaded onto 8%–12% SDS-PAGE gels and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated in blocking buffer (TBST containing 5% skim milk powder) for 1 h at room temperature and immersed in primary antibodies overnight at 4 °C, then incubated with secondary antibodies for 1 h at room temperature. The blot signals were quantified with Odyssey software (LI-COR). The primary antibodies used were as follows: rabbit monoclonal anti-IRF-5 (Abcam, USA), rabbit polyclonal anti-IRF-4 (Santa Cruz, USA) and anti-Myd88 (Abcam, USA). β -Actin (Abcam) served as the loading control.

ELISA

Blood samples were obtained from the right atrium at the end of the experiments and centrifuged at 200 revolutions per minute for 20 min. The serum was collected and stored at -20 °C. Serum levels of iNOS, TNF- α , IL-6, IL-4, IL-10 and TGF- β were measured in each group with ELISA Kits (Xinbosheng, China) according to the manufacturer's instructions.

Statistical analysis

All data were presented as mean $\pm$ standard error of the mean (SEM) and analyzed with a *t*-test for two groups and two-way ANOVA (with Tukey's *post hoc* correction for multiple comparisons when appropriate) for comparison of the means

between the experimental groups. NDS were analyzed with a Mann-Whitney U-test. $P < 0.05$ was considered statistically significant. All *in vivo* and imaging studies were performed in a blinded manner. Because of the higher body weights and severe behavioral deficits in aging mice, MCAO surgeons and behavior test observers were aware of the grouping (aging *vs* young).

Results

Stroke outcomes were different between young and aged mice

To compare stroke outcomes between young and aged animals, we first examined the infarct volumes and neurological behavior at different time points (1, 3, and 7 d) of MCAO in mice of both ages. At each time point, 90 min of MCAO induced significantly larger infarcts in young *vs* aged animals (Figure 1A, 1B). The infarct sizes were equivalent at 1 and 3 d of stroke in both the young and aged mice, and then decreased at d 7 in both cohorts. Behavior deficits were examined by NDS and the corner test, and the results showed a pattern opposite from that of infarct volumes. At each time point, aged animals had significantly higher NDS than their young counterparts (Figure 1C); at d 7 post-stroke, significantly more right turns in the corner test were seen in the aged *vs* young animals (Figure 1D). There were no significant differences in blood levels of pH, pO₂, pCO₂, and glucose between young and aged animals at reperfusion; CBF decreased to equivalent levels after MCAO and returned to nearly 90% of baseline levels after reperfusion in both young and aged mice (Table 1).

Microglia were differentially activated in young *vs* aged animals after stroke

Post-stroke inflammation contributes to secondary neuronal damage and affects recovery from ischemic injury. To investigate whether the age-related difference in stroke outcomes was related to inflammatory responses, we examined the activation of microglia, the resident immune cells in the ischemic brain. MHCII and CD206 are well-established markers for M1 and M2 microglia (or macrophage) activation, respectively^[18], and Iba-1 is a widely used marker for microglial cells^[19]. IRF5 and IRF4 have been reported to induce the expression of pro-/anti-inflammatory cytokines, respectively, in macrophages^[10, 11]. We performed immunofluorescence co-labeling of MHCII/IRF5/Iba-1 and CD206/IRF4/Iba-1 on microglia after stroke to quantify cells of the M1 or M2 phenotype. As shown in Figure 2B, there was only sparse expression of MHCII or IRF5 in the young and aged sham groups. In the stroke groups at either 1 or 7 d, the aged animals had significantly more MHCII/

Table 1. Physiological measurements between young and aged mice.

Group	pH	pO ₂ (mmHg)	pCO ₂ (mmHg)	Glucose (mg/dL)	LDF (%) ischemia	LDF (%) reperfusion
Young	7.30 $\pm$ 0.08	91 $\pm$ 12.2	39 $\pm$ 6.1	176 $\pm$ 18.8	13 $\pm$ 2.1	90 $\pm$ 8.9
Aged	7.26 $\pm$ 0.15	94 $\pm$ 16.3	37.6 $\pm$ 11.6	178 $\pm$ 21.7	12 $\pm$ 3.2	89 $\pm$ 6.2

No significant differences were seen in physiologic variables between young and aged mice 90 min after MCAO.

Figure 1. Stroke outcomes in young and aged mice after MCAO. (A) Representative coronal brain slices stained with TTC at 1, 3, and 7 d of MCAO. (B) Quantification of infarct volumes. (C) Neurological deficit scores at the three time points. (D) Corner test scores calculated by $(R)/(R+L) \times 100\%$, where R and L are right and left turn number, respectively. $n=8/\text{group}$; * $P<0.05$ and ** $P<0.01$.

IRF5/Iba-1 co-labeled cells than the young mice (Figure 2F). The same pattern was observed in MHCII or IRF5 sole staining (Figure 2D, 2E). A temporal change in protein expression was also found, because either MHCII or IRF5 was expressed on significantly more cells at 7 *vs* 1 d in both age groups ($n=8/\text{group}$; $P<0.001$). Interestingly, the results of CD206/IRF4/Iba-1 staining exhibited a completely different pattern: the young mice in either the 1 or 7 d group had significantly more CD206/IRF4/Iba-1 co-labeled cells than the aged cohort (Figure 3A, 3E), and the CD206 or IRF4 sole staining showed the same pattern (Figure 3C, 3D). In addition, CD206/IRF4 expression showed temporal changes opposite from those of MHCII/IRF5 expression after stroke: significantly more positive cells were observed at 1 *vs* 7 d in both age groups ($n=8/\text{group}$; $P<0.001$).

Serum cytokine levels were different between young and aged mice after stroke

Serum cytokine levels are reflective of systemic inflammatory responses to stroke^[20], and circulating cytokines direct the migration and infiltration of peripheral immune cells into the ischemic brain. Because we had observed differentiated activation of microglia after stroke in young and aged mice, we next sought to investigate whether there was also an age-related difference in the systemic inflammatory response. Pro-inflammatory (TNF- α , iNOS, IL-6) and anti-inflammatory

(TGF- β , IL-4, IL-10) cytokines were examined by ELISA. In general, the levels of pro-inflammatory cytokines were higher in the aged *vs* young cohort, and there were significant differences at 1 and 3 d for TNF- α , 3 and 7 d for iNOS, and 3 d for IL-6 (Figure 4A–4C). However, the opposite pattern was found in the results of anti-inflammatory cytokines: the levels were significantly higher in the young *vs* aged cohort at all 3 time points, with the exception of IL-10 and IL-4 at 7 d of stroke (Figure 4D–4F). The cytokine data were consistent with the results of microglial activation in Figures 2 and 3.

Activation of the MyD88-IRF5 signaling pathway was higher in aged ischemic brains

MyD88 is a key adaptor protein that mediates the regulatory effect of IRF5 on M1 activation^[13]. However, IRF4 competes with IRF5 for binding to adaptor MyD88^[18]. Therefore, we next performed Western blotting to determine how the proteins that are related to the MyD88-IRF5 signaling pathway changed after stroke. As expected, the expression of IRF4 was significantly higher at all three time points in young *vs* aged mice; however, IRF5 was more robustly expressed in the aged ischemic brains than in the young brains (Figure 5A–5C). The expression of MyD88 was also significantly higher in aged *vs* young mice at each time point (Figure 5D, 5E), showing the same pattern as that of IRF5 expression.

Figure 2. Immunofluorescence staining of brain slices for M1 microglial phenotype after stroke. (A) Boxed areas in a TTC-stained slice illustrate the three examined regions in each slice. (B) Co-localization of IRF5, MHCII, Iba-1 and DAPI at 1 and 7 d post-stroke. 20 $\times$; scale bar=50 μ m. (C) Representative enlarged images of IRF5, MHCII, Iba-1 and DAPI co-localization. 60 $\times$; scale bar=20 μ m. (D–F) Quantification of MHCII⁺ cells (D), IRF5⁺ cells (E), and merged positive cells (F) in (B). $n=8$ /group; * $P<0.05$ and ** $P<0.01$.

Discussion

Aging causes neurochemical and physiological changes in the brain that may affect ischemic outcomes after a stroke occurs. The present study examined stroke outcomes and inflammatory responses in young and aged mice, revealing several important findings. First, IRF5 and IRF4, the two interferon regulatory factors that regulate macrophage activation, are also related to microglial polarization. IRF5 and IRF4 were highly colocalized with the M1 marker MHCII and M2 marker CD206, respectively, in both the young and aged ischemic microglia, but this result was not seen in sham mice. Second, the aged animals had worse functional outcomes than those of the young mice, independently of the morphological changes in the ischemic brains; worse behavioral deficits corresponded to a more robust inflammatory response in the aged *vs* young cohort. Third, there was an age-related difference in microglial activation after stroke. The young stroke

brains had higher IRF4 expression than the aged brains, and the high expression corresponded to a stronger M2 microglial phenotype in the young *vs* aged ischemic brains, as indicated by an up-regulated membrane CD206 level. In contrast, the more robust inflammatory responses in the aged stroke brains were probably induced by up-regulated expression of IRF5, which colocalized with pro-inflammatory marker MHCII. Finally, there was a temporal difference in M1 and M2 activation of microglia after stroke, because an early M2 phenotype of microglia was observed at d 1, declined at d 7 and was followed by an increased M1 phenotype in both the young and the aged groups.

Post-stroke inflammatory responses play vital roles in deteriorating neuronal injury in ischemic stroke. Recent studies have shown that IRF4 and IRF5, members of the interferon regulatory factors (IRFs), contribute to macrophage polarization in periphery inflammatory diseases^[10, 21]. IRF4 expression

Figure 3. Immunofluorescence staining of brain slices for M2 microglial phenotype after stroke. (A) Co-localization of IRF4, CD206, Iba-1 and DAPI at 1 and 7 d post-stroke. 20 $\times$; scale bar=50 μ m. (B) Representative enlarged images of IRF4, CD206, Iba-1 and DAPI co-localization. 60 $\times$; scale bar=20 μ m. (C–E) Quantification of CD206⁺ cells (C), IRF4⁺ cells (D), and merged positive cells (E) in (A). $n=8$ /group; ** $P<0.01$.

is strongly induced in bone marrow-derived macrophages by IL-4 signaling. IRF4 not only regulates the expression of several secondary DNA binding proteins that are responsible for M1 macrophage phenotypes, such as MHC-II^[12], but also increases the expression of M2 phenotype marker genes, such as Arg1, Ym1, and Fizz1^[10]. In studies of systemic inflammation, IRF4 has been considered a key modulator of M2 polarization of macrophages^[10]. In contrast, IRF5 is a key inducer of M1 macrophage activation^[11, 22]. In peripheral immune diseases, the TLR4-MyD88-IRF5 signaling pathway plays a critical role in regulating inflammation^[21]. TLR4-MyD88 signaling is upstream of IRF5^[23]. The recognition of DAMP by TLR4 leads to the phosphorylation of IRF5 by a complex including TNF receptor-associated factor-6 (TRAF-6), interleukin-1 receptor-associated kinase-1 and -4 (IRAK-1/-4), and adaptor protein MyD88^[24]. Activated IRF5 is then translocated to the nucleus, where it binds IFN-stimulated response elements (ISREs) and promotes the transcription of multiple pro-inflammatory mediators, thus resulting in macrophage polarization toward the M1 phenotype. However, IRF4 selectively competes with IRF-5 for binding to MyD88 and inhibits the transmission of TLR outside-in signaling to NF- κ B and other pro-inflammatory transcription factors^[25]. Because IRF5 is the key mediator for signal transduction of the M1 phenotype, the competition

between IRF5 and IRF4 for MyD88 binding renders suppression of M1 macrophage polarization^[18]. Therefore, the IRF5-IRF4 regulatory axis is a key determinant of macrophage polarization when inflammation occurs^[9]. Microglia have the same myeloid origin as monocytes and become the resident macrophages in the brain after stimulation of inflammatory signals^[26]. Our data of the current study provide the first evidence that the IRF5-IRF4 regulatory axis is also involved in microglial polarization. Microglial activation is regulated by multiple inflammatory mediators and pathways^[27]. However, it is presently unclear whether the IRF5-IRF4 regulatory axis is also the determinant factor of microglial polarization. Further studies are warranted to clarify the role of the IRF5-IRF4 axis in ischemic microglia.

Neurochemical and physiological changes occur with aging^[28]; accordingly, young and aged brains exhibit different stroke phenotypes. Aging is the most important non-modifiable risk factor in stroke, and most stroke victims are over 65 years old^[29]. Therefore, it has more translational value to study the ischemic stroke with aged animal models. In the present study, the aged stroke mice had smaller infarct volumes than did young mice; paradoxically, the aged mice had higher neurological deficit scores and worse behavior test results than those of the young mice. This result was consistent with

Figure 4. Serum levels of cytokines after MCAO measured by ELISA. Pro-inflammatory (A, TNF- α ; B, iNOS; C, IL-6) and anti-inflammatory cytokines (D, TNF- β ; E, IL-4; F, IL-10) were examined. Each sample was probed in triplicate. $n=8/\text{group}$; * $P<0.05$ and ** $P<0.01$.

clinical data showing that older ischemic stroke patients have worse functional outcomes after stroke than younger patients, and these differences remain despite adjustment for baseline differences in stroke risk factors and other comorbidities^[30]. To our knowledge, this is the first report regarding the paradoxical stroke outcomes in aged *vs* young animals, although our previous study using middle-aged mice has shown similar findings^[31]. In our MCAO model, both young and aged mice had an equivalent decrease in CBF after MCAO, and no difference in CBF was seen at baseline, intra-ischemically, and post-reperfusion in young *vs* aged mice^[32]. The mechanisms underlying the differential infarct volumes are not clear and may be partly because aged brains are atrophic and have more space for edema formation and less chance of developing massive infarction after ischemia, as compared with young brains. Another possible mechanism may be related to the lower level of acid toxicity in aged ischemic brains, owing to metabolic drift with aging^[33]. It has been demonstrated that morphological changes in ischemic brains do not necessarily correlate with functional outcomes^[31, 34, 35]. Notably, corresponding to the worse functional outcomes, aged mice exhibited more

robust inflammatory responses, as indicated by significantly more IRF5- and MHCII-positive cells as well as higher serum levels of pro-inflammatory cytokines. Inflammatory responses have deleterious effects on both behavior deficits and functional recovery after stroke^[36]. Our data suggested that up-regulated inflammatory responses correlate less with infarct size *vs* functional outcomes, probably for several reasons: (1) both pro- and anti-inflammatory responses are involved in mediating ischemic injury; (2) the infarct size is initially influenced by the ischemia-induced primary tissue damage, and (3) morphological changes in the ischemic brains are determined by many pathological mechanisms not limited to post-stroke immune responses.

Interestingly, our data showed a temporal difference in M2 and M1 phenotypes of microglial activation that predominated at the early (d 1) and late (d 7) phases of stroke, respectively, in agreement with findings from previous studies^[37]. Microglial activation is the key element in initiating and perpetuating post-stroke inflammation^[27]. The stronger M1 microglial activation and pro-inflammatory responses seen in the aged *vs* young stroke mice may explain why the aged cohort had

Figure 5. Western blots of IRF4, IRF5 and MyD88 in brains after stroke. (A) Representative Western blots of IRF4 and IRF5 protein levels in brain homogenates in sham and stroke mice at different time points after stroke onset. Actin was used as a loading control. (B, C) Optical density is expressed as the ratio of IRF4 (B) or IRF5 (C) bands to control bands. (D) Representative blots of MyD88 protein levels in brain homogenates in sham and stroke mice at different time points after stroke onset. (E) Optical density is expressed as the ratio of MyD88 bands to control bands. Eight stroke and four sham brains were analyzed. * $P<0.05$ and ** $P<0.01$.

poorer functional outcomes. Up-regulated inflammation in the aged mice was clearly not correlated to the infarct size, which was smaller in the aged brains; instead, this inflammation was probably because aged brains have more activated microglia and more CD45^{high} leukocytes at baseline than do young brains^[38], and as a result, aged brains are “primed” to a pro-inflammatory response. In addition, aged microglia and macrophages exhibit deficits in phagocytic and chemotactic functions, as demonstrated by multiple studies on CNS diseases^[39–41]. Several mechanisms may underlie the age-specific phenotype of microglia/macrophages: (1) neurons tend to degenerate and become damaged with aging, and the inhibitory ligand receptor interactions with microglia are lost gradually^[42]; (2) misfolded proteins such as amyloid- β accumulate during normal aging and stimulate the microglia to express pro-inflammatory cytokines; (3) TGF- β expression increases with age, and chronic exposure of microglia to this cytokine may impair their capacity to secrete anti-inflammatory cytokines^[43].

The role of the TLR4-MyD88-IRF5 pathway in microglial activation has not been reported in ischemic stroke studies. Our data showed that there was also an age-related difference in MyD88 signaling after stroke, which matched the pattern of IRF5 expression in the aged *vs* young mice brains. IRF5 is required for TLR-mediated expression of IL-6, TNF, IL-12, and other pro-inflammatory cytokines, in a process controlled by several intermediate proteins, especially MyD88^[44]. Although IRF4 can compete with IRF5 for MyD88 binding^[25], the synchronous increase in MyD88 and IRF5 expression from d 1 to 7 suggested that there may be an innate mechanism for generating ample MyD88 in response to the increase in IRF5 production after an ischemic insult.

There are several limitations to this work that should be

noted when interpreting the results. IRF5/IRF4 knock-out mice were not used to mechanistically study the role of the two transcription factors in inducing M1/M2 microglial polarization, because the main focus of the present study is the age-related differences in post-stroke IRF5/IRF4 signaling. In a future study, we will use IRF5/IRF4 knock-out mice and lenti-IRF5/IRF4 viruses to manipulate the expression of the two IRFs and determine the effects on stroke outcomes. Another caveat is that we examined IRF5/IRF4 for only up to 7 d post-stroke; nevertheless, the ischemic damage matured by d 7, and the examined time period was long enough for the age-related differences to occur. One of the on-going studies in our laboratory is investigating the long-term expression of IRF5/IRF4 after stroke and the roles of these factors in mediating chronic inflammation.

In conclusion, young and aged brains respond differently to ischemic insult. Ischemia is more detrimental to aged *vs* young brains despite the morphological changes, an effect related to differential IRF5/IRF4 signaling and inflammatory responses. Aged ischemic brains exhibit a more robust pro-inflammatory process, thus making post-stroke inflammation an important therapeutic target for stroke in the aged population.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant 81371295) and Science and Technology Huimin Project of Wuhu, Anhui Province, China (2015hm06).

Author contribution

Shou-cai ZHAO and Chun WANG designed the experiments, performed MCAO and drafted the manuscript; Heng XU, Wen-qian WU, Zhao-hu CHU, and Ling-song MA performed

MCAO, IHC, and Western blotting and analyzed the data; Ying-dong ZHANG and Fudong LIU designed the experiments and revised the manuscript.

References

- Perego C, Fumagalli S, De Simoni MG. Temporal pattern of expression and colocalization of microglia/macrophage phenotype markers following brain ischemic injury in mice. *J Neuroinflammation* 2011; 8: 174.
- Stoll G, Jander S, Schroeter M. Inflammation and glial responses in ischemic brain lesions. *Prog Neurobiol* 1998; 56: 149–71.
- Chen Y, Won SJ, Xu Y, Swanson RA. Targeting microglial activation in stroke therapy: pharmacological tools and gender effects. *Curr Med Chem* 2014; 21: 2146–55.
- Perego C, Fumagalli S, De Simoni MG. Three-dimensional confocal analysis of microglia/macrophage markers of polarization in experimental brain injury. *J Vis Exp* 2013; (79). doi: 10.3791/50605.
- Navarrete-Reyes AP, Montana-Alvarez M. Inflammaging. Aging inflammatory origin. *Rev Invest Clin* 2009; 61: 327–36.
- Kohman RA, Bhattacharya TK, Wojcik E, Rhodes JS. Exercise reduces activation of microglia isolated from hippocampus and brain of aged mice. *J Neuroinflammation* 2013; 10: 114.
- Gur AY, Tanne D, Bornstein NM, Milo R, Auriel E, Shopin L, *et al*. Stroke in the very elderly: characteristics and outcome in patients aged ≥ 85 years with a first-ever ischemic stroke. *Neuroepidemiology* 2012; 39: 57–62.
- Liu F, Benashski SE, Persky R, Xu Y, Li J, McCullough LD. Age-related changes in AMP-activated protein kinase after stroke. *Age (Dordr)* 2012; 34: 157–68.
- Gunthner R, Anders HJ. Interferon-regulatory factors determine macrophage phenotype polarization. *Mediators Inflamm* 2013; 2013: 731023.
- Eguchi J, Kong X, Tenta M, Wang X, Kang S, Rosen ED. Interferon regulatory factor 4 regulates obesity-induced inflammation through regulation of adipose tissue macrophage polarization. *Diabetes* 2013; 62: 3394–403.
- Korzeniewska J, Barnes BJ. The COP9 signalosome interacts with and regulates interferon regulatory factor 5 protein stability. *Mol Cell Biol* 2013; 33: 1124–38.
- El Chartouni C, Schwarzfischer L, Rehli M. Interleukin-4 induced interferon regulatory factor (Irf) 4 participates in the regulation of alternative macrophage priming. *Immunobiology* 2010; 215: 821–5.
- Paun A, Reinert JT, Jiang Z, Medin C, Balkhi MY, Fitzgerald KA, *et al*. Functional characterization of murine interferon regulatory factor 5 (IRF-5) and its role in the innate antiviral response. *J Biol Chem* 2008; 283: 14295–308.
- McCullough LD, Zeng Z, Blizzard KK, Debchoudhury I, Hurn PD. Ischemic nitric oxide and poly (ADP-ribose) polymerase-1 in cerebral ischemia: male toxicity, female protection. *J Cereb Blood Flow Metab* 2005; 25: 502–12.
- Guo S, Li ZZ, Jiang DS, Lu YY, Liu Y, Gao L, *et al*. IRF4 is a novel mediator for neuronal survival in ischaemic stroke. *Cell Death Differ* 2014; 21: 888–903.
- Zhang L, Schallert T, Zhang ZG, Jiang Q, Arniago P, Li QJ, *et al*. A test for detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischemia. *J Neurosci Methods* 2002; 117: 207–14.
- Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR. A semiautomated method for measuring brain infarct volume. *J Cereb Blood Flow Metab* 1990; 10: 290–3.
- Lawrence T, Natoli G. Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nat Rev Immunol* 2011; 11: 750–61.
- Mirzaei N, Tang SP, Ashworth S, Coello C, Plisson C, Passchier J, *et al*. *In vivo* imaging of microglial activation by positron emission tomography with [^{14}C]PBR28 in the 5XFAD model of Alzheimer's disease. *Glia* 2016; 64: 993–1006.
- Rallidis LS, Vikelis M, Panagiotakos DB, Rizos I, Zolindaki MG, Kaliva K, *et al*. Inflammatory markers and in-hospital mortality in acute ischaemic stroke. *Atherosclerosis* 2006; 189: 193–7.
- Chen G, Shaw MH, Kim YG, Nunez G. NOD-like receptors: role in innate immunity and inflammatory disease. *Annu Rev Pathol* 2009; 4: 365–98.
- Krausgruber T, Blazek K, Smallie T, Alzabin S, Lockstone H, Sahgal N, *et al*. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat Immunol* 2011; 12: 231–8.
- Schoenemeyer A, Barnes BJ, Mancini ME, Latz E, Goutagny N, Pitha PM, *et al*. The interferon regulatory factor, IRF5, is a central mediator of toll-like receptor 7 signaling. *J Biol Chem* 2005; 280: 17005–12.
- Ban T, Sato GR, Nishiyama A, Akiyama A, Takasuna M, Umehara M, *et al*. Lyn kinase suppresses the transcriptional activity of IRF5 in the TLR-MyD88 pathway to restrain the development of autoimmunity. *Immunity* 2016; 45: 319–32.
- Negishi H, Ohba Y, Yanai H, Takaoka A, Honma K, Yui K, *et al*. Negative regulation of Toll-like-receptor signaling by IRF-4. *Proc Natl Acad Sci U S A* 2005; 102: 15989–94.
- London A, Cohen M, Schwartz M. Microglia and monocyte-derived macrophages: functionally distinct populations that act in concert in CNS plasticity and repair. *Front Cell Neurosci* 2013; 7: 34.
- Patel AR, Ritzel R, McCullough LD, Liu F. Microglia and ischemic stroke: a double-edged sword. *Int J Physiol Pathophysiol Pharmacol* 2013; 5: 73–90.
- Anyanwu EC. Neurochemical changes in the aging process: implications in medication in the elderly. *ScientificWorldJournal* 2007; 7: 1603–10.
- Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, *et al*. Heart disease and stroke statistics—2015 update: a report from the American Heart Association. *Circulation* 2015; 131: e29–322.
- Fonarow GC, Reeves MJ, Zhao X, Olson DM, Smith EE, Saver JL, *et al*. Age-related differences in characteristics, performance measures, treatment trends, and outcomes in patients with ischemic stroke. *Circulation* 2010; 121: 879–91.
- Liu F, Yuan R, Benashski SE, McCullough LD. Changes in experimental stroke outcome across the life span. *J Cereb Blood Flow Metab* 2009; 29: 792–802.
- Manwani B, Friedler B, Verma R, Venna VR, McCullough LD, Liu F. Perfusion of ischemic brain in young and aged animals: a laser speckle flowmetry study. *Stroke* 2014; 45: 571–8.
- Ivanisevic J, Stauch KL, Petrascheck M, Benton HP, Epstein AA, Fang M, *et al*. Metabolic drift in the aging brain. *Aging (Albany NY)* 2016; 8: 1000–20.
- Alexis NE, Back T, Zhao W, Dietrich WD, Watson BD, Ginsberg MD. Neurobehavioral consequences of induced spreading depression following photothrombotic middle cerebral artery occlusion. *Brain Res* 1996; 706: 273–82.
- Grabowski M, Brundin P, Johansson BB. Paw-reaching, sensorimotor, and rotational behavior after brain infarction in rats. *Stroke* 1993; 24: 889–95.
- Kim JY, Kawabori M, Yenari MA. Innate inflammatory responses in stroke: mechanisms and potential therapeutic targets. *Curr Med Chem* 2014; 21: 2076–97.
- Hu X, Li P, Guo Y, Wang H, Leak RK, Chen S, *et al*. Microglia/

- macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. *Stroke* 2012; 43: 3063–70.
- 38 Ritzel RM, Patel AR, Grenier JM, Crapser J, Verma R, Jellison ER, *et al*. Functional differences between microglia and monocytes after ischemic stroke. *J Neuroinflammation* 2015; 12: 106.
- 39 Kumar A, Stoica BA, Sabirzhanov B, Burns MP, Faden AI, Loane DJ. Traumatic brain injury in aged animals increases lesion size and chronically alters microglial/macrophage classical and alternative activation states. *Neurobiol Aging* 2013; 34: 1397–411.
- 40 Miron VE, Boyd A, Zhao JW, Yuen TJ, Ruckh JM, Shadrach JL, *et al*. M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. *Nat Neurosci* 2013; 16: 1211–8.
- 41 Lai AY, Dibal CD, Armitage GA, Winship IR, Todd KG. Distinct activation profiles in microglia of different ages: a systematic study in isolated embryonic to aged microglial cultures. *Neuroscience* 2013; 254: 185–95.
- 42 Perry VH, Holmes C. Microglial priming in neurodegenerative disease. *Nat Rev Neurol* 2014; 10: 217–24.
- 43 Flanary BE, Sammons NW, Nguyen C, Walker D, Streit WJ. Evidence that aging and amyloid promote microglial cell senescence. *Rejuvenation Res* 2007; 10: 61–74.
- 44 Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, *et al*. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* 2005; 434: 243–9.