Accepted Date:25-May-2013

Article Type: Original Paper

Aging aggravates ischemic stroke-induced brain damage in mice with chronic peripheral infection

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Summary

Ischemic stroke is confounded by conditions such as atherosclerosis, diabetes and infection, all of which alter peripheral inflammatory processes with concomitant impact on stroke outcome. The majority of the stroke patients are elderly, but the impact of interactions between aging and inflammation on stroke remains unknown. We thus investigated the influence of age on the outcome of stroke in animals predisposed to systemic chronic infection. Th1-polarized chronic systemic infection was induced in 18-22 month and 4-month-old C57BL/6j mice by administration of *T. muris* (gut parasite). One month after infection, mice underwent permanent middle cerebral artery occlusion and infarct size, brain gliosis and brain and plasma cytokine profiles were analyzed. Chronic infection increased the infarct size in aged but not in young mice at 24 hours. Aged, ischemic mice showed altered plasma and brain cytokine responses while the lesion size correlated with plasma pre-stroke levels of RANTES. Moreover, the old, infected mice exhibited significantly increased neutrophil recruitment and up-regulation of both plasma interleukin-17α and tumor necrosis factor α levels. Neither age nor infection status alone or in combination altered the ischemia-induced brain microgliosis. Our results show that chronic peripheral infection in aged animals renders the brain more vulnerable to ischemic insults, possibly by increasing the invasion of neutrophils and altering the inflammation status in the blood and brain. Understanding the

interactions between age and infections is crucial for developing a better therapeutic regimen for ischemic stroke and when modeling it as a disease of the elderly.

Introduction

Stroke is the second leading cause of death and long term disabilities worldwide. It typically occurs in the elderly and often in patients that are predisposed to comorbid conditions. Coexisting diseases such as atherosclerosis (Ross 1999), hypertension (Savoia & Schiffrin 2006), diabetes (Dandona *et al.* 2004), and infections all involve peripheral inflammation, which is likely to influence stroke outcome. Consequentially, up to a third of diagnosed stroke patients have had a preceding infection (Emsley & Hopkins 2008).

Based on clinical data, pre-existing inflammation is a major contributory factor to the outcome of stroke. In general, stroke incidence is increased during the seasons with high occurrence of respiratory tract infections (Lanska & Hoffmann 1999). Infections, especially of bacterial origin, have been shown to increase the prevalence of brain infarctions in young and middle-aged patients (Syrjanen *et al.* 1988). Specifically, the risk of stroke has been shown to peak 3 days after respiratory or urinary tract bacterial infections (Smeeth *et al.* 2004; Clayton *et al.* 2008). However, infectious pathogens other than bacteria have also been found to increase the susceptibility to stroke (Emsley & Hopkins 2008). Chronic inflammatory stimuli induced by parasite infections (Denes *et al.* 2010) and influenza (Muhammad *et al.* 2011) increase ischemic brain damage in mice, clearly demonstrating the destructive nature of peripheral inflammatory conditions. However, the impact of other confounding factors, such as age, on the susceptibility of infected individuals to ischemic brain damage still remains unclear. This is partially due to the fact that most preclinical studies use young, healthy rodents, even though aging is a non-modifiable risk factor for

stroke. It is thus still unclear whether aging exerts its effects via stimulation of excessive or dysregulated inflammatory responses. This hypothesis is supported by previous studies showing that aged animals have altered immune responses resulting from ischemic insult (Kharlamov *et al.* 2000; Popa-Wagner *et al.* 2007; Dinapoli *et al.* 2010; Sieber *et al.* 2011), intracerebral hemorrhage (Lee *et al.* 2009) or mechanical injury (Kyrkanides *et al.* 2001). However, mechanisms through which aging interacts with systemic inflammation to influence cerebrovascular pathologies remains unclear. Thus, our aim was to investigate the impact of age on ischemic stroke in animals subjected to chronic infection.

Results

Aging with chronic infection exacerbates ischaemic brain injury

Chronic peripheral infection was modeled by *Trichuris muris* (*T. muris)* induced infection. *T.Muris* infection was achieved by administering parasite eggs by oral gavage to young (4-months of age) and old (18-22 months of age) C57BL/6j background mice. This treatment has been shown to lead to a chronic, Th1-polarized immune response characterized by systemic up regulation of several pro-inflammatory cytokines with the peak at 4-5 weeks post infection (Denes *et al.* 2010). All animals underwent permanent middle cerebral artery occlusion (pMCAo) or sham operation one month after *T. muris* infection. At this time point all treated animals showed elevated plasma levels of RANTES and sufficient worm count in the cecum as published previously (Denes *et al.* 2010). The lesion size was imaged *ex vivo* by magnetic resonance imaging (MRI) at 24 hours post injury. Quantification of the MRI images revealed that infection itself did not aggravate the ischemic brain damage in young mice (young uninfected 3.64 +/2.25 mm^2 and young infected 2.64 +/- 1.62 mm^2). In contrast, old infected, mice exhibited dramatically larger (8.29 +/- 3.2 mm^2) ischemic brain damage compared to young uninfected or infected mice (Fig. 1). Aging alone without infection did

not significantly influence the lesion volume. There were no significant differences in the physiological parameters between the groups.

Plasma levels of RANTES prior to ischemia correlate with infarct size.

Plasma levels of "regulated and normal T cell expressed and secreted cytokine", RANTES, also known as CCL5, were measured from blood samples drawn from saphenous vein 1 day prior to ischemia. Chronic infection by *T. muris* caused a significant elevation in plasma RANTES levels both in young and old mice implicating a development of Th1-polarized chronic infection (Denes *et al.* 2010) (Fig. 2A). To test the possible relationship between plasma RANTES levels and the lesion volume, pre-stroke p lasma RANTES levels were correlated with volume of brain infarction (Fig. 2B). Young ischemic mice did not show a significant correlation between these two parameters, however, the lesion size in old ischemic mice correlated statistically significant with plasma RANTES levels (r2 = 0.51 P=0 0.03; Fig. 2B).

Concentrations of plasma cytokines increase within hours after stroke

Aging has been associated with dysregulation of the immune system which is then unable to fully respond to pathological insults (Plackett *et al.* 2004; Raynor *et al.* 2012). We thus evaluated how aging and chronic systemic infection influenced the concentrations of inflammatory cytokines and chemokines in both plasma and brain. Serial plasma samples were drawn from the saphenous vein of young and old, uninfected and infected ischemic mice at 1 and 4 hours after the induction of ischemia for analysis of a panel of cytokines (tumor necrosis factor-α (TNFα), RANTES, monocyte chemoattractant protein-1 (MCP-1),

KC (chemokine C-X-C motif ligand 1), interleukin-6 (IL-6), IL-1β, IL-1α, IL-17α, IL-10, interferon-γ (IFN-γ) and granulocyte colony stimulating factor-1 (G-CSF). Of the cytokines analyzed, only plasma levels of GCSF-1, KC and IL-6 were significantly increased at 4 h after stroke compared to 1 h post stroke in all ischemic study groups, but neither age nor preceding infection affected the plasma cytokine levels at this early time point (data not shown).

Aging and infection did not alter gliosis in the brain after cerebral ischemia

Brain microgliosis in the ischemic animals was assessed by immunohistochemical staining using ionized calcium-binding adapter molecule (Iba-1) antibody at 24 hours after ischemia. As expected, quantification of the immunoreactivity revealed a significant ischemia-induced up regulation of Iba-1 compared to the contralateral side (Fig. 3). However, there were no differences in the peri ischemic Iba-1 immunoreactivity between the treatment groups.

Aging with predisposing chronic peripheral infection leads to massive influx of neutrophils into the ischemic brain

Ischemia induced neutrophil infiltration into the ischemic cortex whereas the contralateral cortex remained devoid of any neutrophils. Quantification of the recruited neutrophils revealed that chronic peripheral infection had no effect on the neutrophil infiltration into the ischemic brain parenchyma in young mice (Fig. 4A, C, D). Aging itself did not increase neutrophil infiltration, as old uninfected ischemic mice had statistically similar levels of infiltrated neutrophils to young ischemic mice, irrespective of their infection status (Fig. 4A, E). However, predisposing chronic peripheral infection aggravated neutrophil infiltration

significantly in aged ischemic mice (Fig. 4A, F). The size of the infarction correlated ($p<0.001$) with neutrophil infiltration (Fig. 4B).

Aged infected mice fail to up regulate G-CSF and MCP-1 in the peri-ischemic area as compared to aged uninfected mice

Levels of Iba-1 immunoreactivity were similar between young and old ischemic mice, although total Iba-1 immunoreactivity is a relatively indefinite marker for inflammation because other cell types contribute to secretion of inflammatory mediators upon stroke. We therefore investigated the inflammatory profile of the brains of the infected, ischemic mice in more detail.

24 h after stroke, cortical brain samples from ischemic young and old, uninfected and infected mice were dissected for the analysis of TNFα, RANTES, MCP-1, KC, IL-6, IL-1β, IL-1α, IL-17α, IL-10, IFN-γ and G-CSF. Cytometric bead array (CBA) analysis revealed that aging or infection alone did not alter the levels of the cytokines in the the contralateral, intact hemisphere (Fig. 5). Ischemia alone increased the peri ischemic levels of G-CSF, MCP-1, KC, and RANTES in both young ischemic uninfected and aged ischemic uninfected mice and IL-1α in aged ischemic uninfected mice when compared to the contralateral intact hemisphere (Fig. 5A-D). Moreover, ischemia-induced increases in cytokine levels were significantly greater in aged uninfected compared to young uninfected mice. Chronic infection induced a differential reduction of ischemia-induced brain cytokines in young and aged mice. In young mice with chronic infection ischemia failed to increase brain levels of IL-1β(Fig. 5D) and RANTES (Fig. 5E) but not G-CSF (Fig. 5A), MCP-1 (Fig. 5B) or KC (Fig. 5C), whilst in aged mice with chronic infection the ischemia-induced increase in the levels of G-CSF (Fig. 5A) and MCP-1 (Fig. 5B) but not KC (Fig. 5C), IL-1β (Fig. 5D) or

RANTES (Fig. 5E) were significantly attenuated. The effect of chronic infection on ischemia-induced levels of brain G-CSF, IL-1β and RANTES in young mice was modest and in the contralateral hemisphere the values were often below the detection limit. Chronic infection in aged mice reduced the ischemia-induced levels of brain KC by 30%, though this change failed to reach statistical significance.

Aging and infection did not induce any alterations on the levels of plasma cytokines 1 or 4 hours after stroke. Because infarct size and leukocyte infiltration were both increased specifically in the old mice predisposed to chronic infection, we measured the plasma levels of pro and anti-inflammatory cytokines 24 h after stroke. IL-17A, a pro-inflammatory cytokine which regulates expression or release of other pro-inflammatory cytokines such as TNFα and KC, was shown recently to contribute to neutrophil infiltration into the brain and ischemic injury after transient MCAo. From the cytokines analyzed, we found alterations in the levels of TNFα (Fig. 6A) and IL-17A (Fig. 6B). The preceding peripheral infection in young mice or aging alone did not increase the plasma TNFα or IL-1α levels. However, old infected mice exhibited significantly higher levels of TNFα compared to young uninfected or infected mice (Fig. 6A) and higher levels of IL-17A compared to young infected mice (Fig. 6B).

Discussion

Here we show for the first time that aging exacerbates ischemic brain injury in mice with chronic peripheral infection. We demonstrate that aged mice with chronic peripheral infection develop larger brain infarcts compared to their young counterparts and this increase is

associated with greater neutrophil recruitment and diminished ischemia-induced up regulation of G-CSF. We also found that pre-stroke concentrations of circulating RANTES anticipated the infarct size 24h after MCAo in aged animals.

In this study we used *T.muris* infection as a well-characterized tool to achieve chronic peripheral inflammation which is an important part of the pathology in several co-morbid conditions such as diabetes (Dandona *et al.* 2004), atherosclerosis (Ross 1999), hypertension (Savoia & Schiffrin 2006) and infection itself, and known to contribute to poor outcome in stroke (Denes *et al.* 2010). The infection paradigm used in this study has been shown to induce Th1-polarized immune response and lead to increased infarct size in mice after MCAo, with concomitant increase in platelet aggregation, leukocyte infiltration and MMP-9 activation (Denes *et al.* 2010). This type of infection paradigm was chosen not to mimic any specific type of infection but rather as a general model of Th1-polarized immune responses known to contribute to impaired outcome in brain ischemia. Indeed, the inflammatory component in common comorbidities, such as atherosclerosis, hypertension (Savoia & Schiffrin 2006) and diabetes (Haskins & Cooke 2011) is often Th1 shifted. However, intestinal parasite infections are relatively common throughout the world.

Aging is a phenomenon associated with altered homeostasis and immune responses in the body (Weiskopf *et al.* 2009). Age has been shown to influence the outcome of stroke in the clinical setting (Nakayama *et al.* 1994). The behavioral outcome in aged animals has been reported to be impaired compared to young animals (Zhang *et al.* 2005; Popa-Wagner *et al.* 2007; Petcu *et al.* 2008). The effect of age on the infarct size in preclinical studies has resulted in somewhat contradictory findings depending on the ischemia model used (Davis *et*

al. 1995; Kharlamov *et al.* 2000; Shapira *et al.* 2002; Rosen *et al.* 2005). In this current study the infarct size in aged mice was similar compared to young mice, a finding that is in agreement with a previously published study using a photothrombotic model of brain ischemia (Zhao *et al.* 2005). The effect of age on stroke outcome may depend on the time or lack of reperfusion, specific site of infarction, species and strain and our study clearly shows that even under circumstances when aging alone has no significant impact on infarct size, preceding chronic infection markedly aggravates ischemic brain injury in aged mice. A deeper understanding of the interactions between aging and infection are particularly important as aging is associated with dysregulated inflammation. Also, chronic infections are more frequent in the elderly suggesting that infections may not be well tolerated by older people. Thus, mechanisms underlying the interactions of aging/infections to stroke outcome could offer the key to novel therapies.

Cerebral inflammation is elevated with aging (Sieber *et al.* 2011) and aged animals have been reported to show an increase in pro-inflammatory mediators after ischemia (Dinapoli *et al.* 2010), intracerebral hemorrhage (Lee *et al.* 2009) or mechanical injury (Kyrkanides *et al.* 2001). In the current study, the brain peri-infarct levels of MCP-1, KC, IL-1α and RANTES were elevated after brain ischemia in aged mice compared to their young counterparts. Stroke-induced responses seem to be model dependent, as one study has shown the aging brain to mount attenuated levels of pro-inflammatory cytokines upon transient MCAo (Sieber *et al.* 2011). Even though we observed an increase in several pro-inflammatory mediators in aged brain upon stroke, brain microgliosis was not altered between the treatment groups. However, glial cells are not the only mediators of inflammation in ischemic brain, as endothelial cells, pericytes and neurons participate in the stroke induced cytokine production (reviewed by del Zoppo (del Zoppo 2010)) and MCP-1, KC and IL-1α can be produced by

various different cell types including endothelial cells, neurons and astrocytes (Cushing *et al.* 1990; Standiford *et al.* 1991; Che *et al.* 2001). Moreover, immunohistological characteristics of microglial activation are certainly inaccurate markers with little information on exact status of the activity of these cells.

Our study showed that old infected mice developed the largest infarcts upon pMCAo and this particular group displayed decreased levels of ischemia-induced increase in MCP-1 and G-CSF in the peri-ischemic area, indicating the development of a dysregulated inflammatory response in aged, infected animals. G-CSF is a glycoprotein originally found to be involved in the proliferation, survival and maturation of granulocytes but later shown to confer neuroprotection against ischemic insult by directly binding its corresponding receptor to neurons (Schabitz *et al.* 2003). G-CSF and its receptor have been shown to be induced upon brain ischemia in the peri-ischemic area (Schneider *et al.* 2005) and G-CSF has been suggested to serve as a defense mechanism against ischemic cell death by multiple mechanisms (Sugiyama *et al.* 2011) (Solaroglu *et al.* 2009). Even though the AXIS-2 clinical trial for G-CSF treatment failed to show efficacy in humans, the mechanisms by which G-CSF could mediate neuroprotection in elderly patients with co-morbidities with elevated systemic inflammatory burden have not been modeled in preclinical studies. Although many of the beneficial effects of G-CSF in brain ischemia models may require a follow-up time longer than 24 h, it is possible that the inability to properly increase G-CSF-mediated pathways in aged infected ischemic mouse brain is one of the reasons for the aggravated ischemic damage observed in our stroke model. Our study shows that aging, together with peripheral infection leads to increased neutrophil recruitment into the ischemic brain. Neutrophils are likely mediators of increased neuronal damage as several studies have highlighted their destructive functions including secretion of matrix metalloproteinases

degrading BBB integrity, inflammation, edema formation and hemorrhagic transformation (McColl *et al.* 2007; Buck *et al.* 2008; Murikinati *et al.* 2010). IL-17A has been recently shown to induce neutrophil infiltration into the ischemic brain parenchyma (Gelderblom *et al.* 2012). Since neutrophils may mediate ischemic brain injury specifically in the cortex (Beray-Berthat *et al.* 2003) we hypothesize that increased neutrophil invasion is one of the factors contributing to greater ischemic brain damage, which evolved into the cortex in our stroke model.

IL-17A is a proinflammatory cytokine and is secreted by γδ T cells in the ischemic brain. Peripheral neutralization of IL-17A has been shown to provide protection against ischemic damage (Gelderblom *et al.* 2012) and elevated levels of IL-17 expressing blood mononuclear cells have been observed in human stroke patients (Kostulas *et al.* 1999). γδ T cells have been shown to infiltrate into the ischemic brain parenchyma at later stages of the injury and the levels of brain IL-17A levels after ischemia are increased at 3 days post stroke (Li *et al.* 2005; Shichita *et al.* 2009). Even though the presence of brain γδ T cells nor the levels of brain IL-17A were not analyzed in this study it may be that the levels of plasma IL-17A at the early 24 h time point in the current study reflect on increased activation of γδ T cells in the periphery. Indeed, the amount of infiltrating T-cells have been shown to dramatically increase at 5 d post injury in the same pMCAO model as used in the current study (Zhou *et al.* 2013). TNFα on the other hand is well characterized proinflammatory cytokine contributing to the ischemic damage (Hallenbeck 2002) and TNFα levels in plasma have been shown to be increased not only in preclinical stroke models but also in ischemic patients (Sotgiu *et al.* 2006; Yousuf *et al.* 2013). The observed increase in the levels of plasma IL-17A and TNFα in the current study thus have clinical relevance.

Chronic peripheral infection by *T.muris* has been reported to increase neuronal damage in young mice after transient middle cerebral artery occlusion (tMCAo) (Denes *et al.* 2010) and the effect was shown to be mediated by systemic increase in plasma RANTES levels. In that particular study, this led to delayed resolution of brain inflammation (48h post stroke) as analyzed by leukocyte recruitment, platelet aggregation and matrix metalloproteinase activation in the ischemic brain parenchyma. It appears that in the permanent pMCAo model, chronic peripheral inflammation exerts detrimental effects on ischemic brain injury only in combination with an additional comorbidity, aging, which is likely to occur clinically in patients presenting with multiple comorbidities. Interactions between comorbidities in stroke patients might also explain the lack of translation of various potential treatments from animal research to the clinic. Nevertheless, the fact that systemic infection in young mice subjected to transient ischemia exacerbated neuronal death (Denes *et al.* 2010) whereas in young mice subjected to permanent ischemia, infection itself had no effect on the lesion volume. Age-induced changes were required to see a similar effect in our study and it highlights the impact of the ischemia model on the outcome of stroke. Transient MCAo (tMCAo) used in the study of Dênes et al (Denes *et al.* 2010) includes reperfusion associated injury contributing to neuronal damage whereas pMCAo lacks this additional pathology. Indeed, these two ischemia models capture different neuroinflammatory profiles as both the amount of neutrophil infiltration and expression of pro-inflammatory cytokines has been shown to be higher in pMCAo model compared to tMCAo (Zhou *et al.* 2013). Since spontaneous reperfusion occurs in less than half of the stroke patients with very little influence on clinical improvement (Bowler *et al.* 1998), the results presented here, together with the previous data (Denes *et al.* 2010), indicate the importance of the use of pathologically diverse animal models, especially with several comorbidities more closely resembling human stroke in preclinical studies.

Taken together, this study demonstrates for the first time that aging aggravates neuronal damage in mice with chronic peripheral infection. Increased neuronal death was associated with exaggerated infiltration of neutrophils in the ischemic brain parenchyma and diminished increase in stroke-induced levels of G-CSF in the peri-ischemic area. As majority of stroke patients are elderly with various predisposing conditions with an inflammatory component, these results have a clinical importance and raise the demand for using more clinically relevant animal models in preclinical stroke research.

Experimental procedures

Animals

All animal experiments were conducted according to the national regulations of the use of laboratory animals and were approved by the National Animal Experiment Board of Finland and followed the Council of Europe Legislation and Regulation for Animal Protection. The mice were housed under a 12-hour light-dark cycle and allowed free access to standard rodent chow and water. A total of 50, 18-22-month-old (referred to as "old") and 62 4-month-old C57BL/6j ("young") background male mice were used in this study. A total of 6 mice died during the surgery. There was no difference in the mortality amongst the study groups.

Randomization and blinding

Animals were randomized into treatment groups using GraphPad Quick Calcs software for random numbers. All data were analyzed blinded to the treatment groups.

Trichuris muris infection

T. muris infection was introduced by oral gavage of ten infective T. muris eggs in 50 µl volume of phosphate buffered saline (PBS). This treatment has been shown to lead to a chronic, Th1-polarized immune response that peaks at 4-5 weeks post infection (Denes *et al.* 2010). Uninfected mice received only vehicle (PBS). The mice of both age groups (young and old) were divided into two subgroups which received either *T. muris* parasite infection (infected mice) or vehicle control (uninfected mice). Thirty-five days later, at the time when chronic Th1-polarised immune response peaks (Denes *et al.* 2010) , the mice underwent pMCAo or sham surgery. The infection induced by *T. muris* did not cause any adverse effects or mortality.

Permanent focal cerebral ischemia

pMCAo was introduced as described earlier (Koistinaho *et al.* 2002). Briefly, the mice were anesthetized by 5 % isoflurane (in 70% N_2O/30% O_2) and the surgical anesthesia was maintained by 1.8% isoflurane. The temperature of the mice was maintained at 37+/-0.5 °C using a homeothermic control system connected to a heating blanket and a rectal probe (PanLab, Harvard apparatus, Spain). The temporal bone of the mice was exposed and approximately a 1-mm hole was drilled on the site of the middle cerebral artery (MCA). Saline was applied during the drilling to prevent heat injury. The dura was carefully removed to expose the MCA. The MCA was gently lifted up and coagulated by using a thermocoagulator (Aron Medical Instruments) at the level of the inferior cerebral vein. The occlusion of MCA was confirmed by cutting the artery, after which the temporalis muscle was lifted back and the wound closed by stitches. Sham-operated mice underwent an identical surgical procedure except for coagulation of MCA. All mice were allowed to recover in a

heated recovery chamber for 30 minutes after which they were returned into individual cages. The mice were sacrificed 24 hours post-ischemia.

Physiological parameters

Physiological parameters were measured immediately after the onset of ischemia from blood samples drawn from the saphenous vein. The partial pressure of carbon dioxide and oxygen and pH were measured using iSTAT analyzer (Abbott, Abbott Park, IL). Blood glucose levels were measured using Freestyle blood glucose monitoring device (Abbott, Alameda, CA).

Magnetic resonance imaging

Lesion size of the ischemic animals was assessed *ex vivo* by MRI with brains submerged in 4% paraformaldehyde (PFA) in 10 mm NMR-tube. Twenty-four hours post-ischemia the mice were anesthetized with 250 mg / kg of Avertin (2,2,2 Tribromoethanol in tertiary amyl alcohol) and transcardically perfused with heparinized (2500 IU / l) saline. The brains were dissected out and placed in 50 ml tubes containing 4% PFA in 0.1M phosphate buffer. *Ex vivo* MRI was done using a vertical 9.4 T magnet (Oxford Instruments PLC, Abingdon, UK), interfaced to a Varian DirectDrive console (Varian Inc, Palo Alto, CA). A quadrature volume RF-coil (diameter 20mm, Rapid Biomedical GmbH, Rimpar, Germany) was used for transmission and reception. T2-weighted multi-slice images were taken with double spin-echo sequence with adiabatic refocusing pulses (TR = 2.5 s, TE = 40 ms, matrix size = 256*128, field of view = 19.2 mm * 19.2 mm, slice thickness 0.8 mm, gap 0 mm). A total of 15 consecutive slices were imaged throughout the brain. The total lesion volume was outlined manually on T2-weighted signal intensity from all images with a visible infarction using an

in-house written program in Matlab R2007b (Math-Works, Natick, MA) and the lesion volume was calculated by multiplying the number of pixels with pixel size and slice thickness. The analysis was done blinded to the study groups. The total infarction volume was measured from the total of 38 mice in the following study groups: young uninfected (n = 8), young infected (n = 10), old uninfected (n = 10) and old infected mice (n = 10).

Immunohistochemistry

Twenty-four hours post ischemia the mice were anesthetized by 250 mg / kg of Avertin and perfused transcardically with heparinized (2500 IU / L) saline. The brains were dissected out and postfixed by 4% paraformaldehyde in 0.1M phosphate buffer for 24 hours followed by cryoprotection in 30% sucrose for 2 days. The brains were then snap-frozen on liquid nitrogen and 20-µm-thick coronal cryosections were cut throughout the lesion starting at the beginning of the lesion area using a cryostat (Leica Microsystems GmH, Germany). For each staining a set of 6 sections at 400 µm intervals spanning through the lesion area was stained.

For the detection of microglia and neutrophils sections were incubated with Iba-1 (ionized calcium-binding adapter molecule 1, 1:250 dilution, Wako, Wako Chemicals GmbH, Germany) and anti-neutrophil (1:5000 dilution, Serotec, Kidlington, UK) antibodies, respectively. After overnight incubation with primary antibodies, the sections were incubated with appropriate biotinylated IgG H+L secondary antibodies (1:200, Vector Laboratories, Burlingame, CA, USA) followed by Vectastain ABC peroxidase system (Vector) and development by Ni-enhanced 3,3'-diaminobenzidine.

Immunoreactivity was imaged at 10x magnification, using an AX70 microscope (Olympus, NY, USA) with an attached digital camera (Color View 12 or F-View, Soft Imaging System,

Germany) running Analysis Software (Soft Imaging System). All immunoreactivites were quantified using ImagePro Plus Software (Media Cybernetics, Silver Spring, MD, USA) blinded to the study groups. Iba-1 immunoreactivity was quantified from a precise peri-ischemic area, defined as a 720 µm x 530 µm cortical region immediately adjacent to the dorsal border of the ischemic lesion spanning across 6 consecutive coronal sections taken at 400 µm intervals starting at the anterior part of the lesion. Corresponding cortical area of the contralateral healthy hemisphere was imaged to reveal the ischemia-induced up regulation of Iba-1. Neutrophils were similarly quantified from 720 µm x 530 µm area at the lesion site from 6 consequtive sections taken at 400 µm intervals. Since the contralateral hemisphere was devoid of any neutrophils, the contralateral side was excluded from neutrophil quantification. Iba-1 immunoreactivities were quantified from a total of 36 ischemic mice in following treatment groups: young uninfected (n = 8), young infected (n = 10), old uninfected (n = 8) and old infected mice (n = 10). Neutrophil immunoreactivity was quantified from total of 37 ischemic mice in following groups: young uninfected (n = 8), young infected (n = 10), old uninfected (n = 9) and old infected mice (n = 10).

Cytokine measurement

An array of cytokines, RANTES, MCP-1, KC, IL-6 , IL-1β, IL-1α, IL-17α, IL-10, IFN-γ and G-CSF were measured from plasma samples taken 1 or 4 h after ischemia as well as from freshly frozen brain samples dissected at 24 h post ischemia. Since plasma RANTES levels have been shown to correlate with the successful Th1 polarized infection status in mice (Denes *et al.* 2010), plasma RANTES levels were measured at 1 day prior to onset of ischemia. Plasma was separated from serial blood samples drawn from the saphenous vein using 1:10 volume of sodium citrate (3.8 %) as an anticoagulant. Plasma was obtained after

centrifugation of the samples at 1500 g for 10 minutes. At 24 h post ischemia the mice were anesthetized with Avertin and transcardially perfused with heparinized (2500 IU / L) saline as described above. The brains were dissected under a dissection microscope into cortical brain samples of the lesion area, peri-ischemic area and the corresponding contralateral side. At the time of tissue dissection the cortical lesion area was clearly visible as a white, roundish area, evidently consisting of dead tissue. Peri-ischemic area was defined as a 1 mm wide area immediately surrounding the lesion. Contralateral tissues were defined as approximate corresponding cortical area on the contralateral cortex. The samples were immediately snap frozen in liquid nitrogen and stored at -70 ^{o}C for analysis. Brain samples were homogenized in lysis buffer at 4 ^{o}C as described earlier (Denes *et al.* 2010). Protein concentrations were calculated using a BCA assay (Pierce/Thermo Fisher Scientific country). Levels of cytokines in the plasma and brain homogenates were measured by using a cytometric bead array (CBA, BD Pharmingen country) according to the manufacturer's instructions. Also, after analyzing the brain cytokines at the end time point of 24 h, additional analysis of selected cytokines (IL-2, IL-4, IL-6, IL-10, IL-17A, INF-γ and TNFα) from plasma was carried out by using CBA flex sets (BD Pharmingen). Samples in which the read out was below the detection limit were omitted from the analysis.

Statistical analysis and exclusion criteria

Statistical analysis was done by GraphPad Prism running one-way ANOVA with Bonferroni posthoc test or by SPSS 19 (IBM SPSS Inc. IL) using the linear mixed model followed by Sidak post hoc correction when appropriate. Original values were converted to logarithmic scale in order to improve model assumptions (normality of residuals and homoscedasticity) when appropriate. Exclusion criteria were pre-determined; animals with bleeding during the

surgery were excluded from the study. As a result, all together 6 mice (1 young sham infected, 1 young ischemic uninfected, 2 young ischemic infected and 2 old ischemic infected) died during the surgery or had to be terminated due to bleeding as a result of a technical error. Statistical outliers as analyzed by Grubb's tests using GraphPad Prism QuickCalcs were excluded from the data set. The data is presented as mean +/- SD.

Acknowledgements

This work was funded by the European Union's 7th Framework Programme (FP7/2008 – 2013, European Stroke Network, agreement numbers 201024 and 202213 and supported by the Academy of Finland.

Author contributions

T.M., J.K., N.R. and A.D planned the study, H.D., T.M., A.D., P.V., S.W., J.M. and E.S. performed the experiments, R.G. and N.H. supplied the infection model, T.M, A.D and J.K wrote the manuscript.

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Figure legends

Figure 1. Infection aggravated neuron loss in aged mice. Quantification of MRI images taken at 24 hours post stroke revealed that infection had no effect on the lesion size in young mice, however, aged mice with systemic *T.muris* infection suffered from significantly larger neuronal death compared to young infected and uninfected mice (A). Lesion size quantification from MRI images was done from total of 38 animals; young uninfected (n =

8), young infected (n = 10), old uninfected (n = 10) and old infected mice (n = 10). Individual values are plotted as a scatter blot. * indicates $p < 0.05$ and ** indicates $p < 0.01$ (one-way ANOVA with Bonferroni posthoc test). Figures B – E represent typical example *ex vivo* T2W1 images of the young uninfected (B), young infected (C), old uninfected (D) and old infected (E) mice.

Figure 2. T.muris infection increased the pre-ischemic plasma levels of RANTES in both young and old mice (A). * indicates $p < 0.05$ and ** indicates $p < 0.01$ (one-way ANOVA with Bonferroni posthoc test). Plasma RANTES levels were plotted against the lesion size (B). The levels of RANTES in plasma showed significant correlation with the lesion volume in aged mice (r2 = 0.51 P=0 0.03) but not in young mice (r^2 = 0.24 P 0.15).

Figure 3. Infection did not affect ischemia induced microgliosis. As expected, ischemia induced a significant increase in Iba-1 (A) immunoreactivity in the peri ischemic area. However, there were no differences in the extent of ischemia induced Iba-1 between the treatment groups. Figures B – E depict typical examples of Iba-1 immunoreactivity in the peri-ischemic area of young uninfected (B) and infected (C) and aged uninfected (D) and infected mice (E), respectively. Iba-1 immunoreactivity was quantified from total 37 ischemic mice in following treatment groups: young uninfected (n = 8), young infected (n = 10), old uninfected (n = 8) and old infected mice (n = 10). Scale bar = 100 µm. Results are presented as mean +/- SD. ** indicates $p < 0.01$ (linear mixed model followed by Sidak post hoc correction).

Figure 4. Aged mice with systemic infection showed increased neutrophil infiltration into the ischemic brain parenchyma. Ischemia caused neutrophil infiltration at the lesion site. Systemic infection induced by *T.muris* did not increase ischemia induced neutrophil infiltration in young mice (A). Age alone had no additive effect in the neutrophil infiltration as aged mice displayed neutrophil infiltration to similar extent compared to young mice however, aged mice with systemic infection showed significantly more neutrophils in the ischemic brain parenchyma (A). The extent of neutrophil infiltration showed significant correlation with the lesion volume ($r^2 = 0.33$, $p < 0.001$; B). Figures C-F depict representative images of neutrophil infiltration in the ischemic brain parenchyma in young uninfected (C), infected (D) and aged uninfected (E) and infected (F) mice. Neutrophil immunoreactivity was quantified from total of 38 ischemic mice in following groups: young uninfected (n = 8), young infected (n = 10), old uninfected (n = 9) and old infected mice (n = 10). Scale bar = 100 µm. Results are shown as mean +/- SEM. * indicates $p < 0.05$ (one-way ANOVA with Bonferroni posthoc test).

Figure 5. Aged infected mice fail to up regulate MCP-1 and G-CSF in the peri ischemic area. The brain cytokine profile was measured at 24 hours post ischemia. Aging or infection alone did not alter the levels of the cytokines as measured from the contralateral intact hemisphere (A – E)). Ischemia alone increased the peri ischemic levels of G-CSF (A) MCP-1 (B), KC (C), and RANTES (E) in both young and aged mice when compared to the contralateral intact hemisphere. Old mice exhibited significantly higher ischemia-induced increase in the levels of G-CSF (A), MCP-1 (B), KC (C), IL-1α (D) and RANTES (E) and aged mice with predisposing chronic infection failed to up regulate G-CSF and MCP-1 to similar degree compared to aged uninfected mice (A, B). Results are shown as mean +/- SEM. * indicates $p < 0.05$ and ** indicates $p < 0.01$ (linear mixed model followed by Sidak

post hoc correction). The n-number in each group: KC n = 8 - 10 in all groups; MCP-1, n = 10 in all groups; RANTES n = 10 in all groups; G-CSF young uninfected contra (n = 6) and ipsi (n = 9), young infected contra (n = 7) and ipsi (n = 10), old uninfected contra (n = 10) and ipsi (n = 10) and old infected mice contra (n = 7) and ipsi (n = 10). For IL-1α young uninfected contra (n = 6) and ipsi (n = 7), young infected contra (n = 2) and ipsi (n = 8), old uninfected contra (n = 5) and ipsi (n = 9) and old infected mice contra (n = 4) and ipsi (n = 10).

Figure 6. Aged infected mice show increased plasma levels of TNF and IL-17A. Plasma levels of TNFα and IL-17α at 24 hours post stroke were not altered upon infection in young animals nor by aging alone. However, old infected mice exhibited significantly higher levels of TNF compared to young uninfected and infected mice (A) and higher levels of IL-17A compared to young infected mice (B). Results are shown as mean +/- SEM. * indicates $p < 0.05$ and *** indicates $p < 0.001$ (one-way ANOVA with Bonferroni posthoc test). For both cytokines n = 8 – 10 in each group.