Neuroscience 290 (2015) 321–331

PIVOTAL ROLE OF CEREBRAL INTERLEUKIN-23 DURING IMMUNOLOGIC INJURY IN DELAYED CEREBRAL ISCHEMIA IN MICE

Y. ZHENG,[†] D. ZHONG,[†] H. CHEN, S. MA, Y. SUN, M. WANG, Q. LIU AND G. LI[*]

Department of Neurology, The First Affiliated Hospital, Harbin Medical University, 23 You Zheng Street, Harbin 150001, Heilong Jiang Province, PR China

Abstract—*Background:* Interleukin-23 (IL-23) is required for T helper 17 (Th17) cell responses and IL-17 production in ischemic stroke. We previously showed that the IL-23/IL-17 axis aggravates immune injury after cerebral infarction in mice. However, IL-23 might activate other cytokines and transcription factor forkhead box P3 (Foxp3) production in cerebral ischemia. We aimed to determine whether IL-23p19 knockdown prevents cerebral ischemic injury by reducing ischemic-induced inflammation.

***Methods:* Ischemic stroke models were established by permanent middle cerebral arterial occlusion (pMCAO) in male C57BL/6 mice. *In vivo* gene knockdown was achieved by intravenous delivery of lentiviral vectors (LVs) encoding IL-23p19 short hairpin RNA (LV-IL-23p19 shRNA). Enzyme-linked immunoassay (ELISA) and quantitative real-time polymerase chain reaction (qRT-PCR) confirmed inhibitory efficiency. Behavioral deficits were evaluated by adhesive-removal somatic-sensory test. Brain infarct volume was measured at day 5 after pMCAO by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Expression of IL-17, IL-4, interferon (IFN)-γ and Foxp3 in ischemic brain tissues were detected by qRT-PCR and Western blotting, respectively. Additionally, immunohistochemical staining located cytokines in ischemic brain tissues.**

***Results:* RNA interference knockdown of IL-23p19 resulted in improved neurological function and reduced infarct volume. IL-23p19 knockdown suppressed IL-17 gene and protein expression. Moreover, IL-23p19 deficiency enhanced IFN-γ and Foxp3 expressions in delayed cerebral ischemic mice, and did not impact IL-4 expression. Immunohistochemical staining showed that IL-17, IL-4, IFN-γ and Foxp3-positive cells were located around ischemic lesions of the ipsilateral hemisphere.**

***Conclusions:* IL-23p19 knockdown prevents delayed cerebral ischemic injury by dampening the ischemia-induced inflammation, and is a promising approach for clinically managing ischemic stroke.**

Key words: IL-23p19 knockdown, cerebral ischemia, pMCAO, IL-17, IFN-γ, Foxp3.

INTRODUCTION

Stroke is one of the most severe neurological diseases threatening millions of lives worldwide (Murray and Lopez, 1997). Ischemic stroke accounts for about 87% of the total number of stroke patients (He et al., 2012). Stroke is a thrombotic disease caused by the occlusion of cerebral arteries following disruption of energy supply to brain tissues (Garcia et al., 1996; Marder et al., 2006). Recent studies have demonstrated that cerebral post-ischemic inflammation is mediated by infiltration of immune cells, in particular macrophages, lymphocytes, and dendritic cells (DCs) (Gelderblom et al., 2009; Inacio et al., 2011; Joo et al., 2013), although these cells and their effector cytokines were previously found in autoimmune diseases (Dhodapkar et al., 2008; Steinman, 2010; Judson et al., 2012; Mieliauskaite et al., 2012; Espigol-Frigole et al., 2013).

Inflammation-mediated brain injury is caused by the interaction of both the innate and adaptive immune systems (Iadecola and Anrather, 2011). In the past years, multiple investigations have discussed the importance of infiltrating cells and their pro-inflammatory cytokines, which lead to secondary injury after cerebral ischemia. When brain ischemia occurs, infiltrated macrophages and DCs are major sources of interleukin (IL)-23 (Carmody et al., 2007; Uemura et al., 2009). IL-23 expands and generates IL-17-producing T cells such as T helper 17 (Th17) cells and γδ T cells, which are capable of inducing secondary injury after initial brain ischemia (Shichita et al., 2009; Brait et al., 2012). Therefore, IL-23 plays a key role upon downstream cells and cytokines in cerebral ischemia. IL-23 is a heterodimeric cytokine comprising the common p40 subunit of IL-12 but binding a specific p19 subunit (Wiekowski et al., 2001; Kastelein et al., 2007). Significantly increased IL-23p19 expression, both at messenger ribonucleic acid (mRNA) and protein levels, was demonstrated in multiple sclerotic lesions (Li

*Corresponding author. Tel./fax: +86-451-85555886.
E-mail address: hydlgz1962@163.com (G. Li).
[†] They contributed equally to this work.

Abbreviations: ANOVA, analysis of variance; DAB, diaminobenzidine tetrahydrochloride; DC, dendritic cell; ELISA, enzyme-linked immunoassay; Foxp3, forkhead box P3; IFN, interferon; IL, interleukin; I/R, ischemia/reperfusion; LV, lentiviral vector; mRNA, messenger ribonucleic acid; NC, negative control; pMCAO, permanent middle cerebral artery occlusion; PMSF, phenylmethylsulfonyl fluoride; qRT-PCR, quantitative real-time polymerase chain reaction; shRNA, short hairpin RNA; TTC, tetrazolium chloride; TUs, transducing units; TBS, Tris bufferred saline.

http://dx.doi.org/10.1016/j.neuroscience.2015.01.041
0306-4522/

et al., 2007). Meanwhile, anti-IL-23p19 antibody is effective in treating certain autoimmune diseases (Elson et al., 2007; Cornelissen et al., 2013; Wang et al., 2013). Thus, from a therapeutic viewpoint, IL-23p19 might represent a new target for ischemic stroke therapy.

Our previous research demonstrated increased IL-17-positive cell numbers and IL-17 mRNA in the ischemic hemisphere of permanent middle cerebral artery occlusion (pMCAO)-operated rats (Li et al., 2005). Further study confirmed that the IL-23/IL-17 axis plays a key role in cerebral infarction in the later phase of development, and its down-regulation may attenuate inflammatory damage (Ma et al., 2013). More importantly, some researchers have found that depletion of the IL-23 gene results in a more obvious protective effect than depletion of γδ T cells in the brain ischemia/reperfusion (I/R) mouse model (Brait et al., 2012), suggesting that IL-23 plays additional roles in brain injury besides IL-17 induction (Konoeda et al., 2010). It is also generally accepted that other T cell-associated cytokines such as interferon (IFN)-γ, IL-4 and the transcription factor forkhead box P3 (Foxp3) are involved in ischemic stroke (Jeong et al., 2003). For example, IL-4 is a major negative regulator of proinflammatory cytokine production by both brain cells and T cells, and appears to play a key role in controlling neuro-inflammation (Xiong et al., 2011). IFN-γ contributes to the maintenance, and perhaps the initiation of inflammatory changes in the post-ischemic brain (Yilmaz et al., 2006). Foxp3, which is arguably the best Treg-associated cell marker, limits neuro-inflammation in permanent cortical stroke (Liesz et al., 2013). However, little is known about the relationship between IL-23 and these cytokines in ischemic stroke.

Here, we report that IL-23p19 knockdown leads to specific changes in downstream factors like IL-17, IL-4, IFN-γ and Foxp3, balancing the host immune homeostasis and ameliorating neurological dysfunction.

EXPERIMENTAL PROCEDURES

Animals and surgical procedures

The local ethics committee for animal research approved the experimental protocol, and all procedures were conducted following national and international guidelines for the use of experimental animals. Male C57/BL6 mice weighing approximately 25 g were included in the study. Animals were housed on a 12-h-light/dark cycle at controlled temperature and humidity with free access to food and water. Mice were randomly assigned to the following four groups: sham-operated group (sham group, $n = 12$), pMCAO experimental group (pMCAO group, $n = 12$), Lentiviral-mediated IL-23p19-specific short hairpin RNA (shRNA) (lentiviral vector (LV)-IL-23p19 shRNA) delivery group (pMCAO + LV, $n = 12$) and a negative control (NC) LV with scrambled shRNA (LV-NC shRNA) delivery group (pMCAO + NC, $n = 12$). The animals in each group were then assigned to subgroups for tetrazolium chloride (TTC) staining, immunohistochemistry, quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analysis. Thirty-three additional mice were randomly divided into eleven groups to assess the infection efficacy of LV-IL-23p19 shRNA before operation: PBS group; LV-IL-23p19 shRNA groups, including 5, 10, 25, 50, and 100 μl/d groups; LV-NC shRNA groups, including 5, 10, 25, 50, and 100 μl/d groups; all $n = 3$). LV-IL-23p19 shRNAs infected more than 80% of cells at a multiplicity of infection (MOI) of 20 and successfully silenced the expression of IL-23p19 *in vitro*.

After anesthetization with chloral hydrate (350 mg/kg, i.p.), mice were placed in a supine position and a midline skin incision was made to expose the right common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA). PMCAO was performed by gently inserting a poly-L-lysine-coated 6-0 monofilament into the right internal carotid artery as described previously (Longa et al., 1989). Sham-operated mice were subjected to the same procedure but without occlusion. The rectal temperature was maintained at 36 °C with a warming lamp. Animals without neurological function deficits immediately after ischemia, those that died within 5 days, and those with subarachnoid hemorrhage at the time of death, were excluded from the analysis.

IL-23p19 shRNA Lentiviral construction, preparation and administration

Four shRNA sequences targeting mouse IL-23p19 (GenBank, Accession NM_031252) and a NC sequence were designed and synthesized according to the manufacturer's instructions (Genechem, Shanghai, China). The best-performing IL-23p19 shRNA sequence was GCAGGCAGCTGGCTAGAGA; TTCTCCGAAC GTGTCACGT was used as NC scrambled shRNA (NC). ShRNAs were cloned into a LV pGCsil-GFP containing a CMV-driven EGFP reporter gene and a U6 promoter upstream of the restriction sites (Genechem, Shanghai, China). All constructs were confirmed by sequence analysis. Recombinant lentiviral constructs were produced by co-transfecting 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA., USA; cat. No. 11668-500) according to standard protocols. Briefly, the culture medium was collected over 48 h, concentrated by ultracentrifugation, aliquoted, and stored at −80 °C until use. Viral titers, expressed as transducing units (TUs) per milliliter, were determined by measuring the GFP expression in 293T cells. This was done by transducing 293T cells with serial dilutions of concentrated lentivirus. The initial titers were approximately 3×10^9 TU/ml.

Predetermined doses of LV-IL-23p19 shRNA (5, 10, 25, 50 and 100 μl) tittered at 2×10^7 TU/ml and equal amounts of LV-NC shRNA (5, 10, 25, 50 and 100 μl) as well as PBS control were injected by caudal vein once daily for seven consecutive days before pMCAO (Zhou et al., 2013). Next, lipopolysaccharide (LPS, 055:B5, Sigma–Aldrich, St. Louis, MO, USA) was injected subcutaneously to stimulate animals for 24 h. Afterward, brain samples were respectively obtained for further study.