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ORIGINAL RESEARCH

Increased Peripheral CD137 Expression in a Mouse Model of Permanent Focal Cerebral Ischemia

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Abstract
Various studies demonstrate that CD137 (TNFRSF9, 4-1BB) promotes atherosclerosis and vascular inflammation in experimental models via interactions with the CD137 ligand (CD137L). However, the exact role of CD137 in ischemic stroke remains unclear. In this study, we analyzed dynamic changes of peripheral CD137 expression on T cells in a mouse model of cerebral ischemia-middle cerebral artery occlusion (MCAO), as well as alternation of neurological function, infarct size and cerebral inflammatory status after inhibition of the CD137/CD137L pathway using an anti-CD137L monoclonal antibody. MCAO mice showed elevated surface expression of CD137 on T cells in both peripheral blood and lymphoid tissues during early cerebral ischemia. Remarkably, blockade of the CD137/CD137L pathway reduced the post-ischemic brain damage. Our findings indicate that enhanced CD137 costimulation occurs in early cerebral ischemia and promotes T cell activation, which in turn upregulates inflammatory immune response and possibly exerting deleterious effects on cerebral ischemia.

Keywords CD137 · CD137L · MCAO · Cerebral ischemia

Introduction

Ischemic stroke (IS) is a common cerebral vascular disease (CVD) with high morbidity and mortality. IS usually occurs as a result of an interrupted blood supply to an area of the brain (Lloyd-Jones et al. 2010). Under the pathophysiological conditions induced by IS, a sudden interruption of cerebral blood flow causes tissue hypoxia and provokes an inflammatory cascade leading to impairment of ion homeostasis, neuronal excitotoxicity, intracellular calcium overload, free radical production, lipid peroxidation, and ultimately resulting in neuronal injury (Petrovic-Djergovic et al. 2016; Bonaventura et al. 2016). Of note, the role of inflammation in IS has attracted attention since neuronal death due to occlusion of cerebral blood flow can trigger both local and systemic immune responses, thereby causing microglia activation and leukocyte recruitment into injured brain tissue (Wang et al. 2007). Among these infiltrating leukocytes, neutrophils and T cells are believed to play crucial roles in tissue damage following cerebral ischemia (Jickling et al. 2015; Perez-de-Puig et al. 2015; Gu et al. 2015). IS-related studies using human and murine models have revealed an increased amount of T cells in lymphoid tissue or the cerebral infarction region (Gelderblom et al. 2009; Planas et al. 2012). In general, two distinct signals are necessary in the process of T cell activation: the first signal is mediated via T-cell receptor (TCR), while costimulatory molecules trigger the second signal. Adequate costimulation, such as engagement of CD28 with B7, is critical in initiating T cell responses and failure of T cells to receive a second signal can result in anergy or apoptosis (Carreno and Collins 2002; Salomon and Bluestone 2001; Scandiuzzi et al. 2011; Hutloff 2011; Esensten et al. 2016). Aside from CD28 costimulation, several molecules belonging to the TNF receptor superfamily, including CD137 (TNFRSF9, 4-1BB) and CD40 (TNFRSF5), can act as CD28-independent costimulators involved in the induction, differentiation,

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and survival of T cells (Watts 2005; Croft 2003; Kwon et al. 2002; Sanchez-Paulete et al. 2016).

Although various studies demonstrate the CD137/CD137L costimulation is implicated in immunopathogenesis of atherosclerosis (Olofsson et al. 2008; Jeon et al. 2010) and acute coronary syndromes (Li et al. 2010; Yan et al. 2011), the exact role of CD137 in ischemic stroke remains unclear. To clarify this issue, we previously performed a study which revealed elevated plasma levels and monocyte-associated expression of CD137L in patients with acute atherothrombotic stroke (Yu et al. 2014). We also recently found that patients with acute atherothrombotic stroke had increased sCD137 levels and CD4+ T cell-associated expression of CD137 in peripheral blood (He et al. 2018). All these findings suggest the existence of a CD137L- or CD137-mediated inflammatory response occurring in early stages of this disease. However, further studies are necessary to ascertain the CD137 expression on T cells and determine its pathogenic role during the early stages of ischemic stroke. Here, we have analyzed peripheral CD137 expression in response to cerebral ischemia using a mouse model of middle cerebral artery occlusion (MCAO).

Materials and Methods

Mice

Female 8-week-old *C57BL/6J* wild-type (WT) mice were purchased from Beijing Vital River (Distributor of Jackson Laboratory, Beijing, China). All mice were bred and kept under specific-pathogen-free conditions in the animal center of Peking University People's Hospital (Beijing, China). The study was approved by the Ethics Committee of Peking University People's Hospital.

Establishment of MCAO Model

Mice were deeply anesthetized with intraperitoneal injection of 2.5% pentobarbital sodium (0.05 g/kg), followed by a midline neck incision and gentle pull-apart of soft tissues. Under a JSZ5B stereomicroscope, a blunt dissection was carried out to expose the trachea and retract the muscles for localization of the carotid artery. The left common carotid artery (CCA), internal common carotid artery (ICA) and external common carotid artery (ECA) were carefully dissected from surrounding tissue, and both the CCA and ICA were temporarily occluded by reverse-action tweezers. A permanent suture was placed around the ECA, as distally as possible, and another temporary suture slightly tight was placed on the ECA distal to the bifurcation. A small hole was then cut into ECA between permanent and temporary sutures, accompanied by a stepwise installment of a 12 mm-long 6-0 silicon-coated monofilament suture (1620, Beijing Cinontech, Beijing, China) into the ECA. After complete cutting of the ECA distal to the permanent suture, the occluder was inserted into the ICA and the reverse-action tweezers were removed. The occluder was introduced to occlude the origin of the MCA in the circle of Willis and stopped when its insertion was around 9–10 mm beyond the bifurcation of ECA and CCA, when the occluder was blocked and unable to move anymore. The suture on the ECA was tightly tied to fix the monofilament in position. For sham operations, all procedures are identical except that the occluder was not inserted. The animals were then awakened, and all the MCAO mice presented with left Horner syndrome, incapability to extend the right forelimb when suspended or veering or circling to the right when walking.

Sample Collection

The spleen and lymph nodes (superficial inguinal and mesenteric) were mechanically dissociated, passed through a 70-μm Cell Strainer, and the resulting suspension was collected by centrifugation. Mononuclear cells were thus obtained by Percoll gradient centrifugation. All the heparinized blood specimens were collected from ophthalmic artery, followed by a stepwise isolation of peripheral blood mononuclear cells (PBMC) by standard density gradient centrifugation. After a brief centrifugation, plasma was stored in small aliquots at − 70 °C for further analysis.

Flow Cytometry

Mononuclear cells were examined for expression of CD137 on T cell subsets by dual-color direct immunofluorescence staining and flow cytometry using a FACScan (Becton Dickinson, CA, USA). Briefly, 200 μl cell suspension containing 1×10^6 cells/ml were prepared and stained with either fluorescein isothiocyanate (FITC)-labeled anti-mouse CD4 monoclonal antibody (MoAb) (Catalog Number: 11-9473-82) and phycoerythrin (PE)-labeled anti-mouse CD137 MoAb (Catalog Number: 12-1371-82), FITC-labeled anti-mouse CD8a MoAb (Catalog Number:MA5-17398) and PE-labeled anti-mouse CD137 MoAb, or FITC-labeled anti-mouse CD45R (B220) MoAb (Catalog Number: 11-0452-82) and PE-labeled anti-mouse CD137L MoAb (Catalog Number: 12-5901-82) (eBioscience, San Diego, CA, USA). After 30-min incubation in dark at 4 °C, these cells were washed twice with staining buffer and analyzed on FACScan with BD CellQuest™ Pro software (© 2002, BD Biosciences, San Jose, CA, USA).

ELISA

Plasma levels of sCD137 were detected with an enzyme-linked immunosorbent assay (ELISA) kit (Catalog Number: EMTNFRSF9) (eBioscience, San Diego, CA, USA) following the manufacturer's recommendations. All assays were conducted simultaneously in a blinded fashion in triplicates.

Administration of Anti-CD137L Monoclonal Antibody (MoAb)

Immediately after MCAO, mice were intraperitoneally injected with single dosage of 200-μg anti-mouse CD137L MoAb (Catalog Number: 14-5901-81) (eBioscience, San Diego, CA, USA) per mouse. The control group ($n = 5$) received same dosage of isotype antibody (eBioscience, San Diego, CA, USA). All of the mice were sacrificed 72 h after the administration for further analysis.

Neurological Function Assessment

Neurological function was assessed at 6 h, 24 h and 72 h after MCAO modeling, respectively. The Zea Longa score (Longa et al. 1989) was determined according to the following scoring system: 0, no deficit; 1.0, unable to extend the right forelimb when suspended; 2.0, circling to the right when walking; 3.0, rolling to the right when walking; 4.0, no spontaneous movement, unconsciousness or coma. Mice with scores 2 to 3 formed the efficacy model and were used in the following experiments.

TTC Staining

Mouse head was removed immediately after sacrifice. The mouse brain tissue was placed on ice for 15 min, and then, 2-mm-thick sagittal sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 15 min at 37 °C. Infarcts were analyzed to clarify the outcome of MCAO model at different time points (Fig. 1).

RNA Preparation and cDNA Synthesis

Isolation of total RNA from infarcted brain tissue was performed according to the standard protocol of RNeasy® Mini Kit (Catalog Number: 74104) (Qiagen, Germany). Reverse transcription was conducted with SUPERSCRIPT III TRANSCRIPT reagents (Catalog Number: 18080093) (Invitrogen, USA) using random primer and primer containing 50 uM oligio (dt). The reaction was carried out at 25 °C for 5 min, 50 °C for 60 min and 70 °C for 15 min

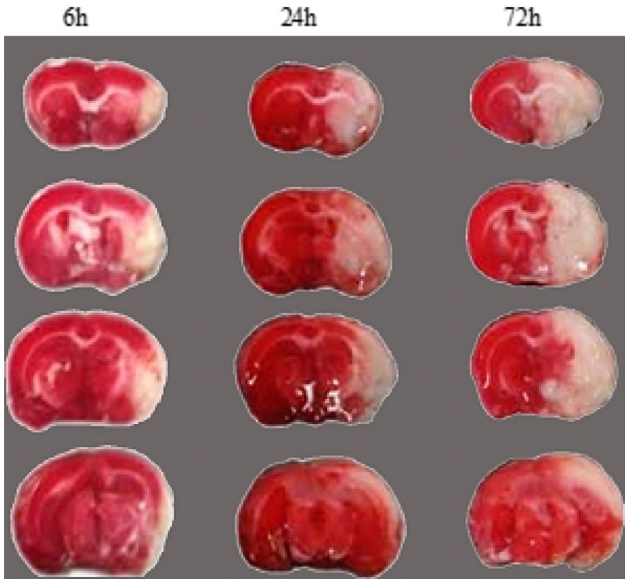

Fig. 1 TTC staining of brain tissue in MCAO model. The mice brain tissue was obtained at 6, 24 and 72 h after MCAO, placed on ice for 15 min, and then 2-mm-thick sagittal sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 15 min at 37 °C. The white area represents the infarct lesions

on a GeneAmp® PCR system 9700 (Applied Biosystems, Norwalk, CT, USA).

Real-Time PCR

PCR was performed in a 20-μl reaction system containing 1 μl cDNA template, 2.5 mM $MgCl_2$, 250 μM each of dNTPs, 250 nM of each primer, 0.3 μl SYBR (20×) and 1 U of Taq polymerase (Catalog Number: 10966-026) (Invitrogen, USA). The primers were synthesized by Invitrogen, USA, according to previously published sequences (Huang et al. 2016; Pan et al. 2015; Zechel et al. 2002) (Table 1). Real-time PCR was done in an ABI 7500fast Sequence Detector. All amplifications were performed at least in triplicates. Amplifications were carried out at 95 °C for 30 s followed by 45 cycles of 30 s at 95 °C and 40 s at 60 °C. The mRNA expression levels of each gene were calculated by using the $2^{-\Delta\Delta CT}$ (comparative threshold cycle, or C_T) method, as detailed by the manufacturer (Technical Bulletin 2; Applied Biosystems).

Statistics

Statistical analysis was performed using SPSS 18.0 statistical software (SPSS Inc., Chicago, IL). All the data were presented as mean ± standard deviation (Zea Longa score, cytokines mRNA) or median with range (CD137 and CD137L surface expression, sCD137). Normally distributed data was analyzed by a one-way analysis of variance

Table 1 Primers used in this study

Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)
IL-6	ATTGGAAATTGGGGTAGGAAG	ACAAGAAAGACAAAGCCAGAGTC
IL-1β	CCACAGCCACAATGAGTGATACT	GAACTCAACTGTGAAATGCCACC
TNF-α	CCCAGACCCTCACACTCAGAT	TTGTCCCTTGAAGAGAACCTG
MMP-9	AGTTTGGTGTCGCGGAGCAC	TACATGAGCGCTTCCGGCAC
β-actin	TGGAATCCTGTGGCATCCATCAAA	TAAAACGCAGCTCAGTAACAGTCC

(ANOVA) using Student–Newman–Keul's post hoc or Student's t test as well as Pearson's correlation test. Non-normally distributed data were analyzed with Kruskal–Wallis ANOVA and Dunn's post hoc as well as Spearman's correlation test. Statistical significance was set at $P < 0.05$.

Results

Cell Surface Expression of CD137 on T Cells

The MCAO mice showed a continuous increase in CD137 expression (Fig. 2) on CD4+ T cells in peripheral blood or lymphoid tissues after 6 h (blood: 0.18%, 0.68 MFI, lymphoid: 0.62%, 0.68 MFI), 24 h (blood: 2.07%, 2.23 MFI, lymphoid: 1.01%, 1.06 MFI), and 72 h (blood: 3.32%, 3.42 MFI, lymphoid: 4.22%, 4.32 MFI) of ischemia, and this increase did reach statistically significant differences ($P < 0.05$ and $P < 0.05$) with Kruskal–Wallis and Dunn's post hoc test to compare these three groups at different time points (6 h, 24 h and 72 h post-ischemia). The CD137 expression at 72 h post-ischemia ($n = 5$) was significantly higher than that at 6 h post-ischemia ($n = 5$) ($P < 0.05$ or $P < 0.01$). In addition, the MCAO group at 72 h post-ischemia ($n = 5$) had increased CD137 expression on CD4+ (4.22%, 4.32 MFI) and CD8+ (2.28%, 2.32 MFI) T cells in lymphoid tissue as compared with the sham-operated group ($n = 5$) [CD4+: 0.27%, 0.32 MFI, CD8+:0.34%, 0.43 MFI] ($P < 0.05$ or $P < 0.01$) (Table 2).

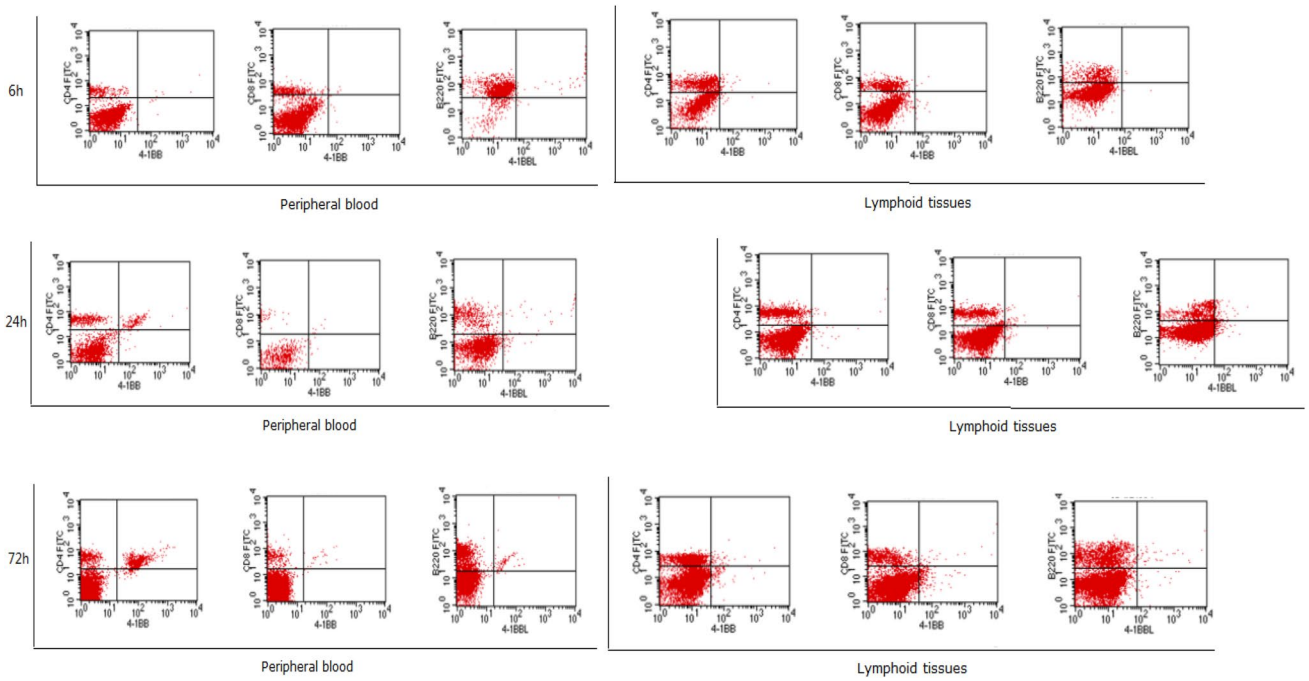

Fig. 2 Flow cytometry mononuclear cells of all mice were examined for surface expression of CD137 on T cell subsets or CD137 ligand (CD137L) on B cells by dual-color direct immunofluorescence and flow cytometry. 2×10^5 cells in a 200-μl cell suspension were prepared and surface stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse CD4 monoclonal antibody (MoAb) and phycoerythrin-labeled anti-mouse CD137 MoAb, FITC-labeled anti-mouse CD8a MoAb and PE-labeled anti-mouse CD137 MoAb, FITC-labeled anti-mouse CD45R (B220) MoAb and PE-labeled anti-mouse CD137L MoAb. After 30-min incubation in dark at 4 °C, these cells were washed twice with staining buffer and analyzed using FACScan

Table 2 Expression of CD137 on CD4+ and CD8+ T cells in peripheral blood and lymphoid tissue of MCAO ($n=5$) and sham-operated group ($n=5$)

	Peripheral blood (median)						Lymphoid tissue (median)					
	CD4+ (%)	CD8+ (%)	CD137 level (%)		CD137 level (MFI)		CD4+ (%)	CD8+ (%)	CD137 level (%)		CD137 level (MFI)	
			CD4+ (%)	CD8+ (%)	CD4+ (%)	CD8+ (%)			CD4+ (%)	CD8+ (%)	CD4+ (%)	CD8+ (%)
6 h post-ischemia												
MCAO group	80.3	10.3	0.2	0.4	0.7	0.5	76.4	18.6	0.6	0.2	0.7	0.2
Range	75.8–85.1	9.2–14.7	0.1–1.3	0.1–0.9	0.3–1.1	0.2–0.9	65.8–78.4	14.4–28.2	0.1–1.5	0.1–0.8	0.3–1.1	0.2–0.9
Sham group	80.6	12.3	0.5	0.2	0.5	0.4	80	15.1	0.3	0.1	0.3	0.4
Range	75.9–85	10–19.1	0.4–0.8	0.1–0.7	0.4–0.8	0.2–0.8	70.2–82	15.6–18.8	0.1–0.5	0.1–0.7	0.1–0.5	0.4–1.3
24 h post-ischemia												
MCAO group	84.3	10.7	2.1	0.5	2.2	0.4	75.1	20.7	1.0	1.1	1.1	1.2
Range	80.1–89.8	5.2–14.9	0.7–6	0.2–2.1	0.8–5.7	0.3–2.1	64.5–81	14–30.2	0.2–2.7	0.6–2.4	0.8–5.7	0.3–2.1
Sham group	85.0	10.5	0.5	0.2	0.5	0.2	70.6	24.4	0.7	0.5	0.8	0.6
Range	77.8–88.5	6.5–17.2	0.1–1.7	0.1–0.97	0.1–1.7	0.1–1	61.6–75.9	19.6–30.4	0.1–2.8	0.1–1	0.2–2.8	0.2–1.9
72 h post-ischemia												
MCAO group	86.5	8.5	3.3^{Δ}	2.1^{Δ}	3.4^{Δ}	2.2^{Δ}	78.7	16.4	$4.2\ *^{\Delta\Delta}$	$2.3*^{\Delta}$	$4.3**^{\Delta}$	$2.3*^{\Delta}$
Range	70.9–90	5.2–28.2	0.7–8.4	0.7–3.3	1.7–8.4	0.8–3.4	66.8–89.2	5.8–19.2	1.5–5.1	0.6–3	1.7–8.4	0.8–3.4
Sham group	85.6	8.6	1	0.8	1.0	1.1	76.2	13.2	0.3	0.3	0.3	0.4
Range	72.3–88.6	2.3–12.5	0.8–1.8	0.2–5.1	0.9–2	0.4–5	74.5–84.8	11.7–22.6	0.1–1.1	0.3–1.4	0.1–1.2	0.3–1.3

MFI mean fluorescence intensity

$**P<0.01$ or $*P<0.05$ for *post hoc* comparison with sham group

$^{\Delta\Delta}P<0.01$ or $^{\Delta}P<0.05$ for *post hoc* comparison with 6 h post-ischemia

Cell surface expression of CD137L on B220+ B cells

Within the MCAO group, there were irregular changes in CD137L expression (Fig. 2) on B220+ B cells at different time points (6 h, 24 h and 72 h post-ischemia) in either peripheral blood or lymphoid tissue. However, there was no significant difference in CD137L expression on B220+ B cell between MCAO ($n=5$) and sham group ($n=5$) at different time point ($P>0.05$) (Table 3).

Plasma sCD137 Levels

The MCAO mice showed a tendency of continuous decrease in plasma sCD137 levels after 6 h (1345 pg/ml), 24 h (1124 pg/ml), and 72 h (647 pg/ml) of ischemia ($P<0.05$), and this decrease did reach statistically significant differences ($P<0.05$) with Kruskal–Wallis and Dunn's post hoc test to compare these three groups at different time points (6 h, 24 h and 72 h post-ischemia). The sCD137 levels at 72 h post-ischemia ($n=5$) were significantly lower than that at 6 h post-ischemia ($n=5$) ($P<0.05$). At 72 h post-ischemia, the plasma sCD137 levels were significantly decreased in the MCAO group ($n=5$) as compared with the sham group ($n=5$) [1066 pg/ml] ($P<0.05$) (Fig. 3).

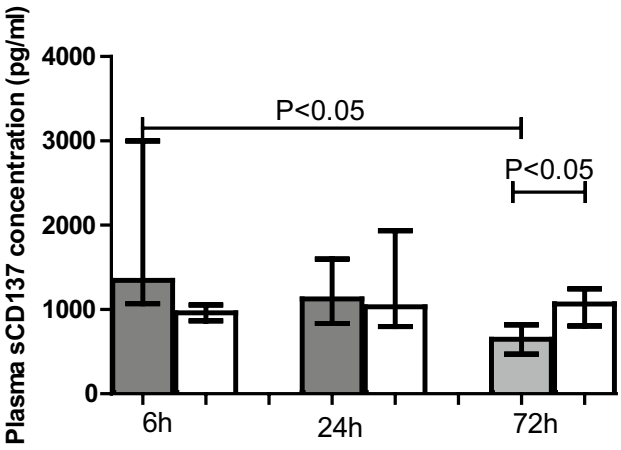

Fig. 3 Comparison of plasma level of soluble CD137 (sCD137) between MCAO mice and sham group at different time point filled square MCAO group ($n=5$), empty square sham group ($n=5$). Plasma levels of sCD137 were detected with an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, USA). All assays were conducted simultaneously in a blinded fashion in triplicates. Statistical analysis was performed with Kruskal–Wallis and Dunn's post hoc test

Zea-Longa Score at All-Time Points of Post-Ischemia

C57/BL mice presented with diseases about 24 h after MCAO, and thereafter their symptoms continued to increase at 48 h and 72 h respectively. Clinical assessment of neurological function demonstrated that the anti-CD137L MoAb-treated mice (1.6 ± 0.5) had lower disease severity scores than both control isotype IgG-treated ($n=5$) and MCAO mice ($n=5$) (2.4 ± 0.5 and 2.6 ± 0.5, $P<0.05$ and $P<0.05$) (Fig. 4).

Table 3 Expression of CD137L on B220+ B cell in peripheral blood and lymphoid tissue of MCAO ($n=5$) and sham-operated group ($n=5$)

	Peripheral blood (median)			Lymphoid tissue (median)		
	B220+ (%)	CD137L level (%)	CD137L level (MFI)	B220+ (%)	CD137L level (%)	CD137L level (MFI)
		B220+	$B220^+$		B220+	B220+
6 h post-ischemia						
MCAO group	19.4	1.3	1.3	23.1	1.4	1.5
Range	14.5–57.8	0–2.2	0.1–2.1	15.2–86.3	0.5–2.7	0.5–2.8
Sham group	28.8	0.5	0.5	18.9	0.3	0.4
Range	15.9–29.2	0.2–1.9	0.3–1.8	13.7–23.6	0.1–1	0.3–1.8
24 h post-ischemia						
MCAO group	15.0	1.5	1.5	23.1	3.1	3.0
Range	7.4–26.3	0.5–2.6	0.5–2.7	15.1–75.1	0.4–5.2	0.8–5.3
Sham group	24.7	0.9	0.9	12.7	1.3	1.4
Range	16.3–39.3	0.3–2.7	0.3–2.8	12.4–32.6	0.3–2.7	0.3–2.8
72 h post-ischemia						
MCAO group	27.8	0.98	0.91	38.2	3.42	3.5
Range	18.5–40.3	1–3.2	0.2–2.6	19.9–79.8	0.5–7.8	0.6–7.8
Sham group	29.0	1.7	1.9	24.7	0.64	0.7
Range	23.3–32.3	0.9–3.3	0.9–3.3	11.6–28.2	0.3–2.4	0.9–3.3

MFI mean fluorescence intensity

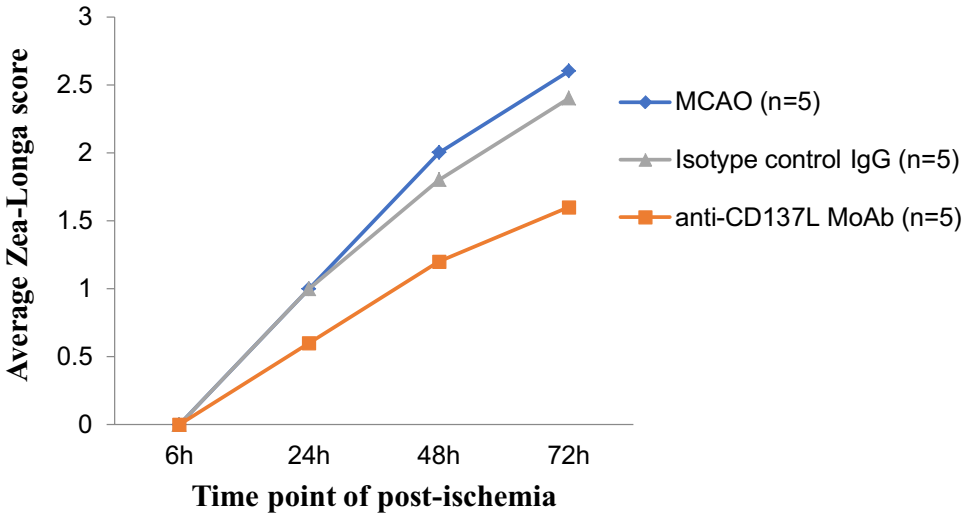

Fig. 4 Changes in mean disease severity scores in control and anti-CD137L monoclonal antibody (MoAb)-treated mice ($n=5$)

Size of Cerebral Infarcts

At 6 h, 24 h, and 72 h post-ischemia, MCAO mice displayed a trend toward extension of infarct zone in the brain parenchyma as visualized through TTC staining. However, after administration with an anti-CD137L MoAb, the MCAO mice presented with a tendency of reduction in infarct size at 72 h post-ischemia as compared with control isotype IgG-treated mice (Fig. 5).

Quantification of IL-1β, IL-6, TNF-α and MMP-9 mRNA Expression

In isolated infarcted brain tissues at 72 h post-ischemia, there were decreased IL-1β, IL-6 and MMP-9 mRNA expression levels in mice injected with anti-CD137L MoAb ($n=4$) as compared with mice treated with isotype control IgG ($n=4$), but no significant difference of TNF-α mRNA expression was found between these two groups (Table 4).

Correlation

In the MCAO group, plasma sCD137 levels were not significantly correlated with surface expression of CD137 on T cell subsets at 6 h, 24 h or 72 h after ischemia.

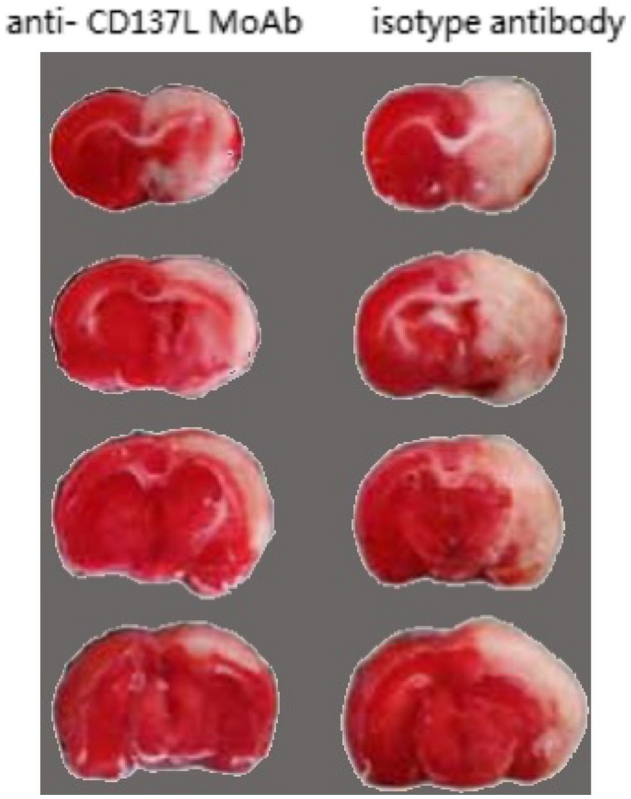

Fig. 5 TTC staining of brain tissue in MCAO mice after treatment with anti-CD137L monoclonal antibody (MoAb) and isotype antibody

Discussion

So far, several studies have shown appearance of lymphocytes (both B and T cells) in the brain as early as 4–6 h post-ischemia, and peaks about 3 days, suggesting their involvement in the development of inflammation within the ischemic area (Gelderblom et al. 2009; Gronberg et al. 2013; Yilmaz et al. 2006). Among different T cell subsets, both CD4+ and CD8+ T cells may exert harmful effects on ischemic injury in the acute phase (Yilmaz et al. 2006; Selvaraj and Stowe 2017). In this study, the MCAO mice demonstrated a trend toward continuous increase in CD137 expression on CD4+ T cells in either peripheral blood or lymphoid tissues during a 72 h period of cerebral ischemia, particularly at 72 h post-ischemia. Together with similar changes observed in lymphoid CD8+ T cells, these findings

Table 4 Comparison of mRNA expression of IL-1β, IL-6, TNF-α and MMP-9 gene between mice injected with anti-CD137L monoclonal antibody (MoAb) ($n=4$) and its isotype control IgG ($n=4$)

Gene	Anti-CD137 MoAb ($n=4$)	Isotype IgG ($n=4$)	P value
IL-1β	0.1714 ± 0.04579	0.3492 ± 0.1138	0.0274
IL-6	0.5486 ± 0.2788	0.9541 ± 0.1510	0.0430
TNF-α	0.6437 ± 0.4509	0.6482 ± 0.4195	0.9890
MMP-9	0.3973 ± 0.1468	0.7582 ± 0.2478	0.0461

suggest that during early stages of cerebral ischemia CD137 expression in T cells is upregulated in the periphery, and as a result, provides costimulatory signals for the activation of T cells, thus promoting a systemic inflammatory response, and possibly exerting deleterious effects on cerebral ischemia. Interestingly, TTC staining also showed that the infarct zone of brain tissue in mice tended to expand with prolonged ischemic time, providing further confirmation of our hypothesis regarding the pathogenic role of aberrant CD137 expression in peripheral during the early stages of ischemic stroke.

Unlike membrane-bound CD137 with a positive costimulatory signal for lymphocytes activation, sCD137 may regulate immune responses through a negative control mechanism, when it is released from cell surface of activated lymphocytes (Michel et al. 1998; Michel and Schwarz 2000). Several studies have reported that sCD137 exerts an antagonistic effect toward membrane CD137 and thus suppresses the proliferation of T cells and secretion of interleukin (IL)-2 (DeBenedette et al. 1995; Hurtado et al. 1995). Our study showed that during the early stages of cerebral ischemia, MCAO mice presented with a tendency toward continuous decrease in plasma sCD137 levels, especially at 72 h post-ischemia. It is possible that the gradually decreasing sCD137 levels may contribute to a downregulation of its immunosuppressive effects on systemic inflammation, and then further worsen the severity of cerebral infarct. Nevertheless, more evidence is needed to address this issue.

Although the detrimental role of non-regulatory T cells in acute IS has been unequivocally proven, the impact of B cells is incompletely understood. Previous reports showed discrepant results: some found a beneficial role of B cells (Ren et al. 2011; Chen et al. 2012; Offner and Hurn 2012), whereas others found no impact on infarct volume and functional outcome (Yilmaz et al. 2006; Kleinschnitz et al. 2010; Schuhmann et al. 2017). Interestingly, Doyle et al. (2015) reported a harmful role of B cells on long-term cognitive function. In this study, however, the level of CD137L expressed on lymphoid or blood B cells showed no marked changes within 72 h period of ischemia. In parallel with our similar results observed in stroke patients (Liu et al. 2006), this suggests that the membrane-bound CD137L may be irrelevant or at least not directly related to B cells-associated immunoactivities in early cerebral ischemia. On the other hand, CD137L is also expressed in other antigen presenting cells (APC) such as dendritic cells (DC) and monocyte/macrophages, and hence elevated CD137 could trigger reverse signaling through ligation of CD137L, thus promoting the proliferation, migration and survival of monocytic cells expressing CD137L (Langstein et al. 1998; Shao and Schwarz 2011). The roles of monocyte/macrophages in stroke remain controversial, as they might act as a double-edged sword via multiple mechanisms of clearing debris, inflammation, causing tissue damage and promoting tissue healing after stroke (Kanazawa et al. 2017). Nonetheless, future studies on CD137L expression on these immune cells will undoubtedly help unravel the exact roles of the costimulatory molecules in ischemic stroke.

To determine the pathogenic effects of CD137/CD137L costimulation on cerebral ischemia, we observed the changes in clinical scores, infarct size and inflammation milieu within brain tissues after inhibition of the costimulatory pathway using an anti-CD137L MoAb as described elsewhere (Li et al. 2014), since two distinct studies reported that the anti-CD137 MoAb inhibited the proliferation of T lymphocytes in vitro (Ju et al. 2003; Liu et al. 2006). As expected, treatment with the anti-CD137L MoAb immediately after MCAO resulted in a remarkable improvement in neurologic deficits, along with reduced infarct size and decreased proinflammatory cytokines levels compared to controls. These findings validate our view that during early cerebral ischemia, the upregulated CD137 expression prompts T cell-associated systemic inflammatory responses, which might consequently worsen brain injury (Anthony et al. 2012; Petrovic-Djergovic et al. 2016). On the other hands, several studies also showed that use of anti-CD137L MoAb enhanced proliferation of monocytes or the differentiation of potent T(h)1-inducing DC from human monocytes by reverse signaling of CD137L (Ju et al. 2003, 2009). This partially explained our results revealing no significant difference of TNF-α mRNA expression between anti-CD137L MoAb-treated and control group, as this proinflammatory cytokine is predominantly produced from APC such as monocyte/macrophages (Lambertsen et al. 2012). Hence, our results suggest that anti-CD137L MoAb treatment might lead to neuroprotective effects on cerebral ischemia through inhibition of CD137 costimulation, rather than CD137L reverse signaling. Intriguingly, several in vivo studies reported that administration of an agonistic anti-CD137 MoAb prompted atherosclerotic lesion inflammation in aorta and carotid artery of hypercholesterolemic ApoE$^{-/-}$ mice, with an increased CD8+ T cells and mRNA levels of TNF-α, IFN-γ and IL-10 (Olofsson et al. 2008; Söderström et al. 2017). Taken together, our findings implicate that CD137 may potentially serve as a promising therapeutic target for acute ischemic stroke, since agonistic MoAb targeting CD137 have been developed and utilized

for cancer immunotherapy in several ongoing clinical trials (Chester et al. 2018).

In conclusion, this study shows that, during early stages of cerebral ischemia, an elevated surface expression of CD137 on T cells in peripheral is associated with increased systemic inflammation, while blockade of the CD137 costimulation markedly ameliorated post-ischemic brain damages. Our findings indicate that enhanced CD137 costimulation occurs in early cerebral ischemia and might promotes T cell activation, which in turn, upregulates the inflammatory response and possibly exerting deleterious effects on cerebral ischemia. On the other hands, more efforts are needed to explore whether presence of CD137 costimulated T cells in the brain as well as the effects of the CD137/CD137L pathway on regulatory T cells in our future study. To our knowledge, this is the first reported animal study examining the expression and pathogenic roles of CD137 in cerebral ischemia. Therefore, our findings pave the way for a new promising immunotherapeutic option in the treatment of ischemic stroke.

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Compliance with Ethical Standards

Conflict of interest Xiao-Qing Li, Yang-Yang Wang, Ting-Ting Yang, Yi-Ning Qian, He Yin, Shan-Shan Zhong, Rong A, Yang He, Bao-Lei Xu, and Guang-Zhi Liu declare that there are no conflicts of interest.

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