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CD93-AA4.1: A Novel Regulator of Inflammation in Murine Focal Cerebral Ischemia

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The stem-cell marker CD93 (AA4.1/C1qRp) has been described as a potential complement C1q-receptor. Its exact molecular function, however, remains unknown. By using global expression profiling we showed that CD93-mRNA is highly induced after transient focal cerebral ischemia. CD93 protein is upregulated in endothelial cells, but also in selected macrophages and microglia. To elucidate the potential functional role of CD93 in postischemic brain damage, we used mice with a targeted deletion of the CD93 gene. After 30 min of occlusion of the middle cerebral artery and 3 d of reperfusion these mice displayed increased leukocyte infiltration into the brain, increased edema, and significantly larger infarct volumes (60.8 ± 52.2 versus 23.9 ± 16.6 mm³) when compared with wild-type (WT) mice. When the MCA was occluded for 60 min, after 2 d of reperfusion the CD93 knockout mice still showed more leukocytes in the brain, but the infarct volumes were not different from those seen in WT animals. To further explore CD93-dependent signaling pathways, we determined global transcription profiles and compared CD93-deficient and WT mice at various time points after induction of focal cerebral ischemia. We found a highly significant upregulation of the chemokine CCL21/Exodus-2 in untreated and treated CD93-deficient mice at all time points. Induction of CCL21 mRNA and protein was confirmed by PCR and immunohistochemistry. CCL21, which was formerly shown to be released by damaged neurons and to activate microglia, contributes to neurodegeneration. Thus, we speculate that CD93-neuroprotection is mediated via suppression of the neuroinflammatory response through downregulation of CCL21. The Journal of Immunology, 2010, 184: 6407–6417.

The stem-cell marker CD93 (AA4.1/C1qRp) has been described as a potential complement C1q-receptor. Its exact molecular function, however, remains unknown. By using global expression profiling we showed that CD93-mRNA is highly induced after transient focal cerebral ischemia. CD93 protein is upregulated in endothelial cells, but also in selected macrophages and microglia. To elucidate the potential functional role of CD93 in postischemic brain damage, we used mice with a targeted deletion of the CD93 gene. After 30 min of occlusion of the middle cerebral artery and 3 d of reperfusion these mice displayed increased leukocyte infiltration into the brain, increased edema, and significantly larger infarct volumes (60.8 ± 52.2 versus 23.9 ± 16.6 mm³) when compared with wild-type (WT) mice. When the MCA was occluded for 60 min, after 2 d of reperfusion the CD93 knockout mice still showed more leukocytes in the brain, but the infarct volumes were not different from those seen in WT animals. To further explore CD93-dependent signaling pathways, we determined global transcription profiles and compared CD93-deficient and WT mice at various time points after induction of focal cerebral ischemia. We found a highly significant upregulation of the chemokine CCL21/Exodus-2 in untreated and treated CD93-deficient mice at all time points. Induction of CCL21 mRNA and protein was confirmed by PCR and immunohistochemistry. CCL21, which was formerly shown to be released by damaged neurons and to activate microglia, contributes to neurodegeneration. Thus, we speculate that CD93-neuroprotection is mediated via suppression of the neuroinflammatory response through downregulation of CCL21.

Brain tissue damage after cerebral ischemia is exacerbated by a complex pattern of pathophysiologic mechanisms including excitotoxicity, peri-infarct depolarizations, inflammation, and apoptosis (1–4). Besides proinflammatory cytokines, such as IL-1β and TNF-α, TLRs and several chemokines have been described to mediate the inflammatory response in the CNS (2, 5, 6). Chemokines are a family of proteins that direct leukocyte migration and activation to inflammatory stimuli. Depending on the position of conserved cysteine residues, chemokines have been subdivided into the families C, CC, CXC, and CXC (5, 7). Chemokines have been characterized as playing a role in balancing the central and peripheral immune responses between lymphoid and nonlymphoid tissues (8). Although the expression of most chemokines is induced during infection and inflammation, some chemokines, including chemokine ligands CCL19 and CCL21, are constitutively expressed and control cell movement during homeostasis (9).

Whereas a plethora of genes linked to the pathophysiology of cerebral ischemia has already been identified using candidate approaches, screening techniques have proved to be valuable in identifying novel neuroprotective signaling pathways in the post-ischemic brain (10–15). Analysis of expression profiles of >39,000 mRNA transcripts in brain hemispheres of C57BL/6 wild-type (WT) mice at various time points after induction of cerebral ischemia using Affymetrix GeneChip arrays revealed nearly 2000 genes, which were significantly upregulated at least 2-fold, with p < 0.05 (G. Trendelenburg and W. Nietfeld, unpublished observations). Several of the identified genes have been associated already with focal cerebral ischemia in candidate approaches or previous screening experiments, but many represent new candidates. Several of these induced genes are related to inflammation or apoptosis (KEGG Pathway Database [www.genome.jp/kegg/pathway.html]; BioCarta Charting Pathways of Life [www.biocarta.com/genes/index.asp]) (15–17). This may also be the case for the CD93 gene (1419589_at).

The 126-kD CD93 protein was initially characterized as one of several putative complement C1q receptors and was given the name C1qRp (18–21). However, subsequent analysis revealed that CD93 interacts with C1q only under nonphysiologic conditions (18, 22). CD93/C1qRp was also characterized as a hematopoietic stem cell marker Ag and named AA4.1 (23). A subset of CD93-positive bone marrow progenitor cells express neural stem cell markers and thus may differentiate into neural cells (24). In addition, surface CD93 expression has proved to be inducible by inflammatory stimuli (25, 26). The molecular function of CD93, however, is still an enigma. CD93 expression has also been found in microglia, as well as in a subset of pyramidal neurons, but the vascular endothelium has

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Abbreviations used in this paper: Affy, Affymetrix GeneChip analysis; C, contralateral; fc, fold change; I, ipsilateral; Illum, Illumina hybridization assay; LTB, lymphotixin β; LTBR, lymphotixin-β receptor; MCAO, middle cerebral artery occlusion; MT, metallothionein; Pdpn, podoplanin; TM, TagMan PCR; U, untreated; WT, wild-type. Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/$16.00

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been described as the predominant site of CD93 expression (18, 27, 28), particularly during the remodeling of the vascular tree (29).

It was hypothesized that CD93 is involved in angiogenesis and endothelial cell migration, and possibly also in leukocyte extravasation at postcapillary venules (18, 22, 30, 31). Nevertheless, CD93-deficient mice (CD93<sup>−/−</sup>) are viable and showed no gross abnormalities in their vascular development. However, CD93<sup>−/−</sup> mice do have a defect in the phagocytic removal of apoptotic cells in vivo (18), demonstrating that CD93 contributes to the in vivo clearance of dying cells. We therefore hypothesized that CD93 participates in the neuroninflammatory changes observed in postischemic brain injury.

In this study, we explored whether CD93 mediates the death of dying cells. We therefore hypothesized that CD93 participates in angiogenesis and endothelial cell migration, and possibly also in leukocyte extravasation at postcapillary venules (18, 22, 30, 31). Nevertheless, CD93-deficient mice (CD93<sup>−/−</sup>) are viable and showed no gross abnormalities in their vascular development. However, CD93<sup>−/−</sup> mice do have a defect in the phagocytic removal of apoptotic cells in vivo (18), demonstrating that CD93 contributes to the in vivo clearance of dying cells. We therefore hypothesized that CD93 participates in the neuroninflammatory changes observed in postischemic brain injury.

Materials and Methods

**Animals**

The CD93<sup>−/−</sup> mice, generated as previously described (18), had been backcrossed onto the C57BL/6 background for 10 generations. Genotyping was determined by PCR as described below. Adult 10–12-wk-old CD93<sup>−/−</sup> mice and age-matched C57BL/6 control mice (BfR, Berlin, Germany) were used. All animal handling and surgery was performed in accordance with the Guidelines for the Use of Animals in Neuroscience Research (Society for Neuroscience, www.sfn.org/index.cfm?pagename=guidelinesPoliciesUseOfAnimalsAndHumans). All experiments were approved by the institutional Animal Care Committee, (LAGeSo No. G0382/05). The mice were bred in a selective pathogen-free environment and under standardized conditions of temperature (21°C), humidity (60%), and light and dark cycles (12 and 12 h each), with food and water provided ad libitum.

**Genotyping**

Genotyping was performed using the REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich, St. Louis, MO) with DNA extracted from mice tails and the following primers: CD93/F2 (5′-AGG CAT CCC AGC GAG GAA GGC CAA GTG-3′), neomycin 3′- (5′-GGG ATC GGC AAT AAA AAG AC-3′), and CD93/U (5′-GTC CTG CCA CTG ATC TAT ACC-3′) (18). Amplification of the WT gene resulted in an amplicon of ~721 bp, whereas the targeted gene corresponded to an ~480-bp fragment.

**Induction of focal cerebral ischemia**

Middle cerebral artery occlusion (MCAO) was induced by inserting a silicone-coated 8/0 nylon monofilament (Xantopren M Mucosa and Acticon, Düsseldorf, Germany) into the internal carotid artery as described by Hara et al. (32). As a sham control, the operation was performed without advancing the filament. Sufficiency of occlusion and reperfusion of the middle cerebral artery was monitored by Laser Doppler flowmetry (Peri Flux 4001 Master, Perimed, Stockholm, Sweden). Mice were anesthetized with 2% isoflurane for induction and 1.5% isoflurane, 70% N<sub>2</sub>O, and 30% O<sub>2</sub> via a face mask. Sufficiency of occlusion and reperfusion of the middle cerebral artery was monitored by Laser Doppler flowmetry (Peri Flux 4001 Master, Perimed, Stockholm, Sweden). Mice were anesthetized with 2% isoflurane for induction and 1.5% isoflurane, 70% N<sub>2</sub>O, and 30% O<sub>2</sub> via a face mask. The period of anesthesia did not exceed 10 min. After 30 min or 1 h of ischemia, the animals were re-anesthetized and the filament was removed to permit reperfusion. During surgery and ischemia, body temperature was measured and maintained between 37.0 and 37.5°C with a heating pad. There was no significant difference in the mean body weight between the different groups.

**Microarray analysis of gene expression**

At specified time points of reperfusion, mice were anesthetized deeply and decapitated. The brains were removed rapidly from the skull. RNA derived from ipsilateral (ischemic) hemispheres, pooled from six WT C57BL/6 mice (after MCAO, respectively sham treatment) at each time point, was used for Affymetrix GeneChip experiments using Affymetrix GeneChip Mouse Expression Set 430 (Affymetrix, Santa Clara, CA). For Illumina Sentrix array (BfR, Berlin, Germany), isolated RNA (Trizol, Invitrogen) was transcribed into cDNA (SuperScript II). Expression Set 430 (Affymetrix, Santa Clara, CA). For Illumina Sentrix array (BfR, Berlin, Germany), isolated RNA (Trizol, Invitrogen) was transcribed into cDNA (SuperScript II), biotinylated, and hybridized to a GeneChip (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM224409). To verify CCL21 expression results obtained by Illumina Sentrix arrays, CCL21 mRNA levels were analyzed by real-time PCR using LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science, Mannheim, Germany) with the following primers: forward 5′-AAT CCT GCT TTT ACC CCG GAA GCA CCA TCC TAA-3′, and reverse 5′-CTC TTG AGG GCT GTG TCA TCG CAG TTT CAG AAC C-3′. Expression was determined according to the protocol of Laemmli et al. (33). Protein was extracted in nonreducing loading buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NaDodSO<sub>4</sub> (SDS), 1% Triton-X-100, 1% deoxycholate acid and 10% (w/v) SDS-polyacrylamide gels. Following electrophoresis and semidry blotting onto nitrocellulose membrane (Serva, Heidelberg, Germany), incubation with a polyclonal rabbit antibody against CCL21 (Santa Cruz Biotechnology, Santa Cruz, CA), was used. A broad range, prestained SDS-PAGE protein standard (Invitrogen) was used to determine protein sizes.

**Assessment of infarct volume**

Two or 3 d after induction of ischemia for 60 min or 30 min, respectively, mice were deeply anesthetized and sacrificed. The brains were removed rapidly from the skull and snap-frozen in 2-methylbutane on dry ice. Brain tissues were sectioned (12 μm each), stained with hematoxylin and eosin, and the area of infarction was quantified by using Sigma Scan Pro Version 5.0.0 Software (Jandel Scientific, San Rafael, CA), and infarct volumes were calculated. A correction for edema (brain swelling) was applied by calculating the indirect infarct volume as the volume of the contralateral hemisphere minus the noninfarcted volume of the ipsilateral hemisphere. The difference between direct and indirect infarct volumes represents brain swelling. Relative infarct size was calculated as a percentage of the size of the contralateral hemisphere. Different volumes were measured to control for edema (brain swelling). Relative infarct size was calculated as a percentage of the size of the contralateral hemisphere. Different volumes were measured to control for edema (brain swelling). The area of infarction was quantified by using Sigma Scan Pro Version 5.0.0 Software (Jandel Scientific, San Rafael, CA), and infarct volumes were calculated. A correction for edema (brain swelling) was applied by calculating the indirect infarct volume as the volume of the contralateral hemisphere minus the noninfarcted volume of the ipsilateral hemisphere. The difference between direct and indirect infarct volumes represents brain swelling. Relative infarct size was calculated as a percentage of the size of the contralateral hemisphere. Different volumes were measured to control for edema (brain swelling).

**Immunohistochemistry**

Staining was performed on fresh-frozen (CD93), respectively PFA-perfused (CCL21), tissue harvested at different times of reperfusion. PFA-perfused brains were fixed in 4% PFA and dehydrated before they were embedded.

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into paraffin and then sectioned in 5-μm slides. From the fresh-frozen tissue, 12-μm coronal cryosections at interaural positions 6.6, 5.3, 3.9, 1.9, and 0 mm were thaw-mounted onto glass slides. Adjacent sections were used to determine stroke volume (see above). Slides were air-dried for 30 min and fixed for 10 min in -20°C methanol and acetone (1:1). The sections were incubated in blocking solution containing 3% normal goat serum and 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 60 min. The slides were incubated for 2 h at room temperature with a polyclonal rabbit anti-CD93 Ab (30) with a dilution of 1:200, or with a rat-anti CD93 Abs (1) and a goat-anti mouse IgG (Invitrogen) diluted 1:300 for 1 h at room temperature. To determine the cell-type of CD93-positive cells, additional staining of endothelial cells was performed using rat anti-CD31 (350274; BD Pharmingen, San Jose, CA) and a polyclonal goat anti-CD34 (1:25; sc-1407; Santa Cruz Biotechnology), whereas anti-CNP (Santa Cruz Biotechnology) was used to stain oligodendrocytes. Further cell type-specific double staining was performed using the following Abs: astrocytes, Guinea pig anti-GFAP (Advanced ImmunoChemical, Long Beach, CA); granulocytes, rabbit anti-myeloperoxidase (MPO; Chemicon, Temecula, CA); microglia and macrophages, goat anti-Iba1 (ab5076; Abcam, Cambridge, U.K.); neurons, mouse anti-NeuN (Chemicon, Temecula, CA). The primary Abs were detected in the brain sections by goat anti-mouse, goat anti-rabbit, goat anti-rat, goat anti-guinea pig, or donkey-anti goat Abs conjugated, respectively, with Texas Red or Cy3 (Molecular Probes, Leiden, The Netherlands). Omission of the primary Ab was used to ensure specificity of the secondary Abs; specificity of the CD93-Ab was verified by control staining of sections derived from CD93-/- mice. A proprietary blocking agent (OMM kit, Vector) was used to minimize background signal from endogenous mouse IgG in the NeuN stains. Confocal detection of double labeling studies was performed by confocal laser-scanning microscopy using Leica DM LFS A (Leica, Solms, Germany).

For CCL2-staining, sections were deparaffinized three times in xylene, followed by ascending alcohol series, and unmasked with trypsin (Abcam) for 20 min at 37°C. Nonspecific binding was blocked with 15% normal donkey serum. Primary goat-anti CCL2 Ab (1:200; R&D Systems, Wiesbaden, Germany) was incubated over night at 4°C followed by staining using donkey anti-goat Cy3 (1:200). Slides were also counterstained with Hoechst 33258, which stains DNA (Invitrogen).

Leukocyte quantification

WT and CD93 knockout mice brain sections (see above) at different time points of reperfusion were stained with CD11b Ab (see above for procedure). CD11b-positive cells in the whole ipsilateral (ischemic) hemisphere at the following positions were counted using a stereo investigator 7 (MicroBrightField Bioscience, Williston) stande to bregma 5.3 mm, 3.9 mm, and 1.9 mm (n = 3–4 animals).

Statistical analysis

Students t test for independent samples was applied to determine statistical significance between the mean values of two study groups if not stated otherwise. Values of p < 0.05 were considered statistically significant. For comparison of infarct volumes between different groups Mann-Whitney U test was used, and significance was accepted when the calculated U value was below the critical U value.

Results

CD93 mRNA is induced after focal cerebral ischemia in the mouse

Expression values for >39,000 mRNA transcripts from six pooled ipsilateral WT brain hemispheres were analyzed at 1, 3, 6, 12, and 24 h of reperfusion after 1 h of MCAO and compared with sham-treated mice by using Affymetrix GeneChip arrays (Set 430). The Affymetrix GeneChip results revealed a significant upregulation of CD93 mRNA during the first day of reperfusion, with a maximal induction at 12 h after MCAO when compared with sham-treated mice (Fig. 1A). Moreover, mRNA of control genes, such as metallothionein-I/II and moesin, which interacts with CD93 (34), was shown to be induced as described by us and other groups before (11, 14), whereas the expression of the CD93-homologous protein thrombomodulin (35) did not exceed a 1.5-fold change as measured by Affymetrix GeneChip and RT-PCR (data not shown). Apart from Affymetrix GeneChip analysis, which was performed to investigate early mRNA expression patterns over time, gene expression was also compared between ipsilateral and contralateral hemispheres (expression patterns over space). Gene expression was independently determined from three pooled ipsilateral and contralateral brain hemispheres, respectively, for each time point (3, 12, 24, and 48 h) to compare gene-specific expression in the ischemic hemisphere not only with the sham-operated mice as reference (as done for the initial Affymetrix screening experiment), but also to allow comparison between ipsilateral and contralateral hemispheres. Upregulation of CD93 mRNA in the ipsilateral, ischemic brain hemisphere was further confirmed by real-time PCR (TagMan LowDensity Arrays, probe ID Mm00440239_g1) in combination with an independent murine tissue preparation (Fig. 1A).

Expression of CD93 protein in ischemic mouse brain

Induction of CD93 protein in the ischemic hemisphere was confirmed by Western blotting. An ~130-kDa band appeared in protein extract derived from both the ipsilateral (ischemic) and contralateral hemispheres of postischemic mice at 3 to 48 h (Fig. 1A). Upregulation of CD93 mRNA in the ipsilateral, ischemic brain hemisphere was further confirmed by real-time PCR (TagMan LowDensity Arrays, probe ID Mm00440239_g1) in combination with an independent murine tissue preparation (Fig. 1A).

Affymetrix GeneChip analysis (Affy), real time TaqMan PCR (TM), and Illumina hybridization assays (Illum) for each time point. RNA was pooled from ipsilateral postischemic hemispheres of C57BL/6 WT mice (n = 4 for Illum and TM; n = 6 for Affy). Expression values of ipsilateral hemispheres were compared with values of sham-treated control animals (Affy) or contralateral hemispheres (TM; Illum). CD93-gene specific primers were used (identifier numbers Mm00440239_g1 [FC TM], scli18542.4.1.65-S [FC Illum], respectively 1419589, at [FC Affy], n.d., not determined. B, C57BL/6 WT mice underwent 1 h MCAO or were left untreated (control). After 12 or 48 h of reperfusion, protein was extracted from the contralateral (C) and ipsilateral (I) hemispheres. Extracts were immunoblotted with a polyclonal rabbit anti-CD93 specific Ab. The arrow denotes the 130-kDa band of the CD93 isoform; 70 μg total protein extract (determined by BCA protein assay) per lane were used. Results are representative of three separate experiments.

FIGURE 1. Up-regulation of CD93-mRNA and protein in postischemic mouse brain tissue. A, Fold change (FC) expression after various reperfusion times in an MCAO model with 1 h of occlusion time determined by Affymetrix GeneChip analysis (Affy), real time TaqMan PCR (TM), and Illumina hybridization assays (Illum). For each time point, RNA was pooled from ipsilateral postischemic hemispheres of C57BL/6 WT mice (n = 4 for Illum and TM; n = 6 for Affy). Expression values of ipsilateral hemispheres were compared with values of sham-treated control animals (Affy) or contralateral hemispheres (TM; Illum). CD93-gene specific primers were used (identifier numbers Mm00440239_g1 [FC TM], scli18542.4.1.65-S [FC Illum], respectively 1419589, at [FC Affy], n.d., not determined. B, C57BL/6 WT mice underwent 1 h MCAO or were left untreated (control). After 12 or 48 h of reperfusion, protein was extracted from the contralateral (C) and ipsilateral (I) hemispheres. Extracts were immunoblotted with a polyclonal rabbit anti-CD93 specific Ab. The arrow denotes the 130-kDa band of the CD93 isoform; 70 μg total protein extract (determined by BCA protein assay) per lane were used. Results are representative of three separate experiments.
of WT animals, compared with the expression in contralateral hemispheres, the expression at shorter reperfusion times, or the expression in brain tissue of untreated animals (Fig. 1B).

Immunohistochemistry

The induction of CD93 protein in postischemic brain tissue of WT mice was also observed by the use of immunohistochemistry at various time points after MCAO, but not in the brains of CD93-deficient mice. A strong upregulation of CD93 was found at various times of reperfusion in the ipsilateral hemisphere, with the highest expression in the peri-infarct region and in the infarct core. Anti-CD93 Ab stained vascular structures of the ischemic hemisphere intensively, whereas vascular structures of the non-ischemic contralateral hemisphere were stained only with low intensity. Double labeling with anti-CD31, anti-GFAP and anti-Iba1 (Fig. 2) identified the majority of CD93-immunopositive cells as endothelial cells and selected infiltrating macrophages and microglia, which mainly occurred at later time points of reperfusion. Some macrophages and microglia were stained with anti-CD93 Ab in the penumbra at 72 h of reperfusion. Infrequently, CD93 colocalized with neurons of the peri-infarct region (data not shown). In the infarct core, CD93 signal was confined to selected macrophages and microglia, but at 48 h of reperfusion anti-CD93 Ab almost always stained vascular CD31-positive structures with high intensity (Fig. 2). Selected CD93-positive cells were located immunohistochemically in the direct vicinity of GFAP-positive cells and were found exclusively at the border of the infarct core. No colocalization was observed with the oligodendrocyte marker CNP (data not shown). Confocal laser-scanning microscopy confirmed CD93 expression in small vessels (CD34-positive cells), but did not reveal a colocalization of CD93 and GFAP (Fig. 2B), despite an expression of GFAP and CD93 in direct vicinity (Fig. 2B, 2D–F), indicating that astrocytes do not express CD93.

FIGURE 2. CD93 expression in ischemic brain tissue. A, Double staining of ischemic mouse brain tissue in the infarct border zone at 48 h after 1 h MCAO in WT mice (a–f, k–m), at 48 h after 1 h MCAO of CD93-deficient mice (o), at 72 h after 30 min MCAO in WT mice (g–j), and brain tissue of untreated WT (CD93+/+) mice (n). Immunohistochemical staining of CD93 and cell specific markers, respectively. Right column (c, f, j, m). Merge of the first (CD93-specific staining [a, d, g, k]) and second column (cellular markers [b, e, h, l]) is displayed. Intensely stained CD93-positive cells in the ischemic hemisphere (a, d, k) were found to be mostly endothelial cells (l and m), rat anti-CD31 (BD Pharmingen, San Jose, CA). Some microglial cells (e, f, h, and j), goat anti-Iba1 (Abcam, Cambridge, U.K.; i) and j, visualization of cell nuclei using Hoechst 33258 DNA stain) also displayed weak CD93-positive staining at 48 h after 1 h MCAO (arrow in f), whereas at 72 h after 30 min various Iba1-positive cells were observed with intense CD93-staining (g–j). Most astrocytes do not colocalize with CD93-positive cells (b, c, guinea pig anti-GFAP [Advanced ImmunoChemical, Long Beach, CA]). In contrast to ischemic tissue of WT mice, staining of nonischemic mouse brain (n) revealed only a weak CD93-staining in endothelial cells. α, control staining using CD93-specific Ab in ischemic mouse brain tissue of CD93-knockout [CD93+−] mice at 48 h after 1 h MCAO. Original magnification ×40 (a–f, k–o) and ×100 (g–j). Scale bar, 50 μm. B, Confocal laser-scanning microscopy (Leica DM LFS, Leica, Solms, Germany) of ischemic brain tissue of WT mice at 48 h after 1 h MCAO using rat anti-CD93 Ab (a, d) in combination with anti-CD34 Ab (b), respectively. Anti-GFAP Ab (c), which demonstrates CD93 expression in CD34-expressing (endothelial) cells, but argues against a colocalization of CD93 in GFAP-expressing cells (f). Double-labeling studies were performed using rat anti-mouse CD93/C1qRp Ab (eBioscience) in combination with goat anti-rat secondary Ab (Alexa 488; Invitrogen), and goat anti-CD34 (Santa Cruz Biotechnology) in combination with Cy3 donkey anti-goat Ab (Molecular Probes). Original magnification ×200 (a–f).
Infarct volumes of CD93−/− mice and WT controls at 2 d of reperfusion after induction of transient focal cerebral ischemia

To test whether the CD93 protein is of functional relevance in the standard murine stroke model, infarct volumes of male and female CD93-deficient mice and matching WT mice after 1 h of MCAO and 48 h of reperfusion were compared. Although there was a tendency toward larger infarct volumes in the CD93 knockout mice when compared with WT mice, no significant difference was detected in the mixed-gender groups (given by mean ± SD; CD93−/−: 137.5 ± 31.0 mm³; CD93+/−: 131.7 ± 36.2 mm³; p = 0.194; median [25th percentile; 75th percentile]; CD93−/−: 130.7 [116.3; 154.8], CD93+/−: 116.7 [93.7; 142.7]), in the female mice (CD93−/−: 145.6 ± 32.7 mm³ versus CD93+/−: 127.3 ± 43.0 mm³; p = 0.316; median [25th percentile; 75th percentile]; CD93−/−: 145.1 [124.0; 162.0], CD93+/−: 124.0 [95.1; 143.3]), or in the male mice (CD93−/−: 125.3 ± 26.1 mm³ versus CD93+/−: 114.8 ± 26.6 mm³; p = 0.473; median [25th percentile; 75th percentile]; CD93−/−: 120.0 [110.1; 138.4], CD93+/−: 111.3 [94.1; 124.8]).

CD93−/− mice of both genders have an increased infarct volume when compared with WT mice at 72 h after induction of transient focal cerebral ischemia

Because of the involvement ascribed to CD93 in phagocytic processes and angiogenesis, we analyzed infarct volumes in a second experimental stroke model, which does not cause infarction (i.e., pannecrosis) and angiogenesis, we analyzed infarct volumes in a second experimental stroke model, which does not cause infarction (i.e., pannecrosis) but rather selective neuronal cell death with a profound neuro-inflammatory response (36, 37). In contrast to the findings after 60 min MCAO (Fig. 3), direct infarct volumes in CD93-deficient mice of mixed gender were significantly increased compared with WT mice at 72 h of reperfusion after 30 min MCAO (difference of direct infarct volume in pooled gender as given by mean ± SD: CD93−/−: 60.8 ± 52.2 mm³; CD93+/−: 23.9 ± 16.6 mm³; p = 0.022 (median [25th percentile; 75th percentile]; CD93−/−: 33.2 [18.3; 104.5], CD93+/−: 18.5 [12.7; 24.3]) (Fig. 4). The difference proved not to be gender-specific, but was only statistically significant in male mice and mixed gender. Direct infarct volumes in male mice as given by mean ± SD: 74.0 ± 54.0 mm³ / CD93−/− versus 27.5 ± 19.2 mm³ / CD93+/−; p = 0.020 (median [25th percentile; 75th percentile]; CD93−/−: 59.2 [27.1; 131.6], CD93+/−: 22.8 [13.1; 28.9]); direct infarct volumes in female mice: 31.7 ± 37.7 mm³ / CD93−/− versus 18.0 ± 4.7 mm³ / CD93+/− (median [25th percentile; 75th percentile]; CD93−/−: 13.7 [13.2; 20.3], CD93+/−: 14.0 [13.2; 20.3]). Differences of calculated indirect infarct volumes did not reach significance, but brain swelling (edema) significantly increases in CD93−/− mice when compared with WT mice (Fig. 4). Thus, brain swelling mainly contributes to increased direct infarct volumes (Fig. 4).

CD93 deficiency leads to increased inflammatory cell infiltration in the postischemic brain tissue

Because of the role attributed to CD93 protein in leukocyte extravasation, its endothelial expression, and its structural homology to

![FIGURE 3. Comparison of CD93-deficient and WT mouse (C57BL/6) at 48 h of reperfusion and 60 min of transient MCAO. Direct infarct volumes (A, D, G) and indirect infarct volumes (B, E, H), and volume of the edema (C, F, I) given as box-and-whisker plots at 48 h of reperfusion after induction of ischemia are visualized for both CD93−/− and WT mice for both genders (A–C) and male (D–F) and female (G–I) mice separately. Statistical analysis was performed using the Mann-Whitney U test. The indirect infarct volume was calculated as the volume of the contralateral hemisphere minus the noninfarcted volume of the ipsilateral hemisphere. There is no significant difference between infarct sizes of CD93−/− and WT mice, albeit there is a small but insignificant tendency in both genders toward larger direct infarct volumes and edema in the CD93−/− mice. CD93 knockout total, CD93-knockout mice of both genders (n = 15); WT total, WT mice of both genders (n = 18); CD93 knockout female, female CD93-deficient mice (n = 9); WT female, female WT mice (n = 10); CD93 knockout male, male CD93-deficient mice (n = 6); WT male, WT male mice (n = 8). In all box plots, the top of the box represents the 75th percentile, the bottom of the box represents the 25th percentile, and the line in the middle represents the 50th percentile. The whiskers (the lines that extend from the top and bottom of the box) represent the highest and lowest values that are not outliers or extreme values.](http://www.jimmunol.org/)

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other adhesion molecules (such as selectin) (30, 36), we asked whether CD93$^{-/-}$ mice have increased leukocyte infiltration into the postischemic brain. Indeed, CD11b staining, which stains invading macrophages, monocytes, and activated microglia, demonstrated a significant increase of CD11b-positive cells in the ischemic hemisphere of CD93$^{-/-}$ mice when compared with WT mice at 48 h (Fig. 5A) and 72 h (Fig. 5B) of reperfusion. The increased inflammatory cell counts in CD93-deficient mouse brains do not appear to be secondary to different infarct sizes, because CD11b-positive cell counts at 48 h differed significantly between the two strains (CD93$^{-/-}$ versus WT) despite similar infarct sizes (Fig. 5C).

**Gene expression analysis of genes differentially expressed between CD93$^{-/-}$ mice and WT mice**

To further decipher CD93-dependent pathways, we compared global expression profiles of ischemic (ipsilateral) and nonischemic (contralateral) hemispheres of CD93-deficient mice with expression profiles of WT mice; 46,374 probes were investigated at each condition by the use of Illumina Sentrix array technology (detailed information is accessible via GEO accession number GSE21002 or www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21002). After 72 h reperfusion and induction of transient focal cerebral ischemia for 30 min (induction for 60 min and 48 h of reperfusion, respectively), transcripts extracted from the ischemic hemisphere of CD93-deficient mice were compared with those derived from the ischemic hemisphere of WT control mice. As shown in Table I, gene expression analysis identified several genes that were differentially regulated depending on the presence or absence of the CD93 gene. Forty-two genes were found to be induced ≥1.5-fold in untreated CD93-deficient mice compared with untreated WT mice, and only nine genes were found to be induced in untreated CD93-deficient animals at least 2-fold when compared with untreated WT mice (Table I). At 48 h of reperfusion after induction of cerebral ischemia for 60 min, 18 genes were differentially expressed at least 2-fold, and 85 genes at least 1.5-fold between the ipsilateral (ischemic) hemispheres of CD93-deficient and WT mice. Comparison of genes in CD93$^{-/-}$ mice with WT mice in the ischemic hemisphere at 72 h reperfusion after induction of cerebral ischemia for 30 min revealed 58 with at least 2-fold induction (data not shown).

In contrast, comparison of the number of genes induced in the ischemic hemisphere at 48 h after 60 min MCAO versus the nonischemic (contralateral) hemisphere revealed 289 genes induced at least 2-fold in CD93$^{-/-}$ mice, 211 of which are also induced in WT mice (Fig. 6). Counting the number of genes induced at least 2-fold in the ischemic hemisphere at 48 h after 1 h MCAO, when compared with the expression level in the non-treated hemisphere, revealed 439 genes in CD93-deficient mice and 360 genes in WT mice, of which 309 are induced in both strains (Fig. 6). **CD93 deficiency leads to induction of CCL21 (Exodus2)-mRNA expression in untreated knockout mice and in postischemic mice**

Interestingly, the mRNA of a single gene, Exodus-2 (also named CCL21), is represented in three different probe sets, was found to be upregulated almost 10-fold in untreated CD93-deficient
mice compared with WT control mice. This dramatic difference in CCL21 expression in brain tissue of naive CD93 knockout mice was also found after MCAO. To evaluate results obtained with microarrays, expression of CCL21 mRNA was confirmed by real-time PCR in separate experiments. Quantitative PCR confirmed expression data derived from Illumina microarrays: CCL21 mRNA is highly induced in the CD93−/− brain tissue of untreated mice and in postischemic brain tissue at all time points after induction of cerebral ischemia, compared with WT brain tissue (data not shown). However, in comparison with microarray expression data, PCR results demonstrate an even higher induction ratio of CCL21 mRNA expression in the ischemic hemisphere of CD93−/− mice when compared with WT animals; an almost 100-fold increase of the CCL21 expression in CD93 knockout mice was detected by PCR when compared with WT mice at 48 h after 1 h MCAO. Moreover, in the normal brain of male untreated CD93-deficient mice, CCL21 mRNA was found to be induced 127-fold compared with the normal WT expression in normal WT brains. As observed previously by other groups, mRNA quantification by PCR revealed higher induction ratios than those measured by hybridization technologies (e.g., 127-fold versus 8-fold induction in untreated animals). In contrast to the dramatic induction of CCL21 expression in knockout animals, we observed only a maximum 2.2-fold increase of the CCL21-mRNA expression in the ischemic hemisphere at 72 h after 30 min MCAO as measured by PCR and compared with the nonischemic hemisphere (data not shown). Furthermore, to detect a potential compensatory regulation of the CCL21 receptors (CXCR3 and CCR7) and any other chemokine signaling system that could contribute to neuroinflammation (CCL2/CCR2), expression of CCL21-associated genes (e.g., CD30 and its ligand) (39), and CXCR3, CCR7, CCL2 and its receptor CCR2 were studied. This revealed no significant differential expression between CD93−/− and WT mice for either CCL2, its receptor CCR2, or the CCL21-receptors CCR7 and CXCR3 (Table II).

Exodus2 (CCL21) protein is induced in cerebral brain tissue of untreated and in postischemic brain tissue of CD93-deficient mice

To analyze whether induction of CCL21-transcripts in CD93-deficient mice provokes a similar induction of CCL21-protein in the mutant (CD93−/−) mice, brain and spleen tissue were stained with CCL21-specific Ab. Specific staining was observed in the spleens of WT mice (Fig. 7A), but an abundant and much more intense CCL21-positive staining in the spleens of CD93-knockout mice (Fig. 7D). In normal brain tissue, weak CCL21 staining was observed in individual cells of CD93-deficient mice, but a CCL21-specific signal was almost absent in the brain tissue of WT mice (data not shown). In contrast to the weak cerebral staining pattern observed in untreated mice, CCL21-positive cell number increased after induction of cerebral ischemia for 60 min and after 48 h of reperfusion in WT mice as well as in CD93-deficient animals (Fig. 7). However, CCL21-positive cells, which were mainly located at the infarct border zone in both animal strains, revealed a highly intense CCL21-specific staining in CD93 knockout mice, but only a low- to medium-intensity CCL21-signal in WT mice. Moreover, CCL21-positive cells were more abundant in CD93 knockout mice than in WT mice.

Discussion

By using a recent Affymetrix microarray platform, we identified CD93 as a significantly induced gene whose role in cerebral ischemia was unknown until now. Both CD93 mRNA and CD93 protein were significantly induced after cerebral ischemia. CD93 was further shown to be neuroprotective in specific conditions: CD93-deficient mice displayed significantly larger infarct volumes in an experimental stroke model with a more selected neuronal death in combination with a profound neuroinflammatory response (37). Interestingly, there was only a minor, nonsignificant tendency toward increased infarct volumes in CD93-deficient mice when...
compared with WT mice in an alternative, more severe stroke model, which causes extensive pan-necrosis.

To unravel potential CD93-mediated neuroprotective mechanisms, we performed global analysis of expression patterns of CD93-deficient and WT mice at different time points before and after cerebral ischemia. Despite the finding that several genes were differentially expressed, only one single gene—\( \text{CCL21} \) (\( \text{Exodus-2} \))—was found to be highly induced in CD93 knockout mice under all conditions when compared with control animals. Thus, we speculate a central role of this chemokine in CD93-mediated neuroprotection. Cerebral ischemia leads to a significant inflammatory immune response. The resulting neuroinflammation is thought to exacerbate postischemic brain injury during the initial phase of reperfusion after cerebral vessel occlusion, although it might sustain restoration of tissue homoeostasis at later time points. Ongoing repair mechanisms require the phagocytosis of large amounts of dead cells. The protein CD93 was shown recently to contribute to the in vivo clearance of dying cells (18). However, no information on CD93 function in cerebral ischemia has been available until. Recently, van der Net et al. (40) confirmed

### Table 1. mRNA expression of genes most significantly upregulated in brain tissue of CD93-deficient mice when compared with WT mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>Illumina Number</th>
<th>Ratio U</th>
<th>Ratio 48 h</th>
<th>Ratio 72 h</th>
</tr>
</thead>
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<tr>
<td>CCL21B</td>
<td>sc0065956.1_31-S</td>
<td>9.659</td>
<td>14.286</td>
<td>12.251</td>
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<td>LOC100041504</td>
<td>sc0018829.1_65-S</td>
<td>8.040</td>
<td>9.744</td>
<td>10.609</td>
</tr>
<tr>
<td>LOC100041516</td>
<td>sc0020298.1_98-S</td>
<td>7.875</td>
<td>10.414</td>
<td>10.536</td>
</tr>
<tr>
<td>RPL23</td>
<td>sc39638.4.1_92-S</td>
<td>4.264</td>
<td>1.260</td>
<td>0.797</td>
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<tr>
<td>PLA2G4E</td>
<td>sc118791.2_626-S</td>
<td>3.343</td>
<td>3.905</td>
<td>3.677</td>
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<tr>
<td>STK4</td>
<td>sc119945.14_178-S</td>
<td>2.955</td>
<td>3.332</td>
<td>3.875</td>
</tr>
<tr>
<td>MT-ND5</td>
<td>sc0017721.1_131-S</td>
<td>2.274</td>
<td>1.222</td>
<td>1.744</td>
</tr>
<tr>
<td>PPIH16B</td>
<td>sc11990.13_167-S</td>
<td>2.160</td>
<td>1.791</td>
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</tr>
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<td>ZCH313</td>
<td>sc067302.8_2-S</td>
<td>2.008</td>
<td>1.008</td>
<td>1.383</td>
</tr>
<tr>
<td>CNTFR</td>
<td>sc0002829.1_12-S</td>
<td>1.993</td>
<td>2.370</td>
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<tr>
<td>CASC4</td>
<td>sc0003123.1_3-S</td>
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<td>2310001H12RIK</td>
<td>GI_21704131-S</td>
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<td>INMT</td>
<td>sc28971.3_6-S</td>
<td>1.821</td>
<td>1.464</td>
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<td>MTF2</td>
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<td>NR1D2</td>
<td>sc0035318.1_48-S</td>
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<td>UBR1</td>
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<td>PRKPS18</td>
<td>sc019144.7_7-S</td>
<td>1.675</td>
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<tr>
<td>LOC100048622</td>
<td>sc056398.1_30-S</td>
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<td>sc117622.1_190.1-S</td>
<td>1.649</td>
<td>1.097</td>
<td>1.393</td>
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<td>EPB4.1LI</td>
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<td>D19ERTD721E</td>
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<tr>
<td>ZFP106</td>
<td>sc0003002.1_194-S</td>
<td>1.626</td>
<td>0.950</td>
<td>0.976</td>
</tr>
<tr>
<td>TPH3</td>
<td>sc37491.11_193-S</td>
<td>1.624</td>
<td>0.751</td>
<td>1.910</td>
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<td>LRRC59</td>
<td>sc39705.9.188.7-S</td>
<td>1.616</td>
<td>1.054</td>
<td>1.308</td>
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<tr>
<td>B93009701RIK</td>
<td>sc093009701PX016162N21</td>
<td>AK047604</td>
<td>3109-S</td>
<td>1.613</td>
</tr>
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<td>LOC100047369</td>
<td>sc000093.1_57-S</td>
<td>1.605</td>
<td>1.048</td>
<td>1.039</td>
</tr>
</tbody>
</table>

Shown are the differentially regulated genes, ranked by their degree of induction in brain tissue derived from CD93-knockout mice when compared with WT brain tissue. Second column: gene identifiers according to the Illumina denotation (Illumina, San Diego, CA). Third column: relative expression, given by the expression level of each gene in CD93-knockout normal male brain tissue as determined by IlluminaBeadChip technology, divided by the expression level in normal (untreated) WT brain tissue. Fourth column (ratio 48 h): relative expression in CD93-deficient brain tissue at 48 h after 1 h MCAO when compared with the expression level in WT brain tissue at 48 h after 1 h MCAO. Last column (ratio 72 h): relative expression in postischemic hemispheres of CD93 knockout mice at 72 h after 30 min MCAO when compared with WT mice. mRNA was pooled from three male mice per group.

MT, metallothionein.

FIGURE 6. CD93 deficiency leads to altered gene expression patterns. Venn diagrams demonstrating the number of genes induced at least 2-fold when measured in the ischemic brain hemisphere of WT or CD93\(^{-/-}\) mice after 1 h MCAO and 48 h reperfusion (A, B) or 30 min MCAO and 72 h reperfusion (C, D), respectively, when compared with the nonischemic contralateral hemisphere (A, C) or the healthy hemisphere of untreated mice (B, D) using Illumina Sentrix arrays. Ratios were calculated using expression level in the ischemic (ipsilateral) hemisphere divided by the expression level in the nonischemic (contralateral) hemisphere (A, C), respectively, in the same hemisphere of untreated mice (B, D). Only male mice were used; \( n = 3 \) for each condition.
Table II. Expression of selected CCL21-related genes in CD93-deficient mice compared with WT mice, respectively, in the contralateral hemisphere

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ratio U CD93/U versus WT/U</th>
<th>Ratio 48 h CD93/48h versus WT/48h</th>
<th>Ratio 72 h CD93/72h versus WT/72h</th>
<th>CD93 Ipsi versus Contra/48h</th>
<th>CD93 Ipsi versus Contra/72 h</th>
<th>WT Ipsi versus Contra/48h</th>
<th>WT Ipsi versus Contra/72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2</td>
<td>0.91</td>
<td>1.23</td>
<td>1.23</td>
<td>1.85</td>
<td>1.69</td>
<td>1.82</td>
<td>1.31</td>
</tr>
<tr>
<td>CXCR3</td>
<td>1.17</td>
<td>0.93</td>
<td>0.85</td>
<td>1.06</td>
<td>1.16</td>
<td>1.13</td>
<td>1.15</td>
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<tr>
<td>CCR7</td>
<td>1.05</td>
<td>1.08</td>
<td>1.01</td>
<td>1.00</td>
<td>1.13</td>
<td>0.76</td>
<td>1.14</td>
</tr>
<tr>
<td>CCR2</td>
<td>0.89</td>
<td>1.02</td>
<td>0.82</td>
<td>0.86</td>
<td>0.78</td>
<td>0.96</td>
<td>0.99</td>
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<tr>
<td>CXCL13</td>
<td>0.91</td>
<td>1.13</td>
<td>1.00</td>
<td>1.25</td>
<td>1.06</td>
<td>0.96</td>
<td>1.05</td>
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<tr>
<td>CD30L</td>
<td>0.92</td>
<td>1.07</td>
<td>1.04</td>
<td>1.16</td>
<td>1.06</td>
<td>1.31</td>
<td>0.95</td>
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<tr>
<td>CD30</td>
<td>0.95</td>
<td>1.13</td>
<td>1.03</td>
<td>1.16</td>
<td>1.04</td>
<td>0.95</td>
<td>1.27</td>
</tr>
<tr>
<td>Pdpn</td>
<td>1.02</td>
<td>1.02</td>
<td>1.39</td>
<td>2.91</td>
<td>3.00</td>
<td>2.94</td>
<td>2.36</td>
</tr>
<tr>
<td>LTB</td>
<td>1.01</td>
<td>1.10</td>
<td>1.05</td>
<td>0.85</td>
<td>0.99</td>
<td>0.92</td>
<td>0.85</td>
</tr>
<tr>
<td>LTBR</td>
<td>0.97</td>
<td>0.97</td>
<td>1.27</td>
<td>1.43</td>
<td>1.43</td>
<td>1.43</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Regulation of the CCL21-related genes as determined by Illumina Sentrix arrays. Values are given as relative expression levels, determined as the ratio of the absolute signal intensity in the ipsilateral (column 2; non-treated; columns 3–6; ischemic) CD93-knockout hemisphere divided by the value obtained in the WT hemisphere (columns 2–4) in the two different infarct models (columns 3, 5, 7: 1 h MCAO and 48 h reperfusion; columns 4, 6, 8: 30 min MCAO and 72 h reperfusion). Columns 5–8 demonstrate the values of the ipsilateral (ischemic) hemisphere divided by the value derived from the contralateral (nonischemic) hemisphere (columns 5–8) of the same mice (CD93-knockout mice: columns 5, 6; WT mice: columns 7, 8) as reference. mRNA was pooled from three mice per group.

Contra, contralateral; Ipsi, ipsilateral; LTB, lymphotoxin-β; LTBR, lymphotoxin-β receptor; Pdpn, podoplanin; U, untreated.

CD93 deficiency leads to upregulation of CCL21/Exodus-2 protein. CCL21 protein expression as revealed by CCL21-specific Ab staining (R&D Systems) in spleens (A–F) of WT mice (A–C) and CD93−/− mice (D–F), and expression in the peri-infarct region of the ischemic brain hemisphere (G–R) of WT mice (G–I, M–O) and CD93-deficient mice (J–L, P–R). Samples were taken at 48 h of reperfusion after 60 min MCAO. First column, representative images of the tissue stained with anti-CCL21 Ab; second column, visualization of nuclei using Hoechst 33258 DNA stain; third column, overlay of columns one and two. Original magnification ×10 (A–F), ×20 (G–L), and ×100 (M–R). Scale bar, 50 μm.

FIGURE 7. CD93 deficiency leads to upregulation of CCL21/Exodus-2 protein. CCL21 protein expression as revealed by CCL21-specific Ab staining (R&D Systems) in spleens (A–F) of WT mice (A–C) and CD93−/− mice (D–F), and expression in the peri-infarct region of the ischemic brain hemisphere (G–R) of WT mice (G–I, M–O) and CD93-deficient mice (J–L, P–R). Samples were taken at 48 h of reperfusion after 60 min MCAO. First column, representative images of the tissue stained with anti-CCL21 Ab; second column, visualization of nuclei using Hoechst 33258 DNA stain; third column, overlay of columns one and two. Original magnification ×10 (A–F), ×20 (G–L), and ×100 (M–R). Scale bar, 50 μm.

a genetic link between CD93 gene polymorphisms and myocardial infarction in a human risk population.

We found the CD93 protein to be highly induced after focal ischemia in endothelial cells and, to a lesser extend, on selected invading macrophages/microglia, which is in accordance with a prevailing endothelial expression of CD93 and its hypothetical role in the immune response and remodeling of the vascular tree (18, 29, 28, 41, 42).

The CD93 molecule is a heavily O-glycosylated type I transmembrane protein consisting of a carbohydrate-recognition domain (called CLECT-thrombomodulin-like domain for C-type lectin-like domain [CTLD] of the type found in thrombomodulin and endothosialin) (41), five epidermal growth factor-like domains, a mucin domain, a single transmembrane domain, a PDZ-binding domain (44), and a short intracellular domain (18, 35). The molecular structure of CD93 is reminiscent of thrombomodulin, a receptor for thrombin that is recognized as a major natural anticoagulant mainly expressed on endothelial cells and platelets (31, 36). CD93 and thrombomodulin seem to be derived from a common ancestor (31). Thrombomodulin serves as a physiologic anticoagulant abundantly and has been shown to regulate endothelial function in inflammation. Furthermore, thrombomodulin polymorphisms have been shown to be associated with an increased risk of early onset ischemic stroke (45). Whereas homozygous thrombomodulin knockout mice are not viable, mice lacking an N-terminal part of thrombomodulin are viable but show a range of abnormalities resulting from a latent inflammatory state (46).

Despite its membrane-bound form, CD93 might be shed from the cell surface upon cellular activation (26, 47, 48). A similar ectodomain release has been reported for t-selectin and CD44, as well as for TNF-α and other cytokines (35). McGreal et al. (22) demonstrated specific binding of a soluble recombinant CD93-Fc chimeraic protein to vascular endothelial cells in sections of inflamed human tonsil, indicating the presence of a CD93 ligand at this site (22). Considering these data, we cannot exclude that part of the endothelial CD93-positive signal might derive from soluble CD93 protein rather than locally expressed CD93 membrane protein. It must still be clarified whether CD93 is cleaved in cerebral ischemia, soluble CD93 might have clinical application as an anti-adhesion molecule to dampen local inflammation (36), or pharmacologic modulation of CD93 expression (49) might improve postischemic outcome must still be clarified.

The exact molecular function of CD93 has not yet been identified. Our finding of a robust induction of chemokine CCL21-expression in CD93-deficient mice could explain how CD93 can protect the brain after cerebral ischemia; it could also explain other CD93-related observations. It was recently demonstrated that CD93 is important for the maintenance of plasma cells in the bone marrow (50). It was further shown that CD93-deficient mice were able to
respond normally with serum IgG to immunization, but showed a statistically significant decrease in IgG levels at later time points (51). Accordingly, one can speculate that these observations may also be influenced by the differentially regulated expression of CCL21/Exodus-2 in CD93−/− mice.

Chemoattractant signals, such as CCL21, were thought to control leukocyte navigation by regulating migration from the blood into tissues and subsequent localization of cells within the tissue microenvironment (51). CCL21 was postulated to be a part of the neuron-microglia signaling system in cerebral ischemia (5, 7). In contrast to most chemokines found in brain tissue, which are expressed by glial cells and by infiltrating leukocytes, CCL21 was found to be expressed exclusively on neurons and not on glial cells (7). CCL21 was shown to be involved in the signaling between endangered neurons and microglial cells (5, 52). Damaged neurons rapidly induce and release CCL21 in vitro and in vivo, and they activate microglia via the chemokine receptor CXCR3 (5, 7, 52), whereas basically all T cells in the cerebrospinal fluid of healthy individuals express CCR7, the second receptor for the chemokine CCL21 (53).

CCL21 and its receptor in the ischemic CNS, CXCR3, were previously found to be induced in ischemic mouse brain (54–56). CXCR3-deficiency was associated with reduced microglial activity and reduced loss of secondary neurons in hippocampal formation in an entorhinal cortex lesion model (57). Furthermore, ectopic CCL21 expression in oligodendrocytes induced a massive brain inflammation that killed the animals within 3 d after onset of the expression (55).

Because CD93-deficiency induces a dramatic increase of CCL21 expression in the brain (but also in other organs, such as the spleen), we hypothesize that the upregulation of this chemokine interferes with leukocyte homing into the brain and microglia activation, thereby promoting posts ischemic inflammation. This hypothesis fits well with our observation of lower leukocyte counts in postischemic brain tissue of WT animals than in CD93−/− animals (Fig. 5). The observation that leukocyte infiltration between CD93-deficient and WT mice significantly differs in a model that does not have significant differences in lesion size argues against a simple secondary effect because of different infarct volumes. Because other chemokines are not induced, or are only marginally induced (e.g., CCL2), in CD93-deficient mice, we postulate a major CCL21-mediated effect on leukocyte extravasation and microglia activation in CD93−/− mice (Table II).

This effect resembles the way in which other receptors act on the initiation of neuroinflammation: following stroke receptors, such as the TLRs, upregulate the expression of adhesion molecules that facilitate the infiltration of lymphocytes into the ischemic brain region, thereby contributing to neuronal damage (58). However, the exact contribution of other signaling pathways (e.g., inflammation mediated by other chemokines, IL-1β-, or TLR-signaling) (6, 51) in CD93-mediated neuroprotection remains to be determined.

Furthermore, we cannot exclude additional effects by altered signaling networks in the transgenic mice. Our experimental design does not allow a causal relationship between CCL21 induction and leukocyte invasion in CD93-deficient mice. Future studies need to determine the exact signaling cascade by which CD93 inhibits the CCL21-expression and to what extend this pathway and those linked to the other differentially expressed genes contribute to the neuroprotection observed in male CD93-deficient mice. The future use of conditional transgenic mice or double-knockout mice should allow us to dissect these pathways in more detail.

We demonstrate a significant upregulation of CD93 mRNA and protein, as well as a significant neuroprotective effect of this gene in specific conditions after cerebral ischemia at 3 d of reperfusion. Besides its induction, we describe a neuroprotective effect of CD93 expression and link CD93 regulation in ischemic brain tissue to altered chemokine CCL21 expression and, therefore, to the inflammatory response.

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Disclosures

The authors have no financial conflicts of interest.

References
