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De novo expression of the hemoglobin scavenger receptor CD163 by activated microglia is not associated with hemorrhages in human brain lesions

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Summary. The main function of CD163 (hemoglobin scavenger receptor) is to bind the hemoglobin-haptoglobin complex, thereby mediating extravasal hemolysis. However, CD163 also has an antiinflammatory function. After CD163-mediated endocytosis, hemoglobin is catabolized further by hemeoxygenase 1 (HO-1). Previously, we found expression of HO-1 to be restricted to microglia/ macrophages at sites of hemorrhages in human traumatic and ischemic brain lesions. We now investigated if CD163 expression is also correlated with hemorrhages in brain lesions. Methods. Autopsy brain tissue from 44 cases with hemorrhagic brain lesions (32 traumatic brain injuries/TBI, 12 intracerebral bleedings/ICB), 56 non-hemorrhagic brain lesions (30 ischemias, 26 hypoxias) and 6 control brains were investigated. The post injury survival times ranged from a few minutes to 60 months. Results. In controls, single perivascular monocytes expressed CD163, but only single CD163+ microglia were found in 3/6 cases. CD163+ cells in the parenchyma (activated microglia/macrophages) increased significantly within 24 hours after trauma and ischemia and within 1-7 days following ICB or hypoxia. Overall, significantly lower and higher levels of parenchymal CD163+ cells occurred in hypoxia and ischemia, respectively. Perivascular CD163+ cells also increased significantly in all pathological conditions. In areas remote from circumscribed brain lesions (TBI, ICB, ischemia), significant changes were only found in ICB and ischemia. Conclusions. De novo expression of CD163 by activated microglia/macrophages and CD163+ infiltrating monocytes are neither restricted to nor predominant in hemorrhagic brain lesions. Thus, the antiinflammatory function of CD163 probably predominates, both in hemorrhagic and non-hemorrhagic brain lesions and points to possible immunomodulatory treatment strategies targeting CD163

Key words: Microglia, Ischemia, Hypoxia, Trauma, Hemorrhage

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Introduction

Macrophages constitute the major cellular compartment for hemoglobin (Hb) degradation and subsequent recycling of heme-iron to erythropoesis (Knutson and Wessling-Resnick, 2003). Two major pathways mediate Hb uptake by macrophages. In the first, aged erythrocytes are phagozytosed and degraded by macrophages within the liver, spleen and bone marrow, whereas the second pathway represents receptor-dependent endocytosis of free Hb, which is released during intravascular hemolysis or as a consequence of red blood cell membrane disruption during hemorrhages and erythrophagocytosis (Schaer et al., 2007b).

CD163 (HbSR, alias M130) is a member of the scavenger receptor cysteine-rich family class B and is specific for cells of the monocytic lineage: monocytes and macrophages including microglia. Similarly to expression of ED2 in rat brains, CD163 expression in normal human brains has been reported in perivascular monocytic cells, but not in resting microglia (Van Den Heuvel et al., 1999; Fabriek et al., 2005; Kim et al., 2006). CD163 is a transmembrane protein with a short cytoplasmic tail and functions as an endocytic receptor for hemoglobin–haptoglobin (Hb–Hp) complexes. As

such, CD163 comprises the only known pathway for uptake of cell-free Hb, as well as for Hb bound to the plasma protein haptoglobin (Kristiansen et al., 2001; Schaer et al., 2006b, 2007a). The high capacity of the CD163 pathway for Hb uptake is related to the receptor's capacity to undergo ligand-independent (constitutive) endocytosis and subsequent recycling to the cell surface (Schaer et al., 2006a). CD163 mediated endocytosis of Hb reveals a response in macrophages, which is dominated by induction of the heme breakdown enzyme heme oxygenase-1 (HO-1). HO-1 catalyzes the rate-limiting step in heme degradation, yielding biliverdin, which is subsequently reduced enzymatically to bilirubin (Tenhunen et al., 1969; Maines et al., 1993; Suttner and Dennery, 1999). Heme-iron released during these steps is then bound to the storage protein ferritin or exported to plasma via the iron exporter ferroportin.

Furthermore, there is evidence to support an immunomodulatory (anti-inflammatory) role of CD163 (Philippidis et al., 2004; Abraham and Drummond, 2006). In particular, the cross-linking of CD163, either by antibodies (Van Den Heuvel et al., 1999) or Hb-Hp complexes (Kristiansen et al., 2001) results in a potent cytokine response. Accordingly, enhanced proteolytic shedding of CD163 has been associated with decreased pro-inflammatory cytokine (Il6, IL12p40) plasma levels, suggesting a possible link of CD163 to cytokine production (Fabriek et al., 2007).

In a previous study on human traumatic and ischemic brain lesions, we found HO-1 expression to be restricted to micoglia/macrophages in hemorrhagic areas (Beschorner et al., 2000). CD163-mediated endocytosis is required for further degradation of Hb by HO-1. We hypothesized that an upregulation of CD163 might also be correlated to bleedings and therefore we investigated the expression of CD163 cases with hemorrhagic (trauma/TBI, intracerebral bleeding/ICB) and with non-hemorrhagic (ischemia, hypoxia) brain lesions.

Materials and methods

Cases

Brain slices from patients with clinical history and histologically confirmed diagnosis of traumatic brain injury/TBI (n=32), intracerebral bleeding/ICB (n=12), focal cerebral ischemia (n=30) and hypoxic encephalopathy (n=26) were investigated. Cases with TBI and ischemia were obtained from updated brain banks reported previously (Beschorner et al., 2007a,b). Cases with ICB and hypoxic encephalopathy were selected from the archives of the Institute for Brain Research, Tuebingen and from the Department of Neuropathology, Zurich. Post-mortem delay to autopsy was documented in 86/106 (81%) of the cases and ranged from 5-228 hours (median 39.5 h). As a standard procedure, the entire brain was fixed in 4.5% buffered formalin (pH 7) for 2-3 weeks. Thereafter, the brain was cut in coronal sections and tissue samples from different brain regions were selected for paraffin embedding.

For statistical analysis, cases were clustered into four groups with increasing survival times after onset of brain damage (group 0, controls; group 1, survival time <24hrs; group 2, 1-7 days; group 3, 1-9 weeks; group 4, >9 weeks; compare figures 2 and 3) characterized by different cellular reactions in traumatic and ischemic brain lesions with disruption of the blood-brain-barrier (Blumbergs et al., 2008; Ferrer et al., 2008): up to 24 h (no/minimal cellular infiltration), 1-7 days (granulocytic infiltration and microglial activation), 1-9 weeks (increasing numbers of macrophages in the lesion and reactive astrocytes in adjacent regions), more than 9 weeks (ongoing resorption by macrophages well circumscribed to adjacent glial scar formation).

Corresponding areas (frontal cortex, frontal white matter, pyramidal cell layer of the hippocampus and hippocampal white matter) from six unaffected brains from an established normal brain bank served as controls (Mittelbronn et al., 2001). Clinical data of all cases investigated are summarized in Tables 1 and 2, respectively.

In cases with focal or circumscribed brain lesions (TBI, ischemia, ICB) samples from the lesion with adjacent brain tissue were routinely stained and analyzed by light-microscopy. Hematoxylin-Eosin (HE) staining was used for evaluation of the typical histological features defined as standard indicators of lesion age. Additionally, tissue samples from areas remote to the lesion (white matter area, preferably from the contralateral hemisphere) were investigated if available. Following trauma, ICB or ischemia, in some older lesions (group 4) an evaluation of parenchymal cells in the lesion was no longer possible due to pseudocystic stage.

According to known differences in microglial cell density in gray and white matter (Mittelbronn et al., 2001) the lesion and adjacent area were preferentially evaluated in the white matter (trauma, ICB, ischemia). Thus, the adjacent area in TBI, ischemia and ICB was defined as the white matter region neighboring the hemorrhagic area (trauma, ICB) or the pan-cellular necrosis (ischemia) with edema and developing reactive astrogliosis, respectively. In hypoxic brain samples, the lesion was defined as the gray matter, namely the frontal cortex and sector CA1-2 of the hippocampus, respectively. The white matter neighboring these gray matter (lesional) areas was defined as the 'adjacent area'. Due to the global brain damage in hypoxia no remote area was available for evaluation in these cases.

In all control cases and in all cases with hypoxic brain damage tissue samples from the frontal cortex and the adjacent subcortical white matter, as well as from the sector CA1-2 of the hippocampus and the adjacent white matter were available. Likewise, in cases with trauma, ICB and ischemic stroke, tissue samples from the lesion (white matter) and the adjacent white matter were available in all cases. Suitable tissue samples from remote areas (white matter) were available in 32/32

cases with TBI, 11/12 cases with ICB and 20/30 cases with ischemic stroke.

In all cases histopathological stages of the brain lesion corresponded to clinical data.

This study was carried out according to the ethical guidelines of the University of Tuebingen, Germany.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections using an automated immunohistochemistry slide staining system (BenchMark®, Ventana Medical Systems, Tucson, Az, USA). Mouse monoclonal CD163 antibody (clone EDHu-1; IgG1; AbD Serotec, Raleigh, NC 27604, USA) was applied at a dilution of 1:1000. The automated standard protocol is based on an indirect biotin-avidin system and uses a universal biotinylated immunoglobulin secondary antibody and diaminobenzidine substrate. The sections were finally counterstained with Mayer's haemalaun. Negative controls consisted of sections incubated in the absence of the primary antibody and in isotype controls (IgG1, Dako-Cytomation, Glostrup, Denmark).

In order to investigate if the CD163+ cells may in part be astrocytes we performed double-labellings. CD163 expression was first visualized as described above. After antigen retrieval, a monoclonal antibody against the glial fibrillary acidic protein (GFAP, Novocastra, dilution 1:2000) was applied using the alkaline phosphatase method and Fast-Red (Enhanced V-Red Detection Kit, Ventana Medical Systems, #760-031), yielding a red reaction product. Finally, the sections were counterstained with Mayer's haemalaun.

Evaluation

Evaluation of CD163 in cases with TBI, ICB or ischemia was done separately within the lesion, in the perilesional tissue directly adjacent to the lesion and (if available) in areas remote from the lesion. All sections were evaluated independently by two observers (KH, RB) blinded to clinical history and diagnosis. To ensure evaluation of the same region by both observers, the appropriate areas were first selected in consensus using a teaching microscope, framed on the HE stained sections and then transferred to the immunostained parallel tissue sections.

In all samples and all regions investigated numbers of CD163+ cells in the brain parenchyma ('parenchymal cells') were counted in ten high power fields (HPF; x400 magnification with an eyepiece grid representing 0.0625 mm^2 each). In the same regions numbers of immunostained 'perivascular cells' (i.e. cells attached to the outer vessel wall and cells in perivascular spaces) were counted in perivascular spaces of blood vessels. In the hippocampal pyramidal cell layer, cell counting was always started in sector CA1. If the evaluable tissue from sector CA1 met less than ten HPF, cell counting was continued in sector CA2.

Inter-observer variability was managed as follows: if cell counts differed less than 5% the mean cell count was calculated and used for statistical analysis; if cell counts differed more than 5% the sections were re-evaluated using a teaching microscope and positive cells were counted in consensus.

Statistical analysis

Analysis of the number of CD163+ cells was done for parenchymal cells as well as for perivascular cells for each group of cases with different survival times (group 1, <24h; group 2, 1-7days; group 3, 1–9 weeks; group 4, >9weeks) and for each investigated region (lesion, adjacent, remote) in comparison to data from corresponding areas of controls. Since the scores nested into the respective groups showed unequal variance, the overall difference between groups was analyzed after Box-Cox-Transformation. ANOVA followed by Tukey-Kramer tests was used to determine differences between each group and the respective control group (positive q-values represent significant differences). Data were then transformed back for graphical illustration as Box plots. JMP software (version 6; SAS Institute Inc., Cary, North

Table 1. Clinical data on controls and cases with hypoxic brain damage.

case#	Pathology	Age (yrs.)	Sex	Survival time (post injury)	Group
1	control	39	m	-	0
2	control	25	f	-	0
3	control	36	f	-	0
4	control	54	m	-	0
5	control	72	m	-	0
6	control	76	f	-	0
7	hypoxia	65	m	1 h	1
8	hypoxia	72	f	1 h	1
9	hypoxia	32	m	6 h	1
10	hypoxia	31	f	8 h	1
11	hypoxia	41	m	8 h	1
12	hypoxia	57	m	12 h	1
13	hypoxia	78	f	14 h	1
14	hypoxia	52	m	21 h	1
15	hypoxia	76	m	24 h	1
16	hypoxia	66	f	1.0 d	2
17	hypoxia	73	f	1.1 d	2
18	hypoxia	67	f	1.9 d	2
19	hypoxia	32	f	2 d	2
20	hypoxia	76	m	2 d	2
21	hypoxia	57	m	2 d	2
22	hypoxia	42	f	3.7 d	2
23	hypoxia	84	f	4 d	2
24	hypoxia	35	m	4 d	2
25	hypoxia	63	m	4.6 d	2
26	hypoxia	59	m	5 d	2
27	hypoxia	71	m	5 d	2
28	hypoxia	38	f	6 d	2
29	hypoxia	60	f	7 d	2
30	hypoxia	74	m	8 d	3
31	hypoxia	14	m	8 d	3
32	hypoxia	83	f	15 d	3

Carolina, USA) was used to obtain the necessary data.

Furthermore, we analyzed whether the increase in CD163+ cells differed between the four investigated pathologies (trauma, ICB, ischemia, hypoxia). Therefore, numbers of CD163+ parenchymal and perivascular cells in the different survival groups (group 1-4) and in the different investigated areas (lesion, adjacent, remote) were compared among the four investigated underlying pathologies using the same statistical approach as described above. For comparison of numbers of CD163+ cells in localized brain lesions (TBI, ICB, ischemia) and in hypoxic brains the following points had to be considered. First, the distribution of microglial cells is heterogeneous in the human CNS (Mittelbronn et al., 2001). Second, in cases with a localized brain lesion (TBI, ICB, ischemia) tissue samples were collected from cerebral white matter areas (lesion, adjacent area) whereas in cases with hypoxia the lesional area was defined as a gray matter region and the adjacent area was defined as the neighboring white matter. Therefore, in cases with hypoxia only data obtained from the frontal lobe (not hippocampus) were compared to data from cases with TBI, ICB or ischemia. Graphic illustrations of data are show as boxplots indicating median, upper (75%) and lower (25%) quartiles and upper (97.5%) and lower (2.5%) whisker (Figs. 2, 3).

Results

CD163 in control brains

In normal human brains, both in gray and white matter regions, CD163+ spindle-shaped cells were constantly found attached to the wall of small and medium-sized vessels, possibly reflecting pericytes (Fig 1). Mean numbers of perivascular CD163+ cells surrounding 10 blood vessels ranged between 3.8 (confidence interval/CI 1.2-6.4) in hippocampal sector CA1-2, 3.9 (CI 2.8-5.1) in frontal white matter, 4.0 (CI 1.3-6.7) in hippocampal white matter and 5.5 (CI 2.2-8.8) in the frontal cortex, respectively. In the brain

Table 2. Clinical data on cases with circumscribed brain lesions (TBI, ICB, ischemia).

case#	Pathology	Age (yrs.)	Sex	Survival time post injury	Group
33	TBI	64	m	0 h	1
34	TBI	57	m	0 h	1
35	TBI	31	m	1.3 h	1
36	TBI	60	m	2.5. h	1
37	TBI	36	m	3 h	1
38	TBI	20	m	5.2 h	1
39	TBI	27	m	5.5 h	1
40	TBI	27	f	6 h	1
41	TBI	43	m	6 h	1
42	TBI	63	m	7 h	1
43	TBI	67	f	10 h	1
44	TBI	46	m	12 h	1
45	TBI	42	f	12 h	1
46	TBI	29	m	12 h	1
47	TBI	28	m	14 h	1
48	TBI	44	m	20 h	1
49	TBI	52	m	20 h	1
50	TBI	87	f	1.5 d	2
51	TBI	64	m	2 d	2
52	TBI	60	m	4 d	2
53	TBI	84	f	4 d	2
54	TBI	58	m	6 d	2
55	TBI	59	m	7 d	2
56	TBI	55	m	8 d	3
57	TBI	41	m	10 d	3
58	TBI	38	m	12 d	3
59	TBI	83	m	14 d	3
60	TBI	80	f	15 d	3
61	TBI	18	f	16 d	3
62	TBI	19	m	27 d	3
63	TBI	56	f	180 d	4
64	TBI	29	m	1825 d	4
65	ICB	69	m	4 h	1
66	ICB	41	f	24 h	1
67	ICB	41	f	24 h	1
68	ICB	79	f	1.5 d	2
69	ICB	81	f	2 d	2
70	ICB	55	m	3 d	2
71	ICB	59	m	7 d	2
72	ICB	47	m	8 d	3
73	ICB	57	f	12 d	3
74	ICB	56	m	16 d	3
75	ICB	73	m	20 d	3
76	ICB	65	m	41 d	3
77	ischemia	67	f	6 h	1
78	ischemia	65	f	10 h	1
79	ischemia	72	m	11 h	1
80	ischemia	62	f	12 h	1
81	ischemia	86	f	24 h	1
82	ischemia	62	m	2 d	2
83	ischemia	56	f	2 d	2
84	ischemia	78	f	2.5 d	2
85	ischemia	68	m	3 d	2
86	ischemia	73	f	3 d	2
87	ischemia	74	f	3 d	2
88	ischemia	75	m	4.5 d	2
89	ischemia	76	f	6 d	2
90	ischemia	77	f	7 d	2
91	ischemia	51	f	7 d	2
92	ischemia	75	f	10 d	3
93	ischemia	63	m	12 d	3
94	ischemia	82	m	49 d	3
95	ischemia	52	f	52 d	3
96	ischemia	52	f	53 d	3
97	ischemia	70	f	53 d	3
98	ischemia	70	f	56 d	3
99	ischemia	69	m	63 d	3
100	ischemia	70	m	74 d	4
101	ischemia	78	f	98 d	4
102	ischemia	32	f	98 d	4
103	ischemia	58	m	133 d	4
104	ischemia	86	f	134 d	4
105	ischemia	73	m	150 d	4
106	ischemia	75	m	960 d	4

TBI, traumatic brain injury; ICB, intracerebral bleeding

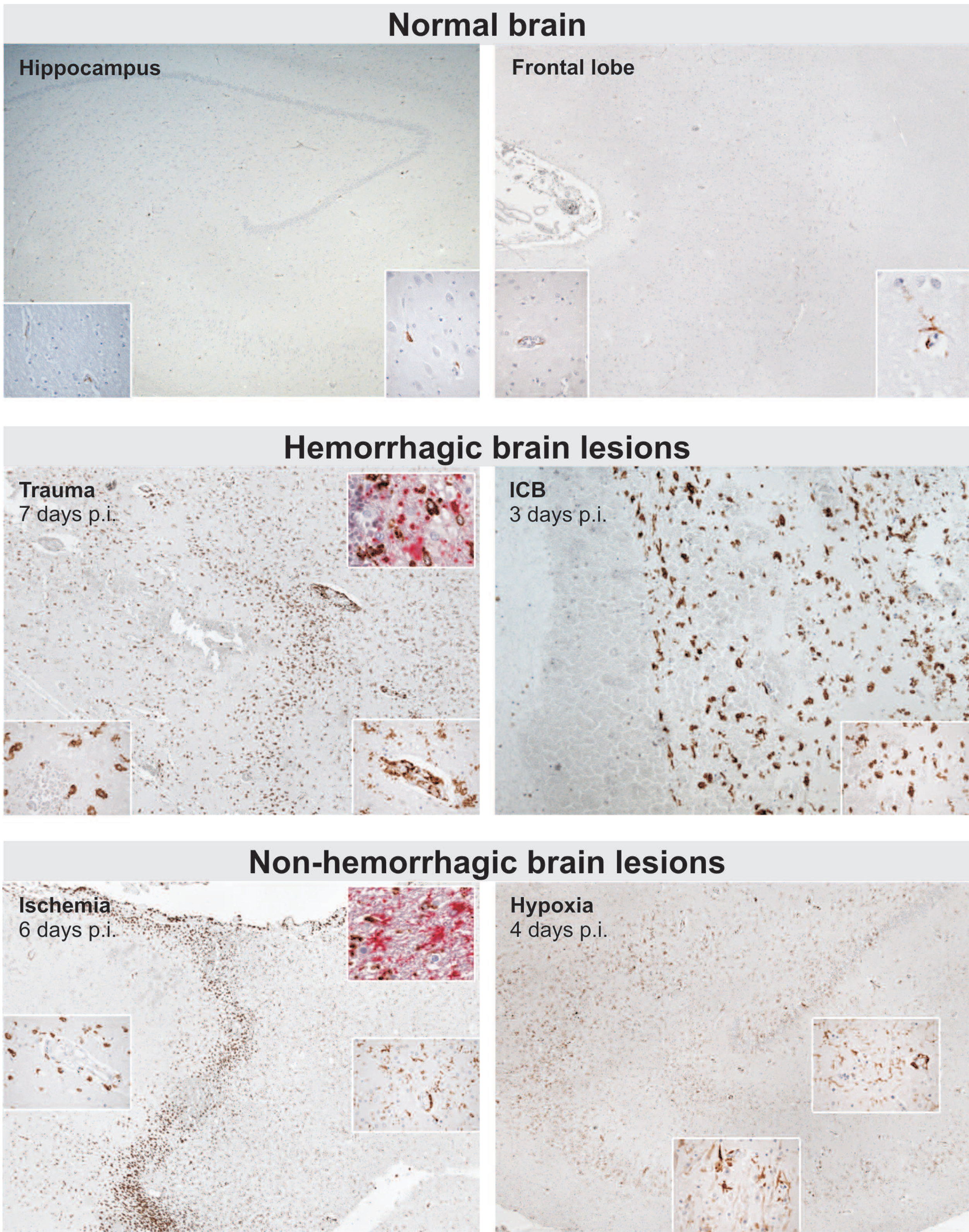

Fig. 1. Expression of CD163 in normal brain is restricted to perivascular cells consistent with pericytes and/or monocytes. Higher magnifications show CD163+ perivascular cells in the gray matter (pyramidal cell layer of the hippocampus and frontal cortex) and in the white matter. The inset showing frontal white matter displays one perivascular cell attached to the wall of a capillary and one ramified microglial cell in the directly neighboring brain parenchyma. In all pathological conditions investigated (trauma, ICB, ischemia, hypoxia), numbers of parenchymal and perivascular CD163+ cells are increased, without reference to hemorrhages. Overviews of brain lesions following trauma and ischemia show the lesion in the left part of the photograph (asterisk) and the adjacent brain tissue on the right side. Note the wall of CD163+ cells at the outer part of the lesion. This area is also shown at slightly higher magnification in a case of ICB (magnifications see below). Photographs from traumatic, ischemic and hypoxic brain lesions also display accumulations of CD163+ cells surrounding blood vessels (perivascular cells, arrows). Photographs at low magnification were taken at an original magnification of x25 (normal brain, trauma, ischemia and hypoxia) and x100 (ICB), respectively. The insets in the upper right corner of photomicrographs from the traumatic and the ischemic lesion show that there was no coexpression of CD163 (brown) and GFAP (red), indicating that astrocytes do not express CD163 (x1000 each). Original magnification for the other insets is x400, except for the left inset in normal hippocampus (x200, white matter), the right inset in normal frontal lobe (x1000, white matter) and the inset in ICB (x1000).

parenchyma single CD163+ ramified microglial cells were found in 4/6 cases (Fig. 1). Thus, only in one case single CD163+ microglial cells were detectable in all four regions investigated (frontal cortex, frontal white matter, CA1-2, hippocampal white matter). In two cases CD163+ microglia occurred in only one region and in one case in three regions. Mean numbers of CD163+ parenchymal cells were 1.50 (CI 0-3.4) in CA1-2, 0.3 (CI 0.2-0.9) in hippocampal white matter, 2.2 (CI 0-6.9) in frontal cortex and 0.8 (CI 0.1-1.6) in frontal white matter.

CD163+ cells in hemorrhagic and non-hemorrhagic brain lesions

Mean numbers and range of CD163+ cells in the different groups of varying survival times and in the different investigated regions (lesion, adjacent, remote) are illustrated in figure 2. Significant changes of mean numbers of CD163+ cells in comparison to controls are marked by an asterisk but the conclusiveness of data for group 4 (>9 weeks) is limited due to low or missing numbers of cases (Tables 1 and 2, Fig. 2). CD163 expression in perivascular cells served as internal control in all cases and was constantly preserved even in cases with prolonged post-mortem interval to autopsy.

CD163+ parenchymal cells in hemorrhagic brain lesions (TBI, ICB)

Following TBI, numbers of CD163+ microglia/macrophages significantly increased in the lesion within the first 24 hours (group 1, mean 7.4, CI 1.0-13.8), further increased in group 2 and reached maximum level in group 3 (mean: 467.0, CI 187.1-746.9). In the adjacent area CD163+ cells increased later and reached level of significance in groups 2-4 (Figs. 1, 2). No significant changes were found in the remote area.

Similarly, following ICB a significant increase in CD163+ cells was found in the lesion and in the adjacent area in groups 2 and 3 (Figs. 1, 2). No significant changes were found in group 1 in any region (Figs. 1, 2). On the basis of only three cases, level of significance was reached in group 3 in the remote area (mean: 11.3, CI 6.0-16.5).

CD163+ parenchymal cells in non-hemorrhagic brain lesions (ischemia, hypoxia)

Following ischemia, similar to traumatic brain lesions, a significant increase in mean numbers of CD163+ cells occurred within 24 hours (group 1, mean: 14.0, CI 0-39.2) in the lesion and within 1-7 days (group 2, mean: 48.2, CI 0-101.7) in the adjacent region, and remained significantly elevated till late stages (Figs. 1, 2). In the remote area level of significance was reached in group 3 (1-9 weeks, n=9).

After hypoxic brain damage, in the lesion areas (frontal cortex and hippocampal pyramidal cell layer) a significant increase in CD163+ cells occurred in group 2 (mean: 52.1, CI 25.0-79.1; mean: 112.4, CI 54.0-170.7, respectively) and in group 3 (Figs. 1, 2). In the adjacent white matter numbers of CD163+ cells were significantly increased only in group 2 (hippocampus) and in group 3 (frontal lobe, Fig. 2).

CD163+ perivascular cells in hemorrhagic and non-hemorrhagic brain lesions

Following hemorrhagic brain lesions (trauma, ICB), perivascular CD163+ cells were significantly increased in the lesion (trauma: mean 16.6, CI 7.8-25.4; ICB: mean 12,.3, CI 0-25.1) and in the adjacent area (trauma: 11.0, CI 0-25.1; ICB: 18.5, CI 6.4-30.6) only in group 3 (Fig. 1) and group 4. In remote areas after trauma a significant increase of CD163+ perivascular cells was also observed in group 3 (mean: 9.0, CI 5.2-12.8).

Following ischemia, no significant changes in numbers of CD163+ perivascular cells occurred in the lesion. In the adjacent area significant changes were found in group 3 (mean: 15.3, CI 8.6-21.9) and 4 (mean: 14.0, CI 1.1-26.9, Fig. 1). Similarly, in remote areas a significant increase in CD163+ perivascular cells was also found in group 3 (mean: 11.2, CI 6.5-15.8) and 4 (mean: 9.3, CI 5.3-13.2). On the basis of three cases a significant increase was also found in remote areas in group 1 (mean: 10.0, CI 3.4-16.6), 3 (mean: 11.2, CI 2.5-13.1) and 4 (mean: 9.3, CI 5.3-13.2).

After hypoxia, a significant increase in CD163+ perivascular cells was found in the frontal cortex (lesion) in groups 1-3 (means: 11.8, 9.7 and 20.3, respectively) and in the hippocampal pyramidal cell layer (lesion) in groups 2 (mean: 11.3) and 3 (mean: 16.0). In adjacent areas significant changes were noted in group 2 (hippocampus, Fig. 1) and group 3 (frontal lobe).

Comparison of CD163 expression following different pathological conditions

In the various lesions, significant differences in the number of CD163+ parenchymal cells were noted only in group 1 and in group 3 (Fig. 3). In group 1 a significantly higher number of CD163+ cells was found in ischemic lesions (mean: 47.0) in comparison to lesions in TBI (mean: 13.0) or ICB (mean: 12.7). In group 3, significantly more CD163+ microglia/macrophages appeared in ischemic lesions (mean: 116.0) in comparison to TBI (mean: 40.0), ICB (mean: 54.8) and hypoxia (mean: 90.4). Furthermore, in group 3 mean numbers of parenchymal CD163+ cells in the lesion were significantly higher in ICB in comparison to TBI and hypoxia, as well as in TBI in comparison to hypoxia. In adjacent areas, significant differences were detected only in group 3 when analyzing data from three cases with hypoxia (mean: 57.2) in comparison to data from ischemia (mean: 19.1), ICB (mean: 17.9) and TBI (mean: 16.4).

Overall, at least in groups 1 and 3, numbers of

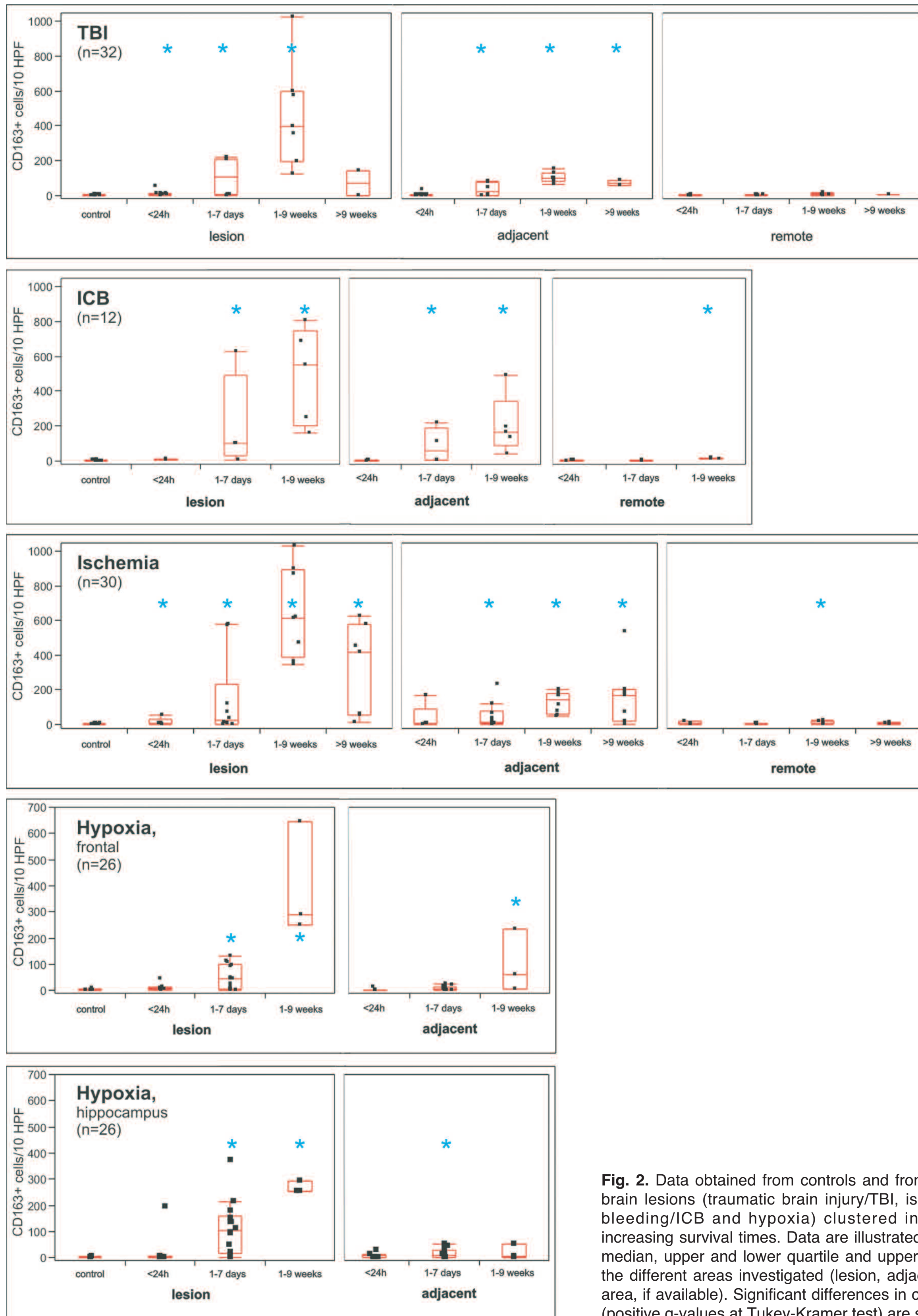

Fig. 2. Data obtained from controls and from cases with different brain lesions (traumatic brain injury/TBI, ischemia, intracerebral bleeding/ICB and hypoxia) clustered into four groups with increasing survival times. Data are illustrated as boxplots showing median, upper and lower quartile and upper and lower whisker in the different areas investigated (lesion, adjacent area and remote area, if available). Significant differences in comparison to controls (positive q-values at Tukey-Kramer test) are shown by asterisks.

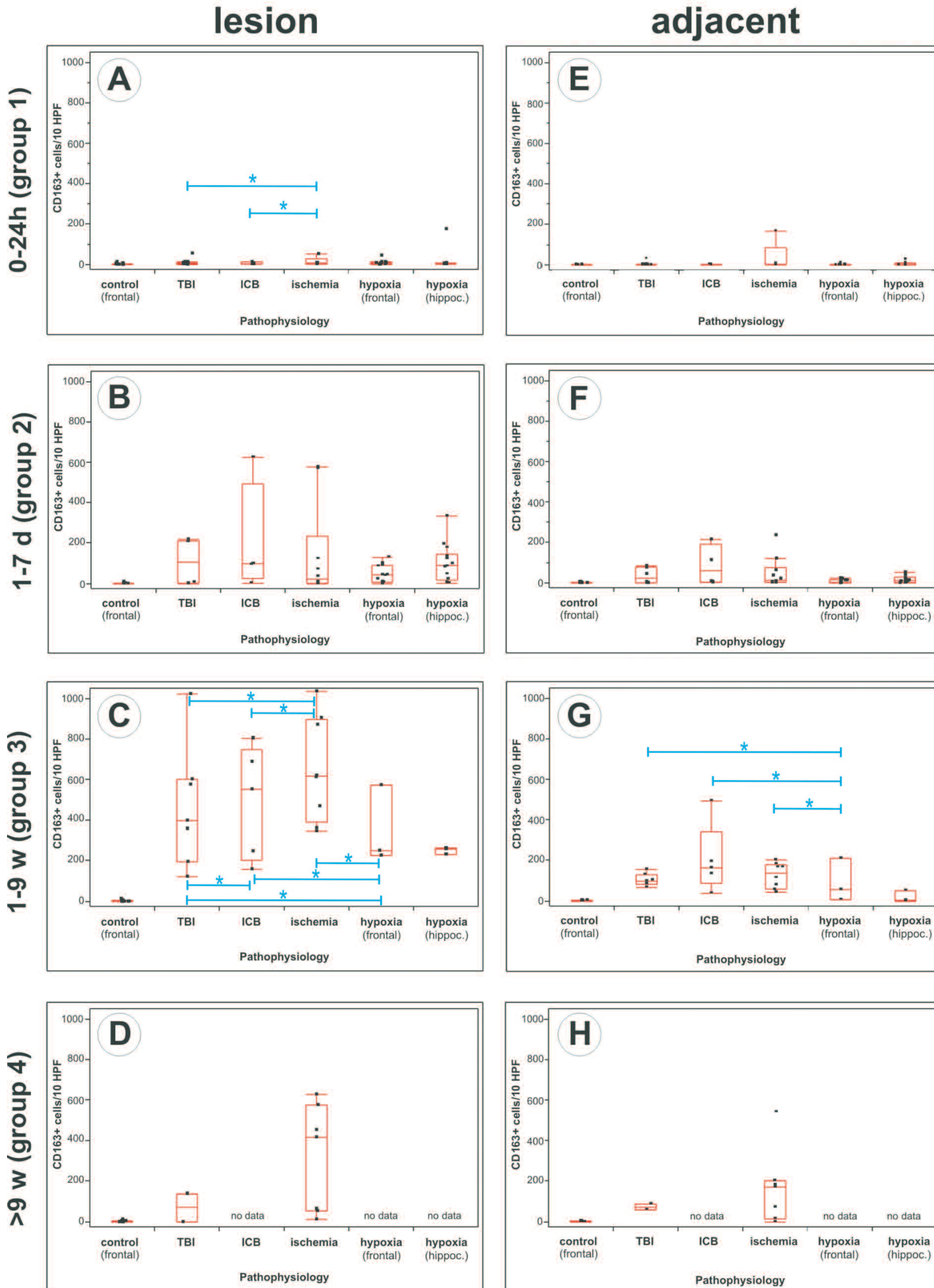

Fig. 3. Data obtained from cases with four different types of brain lesions (traumatic brain injury/TBI, intracerebral bleeding/ICB, ischemia and hypoxia) clustered into four groups with increasing survival times. Data are illustrated as boxplots showing median, upper and lower quartile and upper and lower whisker in the lesion and in the adjacent brain tissue. According to heterogeneous distribution of microglial cells in the human brain (Mittelbronn et al., 2001), in cases of hypoxia only data obtained from the frontal lobe were considered for statistical analysis. Significant differences between different pathologies (positive q-values at Tukey-Kramer test) are shown by asterisks. Data from controls and from hippocampal sections of hypoxia cases are also shown for completeness.

CD163+ microglia/macrophages were significantly lower after hypoxia in comparison to trauma, ICB and ischemia, and the highest levels of CD163+ cells appeared following ischemia when compared to all other pathologies (Fig. 3).

When comparing numbers of CD163+ perivascular cells, no significant differences occurred between the different pathological conditions (trauma, ICB, ischemia, hypoxia) in any group (varying survival time) nor in any investigated region (data not shown).

Double-labelling experiments

Single cases were selected for double-labelling experiments to investigate coexpression of CD163 and GFAP. In brain lesions from all four investigated pathologies no coexpression of CD163 and GFAP was notable, indicating that no astrocytes contributed to the pool of CD163+ cells.

Discussion

In the present study, we found increasing numbers of CD163+ cells following both hemorrhagic (trauma, ICB), as well as non-hemorrhagic (ischemia, hypoxia) brain lesions. The increasing numbers of CD163+ microglia/macrophages in non-hemorrhagic brain lesions point to a functional role of CD163 different from its well known function as receptor for the Hb-Hp complex in extravasal hemolysis.

Significantly lower levels and significantly higher levels of CD163+ cells occurred in non-hemorrhagic types of brain lesion, namely in hypoxia and in ischemia. Significantly higher levels of CD163+ cells following ischemia, trauma and ICB in comparison to hypoxia is in accordance to a obvious breakdown of the blood-brain barrier in the former brain lesions. However, as the highest levels of CD163+ cells are observed in a non-hemorrhagic type of lesion, namely following ischemia, the anti-inflammatory function of CD163 may also predominate in non-hemorrhagic brain lesions (Philippidis et al., 2004; Abraham and Drummond, 2006; Schaer et al., 2006a). As the investigated types of brain lesions result from different pathophysiological conditions, differences in the upregulation of CD163 on microglia/macrophages may, at least in part, reflect pathophysiological differences other than bleeding. For example, in non-hemorrhagic brain lesions the time until onset of tissue damage is shorter and the extend of tissue damage is higher following ischemia when compared to hypoxia. Thus, when comparing data from different pathophysiological conditions, the survival time should also be considered. Furthermore, different treatment strategies may also play a role in the time course and the extend of CD163 upregulation in different types of brain lesions. The medical files of the majority of the investigated cases are limited due to several reasons (e. g. date of death more than 20 yrs. ago; outpatients). For this reason we could not investigate the influence of different therapies (e. g. corticosteroids). Further investigations are needed to estimate in which relation the endocytotic and the immunomodulatory properties of CD163 occur in hemorrhagic brain lesions. From our observation that increasing numbers of CD163+ cells also appeared in areas neighboring (adjacent to) the hemorrhagic lesions, it seems likely that the antiinflammatory function of CD163 also predominates under hemorrhagic conditions.

A significant increase in CD163+ parenchymal cells (microglia/macrophages) occurred within the first 24 hours after trauma and ischemia and within 1-7 days post injury after ICB and hypoxia. Under all four pathological conditions, numbers of CD163+ microglia/macrophages remained significantly elevated till later stages (1-9 weeks or >9 weeks) in the lesion and in the adjacent region. Thus, CD163 signalling is involved in the pathophysiological reaction and consequently might be a target for possible therapeutic intervention throughout a wide period of time, ranging from early to late stages after brain damage.

In contrast to resting (or 'surveying' (Hanisch and Kettenmann, 2007)) microglial cells which lack CD163 expression (Fabriek et al., 2005; Kim et al., 2006; Fischer-Smith et al., 2008), increasing numbers of CD163+ cells were found both in the brain parenchyma (microglia/macrophages) and in perivascular spaces (blood-derives monocytes). Thus, CD163 immunoreactivity does not help to distinguish activated resident microglia from infiltrating blood-derived monocytes. However, as resting microglia are CD163 negative, the *de novo* expression of CD163 is very helpful to demonstrate microglial activation under different pathological conditions (Fabriek et al., 2005; Kim et al., 2006; Fischer-Smith et al., 2008).

Microglial activation attends morphological changes in cell shape from ramified (resting) to amoeboid and foamy macrophages (Kreutzberg, 1996). Several immunohistochemical markers that are commonly applied to demonstrate microglia/macrophages (e. g. CD68 and MHC-II) are already constitutively expressed by microglial cells (Mittelbronn et al., 2001) and are upregulated after activation. For these antibodies, decision-making if microglial activation is present depends on assessment of two gradual features: staining intensity and cell morphology. The de novo expression makes CD163, like CD14 (Beschorner et al., 2002), a highly suitable marker to clearly demonstrate microglial activation, even at early stages.

The number of perivascular CD163+ cells (which are considered blood-derived monocytes) increased somewhat later in comparison to CD163+ parenchymal cells and appeared in group 2 (1-7 days) after ICB and in group 3 (1-9 weeks) after trauma and ischemia. Surprisingly, following hypoxia a significant increase was already observed within group 1 (<24 hours) in the frontal cortex, which preceded the increase in the brain parenchyma. This might be explained by a delayed increase in numbers of activated (CD163+) microglia,

rather than due to an enhanced perivascular infiltration. In the adjacent area after hypoxia, parenchymal and perivascular CD163+ cells increased in parallel.

Recently, it was reported that the tumor necrosis factor-like weak inducer of apoptosis (TWEAK), a member of the tumor necrosis factor (TNF) family of cytokines, also interacts with CD163 (Bover et al., 2007). TWEAK signaling is associated with disruption of the blood-brain barrier (Zhang et al., 2007), as well as activation of the NF-kappaB in the CNS with release of proinflammatory cytokines and matrix metalloproteinases (Yepes, 2007). Inhibition of TWEAK activity after middle cerebral artery occlusion significantly reduced infarct volume, extent of microglial cell activation and apoptotic cell death in the ischemic penumbra. Therefore, it was suggested that inhibition of TWEAK expression or function (e. g. through binding to CD163) may represent a novel neuroprotective strategy to treat ischemic stroke (Yepes et al., 2005).

The expression of CD163 is influenced by several factors. Glucocorticoids, IL-6, IL10 and steroids induce an increase of CD163 expression, whereas TNF-α (tumour necrosis factor alpha) and interferon gamma, LPS and transforming growth factor B lead to decreased expression of this receptor (for review see Onofre et al., 2009). Treatment with phorbol ester and cyclosporine A decreases expression of CD163 on the cell surface (Wenzel et al., 1996; Hogger et al., 1998; Sulahian et al., 2001). Thus, the signal-inducing and immunoregulatory properties of CD163 are interesting aspects, which might be exploited in anti-inflammatory therapy (Graversen et al., 2002). A pharmacological modulation of CD163 expression may have beneficial effects on directly or indirectly (e.g. via TWEAK signalling) mediated cell damage. Furthermore, the limited lifetime of macrophages makes them interesting candidates as a gateway for gene/drug targeting of macrophages in regions with tissue damage (Graversen et al., 2002). However, additional studies including functional data on possible CD163 targeting treatment strategies are required.

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