Acta Neuropathol (2002) 103:541–549
DOI 10.1007/s00401-001-0503-7

REGULAR PAPER

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CD14 expression by activated parenchymal microglia/macrophages and infiltrating monocytes following human traumatic brain injury

Received: 27 July 2001 / Revised, accepted: 13 November 2001 / Published online: 30 January 2002

Abstract The immune response in the central nervous system (CNS) is under tight control of regulatory mechanisms, resulting in the establishment of immune privilege. CNS injury induces an acute inflammatory reaction, composed mainly of invading leukocytes and activated microglial cells/macrophages. The generation of this robust immune response requires binding of receptors such as CD14, a pattern recognition receptor of the immune system. CD14, a surface molecule of monocytic cells, is upregulated after monocyte stimulation and is involved in cellular activation. To examine CD14 expression in human brain lesions we investigated sections of brains obtained at autopsy from 25 cases following closed traumatic brain injury (TBI) and 5 control brains by immunohistochemistry. Detection of CD14 in controls demonstrated constitutive expression by perivascular cells, but not in parenchymal microglial cells, equivalent to known expression pattern of ED2 in rats. Following TBI, numbers of $CD14^+$ cells in perivascular spaces and in the brain parenchyma increased in parallel within 1–2 days, both at the lesion and in adjacent perilesional areas. The number of $CD14^+$ cells in perivascular spaces and in the brain parenchyma reached maximum levels within 4–8 days and remained elevated until weeks after trauma. In contrast to activated parenchymal microglia/macrophages, resting parenchymal microglial cells lacked CD14. Thus, early CD14 expression constitutes an essential part of the acute inflammatory CNS response following trauma.

Keywords Traumatic brain injury · Inflammation · Immune response · CD14

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Introduction

Central nervous system (CNS) injury induces an acute inflammatory reaction, composed mainly of invading leukocytes and activated resident microglia/macrophages [53]. Brain macrophages following injury derive from distinct sources and can be divided into different populations: perivascular cells [23] (also called 'perivascular microglia' [29], pericytes [28], 'fluorescent monocytes' [47], or 'perivascular monocytes' [41, 74]), infiltrating blood monocyte-derived macrophages, and parenchymal microglial cells [6]. Microglial cells are considered bone marrow-derived cells that populate the CNS parenchyma early in embryonic development [34, 43, 69]. In contrast, under adult physiological conditions, there is little or no repopulation of parenchymal microglia by blood-derived monocytes [31, 33, 42]. Activation of microglial cells is considered to be a hallmark of various pathological conditions of the CNS, including infection and inflammation, neurodegenerative disorders, ischemia and traumatic brain injury (TBI) [18, 37, 48, 53]. The generation of these local immune responses requires signal transduction via binding of receptors. CD14 is a key pattern recognition receptor of the innate immune system [27], associated with inflammatory events including: (1) signal transduction (via Toll-like receptors [35, 70]), (2) activation of several intracellular signaling pathways including the IκB kinase-NF-κB pathway and three mitogen-activated protein kinase (MAPK) pathways (ERK1, ERK2, JNK and p53) [25], (3) synthesis of cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)1, -6, -8, -18, and nuclear factor-κB (NF-κB) [15, 46, 49, 58], and (4) increased phagocytic capacity [16], and can also mediate uptake and metabolism of extracellular phosphatidylinositol as a source of arachidonate for leukotriene synthesis [76] and, thus, plays a crucial role in immune response, inflammation and tissue homeostasis.

CD14 is expressed as a 55-kDa glycosylphospatidylinositol-anchored membrane-bound protein (mCD14) on myeloid cells, including macrophages and activated microglial cells [8, 73] and is mainly known to function as a

receptor for lipopolysaccharide (LPS), the endotoxin of Gram-negative bacteria [79]. Beside LPS and components of other pathogens such as Gram-positive bacteria [40, 56], mycobacteria [57], respiratory syncytial virus [39], and membrane structures of apoptotic cells [2, 16], endogenous mediators such as heat shock proteins (hsp60, hsp70), TNF-α, IL-2, phosphatidylinositol and phosphatidylserine are known to activate monocytic cells via CD14 [5, 12, 36, 51, 76, 77].

Recent experiments have shown that treatment with anti-CD14 antibodies produces anti-inflammatory effects in human cell cultures [46] and in animal [21, 59] and human [75] in vivo studies. To provide a pathophysiological basis for the action of CD14 in human traumatic brain lesions, we have analyzed CD14 expression following TBI compared to control brains.

Table 1 Clinical and autopsy data from cases with TBI (*TBI* traumatic brain injury)

Case	Age (years)	Sex	Survival time	Cause of death
1	64	M	<1 h	Disruption of brain stem
2	57	M	<1 h	Polytrauma
3	49	M	1 h	Asystole
4	93	F	5 h	Polytrauma
5	20	M	5.2 h	Polytrauma
6	27	M	5.5 h	Severe TBI
7	63	F	5.7 h	Polytrauma
8	27	F	<6 h	Herniation
9	43	M	<6 h	Herniation
10	63	M	7 h	Severe brain edema
11	46	M	12 h	Severe brain edema
12	28	M	14 h	Hemorrhagic shock
13	44	M	20 h	Severe TBI
14	52	M	20 h	Herniation
15	87	F	36 h	Severe TBI
16	64	M	48 h	Severe brain edema
17	84	F	4 days	Polytrauma
18	59	M	7 days	Herniation
19	55	M	8 days	Anoxic encephalopathy
20	41	M	10 days	Contusion of brain stem
21	38	M	12 days	Herniation
22	83	M	14 days	Pneumonia
23	80	F	15 days	Herniation
24	18	F	16 days	Multiple-organ failure
25	56	F	6 months	Pneumonia

Material and methods

Patients

We investigated brain specimens obtained at autopsy from 25 patients (age 18–93 years, mean 52.9±20.9 years) from a previously described series of cases who died after various survival times following closed TBI [9]. Age, gender and survival times of cases are listed in Table 1 (for further clinical and autopsy data see [9]). In addition to patient data, hematoxylin-eosin, Luxol fast blue (LFB) and iron (Fe) staining was used for evaluation of the typical histological features defined as standard indicators of trauma age [24]. In addition to samples from the lesion site area, sections taken remote from the contusion were investigated in five cases with various survival times post trauma (cases 3, 10, 17, 18 and 25). As controls, the results were compared to tissue of five cases out of a recently described series of neuropathologically unaffected control cases (Table 2) [50].

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections were rehydrated and boiled in a microwave oven (600 W, seven times for 5 min) in citrate buffer (2.1 g sodium citrate/l, pH 6.0). Endogenous peroxidase was inhibited with 1% H_2O_2 in methanol (1:10; 15 min). Sections then were incubated with 10% normal porcine serum (Biochrom, Berlin, Germany) to block nonspecific binding of immunoglobulins.

Immunohistochemistry for CD14 was performed in all cases using a monoclonal antibody (NCL-CD14–223, clone 7; Novocastra Laboratories, Newcastle, UK; dilution 1:100). CD14 antibody was applied overnight at 4°C. Antibody binding was visualized with a biotinylated secondary antibody (rabbit anti-mouse), streptavidin and biotinylated horseradish peroxidase (HRP) complex (StreptABComplex/HRP; Dako, Glostrup, Denmark) and diaminobenzidine (DAB; Dako) as chromogen. Sections were counterstained with Mayer's hematoxylin. Negative controls consisted of sections incubated in the absence of the primary antibody.

Furthermore, to verify CD14 expression patterns, selected paraffin sections were appropriately immunostained using an additional polyclonal antibody detecting the N terminus of human CD14 (CD14-N, sc-6998; Santa Cruz Biotechnology, Santa Cruz, Calif.; goat anti-human). The polyclonal CD14 antibody was applied following microwave pretreatment at 4°C overnight (dilution 1:100). The monoclonal and the polyclonal anti-CD14 antibody revealed no detectable differences in staining patterns in our series.

Blocking procedures

Preabsorption controls were performed to confirm the specificity of CD14 immunostaining patterns of the two anti-CD14 antibodies. In these, prior to application to the tissue sections, the primary antibody (CD14, Santa Cruz Biotechnology) was coincubated for 2 h on ice with tenfold access of one of the following peptides: (1) purified recombinant CD14 blocking peptide (sc-6998 P; Santa

Table 2 Clinical and autoptical data from control cases

Case	Age (years)	Sex	Cause of death	Neuropathological findings
26	21	F	Hemorrhagic shock after car accident	Single eosinophilic hippocampal neurons
27	44	M	Acute posterior myocardial infarct	Inconspicuous
28	56	F	Hemorrhagic shock after recurrent ulcer bleeding	Inconspicuous
29	76	F	Hemorrhagic shock after severe esophageal varicose hemorrhage	Inconspicuous
30	82	F	Hemorrhagic shock after femoro-femoral bypass operation	Minor signs of hypertensive arteriopathy

Cruz Biotechnology), or (2) recombinant endothelial-monocyte-activating polypeptide 2 (EMAP-II) [61], as an irrelevant control peptide. After coincubation of CD14 antibody with cognate CD14 peptide, no immunostaining was detectable, as in sections developed in the absence of primary antibody. In contrast, preabsorption with the recombinant irrelevant control peptide EMAP-II did not affect immunolabeling, demonstrating specificity of the immunostainings.

Double-labeling experiments

In double-labeling experiments, we first stained a cell-type or activation-specific antigen using the avidin-biotin-peroxidase complex (ABC) procedure in combination with alkaline phosphatase conjugates. Specific antigens were labeled with monoclonal antibodies against glial fibrillary acid protein (GFAP; Novocastra, 1:500) to detect astrocytes, and against CD68 (Dako, 1:100) for identification of microglia/macrophages. Activated microglia/macrophages were detected with antibodies against HLA-DR, -DP, -DQ (MHC class II; Dako, 1:50) or MRP8 (BMA, Augst, Switzerland; 1:100). B lymphocytes (CD20; Dako; 1:200) and T lymphocytes (CD3; Novocastra, NCL-CD3-PS1; 1:100) were identified using appropriate antibodies. Endothelial cells were labeled with an antibody detecting CD31 (Dako; 1:50). For visualization of antibody binding Fast-Blue BB salt (Sigma, Deisenhofen, Germany) was used as chromogen, yielding a blue reaction product. Briefly, slices were deparaffinized, irradiated in a microwave oven for antigen retrieval and incubated with nonspecific porcine serum. Visualization was achieved by adding biotinylated secondary antibodies (1:400) for 30 min and alkaline phosphatase-conjugated ABC complex diluted 1:400 in TBS-BSA for 30 min. Consecutively, we developed with Fast-Blue BB salt as chromogen. Before CD14 immunolabeling was applied, sections were irradiated in a microwave oven for 5 min in citrate buffer. CD14 antibody (NCL-CD14-223; Novocastra) was then applied as described above, revealing a brown reaction product with DAB. In double-labeling experiments for identification of T lymphocytes, the CD3 antibody was applied first and visualized with DAB, after which CD14 immunolabeling was performed using Fast-Blue BB salt as chromogen. In double-labeling experiments sections were not counterstained with Mayer's hematoxylin.

For color reproduction, slides were digitally converted using a film scanner (CannonScan FS 2710, Canon Inc., Tokyo, Japan) and contrast enhanced using an image processing program (Adobe PhotoShop 5) to obtain pictures comparable to the original slides.

Evaluation

Immunolabelings for CD14 were evaluated separately in the core of the lesion, in the adjacent perilesional tissue and in areas remote to the contusion. For evaluation of CD14 staining patterns, the

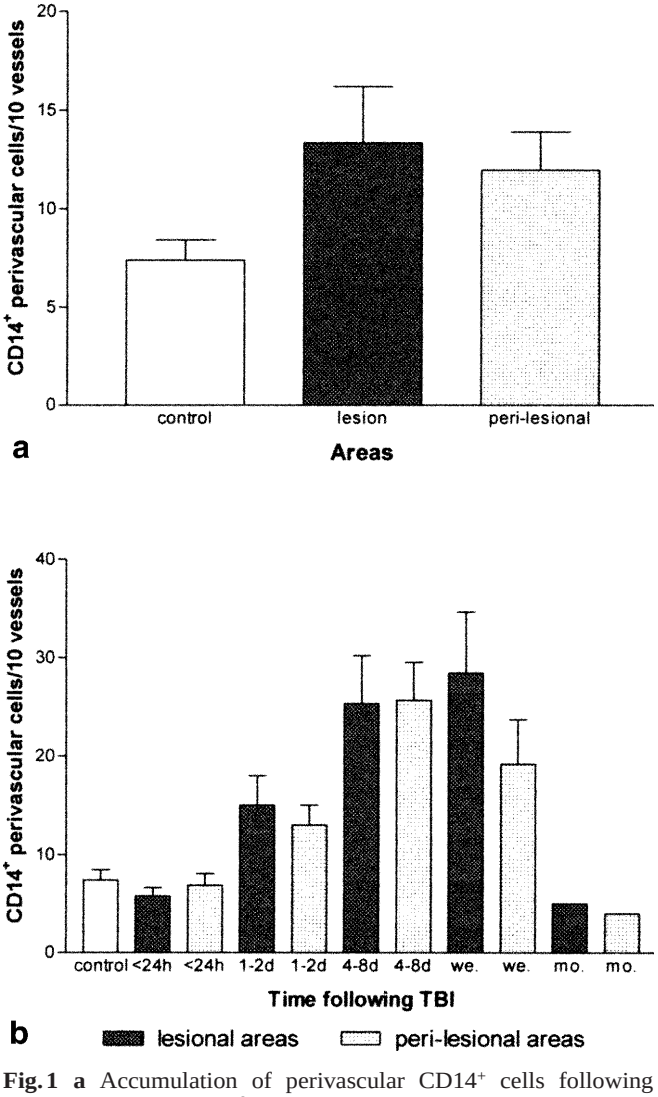

Fig. 1 **a** Accumulation of perivascular CD14+ cells following TBI. **b** Accumulation of perivascular CD14+ cells in lesional and perilesional areas following TBI (*TBI* traumatic brain injury, *d* days, *we*. weeks, *mo*. months)

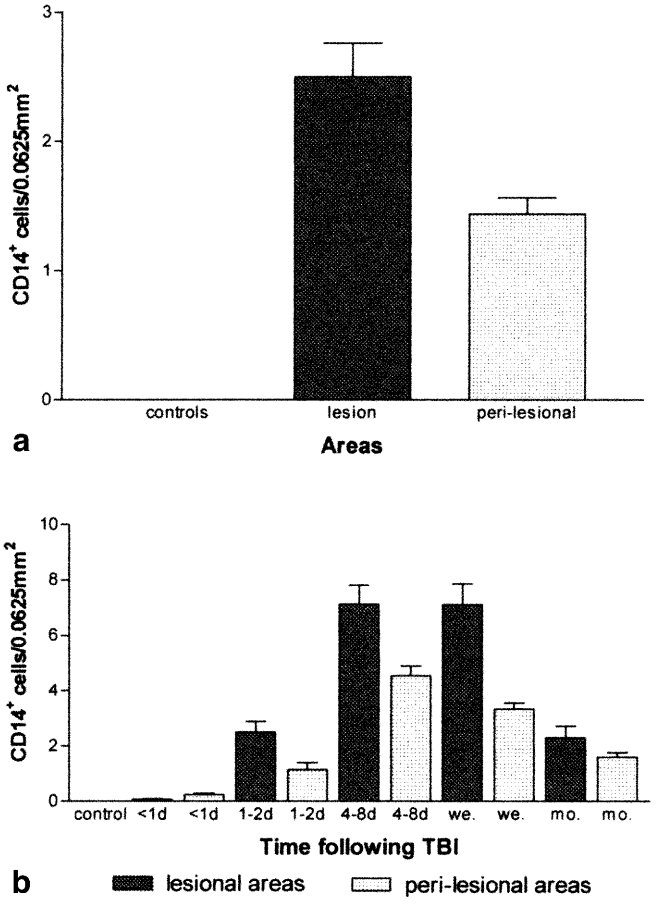

Fig. 2 **a** Parenchymal accumulation of CD14+ cells following TBI. **b** Parenchymal accumulation of CD14+ cells in lesional and perilesional areas following TBI

number of $CD14^+$ perivascular cells (cells nestled against the outer vessel wall in a monolayer fashion and cells in Virchow-Robin spaces) were counted per ten vessels. Numbers of $CD14^+$ parenchymal cells were counted in ten high-power fields (×400 with an eyepiece grid representing 0.0625 mm^2).

Statistical analysis

For statistical analysis cases were clustered into five groups with increasing survival times post TBI (up to 24 h, 1–2 days, 4–8 days, weeks or months; compare Figs. 1a, b and 2a, b). Data obtained from these case groups were compared to controls using the two-tailed unpaired Student's *t*-test. The numbers of $CD14^+$ perivascular cells were calculated as means of labeled cells (MLPVC ± SEM). Data of the density of $CD14^+$ parenchymal cells were calculated as means of labeled parenchymal cells (MLPC ± SEM).

Results

CD14 expression pattern

Immunolabelings with both anti-CD14 antibodies applied revealed a profound membranous expression pattern. Moreover, both anti-CD14 antibodies occasionally revealed a cytoplasmic staining.

CD14 expression in control brains

In control brains no $CD14^+$ cells were present in the brain parenchyma (MLPC 0.0±0.0). Around blood vessels frequently $CD14^+$ perivascular cells were seen (MLPVC 7.4± 1.0) often nestled against the outer vessel wall in a monolayer fashion (Figs. 1a, b, 3A). Occasionally single $CD14^+$ cells that were not attached to vessel walls occurred in Virchow-Robin spaces. Additionally, a weak staining of plexus choroideus epithelium and single $CD14^+$ cells in the subarachnoid space were seen (not shown).

CD14 expression following TBI

$CD14^+$ perivascular cells

Numbers of $CD14^+$ perivascular cells increased significantly following TBI, both at the lesion core (P=0.0015) and in perilesional areas (P=0.062), but not in areas remote from the contusion (P=0.3486). Increasing numbers of $CD14^+$ perivascular cells appeared within 1–2 days post TBI (lesion core: MLPVC 15.0±3.0; perilesional area: MLPVC 13.0±2.0), and further increased reaching significant levels at 4–8 days (lesion core: MLPVC 25.3±4.8, P=0.0362; perilesional area: MLPVC 25.7±3.8, P=0.0357) and weeks post TBI (lesion core: MLPVC 28.4±6.2; perilesional area: MLPVC 19.2±4.5) corresponding to the known time course of invasion of blood-derived macrophages (Figs. 1a, b, 3C) [64].

Parenchymal $CD14^+$ cells

The time course of $CD14^+$ cells occurring in the brain parenchyma paralleled accumulation of $CD14^+$ cells in perivascular spaces (Fig. 2a, b). $CD14^+$ cells increased significantly in the brain parenchyma, both at the lesion core ($P \leq 0.0001$; MLPC 2.5±0.4) and in perilesional tissue ($P \leq 0.0001$; MLPC 1.4±0.1), when compared to controls. In general, significantly higher numbers of $CD14^+$ cells were detected at the lesion core in comparison to perilesional areas (Fig. 2a, P=0.0004). Within 24 h a few parenchymal cells expressing CD14 were present at the lesion and in perilesional tissue. Thereafter, numbers of $CD14^+$ parenchymal cells increased further, reaching significant levels within 1–2 days (lesion core: MLPC 2.5±0.4, P=0.0001; perilesional area: MLPC 1.2±0.3, $P \leq 0.0001$) compared to controls, and increased further significantly (in comparison to levels after 1–2 days) reaching maximum levels at lesional (P=0.0004; MLPC 7.1±0.7) and in perilesional areas (P=0.0004; MLPC 4.5±0.4) after 4–8 days and remained elevated until months following TBI (lesion core: MLPC 2.3±0.4; perilesional area: MLPC 1.6±0.2) (Fig. 3C).

Remote areas following TBI

Sections taken remote from the contusion revealed results similar to sections from control brains. No $CD14^+$ cells could be detected in the brain parenchyma (MLPC 0.0±0.0). $CD14^+$ expressing perivascular cells were frequently nestled against the outer vessel wall in a monolayer fashion seen (MLPVC 5.3±1.1).

Cellular sources of CD14 expression (double-labeling experiments)

CD14 expression was found to be colocalized in $CD68^+$ microglia/macrophages and perivascular cells ($CD68^+$/$CD14^+$) (Fig. 3B). Moreover, CD14 was found to be colocalized with the activation antigens MRP8 (S100A8; Fig. 3D) and MHC class II (Fig. 3E). No colocalization of CD14 expression was detected in endothelial cells ($CD31^+$/$CD14^-$; Fig. 3F), astrocytes ($GFAP^+$/$CD14^-$), B lymphocytes ($CD20^+$/$CD14^-$) or T lymphocytes ($CD3^+$/$CD14^-$).

Discussion

CD14 is a ligand for cell wall molecules from diverse pathogens (e.g., LPS) [39, 40, 56, 57, 79] or membrane structures from apoptotic cells [2, 16] and mediates activation of monocytic cells by cytokines like IL-2, hsp60 or hsp70 [5, 12, 36], triggering production and release of mediators such as TNFα, IL-1, IL-6, IL-8, IL-18 and IFNβ [15, 46, 58] in addition to chemical mediators like NO [22, 58]. CD14-mediated activation of three mitogen-activated protein kinase pathways (ERK1, ERK2, JNK and p53) leads to an increased phagocytic activity [72], resulting in the uptake and metabolism of extracellular phosphatidylinositol as a source of arachidonate for synthesis of leukotrienes, potent agonists for chemotaxis,

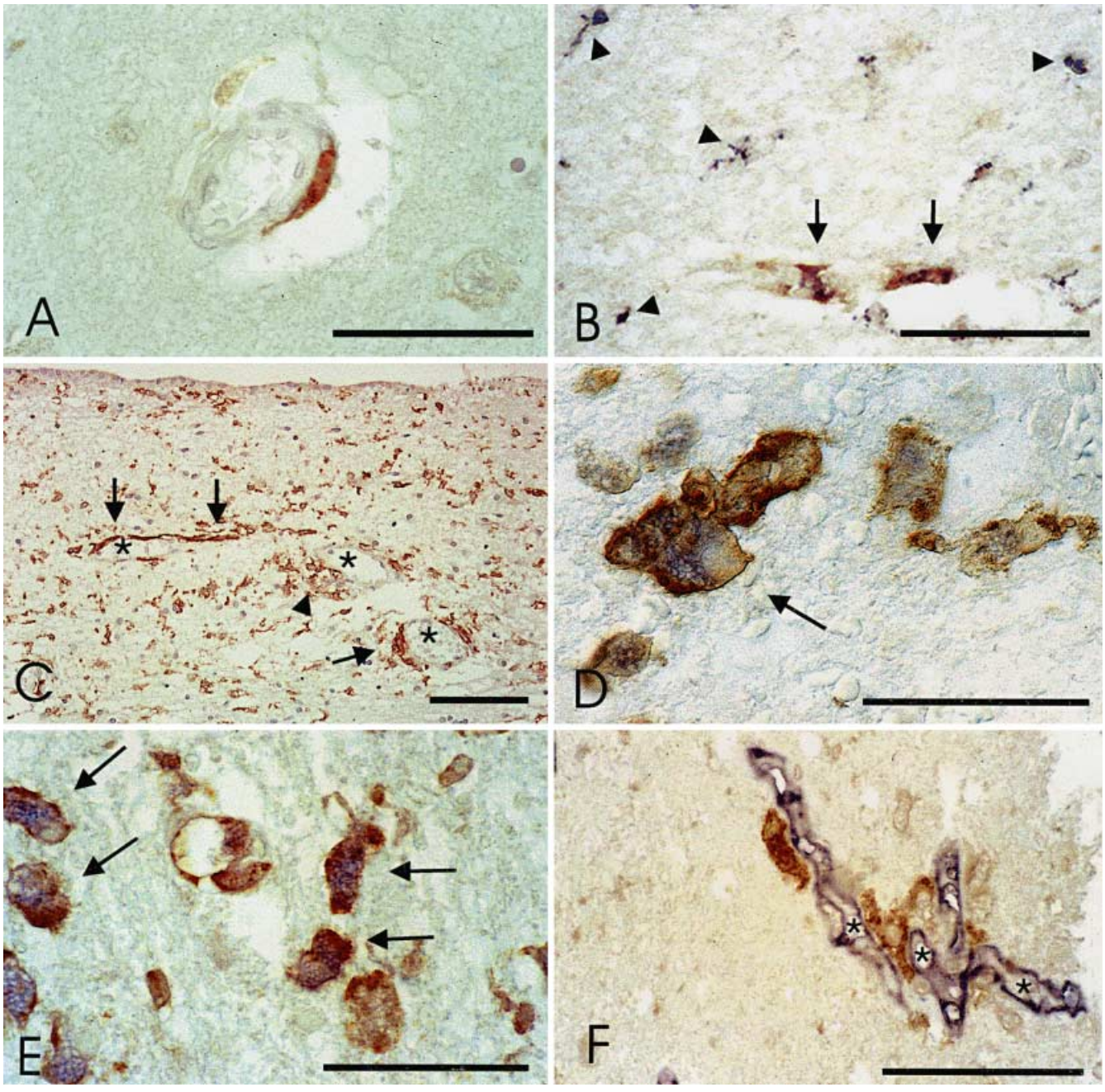

Fig. 3 **A** In control brains CD14 expression is restricted to single perivascular cells. **B** Double-immunolabeling experiments in controls reveal colocalization of CD14 (*brown*) and CD68 (*blue*) in perivascular cells (*arrows*), whereas parenchymal microglia/macrophages, in contrast, constitutively express CD68 (*blue*, *arrowheads*) but not CD14 (*brown*). **C** Following TBI, increasing numbers of $CD14^+$ cells in perivascular spaces around some blood vessels (*asterisk*) occur. In parallel to the increase in numbers of $CD14^+$ perivascular cells, $CD14^+$ cells appear in the brain parenchyma. **D–F** In traumatic brain lesions $CD14^+$ microglia/macrophages (*brown*) coexpress MRP8 (*blue*, **D**) and MHC class II molecules (*blue*, **E**), whereas blood vessels (*asterisks*) reveal no colocalization of CD14 in $CD31^+$ endothelial cells (*blue*, **F**). *Bars* **A**, **D**, **E**, **F** 50 μm; **B** 100 μm; **C** 250 μm

smooth muscle contraction and cell cycle stimulation (proliferation) [76, 77]. These cytokines and mediators have pleiotrophic biological activities and play crucial roles in the immune response and inflammation.

A large variety of pathological conditions or diseases of peripheral organs, including LPS-mediated damage of lung, kidney and liver [20], inflammatory diseases such as diverse types of arthritis [63], Kawasaki disease [68] or respiratory syncytial virus infection [39], myocardial infarction [32], as well as polytrauma and severe burns [38], have been shown to be associated with an up-regulation of CD14 by monocytic cells. In contrast, little is known about CD14 expression in the diseased CNS. Elevated levels of soluble CD14 (sCD14) were found in cerebrospinal fluid (CSF) and in brain microglia/macrophages

in acute bacterial or viral infections [13, 52] and in multiple sclerosis [19, 73]. In a murine model of experimental meningitis dramatically elevated levels of sCD14 in CSF and an active amplification of CD14 and concomitant surface expression (mCD14) by leukocytes in the subarachnoid space were detected, whereas parenchymal astrocytes and microglial cells did not significantly contribute to elevated CD14 levels in this model [13]. Furthermore, parenchymal $CD14^+$ cells were frequently detected in vivo in HIV encephalitis [54].

To investigate contribution and pathophysiological sequence of CD14 in the altered immune privilege following CNS damage, we now analyzed the time kinetic and cellular source of CD14 expression following human closed TBI. A significant early lesion-associated increase in numbers of $CD14^+$ cells, reaching maximum levels within several days to weeks and remaining elevated till months post TBI, was observed. The up-regulation of CD14 by monocytes/macrophages in perivascular spaces and by parenchymal microglia/macrophages correlated positively, indicating that both, infiltrating monocytes and activated parenchymal microglial cells, contribute to the pool of CD14 expressing cells following TBI.

Cellular source of CD14 expression

Double-immunolabeling experiments revealed CD14 expression restricted to invading $CD68^+$ monocytes and $MRP8^+$ ($S100A8^+$), MHC class II^+ activated microglia/macrophages. We could not detect any colocalization of CD14 in astrocytes ($GFAP^+/CD14^-$), in polynuclear leukocytes (clearly recognizable by morphology), B ($CD20^+/CD14^-$) or T lymphocytes ($CD3^+/CD14^-$), nor in endothelial cells ($CD31^+/CD14^-$). This is in accordance to previous studies reporting granulocytes, lymphocytes and endothelial cells to lack intrinsic mCD14 but to be directly activated by complexes of LPS and sCD14 [4, 44, 60, 71].

Both anti-CD14 antibodies applied in this study revealed a predominant membranous staining pattern, according to the expected location of CD14 on the cell membrane of monocyte-derived cells. Additionally, a cytoplasmic CD14 labeling was occasionally observed, possibly due to known large intracellular pools of CD14 in monocytic cells [3]. An enhanced CD14 immunolabeling that possibly could be explained by binding to sCD14 (within the lumen of blood vessels or in edematous brain tissues) did not occur in traumatic brain lesions, when compared to areas taken remote from the lesion or controls, suggesting specific detection of mCD14 by both anti-CD14 antibodies applied in this study. However, other groups have reported that hepatocytes [67], granulocytes [66, 68], B lymphocytes [80], and endothelial cells [30, 45] express CD14.

CD14 expression defines an alteration of CNS immune privilege

Previous in situ and in vitro studies showed quiescent parenchymal microglial cells, like astrocytes [13, 26, 79], to be negative for CD14 [7, 8, 17, 54]. In contrast, as for ED2 in rat brains, a strong constitutive expression of CD14 by perivascular cells was demonstrated [7, 64, 65]. The expression by high numbers of $CD14^+$ parenchymal microglial cells after culture, even under basal conditions [7, 13, 14], points to CD14 being an early marker of microglial activation.

In addition to morphological changes, the up-regulation or de novo expression of diverse antigens, including MHC class II, leukocyte common antigen (LCA/CD45) and the monocytic antigen CD68, has previously been used to demonstrate microglial activation. These antigens are up-regulated by microglial cells under various pathological conditions, but, however, are already expressed constitutively in vivo and in vitro by microglial cells [7, 11, 50, 78]. In contrast, CD14 expression clearly distinguishes between resting ($CD14^-$) and activated ($CD14^+$) parenchymal microglial cells. Therefore, beside macrophage related protein-8 (MRP8/S100A8), which is specific for microglial activation in human brain lesions of various etiology, including HTLV-I-associated myelopathy [1], cerebral malaria [62], ischemia [55] and TBI [10], CD14 represents a key marker of an altered state of the immune privilege in the pathophysiological response following brain damage.

CD14 as a possible pharmacological target

Recently beneficial effects by treatment with an anti-CD14 antibodies have been reported in vivo and in vitro based on suppression of the inflammatory response. In human peripheral blood mononuclear cells IL-18 gene expression and secretion after LPS stimulation was significantly reduced [46]. Schimke et al. [59] reported, in a rabbit model of endotoxic shock, protection from injury to visceral organs and death even when the anti-CD14 antibody was administered after LPS exposure. Recently, Verbon et al. [75] were able to reduce inflammatory host reactions in human volunteers by infusion of a human anti-CD14 antibody (IC14) prior to LPS administration, including (1) reduction of clinical symptoms and signs, (2) decrease of plasma levels of the pro-inflammatory cytokines and attenuation of the increase in serum levels of acute phase proteins, while the release of anti-inflammatory cytokines were only delayed, (3) inhibition of neutrophil and endothelial cells activation, and (4) a modest reduction in the capacity of phagocytizing monocytes and granulocytes [75]. Thus, CD14 is a possible pharmacological target to prevent detrimental effects of inflammatory reactions following brain damage.

In summary, increasing numbers of CD14-expressing cells during the inflammatory reaction following human closed TBI was restricted to the lesion and adjacent tissue,

and was confined to infiltrating monocytes and activated parenchymal microglia/macrophages. Lesional accumulation of $CD14^+$ cells occurred early, reached maximum levels within days and remained elevated until late stages post trauma, both in perivascular spaces and in the brain parenchyma.

Acknowledgements We thank Michel Mittelbronn for providing normal brain tissue sections, and Gudrun Albrecht for excellent graphic and phototechnical work. This study was supported by a grant of the Federal Ministry of Education, Science, Research and Technology (BMBF; 01 K0 9809/8). J.M.S. is supported by the Young Scientists Program of German Israeli Foundation for Scientific Research and Development (G.I.F.) no. G-2024-1065.1/2000.

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