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Neurobiology

Central Nervous System Injury Triggers Hepatic CC and CXC Chemokine Expression that Is Associated with Leukocyte Mobilization and Recruitment to Both the Central Nervous System and the Liver

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The administration of interleukin-1β to the brain induces hepatic CXC chemokine synthesis, which increases neutrophil levels in the blood, liver, and brain. We now show that such hepatic response is not restricted to the CXC chemokines. CCL-2, a CC chemokine, was released by the liver in response to a tumor necrosis factor (TNF)-α challenge to the brain and boosted monocyte levels. Furthermore, a clinically relevant compression injury to the spinal cord triggered hepatic chemokine expression of both types. After a spinal cord injury, elevated CCL-2 and CXCL-1 mRNA and protein were observed in the liver by TaqMan reverse transcriptase-polymerase chain reaction and enzyme-linked immunosorbent assay as early as 2 to 4 hours. Simultaneously, we observed elevated levels of these chemokines and circulating leukocyte populations in the blood. Leukocytes were recruited to the liver at this early stage, whereas at the site of challenge in the central nervous system, few were observed until 24 hours. Artificial elevation of blood CCL-2 triggered dose-dependent monocyte mobilization in the blood and enhanced monocyte recruitment to the brain after TNF-α challenge. Attenuation of hepatic CCL-2 production with corticosteroids resulted in reduced monocyte levels after the TNF-α challenge. Thus, combined production of CC and CXC hepatic chemokines appears to amplify the central nervous system response to injury. ***(Am J Pathol 2005, 166:1487–1497)***

After acute injury in the rodent brain, one of the earliest events is the hepatic release of regulatory acute-phase proteins, which occurs before there is any evidence of an inflammatory response in the brain.[1,2] We have found that one of the first acute-phase proteins to be released from the liver in response to interleukin (IL)-1β microinjection into the brain is the CXC chemokine CXCL-1/CINC-1, which amplifies the hepatic response by initiating a dose-dependent leukocytosis and neutrophil recruitment to the brain.[1] In addition, an IL-1β-mediated challenge to the brain gives rise to neutrophil recruitment to the liver and to hepatocellular damage.[1] The systemic acute-phase response—characterized by hepatic acute phase protein synthesis, leukocyte mobilization, fever, and changes in serum levels of glucocorticosteroids and cytokines[3]—may be viewed as a double-edged sword: whereas an acute-phase response promotes a return to homeostasis, posttrauma recovery may also be impeded by the development of multiorgan dysfunction syndrome.[4] Although many features of multiorgan dysfunction syndrome may be driven by low-grade systemic infection commonly associated with acute brain injury,[5] it is probable that the hepatic chemokine response associated with brain injury may also be involved.

The chemokines can be divided into two main families—CC and CXC—both of which have well-established roles in the control of the specificity of leukocyte recruitment to local inflammation sites.[6] To date, the systemic role of chemokines has been primarily overlooked be-

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cause it does not fit with the generally accepted paradigm that local chemokine gradients are responsible for local leukocyte recruitment in response to inflammation; previous studies describing chemokine regulation in central nervous system (CNS) inflammation have focused only on their local chemoattractant functions.[6] It is of interest that, despite their functional similarity, of all of the CINC chemokines studied so far, only CXCL-1 behaves as a hepatic acute-phase protein in controlling neutrophil-mediated inflammatory damage to the brain.[1] Hitherto, it was not known whether members of the CC chemokine family serve to control, as CXCL-1 controls neutrophils, the monocyte component of the local and systemic inflammatory response to CNS inflammation. The CC chemokine family is large, but CCL-2 [previously known as monocyte chemoattractant protein-1 (MCP-1)] is an archetypal member; we have shown previously that when CCL-2 is directly injected into the brain, it is a potent CNS monocyte chemoattractant,[7] and subsequent CNS studies have described its elevation after endotoxin challenge[8] and in experimental models of either mechanical[9–11] or ischemic[12–14] brain injury. Gene knockout studies[15–17] or studies using chemokine antagonists that interfere with CCL-2 function[18,19] consistently display reduced monocyte recruitment after inflammation.

Expression of the proinflammatory cytokine tumor necrosis factor (TNF)-α is associated with the pathology of a broad spectrum of CNS disease and injury. The microinjection of TNF-α into the brain gives rise to a distinct pattern of leukocyte recruitment characterized by the recruitment of T cells and macrophages,[20,21] a reduction in cerebral blood volume, and brain inflammation as reflected by compromised tissue energy metabolism.[22] In this study, we sought to determine whether the distinct pattern of TNF-α-mediated leukocyte recruitment to the brain was reflected in the elevated expression of CC chemoattractants by the liver. We show that hepatic chemokine synthesis is a generalized inflammatory response to brain inflammation. We demonstrate that, in response to TNF-α-induced experimental brain inflammation, CCL-2 is elevated in the liver and in the blood, that a leukocytosis is induced, and that there is acute hepatic and delayed brain monocyte recruitment, which can be attenuated by systemic administration of the glucocorticosteroid dexamethasone or enhanced by the exogenous administration of CCL-2. Furthermore, we demonstrate that a controlled compression injury to the spinal cord also generates a CXC and CC hepatic chemokine response that is associated with the recruitment of leukocytes to the liver before their recruitment to the spinal cord.

Materials and Methods

Reagents

Rat recombinant TNF-α (rrTNF-α) was obtained from the National Institute for Biological Standards and Controls (Potters Bar, UK). The cytokine was dissolved in endotoxin-free saline (vehicle). The TNF-α contains 100 IU endotoxin/mg (corresponding to 10 ppm by weight), which, in view of previous studies,[23] was considered negligible in the context of these experiments. All other reagents were obtained from Sigma-Aldrich, Dorset, UK, unless otherwise stated.

Animals and Anesthesia

Twelve-week-old (>200 g) male Wistar rats were used throughout (Charles River, Margate, UK). In each experiment, at least three animals were used per group. Rats were anesthetized using isoflurane (Rhodia Organique Fine Ltd., Bristol, UK) for induction and maintenance at 2.5 to 3% in entonox (50% N_2O, 50% O_2). All procedures were performed with ethical committee approval under a UK Home Office License.

Stereotaxic Surgery

All surgical procedures were performed under an operating microscope (Wild M650; Leica, Milton Keynes, UK). Stereotaxic surgery was performed as described previously.[24] Briefly, anesthetized rats were held in a stereotaxic frame. A small hole was drilled in the skull and 1 μg of rrTNF-α in a volume of 1 μl or 1 μl of endotoxin-free sterile saline vehicle, was microinjected into the striatum (an area of brain parenchyma containing both gray and white matter) with a glass capillary needle (tip <50 μm).

Intraperitoneal Injection of Cytokines

Rats were injected intraperitoneally with rrTNF-α (4 μg/kg), 1 μg per 250 g of rat or an equivalent volume of sterile saline and left to recover for 2, 4, 6, 12, or 24 hours.

Spinal Cord Controlled Compression Injury

A partial laminectomy was performed on anesthetized rats as previously described[25] and a controlled compression injury of 15 N per cm^2 for 5 seconds was produced using a modified Newton meter. Animals were killed 2 hours and 4 hours after injury.

Intravenous Injection of CCL-2

Rat recombinant CCL-2 (5 ng, 500 ng, or 2.5 μg) (Becton Dickinson UK Ltd., Oxford, UK), or sterile saline vehicle was injected into the tail vein of anesthetized rats in a final volume of 250 μl. Rats were culled 2 hours after injection. In a separate set of experiments, rats that had received 2.5 μg of rrCCL-2 were microinjected with TNF-α (1 μg) into the striatum. Animals were killed after 4 hours.

Dexamethasone Pretreatment

Two doses of dexamethasone 21-phosphate (Sigma-Aldrich) or sterile saline were administered intraperitoneally, t = 12 hours before surgery (1 mg/kg) and t = 0 hours (1 mg/kg), before the microinjection of 1 μg of

rrTNF-α into the brain. Rats were killed 6 hours after surgery and blood, serum, and tissues were collected for analysis. Cells recruited to the meninges at 6 hours were analyzed per unit length (mm) and the number of ED-1-positive mononuclear cells present within four densely populated, nonoverlapping fields around the injected hemisphere was quantified.

Tissue and Serum Collection

After appropriate survival times, rats were deeply anesthetized with sodium pentobarbitone. Blood was collected and allowed to clot for 2 hours at room temperature and then serum was collected by centrifugation. *Trans*-cardiac perfusions were performed using heparinized saline. Tissue was removed and either frozen in liquid nitrogen or embedded in Tissue Tek (Bayer Diagnostics, Berkshire, UK) and frozen for histology. Alternatively, in a separate set of animals, tissue for histology was perfusion-fixed with either Bouin's fixative or 10% buffered formalin fixative immediately after perfusion with heparinized saline. Fixed tissue was then dehydrated and processed to wax using standard procedures. For enzyme-linked immunosorbent assay (ELISA), the meninges associated with the injected hemisphere of the brain were carefully removed and the striatum microdissected before snap-freezing in liquid nitrogen.

Analysis of Numbers of Circulating Leukocytes

Whole blood was collected in the presence of 5 mmol/L EDTA, pH 8.0, and leukocyte numbers assessed in a Cell-Dyn 1600 hematology analyzer (Unipath, Mountain View, CA) and verified from immunohistochemically labeled blood smears.

Identification of Leukocytes

Frozen, 10-μm-thick serial coronal sections were cut from tissue blocks, through the injection site in the brain, through a representative lobe of liver or in 20-μm-thick parasagittal sections through the compression-injured spinal cord. Using immunohistochemistry, neutrophils were identified using the anti-neutrophil serum HB199,[26] activated macrophages/Kupffer cells and recruited monocytes were identified using the anti-ED-1 serum (Serotec, Oxford, UK). For each tissue section, four representative fields were chosen for quantitation and the average number of positive cells was calculated and expressed as number of cells per mm^2. Tissue sections were counterstained with cresyl-violet and immunopositive labeling was quantitated only when associated with a cell nucleus.

Immunocytochemistry for CCL-2

Before immunohistochemistry, antigens were retrieved from Bouin's-fixed tissue by microwaving for 11 minutes at 650 W. Ten-μm paraffin wax sections were stained with anti-rat CCL-2 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) using standard methodology. In double-labeling studies, sections were initially stained for CCL-2, which was revealed using diaminobenzidine as the chromogen (brown precipitate), before using cell identification markers for ED-1 (Serotec) which was revealed by very intense purple (VIP) (Vector Laboratories, Peterborough, UK) (purple precipitate). Amendments were required for the identification of CCL-2 in liver. Formalin-fixed liver sections were dewaxed and rehydrated using standard procedures. Antigens for the antibodies ED-1 (Serotec) and HB199[26] were revealed using a pressure cooker (121°C for 20 minutes). Sections were incubated for 1 hour in the respective antibodies according to the manufacturers' instructions. Labeling for cell markers was revealed using directly horseradish peroxidase-conjugated secondary antibodies to mouse or rabbit IgG (Sigma-Aldrich) to eliminate labeling of endogenous hepatic biotin. Retrieval of CCL-2 antigens in the liver required an additional pretreatment (0.04% pepsin in 0.1% HCl for 20 minutes) before overnight incubation at 4°C in anti-CCL-2 rabbit polyclonal antibody (AbCam Ltd., Cambridge, UK). CCL-2 antibody was detected using biotinylated anti-rabbit IgG and standard ABC (Vector Laboratories) because amplification was necessary to reveal CCL-2. Single-labeled and double-labeled immunohistochemistry in the liver was revealed using diaminobenzidine and VIP as described above.

mRNA Extraction and Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR)

The principles of TaqMan RT-PCR have been previously reported.[27] RNA extraction and TaqMan RT-PCR assays were performed essentially as previously described.[1,28] Rat CCL-2 TaqMan probe and primers were identical to those published[29] and standard curves were generated from serially diluted cDNA from TNF-α-challenged rat liver. Rat CXCL-1 mRNA assays were performed as we have previously described.[1,28] Results are expressed in arbitrary units corrected to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), analyzed in a similar manner according to the manufacturer's instructions (Applied Biosystems, Warrington, UK).

Protein Extraction and Assay

Liver or brain was homogenized on ice in phosphate buffer (0.5 mol/L NaCl, 2.5 mmol/L NaH_2PO_4, 7.5 mmol/L Na_2HP0_4, 0.1% polyoxyethylene-sorbitan monolaurate, pH 7.3) containing protease inhibitors (100 mmol/L amino-*n*-caproic acid, 10 mmol/L Na_2EDTA, 5 mmol/L benzamidine, 90 mmol/L 4-(2,aminoethyl)benzenesulfonyl fluoride). Homogenates were centrifuged for 1 hour at 100,000 $\times$ *g* in a Beckman TL-100 ultracentrifuge at 4°C and the supernatants were retained and assayed for protein content using the Bio-Rad D_c protein assay against bovine serum albumin standards (Bio-Rad Laboratories Ltd., Herts, UK).

Measurement of Cytokine Production

ELISAs for the measurement of rat TNF-α (R&D Systems, Europe) or rat CCL-2 (BD Biosciences, Pharmingen, San Diego, CA) were performed essentially as described in the manufacturer's instructions (sensitive to 12.5 pg/ml). Rat CXCL-1 ELISAs were performed essentially as we have previously described (sensitive to 3 pg/ml).[1,28] ELISA data are expressed as pg/ml of serum or pg/mg total protein for liver and brain. Recombinant proteins for standard curves were diluted in naïve serum or brain or liver homogenates at concentrations identical to those used in ELISA of test samples.

Statistical Analysis

The data were presented as mean ± SEM at each time point. When statistical analysis was used, data were analyzed compared with the saline vehicle control values at the identical time point by a one-way analysis of variance. In the case of spinal cord injury models (see Figure 4) naïve control data were compared with 2-hour and 4-hour data using a one-way analysis of variance with the Dunnett multiple comparisons test. Results are considered significant when $P < 0.05$.

Results

De Novo *Hepatic CC Chemokine Expression Is Acutely Elevated after TNF-α Challenge to the Brain*

TNF-α or vehicle were microinjected (tip <50 μm) into the rat brain and the liver was then analyzed for CCL-2 mRNA and protein expression throughout a 24-hour time course by TaqMan RT-PCR and ELISA (Figure 1, a and b). Two hours after TNF-α challenge into the brain, hepatic CCL-2 mRNA was significantly elevated above vehicle-injected controls ($P < 0.0007$). CCL-2 mRNA levels decreased throughout the next 12 hours, but remained significantly elevated compared to vehicle-injected controls. By 24 hours CCL-2 mRNA levels had returned to baseline. CCL-2 protein was significantly elevated after 2 hours ($P < 0.05$), peaking at 4 hours ($P < 0.05$) as compared to vehicle-injected controls, and then rapidly falling to basal levels. Double-labeling immunocytochemistry was used to identify the hepatic cell populations responsible for the elevated expression of CCL-2 after TNF-α challenge to the brain (Figure 1c). CCL-2 protein was identified in neutrophils and ED-1-positive activated Kupffer cells/recruited monocytes within the liver.

Hepatic CCL-2 Protein Is Associated with Increased Serum CCL-2 Levels and Subsequent Leukocytosis

The microinjection of TNF-α into the brain resulted in markedly elevated levels of CCL-2 protein in the circulating blood (Figure 1d). Of note was the tight window of

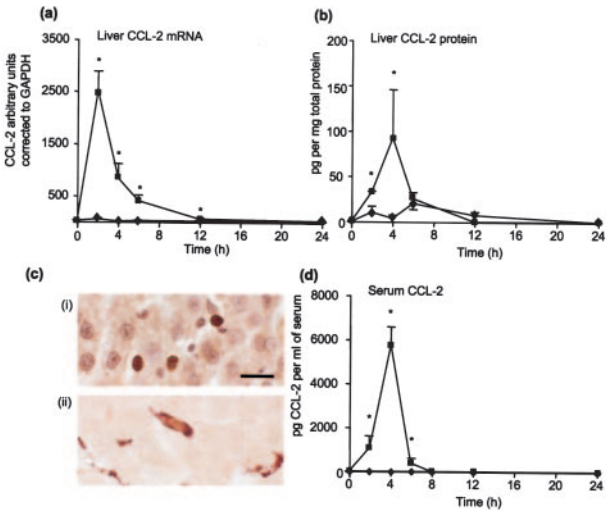

Figure 1. *De novo* hepatic CCL-2 expression is acutely elevated after TNF-α challenge to the brain. Hepatic CCL-2 mRNA (**a**) and protein levels (**b**) were quantitatively assessed by TaqMan RT-PCR and ELISA throughout a 24-hour time course after the injection of saline vehicle (**diamonds**) or TNF-α (**squares**) into the rat brain. Note the peak of hepatic CCL-2 mRNA synthesis 2 hours after TNF-α challenge to the brain (**a**) and the translation into hepatic CCL-2 protein from 2 to 4 hours (**b**). **c:** The source of the hepatic CCL-2 protein is localized to neutrophils (**i**), ED-1-positive Kupffer cells, and recruited monocytes (**ii**) using either single- or double-labeling immunohistochemistry in which CCL-2 is labeled in brown and individual cell types in violet. **d:** Measurement of CCL-2 in the circulating blood by ELISA suggests that the hepatic CCL-2 is initially released into the blood after 2 to 4 hours. **Asterisk** denotes $P < 0.05$ when compared to vehicle-injected controls.

serum CCL-2 expression at 2 hours ($P < 0.05$) and 4 hours ($P < 0.05$), falling dramatically with increasing time. To determine whether the elevated CCL-2 levels were sufficient to stimulate the release of leukocytes into the blood, we analyzed the circulating levels of total leukocytes, monocytes, and neutrophils throughout the 24-hour time course (Figure 2; a to c). Elevations in leukocyte levels, greater than vehicle controls, were observed after 2 hours, falling to baseline throughout the 24-hour time course. This leukocytosis was a result of elevations in circulating levels of both monocytes and neutrophils, which were both significantly elevated initially after 2 hours, peaking after 4 hours.

Inflammation to the Brain Is Not Communicated to the Liver by TNF-α

To investigate whether the hepatic chemokine response to brain inflammation was a simple effect of the microinjected TNF-α draining from brain into the blood, we analyzed circulating TNF-α levels throughout the 24-hour time course by ELISA. Although assay conditions were sufficient to detect concentrations as low as 12.5 pg/ml, no TNF-α was observed in the circulating blood at any time (Figure 2d). In contrast, circulating levels of TNF-α were observed 2 to 4 hours after the injection of a comparable quantity of TNF-α (1 μg/250 g of rat) into the peritoneal cavity. Despite the presence of levels of circulating TNF-α in the blood after the injection into the peritoneum, the resulting leukocytosis was a little delayed

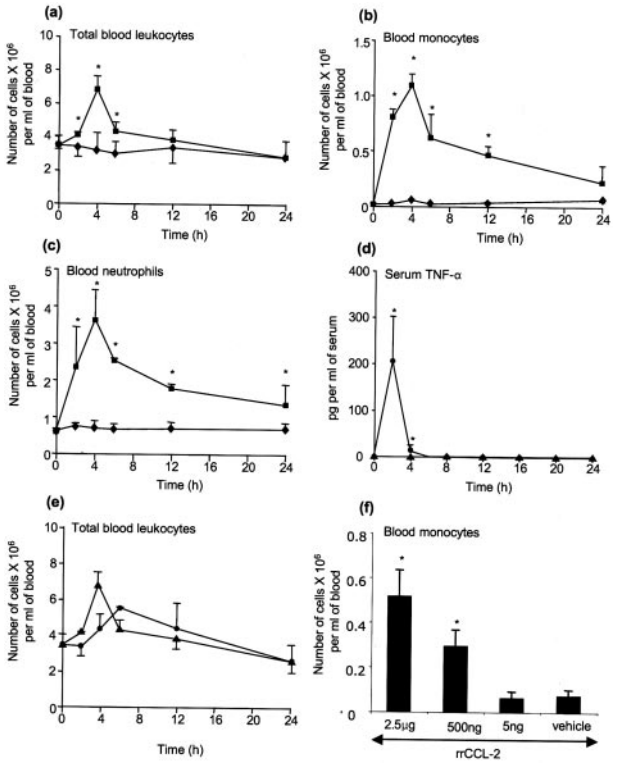

Figure 2. Elevation in serum CCL-2 is associated with the subsequent leukocytosis after brain inflammation. The numbers of circulating leukocytes were assessed using a hematology analyzer throughout a 24-hour time course after the microinjection of TNF-α (**squares**) or vehicle (**diamonds**) into the rat brain. Although increases in the total numbers of leukocytes (**a**), monocytes (**b**), and neutrophils (**c**) were observed 2 hours after TNF-α challenge, peak numbers of each cell type were not observed until 4 hours. ELISA measurement of circulating serum TNF-α (**d**) and total leukocyte numbers (**e**) were compared for a TNF-α challenge administered into the brain (**triangles**) or into the peritoneum (**circles**). Note the later leukocytosis after intraperitoneal challenge despite elevations in circulating TNF-α. **f:** CCL-2 or vehicle was injected intravenously and the number of monocytes in the blood assessed after 2 hours. Note the elevation of circulating monocytes with increasing dose of CCL-2 as compared to vehicle alone. **Asterisk** denotes $P < 0.05$ compared to vehicle-injected controls.

compared to the leukocytosis observed after its injection into the brain (Figure 2e).

Intravenous Injection of CCL-2 Selectively Mobilizes Monocytes into the Blood

To determine whether CCL-2 per se was responsible for the observed leukocytosis we injected rat recombinant CCL-2 intravenously and examined the numbers of circulating monocytes and neutrophils in the blood 2 hours after injection (Figure 2f). Injection of vehicle had no effect on the numbers of either neutrophils or monocytes in the blood. By comparison, intravenous injection of CCL-2 resulted in significantly elevated numbers of circulating monocytes in a dose-responsive manner (500 ng, $P < 0.02$; 2.5 μg, $P < 0.02$) as compared to intravenous injection of vehicle control. The mobilizing effect of CCL-2 was restricted to monocytes because no significant differences in circulating neutrophils were observed (results not shown). We assayed the rat brains to determine whether intravenous CCL-2 alone could stimulate ED-1-positive cell recruitment or macrophage activation within the brain in the absence of injury. We found that ED-1-positive cells were not observed in the brain parenchyma although ED-1-positive cells were observed in small numbers in the meninges and choroid plexus (results not shown).

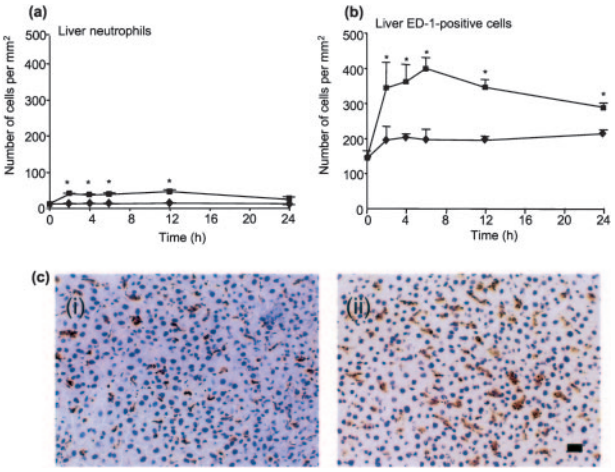

Figure 3. Inflammation to the brain results in acute leukocyte recruitment to the liver. Neutrophils (**a**) and ED-1-positive recruited monocytes and activated Kupffer cells (**b**) in the liver were identified by immunohistochemistry over 24 hours after microinjection of TNF-α (**squares**) or saline vehicle (**diamonds**) into the brain. Note the acute elevation in cell numbers after TNF-α challenge. **c:** Representative photographs of recruited monocytes and activated Kupffer cells after vehicle (**i**) or TNF-α (**ii**). **Asterisk** denotes $P < 0.05$ compared to saline vehicle controls. Scale bar, 40 μm.

Inflammation to the Brain Results in Acute Leukocyte Recruitment to the Liver

To determine whether the TNF-α-induced levels of hepatic CCL-2 production were sufficient to recruit leukocytes, we examined the numbers of neutrophils and ED-1-positive cells (resident and activated Kupffer cells/recruited blood monocytes) by immunohistochemistry (Figure 3; a to c). Compared to vehicle-injected controls, increased numbers of activated Kupffer cells/recruited monocytes were observed as early as 2 hours after the TNF-α challenge to the brain ($P < 0.05$). The number of ED-1-positive cells continued to rise with time peaking after 6 hours ($P < 0.05$) and declining thereafter. Neutrophil numbers increased in the circulating blood, but the numbers of neutrophils recruited to the liver were low compared to the numbers of ED-1-positive cells recruited.

Controlled Compression Injury to the Spinal Cord Generates a Hepatic Chemokine Response

To determine whether a hepatic chemokine response was generated after a more clinically relevant CNS lesion, we performed a mild controlled compression injury to the spinal cord (which generates a Basso-Beatie-Bresnahan score of ~10 after 48 hours) and

examined the resulting hepatic response to the injury. The levels of CCL-2 and the neutrophil chemoattractant CXCL-1, which we have previously shown to be induced in the liver after the stereotaxic microinjection of IL-1β into the CNS parenchyma,[1] were measured in the liver (mRNA) and in the serum (protein) as before. In addition, we looked for evidence of change in the populations of leukocytes in the blood and in the liver. In comparison to resting hepatic CCL-2 mRNA levels, no significant increase in CCL-2 mRNA was observed 2 hours after compression injury to the spinal cord (Figure 4ai), but CCL-2 mRNA levels were significantly elevated 4 hours after the injury (Figure 4ai, $P < 0.01$). An increase in mean CXCL-1 mRNA levels was observed after 2 hours ($P < 0.05$), which was maintained after 4 hours ($P < 0.05$) (Figure 4aii). Elevated levels of both CCL-2 (Figure 4bi, $P < 0.01$) and CXCL-1 (Figure 4bii, $P < 0.05$) were observed in the blood after 2 hours but to a greater degree after 4 hours with a concomitant increase in circulating blood monocytes (Figure 4ci,) and neutrophils (Figure 4cii). Similarly, the numbers of ED-1-positive cells (Figure 4di) and neutrophils (Figure 4dii) observed in the liver were elevated after 2 hours and further elevated after 4 hours (Figure 4e, iv, v, and vi, for representative histology). At these early time points there is very little leukocyte recruitment to the compression-injured spinal cord, despite obvious hemorrhagic areas (Figure 4ei). Isolated ED-1-positive cells and neutrophils were observed; the total number present was very small. Peak ED-1-positive cell numbers (Figure 4eii) and neutrophil recruitment (Figure 4eiii) were evident in the compression-injured spinal cord after a delay of 24 hours.

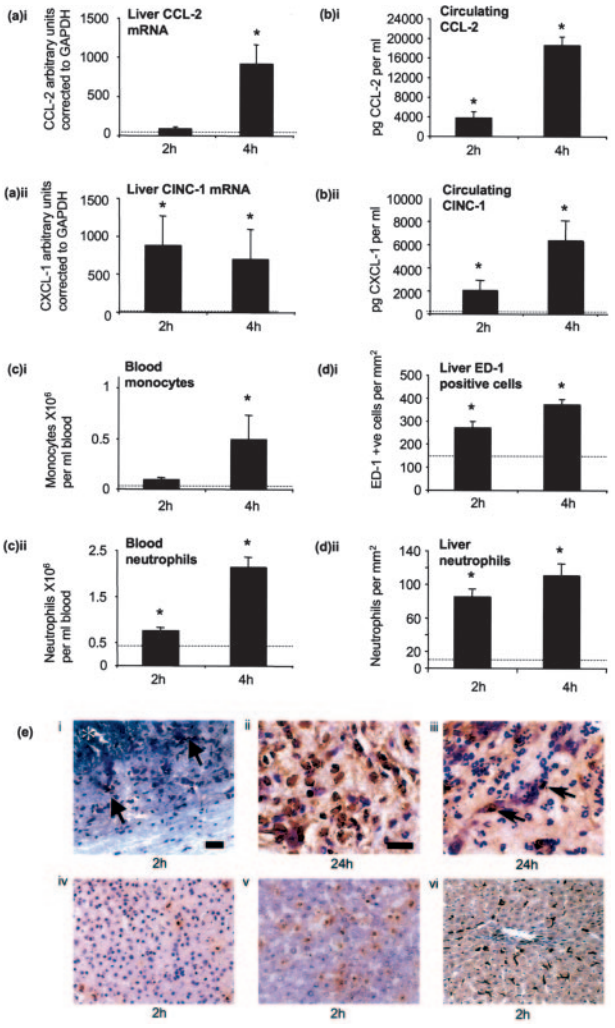

Figure 4. Controlled compression injury to the spinal cord generates a hepatic chemokine response. Liver CCL-2 mRNA (**a**, **i**) and CXCL-1 mRNA (**a**, **ii**) were assessed by TaqMan RT-PCR and serum levels of CCL-2 (**b**, **i**) and of CXCL-1 (**b**, **ii**) were assessed by ELISA 2 hours or 4 hours after a controlled compression lesion to the spinal cord. **c:** The numbers of circulating monocytes (**i**) and neutrophils (**ii**) were assessed using a hematology analyzer. **d:** The numbers of ED-1-positive cells (**i**) and neutrophils (**ii**) present in the liver of the injured rats were assessed by immunohistochemistry. **Dashed line** on each graph represents levels of each component in naïve rats. **Asterisk** denotes $P < 0.05$ compared to naïve controls. **e:** Representative immunohistochemistry pictures show occasional neutrophils (**arrows**) observed at the injury site after 2 hours. **White asterisk** in **e** denotes a site of hemorrhagic injury (**i**). A mixed inflammatory response is observed in the spinal cord after a 24-hour delay with large numbers of both neutrophils (**e**, **ii**) and ED-1-positive cells (**e**, **iii**). Representative immunohistochemistry pictures show isolated neutrophils present in the naïve liver (**e**, **iv**) and large numbers of recruited neutrophils present 2 hours after injury (**e**, **v**). Increased numbers of ED-1-positive cells are observed at 2 hours in the liver (**e**, **iv**). Scale bars, 40 μm.

CCL-2 Is Acutely Elevated in the Meninges and in the Brain Parenchyma after a Delay Following TNF-α Challenge

The temporal and spatial patterns of CCL-2 protein expression were analyzed in the brain parenchyma and meninges by ELISA throughout a 24-hour time course after the microinjection of vehicle or TNF-α into the brain (Figure 5a). Negligible amounts of CCL-2 were detected in the meninges or brain of vehicle-injected rats (results not shown). In comparison with vehicle-injected controls, slight elevations in CCL-2 protein were observed in the TNF-α-injected brain after 2 to 4 hours. These gradually increased with time, peak expression being observed 24 hours after challenge. CCL-2 protein levels in the meninges were strikingly different from those observed in the injected brain. Peak CCL-2 expression was observed in the meninges 4 hours after TNF-α challenge to the parenchyma, falling dramatically by 12 hours. Furthermore, the magnitude of CCL-2 expression in the meninges was higher than that observed in the parenchyma at any time studied.

Immunohistochemistry was used to localize CCL-2 in the brain parenchyma and meninges after TNF-α microinjection into the brain (Figure 5b). No CCL-2 protein was detected in the brain of unchallenged rats (results not shown). After TNF-α injection into the brain, CCL-2 protein was observed adjacent to the TNF-α injection site and was localized as distinct granules within recruited leukocytes (Figure 5bi). This pattern of CCL-2 positivity was also observed throughout the inflamed meninges (Figure 5bii). Double-labeling immunohistochemistry identified CCL-2-positive cells as ED-1-positive cells (Figure 5bii, see inset).

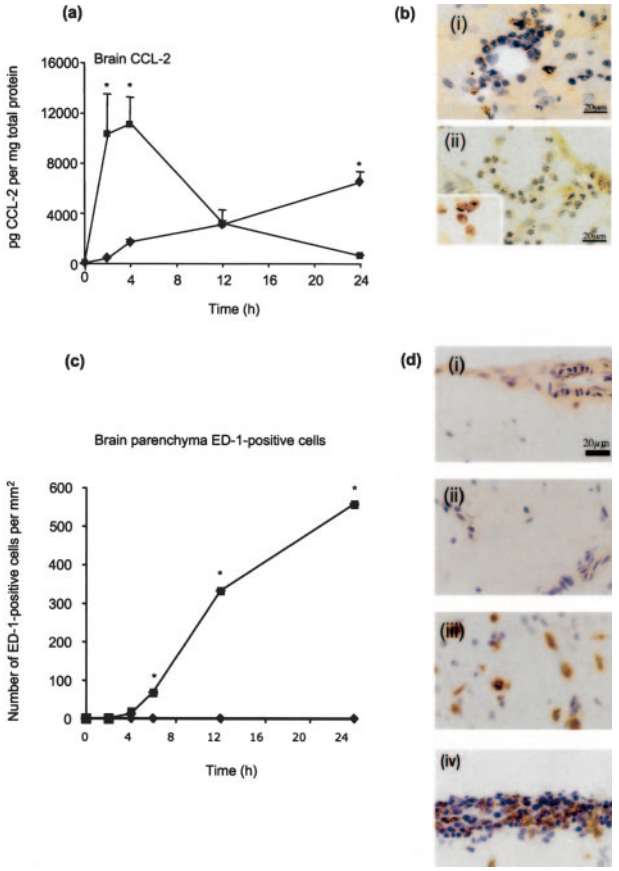

Figure 5. CCL-2 is elevated in individual temporal patterns in different brain compartments after TNF-α challenge. **a:** CCL-2 protein present in the meninges (**squares**) or parenchyma (**diamonds**) was quantitatively assessed by ELISA throughout a 24-hour time course after the injection of TNF-α (1 μg) into the rat brain. Note the early peak in CCL-2 expression after 2 to 4 hours in the meninges and the delayed lower magnitude CCL-2 response in the parenchyma peaking after 24 hours. **b:** CCL-2 protein is immunohistochemically localized in the brain parenchyma close to the injection site (**i**) and also in the meninges (**ii**) in response to the injection of TNF-α into the brain. Double-labeling immunohistochemistry (**b**, **ii**) shown as an **inset**, identifies ED-1-positive cells in the meninges (violet) which are CCL-2-immunopositive (brown). **c:** ED-1-positive cells are present in the brain parenchyma throughout the 24-hour time course after injection of TNF-α (**squares**) or saline vehicle (**diamonds**) into the brain. **f:** Representative photographs showing the absence of leukocytes in the meninges (**i**) or parenchyma (**ii**) 12 hours after vehicle microinjection and their presence in the parenchyma (**iii**) and meninges (**iv**) after injection of TNF-α. **Asterisk** denotes $P < 0.05$ compared to saline vehicle controls. Scale bars, 20 μm.

Leukocytes Are Recruited to the Brain after a Delay Following TNF-α-Induced Inflammation

To establish whether the differential CCL-2 chemokine expression in the brain parenchyma and meninges correlated with the temporal pattern of TNF-α-mediated leukocyte recruitment, we examined the recruitment of ED-1-positive cells and neutrophils by immunohistochemistry (Figure 5, b and c). ED-1-positive cells were recruited to the brain parenchyma or adherent to the lumenal portions of the vasculature throughout the 24-hour period after injection of TNF-α into the brain (Figure 5b). The recruited cells were principally ED-1-positive cells because no neutrophils were observed (results not shown). The recruited ED-1-positive cells could be distinguished as two populations; those which had diapedesed into the parenchyma and those that were associated with lumenal portions of the brain vasculature (Figure 5b). ED-1-positive cells were first detected in the brain associated with the vasculature from 4 hours and steadily increased from 6 hours ($P < 0.0001$) through 12 hours ($P < 0.0001$), peaking at 24 hours ($P < 0.0001$), compared to vehicle-injected controls. Representative histology pictures are included, showing the lack of cellular recruitment in the meninges (Figure 5ci) and parenchyma (Figure 5cii) of vehicle-injected controls and the presence of ED-1-positive cells in the parenchyma (Figure 5ciii) after TNF-α microinjection into the brain.

In parallel with the early CCL-2 expression, recruited ED-1-positive cells were observed in the meninges as early as 2 hours and increased dramatically throughout the 24-hour period (Figure 5civ). Although not observed in the brain parenchyma, neutrophils were found in the meninges. These findings were also true of the choroid plexus where a similar acute inflammatory response was displayed initially after 2 hours increasing to 24 hours (results not shown).

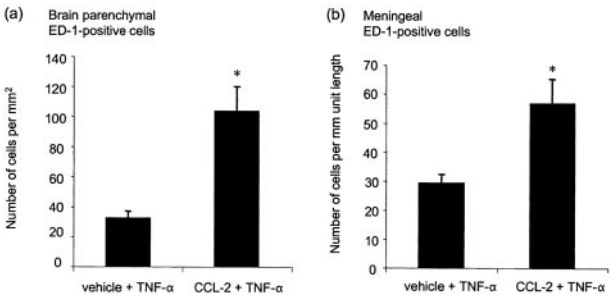

Figure 6. CCL-2 primes monocytes, accelerating their recruitment to the brain. The numbers of ED-1-positive cells present in the brain parenchyma (**a**) and meninges (**b**) were quantitated from immunohistochemically stained sections 4 hours after TNF-α microinjection into the brain together with an intravenous injection of either rrCCL-2 or saline vehicle control. Note the higher numbers of ED-1-positive cells in the brain in response to TNF-α microinjection when rrCCL-2 is intravenously injected into the circulating blood. **Asterisk** denotes $P < 0.05$.

CCL-2 Primes Monocytes, Accelerating Their Recruitment to the Brain

To determine whether the experimental manipulation of the CCL-2 level in circulating blood could affect the monocyte recruitment to the TNF-α-microinjected brain, we intravenously administered CCL-2 or vehicle control at the time of TNF-α microinjection to the brain and assayed the number of recruited monocytes 4 hours afterward (Figure 6). As in our earlier experiments, relatively few ED-1-positive cells were identified in the brain after TNF-α microinjection together with the intravenous injection of saline vehicle. By contrast, a significantly higher number of ED-1-positive cells were observed both in the brain parenchyma and lumenally adherent to the brain vasculature ($P < 0.01$) (Figure 6a) and within the meninges ($P < 0.04$) (Figure 6b) after an intravenous priming injection of CCL-2 at the time of the intracerebral TNF-α microinjection.

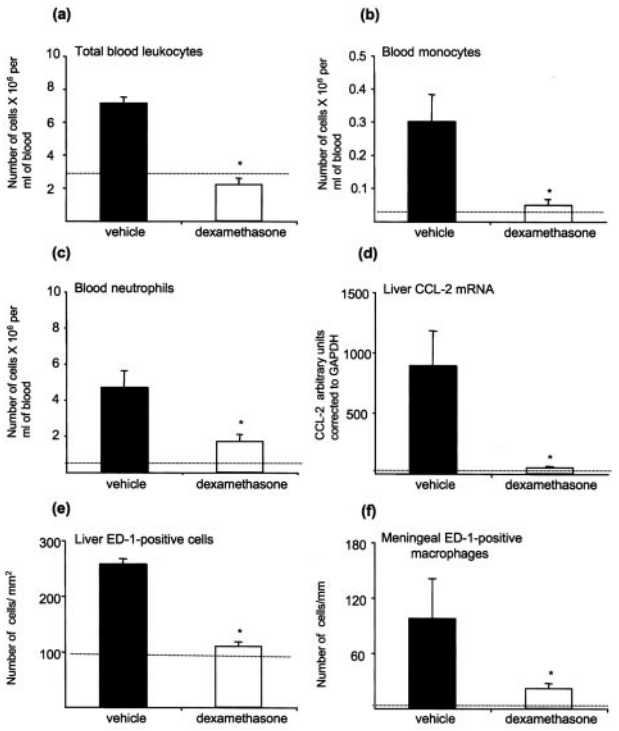

Figure 7. Hepatic amplification of the chemokine response is attenuated by dexamethasone. The numbers of circulating leukocytes were assessed using a hematology analyzer 6 hours after microinjection of TNF-α into the brain, preceded by either saline vehicle (**filled bars**) or dexamethasone (**open bars**). Note the effect dexamethasone has in reducing total leukocytes (**a**), monocytes (**b**), and neutrophils (**c**). **d:** TaqMan RT-PCR shows that dexamethasone also results in reduced levels of CCL-2 mRNA. Note the attenuated recruitment of ED-1-positive cells to both the liver (**e**) and meninges (**f**) in response to dexamethasone. **Dashed line** on each graph represents levels of each component in naïve rats. **Asterisk** denotes $P < 0.05$ compared to saline vehicle controls.

Glucocorticosteroids Block the Hepatic Chemokine Response

Because the principal intervention in the management of acute brain inflammation is the injection of glucocorticosteroids, we sought to identify whether a part of their therapeutic action was to inhibit hepatic chemokine synthesis. To examine this, we administered dexamethasone into the peritoneum before the injection of TNF-α into the brain and examined the response after 6 hours (Figure 7; a to f). Dexamethasone significantly reduced the TNF-α-induced hepatic CCL-2 mRNA response ($P < 0.05$) (Figure 7a). The numbers of ED-1-positive cells recruited to the liver ($P < 0.03$) (Figure 7b), the blood ($P < 0.03$) (Figure 7c), and the meningeal region of the brain ($P < 0.03$) (Figure 7d) were all reduced compared to those observed in control animals injected with saline vehicle before the microinjection of TNF-α.

Discussion

The microinjection of TNF-α into brain parenchyma gives rise to a leukocyte recruitment profile that is dominated by monocytes and T cells.[21] This pattern of recruitment is quite distinct from that induced by the microinjection of IL-1β into the brain, which is characterized by the recruitment of neutrophils. The microinjection of IL-1β into the brain is also associated with the hepatic expression of the CXC chemokine CXCL-1, which appears to control the timing and magnitude of neutrophil mobilization and subsequent recruitment to the brain.[1] As the expression of TNF-α in the brain is associated with a broad spectrum of CNS pathology,[30] we sought to determine whether the distinct pattern of leukocyte recruitment induced by TNF-α might be reflected in the hepatic chemokine response. We found that 2 hours after the microinjection of TNF-α into the brain parenchyma there is rapid, but transient, *de novo* synthesis of CCL-2 mRNA and protein in the liver, which occurs in parallel with marked elevation of CCL-2 levels in the blood. By comparison, CCL-2 protein gradually accumulated in the brain parenchyma throughout a 24-hour period in response to the TNF-α. The presence of CCL-2 in the blood was associated with the mobilization to the circulation of leukocytes, which migrated into the liver and, after a delay, into the brain. The rapid elevation of CCL-2 in the liver, which appears to be expressed by cells of hematopoietic origin, demonstrates that after microinjection of TNF-α into the brain, the hepatic chemokine response to focal brain inflammation is not restricted to the CXC chemokines.[1] Finally, we demonstrate that a CC and CXC hepatic chemokine response is elicited after a controlled compression lesion to the CNS and thus is likely to be a component of the normal response to tissue injury.

Hepatic Chemokine Production Regulates Leukocyte Recruitment

As a consequence of the injection of TNF-α into the brain, elevated hepatic chemokine expression results in the recruitment of leukocytes. The pattern of leukocyte recruitment to the liver was dominated by mononuclear cells, and reflected the pattern of recruitment observed (after a delay) in the brain parenchyma. Thus it would appear that after the microinjection of TNF-α into the brain, hepatic monocyte chemoattractants dominate, and are likely to contribute to the restricted leukocyte recruitment observed in the CNS. In non-CNS tissue the expression of TNF-α results in a mixed inflammatory infiltrate consisting predominantly of neutrophils, but also of monocytes and eosinophils;[31] this is thought to be a result of an interwoven network of local cytokine induction.[32] We have shown that this network of cytokine production is not conserved in the brain.[33] Our discovery that the liver produces CCL-2 in response to an injection of TNF-α into the brain demonstrates that the hepatic chemokine response is more generalized and is not restricted to CXC chemokines. Considering the relative mass of the organs involved, we propose that the liver is the principal source of acute chemokine production after injury and that the liver chemokines amplify the injury response. At early time points CCL-2 is also present in the meninges, but its relatively small mass makes it unlikely to be a major contributor to the CCL-2 pool. We believe the liver to be the dominant contributor as the

numbers of recruited ED-1-positive cells in other organs such as spleen, kidney, and heart displayed no elevations in numbers, implying no increase in chemokine synthesis. The lung did display elevated numbers of ED-1-positive cells, but the percentage increase was much smaller than for the liver (results not shown). There is evidence to suggest that the liver inflammatory response is often linked to changes in the lung and vice versa.[34]

Hepatically Released Chemokines Stimulate a Leukocytosis

Our observation that the liver responds to brain inflammation by producing chemokines is a recent observation,[1] but the ability of blood-borne chemokines to mobilize distinct leukocyte populations has been previously recognized. CXCL-2,[35] CXCL-8,[36] or CXCL-1[1] can mobilize bone marrow neutrophils, resulting in neutrophilia when these chemokines are elevated in the circulating blood. Furthermore, IL-5 and eotaxin are together required for the eosinophilia associated with lung injury.[37] The timing of leukocytosis after the injection of TNF-α into the brain is consistent with systemic increases of CCL-2 in the liver and the blood. CCL-2 has been shown to stimulate migration of bone marrow macrophages *in vitro*,[38] but hitherto there had been no *in vivo* demonstration that CCL-2 can induce a leukocytosis. In this study, we show that intravenous injection of CCL-2 causes a selective elevation in the number of circulating monocytes. The degree of monocyte mobilization, in response to the intravenous injection of CCL-2, was reduced compared to that observed after the TNF-α challenge. The TNF-α challenge to the brain may co-induce expression of a number of other hepatic CC chemokines that also have the ability to mobilize monocytes. It is also likely that endogenous regulatory mechanisms ensured that our single intravenous injection of CCL-2 was rapidly cleared from the circulation. The injection of TNF-α into the brain increased the number of circulating monocytes and, briefly, neutrophils. Neutrophils express CCR1 and CCR3 and have been found to respond to CC chemokines,[39] which may be sufficient to promote mobilization, but insufficient to allow tissue-specific recruitment.

Brain Inflammation Is Unlikely to Be Communicated to the Liver by Blood-Borne TNF-α

The route of communication between brain and liver has not been established, but we show that this communication is unlikely to be due to drainage of the injected cytokine from the brain to the blood because no TNF-α was detected in the blood 2 hours after microinjection of TNF-α into the brain. However, the ELISA that we used to detect TNF-α is sensitive to 12.5 pg/ml, and it is possible that some TNF-α is present in circulation below this limit of detection. Significantly elevated TNF-α levels were observed in the blood after an intraperitoneal injection of TNF-α, but the labile nature of TNF-α is clearly demonstrated by its rapid clearance from the circulation. Indeed, relatively little of the intraperitoneally injected TNF-α can be detected in serum at 2 hours. Furthermore, we show that 2 and 4 hours after a controlled compression injury to the spinal cord, where no cytokines have been microinjected, a hepatic chemokine response is elicited which supports our previous findings[1] of injury-driven hepatic chemokine synthesis. This is the first description of how a traumatic injury response in the CNS can give rise to the hepatic expression of chemokines and the recruitment of leukocytes to the site of injury as well as to the liver. The spinal cord compression lesion is associated with a mixed inflammatory cell infiltrate to the cord, and it is perhaps not surprising that the injury gives rise to both hepatic CXC and CC chemokine synthesis. We do not believe that the hepatic chemokine response is unique to CNS injury; indeed, we have found that the microinjection of IL-1β into a pyrogen testing air-pouch model of focal systemic inflammation also generates a hepatic response (unpublished observations). However, the atypical features of CNS inflammation and the problematic nature of delivering drugs to the CNS give good cause to consider the hepatic response as a new target for therapeutic intervention for CNS injury and disease.

Chemokine Antagonists May Regulate the Hepatic Chemokine Response

Hitherto the target of chemokine antagonist activity has always been considered to be at the local injury site, and studies have ignored the possibility that the antagonists may have been exerting effects elsewhere.[19,40] We have previously shown that the peripheral administration of neutralizing anti-CXC chemokine antibodies inhibits the local inflammatory response in the injured brain, but this effect was accompanied by a marked fall in leukocytosis and in cellular recruitment to the brain and to the liver.[1] Thus the results of chemokine-neutralizing experiments may now require reinterpretation in light of our new observations.

The accepted model of leukocyte recruitment along a chemokine gradient proposes that local chemokines at the site of injury attract a circulating population of phagocytes from the blood up a concentration gradient. This model is problematic because repeated studies have failed to show lumenal expression of chemokines on the vascular endothelium, which is central to the paradigm.[41] Our studies now raise further problems: high levels of chemokines are present in the blood at a time when local chemokines are being produced, thus confounding the establishment of the necessary chemokine gradients. The ratio of local and systemic chemokine production has been found to be a critical factor dictating local leukocyte recruitment in peripheral organs.[42,43] We hypothesize that after acute brain injury, and most probably many other injuries, the absolute level of circulating CCL-2 and other CC chemokines is a pivotal factor governing the process of mononuclear cell recruitment. Transgenic studies would seem to support our hypothesis: chronic overexpression of systemic CCL-2 prevents monocytes

from responding to local CCL-2 at the site of injury.[44] This finding may reflect tachyphylaxis to CCL-2, but it might equally be the result of altered chemokine gradients. Tachyphylaxis is not observed following targeted tissue-specific expression of CCL-2, which leads to the recognized pattern of tissue-specific monocytic infiltration.[15] It is clear that the classical model of leukocyte recruitment requires updating.

Leukocyte Recruitment to the Brain Is Temporally Regulated by CCL-2 Expression

We show that CCL-2 protein is expressed both acutely in the meninges (peaking at 4 hours) and after a delay in the brain parenchyma (24 hours) after the microinjection of TNF-α. Recruited and marginating ED-1-positive cells were positive for CCL-2 both in the meninges and, after a delay, in the brain parenchyma (12 hours). The timing of CCL-2 expression in the different brain compartments was consistent with the observed temporal pattern of ED-1-positive cell recruitment to the brain. Within the brain parenchyma, the majority of ED-1-positive cells were present within the lumen of the microvasculature of the injected hemisphere, which may contribute to the reduction in regional cerebral blood volume associated with the microinjection of TNF-α into the brain.[22] The accumulation of monocytes in vessels was not a feature in the meninges, the majority of the ED-1-positive cells were ablumenal. In studies in which TNF-α has been superfused on to pial vessels, blood flow has been shown to increase,[45] which is consistent with our observations in which lumenal plugging is not a feature.

CCL-2 Can Accelerate TNF-α-Dependent Monocyte Recruitment

Despite the complexity of the CC chemokine family, we were able to show that artificial elevation of circulating CCL-2 alone was sufficient to accelerate the rate of monocyte recruitment to the TNF-α-microinjected brain. These findings have important implications for the management of brain injury because they complement previous studies from our laboratory suggesting that systemic infections in which an accompanying leukocytosis is present may be sufficient to accelerate inflammation in the brain.[46,47]

Glucocorticosteroids Block the Hepatic Chemokine Response

Dexamethasone is a clinically relevant glucocorticosteroid used to treat acute brain injury and disease. In particular, clinical studies have highlighted the value of dexamethasone treatment for meningitis in children,[48] but the mechanism of action is ill-defined. Dexamethasone has previously been shown to decrease CCL-2 transcription and destabilize CCL-2 mRNA[49–51] and probably has similar effects on other CC chemokines. We sought to determine whether part of the action of glucocorticosteroid therapy for acute brain inflammation might be suppression of the hepatic chemokine response, leading to reduced leukocyte recruitment to the brain. After microinjection of TNF-α into the brain parenchyma, dexamethasone significantly reduced hepatic CCL-2 mRNA, leukocytosis, and leukocyte recruitment to both the liver and the brain. Our findings certainly suggest that part of the mode of operation of the glucocorticosteroids may be through the attenuation of the hepatic chemokine response.

Our present findings suggest that anti-inflammatory therapy used to treat brain injury need not necessarily cross the blood-brain barrier, as had been previously thought, and that seemingly efficacious drugs that do not cross the blood-brain barrier may be exerting their action in the periphery through their control of the hepatic chemokine response.

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