Interacting Chemokine Signals Regulate Dendritic Cells in Acute Brain Injury

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Abstract
Brain trauma is known to activate inflammatory cells via various chemokine signals although their interactions remain to be characterized. Mice deficient in Ccl3, Ccr2 or Cxcl10 were compared with wildtype mice after controlled cortical impact injury. Expression of Ccl3 in wildtypes was rapidly upregulated in resident, regularly spaced reactive microglia. Ccl3-deficiency enhanced endothelial expression of platelet selectin and invasion of peripheral inflammatory cells. Appearance of Ccr2 transcripts, encoding the Ccl2 receptor, reflected invasion of lysozyme 2-expressing phagocytes and classical antigen-presenting dendritic cells expressing major histocompatibility complex class II. Ccr2 also directed clustered plasmacytoid dendritic cells positive for the T-cell attracting chemokine Cxcl10. A reduction in Ccr2 and dendritic cells was found in injured wildtype cortex after cyclophosphamide treatment resembling effects of Ccr2-deficiency. The findings demonstrate the feasibility to control inflammation in the injured brain by regulating chemokine-dependent pathways.

Introduction
Traumatic brain injury (TBI) in mice results in distinct upregulation of hundreds of gene transcripts including many linked to inflammation [1,2]. The inflammatory cascade activated by TBI lacks specific pharmacological treatments and is elicited by mechanisms including reactive oxygen species (ROS), hemorrhage and release of nucleotides sensed by upregulated pyrimidinergic receptors such as P2ry6 [1]. This leads to activation of resident microglia offering an instantaneous response [3]. In addition, invading classical (cDCs) and plasmacytoid dendritic cells (pDCs) [4] become engaged in the inflamed central nervous system. The interactions and signals among these inflammatory cells are not fully understood but one possibility includes a balance among chemokine networks.

Chemokines and chemokine receptors are known to orchestrate inflammatory responses via chemotaxis in injured tissues [5,6]. We have previously described the appearance of clustered inflammatory cells expressing chemokine Cxcl10 scattered in grey and white matter of injured wildtype (wt) brains using a controlled cortical impact (CCI) injury model [1,2,7,8]. At the molecular level, strong transcriptional activation after traumatic brain injury can also be seen in chemokine pathways including Ccl3 (with cognate receptors Ccr1 and Ccr5) and Ccr2 (with strongly upregulated ligands Ccl2 and Ccl12) as listed in Table 1. Although this suggests postinjury chemokine functions in brain tissue the interactions among different chemokines, cellular sources of signals and targets for signaling remain unknown. In order to establish a possible interplay among chemokine pathways, we studied knock-out Ccl3−/−, Ccr2−/− and Cxcl10−/− mice subjected to TBI. Based on the findings, a model for hierarchical chemokine activities in the injured cerebral cortex is currently presented.

In a previous report [2] we demonstrated general similarities among inflammatory responses after TBI and neurodegenerative disorders in mouse models. Our results distinguish the brain injury response in resident microglia cells in the brain parenchyma from the contribution of invading immune cells providing phagocytes, antigen-presenting dendritic cells as well as clustered Cxcl10-producing cells. We also investigated posttreatment in injured mice using the immune-suppressing agent cyclophosphamide, clinically administered to patients with severe forms of systemic lupus erythematosus, in order to consider a pharmacological compound for treatment of TBI. Similar to the outcome of TBI in Ccr2-deficient mice, cyclophosphamide dampened the increases of dendritic cells and limited markers of antigen presentation in the injured brain. The study design is presented in Table 2.

Results
Ccl3 and Ccr2 transcripts are differentially regulated in the injured cerebral cortex
TBI rapidly results in local expression of chemokine Ccl3 in the mouse neocortex [1]. By in situ hybridization, responding cells were seen regularly spaced in ipsilateral neocortex, hippocampus and subcortical structures including mesencephalon in a pattern indicating Ccl3 expression in reactive resident microglia (Fig-
Table 1. Chemokine ligands and receptors relevant for this report.

| Chemokine      | Alternate name                                      | Receptor     |
|----------------|----------------------------------------------------|--------------|
| Ccl3           | chemokine (C-C motif) ligand 3                     | Ccr1, Ccr5   |
| Ccl2           | chemokine (C-C motif) ligand 2                     | Ccr2         |
| Cxcl10         | chemokine (C-X-C motif) ligand 10                  | Cxcr3        |
| Csf2rb1        | chemokine (C-C motif) ligand 2                     | Ccr2         |
| Cxcl10         | chemokine (C-X-C motif) ligand 10                  | Cxcr3        |

Ccr2 deficiency results in reduced Lyz2 level and smaller cavity volume

We also examined the outcome of TBI in wt mice compared to homozygous Ccr2-deficient mice [10] three days postinjury. Lysozyme 2 (Lyz2) previously identified by microarray analysis as injury-induced in neocortex [1] was among the transcripts found reduced in the injured Ccr2−/− cortex. From in situ hybridization, Lyz2 expression was obvious in large phagocyte-like cells only partially overlapping with the activated microglia cell-surface marker isolectin B4 (IB4) [1] in injured neocortex and hippocampus (Figure 1E). This pattern contrasts with the clustered appearance of Bst2 (bone marrow stromal cell antigen 2, encoding PDCA-1, plasmacytoid dendritic cell antigen 1) expressed by pDCs [11] (Figure 1E, insert left). qRT-PCR confirmed that Lyz2 injury-increased expression was lower in Ccr2−/− compared to wt cortices. In contrast, Lyz2 was further upregulated in injured Ccl3−/− brains compared to wt brains and thus in parallel with Ccr2 expression levels (Figure 1F).

The injury-induced cortical cavity volume seen in wt brains was significantly reduced in the Ccr2−/− mice seven days after injury (Figure 1G). This is in line with the reduced upregulation of Lyz2 in the injured Ccr2−/− cortex and suggests that fewer tissue-eliminating phagocytic cells invaded the injured brain when the Ccl2/Ccl12 attraction mediated by Ccr2 signaling was interrupted.

Deletion of Ccr2 does not affect injury-evoked Ccl3 but reduces Cxcl10 expression

The Ccl3 expression increased in concert with Ccr2−/− mice (Figure 2A) whereas Cxcl10 injury-induced expression [1,2] was markedly dampened in brains lacking Ccr2 (Figure 2B). In contrast, qRT-PCR showed that Cxcl10 expression was elevated above wt levels in the injured Ccl3-deficient brain, similar to shifts in Ccr2 and Selp transcripts (Figures 1C and D). The increased Cxcl10 expression appears in clustered inflammatory cells [1] and we earlier showed [2] that injured Cxcl10−/− mice [12] lacked this spotted staining. The clusters of positive cells appear with

Table 2. Study design.

| Aim                                      | Experiments                                                                 |
|------------------------------------------|------------------------------------------------------------------------------|
| Outcome of TBI in different chemokine-deficient mouse strains (wildtype versus Ccl3−/−, Ccr2−/−, and Cxcl10−/−) | qRT-PCR, microarray expression analysis, in situ hybridization, isolectin B4 histochemistry, brain cavity volume measurement, flow cytometry, mixed genetic background analysis |
| Characterization of two distinct dendritic cell types in the injured cerebral cortex | Magnetic cell sorting, immune depletion of plasmacytoid dendritic cells, followed by qRT-PCR |
| Testing suppression of dendritic cells in the injured cortex by cyclophosphamide treatment | qRT-PCR, microarray expression analysis, flow cytometry |

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higher intensity in the injured Ccl3−/− cortex compared to wt (Figure 2C). In injured Ccr2−/− mice, expression of Cxcl10 was evident in cell clusters but at reduced labeling intensity in line with the qRT-PCR data (Figure 2B).

Cxcl10 expression did not affect injury-induced upregulation of Ccl3 and Ccr2 transcripts three days post-injury in traumatized Ccl3−/− mice (Figure 2D). A model of chemokine interactions during the initial three days following injury is presented in Figure 2E. The injury resulted in independent increases in cortical levels of Ccl3 and Ccr2 transcripts. Since, Ccr2 affected Cxcl10 levels (Figure 2B), but not the reverse (Figure 2D), Ccl3 is positioned downstream and positively regulated by Ccr2. Furthermore, Ccl3-deficiency increased the injury-evoked Ccr2 levels (Figure 1C) indicating that Ccl3 normally exerts a suppressive effect on Ccr2 and thus indirectly limit Cxcl10 expression in the injured brain.

**Number of dendritic cells is reduced in the injured Ccr2-deficient cortex**

We further investigated the role of Ccr2 signaling in appearance of inflammatory cells in the injured neocortex. Flow cytometry in fractionated dissociates from cortex three days after injury demonstrated the presence of both cDCs and pDCs in the injured neocortex (Figures 3A and B). Cd11c encoded by Itgax (alpha-X integrin) was used as a marker for cDCs [13]. pDCs [11] were characterized by PDCA-1 whereas CD45 (encoded by Ptprc) was used as a pan-leukocyte marker [1,2]. Optimal gating for the two classes of dendritic cells (Figure 3A, left) identified double-positive cells (Figure 3A, right). Isotype controls gave low background signals whereas Cd11c- and PDCA1-positive cells increased less in the Ccr2−/− compared to wt brains (Figure 3B). Uninjured brains showed only few cells double-positive for these markers. The data support the notion of impaired infiltration of peripheral inflammatory cells in mice lacking the Ccr2 chemokine receptor.
The expression of chemokine ligands was detected by qRT-PCR in injured brains, although with different intensities depending on genotype. Expression was stronger in the injured Ccr2−/− mice and weaker in the Ccr2+/− mice compared to wt brains. In contrast, the heme oxygenase (decycling) 1 (Hmox1) transcript located juxta-positioned to the focal injury [1], was similarly upregulated in injured wt and mutant brains with a peak after three days (Figure 3C).

Genetic background does not account for the differential effects of chemokine signaling

We tested for possible contribution from the genetic background to the strikingly opposite effects of Ccl3- and Ccr2-deficiencies (Figure 4). Crossing the inbred knockout strains resulted in expected Mendelian ratios of homozygous Ccl3−/− and Ccr2−/− mice in male F2 offspring, in line with location of these genes on chromosome 11 and 9, respectively. Cortical injury increased Lyz2 levels in F2 Ccl3−/− mice whereas being reduced in F2 Ccr2−/− mice. The injury increases in Ilgax and Cxcl10 transcripts did not reach above wt levels in the F2 Ccl3−/− mice in contrast to Bst2. In the F2 Ccr2−/− cortices, Lyz2, Ilgax, Cxcl10 and Bst2 were robustly reduced confirming results from the parental strains even in a mixed genetic background. It is of note that our genotyping of Ccl3-deficient mice does not
distinguish Ccr2+/+;Ccl3−/− from Ccr2−/−;Ccl3−/− and this heterogeneity may contribute to the lack of upregulation of Itgax and Cxcl10 in F2 Ccl3−/− mice. Irrespective of genotype and genetic background, Gfap levels were similar in all injured cortices.

Antigen presentation marker is limited to classical dendritic cells

Next, we isolated dendritic cell populations from wt neocortex three days after injury using anti-Cd11c and anti-PDCA-1 antibodies coupled to magnetic beads. Subsequent RNA analysis of the inflammatory cells sorted on anti-Cd11c showed enrichment of Itgax and H2-Aa transcripts (Figure 5A). Cells sorted on anti-PDCA-1 beads showed only weak expression of these two transcripts while highly expressing Bst2. In order to examine correlation between pDCs and Cxcl10 expression, antibodies directed against PDCA-1 showed weak expression of these two transcripts while highly expressing Bst2. In order to examine correlation between pDCs and Cxcl10 expression, antibodies directed against PDCA-1 were administered to TBI mice and cells fractionated from the brain for RNA isolation. qRT-PCR showed that Itgax and H2-Aa transcripts were induced to the same extent in the injured neocortex whether the injected antibodies were directed against PDCA-1 or represented control IgG immunoglobulins (Figure 5B, left). In contrast, Bst2 and Cxcl10 transcripts were reduced in mice receiving anti-PDCA-1 antibodies compared to mice given equal doses of normal rat IgG (Figure 5B, right). These data support pDCs as the source of Cxcl10 in injured neocortex.

Cyclophosphamide treatment resembles Ccr2 deletion by limiting injury-induced transcripts

We finally turned to intraperitoneal administration of the chemotherapeutic and anti-inflammatory agent cyclophosphamide to examine possible effects on injury-induced inflammatory cells in wt cerebral cortex three days postinjury. Microarray data from the cyclophosphamide-treated mice were compared to corresponding data from injured Ccr2−/− mice and wt mouse neocortex. Analysis of these three groups identified 20 genes that fulfilled the criteria of being upregulated more than two-fold in injured wt neocortex and reaching levels less than half of this in both Ccr2−/− mice and cyclophosphamide-treated mice (Table 3). Several of these transcripts are involved in inflammatory processes e.g. Ccr2, Chil3, H2-Aa, Ifi204, Mx44c, Plac8, Stat1 and Tgfb1. The Ccr2 reduction by cyclophosphamide in concert with transcripts supporting antigen presentation indicates a major effect on cDCs (H2-Aa, Itgax; Figures 6A and B). Lyz2 and Cxcl10 transcripts (qRT-PCR; n.s.) were not affected whereas a slight reduction of
Bst2 (qRT-PCR; P<0.05) suggests marginal influence of cyclophosphamide on pDCs but not phagocytes/macrophages. The upregulation of Cx3cr1 encoding the fraktalkine receptor and Ccl3 (Figures 6A and B), both characteristic of resident microglia, as well as Gfap and Hmox1 (qRT-PCR; n.s.), were not affected by the cyclophosphamide treatment.

Figure 4. Inflammatory-related transcripts measured in Ccl3−/− and Ccr2−/− mice with F2 hybrid background three days postinjury. Lyz2 expression was upregulated in homoyzogous Ccl3 knockouts but downregulated in Ccr2−/− as in the parental strains, as was Bst2. Both Itgax and Cxcl10 levels were less upregulated in the injured F2 Ccr2−/− brains. Gfap expression increased to the same extent in injured wt and F2 hybrid brains.

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Figure 5. Cell sorting and immunodepletion of dendritic cells in neocortex three days after TBI in wt mice analyzed by qRT-PCR. (a) Cd11c-positive cells sorted on magnetic microbeads expressed enhanced H2-Aa and Itgax levels. In contrast, Bst2 was enriched in cells sorted on anti-PDCA-1 microbeads. (b) Cortical levels of inflammatory transcripts in mice injected with control immunoglobulin or with antibodies directed to PDCA-1. Depletion of pDCs was not accompanied by shifts in Itgax or H2-Aa transcript levels whereas a distinct reduction of Bst2 and Cxcl10 occurred.

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Flow cytometry three days after injury treated with cyclophosphamide showed a robust drop in number of inflammatory CD45 (Ptprc) and Cd11c (Itgax) double-positive cells isolated from neocortex (Figure 6C) supporting RNA data at the protein/cell levels. Double isotype labeling in the area gated for cDCs yielded low background. The reduced Cd11c expression after cyclophosphamide treatment was confirmed by repeated experiments (Figure 6C, bottom right). Overall, these results demonstrate that it is feasible to interact pharmacologically with selected inflammatory cells after brain trauma as summarized in Table 4.

Discussion

The present findings confirm that interactions among chemokines in the injured brain set the stage for inflammatory cell activation. While resident microglia and astrocytes become engaged, a flow of invading immune cells from the periphery after trauma is also evident.

In particular, the current data demonstrate a strong activation of dendritic cells in the injured brain depending on immigration of Ccr2 positive cells from the peripheral circulation [14]. Our data support a pivotal role of infiltrating cells of monocyte lineage in disease progression as demonstrated in previous reports using parabiotic mice and genetic deletion of the Ccr2 receptor [15]. We show that this diapedesis is valid for cDCs [4], pDCs [16] and large phagocytes expressing Lyz2. The activation of dendritic cells in the injured cortex is distinct from the inflammatory response among resident, locally renewing microglia [17–19] characterized by Cx3cr1 expression [20] and found to exert important surveillance functions in the brain [3].

cDCs express integrin alpha X (Itgax/Cd11c) and major histocompatibility class II complex (MHC II, e.g. H2-Aa) [21–23]. These cells coordinate a host of immune responses by capturing and processing proteins to peptides that are presented to T cells on MHC II [4]. Our data link H2-Aa with Itgax-expressing DCs serving as professional antigen presenting cells. These cells have been demonstrated to sufficiently trigger auto-reactive encephalitogenic T cells and to initiate CNS inflammation [24]. A distinct focal co-localization of cDCs and T cells in the inflamed brain, highly reminiscent of the current injury-induced inflammatory cell clusters, was shown and suggested to be indicative of CNS acting as a neolymphoid tissue [24]. The present results also connect pDCs, characterized by the Bst2 transcript [11,25] and its encoded protein PDCA-1, with the expression of Cx3cr1 in injured...
mouse cortex, in agreement with reports on CXCL10 production in activated human pDCs [26,27].

Injury-induced activation of the chemokine Ccl2/Ccr2 axis is demonstrated by the current data. Previously, monocytes have been identified to express Ccr2 as well as high levels of Ly-6C and found to be crucial for autoimmune inflammation in the CNS [28], offering a target for treatment strategies [29]. An important role for the Ccr2 receptor in Ccl2 attraction of monocytes has been demonstrated [10]. Moreover, using a selective Ccr2 antagonist reduced apoptosis while improving behavioral performance in a rat TBI model [30]. Currently, a drastic increase in Ccr2-linked transcripts was observed in the injured Ccl3-deficient brains. This is not attributable to increased Ccl2 levels attracting inflammatory Ccr2 positive cells. A plausible explanation is offered by enhanced upregulation of platelet selectin, encoded by Selp, found in the vasculature of the injured Ccl3−/− brains. The Ccl3−/− mice have been shown to exhibit normal numbers of hematopoietic progenitor cells in peripheral blood and bone marrow and of T cells in lymph nodes and spleen [9]. The pathway by which Ccl3 is regulating Selp expression remains unknown but may result in curtailed endothelial expression of Selp and position Ccl3 as a suppressor of Ccr2-expressing cells in the injured neocortex.

The present data also demonstrate that large phagocytic cells expressing Lyz2 depend on invading Ccr2-positive cells and do not overlap with activated endogenous microglial cells. The injury-induced cortical cavity formation was reduced in our Ccr2−/− mice after seven days. This is in parallel with the reduction of Lyz2 and adds to the idea that Ccr2 is a key receptor in recruitment of different invading peripheral cells. A knock-in strategy to express Cre recombinase from the Lyz2 locus in mice [31] showed high expression of Lyz2 in macrophages and only low expression in Cd11c+ splenic dendritic cells. Also, absence of Ccr2 reduced infarct sizes in a mouse model of cerebral ischemia/reperfusion injury [32], correlating with the current demonstration of reduced cortical cavity after TBI in Ccr2-deficient mice.

The alkylating agent cyclophosphamide affects DNA synthesis e.g. in immune cells in mice [33], acting as an immunosuppressant with clinical applications in autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. Presently, an impaired invasion of inflammatory cells in injured wt brain

| Table 3. Transcripts increased at least two-fold three days postinjury in wt cerebral cortex and with upregulation reduced by at least 50% in Ccr2−/− mice as well as in wt mice treated with cyclophosphamide. |
|-----------------|-----------------|-----------------|-----------------|
| **Gene Symbol** | **Gene Title**  | **GenBank**     | **Probe Set ID** |
| Apoc2           | apolipoprotein C-II | NM_009695         | 1418069_at       |
| Ccr2            | chemokine (C-C motif) receptor 2 | NM_009915         | 1421186_at       |
| Chi3L3          | chitinase 3-like 3 | NM_009892         | 1419764_at       |
| Ddx60           | DEAD (Asp-Glu-Ala-Asp) box polypeptide 60 | NM_001081215      | 1451777_at       |
| H2-Aa           | histocompatibility 2, class II antigen A, alpha | NM_010378         | 1435290_x_at     |
| Ifi271a         | interferon, alpha-inducible protein 27 like 2A | NM_029803         | 1426278_at       |
| Ifi35           | interferon-induced protein 35 | NM_027320         | 1445897_s_at     |
| Ifi44           | interferon-induced protein 44 | NM_133871         | 1423555_a_at     |
| Ifi203          | interferon activated gene 203 | NM_001045481      | 1452231_x_at     |
| Ifi204          | interferon activated gene 204 | NM_008329         | 1419603_at       |
| Ifi205          | interferon activated gene 205 | NM_172648         | 1452349_x_at     |
| Igf15           | IGF15 ubiquitin-like modifier | NM_015783         | 1431591_s_at     |
| Mx4a4c          | membrane-spanning 4-domains, subfamily A, member 4C | NM_022429         | 1450291_s_at     |
| Mx1             | myxovirus (influenza virus) resistance 1 | NM_010846         | 1451905_a_at     |
| Plac8           | placenta-specific 8 | NM_139198         | 1451335_at       |
| Pyhin1          | pyrin and HIN domain family, member 1 | NM_175026         | 1435331_at       |
| Sirpb1a         | signal-regulatory protein beta 1A | NM_001002898      | 1448025_at       |
| Stat1           | signal transducer and activator of transcription 1 | NM_009283         | 1450034_at       |
| Tgfbi           | transforming growth factor, beta induced | NM_009369         | 1415871_at       |
| Usp18           | ubiquitin specific peptidase 18 | NM_011909         | 1418191_at       |
| **Table 4. Responding immune cells/cells responding in TBI.** |
| **Cell type**   | **Expressed markers and molecules** |
| Activated microglia | Ccl3, Cx3cr1, Ib4-binding |
| Phagocytes/macrophages | Ccr2, Lyz2 |
| Plasmacytoid dendritic cells | Ccr2, Bst2/PDCA-1, Cxcl10, CD45 |
| Classical dendritic cells | Ccr2, Itgax/Cd11c, H2-Aa, CD45 |

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receiving cyclophosphamide administration was shown by restriction of injury-evoked Ccr2, Il17a and H2-Aa expression linked to a reduction of inflammatory Cd11c/Il17a positive cDCs. In contrast, cyclophosphamide did not affect the injury-elicted upregulation of Ccl3 or Cx3cr1 in resident microglia, had only marginal effects on pDCs and showed no manifest influence on phagocytes.

The current inflammatory response in the injured brain does not obviously fit the concept of Th1/M1 versus Th2/M2 divergence in macrophage functions [34]. Our microarray data show injury-evoked changes in transcripts assigned to pro-inflammatory, classically activated M1 (characterized by e.g., Cxcl10, Il6 and H1) and anti-inflammatory, alternatively activated M2 macrophages (associated with Arg1, Chi3l3, Il4ra, Il13ra1, Tgfβ1, Msr1 and Cebpδ) [35,36]. Among Th2/M2-transcripts, Chi3l3 was less upregulated both in injured Ccr2−/− brains and cyclophosphamide-treated wt brains. Thus, the present injury model engaged transcripts in both of these pathways.

To what extent do the current findings from the injured mouse brain apply to human clinical conditions? A poor resemblance of inflammatory responses in humans and mice has been suggested from large scale genomic studies of systemic inflammation [37]. One particular case is the Selp promoter that is strongly upregulated in the mouse by various insults but unresponsive to inflammation in humans [38]. However, Selp may have other functional counterparts in the endothelia of patients. Nevertheless, in general the present findings are in line with observations of both cDCs and pDCs in patients with neurological conditions involving inflammation [39]. Also, several of the currently identified inflammatory transcripts are associated with human injuries. Thus, CCL2 and CCL3 transcripts were both increased in patients with posttraumatic brain contusion [40]. CXCL10, CCL3 and CCL2 were upregulated in plasma and microdialysis perfusates in TBI patients [41,42] and CCL2 found elevated in cerebrospinal fluid [43]. Moreover, increased levels of the CCL2 protein in serum of both civilian TBI patients and military blast-induced mild TBI cases have been demonstrated and found to be a potential risk factor for subsequent dementia [44]. Finally, the human orthologue of mouse H2-Aa, known as HLA-DQA1, encodes one of the HLA class II alpha chains and has been associated with autoimmune conditions including asthma, myasthenia gravis and celiac disease [45–47]. Taken together, our results suggest similarities in the studied systems between man and mouse.

The present data demonstrate separate repertoires among invading inflammatory cells of monocytic lineage distinct from those of activated resident cells. Moreover, a potential of dampening specific inflammatory cells invading the injured brain by pharmacological means is revealed. Thus, our findings suggest a time-window for therapeutic interference of invading Ccr2-positive, antigen-presenting cells after traumatic brain injury.

Materials and Methods

Traumatic brain injury (TBI)

Male C57BL/6 (B6) mice with a body weight of 25–35 g were used for the traumatic brain injury (TBI). The mice were anaesthetized with 3.5% isoflurane (in combination of 70% nitrous oxide and 30% oxygen) before being transferred to a stereotactic frame. Anaesthesia was continued with 1–2% isoflurane, using mask ventilation and spontaneous breathing for the rest of the procedure. Bupivacaine (0.3 mg) was injected subcutaneously in the neck providing local analgesia. Body temperature (37°C) was controlled rectally throughout surgery. A craniotomy (approximately 3 cm2) was made over the right parietal cortex between midline, bregma and lambda. The mice were subjected to controlled cortical impact (CCI) injury [7,9] by a pneumatic impact device (model AMS 201, AmScien Instruments, Richmond, Virginia, USA). The compression depth was 0.5 mm, compression duration 100 ms and the velocity 3.1 m/s, resulting in a severe, focal injury. Control tissues were from uninjured mice without craniotomy. All efforts were made to minimize animal suffering in compliance with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Uppsala Ethical Committee on Animal Experiments evaluated and authorized all experimental protocols.

In situ hybridization

Mice were deeply anaesthetized and perfused by a cardiac infusion of sodium chloride followed by 4% paraformaldehyde. Brains were postfixed in formaldehyde overnight. A vibratome was used to cut 60 μm coronal slices collected in phosphate-buffered saline, dehydrated in methanol and stored at −20°C over night. Sections were rehydrated in methanol and saline containing 0.1% Tween-20, bleached in 6% H2O2, treated with 0.5% Triton X-100 and permeabilized with proteinase K and postfixed in formaldehyde. Antisense riboprobes encoding Ccl3, Cxcl10, L Ly2 and Bst2 were synthesized from linearized IMAGE clones using SP6, T3 or T7 polymerase and DIG RNA Labeling Kit (Roche, Mannheim, Germany). Analysis was based on several sections representing at least two injured brains subjected to repeated hybridizations giving similar results. Sections were prehybridized two hours at 55°C. Probes (1 μg/ml) were denatured at 80°C and added to the sections for incubation at 55°C over night. Anti-DIG antibody was diluted 1:5,000 in blocking solution for an overnight incubation at 4°C. Levamisole was used to inhibit endogenous phosphatase. Finally, sections were developed with BM-purple alkaline phosphatase substrate (Roche) at 37°C before being washed in saline, mounted onto microscope slides and photographed.

RNA preparation

Neocortex from the injured side of the brain was dissected and stored in the RNAlater reagent (Qiagen Inc., Valencia, California, USA). The tissue was homogenized using a Polytron homogenizer and total RNA isolated by RNaseasy Mini kit (Qiagen) with absorbance determined at 260 and 280 nm. RNA was prepared from uninjured wt mice (n = 10) and at the following time points after injury: one hour (n = 3), four hours (n = 4), twenty-two hours (n = 4), three days (n = 13), seven days (n = 11), two weeks (n = 12), three weeks (n = 9) and three months (n = 9). Moreover, the Cxcl10+/−/− RNA consisted of uninjured mice (n = 2) and injured mice at three days (n = 7) and seven days (n = 9) postinjury. RNA from Ccl2+/−/− brains were represented by uninjured mice (n = 2) and injured at three days (n = 9) and seven days postinjury (n = 5). The Ccr2−/− RNA comprised of two uninjured brains and postinjury at three days (n = 9), seven days (n = 11), two weeks (n = 11) and three weeks (n = 6). The RNA from the injured F2 hybrids consisted of five brain samples of Ccl2−/− mice and six samples of Ccr2−/− mice that were compared with five injured wt mice.

Quantitative reverse-transcriptase PCR (qRT-PCR)

Total RNA (10 ng) was analyzed and measurements were repeated at least twice using duplicated microwells (25 μl reaction volume). Injury-induced transcriptional changes were studied using the following primer pairs (reference sequence number and upper as well as lower primer stated): Ccl3 (NM_011337, 5'-
and yielded a 650 base-pair fragment. The Ccr2

5

(GM_021334, 5

Itgax

3

run for 10 min at 50

C. For DNA-precipitation, equal volume of
dH2-Ao

NM_010378, 5

G-AAG GAG GGA AGA TGT TG TGT C 3

and

Hmox1

NM_010442, 5

ACC TTC CCG AAC ATG AGC AG-3

and

5-GAG GAC GAA GGA AGA TGT TG TGT C-3

were taken and the pups marked by ear tags. The tail-tips were put

the primers specified below using AmpliTaq Gold polymerase

isopropanol was added. Genotyping by PCR was performed with

obtained commercially. In order to test for any contribution from

control wt B6 males were from our local breeding colony or

mice. The three chemokine-defect strains of mice exhibit no overt

knockout mice backcrossed for at least nine generations with B6 wt

number of homozygous males for TBI. All experiments used

crossing heterozygotes or homozygotes to produce the required

USA) [12] was obtained as homozygous breeding pairs as

control B6 mice. Cxcl10 knockout B6.129S4-

#002687) [9] and Ccr2 knockout B6.129S4-

#006087 Jackson Laboratories, Bar Harbor, Maine,

USA) [12] was obtained as homozygous breeding pairs as

control B6 mice. Cxcl10 knockout B6.129S4-Cxcl10(+/+;JAX

strain #006087 Jackson Laboratories, Bar Harbor, Maine,

USA) [12] was obtained as homozygous breeding pairs as described previously [2]. The strains were then maintained by crossing heterozygotes or homozygotes to produce the required number of homozygous males for TBI. All experiments used

number of knockout mice backcrossed for at least nine generations with B6 wt

mice. The three chemokine-defect strains of mice exhibit no overt

phenotypic deficiencies, impaired development or fertility, nor did

mice. The three chemokine-defect strains of mice exhibit no overt

was carried out at The Uppsala University Hospital Array Platform using Affymetrix Microarray Suite version 5.1 (MAS 5.1) applying the percentile algorithm (Parameters = Percentile:75, Mass-Detection P = 0.05). The analysis excluded data that were unassigned ESTs, probes with signal intensities below 38 and also eliminating signals indicated as Absent. Microarray data can be retrieved in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database [http://www. ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE58485], accession number GSE58485.

Flow cytometry

The neocortex of uninjured and TBI mice (three days postinjury) were dissected (n = 1–2, wt or Ccr2+/− mice, experiments in triplicate) and treated with collagenase D. Cells from the brain tissue were mechanically isolated using a loose

The mRNA of uninjured mice was isolated from the brain cortex using the Plurion RNAeasy Tissue Kit (Qiagen, USA) according to the manufacturer’s instructions. The amplified RT-PCR products were separated on 2% agarose gels with 5 μg/ml ethidium bromide, visualized with UV light and photographed. The concentration of the cDNA was determined by spectrophotometry at 260 nm.

Histochemistry

To detect activated microglia, peroxidase-labeled isocyan B4 from Bandeiraea simplicifolia (L3391, Sigma, Saint Louis, Missouri, USA) was applied to sections, previously hybridized with the Ly2 probe. Binding of isocyan B4 was examined in duplicate sections by adding DAB as a peroxidase substrate before being mounted.
Injury were perfused by 4% paraformaldehyde one week after injury. The brains were removed, cryoprotected in 30% sucrose, snap-frozen in ice-cold isopentane and stored at -80°C. Brains were embedded in Tissue-Tek (Histobal, Gothenburg, Sweden) and cut in 20 μm coronal sections with a cryostat, beginning at bregma. Every 25th section was saved on glass slides (SuperFrost Plus, Menzel-Gläser, Braunschweig, Germany). After nuclear staining in Harris hematoxyn (Histolab), slides were dehydrated and mounted. Images of sections of the injured brains were stored in a digitized format for area measurements in order to reconstruct the cavity volume. Ipsilateral area was subtracted from the corresponding contralateral and area difference was transformed into a circular area to calculate the radius. The total cavity volume was approximated as the sum of the volume of each truncated cone, stacked in the injury. Measurements were performed in six Ccr2+/− mice and five wt mice.

**Magnetic cell sorting**

Inflammatory cells fractionated on Percoll gradients, were incubated with magnetic microbeads coated with anti-Cd11c or anti-PDCA-1 antibodies (Miltenyi Biotech, Bergisch Gladbach, Germany). Four independent experiments were performed, each analysis based on cells pooled from neocortex from two up to six injured mice. The magnetic cell sorting was accomplished using MS Columns and a MiniMACS separation unit (Miltenyi Biotech) for subsequent recovery of RNA. RNA was analyzed in duplicate with qRT-PCR using H2-Aa, Bst2 and Cxcl10 probes and normalized against 28S ribosomal RNA.

**Depletion of plasmacytoid dendritic cells**

Wt mice (n = 6) were injected intraperitoneally 30 minutes after injury with 500 μg rat antibodies directed against mouse PDCA-1 (# 130-091-978, Miltenyi Biotech, Bergisch Gladbach, Germany) to achieve depletion of pDCs. As reference, the same amount of normal rat IgG (14131, Sigma Aldrich Sweden AB) were administered to wt mice (n = 8). Brains were collected for RNA preparation three days after injury.

**Cyclophosphamide treatment**

Wt mice were given phosphate buffered saline (n = 6) or cyclophosphamide monohydrate (n = 8), 200 mg/kg; C0768, Sigma Aldrich) intraperitoneally 30 minutes after the TBI and tissue collected three days later for RNA preparation for qRT-PCR and microarray analysis. In addition, injured mice given saline (PBS) or cyclophosphamide were analyzed by flow cytometry.

**Statistical analysis**

The SigmaStat version 3.1 software (SPSS, Inc., Richmond, CA, USA) was used to perform One Way Analysis of Variance (ANOVA) or Student’s t-test for pair-wise comparisons of groups. Non-parametric data were analyzed using Kruskal-Wallis One Way Analysis of Variance on Ranks and Mann-Whitney U test as advised by the program. P = 0.05 or lower was considered to represent statistically significant differences. Data in the text and graphs are presented as mean values ± standard error of the mean (SEM).

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**Author Contributions**

Conceived and designed the experiments: CI LH TE. Performed the experiments: CI AK HB TE. Analyzed the data: CI AK HB TE. Contributed reagents/materials/analysis tools: CI AK HB TE. Conceived and designed the experiments: CI LH TE. Performed the CCI experiments: CI AK HB TE. Genotyp- ing: AK. RNA preparation: CI AK TE. qRT-PCR: CI AK TE. Flow cytometry: CI AK TE. Cell fractionation: CI AK TE. Magnetic bead isolation: CI AK TE. In situ hybridization: HB. Preparation of figures: CI TE.

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