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DAMP Signaling is a Key Pathway Inducing Immune Modulation after Brain Injury

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Acute brain lesions induce profound alterations of the peripheral immune response comprising the opposing phenomena of early immune activation and subsequent immunosuppression. The mechanisms underlying this brain-immune signaling are largely unknown. We used animal models for experimental brain ischemia as a paradigm of acute brain lesions and additionally investigated a large cohort of stroke patients. We analyzed release of HMGB1 isoforms by mass spectrometry and investigated its inflammatory potency and signaling pathways by immunological in vivo and in vitro techniques. Features of the complex behavioral sickness behavior syndrome were characterized by homecage behavior analysis. HMGB1 downstream signaling, particularly with RAGE, was studied in various transgenic animal models and by pharmacological blockade. Our results indicate that the cytokine-inducing, fully reduced isoform of HMGB1 was released from the ischemic brain in the hyperacute phase of stroke in mice and patients. Cytokines secreted in the periphery in response to brain injury induced sickness behavior, which could be abrogated by inhibition of the HMGB1-RAGE pathway or direct cytokine neutralization. Subsequently, HMGB1-release induced bone marrow egress and splenic proliferation of bone marrow-derived suppressor cells, inhibiting the adaptive immune responses in vivo and vitro. Furthermore, HMGB1-RAGE signaling resulted in functional exhaustion of mature monocytes and lymphopenia, the hallmarks of immune suppression after extensive ischemia. This study introduces the HMGB1–RAGE-mediated pathway as a key mechanism explaining the complex posts ischemic brain-immune interactions.

Key words: alarmins; HMGB1; immunomodulation; myeloid-derived suppressor cell; RAGE; stroke

Introduction

Acute brain injuries, including stroke, induce profound alterations of the peripheral immune system (Chamorro et al., 2012), which contribute substantially to morbidity and mortality of patients. The best described component of these systemic immune alterations is an acquired immune deficiency in the subacute phase after ischemic stroke, which predisposes patients to infectious complications, the identification of the signaling pathways underlying systemic immune modulation is of great translational relevance. Previous reports have suggested a role of the autonomic nervous system in mediating subacute immunosuppression after acute brain injuries because elevated catechol-
amine levels were observed in some of the previous experimental stroke studies. Blocking of adrenoreceptors reversed aspects of poststroke immunodepression, including susceptibility to bacterial infections in experimental stroke (Prass et al., 2003; Meisel et al., 2005). However, recent clinical (Liesz et al., 2013a) as well experimental (Mrcakso et al., 2014) studies have yielded mixed results regarding the implications of catecholamines in immunodepression.

Therefore, we focused in this study on soluble mediators released from the necrotic brain tissue as an alternative pathway in poststroke brain-immune interaction. Previous studies suggested a strong correlation between the volume of damaged brain tissue and the extent of systemic immune alterations, regardless of infarct location (Gendron et al., 2002; Hug et al., 2009). Accordingly, we speculated that soluble mediators would be released from dying neurons in proportion to the extent of necrotic tissue. As a prototypic alarmin, high mobility group box 1 (HMGB1) is a nuclear protein, which is released from necrotic cells with high inflammatory potency (Lotze and Tracey, 2005). As such, HMGB1 can act itself as a cytokine or amplify the binding of cytokines and nucleosomes to Toll-like receptors (TLRs) (Bianchi, 2009). The receptor of advanced glycation end-products (RAGE) has been identified as a key receptor (in addition to TLR2 and TLR4) for HMGB1, mediating activation pathways to a variety of cell types in an organ-specific manner (Lotze and Tracey, 2005).

Our study introduces the signaling of brain-released alarmins, such as HMGB1, through RAGE as an important link between acute brain injury and systemic immune alterations. We demonstrate a biphasic scenario where this signaling cascade initially activates peripheral monocytes and dendritic cells, resulting in a massive release of proinflammatory cytokines. Subsequently, initial (over)activation of the peripheral immune system results in exhaustion of monocytes and recruitment of immature monocytes. Suppressive signaling by the altered monocyte population leads to apoptosis of lymphocytes, the hallmark of poststroke immunosuppression.

Materials and Methods

Animals. The study was conducted in accordance with national guidelines for the use of experimental animals, and the protocols were approved by the governmental committee (Regierungspraesidium Karlsruhe, Germany). Animal experiments were designed, performed, and data reported according to the ARRIVE guidelines (Kilkenny et al., 2010). We used age-matched, male mice (C57BL/6J, 10–12 weeks, 23–25 g body weight, Charles River Laboratories). RAGE−/− (Liliensiek et al., 2004) and MyD88−/− (Adachi et al., 1998) mice were bred in the core animal facility of the University of Heidelberg under specified pathogen-free conditions.

Patient recruitment and blood sampling. Blood samples used for analysis in this study were collected during a previous study (Liesz et al., 2013a) conducted between October 2011 until November 2012. In brief, inclusion criteria were study enrollment within 24 h after symptom onset; a chronic immunological disorder, preexisting immunosuppression, can-

cer, and pregnancy. Blood samples from 104 patients obtained within 24 h after symptom onset were analyzed. Patient characteristics are depicted in Tables 1 and 2. Stroke patients received routine cranial imaging by CT (Somatom Sensation 16) and/or 3T MRI (Trios and/or Verio; all Siemens Medical Systems) to detect ischemic brain lesions. Volume was calculated by integrating areas on each image multiplied by section diameter using a Syngo Multimodality workspace with syngoMMWP-software (version VE31A, Siemens). For the control group, patients were included that presented at the Department of Ophthalmology for pre-
surgical screening for cataract operation. Patients were selected to be in the same range for age and gender of patients with cerebrovascular disorders. All procedures were performed after written informed consent was obtained by the patient or a legal representative.

Animal experiments. Animals received intraperitoneal injection of 100 µg monoclonal anti-HMGB1 antibodies (clone 2g7) (Qin et al., 2006) 30 min before ischemia induction or an isotype control antibody (BioX-Cell); 100 µg soluble RAGE (full-length recombinant human sRAGE) was injected before middle cerebral artery occlusion (MCAO) induction and 4 h after MCAO. The β2-adrenoreceptor inhibitor ICI 118,551 (Sigma) was administered intraperitoneally at 4 mg/kg body weight before MCAO and at 4, 8, and 12 h after MCAO (Mrcakso et al., 2014). Neutralization of circulating cytokines was achieved by intraperitoneal injection of 200 µg each of anti-IL-1β, anti-TNF-α, or anti-IL-6 antibodies (all from eBioscience, functional grade purified) in a total volume of 200 µl PBS intraperitoneally. Control mice received 300 µg of isotype control antibody. Organ sampling (brain, spleen, thymus, bone marrow, blood from retro-orbital sinus) for further analysis was performed in anesthesia with ketamin/xylazine. Rectal temperature and body weight were measured daily. Neurological deficits were assessed by a modified “Bederson score” (Bederson et al., 1986): 0, no deficit; 1, forelimb flexion;
2, decreased resistance to lateral push; 3, unidirectional circling; 4, longitudinal spinning; 5, no movement. Sensorimotor deficit was measured using the Rotarod test as previously described (Jones and Roberts, 1968; Liesz et al., 2013b). Briefly, mice were trained at first at constant 4 rpm, then three times at acceleration from 4 to 40 rpm in 210 s. Baseline assessment was performed as for training with acceleration and the mean calculated from three runs with a 10 min break in between. Time was stopped if the mouse fell off the beam or passively rotated twice on the beam. Assessment 24 h after MCAO was performed similar to baseline measurement.

Experimental stroke models
Coagulation model. We used this stroke model for experiments shown in Figure 1A, B. Mice were anesthetized with 1.0%–2.0% halothane in O2/...
N₂O. After making a 1 cm skin incision between the left eye and ear, we drilled a burr hole through the temporal skull. We removed the dura mater and occluded the MCA permanently using a bipolar electrocoagula-
tion forceps (ERBOTTOM, Erbe). During the operation, body tempera-
ture was kept at 37°C using a feedback controlled heating pad. We sutured the skin lesion and placed the mouse in a cage under an inffee-
hed heating lamp until recovery from anesthesia. Sham treatment was given according to the same surgical protocol as for MCAO but without coag-
ulation of the MCA. The overall mortality in the coagulation model was <5% for the 3 d observation period.

Filament model. We used this model for all remaining experiments. Mice were anesthetized with 1.0%–2.0% halothane in O₂/N₂O-enriched air. We placed the laser Doppler probe over the cortical area supplied by the MCA. Baseline CBF was measured as relative perfusion units and defined as 100% flow. After neck dissection, we made an incision into the left external carotid artery between two ligations and advanced a silicon-
covered 8-0 nylon monofilament through the internal carotid artery to occlude the MCA. MCA occlusion was documented as a decrease in relative perfusion values by laser Doppler to <20% of original flow, a less pronounced decrease in CBF was used as an exclusion criterion. We fixed the filament in this position by ligation, closed the neck, removed the Doppler probe, and placed the mouse in its cage. Then, 30 min (only for experiments in Fig. 1A, B) or 90 min, respectively, after filament inser-
tion, the mouse was anesthetized again and the filament removed. After we closed the surgical wound, the mice were transferred to their cages with free access to water and food. During the operations body tempera-
ture was kept at 37°C with a feedback-controlled heating pad. We main-
tained normothermic body temperature of the mice between operations and until recovery after the procedure by an infrared heating lamp. Sham operation was performed in exactly the same manner as described above, including repeated anesthesia 30 or 90 min after sham surgery, except that the filament was only introduced briefly into the external carotid artery. The overall mortality in the 30 min occlusion model was <5% for the 3 d observation period; mortality for the 90 min occlusion model is reported in Figure 1H.

Assessment of infarct volume. We anesthetized the mice and perfused them transcardially with 20 ml normal saline. We removed the brains from the skull and froze them immediately in isopentane (−80°C). We cut 20-μm-thick coronal cryosections every 400 μm, sliced the sections using a standard cryo-violet staining protocol, scanned them at 600 dpi, and analyzed the infarct area on each section (ImageJ). The Swanson method was applied to indirectly measure the infarct area and to correct for cortical swelling (Swanson et al., 1990). The total infarct volume was determined by integrating measured areas and distances between sections.

Analysis of HMGB1 by electrospay ionization liquid chromatography mass spectrometry. All chemicals and solvents were of the highest available grade (Sigma). Samples were preclarified with 50 μl protein G-Sepharose beads for 1 h at 4°C. Supernatant HMGB1 was immuno-
precipitated with 5 μg rabbit anti-HMGB1 (Abcam; ab182526) for 16 h at 4°C as previously described (Antoine et al., 2009). Free thiols groups within HMGB1 were alkylated for 90 min with 10 mM iodoacetamide at 4°C. Cysteine residues in disulfide bonds were then reduced with 30 mM DTT at 4°C for 1 h followed by alkylation of newly exposed thiols groups with 90 mM NEM at 4°C for 10 min. Samples were subjected to trypsin (Promega) or GluC (New England Biolabs) digestion according to man-
ufacturer’s instructions and desalted using ZipTip C18 pipette tips (Mil-
lipore). Characterization of whole protein molecular weights, acetylated lysine residues, or redox modifications on cysteine residues within HMGB1 were determined as described previously by whole protein electro-
spray ionization or tandem mass spectrometry (Antoine et al., 2012; Nyström et al., 2013) using either an AB Sciex QTRAP 5500 or an AB Sciex TripleTOF 5600 (Sciex Inc.). Peptide analysis was determined using an AB Sciex QTRAP 5500 equipped with a NanoSpray II source by in-line liquid chromatography using a U3000 HPLC System (Dionex), con-
ected to a 180 μm × 20 mm nanoAcquity UPLC C18 trap column and a 75 μm × 15 cm nanoAcquity UPLC BEH130 C18 column via reducing unions. A gradient from 0.05% TFA (v/v) to 50% ACN/0.08% TFA (v/v) in 40 min was applied at a flow rate of 200 nl/min. The ionspray potential was set to 2200–3500 V, the nebulizer gas to 19, and the interface heater to 150°C.

ELISA. Human and mouse serum and cell culture supernatants were collected and immediately frozen at −80°C until analysis. Commercial ELISA kits were used for the quantitative assessment of HMGB1 (IBL) and cytokine expression of IL-1β, TNF-α, IL-6, and IL-12p40 (all R&D Systems).

Homemade behavior analysis. Mice were held individually in 37 × 16 × 13 cm transparent plastic cages as homecages. Mice were recorded for 17–24 h for each day/night cycle before MCAO and at day 1 and/or day 3 after MCAO using CCD cameras and an infrared illumination system during the dark cycle. The camera angle was set up for recording of the long side of cages to observe horizontal as well as vertical activities, such as rearing and hanging. Ethovision (EthovisionXT, version 4.1, by Nol-
dus Information Technologies) video tracking software was used to digitize the trials at a rate of 12.5 frames/s. Total distance moved, rearing time, and rearing frequency were determined using animal center point analysis according to areas indicated in Figure 2F.

Bacterial culture. For bacterial blood cultures, 30 μl blood was drawn from each mouse and diluted in 270 μl sterile PBS. For bacterial lung cultures, both lung lobes were homogenized in PBS in a total volume of 300 μl; 50 μl of each blood sample or 100 μl of each lung homogenate was plated on Columbia agar with 5% sheep blood (BD Biosciences). Plates were incubated at 37°C in the presence of 5% CO₂ and colony forming units were evaluated after 24 h.

Determination of bacterial DNA in lung homogenates by quantitative PCR. Bacterial load in lung homogenates was determined applying a quantitative real-time PCR that amplifies 16S ribosomal DNA. Both lung lobes were homogenized, and DNA was extracted using the QIAamp DNA Mini kit (QIAGEN) according to the manufacturer’s instructions with an eluate volume of 100 μl. Samples were amplified with primers targeting conserved regions within the 16S RNA gene (forward, 5′-TGG AGC ATG TGG TTT AAT TCG A-3′; reverse, 5′-TGC GGG ACT TAA CCC AAC A-3′). A defined plasmid standard (16S RNA amplicon from Esch-
erichia coli, cloned in pCR2.1, Invitrogen; plasmids quantified by spect-
trophotometry) was used as positive control for qPCR. Samples were assayed in triplicate in a 2 μl reaction mixture containing 2 μl of tem-
plate DNA, 12.5 μl of 2× Fast SYBR Green Master Mix (Applied Biosys-
tems) and 12.5 pmol of forward primer and reverse primer (MWG). Cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min each. For quantification, background levels of the nontemplate control were subtracted from the specific samples.

Western blotting. Proteins were isolated for HMGB1 and ARG1 West-
ern blotting from samples using ice-cold RIPA buffer as previously re-
ported (Liesz et al., 2013b). Lysates were electrophoresed, transferred to membrane (Novex) and blocked with skim milk. Blocked mem-
branes were probed with rabbit anti-HMGB1 (1:1000) or incubated with sheep anti-ARG1 (1:500; R&D Systems) in TBS-T plus 5% milk

Stress mediator analysis. Serum cortisol and metanephrine/normeta-
nephrine concentrations were measured in the central laboratory of the University Hospital Heidelberg by the same procedures used and vali-
dated for routine diagnostic analysis. Cortisol concentrations were mea-
sured by chemiluminescence immunoassays, and serum metanephrine/ normetanephrine were detected by ELISA.

Magnetic cell sorting. MACS sorting using magnetic beads was used for isolation of CD11b-, CD11c-, and CD3-positive cells for in vitro studies. Single-cell suspensions were prepared from spleens of the respective na-
dive donor animals or after experimental brain ischemia as indicated in the manuscript. Single-cell suspensions were then processed for further purification of the subpopulation according to the manufacturer’s pro-
tocol using commercial MACS kits (Miltenyi Biotec) for the purification of the indicated population (total T cells, monocytes, dendritic cells; or myeloid-derived suppressor cells).

Real-time PCR. RNA was isolated from spleens or isolated cells using the RNeasy kit (QIAGEN). Reverse transcription was performed with the
Figure 2. HMGB1 is released from necrotic brain tissue and matures to its cytokine-inducing form. A, Mass spectrometric characterization of the critical HMGB1 isoform with cytokine-inducing properties as categorized by the C23–C45 disulfide (NEM adduct) redox form. B, Representative spectrum of the tandem mass spectrometric characterization of a peptide (amino acids 180–188) covering the lysine (K) residues within nuclear localization sequence (NLS) 2 of HMGB1, depicting the hyperacetylated state of HMGB1-NLS2. Acetyl modifications are represented as (ac) on specific lysine residues (K181, K182, K183, and K184). C, D, Tabular overview of the detected isoforms of post-translationally modified HMGB1 in ipsilateral brain hemispheres and serum samples at 2 h (C) and 24 h (D) after 90 min MCAO. Gray shading represents the presence of redox isoforms at cysteine residues and of the acetylation state at lysine residues within both nuclear localization signals (NLS) of HMGB1. C23–C45 disulfide isoforms mediate the cytokine-inducing HMGB1 activity. Hypoacetylation of the NLS regions indicates the nuclear localization and origin of HMGB1. Hyperacetylation of HMGB1’s NLS regions is involved in cytoplasmatic shuttling of HMGB1.
High Capacity complementary DNA Archive Kit and real-time PCR with SYBR-Green assays on an ABI7500 real-time PCR system (Applied Biosystems). Primers were purchased as ready-to-use primer sets for each gene (Super Array). All assays were run in duplicate. Expression of each gene was quantified according to the relative standard curve method relative to the expression of the housekeeping gene encoding for β-actin.

In vitro assays

T-cell stimulation assay. For intracellular cytokine staining of isolated T cells, we plated single-cell suspensions of 300,000 mononuclear cells on 96-well plates (RPMI-1640, + 10% FCS, + glutamine. + HEPES, + penicillin/streptomycin, + mercaptoethanol). Each sample was processed in triplicate. We primed the cells for 48 h with plate-bound anti-mouse CD3ε (clone 145-2C11, BD Biosciences) and 2 µg/ml soluble anti-mouse CD28 (clone 37, clone 51, BD Biosciences), followed by restimulation with 500 ng calcinumycin (Sigma-Aldrich), 5 ng/ml PMA (Sigma-Aldrich), and protein transport inhibitor (BD GolgiPlug, BD Biosciences) for 5 h.

Dendritic cell stimulation assay. CD11c⁺ splenic dendritic cells were isolated by MACS following the manufacturer’s protocol (Miltenyi Biotec). CD11c⁺ cells were cultured at a concentration of 100,000 cells/well for 24 h. Fully phosphorylated modified CpG-ODN 1668 (TIB Molbiol) was used at a concentration of 100 nM by adding the stimulus directly into the culture medium.

Marrow-derived suppressor cell (MDSC) suppression assay. CD11b⁺Ly-6Ghigh cells were isolated by MACS and cocultured with CD3⁺ T cells (MACS-isolated) at the indicated ratio with a total cell count of 200,000 cells per well of a 96-well round-bottom plate. T cells were labeled with CFSE (CFSE cell proliferation kit, Invitrogen), and their proliferation (MACS-isolated) at the indicated ratio with a total cell count of 200,000 cells per well of a 96-well round-bottom plate. T cells were labeled with CFSE (CFSE cell proliferation kit, Invitrogen), and their proliferation was assessed by measuring the CFSE dilutions and analyzed using FlowJo 4.2 software (TreeStar).

HMGB1 stimulation assay. For assessment of the cytokine-inducing activity of HMGB1, mixed splenocyte cultures (Fig. 2D) or cocultures of T cells with antigen-presentation cells (APCs) (Fig. 2E) were used. Erythrocyte-depleted primary cell suspensions of spleens from naive animals were used for mixed splenocyte cultures. For cocultures, T cells were isolated by MACS (T cell isolation kit I, Miltenyi Biotec). CD11c⁺ cells were cultured at a concentration of 100,000 cells/well for 24 h. Fully phosphorylated modified CpG-ODN 1668 (TIB Molbiol) was used at a concentration of 100 nM by adding the stimulus directly into the culture medium.

Flow cytometry. We collected organs (spleen, blood, mesenteric lymph nodes, and bone marrow) after transcardial perfusion with saline at various time points after MCAO for flow cytometric analysis. We stained single-cell suspensions for anti-mouse CD3 (clone 17A2), CD4 (clone RM 4–5), CD8 (clone 53–6.7), B220 (clone RA3–682), NK1.1 (clone PK136), Ly-6C (clone HK1.4), Ly-6G (Gr-1; clone RB6–8C5), CD80 (clone 16–10A1), CD86 (clone GL1), CD25 (clone PC61), CD69 (clone H1.2F3), CD11b (clone M1/70), MHC-II (M5/114.15.2), and CD11c (clone N418). Expression of IFN-γ (clone XMG1.2) and IL-4 (clone H4-4I) (anti-mouse CD28 (clone 37, clone 51, BD Biosciences), followed by restimulation with 500 ng calcinumycin (Sigma-Aldrich), 5 ng/ml PMA (Sigma-Aldrich), and protein transport inhibitor (BD GolgiPlug, BD Biosciences) for 5 h.

Blood samples from stroke patients for FACS analysis were collected in EDTA tubes and immediately processed. Mononuclear cells were isolated by Ficoll gradient centrifugation and single-cell suspensions stained for anti-human CD11b (clone ICRF44), CD33 (clone P67.6), and HLA-DR (clone L243). We performed flow cytometry on a Becton Dickinson FACSCalibur and analyzed the data by CellQuest Pro software.

Statistical analysis. Sample size of the experiments was determined by a priori calculation presuming Type I error of 0.05, power of 0.8, and specific variance based on according preexperiments using $^g$ power statistical software. All values in bar graphs are expressed as mean ± SD. We analyzed infarct volumes and functional outcome tests by two-tailed post hoc analysis for two groups or the Kruskal-Wallis test with Dunn’s multiple comparison test for more than two groups, using GraphPad Prism 5 software. $p < 0.05$ was considered statistically significant.

Results

HMGB1 is released from necrotic brain lesions

We analyzed HMGB1 serum concentrations by ELISA in different experimental stroke models varying by location and volume of the resulting infarct (Fig. 1A) and detected a significant increase only after large infarcts at early time points after stroke (Fig. 1B). This was verified by HMGB1 Western blotting at 2 h after stroke onset (Fig. 1C). We also found massively increased HMGB1 serum concentrations in stroke patients within the first hours after symptom onset (Fig. 1D). To verify HMGB1 release in human stroke, we further evaluated HMGB1 concentrations in blood samples from 104 patients (patient characteristics summarized in Table 1) within 24 h after symptom onset of small or large lesions (dichotomized by the median infarct volume, in this cohort 30 ml) and compared it with control patients (Fig. 1E). Because human and murine results showed a consistent pattern of early HMGB1 release into the circulation after extensive ischemic brain lesions, further experimental studies were performed exclusively in the 90 min transient MCAO model.

We analyzed the impact of blocking HMGB1 by neutralizing antibodies or by elimination of its receptor RAGE in knock-out mice on stroke outcome. Infarct volumes (Fig. 1F) and basic sensory-motor deficits measured by the Bederson score (Fig. 1G) did not differ significantly among anti-HMGB1 treated animals, RAGE−/− mice, and controls in our experiments. Intriguingly, despite the lack of effect on infarct size and neurological deficit, 7 d mortality was substantially reduced in RAGE−/− mice ($p = 0.008, n = 22$) and tended also to be lower in anti-HMGB1-treated mice ($p = 0.07, n = 17$) (Fig. 1H). Both HMGB1 neutralization and RAGE deficiency also attenuated loss of body weight and hypothermia after stroke (Fig. 1I). This dissociation between similar lesion size and differing mortality might indicate that HMGB1-RAGE blockade modified important systemic consequences of brain infarction rather than resulting in immediate structural neuroprotection.

HMGB1 matures in the circulation to its cytokine-inducing isofrom

Because the biological activity of HMGB1 is critically dependent on the redox state of cysteine residues at C23, C45, and C106 (Yang et al., 2013), we analyzed post-translational HMGB1 modifications by tandem mass spectrometry (Fig. 2A,B). At 2 h after ischemia, the fully reduced isoforms with no cytokine-inducing but chemoattractant activity predominated in brain and serum samples as the disulphide form was not detected in any of the investigated brains and only in two often serum samples. Moreover, the exclusively hypoacetylated state confirmed the passive release of HMGB1 from necrotic cells (Fig. 2C). In contrast, the cytokine-inducing C34-C45 disulphide HMGB1 isoform (Yang et al., 2010) was found as the prevalent isoform in serum samples at 24 h but could still not be detected in brain samples of the corresponding animals (Fig. 2D). These results indicate HMGB1 maturation in the blood circulation toward its cytokine-stimulating isoform after initial (passive) release from the necrotic brain tissue.
The early phase after brain injury is characterized by massive systemic cytokine secretion mediating sickness behavior

To determine the contribution of HMGB1 signaling on sterile systemic inflammation, cytokine concentrations were determined within the acute phase after brain ischemia. Expression levels of the proinflammatory cytokines IL-1β, TNF-α, and IL-6 were markedly increased in the acute phase after stroke onset (Fig. 3A). Antibody-mediated neutralization of HMGB1 significantly attenuated splenic expression and serum concentrations of these proinflammatory cytokines (Fig. 3A, B). Supporting the immune-stimulatory role of HMGB1, in vitro stimulation of splenocyte cultures with the cytokine-stimulating isoform of HMGB1 resulted in increased expression of T cell- (IFN-γ) and monocyte- (IL-12) derived cytokine secretion (Fig. 3C). Moreover, we detected a dose–response relation for HMGB1 concentration and IFN-γ secretion by isolated T cells, which depended on the presence of viable APCs (Fig. 3D).

Weight loss and hypothermia, as shown in Figure 1F, are considered as basal markers of sickness in models of immune-mediated diseases (Dantzer, 2006). We further investigated this phenomenon in a homecage behavior analysis setup, which enabled the automated analysis of circadian rhythm and complex mouse behavior (Jhuang et al., 2010; Spulber et al., 2012) (Fig. 4A). Although no behavioral difference was detected between RAGE−/− animals and WT controls at baseline before MCAO (data not shown), we detected a disturbance in circadian rhythm at day 1 after stroke. This was more pronounced in WT compared with RAGE−/− animals with respect to arousal after the start of the day cycle (6 A.M.) and disparity between overall night and day activity (Fig. 4B). Furthermore, mobility and exploratory behavior (total distance moved), targeted movements to reach the water bottle (rearing time and frequency), and challenging physical activities (hanging time) were significantly less affected during the early phase after stroke induction in RAGE−/− compared with WT mice (Fig. 4C).

To investigate the role of the peripheral cytokine release in the acute phase after brain injury as the potential link between RAGE deficiency and improved behavior, we antagonized IL-1β, TNF-α, and IL-6 by neutralizing antibodies and analyzed key features of sickness behavior as introduced above. Infarct volumetry revealed similar infarct volumes in control- and anti-cytokine-treated mice (Fig. 4D). Additionally, focal sensorimotor dysfunction analyzed by the Rotarod test did not differ between the treatment groups (Fig. 4E). This demonstrated that peripheral cytokine neutralization had no immediate impact on the primary brain lesion. In contrast, homecage behavior analysis revealed a similar treatment effect of cytokine antagonization as previously seen in RAGE-deficient mice. The cytokine neutralization improved the stroke-induced disturbances in circadian rhythm with reduced variation between light and dark periods (Fig. 4F). It also improved overall motility as depicted by analysis of total distanced moved in the first 2 h of the light period (6 A.M. to 8 A.M.) (Fig. 4G).

RAGE signaling promotes MDSC expansion

Based on the finding that APC viability is critical for the impact of HMGB1 on lymphocyte function (Fig. 3D), we further analyzed the role of monocytes in this model. We found an expansion of a specific immature monocyte subpopulation in the spleen, characterized by the CD11b+Ly-6C−MHC-IIlow expression pattern after stroke (Fig. 5A), which was abrogated by anti-HMGB1 treatment (Fig. 5B). Further analysis of this population in spleen and bone marrow revealed both increased splenic proliferation (marker Ki-67) and a reduced proportion of this subpopulation...
in the bone marrow (Fig. 5B). These findings are compatible with a combination of local splenic proliferation as well as bone marrow egress of immature monocytes. Moreover, stroke in WT mice resulted in an increase in CD80 expression and a reduction of MHC-II expression on monocytes (Fig. 5C). The stroke-induced rise of immature splenic monocytes compared with sham-operated mice was significantly reduced in RAGE/H11002/H11002 animals 3 d after MCAO, supporting the role of HMGB1-RAGE signaling on immature monocyte recruitment (Fig. 5D).

To assess functional capabilities of splenic monocytes after stroke that were independent of the RAGE signaling pathway, we performed stimulation assays with the TLR9-ligand CpG-ODN.
This revealed substantially reduced IL-12 and TNF-α secretion by monocytes 3 d after brain ischemia consistent with a reduced responsiveness of splenic monocytes resulting from functional exhaustion. In vivo anti-HMGB1 treatment partially attenuated monocyctic hyporesponsiveness to in vitro stimulation as shown by increased IL-12 secretion in the anti-HMGB1-treated group compared with control mice, although it had no effect on TNF-α production (Fig. 5E). Next, we analyzed the frequency of immature monocytes, delineated by expression of CD11b+ CD33+ HLA−, in stroke patients with extensive brain lesions (>100 ml lesion volume) and matched control patients without neurological disease (Fig. 5F; Table 2). We detected a significant expansion of immature monocytes also after human stroke (Fig. 5G). We further analyzed the functional properties of the expanded CD11b+ Ly-6C+ monocyte population to stimulate or inhibit T cells. Splenic CD11b+ monocytes isolated 3 d after stroke used as APCs in coculture with CD3+ T cells had a suppressive effect on T-cell proliferation (Fig. 5H, I). Additionally, arginase-1 (Arg1) expression, a marker of myeloid-derived suppressor cells (Rodríguez and Ochoa, 2008; Gabriovich and Nagaraj, 2009), was massively increased in poststroke splenic monocytes, which was prevented by in vivo pretreatment with anti-HMGB1 (Fig. 5J).

Poststroke lymphocytopenia in the subacute phase is partially mediated by HMGB1 signaling

We investigated the role of the HMGB1-RAGE pathway on the previously documented decrease in immune cellularity in the subacute phase after stroke (Prass et al., 2003; Offner et al., 2006b; Liesz et al., 2009) (Fig. 6A–D). RAGE deficiency, but not anti-HMGB1 treatment, reduced splenic lymphocyte apoptosis after brain ischemia (Fig. 6A, B). Similarly, the stroke-induced reduction of circulating blood T cells was prevented in RAGE-deficient mice (Fig. 6C). The postischemic effect on B-cell and granulocyte counts, however, was not influenced by either anti-HMGB1 treatment or by RAGE- or MyD88 deficiency, respectively (data not shown). Furthermore, we observed a relative increase of activated T cells (activation markers CD25 and CD69) in RAGE+/− mice compared with WT mice after stroke (Fig. 6D). Because of the incomplete antagonization of the immunosuppressive phenotype by RAGE deficiency, we further investigated the impact of TLR-mediated pathways in stroke-induced immune disturbances using MyD88−/− mice. We detected a similar pattern for T-cell counts in spleen and blood as for RAGE+/− mice after stroke with partial abrogation of lymphopenia in MyD88−/− mice without further augmentation by anti-HMGB1 treatment (Fig. 6E). In summary, these results indicate that the immunosuppressive and lymphotrophic effects of extensive brain ischemia are mediated at least in part by HMGB1 via the RAGE and the TLR-MyD88 pathways.

HMGB1-RAGE signaling is a distinct alternative signaling pathway after acute brain injury

To control for potential confounding factors known for the used stroke model of large brain lesion, we performed additional analyses to investigate alternative explanations for the observed effects independent of the HMGB1-RAGE pathway. Previous reports have demonstrated bacterial infections in this model, which could have interfered with the immunological and behavioral readout parameters investigated in this study. However, we did not detect bacteria in lung homogenates or blood of any mice (Fig. 7A). Furthermore, catecholamines released by activation of the sympathetic autonomous nervous system have been reported as mediators of poststroke immunosuppression (Prass et al., 2003; Meisel et al., 2005). Therefore, we measured serum concentrations of glucocorticoid and catecholamine stress mediators in WT control mice, anti-HMGB1 treated, and RAGE-deficient mice after stroke without detecting significant differences among groups (Fig. 7B). Next, we aimed to analyze the functional role of catecholamine signaling on critical parameters affected by the HMGB1-RAGE pathway in the previous experiments, such as cytokine release, spleen cellularity, and MDCS expansion. Therefore, we treated mice either with the β2-adrenoceptor blocker (b2-INH) ICI 118,551 (Mracsko et al., 2014), sRAGE, or the combination of both. We observed that the inhibition of the RAGE pathway by sRAGE-treatment, but not blockade of the adrenergic β2 receptor by b2-INH, abrogated the increased systemic IL-6 secretion after brain ischemia (Fig. 7C). Both sRAGE treatment and b2-INH injection prevented the expansion of the CD11b+ Ly-6C+ monocyte fraction (compare with Fig. 5B). However, b2-INH injection also caused a significant reduction in this cell population compared with control-treated mice (Fig. 7D). On the other hand, the marked reduction of spleen cellularity (Fig. 7E) was not affected by b2-INH but significantly abrogated by sRAGE treatment. Interestingly, the combination of the RAGE as well as the catecholamine signaling had an additive effect on this parameter. These results indicate that the HMGB1-RAGE and the stress-mediator pathways might be largely independent but differentially involved in specific signaling cascades, such as acute cytokine release and immunosuppressive pathways.
Discussion

This study presents data supporting a novel concept of brain-immune interaction after acute brain lesions in which soluble danger signals from the brain mediate a complex immunomodulatory reaction in the peripheral immune system. Previous studies have focused on the role of HMGB1 and other DAMPs on immediate tissue injury within a defined organ, such as the heart and the brain (Andrassy et al., 2008; Muhammad et al., 2008; Shichita et al., 2012). This is the first study uncovering supersystemic cross signaling via RAGE largely by cytokine-inducing isoforms of the soluble mediator HMGB1 released from the necrotic brain. Our findings provide new insights into the peripheral immune alterations in response to brain injury and propose brain-released alarmins as important mediators of the systemic...
immune response leading to complex behavioral and metabolic sequelae.

Our results are of clinical relevance for patients presenting with severe acute brain injury because their prognosis is strongly influenced by secondary immune-mediated complications. In the early phase after clinical stroke and traumatic brain injury features of sickness behavior, including sleep disorders, fatigue, and depression, are common (Dantzer, 2006; Crosby et al., 2012), which affect secondary morbidity and physical recovery (Willey et al., 2010; Lerdal and Gay, 2013). The concept of proinflammatory cytokines as the cause of clinical sickness behavior and peripheral inflammation is well established, and IL-1β, IL-6, and TNF-α are seen as the main mediators (Dantzer et al., 2008). These cytokines are also strongly upregulated in the periphery during the acute phase after brain ischemia as shown in the present and previous reports (Offner et al., 2006a; Chamorro et al., 2012), but the underlying signaling and the resulting pathophysiological implications were unknown. In this study, we identified the HMGB1-RAGE signaling pathway as the critical route inducing this overshooting immune reaction after brain injury. Inhibiting this signaling pathway improved the systemic metabolic and behavioral consequences of stroke and reduced mortality but did not affect the size of the brain lesions. Similarly, direct neutralization of the overshooting cytokine-release in the acute phase after MCAO improved similar behavioral parameters, such as circadian rhythm and mobility as for inhibition of the HMGB1-RAGE pathway, supporting the concept of HMGB1-RAGE mediated cytokine-induced behavioral alterations. Because cytokine-induced sickness behavior is still not well established as a distinct syndrome in the clinical setting and also requires more comprehensive characterization in experimental models, future studies will have to further validate this concept. Additionally, we cannot exclude immediate modulatory effects of HMGB1-RAGE signaling on brain structures that are not primarily affected by the brain ischemia (e.g., hippocampus). Also, stress mediators, which are released after acute brain injury, might have a currently unknown direct impact on poststroke behavior mimicking cytokine-induced sickness behavior (see below).

Immunosuppression predisposes patients to bacterial infections, which contribute substantially to poststroke morbidity (Katzan et al., 2003). Based mainly on an experimental landmark study using the β blocker propranolol (Prass et al., 2003), catecholamines have been suggested as the key mediators of poststroke immune suppression and infectious complications (Dirnagl et al., 2007; Iadecola and Anrather, 2011). Furthermore, a previous study reported the modulation of hepatic iNKT cells by noradrenergic signals after experimental stroke (Wong et al., 2011). Indeed, most immune cells express catecholaminergic receptors and may undergo cell death after in vitro stimulation with metanephrines, but blockade of β adrenergic receptor in vivo and

Figure 7. The HMGB1-RAGE pathway is distinct from infectious or catecholamine-mediated effects. A, Microbiological analysis of blood samples and lung homogenates 3 d after 90 min Filament-MCAO for presence of bacteria. No significant growth of colony forming units (CFU) was detected after 24 h of incubation time on blood agar plates in any sample (n = 21, 2 individual experiments). B, Serum concentrations of cortisol and the catecholamines metanephrine and normetanephrine were measured 24 h after stroke induction (n = 7 per group, 2 individual experiments). Mice were treated with the β2-adrenergoreceptor inhibitor IC 118,551 (b2-INH) and/or sRAGE as indicated in Materials and Methods. C, sRAGE treatment, but not b2-INH, significantly reduced plasma IL-6 concentrations 24 h after F90 min-MCAO. D, The expansion of CD11b+Ly-6C-MDSCs at 24 h after F90 min-MCAO was significantly reduced by sRAGE and, to a lesser extent, by b2-INH and without a measurable additive effect. E, The reduction in spleen cellularity induced by MCAO was abrogated by b2-INH as well sRAGE treatment with an additive effect. C–E, Data were obtained in three individual experiments; n = 5 per group.
in vitro fails to prevent lymphocytopenia, the hallmark of immunodepression (Mracsko et al., 2014). Although increased catecholamine blood levels have been reported after experimental and clinical stroke (Chamorro et al., 2007; Urra et al., 2009b), their correlation with lymphopenia, humoral immunosuppression or bacterial infections was not confirmed in a recent study in stroke patients (Liesz et al., 2013a). Moreover, the catecholaminergic concept of poststroke immune depression suggests the in-
volvement of structures of the autonomic nervous system (e.g., the insula) and a laterality of the observed effect (right vs left hemisphere). However, the evidence from clinical and experimental studies rather points to lesion size instead of lesion location as the primary determinator of immune depression (Gendron et al., 2002; Hug et al., 2009; Liesz et al., 2013a). Therefore, the link between acute ischemic brain injury and the impact on the peripheral immune system remained to be identified. Genetic or pharmacological blockade of pattern recognition receptor signaling via HMGB1 and RAGE in this study abrogated cellular immunosuppression and also restored the activation of lymphocytes in the subacute phase after stroke. We have performed experiments comparing the differential effects of inhibiting the RAGE signaling cascade and beta2-adrenergic signaling on key outcome parameters of the acute immunostimulatory phase (cytokine release) and the subsequent immunosuppressive state in the subacute phase (spleen cellularity and MDSC expansion). Our findings suggest that alarmin-mediated pathways and the poststroke stress-related catecholaminergic effects are largely independent. While brain-released alarmins are released early after brain injury and might cause peripheral immune activation and subsequent immune exhaustion, catecholamines might represent an additional signaling pathway in the subacute phase compromising poststroke immune function (Fig. 8).

The concept of “stroke-induced immunodepression” (Meisel et al., 2005; Dirnagl et al., 2007) has been perceived as an independent and isolated immunological phenomenon after acute brain injury. However, our results suggest that this immunosuppressive state in the subacute phase after stroke has to be integrated as only one feature of the complex systemic immunosuppressive state in the subacute phase after stroke has to be identified. Geometric or pharmacological blockage of pattern recognition receptor signaling via HMGB1 and RAGE in this study abrogated cellular immunosuppression and also restored the activation of lymphocytes in the subacute phase after stroke. We have performed experiments comparing the differential effects of inhibiting the RAGE signaling cascade and beta2-adrenergic signaling on key outcome parameters of the acute immunostimulatory phase (cytokine release) and the subsequent immunosuppressive state in the subacute phase (spleen cellularity and MDSC expansion). Our findings suggest that alarmin-mediated pathways and the poststroke stress-related catecholaminergic effects are largely independent. While brain-released alarmins are released early after brain injury and might cause peripheral immune activation and subsequent immune exhaustion, catecholamines might represent an additional signaling pathway in the subacute phase compromising poststroke immune function (Fig. 8). For the cytokine-stimulating function of HMGB1, its residues at C23 and C45 must form a disulfide bond, whereas the unpaired C106 within BoxB must be in the reduced thiol state (Yang et al., 2010). This isoform is a very strong stimulus for various types of immune cells comparable with the stimulatory function of bacterial antigens, such as LPS (Lotze and Tracey, 2005). In this study, we have identified the induction of an “exhausted phenotype” of mature splenic monocytes after the initial (over)activation of the peripheral immune cells by brain-released HMGB1 in the acute phase (Fig. 8, B, C). The induction of reduced responsiveness of monocytes/macrophages to endotoxins or bacterial stimuli after a strong inflammatory reaction has been described in immunological conditions, such as sepsis or sterile inflammatory response (Randow et al., 1995; Haupt et al., 1998; Cavaillon et al., 2005). This hyporesponsive state of innate immune cells encompasses the reduced expression of MHC and costimulatory molecules as well as the impaired secretion of proinflammatory cytokines (TNF-α and IL-12). These features have also been reported after brain ischemia (Hug et al., 2009; Urra et al., 2009b; Vogelgesang et al., 2013). Inhibition of the initially overshooting immune activation in the hyperacute phase by neutralizing HMGB1 interaction with its receptors ameliorated also the secondary monocyte exhaustion, indicating a functional link between the distinct and opposing immunological phenotypes observed in the acute and subacute phase after brain lesion.
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