Depletion of Bone Marrow–derived Macrophages Perturbs the Innate Immune Response to Surgery and Reduces Postoperative Memory Dysfunction

Vincent Degos, M.D., Ph.D.,* Susana Vacas, M.D.,† Zhenying Han, M.D.,‡ Nico van Rooijen, Ph.D.,§
Pierre Gressens, M.D., Ph.D.,‖ Hua Su, M.D.,# William L. Young, M.D.,** Mervyn Maze, M.B., Ch.B.‡

ABSTRACT

Background: According to rodent models of postoperative cognitive decline, activation of the innate immune response following aseptic surgical trauma results in the elaboration of hippocampal proinflammatory cytokines, which are capable of disrupting long-term potentiation, the neurobiologic correlate of memory. The authors hypothesize that hippocampal

What We Already Know about This Topic

- Animal models of postoperative cognitive dysfunction implicate an innate immune response, with increased circulating inflammatory mediators and migration of bone marrow–derived cells into the brain
- Inhibitors of inflammatory mediators reduce postoperative memory deficits in mice but also reduce wound healing

What This Article Tells Us That Is New

- In mice, treatment with a drug that depletes bone marrow–derived macrophages reduced circulating inflammatory mediators after surgical orthopedic injury, reduced migration of immune cells into the brain, and reduced postoperative memory deficits

* Associate Researcher, Center for Cerebrovascular Research, Department of Anesthesia and Perioperative Care, University of California, San Francisco, San Francisco, California, INSERM, U676, Hôpital Robert Debré, and Department of Anesthesiology and Critical Care, Groupe Hospitalier Pitié-Salpêtrière, Assistance Publique-Hôpitaux de Paris, Université Pierre et Marie Curie, Paris, France. † Postdoctoral Fellow, Department of Anesthesia and Perioperative Care, University of California, San Francisco, San Francisco, California, and Program for Advanced Medical Education, Lisbon, Portugal. ‡ Assistant Researcher, Center for Cerebrovascular Research, Departments of Anesthesia and Perioperative Care, University of California, San Francisco, San Francisco, California. § Professor, Vrije Universiteit, VUMC, Department of Molecular Cell Biology, Faculty of Medicine, Amsterdam, The Netherlands. ‖ Professor, INSERM, U676, Hôpital Robert Debré, Paris, France. # Associate Professor, Center for Cerebrovascular Research, Departments of Anesthesia and Perioperative Care, University of California, San Francisco, San Francisco, California. ** Professor and Vice Chair, Center for Cerebrovascular Research, Departments of Anesthesia and Perioperative Care, Neurological Surgery, and Neurology, University of California, San Francisco, San Francisco, California. †† Professor and Chair, Department of Anesthesia and Perioperative Care, University of California, San Francisco, San Francisco, California.

recruitment of bone marrow–derived macrophages plays a causal role in these processes, resulting in memory dysfunction.
Methods: Clodrolip injection (liposomal formulation of clodronate) before stabilized tibial fracture under general anesthesia was used to deplete bone marrow–derived macrophages. Systemic inflammation and neuroinflammation were studied on postoperative day 1, and memory in a fear-trace conditioning paradigm was assessed on postoperative day 3. $CX3CR1^{GFP/+}$ $CCR2^{RFP/+}$ mice were used to identify bone marrow–derived macrophages.
Results: Clodrolip effectively depleted splenic $CCR2^+$ bone marrow–derived macrophages. It also attenuated the surgery-induced increase of interleukin-6 in the serum and the hippocampus, and prevented hippocampal infiltration of $CCR2^+$ cells without affecting the number of $CX3CR1^+$ microglia. It did not alter the surgery-induced increase in hippocampal monocyte chemoattractant protein-1, the recruitment signal for $CCR2^+$ cells. Clodrolip prevented surgery-induced memory dysfunction, as evidenced by a significant increase in freezing time (29% [95% CI, 21–38%] *vs.* 48% [95% CI, 38–58%], n = 20, $P = 0.004$), but did not affect memory in nonsurgical mice.
Conclusion: Depletion of bone marrow–derived macrophages prevents hippocampal neuroinflammation and

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memory dysfunction after experimental tibial fracture. These data suggest that the hippocampal recruitment of bone marrow–derived macrophages is a necessary mechanism in murine postoperative cognitive dysfunction. Interventions designed to prevent its activation and/or migration into the brain may represent a feasible preemptive strategy.

ACUTE postsurgical memory deterioration leads to persistent cognitive decline[1] that can result in considerable morbidity and increased mortality.[2] The specter of memory dysfunction, including acute delirium, postoperative decline, and dementia, is a source of anxiety for patients and their families.[3] Knowledge about the molecular and cellular pathways involved in postoperative memory dysfunction may provide a launching pad for the development of biomarkers to identify the most vulnerable patients as well as preventive strategies.

Using a murine model of aseptic surgical trauma with a long-bone fracture, we previously demonstrated that postoperative cognitive decline requires the engagement of the innate immune response. This engagement includes increased systemic expression of alarmins and proinflammatory cytokines such as interleukin (IL)-6 in the blood[4,5]; increased ratio of CD11b⁺ cells corresponding to macrophages/microglia cells,[4] and specifically the ratio of CCR2⁺ bone marrow-derived macrophages[6]; and elaboration of proinflammatory cytokines that are capable of disrupting hippocampal long-term potentiation, a neurobiologic correlate of learning and memory.[3,7–10]

Strategies designed to block the effect of proinflammatory cytokines with IL-1 receptor antagonist (anakinra) or tumor necrosis factor (TNF)-α antibody (etanercept) prevented murine postoperative memory dysfunction.[4,5] These interventions also prevented inflammation-dependent wound healing.[11,12]

In this study, we tested the hypothesis that mediation of postoperative memory decline requires recruitment of systemic bone marrow–derived macrophages into the brain, using a specific pharmacologic strategy to acutely deplete systemic phagocytes before an aseptic surgical trauma with an experimental tibial fracture.

Materials and Methods

Animals

All experimental procedures involving animals were approved by the University of California, San Francisco Institutional Animal Care and Use Committee, and conformed to National Institutes of Health guidelines. Twelve 8- to 12-week-old $CCR\ 2^{RFP/+}CX3CR1^{GFP/+}$ male mice[6,13] (fig. 1A) were used to identify bone marrow–derived macrophages. CCR2 and CX3CR1 are acronyms for chemokine (C-C motif) receptor 2 (whose cognate ligand is monocyte chemoattractant protein [MCP]-1) that is highly expressed in bone marrow–derived macrophages, and CX3C chemokine receptor 1 (CX3CR1, fractalkine receptor) that is highly expressed in resident microglia. Eighty-nine
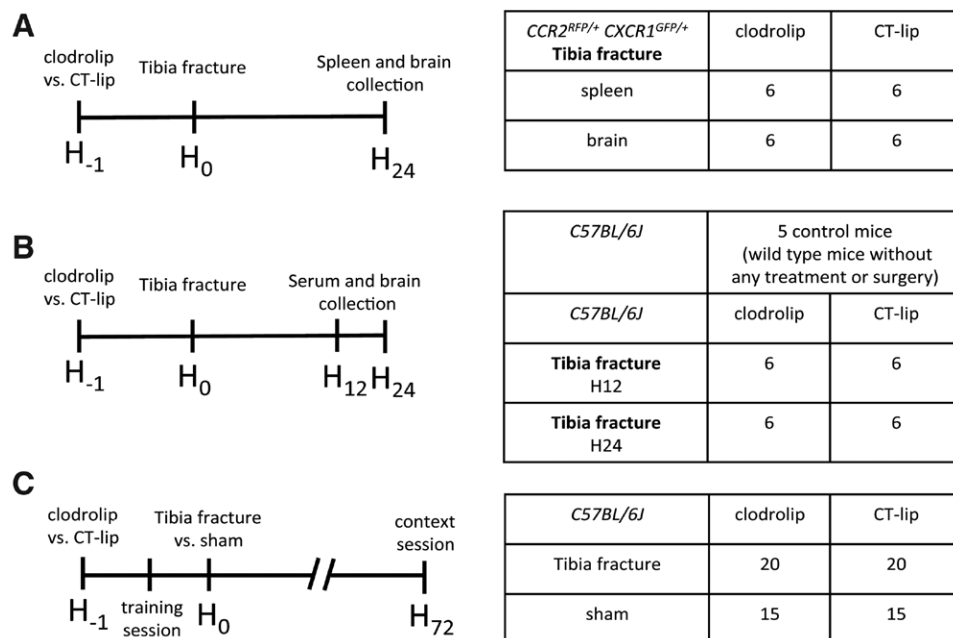
Fig. 1. Study design and splenic macrophage depletion with clodrolip. (*A*) First experiment with 12 *CCR2^RFP/+ CX3CR1^GFP/+* mice divided into two groups of six mice each treated with intraperitoneal (IP) injection of clodrolip *versus* CT-lip 1h before the tibia fracture model and 25h before tissue collection. (*B*) Second experiment with 24 *C57BL/6J* mice divided into four groups of six mice treated with IP injection of clodrolip *versus* CT-lip 1h before the tibia fracture and sacrificed 12 or 24h after the tibia fracture. (*C*) Third experiment with 70 *C57BL/6J* mice divided into four groups treated with IP injection of CT-lip *versus* clodrolip 1h before the tibia fracture (20 mice per group) *versus* sham procedure (15 mice per group). The training session of the memory test was performed 30min after the IP injection and 30min before surgery, and the context session was performed 72h after surgery. CT-lip = control liposome.

wild-type male mice (C57BL/6J, 10–12 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME): 29 for the cytokine expression (fig. 1B) and 70 for the behavior tests (fig. 1C). Mice did not experience unexpected lethality in the study and were euthanized according to our institutional animal care and use committee guidelines.

In Vivo Systemic Phagocyte Depletion with Clodrolip

Clodrolip is a liposomal formulation of clodronate (dichloromethylene bisphosphonic acid), a nontoxic bisphosphonate. Liposomes are lipid vesicles consisting of concentric phospholipid bilayers surrounding aqueous compartments. In this case, liposomes are used as "Trojan horses" encapsulating clodronate, which are then ingested and digested by phagocytes, followed by an intracellular release and accumulation of clodronate. At a certain intracellular concentration, clodronate induces apoptosis of the phagocytes. Clodronate liposomes were obtained from clodronateliposomes. org** (Vrije Universiteit, Amsterdam, The Netherlands) at a concentration of 7 mg/ml and prepared as described previously.[14,15] Clodrolip (200 μl, approximately 100 mg/kg) was injected intraperitoneally 60 min before the bone fracture. Control animals received 200 μl of control liposomal solution (CT-lip). No intraperitoneal or extraperitoneal damage was observed after clodrolip intraperitoneal administration.

Long-bone Fracture with Tibia Fracture Surgery

Anesthesia was induced and maintained with isoflurane by inhalation. We used a dedicated chamber for induction with 5% isoflurane for 3 min, and the operation was performed under 2% isoflurane for 10–12 min. Under aseptic surgical conditions, an open tibial fracture of the right hind limb with intramedullary fixation was performed as described previously.[4] Body temperature was maintained at 37° ± 0.5°C using a thermal blanket throughout the surgical procedure, and analgesia was provided by injection of buprenorphine (0.3 mg in 100 μl of saline). Sham mice for bone fracture (sham group) received the same anesthesia and analgesia as the bone fracture mice.

Measurement of IL-6 in Serum

Mouse blood was collected using cardiac puncture under general anesthesia (isoflurane, 3%) in separate cohorts 12 and 24 h after the bone fracture procedure (fig. 1B). Blood samples were centrifuged at 1300 rpm for 10 min at room temperature, and the serum was collected and frozen at –80°C. IL-6 is secreted by bone marrow–derived macrophages in response to alarmins,[16] and the IL-6 level in the serum is increased within the first 24 h after the tibia fracture.[4,5] The IL-6 level in the serum is also associated with the postoperative memory dysfunction phenotype[5] and is affected by clodrolip in response to lipopolysaccharide infusion.[17] For these reasons, we decided to quantify IL-6 levels in the serum of mice exposed to clodrolip or CT-lip using the IL-6 enzyme-linked immunosorbent assay kit (KMC0062; Invitrogen, Grand Island, NY). Results are expressed as fold increase compared with that measured in five control mice that did not receive any treatment or surgery.

Measurement of Cytokines in the Hippocampus

The hippocampi of the mice were collected rapidly under a dissecting microscope, 12 and 24 h after the tibia fracture (fig. 1B), and placed in RNAlater solution (Qiagen, Valencia, CA). To avoid blood contamination, mice were perfused with saline for 5 minutes before sample collection. Total RNA was extracted using the RNeasy Lipid tissue Kit (Qiagen) treated with recombinant DNase I using a RNase-Free Dnase set (Qiagen), and reverse-transcribed to complementary DNA with a High Capacity RNA to Complementary DNA Kit (Applied Biosystems, Bedford, MA). TaqMan Fast Advanced Master Mix (Applied Biosystems) and gene-specific primers and probes used for quantitative polymerase chain reaction are as follows: β-actin (NM_007393.1), IL-6 (Mm00446190_m1), TNF-α (Mm00443258_m1), IL-1β (Mm01336189_m1), and MCP-1 (Mm00441242_m1). Quantitative polymerase chain reaction was performed using StepOnePlus (Applied Biosystems). Each RNA sample was run in triplicate, and relative gene expression was calculated using the comparative threshold cycle (ΔCT) method and normalized to β-actin. Results are expressed as fold increase compared with that observed in five control mice that did not receive any treatment or surgery.

Quantification of Bone Marrow–derived Macrophages and Microglia

Twenty-four hours after the tibia fracture surgery, the brain and spleen of the $CCR2^{RFP/+}CX3CR1^{GFP/+}$ mice were collected after intracardiac perfusion with paraformaldehyde 4% (fig. 1A). Spleen and brain (bregma, –1.0 to –1.4 mm, corresponding to interaural 2.7 to 2.3 mm in coronal orientation) were sectioned into 20-μm-thick slices and mounted with Vectashield DAPI (Vector Laboratories, Burlingame, CA). The expression of CCR2-RFP and CX3CR1-GFP cells was assessed using confocal images, performed with a Spectral Confocal microscope (Nikon Instruments, Melville, NY) using three laser lines (405, 488, and 561 nm). Z-stacks were rendered into a three-dimensional image using the NIS-Elements AR 3.0 software (Nikon), and the expression of CCR2-RFP and CX3CR1-GFP cells was quantified using ImageJ (National Institutes of Health, Bethesda, MD), with three different photographs per mouse taken with a 20× objective. Data are expressed as relative cell percentages normalized to the average value of the CT-lip group.

Behavioral Test for Hippocampus-dependent Memory with Trace Fear Conditioning

Fear conditioning is used to assess memory in rodents, which are trained to associate a conditional stimulus, such as a conditioning chamber, with an aversive, unconditional stimulus,

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** http://www.clodronateliposomes.org/ashwindigital. asp?docid=26. Accessed January 7, 2013.

such as a foot shock. Freezing behavior is an indicator of aversive memory that is measured when subjects are reexposed to the conditional stimulus. With this model, lesions of the hippocampus disrupt recall of fear responses to the presentation of the context, resulting in a diminution in freezing.[18,19]

For this study, we used a previously published paradigm.[4–6,20] Briefly, the behavioral study was conducted using a conditioning chamber (Med Associates, Inc., St. Albans, VT) and an unconditional stimulus (two periods of foot shock of 0.75 mA during 2 s). An infrared video camera, mounted in front of the chamber, captured motion speed (Video Freeze; Med Associates).

All of the animals underwent the same training session, regardless of the specific intervention, and received their training 30–40 min after the liposomal intraperitoneal injections (whether clodrolipid or CT-lip) that occurred 30 min before surgery (fig. 1C). Three days after conditioning, mice were returned to the same chamber where training had occurred for a context test. During the context test, mice were exposed just to the context and no tones or foot shocks were delivered. Freezing was recognized by the software as a total lack of movement, excluding breathing and movement of vibrissae (linear detection with a minimal freeze duration of 20 frames corresponding to 0.7 s and a motion threshold of 20 arbitrary units).[4–6,20] Decrease in the percentage of time spent freezing indicated impairment of memory.

Body Weight and Maximal Motion Speed

The body weight of the animals was measured 3 days after surgery, following assessment of freezing behavior. An infrared video camera (Video Freeze) captured and quantified motion speed during the context test, and the maximal motion speed was recorded for each mouse.

Statistical Analysis

Data are presented as mean ± 95% CI. Normality was tested with the d'Agostino–Pearson omnibus normality test. Equality of variances was tested with the F test. For two-sample comparisons, Student t tests were used (using the Welch correction if necessary); Mann–Whitney U tests were used if data were not normally distributed. For comparisons of more than two groups, means were compared using one-way ANOVA followed by Student t tests with a Bonferroni-corrected alpha level.

We used the two-way ANOVA procedure to determine whether or not time and treatment were significant factors in predicting IL-6 concentration in the serum, and IL-6, IL-1β, TNF-α, and MCP-1 messenger ribonucleic acid (mRNA) expression in the hippocampi. Given the highly skewed nature of the mRNA expression, we checked the distribution of the residuals. We applied a log transformation (ln[X]) to the response of the mRNA expression before performing analysis to better adhere to the ANOVA model's assumptions of normally distributed residuals and homoscedasticity of residuals.

For the behavior tests, animals were tagged and allocated randomly to each group before any treatment, and researchers were blinded to the group assignment that was revealed only after the analysis phase. A repeated measures ANOVA was performed to determine whether treatment (CT-lip and clodrolip) and the three time periods (baseline, first shock, and second shock) were significant predictors of percentage freezing time during the training session.

For this study, our primary outcome was percentage of freezing time during the context session. Based on previous freezing time data,[4] we estimated that a sample of 18 C57BL/6J surgical mice per group was necessary to demonstrate a 20% increase in percentage freezing time, with 80% power at the 0.017 alpha level (after adjusting for three comparisons) to find a significant difference between clodrolip and CT-lip.

A two-tailed value of $P < 0.05$ was considered statistically significant for two-group comparisons, and the significance threshold was adjusted for multiple comparisons with a Bonferroni correction. Prism 5 (GraphPad Software, Inc., La Jolla, CA) was used to conduct the statistical analyses.

Results

Clodrolip Depletes Splenic Bone Marrow–derived Macrophages and Prevents Hippocampal Bone Marrow–derived Macrophage Infiltration

Using $CCR2^{RFP/+}CX3CR1^{GFP/+}$ mice (fig. 1A), in which RFP+ bone marrow–derived macrophages and GFP+ resident microglia can be tracked,[6,13] we found that clodrolip depleted splenic macrophages and surgery-induced bone marrow–derived macrophage infiltration into the hippocampus. The CCR2+ cells, which are mainly present in the splenic red pulp (fig. 2A), decreased by 96% in the clodrolip-exposed mice (fig. 2B) (95% CI, 95–97%, $P < 0.001$). As shown in figure 3, the number of CCR2+ cells was also significantly reduced in the hippocampi of clodrolip-treated mice compared with CT-lip–treated mice 24 h after surgery (decrease of 76% for the dentate gyrus and 87% in the cornu ammonis 3). However, clodrolip treatment did not change the number of CX3CR1+ cells in the dentate gyrus and cornu ammonis 3 hippocampal regions (fig. 3).

### Clodrolip Reduces Systemic and Hippocampal Proinflammatory Cytokines

We previously showed that proinflammatory cytokines in the blood and hippocampus increased within the first day after surgery.[4] To test whether clodrolip treatment would reduce the proinflammatory cytokines, we studied serum and hippocampal expression 12 and 24 h after surgery (fig. 1B). Twelve hours after surgery, the rise in IL-6 in the serum was significantly attenuated in mice exposed to clodrolip (two-way ANOVA, $P = 0.004$ for the treatment, $P = 0.003$ for the time effect, and $P = 0.19$ for interaction) (fig. 4).

Between 12 and 24 h after surgery, the increase in mRNA hippocampal expression of IL-6, TNF-α, and IL-1 induced by
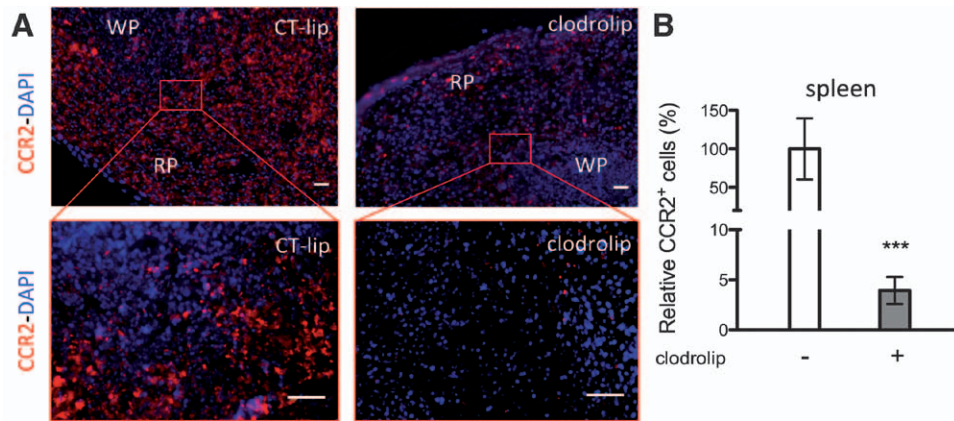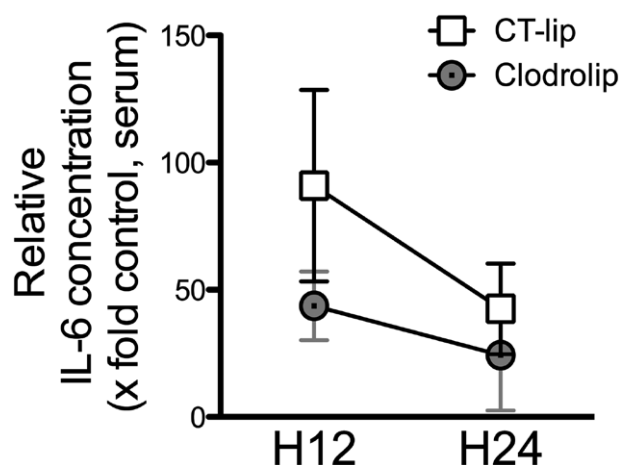
Fig. 2. Effects of systemic macrophage depletion with clodrolip on the CCR2⁺ splenic cells. (*A*) Representative photographs of spleen section showing CCR2⁺ cell repartition mainly in the red pulp (RP) and less in the white pulp (WP), 24 h after tibia fracture. Top photographs are of low magnification (*scale bar* = 100 μm) and bottom photographs are highly magnified images (*scale bar* = 50 μm) in the CT-lip and the clodrolip mice. (*B*) Quantification of the relative percentage of CCR2⁺ cells in the spleen after clodrolip (n = 6, ***$P < 0.001$ with unpaired Student *t* test). CCR2 = chemokine (C-C motif) receptor 2; CT-lip = control liposome; DAPI = 4',6'-diamidino-2-phenylindole; IP = intraperitoneal (*bars* = mean ± 95% CI).
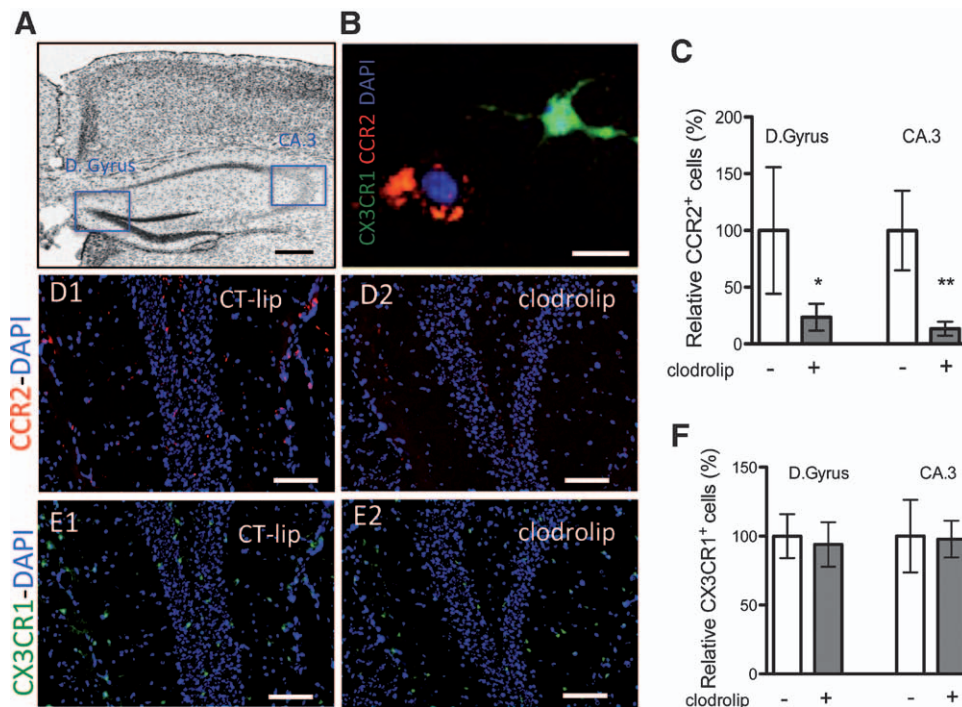
Fig. 3. Effects of systemic macrophage depletion with clodrolip on the hippocampal CCR2⁺ and CX3CR1⁺ cells. (*A*) Representative photographs of the section of interest corresponding to bregma, −1.2 mm (*scale bar* = 500 μm), showing the dentate gyrus (D.Gyrus) and the *cornu ammonis* subdivision 3 (CA.3). (*B*) Representative highly magnified photograph (*scale bar* = 20 μm) of a ramified CX3CR1⁺ green cell and an amoeboid CCR2⁺ red cell in the hippocampus. (*C*) Bar graph shows quantification of the relative percentage of CCR2⁺ cells in the dentate gyrus and the CA.3 regions after clodrolip treatment (n = 6, significant F test for both comparisons, *$P = 0.02$; **$P = 0.002$ with unpaired Student *t* tests with Welch's correction). (*D*) Representative photographs of the dentate gyrus hippocampal sections in the CT-lip (*D1*) and the clodrolip (*D2*) mice (*scale bar* = 100 μm) showing the decrease of CCR2⁺ cells after clodrolip treatment. (*E*) Representative photographs of the dentate gyrus hippocampal section in the CT-lip (*E1*) and the clodrolip (*E2*) mice (*scale bar* = 100 μm) showing the absence of CX3CR1⁺ cell depletion after clodrolip treatment. (*F*) Bar graph shows quantification of the relative percentage of CCR2⁺ cells in the dentate gyrus and the CA.3 regions after clodrolip (n = 6, $P = 0.51$ for dentate gyrus and $P = 0.51$ for CA.3). CT-lip = control-liposome; CCR2 = chemokine (C-C motif) receptor 2; DAPI = 4',6'-diamidino-2-phenylindole (*bars* = mean ± 95% CI).

Fig. 4. Effects of systemic macrophage depletion with clodrolip on the IL-6 serum concentration after the tibia fracture 12 and 24 h after tibia fracture in the clodrolip and CT-lip groups. Data are normalized to the average value of the control mice group that did not receive any treatment or any surgery and expressed as fold time increase (n = 6 per group and per timing, two-way ANOVA, $P = 0.004$ for the treatment effect, $P = 0.003$ for the time effect, and $P = 0.19$ for interaction). CCR2 = chemokine (C-C motif) receptor 2; CT-lip = control liposome; DAPI = 4',6'-diamidino-2-phenylindole; IL = interleukin-6; IP = intraperitoneal (*boxes* and *circles* = mean ± 95% CI).

surgery returned to almost baseline values at 24 h (fig. 5). Clodrolip exposure significantly inhibited the surgery-induced increased expression of mRNA IL-6 (two-way ANOVA, $P < 0.001$ for the treatment, $P = 0.002$ for the time effect, and $P = 0.51$ for interaction), and interacted with the time-dependent decrease for TNF-α (two-way ANOVA, $P = 0.03$ for interaction). Clodrolip treatment did not change IL-1β mRNA expression (two-way ANOVA, $P = 0.42$ for the treatment, $P < 0.001$ for the time effect, and $P = 0.66$ for interaction) (fig. 5).

Systemic macrophages are recruited into tissues by the chemoattractant MCP-1 that binds to CCR2, which is expressed on the surface of bone marrow–derived macrophages.[21,22] Following surgery, MCP-1 mRNA expression increases and is unaffected by prior exposure to clodrolip (two-way ANOVA, $P = 0.64$ for the treatment, $P < 0.001$ for the time effect, and $P = 0.67$ for interaction) (fig. 5D).

Clodrolip Prevents Surgery-induced Memory Impairment
During the preoperative training period, learning was similar in the clodrolip-exposed and the control (nonexposed) groups, with the percentage of freezing being highly associated with time (fig. 6). During the context session, surgery significantly decreased percentage of freezing time in comparison with the sham group (52% [95% CI, 41–63%] *vs.* 29% [95% CI, 21 to 37%], $P = 0.0012$); preoperative exposure to clodrolip resulted in significantly greater freezing time than in the nonexposed surgical cohort (29% [95% CI, 21–38%] *vs.* 48% [95% CI: 38–58%], $P = 0.004$), reaching a level similar to that observed in the sham-operated clodrolip-exposed mice (49% [95% CI, 36–63%], $P = 0.86$) (fig. 7, A and B).

Clodrolip did not affect the body weight of the mice 3 days after the injection (fig. 7C). As for maximal motion speed, the clodrolip-treated groups were no different from the CT-lip groups, even though the maximal motion speed of the surgical groups was significantly slower than the sham groups (fig. 7D).

Discussion
In this study, we report for the first time that bone marrow–derived macrophages are required in the pathogenesis of the neuroinflammatory and memory dysfunction induced by surgery. Also, we report that a possible hippocampal signal through MCP-1 is involved in the recruitment of bone marrow–derived macrophages to this brain region. Data from rodent surgical models have provided insight into the neuroinflammatory basis for postoperative cognitive decline. This usually transient process appears to be part of a motivational system that reorganizes the organism's priorities to facilitate recovery. To date, we have established a pivotal early role for the proinflammatory cytokine TNF-α,[6] and our study demonstrated that hippocampal infiltration of bone marrow–derived macrophages also plays a role in the initiation of neuroinflammation.

Hippocampal Infiltration of Bone Marrow–derived Macrophages after Surgery
Monocyte infiltration into the brain is mainly described in acute brain injuries such as stroke[23] and traumatic brain injuries,[24] as well as chronic inflammatory brain injuries such as multiple sclerosis.[13] Using long-bone fracture as a surrogate for a peripheral orthopedic surgical insult, we previously reported that CCR2+ cells were present in the hippocampus.[6] Because microglia can also express CCR2 under certain conditions,[25,26] we could not ascertain whether these CCR2-expressing cells arose from the resident macrophage population (microglia) or through an infiltration from outside of the central nervous system. Using clodrolip to specifically deplete the systemic pool of phagocytes, including bone marrow–derived macrophages, we were able to demonstrate that the CCR2+ cells in the hippocampus are a result of the recruitment of bone marrow–derived macrophages into the brain.

For passage into the brain, monocytes are required to overcome the blood–brain and/or blood–cerebral spinal fluid barrier[27,28]; these barriers can be disrupted by direct acute brain injury.[29,30] Interestingly, after peripheral surgery, the blood–brain barrier is disrupted, although there is no discernible brain lesion.[6] Now we show that after surgery, the hippocampus expresses MCP-1, which is capable of attracting CCR2+-expressing cells migrating through the disrupted blood–brain barrier. This increased expression of MCP-1 is unaffected by clodrolip treatment, indicating that bone marrow–derived macrophages are not a self-perpetuating source of this chemoattractant for its own recruitment. Future understanding of the source and the triggers for hippocampal MCP-1 following peripheral surgery may result
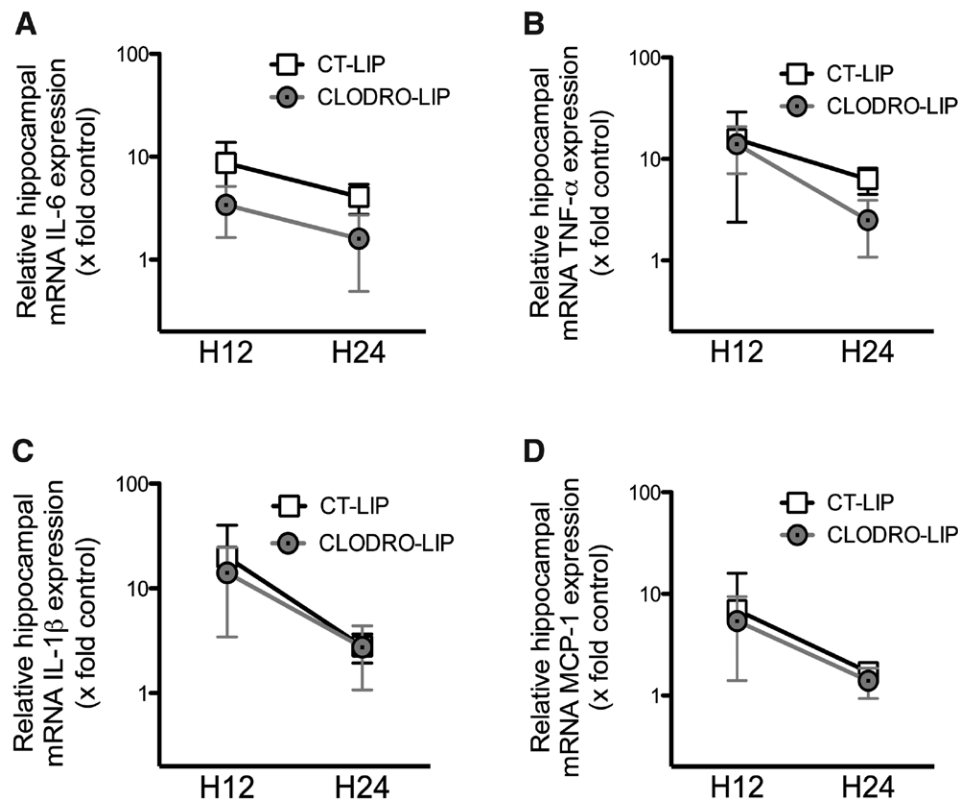
Fig. 5. Clodrolip effect and early kinetic of the mRNA hippocampal expression of IL-1β, TNF-α, IL-6, and MCP-1 after tibia fracture. (*A*) Hippocampal mRNA expression of IL-6 relatively expressed as fold increase compared with control brain expression 12 and 24 h after tibia fracture in the CT-lip and the clodrolip-treated mice (n = 6 per group and per timing, two-way ANOVA, $P = 0.0003$ for the treatment effect, $P = 0.0023$ for the time effect, and $P = 0.51$ for interaction). (*B*) Hippocampal mRNA expression of TNF-α relatively expressed as fold increase compared with control brain expression 12 and 24 h after tibia fracture in the CT-lip and the clodrolip-treated mice (n = 6 per group and per timing, two-way ANOVA, $P = 0.06$ for the treatment, $P < 0.0001$ for the time effect, and $P = 0.035$ for interaction). (*C*) Hippocampal mRNA expression of IL-1β relatively expressed as fold increase compared with control brain expression 12 and 24 h after tibia fracture in the CT-lip and the clodrolip-treated mice (n = 6 per group and per timing, two-way ANOVA, $P = 0.42$ for the treatment effect, $P < 0.0001$ for the time effect, and $P = 0.66$ for interaction). (*D*) Hippocampal mRNA expression of MCP-1 relatively expressed as fold increase compared with control brain expression 12 and 24 h after tibia fracture in the CT-lip and the clodrolip-treated mice (two-way ANOVA, $P = 0.64$ for the treatment, $P = 0.0005$ for the time effect, and $P = 0.67$ for interaction). CT-lip = control liposome; IL = interleukin; MCP-1 = monocyte chemoattractant protein-1; mRNA = messenger ribonucleic acid; TNF = tumor necrosis factor (*boxes* = mean ± 95% CI and data are presented with a logarithmic scale).

in interventional strategies designed to prevent recruitment of bone marrow–derived macrophages into the brain.

Hippocampal Bone Marrow–derived Macrophage Infiltration and Memory Dysfunction

Our recent data suggest that transient hippocampal inflammation is the key element in postoperative memory dysfunction because (1) hippocampal areas are known to be involved in memory tasks[7]; (2) hippocampal neuroinflammation profile correlates with the level of memory dysfunction[4,5]; and (3) hippocampal neuroinflammation leads to long-term potentiation disruption.[31,32] Now we report here that the absence of bone marrow–derived macrophage infiltration, produced by systemic depletion by clodrolip, decreases surgery-induced hippocampal inflammation and memory dysfunction. Therefore, postoperative bone marrow–derived macrophage recruitment into the

hippocampus plays a key role in the initiation of postoperative memory dysfunction.

In the context of postoperative cognitive decline, determining which cells are involved in the initiation of the inflammation response is important because, when exaggerated, this could overwhelm resolving responses and produce persistent postoperative cognitive decline. Earlier, we described that surgical trauma induces systemic release of alarmins (*i.e.*, high-mobility group protein 1) and proinflammatory cytokines (*i.e.*, TNF-α and IL-6).[4,5] Improving our knowledge of cellular and molecular initiation mechanisms will allow insight into an *ex vivo* bioassay to prospectively determine whether patients are at risk.

Limitations of the Study

We used experimental tibial fracture to generate animal postoperative memory acute dysfunction. With this model, we traumatized the bone marrow directly, which could play
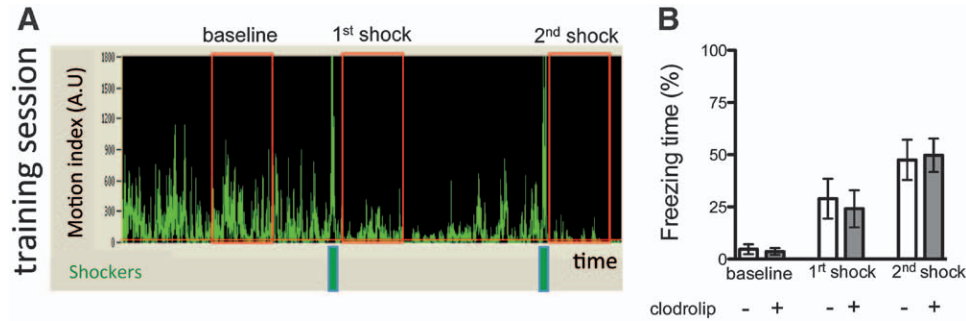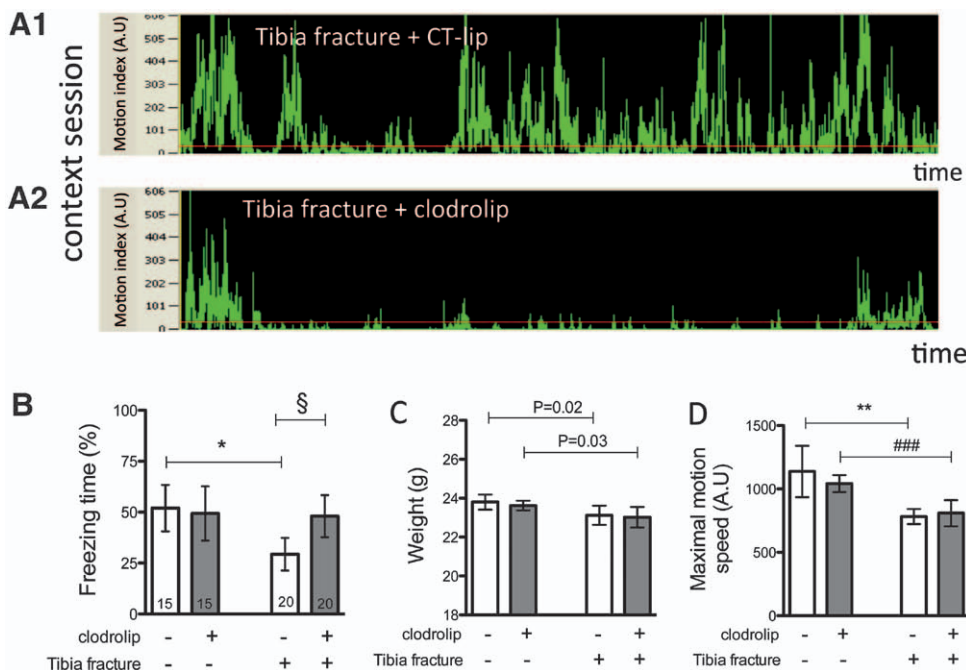
Fig. 6. Clodrolip effect on the training session. (*A*) Representative record of a training session showing the motion (motion index expressed in arbitrary units) of the mouse according to the time. The two *green bars* represent the two shocks and the three *red rectangles* represent the 40-s periods used to quantify the baseline, first, and second shock freezing responses. Bar graph (*B*) quantifies the percentage of freezing time (n = 35), and two-way ANOVA shows a significant effect of time (*P* < 0.0001), no significant effect of treatment (*P* = 0.68), and no interaction (*P* = 0.61).

a key role. However, other models that did not damage bone marrow with a splenectomy[33] also showed that surgery generated postoperative cognitive dysfunction.

Fibrin is deposited in the hippocampus after tibia fracture,[6] suggesting that the blood–brain barrier becomes disrupted and may allow the passage of clodrolip to act directly on the microglia population.[34] However, we found that the systemic administration of clodrolip acts only on the number of CCR2+ cells without significantly affecting the number of CX3CR1+ cells (fig. 3); if clodrolip has an effect on

microglia, it may be to functionally modify them. For this reason, we cannot exclude the possibility that clodrolip does not affect the function of microglia, and that microglia do not play a key role in postoperative cognitive dysfunction.

For this study, we used a pharmacologic strategy to quickly deplete the pool of systemic macrophages. However, because clodrolip is highly toxic for monocytes and macrophages,[35] it can increase the risk of postsurgical infections, generating a phenotype of its own. With a single dose, we did not observe loss of weight or other signs of sickness within the

Fig. 7. Depletion of systemic macrophages reduces surgery-induced memory dysfunction. (*A*) Representative records of context sessions of tibia fracture mice treated with CT-lip (*A1*) and with clodrolip (*A2*) showing the motion (motion index expressed in arbitrary units) of the mice according to the time. (*B*) Quantification of the freezing time percentage according to the four groups (n = 15–20, *P* = 0.0012 and §*P* = 0.004, respectively, with one-way ANOVA and Bonferroni *post hoc* analysis). (*C*) Quantification of the body weight in the four groups 3 days after surgery (n = 15–20, *P* = 0.02 and *P* = 0.03, respectively, not significant after adjustment for multiple comparisons with one-way ANOVA and Bonferroni *post hoc* analysis and no significant effect of the clodrolip treatment. (*D*) Quantification of the maximal motion speed in the four groups 3 days after surgery (n = 15–20, **P* = 0.012 and ###*P* = 0.0003, respectively, with one-way ANOVA and Bonferroni *post hoc* analysis and no significant effect of the clodrolip treatment; *bars* = mean ± 95% CI). AU = arbitrary units; CT-lip = control-liposome.

3 days. We performed a very short-term study, focusing on the acute exaggeration phase of neuroinflammation, and did not perform any long-term study with clodrolip. Clodrolip should be considered as a tool for mechanistic studies but cannot be proposed for clinical therapy.

To distinguish whether these recruited cells were resident or recruited systemic macrophages, we used *CCR2^RFP/+CX3 CR1^GFP/+* mice. CCR2 is receptor for MCP-1 and is mainly expressed in bone marrow–derived monocytes–macrophages. We previously showed that CD11b⁺ macrophages–microglia cells were recruited in the hippocampus after tibial fracture.[4] In this study, however, we did not determine that CCR2⁺ cells were only bone marrow–derived macrophages. Further study on the role of other systemic phagocytes, including neutrophils, in our phenotype will be of most interest.

Hippocampal cytokine expression was performed with mRNA and not with protein. This is a limitation, but we considered that the potential extravasation from blood may affect protein levels. Indeed, in this model we found blood–brain barrier leakage after the tibia fracture,[6] and the increase of circulating IL-6 protein in the serum could contaminate the hippocampal samples with passive movement in the parenchyma (this phenomenon could be amplified by the perfusion itself). By analyzing mRNA expression in the brain collected after purging blood from the vessels, we ensured that hippocampal cells were the source of proinflammatory cytokines.

In conclusion, we showed in this study that bone marrow–derived macrophage activation after experimental tibial fracture is directly involved in the tibia fracture–induced hippocampal bone marrow–derived macrophage infiltration and animal memory dysfunction. Understanding the cellular and biologic pathways involved in postoperative cognitive decline is a key element in designing interventions to prevent this disease. Reducing activation and/or migration of innate immune cells, such as systemic macrophages, into the brain represents a viable preemptive strategy.

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Liebig Explodes Heppenheim's Apothecary Shop … or Did He?
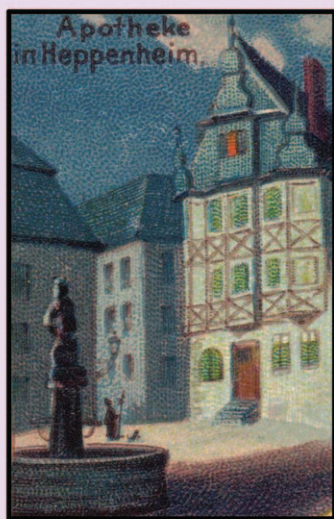
After his chemical tinkerings exploded, schoolboy Justus Liebig (1803–1873) was expelled from his hometown's "gymnasium" in Darmstadt, Germany. Relocating 19 miles south from 1819–20, Justus apprenticed in an apothecary shop (*left*) run by Gottfried Pirsch of Heppenheim. Growing bored with his apothecary mentor, Justus resumed his previous experiments with unstable chemicals. His pharmacy apprenticeship terminated abruptly, after only 10 months, due to a violent explosion (*right*) and/or his family's inability to keep paying Pirsch. The mischievous young Liebig would grow up to become a founder of organic chemistry and a codiscoverer of chloroform. (Copyright © the American Society of Anesthesiologists, Inc.)

George S. Bause, M.D., M.P.H., Honorary Curator, ASA's Wood Library-Museum of Anesthesiology, Park Ridge, Illinois, and Clinical Associate Professor, Case Western Reserve University, C leveland, Ohio. UJYC@aol.com.