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Activation of Myeloid TLR4 Mediates T Lymphocyte Polarization after Traumatic Brain Injury

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Traumatic brain injury (TBI) is a major public health issue, producing significant patient mortality and poor long-term outcomes. Increasing evidence suggests an important, yet poorly defined, role for the immune system in the development of secondary neurologic injury over the days and weeks following a TBI. In this study, we tested the hypothesis that peripheral macrophage infiltration initiates long-lasting adaptive immune responses after TBI. Using a murine controlled cortical impact model, we used adoptive transfer, transgenic, and bone marrow chimera approaches to show increased infiltration and proinflammatory (classically activated [M1]) polarization of macrophages for up to 3 wk post-TBI. Monocytes purified from the injured brain stimulated the proliferation of naive T lymphocytes, enhanced the polarization of T effector cells (T_H1/T_H17), and decreased the production of regulatory T cells in an MLR. Similarly, elevated T effector cell polarization within blood and brain tissue was attenuated by myeloid cell depletion after TBI. Functionally, C3H/HeJ (TLR4 mutant) mice reversed M1 macrophage and T_H1/T_H17 polarization after TBI compared with C3H/OuJ (wild-type) mice. Moreover, brain monocytes isolated from C3H/HeJ mice were less potent stimulators of T lymphocyte proliferation and T_H1/T_H17 polarization compared with C3H/OuJ monocytes. Taken together, our data implicate TLR4-dependent, M1 macrophage trafficking/polarization into the CNS as a key mechanistic link between acute TBI and long-term, adaptive immune responses. ***The Journal of Immunology*****, 2017, 198: 3615–3626.**

Traumatic brain injury (TBI) is a leading cause of death and disability worldwide. Impact and/or coup–contrecoup injuries produce immediate mechanical injury, including cellular necrosis, diffuse axonal shearing, and tissue loss, which contributes to poor neurologic outcomes after TBI (1); however, one third of hospitalized TBI patients die of secondary pathological processes that manifest in the days after the initial traumatic event. In particular, most axonal damage is not due to physical shearing at the time of TBI (2). Rather, delayed atrophy of white matter tracts develops for >1 y post-TBI in rodents and progresses for decades in TBI patients to produce chronic cognitive, psychiatric, and motor deficits (1, 3).

Routine surveillance of the CNS is limited by the immunoprivileged status of the brain, yet a robust and sequential set of systemic immune responses develops after TBI (4, 5). Innate immune activation, which provides immediate, nonspecific host responses to tissue injury, temporally correlates with widespread cellular necrosis after TBI (6). Surgical excision of necrotic brain tissue during the acute injury phase improved clinical outcomes at 6 mo in TBI patients (7). Notably, damage associated molecular patterns, host molecules that are passively released after trauma, activate pattern recognition receptors to initiate innate immune responses (8). We reported that activation of myeloid TLR4, a prototypical pattern recognition receptor, induced proinflammatory responses and exacerbated neurologic injury after TBI (9); however, the mechanisms linking acute neurotrauma with the development of chronic inflammatory responses remain poorly defined.

The CNS was long believed to lack a classical lymphatic system; yet, recent studies documented the presence of specialized meningeal lymphatic vessels that preferentially drain cerebrospinal fluid into the deep cervical lymph nodes (10, 11). These vessels also provide a conduit for the bidirectional movement of immune cells between the CNS and lymph nodes (10, 11). Activated macrophages, an important component of the mononuclear phagocyte system, exhibit an Ag-presenting capability during a primary immune response, and Ag-loaded macrophages travel to draining lymph nodes to initiate adaptive immune responses via an MHC class II–dependent process. Notably, T lymphocytes are activated within deep cervical lymph nodes, rather than at sites of nerve injury (12), and pharmacological inhibition of MHC class II–dependent Ag processing reduced neurodegeneration after TBI (13). Because macrophage activation temporally preceded brain T lymphocyte infiltration and neurologic deterioration after experimental and clinical TBI (14, 15), early macrophage activation may initiate deleterious adaptive immune responses after TBI.

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Abbreviations used in this article: BBB, blood–brain barrier; HMGB1, high mobility group box protein 1; M1, classically activated; M2, alternatively activated; MFI, mean channel fluorescence intensity; RFP, red fluorescent protein; TBI, traumatic brain injury; T_{REG}, regulatory T lymphocyte.

Macrophages polarize based on microenvironmental cues to generate divergent, context-specific functions. Along these lines, classically activated (M1) macrophages release proinflammatory cytokines and remove damaged cells from sites of injury; however, chronic M1 activation exacerbates secondary damage and impairs tissue repair (16). Conversely, alternatively activated (M2) macrophages release counterinflammatory cytokines to dampen immune responses and to promote wound healing (17). A heterogeneous mixture of M1 and M2 phenotypes are observed after TBI, with an early, transient M2 phenotype yielding to a predominant M1 phenotype over the first days post-TBI (18, 19). Accumulation of M1 macrophages within white and gray matter temporally preceded oligodendrocyte apoptosis and widespread myelin loss for weeks after experimental TBI (20). Similarly, a progressive increase in the ratio of M1/M2 macrophages, including the chronic accumulation of HLA-DR$^+$ M1-like macrophages within the corpus callosum of >25% TBI patients, temporally correlated with white matter loss and neurologic injury for two decades after TBI (19, 21–23). Moreover, conditioned media from M1 macrophages increased oligodendrocyte cell death after oxygen-glucose deprivation, whereas conditioned media from M2 macrophages was protective (19). Thus, polarized macrophages may represent a critical determinant of outcomes after TBI.

The adaptive arm of the immune system is composed of CD4$^+$ T_H lymphocytes, CD8$^+$ CTLs, and regulatory T lymphocytes (T_{REGs}). Functionally, T_H cells potentiate immune responses after Ag recognition by stimulating Ab production and by releasing cytokines to enhance the activation of CTLs and macrophages. Conversely, counterinflammatory T_{REGs} attenuate immune responses to prevent chronic inflammation and autoimmunity. Notably, release of context-specific cytokines by APCs modulates adaptive immune responses to provide long-lasting, Ag-specific host protection (24). For instance, phenotype-specific cytokines released by activated macrophages differentiate naive T_H cells (T_H0) into polarized T_H subtypes, such as T_H1, T_H2, and T_H17, which exhibit distinct cytokine repertoires and different functional phenotypes (24). Polarization toward a proinflammatory T_H1 phenotype augments cell-mediated immunity, amplifies M1 macrophage activation, and perpetuates neurodegeneration (25, 26). Conversely, T_H2 polarization is associated with humoral immunity, support of resting microglia, and delayed neurodegeneration (27). Although the T_H1/T_H2 ratio remained unchanged in pediatric TBI patients (28), the presence and activity of IL-17–producing T_H17 cells have not been explored after TBI. In this study, we tested the hypothesis that activation of myeloid TLR4 is critical for T_H polarization after TBI.

Materials and Methods

Controlled cortical impact

The Institutional Animal Care and Use Committee at Augusta University approved all animal studies, in compliance with National Institutes of Health guidelines. Adult male CD-1 mice (Charles River, Wilmington, MA), C57BL/6 mice (the Jackson Laboratory), C3H/OuJ mice (wild-type, stock no. 000635; the Jackson Laboratory), C3H/HeJ mice (TLR4 mutant, stock no. 000659; Jackson Laboratories), and CX3CR1-eGFP mice (stock no. 005582; the Jackson Laboratory) were subjected to a sham injury or moderate controlled cortical impact, as detailed by our laboratory with minor modifications (29). Briefly, mice were anesthetized using 3% isoflurane and placed in a stereotaxic frame, and a craniotomy was made in the right parietal bone midway between bregma and lambda with the medial edge 1 mm lateral to the midline, leaving the dura intact. Mice were impacted at 3 m/s with a 100 ms dwell time and 3-mm depression using a 3-mm diameter convex tip (PinPoint PCI3000 Precision Cortical Impactor; Hatteras Instruments, Cary, NC). Sham-operated mice underwent the identical surgical procedures but were not impacted. The skin incision was closed, and mice were allowed to recover in a clean, warm cage. Body temperature was maintained at 37°C using a small animal temperature controller throughout all procedures (Kopf Instruments, Tujunga, CA). Food and water were provided ad libitum.

Tissue collection

At designated time points, blood was collected by cardiac puncture from deeply anesthetized mice and placed into ice-cold heparinized tubes. Mice were perfused with 30 ml of ice-cold PBS, and whole brains were carefully harvested. A 3-mm coronal brain section centered on the contusion was prepared using an acrylic brain matrix. Brain hemispheres were further subdivided into ipsilateral (injured) and contralateral (uninjured) cerebral cortices for analysis, as detailed below.

Preparative and analytical flow cytometry

Freshly harvested brain tissue was sieved through a 100-μM cell strainer (BD Biosciences, San Diego, CA), followed by centrifugation (1000 rpm, 10 min) to prepare single-cell suspensions. Blood (100 μl) was collected via cardiac puncture, as above. Cells were incubated with Abs against cell surface markers CD3, CD4, CD8, CD45, CD25, CD71, CD11b, CD68, F4/80, CD206, and TLR4 (all from eBioscience, San Diego, CA). Following a PBS wash, cells were fixed and permeabilized using a Fixation/Permeabilization Concentrate (Affymetrix eBioscience) and incubated with Abs for intracellular labeling of TNF-α, TGF-β, IL-17, IL-12, IL-10, and Foxp3 (BD Biosciences, Bedford, MA). After a final wash, cells were analyzed using a four-color flow cytometer (FACSCalibur) and CellQuest software (both from BD Biosciences, San Jose, CA), as we described previously (30, 31). Isotype-matched controls were analyzed to set the appropriate gates for each sample. For each marker, samples were analyzed in duplicate. To minimize false-positive events, the number of double-positive events detected with the isotype controls was subtracted from the number of double-positive cells stained with the corresponding Abs (not isotype control). Cells expressing a specific marker were reported as a percentage of the number of gated events. Mean channel fluorescence intensity (MFI) derived from a fluorescence graph was used to study the level of cell surface TLR expression. ΔMFI was calculated as MFI of the TBI group − MFI of the sham group.

Adoptive transfer of CFSE-labeled macrophages

Total splenocytes and bone marrow were collected and enriched, and CD11b$^+$ CD68$^+$ F4/80$^+$ macrophages were consecutively sorted three times by magnetic bead isolation (Miltenyi Biotec) to achieve >95% purity. Purified macrophages were labeled with 5 μM CFSE (Molecular Probes, Eugene, OR), a green fluorescent cell staining dye, and resuspended in sterile PBS, as we described (32). A total of 6×10^5 cells per mouse was injected via the tail vein immediately after sham/TBI injury. Trafficking, distribution, and phenotype of adoptively transferred CFSE$^+$ macrophages were analyzed by ex vivo fluorescence imaging, confocal imaging, and flow cytometry, respectively. To quantify the polarization and CNS infiltration of CFSE$^+$ macrophages, 100 μl of blood was collected from deeply anesthetized mice via cardiac puncture, and macrophage polarization was assessed by flow cytometry, as detailed above. Mice were then perfused with ice-cold PBS, and brains were carefully harvested into a sterile petri dish. Brains were imaged on a SPECTRAL Ami X imager (Spectral Instruments Imaging, Tucson, AZ) using an excitation wavelength = 430 nm, emission wavelength = 570 nm, excitation power = 20 keV, exposure time = 6 s, and field of view 10 cm. Fluorescence intensity, calculated as the mean intensity difference between ipsilateral and contralateral hemispheres, was quantified within regions of interest in triplicate using AMIView software. Data were expressed as mean photons per unit area per group. To provide spatial analysis of macrophage trafficking into the brain, deeply anesthetized mice were perfused with PBS, followed by fixation with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were postfixed overnight in paraformaldehyde, followed by cryoprotection with 30% sucrose (pH 7.4) until brains were permeated. Serial coronal sections (12 μm) were prepared using a cryostat microtome and directly mounted onto glass slides. Anti-fade mounting medium was added, and glass cover slips were placed atop the slide. CFSE immunofluorescence was imaged using a LSM 780 Meta confocal laser microscope and vendor-supplied software (Carl Zeiss).

Bone marrow chimera

C57BL/6 recipient mice received a sublethal dose (6 Gy; [^{137}Cs]) of total body irradiation. Twenty-four hours later, they were injected i.v. with 5×10^6 bone marrow cells collected from transgenic mice expressing monomeric red fluorescent protein (RFP) (mCherry) under the direction of the human ubiquitin C promoter [B6(Cg)-Tyrc-2J Tg(UBC-mCherry)1Phbs/J, stock no. 017614; the Jackson Laboratory], as detailed previously (33). Beginning at 2 wk posttransplantation, 10 μl of blood was collected from the orbital sinus, and RFP expression was measured in peripheral

blood using flow cytometry. Blood from C57BL/6 mice that did not undergo irradiation or RFP$^+$ cell transplantation was used as a control. On day 30, following confirmation of efficient bone marrow engraftment, mice were subjected to sham/TBI. The presence of infiltrated RFP$^+$ myeloid cells was analyzed in brain tissue by flow cytometry.

MLRs

MLRs were performed, as we described, with minor modifications (31, 34). Responder T lymphocytes were initially enriched using MACS, labeled with 5 μM CFSE for 10 min at 37°C, and plated at 1×10^4 cells per well. Brain tissue was harvested at 24 h postsham/post-TBI, and CD11b$^+$ CD68$^+$ F4/80$^+$ brain macrophages were consecutively sorted three times by magnetic bead isolation (Miltenyi Biotec). Purified brain macrophages were used as stimulators following plating at 5×10^4 cells per well. Combinations of responders (naive T lymphocytes) and stimulators (brain macrophages) were prepared in triplicate wells. Cells were cultured in 200 μl per well of RPMI 1640 medium supplemented with FBS, penicillin, streptomycin, L-glutamine, and 2-ME. After 72–96 h of incubation at 37°C in a humidified, 5% CO_2 incubator, cells were harvested into flow cytometry tubes. Following a PBS wash, samples were incubated at 4°C for 20 min in the dark with anti-rat CD71-PE–conjugated Ab to label activated and dividing T cells. Samples were then washed with PBS, and T cell proliferation and polarization phenotype were quantified in triplicate by flow cytometry.

Macrophage depletion

A Clodrosome Macrophage Depletion Kit, containing control liposomes (Encapsome) and clodronate liposomes (Clodrosome) (Encapsula NanoSciences, Brentwoood, TN), was used to deplete endogenous myeloid cells. Intraperitoneal administration of 200 μl of placebo or clodronate liposomes (5 mg/ml) was performed once daily for three consecutive days. At 24 h after the final injection, blood was collected via the retro-orbital sinus, and myeloid cell (CD11b$^+$ F4/80$^+$) depletion was confirmed by flow cytometry. Sham treatment was performed or TBI was induced upon the confirmation of myeloid cell depletion.

Statistical analysis

Multigroup comparisons were made using one-way ANOVA, followed by the Student–Newman–Keul post hoc test. Two-group comparisons were analyzed using the *t* test. Results are expressed as mean ± SEM. A p value < 0.05 was considered statistically significant.

Results

Chronic M1 macrophage activation within the CNS after TBI

CX3CR1-eGFP mice were subjected to TBI. Scattered eGFP expression, which likely represented resident microglia, was noted

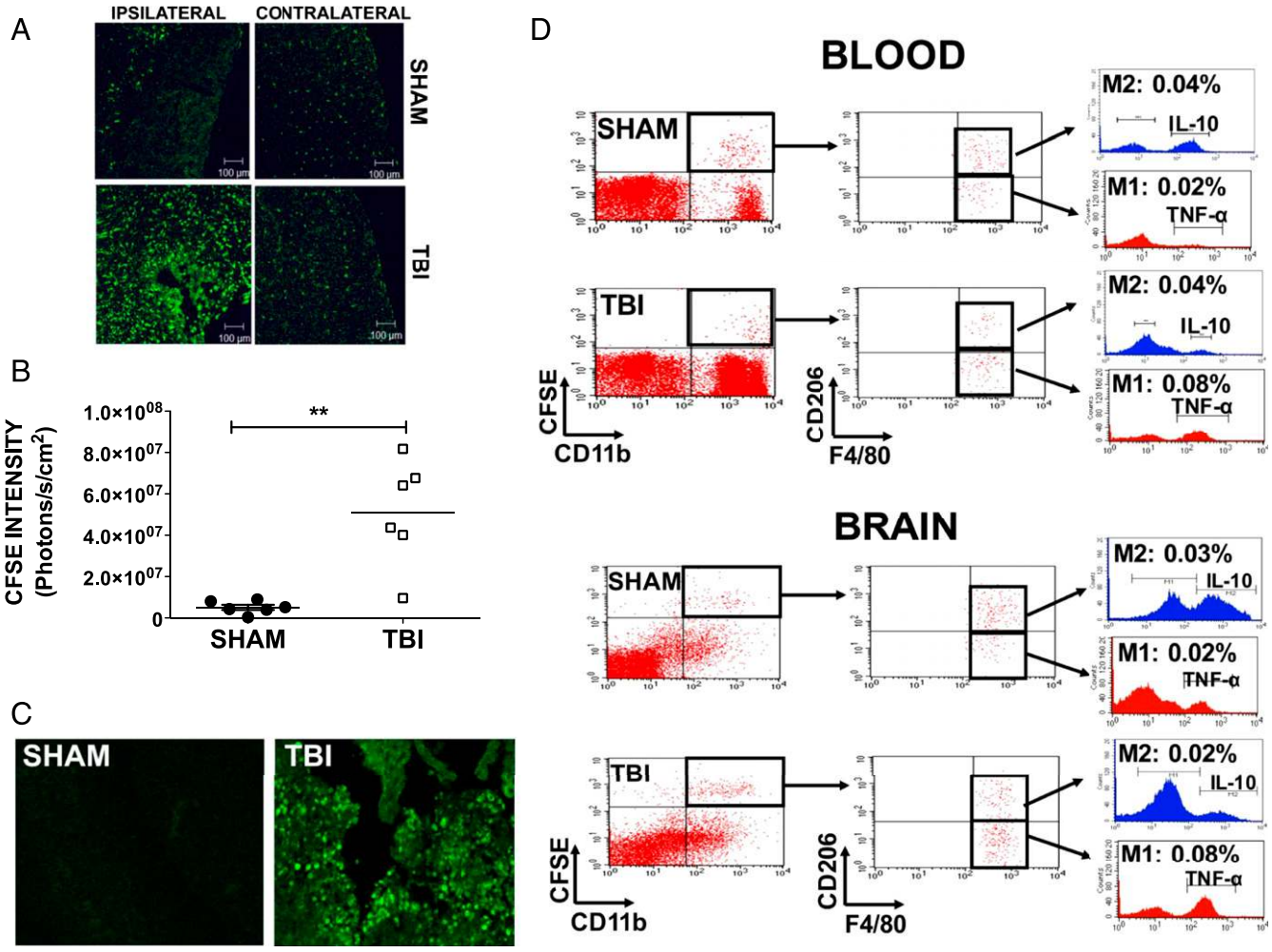

FIGURE 1. Peripheral macrophage trafficking into the CNS after TBI. (**A**) Sham or TBI was induced in CX3CR1-eGFP reporter mice, which express green fluorescence in monocytes, dendritic cells, and microglia. Representative confocal images are depicted showing increased macrophage infiltration/activation within the injured cortex at 72 h post-TBI compared with the contralateral cortex or the cortex of sham-injured mice. Data are representative of $n = 5$ mice per group. (**B**–**D**) CFSE-labeled macrophages (6×10^5 cells per mouse) were delivered via the tail vein at 1 h after sham/TBI. (B) At 72 h post-TBI, ex vivo spectral imaging of brains was performed, and fluorescence was calculated within predefined regions of interest. Scatterplots depict mean ± SEM of CFSE intensity, as represented by photons per second per square centimeter, from $n = 6$ mice per group. (C) Confocal imaging of infiltrated CFSE$^+$ macrophages adjacent to the peri-contusional cortex. Note the strong increase in fluorescence after TBI. Original magnification ×200. (D) CFSE$^+$ cells were recovered from blood or brain tissue after sham/TBI and subjected to flow cytometry to quantify M1 (CFSE$^+$ CD11b$^+$ F4/80$^+$ TNF-α$^+$) and M2 (CFSE$^+$ CD11b$^+$ CD206$^+$ IL-10$^+$) polarization. As shown, TBI strongly increased M1 polarization in blood and brain tissue and blood at 72 h postinjury. In contrast, there was no change in M2 polarization after TBI. **$p < 0.01$ versus sham, *t* test.

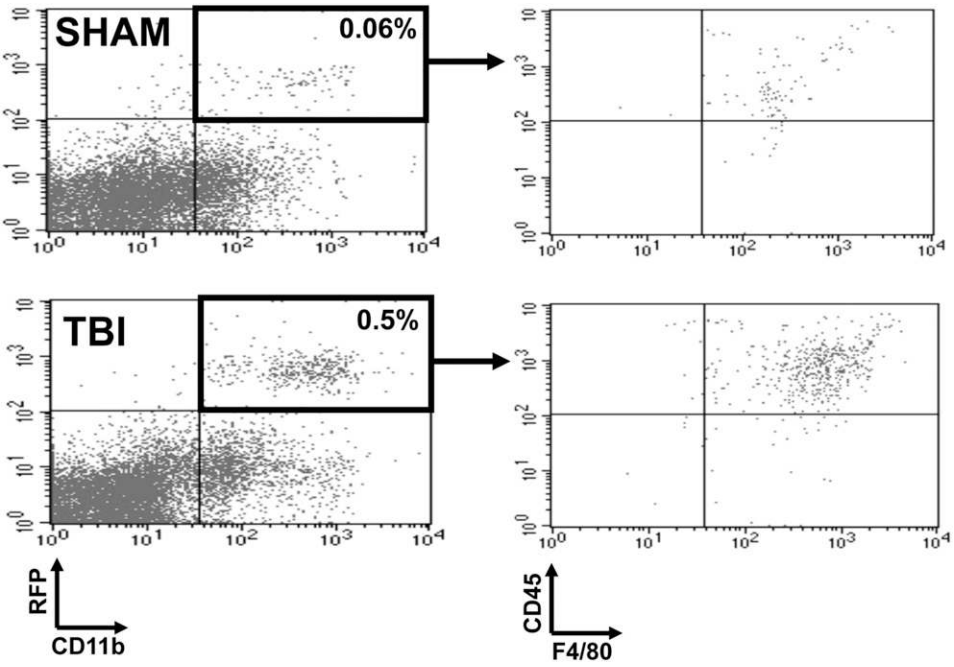

FIGURE 2. Macrophage trafficking in bone marrow chimera mice. Irradiated wild-type (C57BL/6) mice were transplanted with bone marrow from donor transgenic mice that express monomeric RFP under the direction of the human ubiquitin C promoter. At 72 h postsham/post-TBI, RFP^+ $CD11b^+$ macrophages were assessed in brain tissue by flow cytometry. Numbers represent the percentage of total cells. More than 99% of infiltrated RFP^+ macrophages were also $CD45^+$ $F4/80^+$, supporting the infiltration of peripheral macrophages into the brain after TBI.

throughout the cerebral cortex of sham-operated mice and in the contralateral cortex after TBI, whereas intense fluorescence was observed throughout the peri-contusional cortex at 72 h post-TBI (Fig. 1A). Although providing a valuable tool, interpretation of data using CX3CR1-eGFP mice is confounded by the expression of fluorescence in monocytes, as well as in dendritic cells,

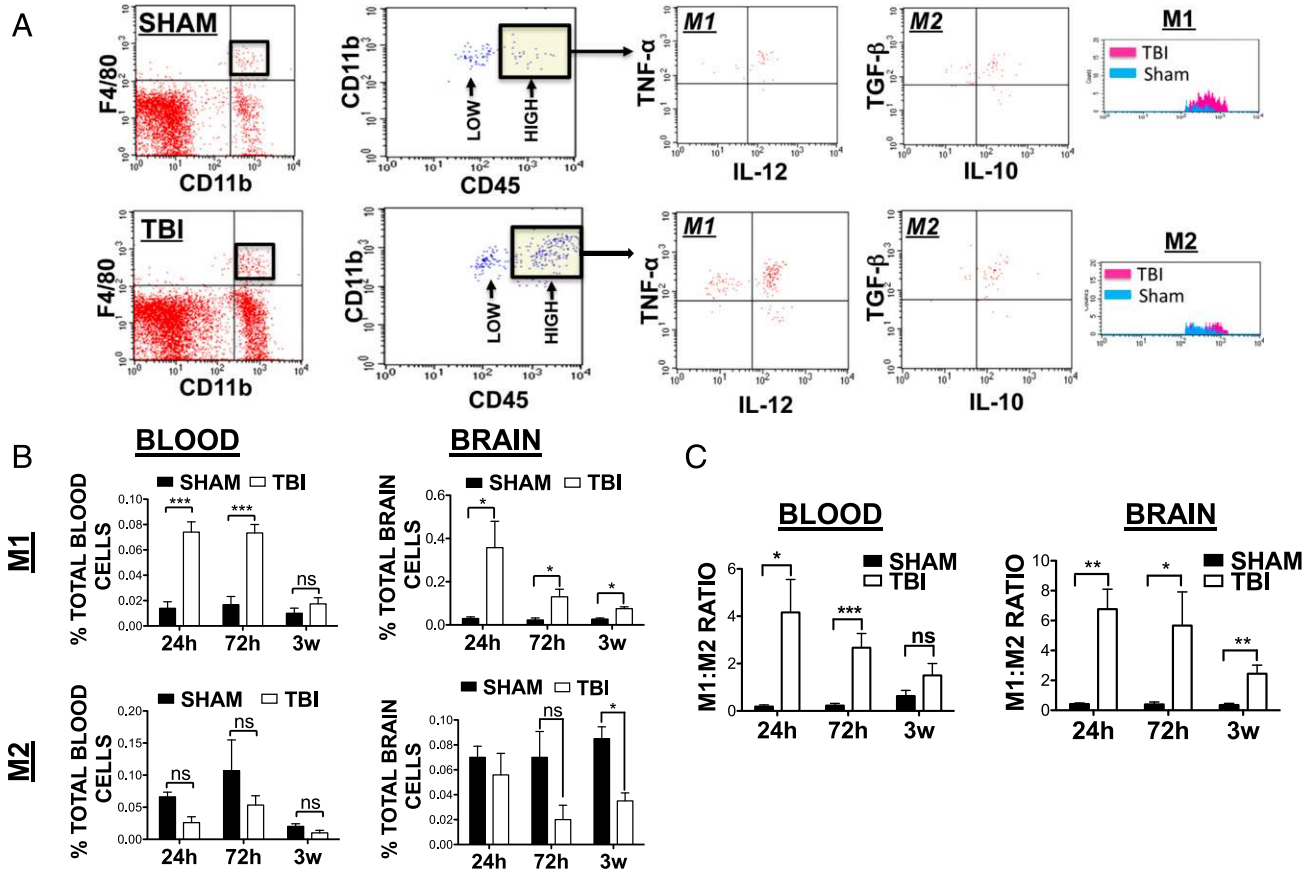

FIGURE 3. Increased M1 polarization after TBI. (**A**) Quantification of macrophage polarization by flow cytometry in peri-contusional brain tissue or in anatomically matched sham brain tissue at 72 h. Activated myeloid cells were selected using CD11b and F4/80 (boxes; upper left panels) and analyzed using CD45 to differentiate infiltrating macrophages ($CD11b^{GH}$ $CD45^{HIGH}$; HIGH arrows, yellow shaded box) from resident microglia ($CD11b^+$ $CD45^{LOW}$; LOW arrows). Polarization of infiltrating macrophages was assessed using functional cytokines for M1 (TNF-α, IL-12) or M2 (TGF-β, IL-10) phenotypes. Representative scatterplots are provided. (**B**) Quantification of macrophage polarization in blood and brain tissue at 24 h, 72 h, or 3 wk after sham or TBI. Data are mean ± SEM of $n = 8$–10 mice per group. (**C**) Graphic representation of the M1/M2 ratio in blood or brain after sham or TBI. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ns, not significant.

NK cells, and microglia. Thus, to more specifically determine whether peripheral macrophages traffic into the CNS, bone marrow–derived monocytes were fluorescently labeled with CFSE and administered i.v. to recipient mice immediately after sham or TBI. Ex vivo spectral imaging showed a 10-fold increase in brain macrophage infiltration after TBI compared with brains from sham-operated mice (Fig. 1B). Histological analysis revealed increased fluorescence throughout the peri-contusional cortex at 72 h post-TBI, whereas few fluorescent cells were observed in the contralateral hemisphere or in the brain of sham-operated mice (Fig. 1C). In a parallel cohort, $CFSE^+$ macrophages were recovered from peri-contusional brain tissue (or anatomically matched sham-operated brain tissue) or blood. FACS analysis of $CFSE^+$ cells revealed an elevated number of M1 macrophages compared with M2 macrophages in brain and blood after TBI (Fig. 1D, 1E).

To ensure that the observed effect of TBI on macrophage polarization was not an artifact of CFSE labeling or due to exogenous administration, an irradiation bone marrow chimera approach was used. Transplantation of RFP^+ bone marrow into irradiated wild-type mice resulted in successful engraftment at day 28 posttransplantation. Mice were then subjected to sham or TBI, and the CNS infiltration of RFP^+ myeloid cells was analyzed by flow cytometry. In agreement with the CFSE experiment, TBI increased the infiltration of RFP^+ $CD11b^+$ $CD45^+$ $F4/80^+$ macrophages into the peri-contusional cortex compared with sham injury (Fig. 2B).

Next, macrophage infiltration and polarization were analyzed in native (nontransgenic, nonlabeled) cells. TBI prominently increased the number of $CD11b^+$ $F4/80^+$ $CD45^{HIGH}$ infiltrating macrophages in the injured cortex, whereas a smaller elevation in the activation of $CD11b^+$ $F4/80^+$ $CD45^{LOW}$ resident microglia was noted (Fig. 3A). M1 polarization was observed within blood at 24 h postinjury compared with sham-operated mice ($p < 0.001$ versus sham). These changes persisted at 72 h ($p < 0.001$ versus sham) and returned to baseline by 3 wk (Fig. 3B). In contrast, no significant changes in M2 polarization were noted in blood at any time point within this study (Fig. 3B). A similar increase in M1 polarization of peripheral macrophages was observed within peri-contusional brain tissue at 24 h post-TBI ($p < 0.05$ versus sham). M1 polarization also was significantly elevated at 72 h and at 3 wk postinjury ($p < 0.05$ versus sham), albeit at a reduced magnitude compared with the 24-h time point (Fig. 3B). M2 polarization was not significantly changed at 24 or 72 h after TBI; however, a significant reduction in M2 polarization was observed within peri-contusional brain tissue at 3 wk (Fig. 3B). Overall, an increased ratio of M1/M2 polarized macrophages was observed in blood and brain over the first 72 h post-TBI; this effect persisted within the brain for 3 wk after injury, which was indicative of a proinflammatory shift within the injured brain (Fig. 3C).

Macrophages differentially stimulate T_H polarization after TBI

Although considerable effort has been put forth to define the temporal and phenotype changes of macrophages after TBI, the functional consequences of macrophage polarization remain largely unstudied. To address this issue, infiltrated macrophages from the brains of sham- or TBI-injured mice were used as stimulators in an MLR with naive T lymphocytes (Fig. 4A). The

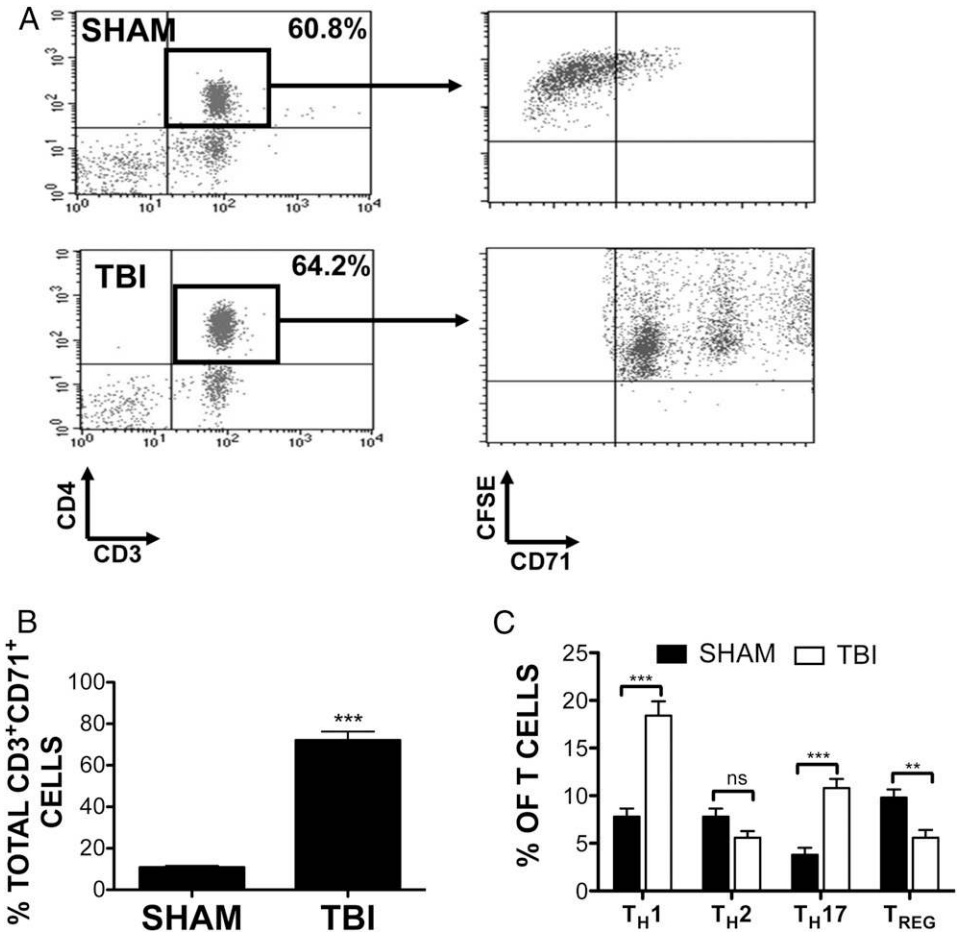

FIGURE 4. Brain-derived monocytes stimulate T cell proliferation and differentiation after TBI. (**A**) Purified brain monocytes were obtained from brain at 72 h after sham or TBI, and cells were added to naive T lymphocytes labeled with CFSE in an MLR. Representative scatterplots depict cellular proliferation after a 72-h incubation. The percentage of $CD3^+$ $CD4^+$ cells within each well, prior to the addition of brain-derived monocytes, is provided. (**B**) Quantification of proliferation data depicted in (A). Data are expressed as the percentage total $CD3^+$ $CD71^+$ T lymphocytes. Data are mean ± SEM from $n = 6$ mice per group; all experiments were performed in triplicate wells. (**C**) Assessment of T lymphocyte polarization at the conclusion of the MLR. T_H1 cells ($CD3^+$ IFN-γ^+), T_H2 cells ($CD3^+$ IL-4^+), T_H17 cells ($CD3^+$ IL-$17A^+$), and T_{REGs} ($CD3^+$ $Foxp3^+$) were quantified as the percentage of total T lymphocytes. $^{**}p < 0.01$, $^{***}p < 0.001$ versus sham, *t* test. ns, not significant.

proportion of $CD3^+$ $CD4^+$ cells relative to the total number of cells in the well was not statistically different between sham- and TBI-treated groups at study initiation (Fig. 4B), suggesting that any observed changes were due to T lymphocyte proliferation rather than plating differences. Addition of brain macrophages from TBI-injured mice induced a 7.2-fold increase in the proliferation of $CD3^+$ $CD71^+$ naive T lymphocytes compared with macrophages isolated from the brains of sham mice ($p < 0.001$ versus cultures treated with brain macrophages from sham mice) (Fig. 4A, 4C). Phenotypic analysis of recovered T lymphocytes revealed a significant increase in T_H1 polarization ($p < 0.001$ versus sham) and T_H17 polarization ($p < 0.001$ versus sham) following treatment with TBI brain macrophages (Fig. 4D). In contrast, no significant changes in T_H2 polarization were observed, whereas a statistically significant reduction in T_{REG} production ($p < 0.01$ versus sham) was noted (Fig. 4D).

To determine whether the MLR reflected changes in vivo, we next explored T_H polarization in blood and brain tissue after TBI. A peak increase in T_H1 polarization was observed in brain and blood at 24 h ($p < 0.001$ versus sham), with persistent elevation at 72 h ($p < 0.001$ versus sham in brain; $p < 0.01$ versus sham in blood) and 3 wk ($p < 0.05$ versus sham in blood and brain) post-TBI (Fig. 5). The pattern of T_H17 polarization mirrored that of T_H1 polarization, with peak T_H17 polarization found at 24 h post-TBI in blood and brain ($p < 0.001$ versus sham), followed by increased expression at 72 h postinjury ($p < 0.001$ versus sham in blood and brain). These changes were still observed at 3 wk post-TBI ($p < 0.05$ versus sham in blood, $p < 0.001$ in brain) (Fig. 5). Conversely, T_H2 polarization was not significantly different between sham and TBI groups at 24 h in blood or brain. At 72 h postinjury, we found a suppression of T_H2 polarization in brain ($p < 0.05$ versus sham) and blood ($p < 0.01$ versus sham), whereas only a reduction in brain was observed at 3 wk ($p < 0.05$ versus sham) (Fig. 5). Finally, a consistent and prolonged reduction in T_{REG} production was seen between 24 h and 3 wk post-TBI in blood ($p < 0.05$, $p < 0.01$, $p < 0.05$ versus sham at 24 h, 72 h, and 3 wk, respectively) and brain ($p < 0.001$, $p < 0.01$, $p < 0.05$ versus sham at 24 h, 72 h and 3 wk, respectively) (Fig. 5). In line with a proinflammatory myeloid shift (Fig. 4C), a pronounced, statistically significant increase in the T_H17/T_{REG} ratio was observed throughout the study in blood and brain tissue after TBI, with a more dramatic change noted in the brain (Fig. 5).

We next used a myeloid cell–depletion strategy to determine whether activated macrophages are critical for T_H polarization after TBI. Administration of Clodrosome significantly reduced the number of circulating $CD11b^+$ $F4/80^+$ myeloid cells (Fig. 6A) compared with Encapsome-treated mice. These changes in blood were similarly reflected by an ~97% reduction in $CD11b^+$ $F4/80^+$ myeloid cells within the brain (Fig. 6B), validating the functional depletion of macrophages. Clodrosome administration significantly reduced T_H1 polarization ($p < 0.05$ versus placebo in blood, $p < 0.01$ versus Encapsome in brain) and T_H17 polarization ($p < 0.01$ versus Encapsome in blood and brain) after TBI (Fig. 6C). In contrast, Clodrosome had no significant effect on T_H2 polarization in blood or brain, whereas T_{REG} production was slightly, but significantly, increased in blood and brain ($p < 0.05$ versus Encapsome treatment) (Fig. 6C).

TLR4 activation is essential for proinflammatory activation after TBI

Elevated expression of $TLR4^+$ macrophages was observed in $CD11b^+$ $F4/80^+$ $CD45^{HIGH}$ infiltrated peripheral macrophages within the peri-contusional cortex after TBI (Fig. 7A). C3H/HeJ mice, which contain an inactivating point mutation in the TLR4

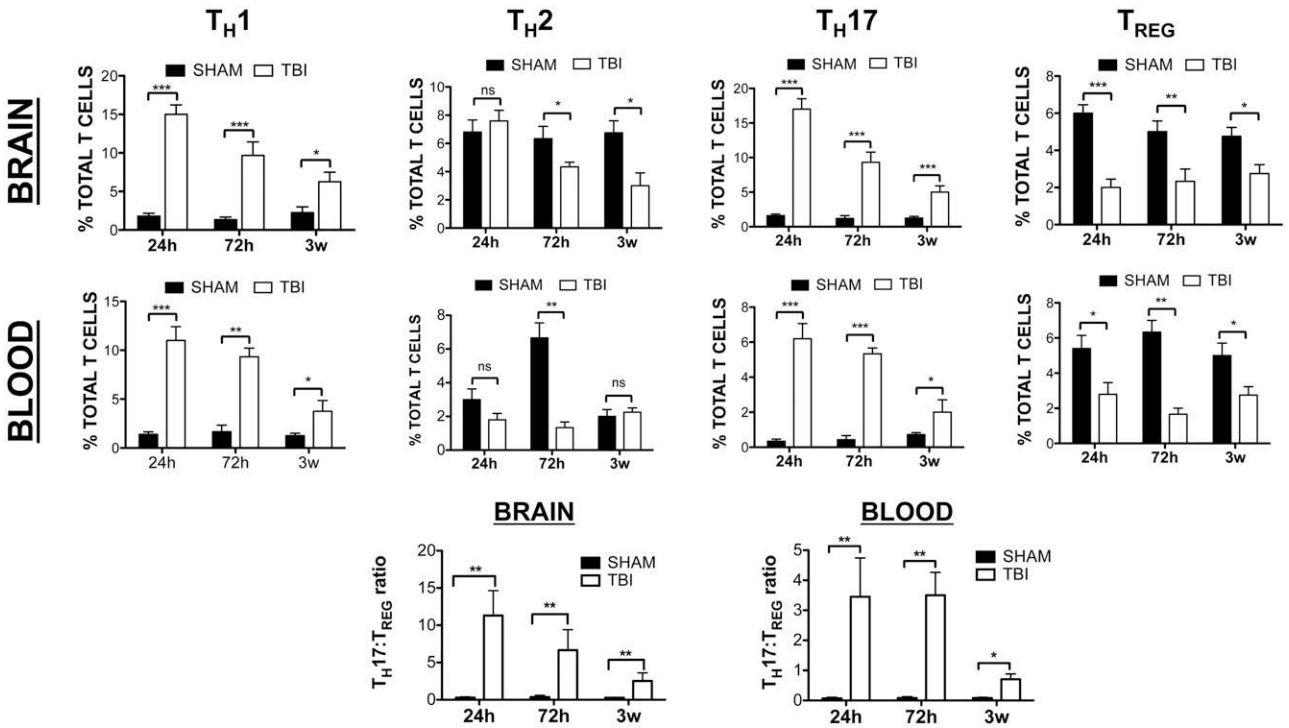

FIGURE 5. TBI promotes T_H1/T_H17 polarization. Blood and brain tissue was collected at 24 h, 72 h, or 3 wk after sham/TBI. T_H1, T_H2, T_H17, and T_{REG} polarization was assessed by flow cytometry. Graphs depict percentage of total T cells within the brain or total T cells within the blood. Bottom panels depict the T_H17/T_{REG} ratio in brain and blood across time. Data are mean ± SEM from n = 6–8 mice per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus sham.

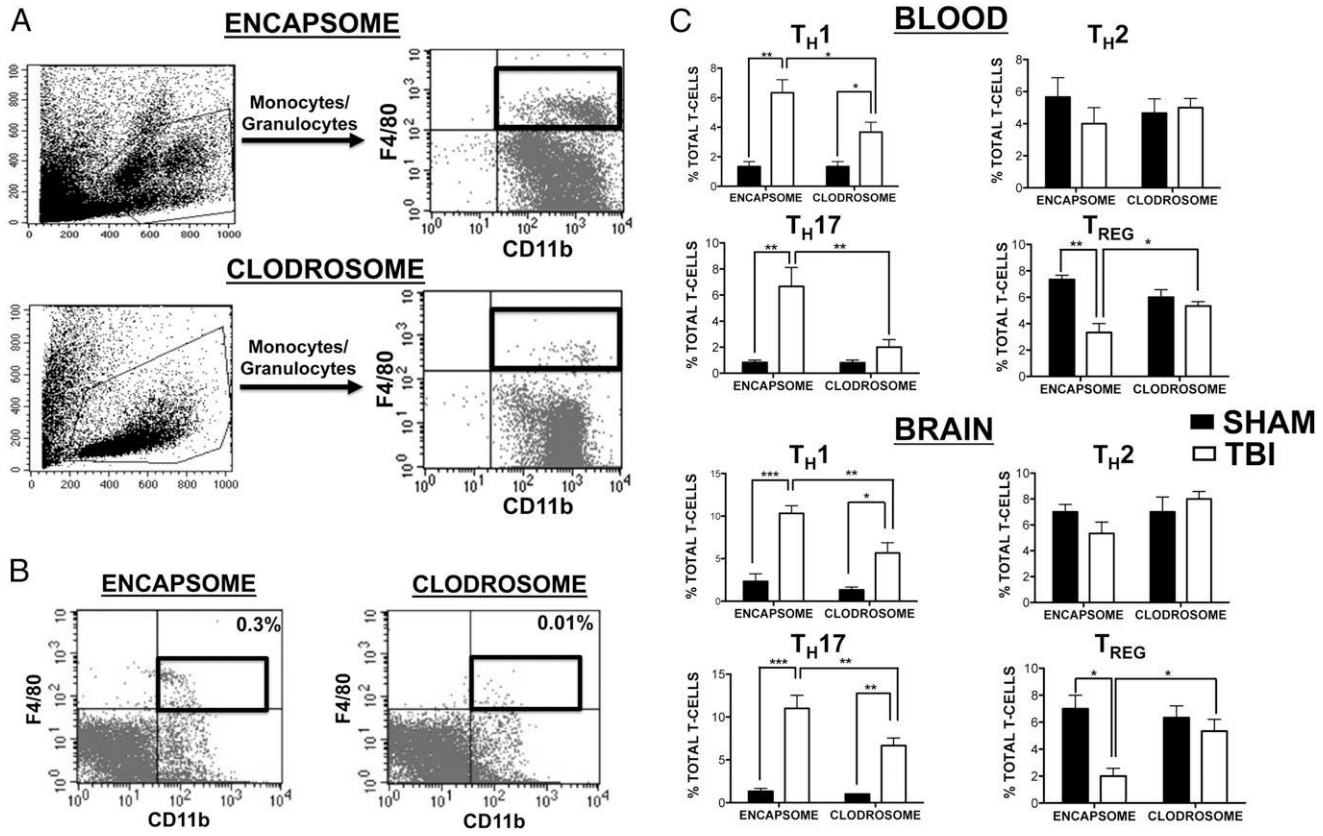

FIGURE 6. Myeloid cell depletion attenuates T_H1/T_H17 polarization after TBI. (**A**) Control liposomes (Encapsome) or clodronate liposomes (Clodrosome) were administered i.p. for three consecutive days. Blood (A) or brain tissue (**B**) was obtained following the final injection, and myeloid cells ($CD11b^+$ $F4/80^+$) were quantified by flow cytometry. Representative scatterplots are provided. (**C**) Sham or TBI was induced at 24 h following the third clodronate injection. At 72 h post-TBI, blood and brain tissue were collected, and T lymphocyte polarization was assessed by flow cytometry. Data are mean ± SEM from $n = 6$–8 mice per group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

signaling domain, reduced M1 macrophage polarization at 72 h post-TBI in blood ($p < 0.05$ versus wild-type) and brain ($p < 0.001$ versus wild-type) compared with C3H/OuJ (wild-type) mice (Fig. 7B). Similarly, M1 polarization was attenuated in the blood and brain of $TLR4^{-/-}$ mice ($p < 0.01$) compared with wild-type mice ($p < 0.01$); however, M2 polarization was unchanged in the blood and brain of $TLR4^{-/-}$ mice compared with wild-type mice (Fig. 7C).

Next, macrophages isolated from the brain of C3H/HeJ or C3H/OuJ mice after TBI were used to stimulate naive T lymphocytes in an MLR. As observed in Fig. 4, macrophages derived from the brain of sham-operated mice did not significantly enhance T lymphocyte polarization (Fig. 8A, 8B). In contrast, macrophages derived from C3H/OuJ or C3H/HeJ mice subjected to TBI significantly increased T lymphocyte proliferation ($p < 0.01$ versus genotype-matched, sham-treated cultures); however, the stimulatory effect of C3H/HeJ macrophages after TBI was reduced compared with macrophages recovered from C3H/OuJ mice after TBI ($p < 0.001$) (Fig. 8A, 8B). T lymphocytes recovered after the MLR were used to assess whether deletion of myeloid TLR4 affected T_H polarization. No differences in T_H polarization were observed following stimulation by macrophages from either genotype after sham injury (Fig. 8C). In contrast, macrophages recovered from C3H/OuJ or C3H/HeJ mice after TBI significantly increased T_H1 and T_H17 ($p < 0.001$ versus sham for both phenotypes in C3H/OuJ mice; $p < 0.01$ versus sham for T_H1 and $p < 0.001$ versus sham for T_H17 polarization in C3H/HeJ mice) (Fig. 8C); however, the stimulatory effect of C3H/HeJ macrophages on T_H1/T_H17 polarization was significantly reduced compared with macrophages from C3H/OuJ mice ($p < 0.001$ versus C3H/OuJ mice for both phenotypes). T_H2 polarization was not significantly affected by injury or genotype. Conversely, T_{REG} production was suppressed by macrophages obtained from C3H/OuJ mice ($p < 0.01$ versus sham), whereas macrophages derived from C3H/HeJ mice had no affect on T_{REG} polarization (Fig. 8C).

Finally, we determined whether the observed changes in the MLR reflected changes in T_H polarization after TBI. In line with ex vivo data, T_H1 polarization ($p < 0.01$ versus sham) and T_H17 polarization ($p < 0.01$ in blood, $p < 0.001$ in brain) were increased in C3H/OuJ mice after TBI (Fig. 9). C3H/HeJ mice exhibited a comparatively smaller, yet significant, increase in T_H1 polarization ($p < 0.05$ versus sham). More importantly, C3H/HeJ mice displayed a reduction in T_H1 polarization after TBI compared with C3H/OuJ mice ($p < 0.05$ versus C3H/OuJ mice). Notably, T_H17 polarization was completely reversed to sham levels in C3H/HeJ mice (not significantly different from sham, $p < 0.01$ versus C3H/OuJ mice after TBI in blood, $p < 0.001$ versus C3H/OuJ mice after TBI in brain). A reduction in T_H2 polarization was found in the blood, but not the brain, of C3H/OuJ mice ($p < 0.001$ versus sham C3H/OuJ mice), whereas this effect was not observed in C3H/HeJ mice. Finally, C3H/OuJ mice displayed a consistent reduction in T_{REGs} in blood and brain after TBI ($p < 0.05$ versus sham). In contrast, T_{REGs} were not significantly changed in C3H/HeJ mice after TBI. In keeping with a proinflammatory role for TLR4, the increased T_H17/T_{REG} ratio observed in the blood and brain of C3H/OuJ mice after TBI was completely reversed to sham levels in C3H/HeJ mice (Fig. 9).

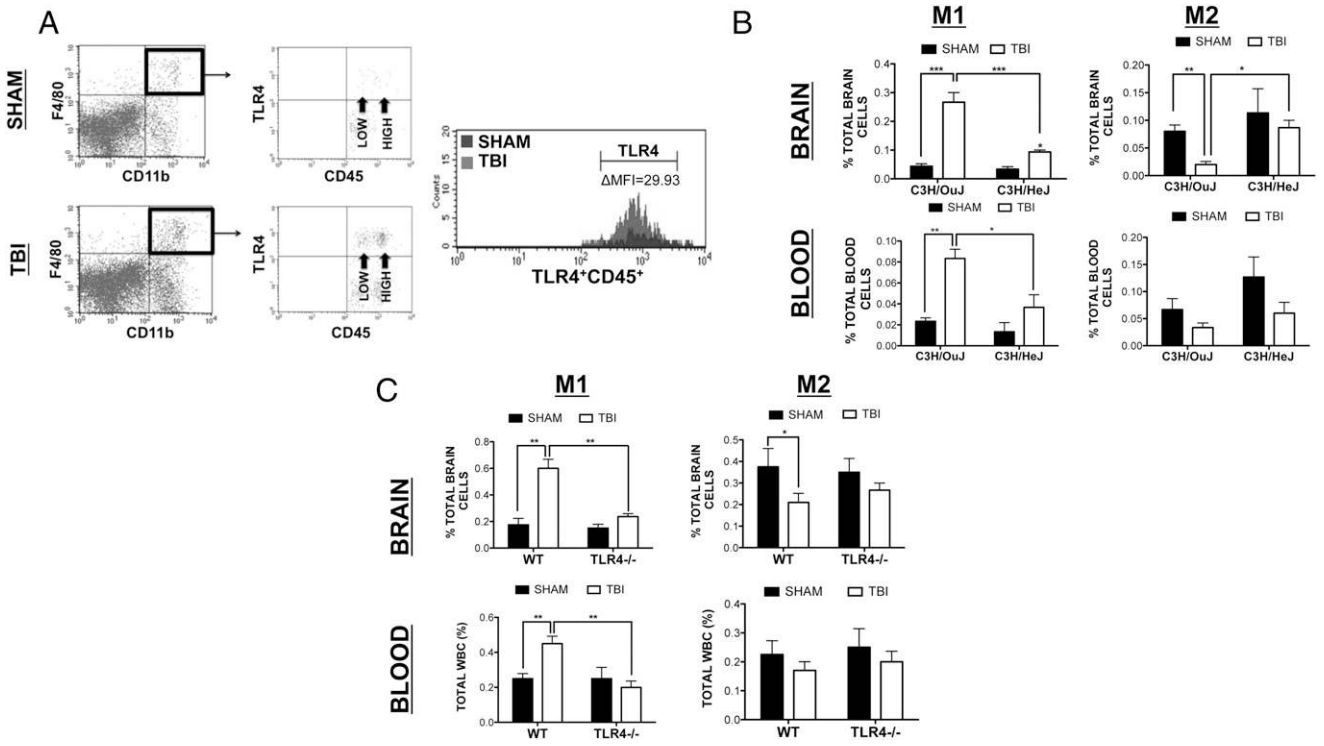

FIGURE 7. TLR4 mediates macrophage polarization after TBI. (**A**) Representative scatterplots show increased expression of TLR4 in brain myeloid cells at 72 h postsham/post-TBI. TLR4 was expressed in infiltrated peripheral macrophages ($CD11b^+$ $F4/80^+$ $CD45^{HIGH}$) and, to a lesser extent, in resident microglia ($CD11b^+$ $F4/80^+$ $CD45^{LOW}$). Graph of MFI of TLR4 in $CD45^+$ cells after sham (MFI = 207.21) or TBI (MFI = 237.14). Macrophage polarization was quantified in (**B**) C3H/OuJ (wild-type) or C3H/HeJ (TLR4 mutant) mice or in (**C**) C57BL/6 (wild-type) or $TLR4^{-/-}$ mice at 72 h postsham/post-TBI. Data are mean ± SEM from n = 6–8 mice per group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Discussion

In this article, we make three important mechanistic observations that implicate infiltrating macrophages as key initiators of adaptive immune responses after TBI. First, we demonstrate that peripheral macrophages polarize into an M1 phenotype, traffic into the CNS, and remain persistently elevated for weeks after TBI. Second, we show that CNS-infiltrated macrophages isolated early after TBI potently stimulate T lymphocyte proliferation and polarization toward proinflammatory T_H1 and T_H17 phenotypes, with a concomitant reduction in counter-inflammatory $Foxp3^+$ T_{REG} production. These changes produced a persistently elevated T_H17/T_{REG} ratio for weeks after TBI. Finally, we provide evidence that activation of myeloid TLR4 mediates, at least in part, T_H polarization after TBI, establishing a molecular link between acute trauma and long-term adaptive immune responses.

We (9, 36) and other investigators (35) determined that increased plasma or cerebrospinal fluid levels of the damage associated molecular pattern, high mobility group box protein 1 (HMGB1), were independent predictors of 1-y mortality and unfavorable outcomes in severe TBI patients (9, 35, 36). We also reported acute neuronal release of HMGB1-induced proinflammatory responses and exacerbated neurologic damage via activation of myeloid TLR4 after experimental TBI (9). Consistent with a report showing that activation of TLR4 increased the polarization of bone marrow–derived macrophages toward a M1 phenotype and reprogrammed M2 macrophages toward a M1 phenotype (37), we observed that M1 polarization was attenuated in TLR4 mutant and knockout mice after TBI. Although we acknowledge that macrophages likely exist along a dynamic continuum, rather than as rigid binary polarization phenotypes (38), activation of myeloid TLR4 may establish a deleterious proinflammatory environment within the CNS after TBI.

TLR4-dependent M1 polarization meets the current operational definition of macrophage polarization; however, changes in the expression of surface markers and cytokines do not necessarily reflect functional significance. Activation of TLR4, which accelerated the clearance of myelin debris (39), increased MHC class II expression and enhanced the migration of MHC class II^+ APCs to secondary lymphoid organs (40). HLA-DR, an MHC class II cell surface receptor that initiates adaptive immune responses after binding myelin basic protein (41, 42), is expressed by chronically infiltrated M1-like macrophages after TBI (21). Interestingly, HLA-DR increased the responsiveness to TLR4 in HEK293 cells, whereas peritoneal macrophages from MHC class II–knockout mice exhibited impaired responsiveness to TLR4 activation (43). Consistent with these reports, macrophages obtained from the brains of wild-type mice potently stimulated naive T lymphocyte proliferation, whereas macrophages derived from TLR4 mutant mice were ineffective. Coupled with the observation that myeloid TLR4 activation increased oligodendrocyte injury and exacerbated hypomyelination in immature rodents (44), acute activation of TLR4 on infiltrating macrophages may initiate adaptive immune responses after TBI.

$Rag1^{-/-}$ mice, which are devoid of mature lymphocytes, exhibited no differences in outcomes for up to 1 wk after TBI (45); however, T lymphocytes consist of functionally diverse subsets that exert protective and detrimental roles in the CNS (46–48). In addition to Ag presentation, macrophages release phenotype-specific cytokines that polarize naive T_H0 cells into distinct T_H subtypes to provide long-lasting context- and Ag-specific immunity (49). In keeping with a proinflammatory myeloid shift, we observed a TLR4-dependent increase in T_H1 and T_H17 polarization,

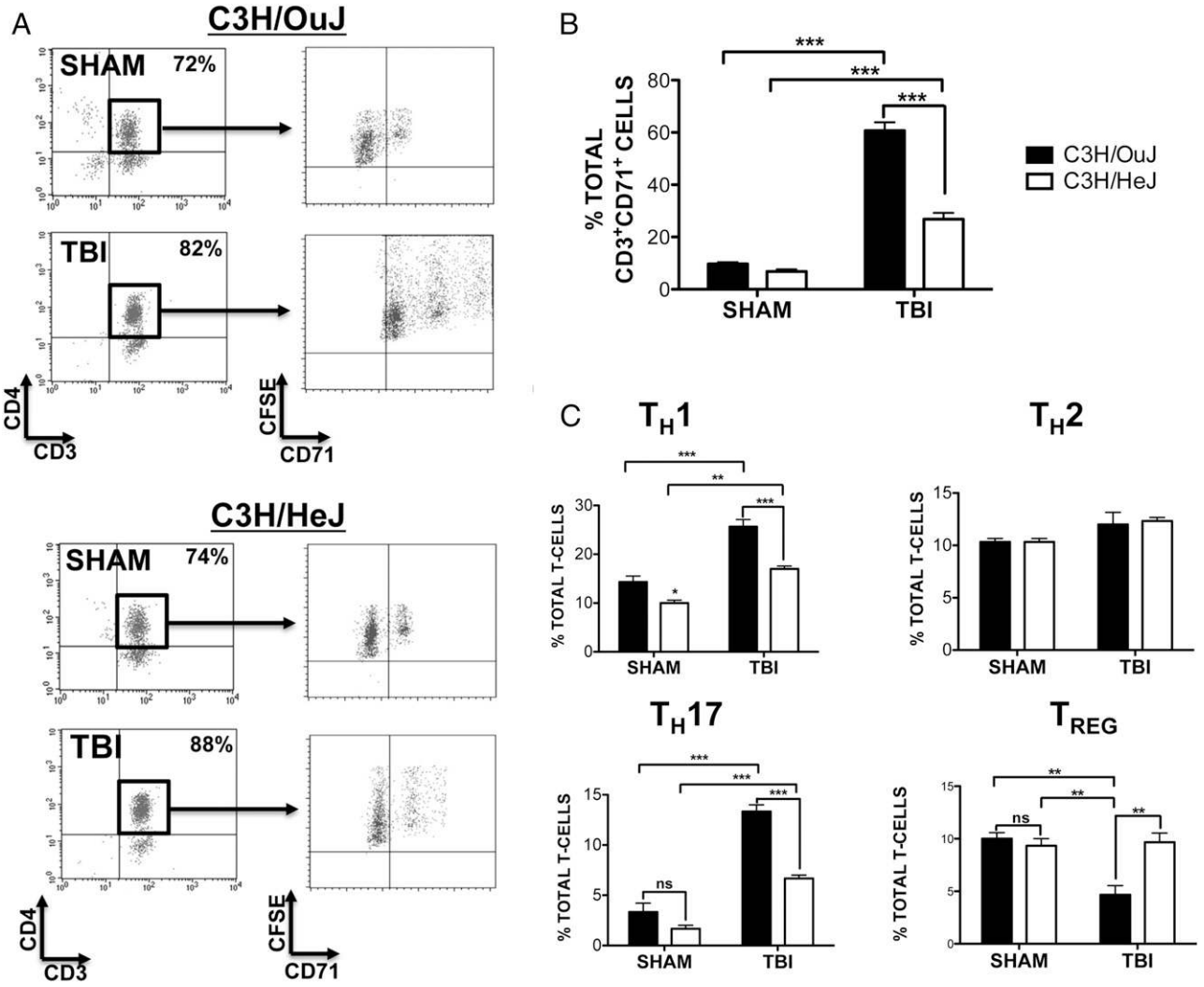

FIGURE 8. Myeloid TLR4 stimulates T lymphocyte polarization after TBI. (**A**) Purified brain monocytes were isolated from C3H/OuJ (wild-type) or C3H/HeJ (TLR4 mutant) mice at 72 h after sham/TBI and added to naive T lymphocytes in an MLR. Representative scatterplots depict cellular proliferation after a 72-h incubation. The percentage of $CD3^+$ $CD4^+$ cells within each well, prior to the addition of brain-derived monocytes, is provided. (**B**) Quantification of T lymphocyte proliferation following stimulation with brain monocytes derived from C3H/OuJ and C3H/HeJ mice, as shown in (A). Data are presented as the percentage total $CD3^+$ $CD71^+$ T lymphocytes. Data are mean ± SEM from $n = 6$ mice per group; all experiments were performed in triplicate wells. (**C**) T lymphocyte polarization was assessed in cells obtained at the termination of the MLR. T_H1 cells ($CD3^+$ IFN-γ^+), T_H2 cells ($CD3^+$ IL-4^+), T_H17 cells ($CD3^+$ IL-$17A^+$), and $T_{REG}s$ ($CD3^+$ $Foxp3^+$) were quantified as the percentage of total T lymphocytes. $^{**}p < 0.01$, $^{***}p < 0.001$. ns, not significant.

with a concomitant reduction in $T_{REG}s$, in blood and brain for 3 wk after TBI. Notably, the exogenous TLR4 agonist LPS was the most potent stimulus for Ag-loaded macrophages to drive T_H17 polarization in human autologous cocultures (50). Although T lymphocytes do not routinely cross the blood–brain barrier (BBB) (51), i.p. LPS administration increased the differentiation and brain influx of T_H17 cells after neonatal hypoxia-ischemia (52). T_H17 cells also make up a larger proportion of total T lymphocytes within the brain compared with blood, suggesting that T_H17 cells may locally polarize and/or preferentially traffic into the CNS after TBI. Consistent with the former possibility, activation of TLR4 in primary microglia or in peritoneal macrophages increased the expression of IL-23, a cytokine required for expansion and survival of T_H17 cells and for suppression of Foxp3 (37, 53, 54). Also, astrocytes induced IFN-γ and IL-17 expression in T lymphocytes via a mechanism involving IL-23 (55). In addition to central regulation, T_H17 cells migrated across the BBB to exacerbate CNS inflammation via activation of IL-17 and IL-22 receptors on BBB endothelial cells (56).

The mechanisms by which myeloid TLR4 activation enhanced T_H1/T_H17 polarization and simultaneously reduced T_{REG} production after TBI remain undefined. Notably, we observed a TLR4-dependent release of IL-6 from primary human macrophages after HMGB1 treatment or after experimental TBI (9). HMGB1 increased the T_H17/T_{REG} ratio in atherosclerotic patients (57), whereas HMGB1 increased T_H1 polarization (58) and stimulated T_H17 polarization via an IL-6–dependent mechanism during acute allograft rejection (59). IL-6 also inhibited TGF-β1–induced $Foxp3^+$ T_{REG} generation while simultaneously synergizing with TGF-β1 to stimulate the differentiation of pathogenic T_H17 cells from naive T cells (60). Although incompletely explored after TBI, an elevated IL-6/IL-10 ratio was associated with unfavorable outcomes at 6 mo in patients with severe TBI (61). In addition, IL-12, another TLR4-regulated cytokine produced by activated macrophages that is necessary for T_H1 polarization (62), was acutely elevated in the cerebrospinal fluid of pediatric patients with severe TBI (28).

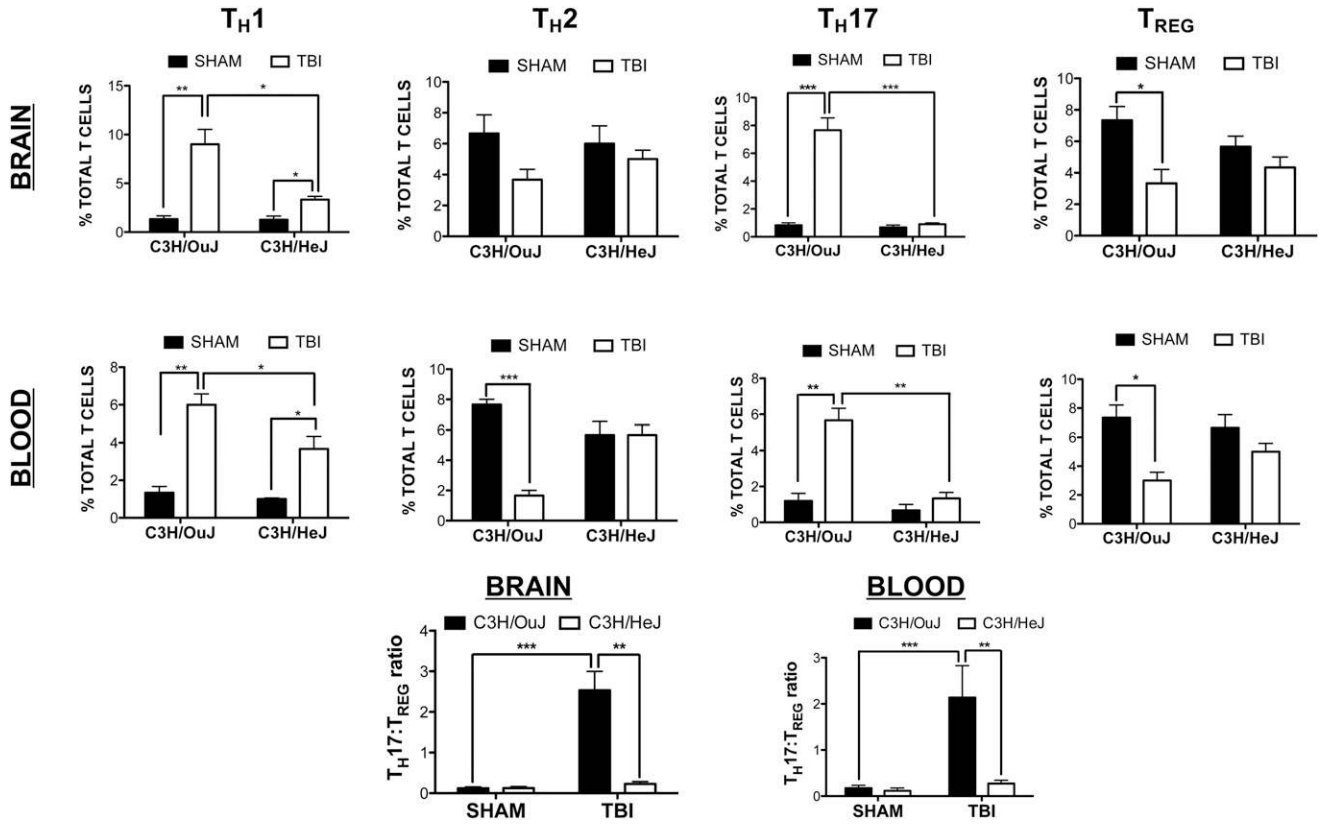

FIGURE 9. TLR4 mediates T cell polarization after TBI. T_H polarization was assessed by flow cytometry in blood and brain tissue from C3H/OuJ (wild-type) and C3H/OuJ (TLR4 mutant) mice at 72 h after sham/TBI. Graphs depict percentage of total T cells within the brain or blood. Bottom panels depict the T_H17/T_{REG} ratio in brain and blood of C3H/OuJ and C3H/HeJ mice at 72 h postsham/post-TBI across time. Data are mean ± SEM from $n = 6–8$ mice per group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

IL-17 promotes proinflammatory macrophage activation, and brain infiltration of T_H17 cells coincided with microglial activation and clinical symptomology after experimental autoimmune encephalopathy (63, 64). Likewise, FTY720, which prevents lymphocyte egress from lymphoid organs, reduced CNS trafficking of leukocytes and $CD4^+$ $IL\text{-}17^+$ T lymphocytes while simultaneously reducing neuroinflammation and white matter loss after neonatal hypoxia-ischemia (52). FTY720 similarly attenuated the accumulation of MHC-II^+ macrophages and microglia after TBI (65). In keeping with these findings, a reduction in circulating T_{REGs}, which suppress the proinflammatory effects of human T_H17 cells (66), correlated with a higher incidence of white matter microstructural abnormalities in ischemic stroke patients (67). Coupled with the observation that myelin basic protein increased IL-17 production in $CD4^+$ T lymphocytes from MS patients (68), an increased T_H17/T_{REG} ratio may establish a proinflammatory feedback loop to perpetuate chronic neurodegeneration after TBI (23).

Although we postulate a detrimental role after TBI, T_H17 cells are not pathogenic per se. Adoptive transfer of myelin-specific T_H17 cells increased basal hippocampal neurogenesis via actions in peripheral lymphoid tissues, whereas the same myelin-reactive T_H17 cells impaired endogenous remyelination in cuprizone-fed mice (69, 70). An explanation for these paradoxical effects is unclear, but the activation status of human Ag-presenting macrophages may dictate the efficiency of T_H17 responses (50). Adoptive transfer of T_H17 cells derived after TGF-β and IL-6 treatment are not detrimental, whereas T_H17 cells generated by treatment with TGF-β, IL-6, and IL-23 induced symptoms of experimental autoimmune encephalopathy (71). Whether similar effects occur after TBI remains unstudied; however, an IL-23–IL-17 signaling pathway increased neuronal apoptosis, whereas IL-23 deletion protected against cerebral ischemia-reperfusion injury (72, 73). In addition to differential regulation, T_H17 cells may transdifferentiate into T_{REGs} in the presence of TGF-β during the resolution of inflammation (74). Thus, the repertoire of cytokines released from polarized macrophages may direct T_H polarization and pathological inflammation after TBI.

Finally, we cannot exclude the possibility that other professional APCs contribute to our observed effects. Of note, Clodrosome selectively depletes macrophages, yet phagocytic dendritic cells may also be affected (75). Trauma reportedly favors the differentiation of monocytes to macrophages, rather than to dendritic cells (76); thus, our future work will investigate the importance of dendritic cells after TBI. We also will explore whether chronically elevated production of T_H17 cells functionally mediates delayed white matter loss after TBI. Similarly, we will establish whether T_H17 polarization represents a biomarker to prospectively identify patients who may be susceptible to chronic white matter injury after TBI.

Disclosures

The authors have no financial conflicts of interest.

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