Tumor necrosis factor-α triggers a cytokine cascade yielding postoperative cognitive decline

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Cognitive decline following surgery in older individuals is a major clinical problem of uncertain mechanism; a similar cognitive decline also follows severe infection, chemotherapy, or trauma and is currently without effective therapy. A variety of mechanisms have been proposed, and exploring the role of inflammation, we recently reported the role of IL-1β in the hippocampus after surgery in mice with postoperative cognitive dysfunction. Here, we show that TNF-α is upstream of IL-1 and provokes its production in the brain. Peripheral blockade of TNF-α is able to limit the release of IL-1 and prevent neuroinflammation and cognitive decline in a mouse model of surgery-induced cognitive decline. TNF-α appears to synergize with *MyD88*, the IL-1/TLR superfamily common signaling pathway, to sustain postoperative cognitive decline. Taken together, our results suggest a unique therapeutic potential for preemptive treatment with anti-TNF antibody to prevent surgery-induced cognitive decline.

innate immunity | surgical complications | delirium | dementia

Inflammation triggered by innate mechanisms plays a pivotal role in response to infection, injury, and trauma (1–3). The severity of the consequent inflammatory response may be important in many disease states, and determine whether the disease resolves or becomes chronic (4). Peripheral inflammation can profoundly affect the function of the CNS, including memory and cognition (5–7). Critical illness and postoperative recovery are often associated with cognitive decline, including memory dysfunction, especially in the elderly, a segment of the population that is increasing rapidly (8). Fluctuations in cognitive function and early-onset impairment in memory abilities are frequently observed in the intensive care unit. With an incidence ranging between 28 and 92% in hospitalized medical patients, depending on age, patient comorbidity, and the type of surgery, postoperative delirium is frequently diagnosed with bedside clinical testing (confusion assessment method) (9, 10). Although this acute confusion state is typically limited in duration and potentially reversible, postoperative delirium associates with significant increase in functional disability, length of hospital stay, rates of admission to long-term care institutions, and rates of death (11–13). Postoperative cognitive dysfunction (POCD) refers to longer-lasting cognitive decline that is detected through a battery of neuropsychological tests (14). After major noncardiac surgery, POCD occurs in 7 to 26% of patients, and is independently associated with poor short-term and long-term outcomes, including an increased risk of mortality, inability to cope independently, premature unemployment, and possible permanent dementia (15–17). In this study we used a mouse model of orthopedic surgery (a common type of surgery in the elderly) to extend our earlier laboratory studies of postoperative cognitive decline, documenting the innate immune and inflammatory pathways in more detail, and to identify the pivotal role of TNF-α in inducing the postoperative inflammatory response.

Results

Early Increase in Systemic TNF and High-Mobility-Group Box Chromosomal Protein-1. Following surgery under general anesthesia, TNF-α was the first cytokine to be released and peaked ($P < 0.05$) at 30 min after surgery (Fig. 1A); in contrast, other proinflammatory cytokines, such as IL-1β and IL-6, were not detected until 6 h postsurgery (18). To assess whether the inflammatory response was preceded by systemic release of damage-associated molecular-pattern (DAMPs) molecule, we measured high-mobility group-box chromosomal protein 1 (HMGB-1), which is released after cell necrosis (19). HMGB-1 blood levels were increased at 1 h ($P < 0.05$) and peaked at 6 h ($P < 0.001$) before returning to baseline (Fig. 1B). Significant differences in cytokine kinetics could be observed following infection (endotoxemia) (20) (Fig. S1). No cytokine or DAMP changes were observed following general anesthesia only (Fig. 1 C and D).

Anti-TNF Prophylaxis Prevents Neuroinflammation and Cognitive Decline. In a number of systems TNF triggers the production of proinflammatory cytokines, reported first in mouse "sepsis" (21) and human rheumatoid arthritis (22). We next investigated the effects of TNF-α blockade on systemic cytokine levels, neuroinflammation, and cognitive dysfunction. Preoperative administration of anti-TNF effectively reduced the amount of systemic IL-1β both at 6 and 24 h following surgery ($P < 0.01$, $P < 0.001$) (Fig. 2 A and B). To corroborate the findings and ascertain the kinetics and specificity of TNF-α blockade, we delayed the injection of the antibody until 1 h after surgery and then measured levels of IL-1β and IL-6; administration at this time with TNF-α blockade had no effect (Fig. 2 A and B).

Although IL-1β is pivotal for hippocampal learning and memory, high levels can interfere with long-term potentiation and synaptic plasticity (23). Prophylaxis with anti-TNF antibody attenuated the surgery-induced up-regulation of hippocampal IL-1β (Fig. 2C) ($P < 0.01$). Microglia, the innate immune cells of the CNS, usually reside in the quiescent state; these cells are tightly regulated and, upon activation, release cytotoxic compounds that disrupt homeostatic processes and neuronal functions (24–26). Microglia are activated following surgery, changing their small cell bodies and thin, long, ramified pseudopodia into an amoeboid morphology, with enlargement of the cell body (features described as microgliosis). Preoperative administration of anti-TNF antibody significantly reduced microgliosis after surgery (Fig. 2D) ($P < 0.01$).

To relate the inflammatory response to cognitive behavior, we used trace fear conditioning in which mice are trained to associate a tone with a noxious foot-shock stimulation (27). Contextual fear response shows reduced immobility (freezing) at postoperative day 3, revealing hippocampal-dependent memory impairment (Fig. 2E) ($P < 0.05$). Pretreatment with anti-TNF significantly ameliorated this cognitive dysfunction ($P < 0.05$).
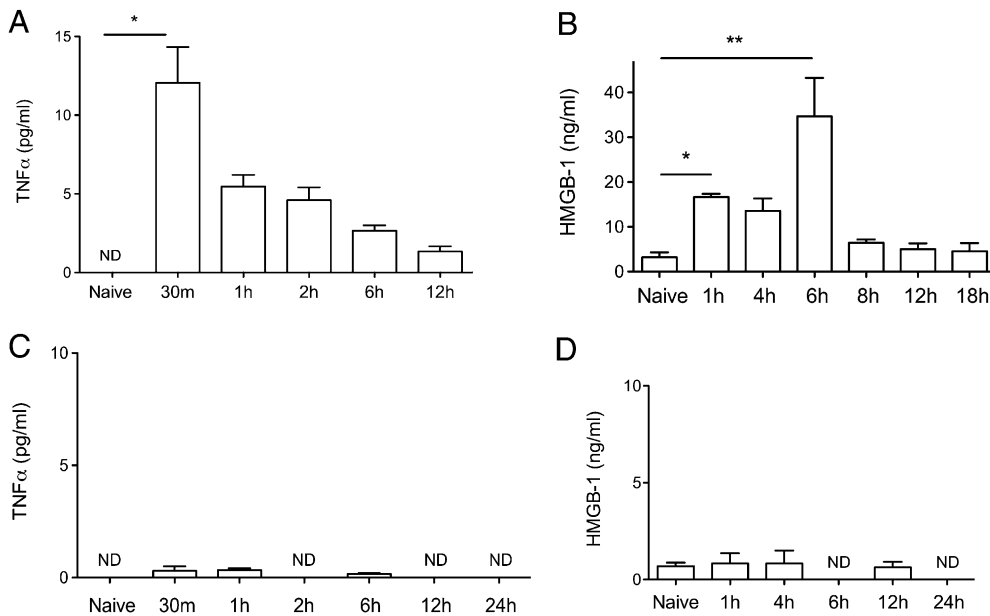
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Fig. 1. TNF-α and HMGB-1 measured by ELISA were increased following tibial surgery. (*A*) Plasma levels of TNF-α were significantly increased after 30 min from skin incision; (*B*) HMGB-1 was up-regulated after 1 h, peaking at 6 h, and returning to baseline thereafter. Plasma levels of both TNF-α (*C*) and HMGB-1 (*D*) remained at baseline following exposure to general anesthesia (2.1% isoflurane) and analgesia (buprenorphine, 1 mg/kg s.c.). Results are expressed as mean ± SEM (*n* = 6). *, $P < 0.05$; **, $P < 0.001$ versus naive by one-way ANOVA followed by Student-Newman-Keuls test. ND, not determined.

MyD88 Mediates Cognitive Decline Following Surgery. We had previously shown that IL-1 perpetuates the inflammatory response in our model, and is prevented by blockade of IL-1 by IL-1 receptor antagonist pretreatment (18). To assess the relationship between the TNF- and the IL-1-dependent pathways, we studied the effects of surgery in mice lacking *MyD88*, the key signaling
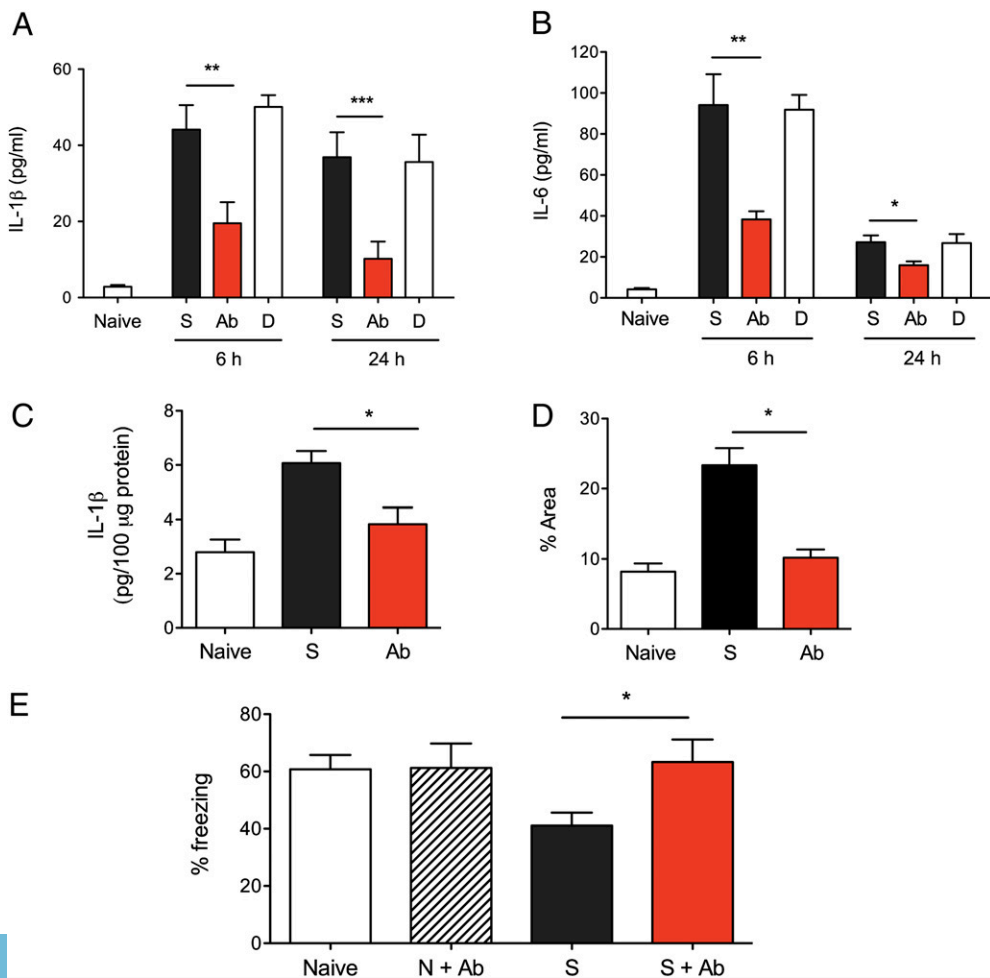

Fig. 2. Effects of anti-TNF prophylaxis on systemic cytokines, neuroinflammation and cognitive behavior. (*A*) Systemic IL-1β and (*B*) IL-6 levels following anti-TNF administration 18 h preoperatively. Delayed administration (1 h) of the antibody did not provide any reduction in plasma cytokines. (*C*) Hippocampi were extracted 6 h after surgery. Surgery with preemptive administration of anti-TNF resulted in no changes in expression of IL-1β compared with naive mice. (*D*) Densitometry of microglial immunostaining with CD11b. One day after surgery, mice showed significant microgliosis compared with naive and surgical mice treated with anti-TNF. (*E*) Mice subjected to surgery exhibited reduced freezing to context when compared with naive mice; preoperative administration (18 h) of anti-TNF mitigated the contextual fear memory impairment. Error bars represent the means ± SEM (*n* = 6, *n* = 10 for acute behavior). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by repeated measures ANOVA followed by Student-Newman-Keuls test. Kruskal-Wallis followed by the Dunn's multiple comparison test was used for categorical data. Ab, antibody; D, 1 h delayed administration of antibody; N + Ab, naive + Ab; S, surgery.
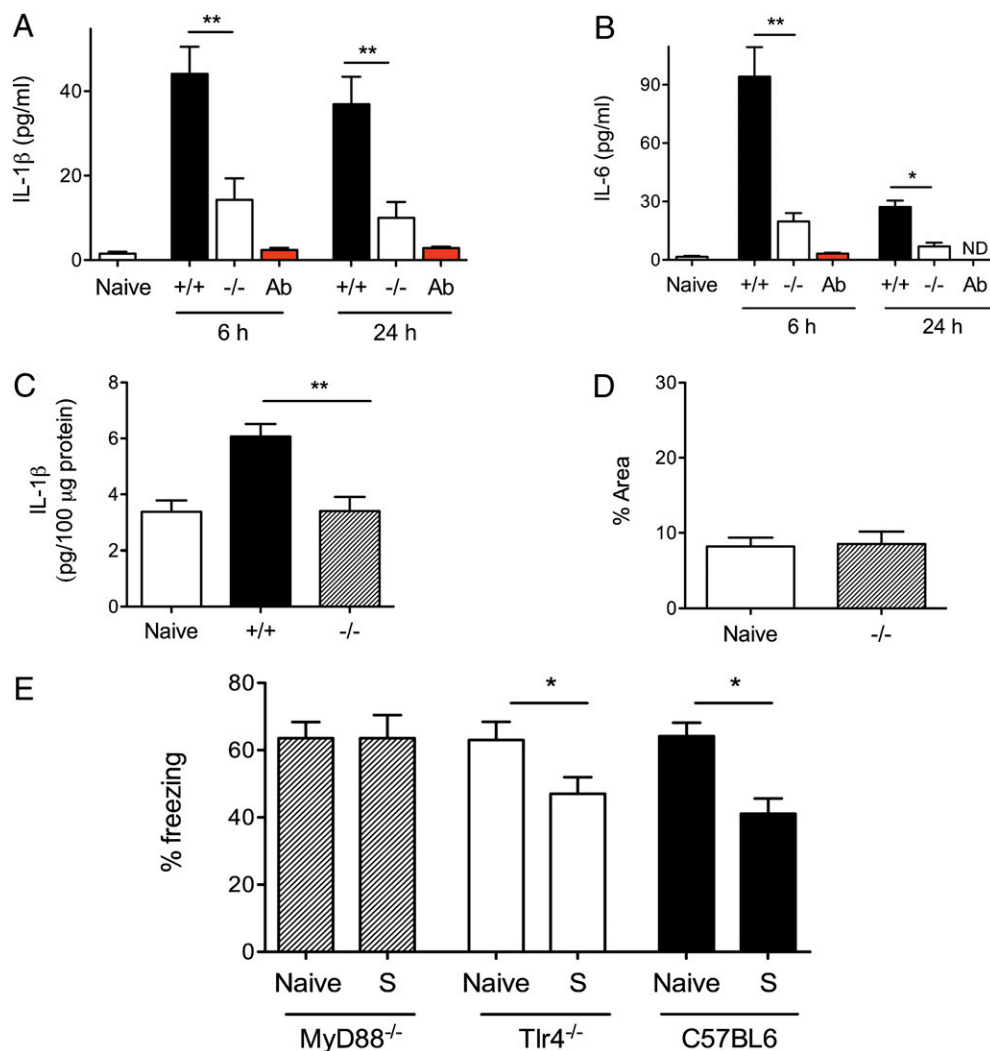
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Fig. 3. Effects of surgery-induced inflammation in *MyD88*$^{-/-}$. (*A* and *B*) Surgery resulted in reduced plasma levels of IL-1β and IL-6 both at 6 and 24 h, as measured by ELISA. Anti-TNF prophylaxis in *MyD88*$^{-/-}$ reduced the amount of systemic cytokines to baseline levels. (*C*) Hippocampal collection was carried out 6 h after treatment. Surgery resulted in no changes in the expression of IL-1β compared with naive *MyD88*$^{-/-}$ (KO). (*D*) Densitometry of microglial immunostaining showed no significant changes in microgliosis. (*E*) Contextual fear response is the same in *MyD88*$^{-/-}$ animals receiving surgery compared with naive and *Tlr4*$^{-/-}$. Error bars represent the means ± SEM (*n* = 6, *n* = 10 for acute behavior). *, *P* < 0.05; **, *P* < 0.01 by repeated measures ANOVA followed by Student-Newman-Keuls test. Kruskal-Wallis followed by the Dunn's multiple comparison test was used for categorical data. Ab, antibody; $^{-/-}$, *MyD88*$^{-/-}$; $^{+/+}$, *MyD88*$^{+/+}$; ND, not determined; S, surgery.

adaptor of the IL-1 and TLR superfamily, but not involved in TNF signaling (28). Compared with wild-type, the systemic cytokine response to surgery was reduced, but not obliterated, in *MyD88*$^{-/-}$ both at 6 and 24 h (Fig. 3 *A* and *B*) (*P* < 0.01, *P* < 0.01). However, when *MyD88*$^{-/-}$ were administered with anti-TNF antibody, surgery-induced incremental change in IL-1β and IL-6 were abrogated, although TNF-α levels were unaffected (Fig. 3 *A* and *B*). Neither signs of neuroinflammation (Fig. 3 *C* and *D* and Fig S2) nor a reduction in freezing behavior was observed in *MyD88*$^{-/-}$ following surgery (Fig. 3*E*).

Anti-TNF Prevents Surgery-Induced Inflammation in *Tlr4*$^{-/-}$ Mice.
Toll-like receptor 4 (TLR4) is involved in a large proportion of known danger signals, and more are emerging, such as tenascin-C in inflamed joints (29). Having established pivotal roles for TNF-α and *MyD88* signaling in surgery-induced inflammation and cognitive dysfunction, we targeted *TLR4* to understand whether this receptor could account for a *MyD88*-dependent signal in POCD (30). Surgery in *Tlr4*$^{-/-}$ induced similar systemic and central inflammation, as well as memory decline, as that seen in the wild-type, indicating that TLR-4 signaling is not required for establishment of cognitive decline (Fig. 4*A*) (*P* < 0.05). When *Tlr4*$^{-/-}$ mice were pretreated with anti-TNF 18 h before surgery, the systemic cytokine response, hippocampal inflammation and memory impairment were abrogated (Fig. 4 *B–E*).

Discussion

Postoperative cognitive decline is a poorly understood disorder, very common even after noncardiac surgery, which can be mimicked by surgery in mice. We have explored the inflammatory pathways and these results, taken together, demonstrate that TNF-α is very important in this process. TNF-α acts upstream of IL-1β and initiates the peripheral cytokine cascade leading to cognitive decline (Fig. 2). Prophylaxis with anti-TNF antibody prevents postoperative cognitive decline. TNF-α blockade successfully reduces further elaboration of IL-1, which has been extensively reported to modulate symptoms of "sickness behavior," including memory dysfunction (20, 31, 32). With a single dose of anti-TNF monoclonal antibody, we have also effectively interfered with the IL-1-dependent amplification mechanism and cognitive function (freezing) was normalized.

Cytokines are central mediators of inflammatory events following all types of events, including peripheral trauma or infection, and have broad physiological effects both on the periphery and in the CNS (33). TNF-α has a pivotal role in the initiation and amplification of the inflammatory cascade; it is involved in regulating chemokines and cytokines release, oxidative stress, recruitment of immune cells and adhesion molecules, apoptosis, healing, and tissue-specific repair mechanism. This function has been extensively documented during the analysis of the pathogenesis of rheumatoid arthritis (34). TNF-α also exerts neuro-

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Fig. 4. Anti-TNF prophylaxis in $Tlr4^{-/-}$. (*A*) Contextual fear response is impaired in $Tlr4^{-/-}$ 3 d after surgery compared with untreated. Prophylaxis with anti-TNF 18 h before surgery prevented the cognitive abnormality. (*B* and *C*) Preemptive administration of anti-TNF in $Tlr4^{-/-}$ reduced the amount of systemic IL-1β and IL-6 to baseline levels, as measured by ELISA. (*D*) $Tlr4^{-/-}$ showed signs of neuroinflammation with increased expression of hippocampal IL-1β, which was reduced by anti-TNF. (*E*) Densitometry of microglial immunostaining with CD11b. One day after surgery, $Tlr4^{-/-}$ showed significant microgliosis compared with naive and surgical mice preoperatively treated with anti-TNF. Error bars represent the means ± SEM ($n = 6$, $n = 10$ for acute behavior).

*, $P < 0.05$; **, $P < 0.01$ by one-way ANOVA followed by Student-Newman-Keuls test. Kruskal-Wallis followed by the Dunn's multiple comparison test was used for categorical data. Ab, antibody; S, surgery; $^{-/-}$, $TLR4^{-/-}$; $^{+/+}$, $TLR4^{+/+}$.
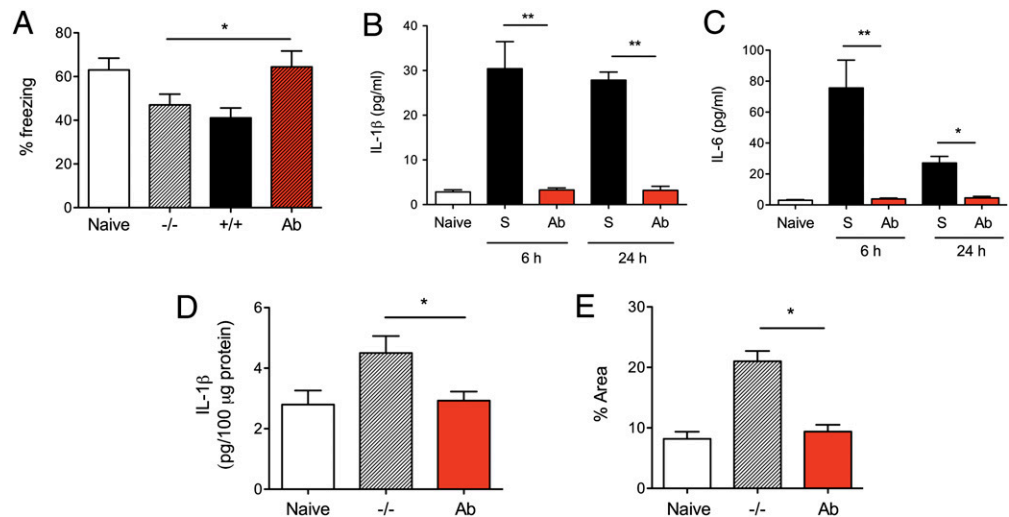
modulatory functions, in particular in regulating microglia and astrocytes activation in the brain (35).

Systemic cytokines exert effects in the CNS both via direct and indirect pathways. TNF-α has been shown to enter the brain through relatively permeable areas in the blood–brain barrier (BBB) (36). In our model, it is possible that transient changes in BBB permeability, either caused by the systemic inflammatory response or other factors including anesthesia (37), could enable direct access to the brain. Neural afferents may also account for signs of CNS inflammation (38). Because of the changes in the peripheral inflammatory effects that were noted in anti-TNF antibody-treated surgical animals, and with the lack of evidence of acute changes in the BBB postsurgery, we consider it likely that the antibody exerts its ameliorative action in the periphery. This finding suggests a pivotal role for systemic inflammation in producing neuroinflammation and cognitive decline. Various indirect routes for cytokine effects need to be excluded, and the key site of anti-inflammatory effects needs further elucidation.

In the brain, microglia can actively secrete cytokines and neurotoxins upon activation (39). Using another model of peripheral organ inflammation, peripheral TNF-α was necessary to stimulate microglia activation and to allow recruitment of circulating monocytes into the brain (40). With this model of surgical trauma we are yet unable to define the precise origin of this neuroinflammatory response, in particular whether it is mediated by peripheral monocytes or resident microglia. Resulting neuroinflammation accounts for behavioral changes, in particular in vulnerable areas, such as the hippocampus (41). Local inflammation directly interferes with the processes of memory consolidation, long-term potentiation, synaptic plasticity, and neurogenesis, resulting in what has been termed "sickness behavior"; inflammatory blockade has been reported to restore hippocampal neurogenesis (42).

Having defined a role for TNF-α following surgery, we investigated whether $MyD88$ signaling, important for the TLR and IL-1 families, was involved in surgery-induced cognitive dysfunction. There was no surgery-induced freezing impairment, as a marker of cognitive decline in $MyD88^{-/-}$ (Fig. 3*E*). This finding is in keeping with our recently reported role of IL-1β in the same model (18). Because the $Tlr4^{-/-}$ mice were not protected in any way from surgery-induced inflammation or POCD, it appears that TLR4 has redundant role in this disease pathway. DAMPs, such as HMGB-1, are recognized by multiple receptors (e.g., RAGE, as well as $Tlr4$ and $Tlr2$) and one of the other receptors might be able to induce signaling in the absence of the other (43). Administration

of anti-TNF to $MyD88^{-/-}$ or $Tlr4^{-/-}$ completely abrogated the surgery-induced IL-1β response and downstream IL-6 production, suggesting a perpetuating role for both these cytokines in the inflammatory response and, possibly, synergism with one another.

Anti-TNF antibody was the first cytokine-selective therapy that was shown to offer clear benefits in the setting of common disease in rheumatoid arthritis, being both effective and acceptably safe (44). Based upon our data, prophylaxis with anti-TNF antibody is a feasible therapeutic option that is ready to be exploited in the elective surgical setting. There have been attempts to use anti-TNF in acute indications in bacterial sepsis almost 20 y ago (45). Sepsis contrasts markedly with POCD, as therapy with anti-TNF, effective prophylactically in septic mice, could not be given early in humans because of the clinical reality of sepsis. This finding is in marked contrast to the preemptive manner in which this biologic can be used before surgery. Because other injured states, such as major trauma and especially chemotherapy, lead to similar states of cognitive decline as POCD, it is possible that similar up-regulation of cytokines may underpin these clinical syndromes, and this is worth investigating. It is possible that by administering anti-TNF antibody surgical patients may be at higher risk for opportunistic infections and other postoperative complications; however, most long-term randomized clinical trials have not reported any differences in the incidence of infections in patients treated with anti-TNF. Tuberculosis relapse is the chief exemption, with long-term treatment increasing its prevalence (46). For POCD, anti-TNF would be very short term: a single injection. Another possible target to prevent postoperative cognitive decline may be HMGB-1, which was significantly increased following surgical trauma (Fig. 1*B*) (47). Overall, we can conclude that TNF-α acts upstream of IL-1β and initiates the peripheral cytokine cascade that results in cognitive decline. Prophylaxis with a single dose of anti-TNF antibody attenuates the downstream activation of both $MyD88$-dependent and independent inflammatory pathways. Therapy with TNF-α inhibitors is clinically well established and already offers beneficial effects in inflammatory conditions, such as rheumatoid arthritis, Crohn's disease, and ankylosing spondylitis (22, 48), and may thus be useful for the prevention of postoperative cognitive decline in susceptible individuals.

Methods

Animal Experiments. Experiments were performed in accordance to the United Kingdom Home Office-approved license. We randomly grouped 12- to 14-wk-old male C57BL/6 mice and assigned them to a specific experiment. Homozygous $MyD88^{-/-}$ on a C57BL/6 background were provided by the the

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Sanger Institute (49). Tlr4$^{-/-}$ mice were obtained from B&K Universal (50). Age-matched congenic inbred wild-type C57BL/6 mice were obtained from Charles River. All animals were fed standard rodent chow and water ad libitum, and were housed (< five mice per cage) in sawdust-lined cages in an air-conditioned environment with 12-h light/dark cycles. All of the animals were checked on a daily basis and if they evidenced poor grooming, huddling, piloerection, weight loss, back arching, and abnormal activity, they were eliminated from further consideration. Investigators who treated animals knew the treatment groups and collected samples, which were then analyzed by other investigators blinded to the specific treatment.

Surgery. We performed an open tibial fracture as previously described (51). Briefly, we anesthetized mice with 2.1% isoflurane and analgesia with buprenorphine (Buprenex, 0.1 mg/kg s.c). A middle incision was performed on the left hind paw and a 0.38-mm pin was inserted in the intramedullary canal, the periosteum stripped, and osteotomy performed. Aseptic conditions were maintained throughout. We subjected mice to vehicle (saline) or TNF-neutralizing antibody (52) (clone TN3, 100 μg per mouse; Sigma) 18 h preoperatively. Blood was collected by cardiac puncture. Plasma cytokines and hippocampal IL-1β were measured by ELISA according to the manufacturer's instructions. Fixed brains were collected for immunohistochemical DAB staining for microglia activation using CD11b (SI Methods).

Behavior. The behavioral study was conducted using a dedicated conditioning chamber (Med Associates Inc.). Mice were trained and tested on separate days. The fear-conditioning paradigm was used as previously described (18). Freezing behavior was recorded 3 d after training. Mice from each treatment group were randomly assigned for assessment of either cytokine response or cognitive behavior to obviate possible confounding effects of behavioral testing on inflammatory markers (53) (SI Methods).

Data Analysis. We used GraphPad v3.0 (GraphPad Software) to calculate the mean, SD, and SEM, and perform statistical tests. We analyzed multiple group means by one-way analysis of variance, followed by Newman-Keuls post hoc test wherever appropriate. The nonparametric test of Kruskal-Wallis followed by the Dunn's multiple comparison test was used for categorical data. P values less than 0.05 were considered significant.

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