CX3CR1\textsuperscript{+} monocytes modulate learning and learning-dependent dendritic spine remodeling via TNF-\(\alpha\)

Juan Mauricio Garré\textsuperscript{1}*, Hernandez Moura Silva\textsuperscript{2}, Juan J Lafaille\textsuperscript{2} & Guang Yang\textsuperscript{1}*

Impaired learning and cognitive function often occurs during systemic infection or inflammation. Although activation of the innate immune system has been linked to the behavioral and cognitive effects that are associated with infection, the underlying mechanisms remain poorly understood. Here we mimicked viral immune activation with poly(I:C), a synthetic analog of double-stranded RNA, and longitudinally imaged postsynaptic dendritic spines of layer V pyramidal neurons in the mouse primary motor cortex using two-photon microscopy. We found that peripheral immune activation caused dendritic spine loss, impairments in learning-dependent dendritic spine formation and deficits in multiple learning tasks in mice. These observed synaptic alterations in the cortex were mediated by peripheral-monocyte-derived cells and did not require microglial function in the central nervous system. Furthermore, activation of CX3CR1\textsuperscript{high}Ly6C\textsuperscript{low} monocytes impaired motor learning and learning-related dendritic spine plasticity through tumor necrosis factor (TNF)-\(\alpha\)-dependent mechanisms. Taken together, our results highlight CX3CR1\textsuperscript{high} monocytes and TNF-\(\alpha\) as potential therapeutic targets for preventing infection-induced cognitive dysfunction.

Activation of the immune system has profound effects on the function of the central nervous system (CNS)\textsuperscript{1–5}, and it is thought to have an important role in schizophrenia\textsuperscript{6}, autism-spectrum disorder\textsuperscript{7} and the neuropsychological impairment associated with infectious diseases\textsuperscript{8}. Indeed, changes in mood and cognitive function are frequently observed in people who are infected with viruses, such as influenza and HIV, or bacteria\textsuperscript{9–13}. The similarity of behavioral effects that are associated with different infectious diseases suggests that immune responses to infectious agents, rather than a specific pathogen \textit{per se}, may have a critical role in cognitive decline. Administration of polyinosinic-polycytidylic acid (poly(I:C)) is a widely used model to study innate immune activation\textsuperscript{14}. Double-stranded RNA is produced during viral replication\textsuperscript{15} and recognized by the mammalian innate immune system primarily through pathogen recognition receptors (PRRs), such as toll-like receptor 3 (TLR3)\textsuperscript{16}. The binding of poly(I:C) to PRRs leads to the expression of a series of immune mediators (such as type 1 interferons (IFNs), pro-inflammatory cytokines and chemokines), a process that efficiently mimics the acute phase of viral infection\textsuperscript{17}. Similar to that observed in viral infection, peripheral immune stimulation through poly(I:C) has been shown to cause behavioral and cognitive impairments in both developing and adult animals\textsuperscript{18–21}; however, the underlying mechanisms remain unclear.

Various host immune cells are involved in innate defense against infection. Monocytes are a population of leukocytes that are derived from progenitors in the bone marrow and that circulate in the bloodstream\textsuperscript{22}. They develop into macrophages or dendritic cells after entering peripheral tissues. In response to infection, monocytes take part in the inflammatory response by producing and releasing pro-inflammatory cytokines, such as TNF-\(\alpha\), and chemokines in the infected areas\textsuperscript{23–25}.

In the CNS, microglia are the major innate immune cells that become activated during systemic infection\textsuperscript{26}. Like monocytes, activated microglia produce a number of inflammatory molecules and have been implicated in the development of neurodegenerative diseases\textsuperscript{5}. Despite the fact that monocytes and microglia are crucial players in innate immunity, which of these populations is involved in the alteration of CNS functions during virus-associated immune activation remains unknown. It is also unclear how activation of these cells alters neuronal circuits and leads to learning and behavioral deficits. Here we show that peripheral immune activation causes deficits in learning tasks, synapse loss and a reduction in learning-dependent synapse formation. These synaptic and behavioral changes are mediated by CX3CR1\textsuperscript{high} monocytes via TNF-\(\alpha\)-dependent mechanisms.

**RESULTS**

**Systemic immune challenge increases dendritic spine dynamics in the cortex**

To investigate how peripheral immune activation affects the brain, we intraperitoneally (i.p.) administered poly(I:C) to adolescent (postnatal day (P) 30) transgenic mice that expressed yellow fluorescent protein in layer V (L5) pyramidal neurons (Thy1-YFP mice) and examined structural changes in the postsynaptic dendritic spines of the primary motor cortex using transcranial two-photon microscopy (Fig. 1a)\textsuperscript{27}. After a single injection with either 5 or 50 mg per kg body weight (mg/kg) poly(I:C), we observed more dendritic spine remodeling over 2 days in poly(I:C)-treated mice than in vehicle-treated control mice (Fig. 1a,b). In control mice, 8.3 \(\pm\) 0.6% of dendritic spines were eliminated and 7.2 \(\pm\) 0.7% were formed, whereas in mice that were treated with 5 mg/kg poly(I:C), 17.5 \(\pm\) 1.1% and 14.0 \(\pm\) 0.9% of dendritic spines

\textsuperscript{1}Department of Anesthesiology, Perioperative Care and Pain Medicine, New York University (NYU) School of Medicine, New York, New York, USA. \textsuperscript{2}Skirball Institute of Biomolecular Medicine, Departments of Pathology and Medicine, New York University School of Medicine, New York, New York, USA. Correspondence should be addressed to G.Y. (guang.yang@med.nyu.edu).

Received 12 May 2016; accepted 11 April 2017; published online 15 May 2017; doi:10.1038/nm.4340
were eliminated and formed, respectively. A significant increase in spine elimination and formation was also detected 24 h after poly(I:C) injection (Fig. 1c and Supplementary Table 1). The rate of spine elimination after poly(I:C) injection was higher than that of spine formation (Supplementary Fig. 1), indicating a net loss of spines over 1–2 d (Fig. 1d). Four days after poly(I:C) injection, no continued increase in spine remodeling was detected relative to that in control mice (Fig. 1b), indicating an acute effect of poly(I:C) treatment on spine dynamics. Consistent with previous reports, we found that dendritic spine remodeling was lower in 2-month-old mice than in 1-month-old mice. However, similar to what was observed in 1-month-old mice, poly(I:C) injection (5 mg/kg) at P60 significantly increased the rates of spine elimination and formation over 2 d (Fig. 1e,f).

Systemic immune challenge impairs the dendritic spine plasticity associated with learning

The remodeling of synaptic connections is important for learning and memory formation. Previous studies have shown that the learning of motor skills causes a learning-dependent increase in dendritic spine formation in the motor cortex and that the extent of new spine formation correlates with an improvement in the animal’s performance after learning. We next investigated whether poly(I:C) injection similarly altered learning-dependent spine remodeling. We trained 1- or 2-month-old mice to run on an accelerating rotating rod (which is referred to as a rotarod) and imaged the same dendrites in the motor cortex before and after a 2-d period of training (Fig. 2a). Two days after rotarod training, we observed a significant reduction in learning-dependent spine formation in poly(I:C)-treated mice as compared to that in the age-matched controls (Fig. 2b,c and Supplementary Table 1). Conversely, we found a significant increase in spine elimination in poly(I:C)-treated mice as compared to that of vehicle-treated controls. Thus, in poly(I:C)-treated mice the combined result of these modifications is a net loss of spines after learning, while vehicle-treated mice exhibit a net gain in spines after learning (Fig. 2d,e). Furthermore, although poly(I:C) treatment had no effects on their body weight (Supplementary Fig. 2) as compared to those observed in vehicle-treated mice, poly(I:C) treatment did cause a significant reduction in motor performance improvements after a 2-d training period in both 1- and 2-month-old mice (Fig. 2f,i).

To evaluate the long-term effect of poly(I:C) treatment on learning-related spine remodeling and motor performance, we reimaged and tested 2-month-old mice on P67, after a 5-d period without further rotarod training (Fig. 2a). We found that the new spines formed during the 2-d training interval (from P60 to P62), fewer persisted at P67 in the poly(I:C)-treated mice than in the control mice (Fig. 2j). Furthermore, motor performance improvement continued to remain lower in the poly(I:C)-treated mice (Fig. 2k). There was a significant
correlation between the percentage of training-related persistent new spines and rotarod performance on P67 (Fig. 2). We also compared the performance of poly(I:C)- and vehicle-treated mice in a novel object recognition (NOR) test (Supplementary Fig. 3). NOR training was performed 24 h after poly(I:C) administration. The training and test sessions were separated by either a 1-h or a 24-h interval to examine the short-term and long-term memory, respectively, of the mice. Although vehicle-treated control mice showed a preference for the novel object during the NOR test, mice that were pretreated with poly(I:C) showed no such preference at either 1 h or 24 h after training, indicating impaired recognition memory.

**CX3CR1**+ cells are crucial for poly(I:C)-induced dendritic spine instability**

The chemokine receptor CX3CR1 is widely expressed in monocytes and monocyte-derived cells in peripheral tissues, and it is exclusively expressed in microglia in the CNS parenchyma34. To determine whether the observed effects of poly(I:C) treatment on dendritic spine plasticity require the involvement of CX3CR1+ cells, we performed in vivo diphertheria toxin receptor (DTR)-mediated cell depletion. Specifically, Cx3cr1CreER mice, which express a tamoxifen-inducible Cre recombinase (CreER) under the control of the endogenous Cx3cr1 promoter, were crossed with mice harboring the ubiquitous Rosa26-stop-DTR (R26DTR) allele, permits DTR expression where Cre is present. For in vivo cell depletion, Cx3cr1CreER;R26DTR+ mice were given tamoxifen for 3 d (P54 to 56) and subsequently treated with diphertheria toxin (DT) for 3 d (P57 to 59) (Fig. 3a). We found that the majority of CX3CR1+ cells, including peripheral CD11b+ cells and CNS microglia, were depleted after the last DT treatment (Fig. 3b–d). One day after the last DT treatment, we administered poly(I:C) and examined the rates of spine remodeling in the cortex over the next 2 d. Similarly to that seen in wild-type (WT) mice, we found a significant increase in spine elimination and formation in control mice that were not depleted for CX3CR1+ cells (Cx3cr1CreER+/+ at 2 d after the administration of poly(I:C) (Fig. 3e). In contrast, in mice that were depleted of CX3CR1+ cells (Cx3cr1CreER+;R26DTR+ poly(I:C) treatment had no significant effects on the rates of dendritic spine elimination and formation (Fig. 3f), indicating the requirement of CX3CR1+ cells in dendritic spine remodeling in the cortex.

Although CX3CR1 is mostly expressed in cells of the myeloid lineage, a small fraction of T cells (which are cells of the lymphoid lineage) may also express CX3CR1 (ref. 34). To determine whether T cells were involved in poly(I:C)-induced spine remodeling, we examined dendritic spine plasticity in Rag1−/− mice, which are deficient for T cells and B cells. We found that administration of poly(I:C) significantly increased the rates of spine elimination and formation in Rag1−/− mice, relative to those observed after treatment with vehicle only, and that these were comparable to the rates observed in WT mice (Fig. 3g), suggesting the role of CX3CR1+ myeloid cells but not of CX3CR1+ T cells in poly(I:C)-induced synapse atarhalization.

**CNS microglia are not required for poly(I:C)-induced dendritic spine instability**

Previous studies have shown that microglia are important for synaphe formation and elimination under both physiological and pathological conditions35–37. To determine which population of CX3CR1+ cells (central or peripheral) regulates cortical spine plasticity during peripheral immune activation, we restricted cell depletion to that of CNS microglia by taking advantage of a previously established strategy in which Cx3cr1CreER+j mice that are
used ~30 d after tamoxifen administration permit Cre-dependent manipulation of gene expression almost exclusively in CNS-resident macrophages, including microglia. This strategy is based on the fact that yolk-sac-derived microglia are a self-renewing population with low turnover rates (months to years), whereas most peripheral circulating CX3CR1+ populations have a high turnover rate (~1 week) and are rapidly replenished through bone marrow (BM) precursors. Consistent with previous studies, we found that CNS microglia were largely depleted, whereas the peripheral CX3CR1+ monocytes were mostly intact, after the last DT (~1 month later) from control (top) and CX3CR1+-cell-depleted (bottom) mice that were stained with the microglia marker Iba1 1 d after administration of the last dose of DT. Scale bar, 50 µm. (e) Quantification of dendritic spine elimination and formation over 2 d in Cx3cr1CreER/+ control mice that were injected with vehicle or poly(I:C) (n = 4 mice per group). (f) Dendritic spine elimination and formation after 2 d in Cx3cr1CreER/+;R26DTR/+ mice which are depleted of CX3CR1+ cells that were injected with vehicle (n = 6 mice) or poly(I:C) (n = 4 mice). (g) Dendritic spine elimination and formation after 2 d in Rag1−/− mice that were injected with vehicle or poly(I:C) (n = 4 mice per group). Throughout, individual circles represent data from a single mouse. Summary data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001; n.s., not significant; by Mann–Whitney test. See also Supplementary Table 1.

Figure 3 CX3CR1+ cells are required for altered dendritic spine plasticity after systemic immune challenge. (a) Schematic representation of the strategy to deplete CX3CR1+ cells, which include peripheral monocytes and CNS-resident microglia. (b) Representative flow cytometry analysis showing the percentages of CX3CR1–EYFP+CD11b+ cells in the cortex (left), liver (middle) and blood (right) of Cx3cr1CreER/+ (top) or Cx3cr1CreER/+;R26DTR/+ (bottom) mice after administration of the last dose of DT. Gate: singlets, DAPI−LIN−CD45low (cortex) or CD45 high (periphery). (c) Quantification of the data shown in b (n = 6 mice per group). (d) Representative coronal sections (n = 8 sections per group) of the motor cortex from control (top) and CX3CR1+-cell-depleted (bottom) mice that were stained with the microglia marker Iba1 1 d after administration of the last dose of DT. Scale bar, 50 µm. (e) Quantification of dendritic spine elimination and formation over 2 d in Cx3cr1CreER/+ control mice that were injected with vehicle or poly(I:C) (n = 4 mice per group). (f) Dendritic spine elimination and formation after 2 d in Cx3cr1CreER/+;R26DTR/+ mice (which are depleted of CX3CR1+ cells) that were injected with vehicle (n = 6 mice) or poly(I:C) (n = 4 mice). (g) Dendritic spine elimination and formation after 2 d in Rag1−/− mice that were injected with vehicle or poly(I:C) (n = 4 mice per group). Throughout, individual circles represent data from a single mouse. Summary data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001; n.s., not significant; by Mann–Whitney test. See also Supplementary Table 1.

CX3CR1+ monocytes mediate poly(I:C)-induced synaptic and learning deficits

To further establish the function of peripheral CX3CR1+ monocytes on dendritic spine remodeling during systemic immune challenge, we transplanted BM cells obtained from Cx3cr1CreER/+;R26DTR/+ mice into irradiated WT recipients (referred to as Cx3cr1CreER/+;R26DTR/+→WT chimeras) to generate chimeric mice that express Cx3cr1CreER/+;R26DTR/+ only in peripheral tissues. One month after BM reconstitution, mice were given tamoxifen and subsequently treated with DT (Fig. 4d). Flow cytometry analysis revealed a substantial reduction in the number of CX3CR1+CD11b+ cells in the blood and liver tissues of the Cx3cr1CreER/+;R26DTR/+→WT chimeras as compared to that in their control counterparts that received BM cells from Cx3cr1CreER/+ or R26DTR/+→WT chimeras, indicating a reduction of CX3CR1+ monocytes and monocyte-derived cells. In contrast, DT treatment had no effect on the number of CNS microglia in the cortex of the Cx3cr1CreER/+;R26DTR/+→WT chimeras (Fig. 4e and Supplementary Fig. 6).

Next we examined the effect of poly(I:C) treatment on the baseline and learning-induced remodeling of dendritic spines in the motor cortex. Poly(I:C) treatment significantly increased the baseline spine elimination and formation rates in nondepleted control mice (Fig. 4f), whereas poly(I:C) treatment had no significant effects on the baseline rates of spine turnover in mice that were depleted of CX3CR1+ monocytes (Fig. 4g). Furthermore, in contrast to control...
Figure 4 CX3CR1⁺ monocytes, but not microglia, mediate synaptic and learning deficits after systemic immune challenge. (a) Schematic of the strategy to selectively deplete CNS-resident microglia. (b) Quantification of CX3CR1⁺CD11b⁺ cells in the cortex, liver and blood of Cx3cr1CreER/+/WT and Cx3cr1CreER/+-R26DT/Tm mice 1 day after administration of the last dose of DT (n = 4 mice per group). (c) Effect of poly(I:C) treatment on dendritic spine elimination and formation after 2 days in Cx3cr1CreER/+/+R26DT/+ mice that were depleted of microglia (n = 4 mice per group). (d) Schematic of the strategy to selectively deplete CX3CR1⁺ monocytes using Cx3cr1CreER/+/+, R26DT/+ to WT chimeras. Cx3cr1CreER/+/WT mice were used as controls. Irradiation was performed with or without head protection (HP). (e) Percentages of CD11b⁺CD45low cells (microglia) in the cortex and Cx3CR1⁺CD11b⁺ cells in blood and liver after administration of the last DT injection (n = 4 mice per group). (f,g) Effect of poly(I:C) treatment on baseline spine elimination and formation after 2 days in Cx3cr1CreER/+/+WT (f) or Cx3cr1CreER/+-R26DT/+WT (g) chimeras (n = 4 mice per group). (h,i) Effect of poly(I:C) treatment on dendritic spine elimination and formation after a 2-day motor-training period in Cx3cr1CreER/+/+WT (n = 6 mice per group) (h) or in Cx3cr1CreER/+/+R26DT/+WT chimeras (vehicle-treated, n = 8; poly(I:C)-treated, n = 9 mice) (i). (j,k) Net change in spine number after a 2-day motor-training period for the mice in h (j) and i (k). (l–o) Effect of poly(I:C) treatment on rotarod performance in Cx3cr1CreER/+/+WT (vehicle-treated, n = 5 mice; poly(I:C)-treated, n = 6 mice) (l,m) or in Cx3cr1CreER/+/+R26DT/+WT chimeras (vehicle-treated, n = 9 mice; poly(I:C)-treated, n = 11 mice) (n,o) that were injected with vehicle or poly(I:C) on P60. Throughout, individual circles represent data from a single mouse. Summary data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01; n.s., not significant; by Mann–Whitney test. See also Supplementary Table 1.

**ARTICLES**

...monocytes, but not microglia, mediate synaptic and learning deficits after systemic immune challenge. (a) Schematic of the strategy to selectively deplete CNS-resident microglia. (b) Quantification of CX3CR1⁺CD11b⁺ cells in the cortex, liver and blood of Cx3cr1CreER/+/+ (control) or Cx3cr1CreER/+/+R26DT/+ mice 1 day after administration of the last dose of DT (n = 4 mice per group). (c) Effect of poly(I:C) treatment on dendritic spine elimination and formation after 2 days in Cx3cr1CreER/+/+R26DT/+ mice that were depleted of microglia (n = 4 mice per group). (d) Schematic of the strategy to selectively deplete CX3CR1⁺ monocytes using Cx3cr1CreER/+/+, R26DT/+ to WT chimeras. Cx3cr1CreER/+/WT mice were used as controls. Irradiation was performed with or without head protection (HP). (e) Percentages of CD11b⁺CD45low cells (microglia) in the cortex and Cx3CR1⁺CD11b⁺ cells in blood and liver after administration of the last DT injection (n = 4 mice per group). (f,g) Effect of poly(I:C) treatment on baseline spine elimination and formation after 2 days in Cx3cr1CreER/+/+WT (f) or Cx3cr1CreER/+-R26DT/+WT (g) chimeras (n = 4 mice per group). (h,i) Effect of poly(I:C) treatment on dendritic spine elimination and formation after a 2-day motor-training period in Cx3cr1CreER/+/+WT (n = 6 mice per group) (h) or in Cx3cr1CreER/+/+R26DT/+WT chimeras (vehicle-treated, n = 8; poly(I:C)-treated, n = 9 mice) (i). (j,k) Net change in spine number after a 2-day motor-training period for the mice in h (j) and i (k). (l–o) Effect of poly(I:C) treatment on rotarod performance in Cx3cr1CreER/+/+WT (vehicle-treated, n = 5 mice; poly(I:C)-treated, n = 6 mice) (l,m) or in Cx3cr1CreER/+/+R26DT/+WT chimeras (vehicle-treated, n = 9 mice; poly(I:C)-treated, n = 11 mice) (n,o) that were injected with vehicle or poly(I:C) on P60. Throughout, individual circles represent data from a single mouse. Summary data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01; n.s., not significant; by Mann–Whitney test. See also Supplementary Table 1.

**CX3CR1⁺ monocytes, but not microglia, mediate synaptic and learning deficits after systemic immune challenge.**

To further investigate the role of monocytes in poly(I:C)-induced dendritic spine alteration, we collected leukocytes from blood and spleen tissues and magnetically sorted them on the basis of the cell surface expression of CD11b, a marker for myeloid cells (including monocytes). Mice that were depleted of CX3CR1⁺ cells (in both the CNS and periphery) were injected with various amounts of CD11b-sorted cells. We found that poly(I:C) treatment significantly increased spine elimination and formation in mice that were injected with 2 × 10⁶ CD11b-sorted cells, as compared to those in vehicle-treated mice (Supplementary Fig. 8). The rates of spine elimination and formation after poly(I:C) administration were significantly lower in mice that were adoptively transferred with a lower number (0.5 × 10⁶) of CD11b-sorted cells than in those that were adoptively transferred with 2 × 10⁶ CD11b-sorted cells. We did not find changes in spine turnover in mice that were injected with the fraction of cells that did not express CD11b.

**CX3CR1⁺ monocytes are required for poly(I:C)-induced synaptic and learning deficits.**

Previous work has identified that two distinct subsets of peripheral blood monocytes exist in mice, based on the level of CX3CR1 expression (CX3CR1low and CX3CR1high). When we examined the expression of Ly6C, a marker for inflammatory monocytes, in CX3CR1⁺ cells, we found that systemic or peripheral depletion of CX3CR1⁺ cells substantially reduced the number of CX3CR1highLy6Clow monocytes in the blood, whereas CX3CR1lowLy6Chigh monocytes were not affected (Supplementary Fig. 9). These results suggest that CX3CR1highLy6Clow monocytes, a population of monocytes that patrol blood vessels, are essential for poly(I:C)-induced cortical changes.

To test the role of CX3CR1high monocytes in poly(I:C)-induced synaptic deficits, we took advantage of the fact that CX3CR1 deficiency...
leads to a substantial reduction of CX3CR1<sup>high</sup> monocytes and generated CX3cr1<sup>+/−</sup>→WT chimeric mice, which have a deficiency of CX3CR1 in peripheral leukocytes (Fig. 5a). In CX3cr1<sup>+/−</sup>→WT chimeric mice, we found that poly(I:C) administration had no significant effects on the baseline rates of dendritic spine elimination and formation in the motor cortex (Fig. 5b,c). Moreover, motor-learning-induced spine formation and elimination were not affected by the administration of poly(I:C) before the training period (Fig. 5d,e). As a result, there were no changes in total spine number after poly(I:C) administration in the CX3cr1<sup>+/−</sup>→WT chimeric mice (Fig. 5f,g). Furthermore, administration of poly(I:C) in CX3cr1<sup>+/−</sup>→WT chimeric mice had no significant effects on baseline performance in the rotarod test and on performance improvement after training, as compared to that observed after treatment with vehicle (Fig. 5h–k).

Thus, systemic immune challenge impairs dendritic spine plasticity and learning through the involvement of CX3CR1<sup>high</sup> monocytes.

**CX3CR1<sup>high</sup> monocytes contribute to increased TNF-α levels in the cortex**

CX3CR1 signaling in CX3CR1<sup>high</sup> monocytes has been linked to the production of TNF-α and chemokines. To explore the molecular mechanisms that mediate the effects of CX3CR1<sup>high</sup> monocytes on dendritic spine plasticity, we measured the protein levels of pro-inflamatory cytokines at various time points after poly(I:C) injection. In WT mice, poly(I:C) treatment caused a transient increase in cytokine production in the peripheral circulation (Fig. 6a and Supplementary Fig. 10). Plasma levels of TNF-α, interleukin (IL)-6, IL-1β and IFN-β were substantially increased at 3 h after poly(I:C) injection, relative to those in vehicle-injected mice (Fig. 6a and Supplementary Fig. 10). Notably, we found a significant increase in TNF-α levels in the cortex at 3–24 h after poly(I:C) injection (Fig. 6b and Supplementary Fig. 11), whereas there were no changes in IL-6 and IL-1β levels (Supplementary Fig. 10); the amount of IFN-β in the cortex was below that detectable by the ELISA.

To determine whether CX3CR1<sup>high</sup> monocytes are required for the poly(I:C)-induced increase in TNF-α levels in the cortex, we performed ELISA measurements in mice that were depleted of either CNS microglia or peripheral monocytes (Supplementary Fig. 9). We found that there were significantly more amounts of TNF-α in the cortex of mice that were depleted of microglia but that had an intact blood monocyte population at 24 h after poly(I:C) administration than in mice that were treated with vehicle. In contrast, in mice that were depleted of CX3CR1<sup>+</sup> monocytes but that had intact CNS microglia, poly(I:C) treatment had no significant effect on the TNF-α levels in the cortex, relative to the levels observed in the vehicle-treated controls. Together with the finding that mice depleted of CX3CR1<sup>+</sup> monocytes had reduced numbers of CX3CR1<sup>high</sup>Ly6C<sup>low</sup> blood monocytes (Supplementary Fig. 9), these results suggest that CX3CR1<sup>high</sup> monocytes are required for the increase in brain TNF-α levels during systemic immune challenge.

**TNF-α mediates poly(I:C)-induced synaptic and learning deficits**

Previous studies have shown that TNF-α has an important role in synapse plasticity. Given our findings that CX3CR1<sup>high</sup> monocytes are required for poly(I:C)-induced spine remodeling and increased TNF-α levels in the cortex, we hypothesized that the effect of systemic immune challenge on learning and learning-dependent synapse plasticity are mediated by this monocyte subset through TNF-α signaling. In Tnf<sup>−/−</sup> mice (which do not produce TNF-α), we found that poly(I:C) administration had no significant effects on the rates of dendritic spine elimination and formation over 2 d, whereas intravenous administration of exogenous TNF-α significantly increased the rates of spine elimination and formation (Fig. 6c). Furthermore, administration of the dominant-negative form of TNF-α (hereafter referred to as DN-TNF), which binds to soluble TNF-α, abolished poly(I:C)-induced increase in baseline spine turnover in WT mice (Fig. 6d).

Several lines of evidence have suggested that systemic inflammation or increased amounts of TNF-α may change the permeability of...
the blood–brain barrier (BBB)\textsuperscript{30}. To control for the potential effects of BBB disruption on spine remodeling, we administered poly(I:C) to mice that were treated with SB-3CT, which protects the BBB by inhibiting matrix metalloproteinase 9 (MMP9)\textsuperscript{51} (Supplementary Fig. 14), and found that treatment with poly(I:C) substantially increased spine remodeling in SB-3CT-treated mice. Furthermore, there were no changes in the number of CD45\textsuperscript{high}CD11b\textsuperscript{+} cells in the cortex 2 d after poly(I:C) administration (Supplementary Fig. 14). Taken together, these results suggest that the deleterious effects of poly(I:C) on spine remodeling are mediated by increased TNF-α production, rather than due to BBB disruption and subsequent entry of blood monocytes into the cortex.

To investigate whether diminishing TNF-α production by monocytes could protect the mice from synaptic and learning impairments, we generated Tnf\textsuperscript{−/−}→WT chimeras that were unable to produce TNF-α in leukocytes. One month after BM transplantation, we administered poly(I:C) to these mice and examined baseline spine turnover, motor-learning-dependent spine plasticity, as well as performance improvement after motor training (Fig. 6e).

We found that poly(I:C) treatment had no significant effects on the rates of spine elimination and formation under both baseline (Fig. 6f) and motor-learning conditions (Fig. 6g,h and Supplementary Fig. 15) in Tnf\textsuperscript{−/−}→WT mice. Furthermore, mice that were deficient for TNF-α production by leukocytes did not show motor learning deficits after poly(I:C) administration (Fig. 6i,j). As a control, we administered poly(I:C) to Il6\textsuperscript{−/−}→WT chimeras, in which IL-6 production by leukocytes was abolished, and examined spine plasticity in the cortex. We found a marked increase of spine elimination and formation 2 d after poly(I:C)-treated mice as compared to that in vehicle-treated controls (Supplementary Fig. 16). The relative increase in spine turnover after poly(I:C) administration was comparable between Il6\textsuperscript{−/−}→WT chimeras and WT mice, suggesting that increased amounts of IL-6 in the serum are not required for synaptic alteration in the cortex.

**DISCUSSION**

Despite the compelling evidence for an association between immune activation and cognitive disorders\textsuperscript{4–7}, the underlying mechanisms remain elusive. Here we identified an important role of CX3CR1\textsuperscript{+} monocytes and TNF-α in the modulation of learning-dependent synapse structural plasticity during peripheral immune activation (Supplementary Fig. 17). Specifically, we showed that the initiation of peripheral innate immunity with a viral mimetic increased postsynaptic dendritic spine turnover in the mouse motor cortex and decreased the dendritic spine formation associated with motor learning. These synaptic changes were prevented by the in vivo ablation of peripheral monocytes but not by depletion of brain-resident microglial cells. By using BM chimeric mice and transgenic mice, we further showed that both synaptic and learning impairments require CX3CR1\textsuperscript{high} blood monocytes and TNF-α production.
Because most dendritic spines in the adult cortex persist throughout life\textsuperscript{28,29}, the loss of dendritic spines after immune challenge is likely detrimental to the functions of synaptic networks. Previous studies have shown that motor-learning-dependent synapse formation strongly correlates with improvements in performance after learning\textsuperscript{29,32}. Consistent with these studies, we observed a marked decrease in performance improvement after a period of rotarod learning in mice with impaired spine formation. Additionally, we observed that peripheral immune activation impaired the mouse's short- and long-term memory in NOR tasks. These results in mice resonate with clinical observations showing that patients with acute or chronic infectious diseases often have weaker performance on motor skills and experience memory decline relative to that in uninfected individuals\textsuperscript{8–13}. Furthermore, because immune activation is involved in many neuropsychiatric and neurological diseases\textsuperscript{5–7}, the synaptic abnormalities observed in our studies may also occur under these medical conditions and contribute to cognitive deficits.

Our findings underscore the importance of CX3CR\textsuperscript{1high} monocytes for synaptic deficits and learning impairment during peripheral immune activation. This was demonstrated by a series of in vivo cell-depletion experiments using \textit{Cx3cr1\textsuperscript{CreER/++;R26DTR/+}} mice. First, immune-challenge-induced synaptic alteration could be prevented by the depletion of the entire CX3CR\textsuperscript{1} population or by the ablation of CX3CR\textsuperscript{1high} blood monocytes, but not by the removal of CNS microglia. Second, adoptive transfer of CD11b\textsuperscript{+} cells, which include monocytes, reconstituted the phenotype in \textit{Cx3cr1\textsuperscript{CreER/++;R26DTR/+}} mice that were depleted of the entire CX3CR\textsuperscript{1} population. Third, consistent with the finding that CX3CR1-deficient mice have a reduced number of Gr1\textsuperscript{low} (CX3CR1\textsuperscript{high}) monocytes\textsuperscript{45}, mice with a CX3CR1 deficiency in peripheral leukocytes did not show synaptic and learning deficits during immune activation, further suggesting that CX3CR\textsuperscript{1high} monocytes are essential for peripheral-immune-challenge-induced cortical changes.

CX3CR1 is also expressed by human monocytes\textsuperscript{44}, in which CX3CR1\textsuperscript{low} cells correspond to classical CD14\textsuperscript{+}CD16\textsuperscript{−} monocytes, whereas nonclassical human CD14\textsuperscript{dimCD16\textsuperscript{+}} monocytes resemble mouse CX3CR\textsuperscript{1high}Ly6C\textsuperscript{low} blood-resident monocytes\textsuperscript{52}. It has been shown that CD14\textsuperscript{low} human monocytes patrol blood vessels in steady state and produce high amounts of TNF-\textgreek{a} and IL-1\textgreek{b} in response to infection by the measles virus or herpes simplex virus (HSV)-1 (ref. 52). In patients with an HIV infection, the CD16\textsuperscript{+} monocyte subset preferentially harbors the virus\textsuperscript{53}, and activation of this subset is associated with neurocognitive impairment after viral infection\textsuperscript{54,55}. Future studies of this monocyte population in humans will help explain their contribution to the cognitive decline associated with viral infection that is observed.

Our study links the activation of CX3CR\textsuperscript{1high} monocytes to synaptic and learning deficits via TNF-\textgreek{a}-dependent mechanisms. TNF-\textgreek{a} can be produced by peripheral monocytes and endothelial cells, as well as by glia and neurons in the brain. Our studies strongly suggest that monocyte-dependent TNF-\textgreek{a} production is a causative event in the initiation of dendritic spine alterations in the cortex (Supplementary Note 1). TNF-\textgreek{a} may affect synaptic plasticity directly through neurally expressed TNF receptor 1 (TNFR1)\textsuperscript{47–49} and/or indirectly through astrocyte–neuron signaling\textsuperscript{56} (Supplementary Note 2). Future use of genetic approaches to manipulate TNF signaling in specific cell types is needed to better understand how TNF-\textgreek{a} alters dendritic spine remodeling.

In summary, our present findings provide an important mechanism by which innate immune responses modulate the synapse structural plasticity associated with learning, which is likely relevant to cognitive disturbances that are associated with systemic infectious states, such as HIV-associated neurocognitive disorders, viral sepsis and meningitis. Targeting the human correlates of mouse CX3CR\textsuperscript{1high}Ly6C\textsuperscript{low} monocytes and preventing the production of TNF-\textgreek{a} and synaptic deficits may be a potential therapeutic strategy to prevent cognitive disturbances in these medical conditions.

**METHODS**

Methods, including statements of data availability and any associated access codes and references, are available in the online version of the paper.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

**ACKNOWLEDGMENTS**

We thank W.B. Gan (New York University) for the Cx3cr1\textsuperscript{CreER/+;iDTR/+} mice and D.R. Lee (New York University) for the Cx3cr1\textsuperscript{CreER/+;iDTR/-} mice. We also thank W.B. Gan and M.V. Bennett for critical reading of the manuscript. This work was supported by a Whittemore Foundation Research Grant (G.Y.), the US National Institutes of Health grants R01GM107469 (G.Y.) and R21A048410 (G.Y.) and a National Council for Scientific and Technological Development (CNPq) (Brazil) fellowship (H.M.S.).

**AUTHOR CONTRIBUTIONS**

J.M.G., H.M.S., J.L.L. and G.Y. designed the experiments; J.M.G. performed the in vivo imaging experiments, animal behavior assays and biochemical experiments; J.M.G. and H.M.S. performed the flow cytometry experiments; all authors contributed to the data analysis and interpretation; and J.M.G. and G.Y. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Yirmiya, R. & Goshen, I. Immune modulation of learning, memory, neural plasticity and neurogenesis. Brain Behav. Immun. 25, 181–213 (2011).
2. Ransohoff, R.M. & Engelhardt, B. The anatomical and cellular basis of innate immune surveillance in the central nervous system. Nat. Rev. Immunol. 12, 623–635 (2012).
3. Marin, I. & Kipnis, J. Learning and memory ... and the immune system. Learn. Mem. 20, 601–606 (2013).
4. Hodes, G.E., Kana, V., Menard, C., Merad, M. & Russo, S.J. Neuroimmune mechanisms of depression. Nat. Neurosci. 18, 1386–1393 (2015).
5. Perry, V.H., Cunningham, C. & Holmes, C. Systemic infections and inflammation affect chronic neurodegeneration. Nat. Rev. Immunol. 7, 161–167 (2007).
6. Brown, A.S. & Derkits, E.J. Prenatal infection and schizophrenia: a review of epidemiologic and translational studies. Am. J. Psychiatry 167, 261–280 (2010).
7. Estes, M.L. & McAllister, A.K. Immune mediators in the brain and peripheral tissues in autism spectrum disorder. Nat. Rev. Neurosci. 16, 469–486 (2015).
8. Schmidt, H. et al. Neuropsychological sequelae of bacterial and viral meningitis. Brain 129, 333–345 (2006).
9. Bucks, R.S. et al. Selective effects of upper respiratory tract infection on cognition, mood and emotion processing: a prospective study. Brain Behav. Immun. 22, 399–407 (2008).
10. Thomas, P. et al. Exposure to herpes simplex virus, type 1 and reduced cognitive function. J. Psychiatr. Res. 47, 1680–1685 (2013).
11. Clifford, D.B. & Ances, B.M. HIV-associated neurocognitive disorder. Lancet Infect. Dis. 13, 976–986 (2013).
12. Hilsabeck, R.C., Perry, W. & Hassanein, T.I. Neuropsychological impairment in patients with chronic hepatitis C. Hepatology 35, 440–446 (2002).
13. Sadek, J.R. et al. Persistent neuropsychological impairment associated with West Nile virus infection. J. Clin. Exp. Neuropsychol. 32, 81–87 (2010).
14. Meyer, U. Prenatal poli(y)I:poli(y)C exposure and other developmental immune activation as HIV-associated neurocognitive disorders. Hepatology 43, 276–286 (2006).
15. Majde, J.A. Viral double-stranded RNA, cytokines and the flu. J. Interferon Cytokine Res. 20, 259–272 (2000).
16. Alexopoulos, L., Holt, A.C., Medzhitov, R. & Flavell, R.A. Recognition of double-stranded RNA and activation of NF-\textgreek{B} by Toll-like receptor 3. Nature 413, 732–738 (2001).
17. Konat, G. Cerebral response to peripheral challenge with a viral mimetic. Neurochem. Res. 41, 144–155 (2016).
Analysis of dendritic spine plasticity was performed on L5 pyramidal neurons, Rosa26-stop-DTR mice (stock 007900), which express a conditional DTR-encoding allele in the ROSA locus, Rag1−/− mice (stock 002126), Thy1−/− mice (stock 005540), Il6−/− mice (stock 002650) and B6 CD45.1 mice (stock 002014) were purchased from the Jackson Laboratory. Cx3cr1CreER mice were obtained from the laboratory of Wen-Biao Gan (New York University). Cx3cr1CreERT2 mice were obtained from the laboratory of Dan Littman (New York University). To image dendritic spine plasticity in Cx3cr1CreER;R26DTR+/− and Thy1−/− mice, these mice were crossed to Thy1-YFP-H mice. For systemic immune challenge, mice were administered poly(I:C) (Sigma P1530; by i.p. injection) that was dissolved in DPBS. Mice were group-housed in temperature-controlled rooms on a 12-h light–dark cycle and were randomly assigned to different treatment groups. The group size was determined based on previous studies using the same methodologies. Both male and female mice were used. All mice were maintained at the NYU Skirball Institute specific-pathogen-free animal facility and handled in accordance with the institutional guidelines for animal care and use.

**ONLINE METHODS**

**Mice.** Thy1-YFP-H mice (stock 003782), which express yellow fluorescent protein (YFP) in L5 pyramidal neurons, Rosa26-stop-DTR mice (stock 007900), which express a conditional DTR-encoding allele in the ROSA locus, Rag1−/− mice (stock 002126), Thy1−/− mice (stock 005540), Il6−/− mice (stock 002650) and B6 CD45.1 mice (stock 002014) were purchased from the Jackson Laboratory. Cx3cr1CreER mice were obtained from the laboratory of Wen-Biao Gan (New York University). Cx3cr1CreERT2 mice were obtained from the laboratory of Dan Littman (New York University). To image dendritic spine plasticity in Cx3cr1CreER;R26DTR+/− and Thy1−/− mice, these mice were crossed to Thy1-YFP-H mice. For systemic immune challenge, mice were administered poly(I:C) (Sigma P1530; by i.p. injection) that was dissolved in DPBS. Mice were group-housed in temperature-controlled rooms on a 12-h light–dark cycle and were randomly assigned to different treatment groups. The group size was determined based on previous studies using the same methodologies. Both male and female mice were used. All mice were maintained at the NYU Skirball Institute specific-pathogen-free animal facility and handled in accordance with the institutional guidelines for animal care and use.

**In vivo cell depletion.** For in vivo cell depletion, Cx3cr1CreERT2 mice were used as the nondepleted controls, and Cx3cr1CreER;R26DTR− mice were used for cell depletion. All mice (control and cell-depleted mice) were given both tamoxifen and DT. To induce CreER recombination, mice were administered tamoxifen (0.35 mg per g body weight per day; by oral gavage) that was dissolved in corn oil (Sigma) for three consecutive days. For cell depletion, DT (Sigma) was diluted in DPBS, and 1 μg of toxin was given i.p. to adult mice (14–18 g body weight) for three consecutive days.

**In vivo transcranial two-photon imaging.** Dendritic spines in the cortex were imaged through a thinned-skull window using a two-photon microscope. Briefly, surgical anesthesia was achieved using an intraperitoneal injection of 100 mg/kg ketamine and 15 mg/kg xylazine. A midline scalp incision exposed the skull. The area to be imaged was identified based on stereotaxic coordinates for the primary motor cortex (1 mm lateral from midline, 0.5 mm posterior from bregma). The head was immobilized, and a cranial window was created by thinning a circular area (~200 μm in diameter) of the skull to ~20 μm in thickness by using a high-speed drill and/or a microsurgical blade. After completion of the skull thinning, the animal was placed under a two-photon microscope while still under anesthesia. The two-photon laser was tuned to 920 nm. All experiments were performed using a 1.1–numerical aperture (NA) 60× objective lens immersed in artificial cerebrospinal fluid to obtain high-magnification (66.7 μm × 66.7 μm; 512 × 512 pixels; 0.75-μm step) images for dendritic spine analysis. After imaging, the scalp was sutured with 6-0 silk, and the animal was returned to the home cage until the next viewing.

**Imaging data analysis.** Analysis of dendritic spine plasticity was performed using the NIH ImageJ software, as described previously. Briefly, for each dendritic segment, filopodia were identified as long, thin protrusions without bulbous heads, and the remaining protrusions were classified as spines. Images of the same dendritic segments identified from two views were compared. Spines were considered stable if they were present in both views, eliminated if they were present in the first view but not in the second view, or were considered to be newly formed if they were present in the second view but not in the first view. The elimination and formation rates were measured as the number of spines newly formed if they were present in the second view but not in the first view, divided by the number of spines existent in the first view. The percentage of persistent new spines was measured as the number of new spines that were present in both the second and third views, divided by the number of spines seen in the first view. For the analysis of spine morphology (Supplementary Fig. 1), eliminated or newly formed spines were classified into three categories: mushroom spines (bulbous head; neck length ≥ head diameter), thin spines (bulbous head; neck length > head diameter) and stubby spines (no visible neck).

To analyze microglial morphology, z-projections of confocal-imaging stacks (100 μm × 100 μm × 15 μm) were generated using ImageJ software. The percentage of area covered by microglia was calculated by making a binary image (gray value 0 for background and 255 for GFP-labeled microglia) from the z-projection and counting the number of white pixels within the area. Investigators were blinded to the experimental condition when performing data analysis.

**Behavioral assays.** All behavioral experiments were performed in a double-blind fashion. Rotarod motor training was performed using an EZRod system. Animals were placed on a motorized rod (30 mm in diameter) in the test chamber (44.5 cm × 14.0 cm × 51.0 cm). The rod stayed stationary for 30 s, and then the rotation speed linearly increased from 0 to 80 r.p.m. over the course of 2 min. The latency to fall and rotation speed were recorded when the animal was unable to keep up with the increasing speed. Rotarod training and testing were conducted in three 20-trial sessions per day over two consecutive days. Mice were tested again in two 20-trial sessions after 7 d. Performance was calculated as the average speed achieved by the mice in each day. The first training session started 24 h after vehicle or poly(I:C) injection.

For the NOR test, mice were tested in custom-built opaque Plexiglass boxes (25 cm × 25 cm × 25 cm). Behavior was videotaped using standard webcams, and images were analyzed with Quicktime software (Apple). Mice were handled by the same researcher 2 min/d for three consecutive days and then habituated to the testing environment for 30 min with no objects in the chamber. Twenty-four hours after the habituation, mice were placed in a testing environment, with two identical sample objects (the familiar object) placed in opposite corners, for 10 min. Mice were removed and returned to their home cages. One or 24 h later, mice were returned to the testing environment, where one of the sample objects was replaced by a novel object that differed from the familiar object in shape and texture, for 5 min. The testing session was scored by an experienced observer who was blinded to the experimental condition. Interaction with the familiar or novel object was considered to be the exploration time in which the animal spent with its head and nose oriented toward and within 2 cm of the object. Different types of interactions, such as the animal accidentally touching, sitting or standing on the object, were not considered exploratory activity. The discrimination ratio was calculated as the time spent exploring the novel object divided by the total time spent with both novel and familiar objects.

**Cell isolation and flow cytometry.** Mice were deeply anesthetized, and blood was collected from the retro-orbital vein. Mice were perfused with 25 ml of Ca2+/Mg2+-free DPBS (Sigma). Brain and liver were removed and placed in DPBS with 2% FCS. Single-cell suspensions from blood, liver and cortex were prepared. The liver was mechanically dissociated and filtered through 70-μm cell strainer. Cells were suspended in 40% Percoll layered over 80% Percoll for density centrifugation (2,000 r.p.m., 30 min). Enriched cells were collected from the interphase. The cortex was minced with scissors and incubated with 300 U of collagenase D (Roche, 1108858001) at 37 °C for 30 min. Collagenase was inactivated by adding 12.5 mM EDTA for an additional 5-min incubation at 37 °C. Digested material was then passed through a 70-μm cell strainer, followed by a centrifugation at 2,000 r.p.m. in a continuous 38% Percoll gradient. Cell pellets were resuspended in FACs buffer (PBS containing 0.5% BSA, 1 mM EDTA and 0.05% sodium azide), and nonspecific binding to FC receptors was blocked by incubation with a CD16- and CD32-specific antibody (BioXcell, 2.4G2, 2.5 μg/ml) for 15 min. Cells were washed and stained with antibodies to the following proteins: CD11b (BioLegend, 102126, 1:400), CD45 (BioLegend, 103128, 1:200), CD45.1 (clone A20, 1:200), CD45.2 (clone 104, 1:200), Ly6C (BioLegend, 128011, 1:1000) and a lineage-specific antibody cocktail (LIN: CD19 (BioLegend, 152407, 1:200), CD13 (BioLegend, 100307, 1:200), TCR-β chain (BioLegend, 109207, 1:200)). Flow cytometry was performed on an LSRII (BD) and analyzed with FlowJo v. 8.7 (Treestar).

**Generation of bone marrow chimera.** One-month-old recipient mice (10–12 g) were exposed to 1,200 rad of whole-body γ-irradiation in two sessions of 7.5 min, 3 h apart. Head protection with lead shield was used in separate cohorts of recipient mice during the process of irradiation to exclude the potential artifacts caused by cranial irradiation. Recipient mice were injected with donor BM cells (3 × 106 to 5 × 106 cells) through the retro-orbital venous sinus 1–3 h after the second session of irradiation. Donor BM cells were extracted from the femur of donor mice and passed through a 70-μm cell strainer. The body weights of the mice were monitored every other day. For the first 2 weeks after irradiation,
animals' drinking water was supplemented with 2 mg/ml sulfamethoxazole and 0.4 mg/ml trimethoprim. Four to six weeks after BM reconstitution, most of BM chimeric mice (from all genotypes tested) were healthy (normal body weight, gait and absence of clasping reflex) and exhibited normal motor function and a preference for the novel object in the NOR test. Flow cytometry analysis of blood samples from congenic (CD45.1→CD45.2) chimeras confirmed that 75±10% (n = 2 mice) of circulating leukocytes were of donor origin 4–6 weeks after BM reconstitution.

Previous studies suggest that cranial irradiation may harm the BBB and cause recruitment of BM cells into the CNS parenchyma39,62. To examine the possibility that BM cells may be recruited into the motor cortex of irradiated mice, we generated chimeric mice with Cx3cr1GFP+/BM and examined the mouse cortex using two-photon microscopy. One month after BM transplantation, we did not observe GFP-expressing cells in the parenchyma of the motor cortex, indicating limited parenchymal recruitment of BM cells into the cortex during the time window relevant to the current study. Moreover, we found no significant differences in dendritic spine plasticity and motor performance between head-protected chimeric mice and chimeras without head protection during irradiation (Supplementary Figs. 7 and 15).

Magnetic cell sorting. CD11b+ cells were harvested from the blood and spleen by magnetic cell sorting. Blood was collected from anesthetized mice through the retro-orbital vein using heparinized capillary tubes and then diluted in DPBS (1:1 vol/vol). Leukocytes were isolated by centrifugation in an iso-osmotic polysucrose and diatrizoate solution (lymphocyte separation medium; Corning) at 300g for 30 min at 4 °C. For the isolation of splenocytes, spleens from anesthetized mice were dissected and smashed in magnetic cell sorting (MACS) buffer containing 0.5% BSA and 2 mM EDTA in PBS, pH 7.2, at 4 °C. The cell suspension was passed through a 70-µm cell strainer, centrifuged at 300g for 10 min at 4 °C, resuspended in ACK lysis buffer (Lonza) for 3 min and then washed in MACS buffer. Blood and spleen leukocytes were resuspended in 400 µl of MACS buffer and incubated with 40 µl of CD11b Microbeads (Miltenyi Biotec) for 15 min at 4 °C. Magnetic separation was performed in appropriate MACS separators. Cell viability was evaluated by a trypan blue exclusion assay, in which cells were incubated in 0.4% trypan blue in isotonic salt solution and evaluated in a hemocytometer. Sorted CD11b+ cells were transferred to the recipient mice through the retro-orbital vein.

Immunohistochemistry. Mice were deeply anesthetized and perfused with 20 ml Ca2+/Mg2+-free DPBS. The brain was removed and fixed overnight in 4% paraformaldehyde at 4 °C. The tissue was rinsed three times with PBS and sectioned at 200 µm with a Leica vibratome (VT 1000 S). Sections were permeabilized in 1% Triton X-100 in PBS for at least 3 h and blocked with a solution containing 0.1% Triton X-100 and 5% normal goat serum for 1 h. Sections were incubated overnight with primary antibodies: rabbit anti-Iba1 (Wako 019-19741, 1:300), chicken anti-αGFAP (Millipore AB5541, 1:200), mouse anti-NeuN (Millipore MAB377, 1:200), or rat anti-TNF-α (Biologend 506302, 1:50). Sections were then washed three times with 0.05% Tween-20 in PBS and then incubated for 1 h with the appropriate secondary antibodies: Alexa-Fluor-405-conjugated goat anti-rat IgG (Abcam 175671), Alexa-Fluor-647-conjugated goat anti-rabbit-IgG (Invitrogen A32733) or goat anti-mouse-IgG (Invitrogen A21236). Sections were washed as described before, incubated with or without DAPI (1 ng/ml) for 10 min, washed and mounted in MoWiol 4-88/glycerol for imaging. For the double staining of TNF-α and the neuronal marker NeuN, sections were pre-treated with sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 5.8) and heated at 100 °C for 3 min before proceeding with the procedure described above. Confocal images were obtained on a Zeiss 700 confocal microscope.

Enzyme-linked immunosorbent assays (ELISAs). Whole blood was collected by ventricular puncture while mice were under deep anesthesia. Blood samples were centrifuged at 1,400g for 10 min to generate plasma fractions. Brain samples were prepared in separate experiments, in which mice were perfused with 25 ml of Ca2+/Mg2+-free DPBS to remove any remaining blood. CNS tissues were harvested and disrupted by sonication in NP-40 lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% Triton X-100 and 0.1% SDS) containing protease inhibitors (Thermo Scientific). The homogenized material was centrifuged at 20,000g for 15 min, and the cleared supernatant was collected for analysis. Total protein levels in CNS homogenates were determined by the BCA assay (Pierce). TNF-α, IL-6 and IL-1β protein levels were measured using the Mouse Platinum ELISA kit (eBioscience), and IFN-β levels were measured using the Mouse IFN-β ELISA kit (Life Technologies). Samples were processed according to the manufacturer's instructions.

Statistical analyses. Prism software (GraphPad 6.0, La Jolla, CA) was used to conduct the statistical analysis. Summary data are presented as mean ± s.e.m. Tests for differences between two populations were performed using the Mann–Whitney test or Wilcoxon test. Multiple-group comparison was performed with nonparametric Kruskal–Wallis test or one-way ANOVA followed by a Bonferroni’s post hoc test as specified in the figure legends. No data points were excluded from the statistical analysis, and variance was similar between the groups being statistically compared. Significance was set at P < 0.05.

Data availability. All data used in this study are available upon request.

57. Feng, G. et al. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron 28, 41–51 (2000).
58. Buch, T. et al. A Cre-inducible diphtheria toxin receptor mediates cell-lineage ablation after toxin administration. Nat. Methods 2, 419–426 (2005).
59. Mombaerts, P. et al. RAG-1-deficient mice have no mature B and T lymphocytes. Cell 68, 869–877 (1992).
60. Pasparakis, M., Alexopoulou, L., Episkopou, V. & Kollias, G. Immune and inflammatory responses in TNF-α-deficient mice: a critical requirement for TNF-α in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. J. Exp. Med. 184, 1397–1411 (1996).
61. Garré, J.M., Yang, G., Bukauskas, F.F. & Bennett, M.V. FGF-1 triggers pannexin-1 hemichannel opening in spinal astrocytes of rodents and promotes inflammatory responses in TNF-α. J. Neurosci. 36, 4785–4801 (2016).
62. Mildner, A. et al. Microglia in the adult brain arise from Ly-6ChCCR2+ monocytes only under defined host conditions. Nat. Neurosci. 10, 1544–1553 (2007).