A neuroprotective role for microglia in prion diseases

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Microglial activation is a hallmark of most neurodegenerative disorders, and is particularly conspicuous in prion diseases. However, the role of microglia, which function as both primary immune effector cells and professional phagocytes in the central nervous system, remains contentious in the context of neurodegeneration. Here, we evaluated the effect of microglial depletion/deficiency on prion pathogenesis. We found that ganciclovir-mediated microglial ablation on tga20/CD11b-thyminidine kinase of Herpes simplex virus (HSVTK) cerebellar organotypic cultured slices markedly aggravated prion-induced neurotoxicity. A similar deterioration of disease was recapitulated in vivo microglial depletion in prion-infected tga20/CD11b-HSVTK mice. Additionally, deficiency of microglia in interleukin 34 knockout (IL34−/−) mice again resulted in significantly augmented proteinase K–resistant prion protein deposition and accelerated prion disease progression. These results provide unambiguous evidence for a general protective role of microglia in prion pathogenesis.

Microglia are the primary immune cells in the CNS, arising from c-kit+ erythromyeloid precursors in the yolk sac, and then migrating to the developing neural tube to mature (Alliot et al., 1999; Ginhoux et al., 2010; Schulz et al., 2012; Kiendorf et al., 2013). In the brain, microglia interact with neurons and exert a wide variety of physiological functions (Chen et al., 2010; Paolicelli et al., 2011; Rogers et al., 2011; Derecki et al., 2012; Schafer et al., 2012; Parkhurst et al., 2013; Zhan et al., 2014). As an integral part of the innate immune system, microglia constantly patrol the brain parenchyma and subsequently clear pathogens, apoptotic cells, and cellular debris by phagocytosis under physiological and pathological conditions (Davalos et al., 2005; Nimmerjahn et al., 2005; Mildner et al., 2011). However, microglia can be activated by various triggering factors, such as injury, infection, or neurodegeneration, leading to morphological and molecular changes and release of cytokines (Aguzzi et al., 2013a). Depending on the type of molecular stimuli and cues received from the surrounding microenvironment, microglia can take on many different activation profiles and are thought to exert both beneficial and detrimental effects (Hanisch and Kettenmann, 2007).

Prion diseases are progressive neurodegenerative disorders associated with striking microglial activation (Aguzzi et al., 2013b). In animal models of prion disease, microglial activation and cytokine release occur at an early stage of the disease and are found in areas of proteinase K (PK)–resistant prion protein (PrPSc) deposition, but precede spongiosis and neuronal loss (Williams et al., 1997; Giese et al., 1998). As in other protein misfolding diseases, microglia are found surrounding PrPSc plaques and intracellular PrPSc can be detected in microglia (Jeffrey et al., 1994; Baker et al., 2002). The presence of high prion infectivity in microglia suggests that microglia may disseminate prions within the CNS (Baker et al., 2002). In addition, the cytokines produced by activated microglia may contribute to progression of the disease.

In contrast, microglia are also professional phagocytes in the brain and can engulf and degrade PrPSc. Indeed, microglial depletion from CD11b-thyminidine kinase of Herpes simplex virus (HSVTK) cerebellar organotypic cultured slices (COCS) infected with RML6 prions (Rocky Mountain Laboratories, scrapie strain passage #6) results in increased deposition of PrPSc and augmented prion titers, indicating that microglia play an important role in the clearance of prions (Falsig and Aguzzi, 2008; Falsig et al., 2008). Nevertheless, because of the dearth of appropriate animal models, the exact role of microglia in prion pathogenesis remains unknown.

In this study, we first assessed the role of microglia in prion pathogenesis using pharmacogenetic microglia depletion ex vivo and in vivo (Heppner et al., 2005). We found that microglia ablation drastically enhanced the neurotoxicity of prions ex vivo and shortened the lifespan of prion-infected mice. We also administered prions to IL-34–ablated (IL34−/−) mice, which have a reduced number of microglia (Greter et al., 2012; Wang et al., 2012). We found enhanced PrPSc deposition and accelerated prion progression compared with WT littermates. These results indicate that microglia play an overall protective rather than deleterious role in prion disease.
RESULTS
Microglial ablation increases the neurotoxicity of prions in the ex vivo CD11b–HSVTK COCS system

Prion pathogenesis is faithfully reproduced in the COCS system (Falsig et al., 2012; Herrmann et al., 2015). Hence, we evaluated the role of microglia in COCS prepared from tga20:CD11b-HSVTK mice (tga20TK+), which were obtained by intercrossing mice overexpressing the cellular prion protein PrPC (Fischer et al., 1996) and mice expressing the HSV TK under the control of the CD11b promoter (Heppner et al., 2005). Administration of ganciclovir (GCV) to tga20TK+ COCS leads to complete microglial ablation after 14 d of treatment (Falsig et al., 2008). Therefore, tga20TK+ COCS were exposed to RML6 or noninfectious brain homogenate (NBH), and GCV-mediated microglial ablation was performed. At 49 d postinoculation (dpi), RML6-infected non–GCV-treated tga20TK+ COCS showed a significant decrease in the density of NeuN+ cerebellar granule neurons (CGN) compared with NBH-treated tga20TK+ COCS (Fig. 1, A and B). However, depletion of microglia (RML6-infected, GCV-treated tga20TK+ COCS) resulted in a dramatic increase in neurotoxicity, leading to subtotal CGN depletion in COCS (Fig. 1, A and B). Therefore, GCV treatment significantly increased neurotoxicity in RML6-infected tga20TK+ cultures compared with RML6-infected, non–GCV-treated tga20TK+ COCS.
The exacerbation of neurotoxicity may be a result of GCV-mediated microglial ablation or of direct toxicity of GCV to COCS. To discriminate between these possibilities, NBH-exposed tga20TK+ COCS were treated with GCV. The viability of NeuN+ CGN was similar to that of NBH-exposed non–GCV-treated tga20TK+ COCS (Fig. 1 B). Additionally, GCV treatment of RML6-infected tga20 (tga20TK+) COCS had no effect on neurotoxicity (Fig. 1 B). These results indicate that microglia are not essential for the long-term survival of COCS, but play a protective role in prion pathogenesis in COCS.

We then asked if phosphorylated GCV released from dying microglia might affect the viability of bystander neurons in the context of prion infection. We co-cultured prion-infected tga20TK− and tga20TK+ slices in the presence of GCV. Cultures were separated by a grease barrier to prevent microglia migration from TK− to TK+ cultures but allowing for the exchange of solutes, including GCV and its metabolites. GCV-treated tga20TK− slices co-cultured with tga20TK+ slices developed pathology similarly to GCV-treated tga20TK− slices cultured separately (Fig. 1 B). Hence, we failed to detect any evidence that dying microglia in tga20TK− slices release soluble neurotoxic factors. Similarly, co-cultured GCV-treated tga20TK+ slices showed an equivalent pathology to GCV-treated tga20TK+ slices cultured separately (Fig. 1 B), suggesting that microglia in tga20TK− slices did not release soluble neuroprotective factors into the medium.

We previously found that microglia-depleted COCS can be reconstituted with peritoneal lavage cells (PLCs), and that this decreases PrPSc accumulation (Falsig et al., 2008). Reconstitution of prion-infected microglia-depleted tga20TK− slices with tga20 PLCs (70,000 cells/COCS) completely repressed the increased toxicity in GCV-treated COCS (Fig. 1 C).

Furthermore, we used magnetic cell sorting (MACS) to purify CD11b+ cells from PLCs. Using this procedure, we achieved a 96.4% purity of the CD11b+ fraction, whereas the unsorted PLCs contained only 60.9% CD11b+ cells. Prion-infected, microglia-depleted GCV-treated tga20TK− cultures were reconstituted with purified CD11b+ cells, and the degrees of reconstitution and cell death were assessed at 49 dpi. Again, reconstitution of RML6-infected tga20 COCS with CD11b+ PLCs restored the neuroprotection that had been abolished by GCV treatment (Fig. 1 D). The variability in NeuN values (Fig. 1, B–D) results from intrinsic variations in primary slice viability.

As previously reported (Falsig et al., 2008), microglial ablation leads to increased accumulation of PrPSc in COCS infected not only by RML6 but also by 22L, whereas non-GCV-treated and GCV-treated tga20TK− COCS show similar accumulation of PrPSc (Fig. 1 E). In addition, prion infection up-regulated the expression of proinflammatory markers, including cytokines (TNF and IL-1β) and chemokines (MCP-1 and RANTES) in COCS at late time points (42 dpi; Falsig et al., 2012). In prion-infected tga20TK+ COCS (42 dpi), TNF, IL1β, RANTES, and MCP-1 were up-regulated (Fig. 1, F–I), but microglial depletion only abolished the expression of TNF and IL-1β in prion-infected COCS (Fig. 1, F and G), whereas up-regulation of RANTES and MCP-1 was accentuated after microglia depletion (Fig. 1, H and I). In tga20TK+ COCS, the up-regulation of TNF, IL-1β, RANTES and MCP-1 upon RML6 infection was not affected by GCV treatment (Fig. 1, J–M). Hence, microglia-depleted tissue accumulates more PrPSc but does not up-regulate TNF and IL-1β. Notwithstanding the significant reduction of proinflammatory cytokines (TNF and IL-1β), microglial ablation still enhanced prion pathogenesis, suggesting that microglia play a protective role in prion pathogenesis in COCS and activated microglia-derived cytokines are not important contributors to prion disease (Aguzzi et al., 2013b).

**Microglial ablation increases the neurotoxicity of prions in the in vivo CD11b-HSVTK system**

Intraventricular administration of GCV to CD11b-HSVTK mice using osmotic minipumps efficiently depletes microglia in vivo (Grathwohl et al., 2009). Because microglia-depleted mice die after ~4 wk of GCV administration, possibly because of intracerebral hemorrhages (Grathwohl et al., 2009), we intercrossed CD11b-HSVTK mice and tga20 mice to obtain a homozygous tga20 transgene (tga20+/+;TK−) and to enable microglial depletion in an accelerated prion disease model (Fischer et al., 1996). We inoculated tga20+/+;TK− and tga20+/+;TK+ mice with RML6 or NBH intracerebrally. At 38 dpi, we implanted osmotic minipumps loaded with GCV (microglia depletion) or PBS (for control) that delivered the compounds into the ventricle. RML6-infected tga20+/+;TK− mice treated with PBS or GCV and tga20+/+;TK+ mice treated with PBS developed terminal scrapie with median incubation times of 56–57 dpi (Fig. 2 A). In contrast, tga20+/+;TK+ mice treated with GCV showed a median incubation time of 50 dpi, with a highly significant acceleration of progression compared with PBS-treated tga20+/+;TK+ mice (7 d; P < 0.0001; Fig. 2 A); under no other conditions were tga20 mice reported to develop scrapie so rapidly. Life span reduction was caused by neither microglia depletion alone nor by the surgical implantation of osmotic pump; PBS-treated tga20+/+;TK+ mice inoculated with NBH survived up to 100 d postimplantation (138 dpi) and GCV-treated tga20+/+;TK+ mice inoculated with NBH died at 24 d postimplantation, whereas RML6-infected GCV-treated tga20+/+;TK+ mice became terminally sick at 12 d postimplantation (Fig. 2 A).

RML6-infected tga20+/+;TK+ mice treated with GCV showed a marked depletion of microglia in the cortex (82% reduction), compared with RML6-infected PBS treated tga20+/+;TK+ or GCV-treated tga20+/+;TK+ mice (Fig. 2, B and C). Noninfected tga20+/+;TK+ mice showed a slightly less efficient microglial depletion (63% reduction), suggesting that prion infection enhanced GCV-mediated microglia depletion (Fig. 2 C). Similarly, microglia depletion was observed in the cerebellum, showing that widespread microglia depletion was efficiently attained (Fig. 2 D). In agreement with previous
studies (Grathwohl et al., 2009), we did not observe aplastic anemia as a result of GCV delivery directly to the brain of HSVTK-expressing mice (Fig. S1).

Accelerated prion pathogenesis in microglia-deficient IL34−/− mice

To further validate the protective role of microglia in prion pathogenesis, IL34−/− mice that contain a reduced number of microglia (Wang et al., 2012), but express a similar amount of PrPSc as WT mice (Fig. 3, A and B), were intracerebrally inoculated with RML6 prions. Mice were monitored for clinical signs of scrapie. The incubation time was defined as the time lag between prion inoculation and the time at which mice were euthanized, which corresponded to the terminal stage of prion disease. We found that both female and male IL34−/− mice showed significantly accelerated prion progression when compared with age- and gender-matched WT littermates (median survival 162 dpi for female IL34−/− vs. 176 dpi for female IL34+/+, P = 0.0025; median survival 170 dpi for male IL34−/− vs. 191 dpi for male IL34+/+, P < 0.0001; Fig. 3 C).

To address whether the accelerated prion progression in IL34−/− mice resulted from the deficiency of microglia and impaired PrPSc clearance, we compared PrPSc levels at a presymptomatic and at a clinically overt stage (105 dpi at ∼5 mo of age and 150 dpi at ∼7.5 mo of age, respectively). Clinically sick mice showed a characteristic scrapie phenotype with waddling gait, mildly reduced grooming, and initial paresis. We observed significantly more PrPSc deposition in IL34−/− brains, suggesting that microglia play a protective role in prion pathogenesis by, at least partially, clearing PrPSc in the brain (Fig. 3, D–G).

Surprisingly, the density of microglia did not differ between IL34−/− and their WT littermates at 105 dpi and 150 dpi (Fig. 4, A–D). Because IL−34−/− mice contain less mi-
This finding suggests that the proliferative stimulus exerted by prion infection can partially compensate for the lack of IL-34. However, in this situation, microglia may be less effective at clearing PrPSc (Hughes et al., 2010; Gómez-Nicola et al., 2013).

Cytokine profiling was also performed and no significant difference was observed between these two groups, indicating that IL-34 is not involved in prion-induced cytokine expression (Fig. 4, E–J). On the contrary, we observed significantly more astrogliosis (GFAP immunoreactivity) in IL34−/− brains (Fig. 4, K–N), probably as a result of enhanced PrPSc accumulation. In terminally sick IL34−/− (~8-mo-old) and WT (~8.5-mo-old) littermates, which showed scrapie symptoms including ataxia, paresis, and reduced activity, no obvious difference in PrPSc deposition (Fig. 5, A and B), microglial activation (Fig. 5, C and D), or astrogliosis was observed (Fig. 5, E–F). In summary, in the microglia deficiency IL34−/− mouse model we confirmed that microglia play an overall protective role in prion pathogenesis.

DISCUSSION

Neuroinflammation is a term that was originally coined to denote lymphocytic and purulent CNS infiltrates that

Figure 3. Microglia-deficient IL34−/− mice demonstrated accelerated prion pathogenesis. (A) qRT-PCR for Prnp mRNA in one hemisphere of IL34−/− and IL34+/+ brains. n = 4. n.s, P > 0.05. (B) PrPSc Western blot for one hemisphere from IL34−/− and IL34+/+ (WT) mice. Median survival of IL34−/− females 162 dpi (n = 17) versus IL34+/+ (WT) females 176 dpi (n = 17). ***, P < 0.01; median survival of IL34−/− males 170 dpi (n = 8) versus IL34+/+ (WT) males 191 dpi (n = 15). ****, P < 0.0001. (C) Survival curves of RML6 infected IL34−/− and IL34+/+ (WT) mice at 105 dpi (D) and 150 dpi (E). Bars, 50 µm. (F and G, left) PrPSc Western blot of homogenates prepared from one brain hemisphere of RML6-infected IL34−/− and IL34+/+ (WT) mice at 105 dpi (F) and 150 dpi (G). Samples were digested with PK as indicated and detected with POM1. (right) Densitometric quantification of the PrPSc Western blot. n = 4 (105 dpi) or 5 (150 dpi) for IL34−/−; n = 4 (105 dpi) or 6 (150 dpi) for IL34+/+. **, P < 0.01; *, P < 0.05. Error bars represent SEM. Survival curves summarize two independent i.c inoculation experiments. Statistical significance in A, F, and G was determined using unpaired Student’s t test. Statistical significance in C and D was determined using Log-rank (Mantel-Cox) test. qRT-PCR, histology, and Western blot results represent at least four independent experiments.
Figure 4. Microglia-deficient IL34−/− mice displayed similar microglial activation, but augmented astrogliosis. (A, left) Iba1 immunohistochemistry of brain tissues from RML6-infected IL34−/− and IL34+/+ (WT) mice at 105 dpi. (right) Quantification of the Iba1 immunostaining. n = 4; n.s, P > 0.05. Bars, 20 µm. (B, left) Iba1 immunohistochemistry of brain tissues from RML6-infected IL34−/− and IL34+/+ (WT) mice at 150 dpi. (right) Quantification of the Iba1 immunostaining. n = 3; n.s, P > 0.05. Bars, 20 µm. (C, left) Iba1 Western blot of one hemisphere from RML6-infected IL34−/− and IL34+/+ (WT) mice at 105 dpi. (right) Densitometric quantification of the Iba1 Western blot. n = 4; n.s, P > 0.05. (D, left) Iba1 Western blot of one hemisphere from RML6-infected IL34−/− and IL34+/+ (WT) mice at 150 dpi. (right) Densitometric quantification of the Iba1 Western blot. n = 5; n.s, P > 0.05. (E–J) qRT-PCR of cytokines from one hemisphere of RML6-infected IL34−/− and IL34+/+ (WT) mice at 105 dpi (E–G) and 150 dpi (H–J). (E and H) TNF mRNA, (F and I) IL-1β mRNA, and (G and J) IL-6 mRNA. Relative expression normalized to GAPDH expression and represented as fold change compared with WT mice ± SEM. n = 4 (105 dpi) or 5 (150 dpi) for IL34−/−; n = 4 (105 dpi) or 6 (150 dpi) for IL34+/+. n.s, P > 0.05. (K, left) GFAP immunohistochemistry of brain tissues from RML6-infected IL34−/− and
accompany infections and autoimmune diseases. More recently, however, the term has been expanded to include microglial activation typically encountered in neurodegenerative diseases that do not have a primary inflammatory pathogenesis, such as Alzheimer's disease, Parkinson's disease, and prion disease (Aguzzi and Falsig, 2012). The contribution of microglial activation to the pathogenesis of these diseases has emerged as a central question and may encompass both beneficial and detrimental aspects. Microglia are immune cells, which respond to various stimuli that trigger the release of proinflammatory factors that could potentially damage the brain. However, microglia are professional phagocytes that engulf and degrade pathogens and apoptotic bodies, and therefore also exert protective and defensive functions in the CNS.

The overall role of microglia in neurodegeneration is likely to depend on environmental cues, many of which remain deciphered, which can turn microglia into either friends or foes in disparate conditions.

Among the neurodegenerative disorders, prion diseases are associated with the most dramatic microglial activation. Although a role for microglia in prion diseases was implicated in a study using prion protein–derived peptide PrP106–126 (Brown et al., 1996), that peptide has never been detected in vivo and is not infectious per se, which raises serious doubts about the validity of those findings. Another study demonstrated that blocking microglial proliferation with a CSF1R inhibitor could slow neuronal damage and disease progression (Gómez-Nicola et al., 2013), suggesting a harmful contribution of microglia to prion disease. However, the overall role of microglia in prion diseases has remained unclear, largely because of the lack of an appropriate mouse model.

Here, we reasoned that the aforementioned question might be best studied by using several model systems that modify microglial functions in independent ways. Because organotypic CD11b-HSVTK COCS allow for the most radical—yet exquisitely specific—ablation of microglia, we opted to investigate prion pathogenesis in this system. We previously reported that microglial ablation from short-term COCS unleashes prion replication (Falsig et al., 2008), but at that time we had not yet discovered that prion infection leads to neurodegeneration in long-term COCS. Consequently, we had been unable to investigate whether microglia ablation would affect neurodegeneration, either positively or negatively. The improved COCS culture system used here allowed us to address the latter question. Not only did we confirm our previous study that depletion of microglia in COCS leads to enhanced PrPSc deposition, but we also detected a dramatic acceleration in prion-induced neurodegeneration of the cerebellar granule layer. Notably, microglia ablation does not affect antiprion antibody-induced neurotoxicity (Sonati et al., 2013), suggesting that microglia exert neuroprotection by reducing the prion load rather than by acting on downstream events shared by prion infections and antiprion antibody treatment (Herrmann et al., 2015).

The notion that microglia protects the brain against prion-induced neurotoxicity was confirmed in vivo using CD11b-HSVTK transgenic mice. This model results in drastic microglia reduction, yet it suffers from limitations in the observational time span. Because the CD11b transgene is ectopically expressed in hematopoietic stem cells, administration of GCV to CD11b-HSVTK transgenic mice leads to aplastic anemia and hemorrhagic diathesis. This issue can be alleviated (but not eliminated) by administering smaller doses of GCV intracerebrally through osmotic minipumps, thereby minimizing systemic exposure to the drug. These limitations forced us to devise a particularly rapid model of prion disease, i.e., the inoculation of mice carrying homozygous arrays of the tga20 transgene. Although this experimental design exaggerates certain aspects of prion pathology, the salient characteristics of the disease (including spongiform encephalopathy, astroglisis, and PrPSc deposition) are faithfully reproduced, and we found that microglial depletion has a major deleterious effect on prion-induced neurodegeneration.

We then wondered whether the conclusions drawn from the aforementioned studies would be verifiable in a system in which microglia are compromised. Toward that goal, we used IL34−/− mice that suffer from impaired microglial development or maintenance and reduced microglia numbers (Greter et al., 2012; Wang et al., 2012). We again observed microglial deficiency resulting in accelerated prion progression and enhanced PrPSc deposition. All of these results suggest that microglia play an overall protective role in prion pathogenesis.

Interestingly, although microglia numbers were reduced in IL34−/− mice, upon prion infection microglia reached numbers similar to WT littermates. We conclude that IL-34 is not required for prion-induced microglial activation and proliferation. The increased deposition of PrPSc and accelerated prion disease in IL34−/− brains may therefore be caused by the scarcity of microglia at early stages of prion infection, or alternatively, to poorly understood defects in the effector functions of microglia.

IL34−/− mice exhibited more astrogliosis than WT mice at 105 and 150 dpi, probably as a consequence of enhanced PrPSc deposition. Alternatively, astrogliosis may conceivably...
influence PrPSc deposition directly in ways that were not investigated here. We favor the former interpretation because terminally sick IL-34−/− mice and WT littermates displayed similar levels of PrPSc and a similar extent of astrogliosis.

The reduction in microglia in the IL−34−− system was less pronounced than in GCV-treated tga20/CD11b-HSV TK mice, and microglia numbers were not significantly different between prion-infected IL−34+/− and IL−34−− mice at 105 and 150 dpi. This unexpected finding may be a result of conspicuous microglia activation (and possibly proliferation) after prion infection. Nevertheless, exacerbation of prion progression was found in both models, thereby providing orthogonal lines of evidence for the protective role of microglia.

The results reported here indicate that microglia, far from mediating prion pathogenesis, are valiant defenders of the brain against prions. What might be the mechanisms by which microglia fight prion infections? The aforementioned results in concert with several previous studies converge to...
organotypic slices culture preparation and microglial depletion. Cerebellar organotypic slices were prepared from 9–11-d-old pups and maintained according to previously published protocols (Falsig and Aguzzi, 2008). GCV-mediated (val-ganciclovir; Roche) microglia depletion experiments were performed on brain slices prepared from CD11b-HSVTK mice intercrossed with tga20 mice (tga20TK+). Cultures were inoculated with 100 µg brain homogenate per 10 slices from terminally sick prion-infected (RML6 and 22L) or NBH from CD1 mice, diluted in 2 ml physiological Grey’s balanced salt solution (GBSS; 8 g l–1 NaCl, 0.37 g l–1 KCl, 0.12 g l–1 Na2HPO4, 0.22 g l–1 CaCl2, 2H2O, 0.09 g l–1 KH2PO4, 0.07 g l–1 MgSO4, 7H2O, 0.210 g l–1 MgCl2 6H2O, and 0.227 g l–1 NaHCO3) supplemented with the glutamate receptor antagonist kynurenic acid (1 mM; GBSSK). Slices were incubated with infectious brain homogenates as free-floating sections for 1 h at 4°C. Slices were washed twice in 6 ml GBSSK and 5–10 slices were placed on a 6-well Millicell-CM Biopore PTFE membrane insert (EMD Millipore). Residual buffer was removed and the inserts were transferred to a cell culture plate and cultured in slice culture medium (50% [vol/vol] MEM, 25% [vol/vol] basal medium Eagle, and 25% [vol/vol] horse serum supplemented with 0.65% [wt/vol] glucose, penicillin/streptomycin, and glutamax; Invitrogen). Cultures were kept in a standard cell incubator (37°C, 5% CO2, 95% humidity), and the culture medium was exchanged three times weekly. For conditional microglia depletion, GCV (5 µg ml–1) was added from 0–21 dpi reading GCV at each medium change. PLCs were obtained from 3–4-mo-old mice by peritoneal lavage using ice-cold PBS. 700,000 PLCs (counting cells with a large cell body) were added to each insert containing 10 freshly plated preinfected brain slices (70,000 PLCs/COCs). Alternatively, PLCs were sorted by MACS according to the manufacturer’s (Miltenyi Biotec) protocol using antibodies raised against CD11b and CD19. Macrophages (CD19+, CD11b+) were added at 700,000 cells/insert. The purity of the sorted cell populations and the total PLCs were analyzed by flow cytometry analysis according to standard protocols using antibodies against CD3, CD19, and CD11b. Slices were harvested and analyzed for mRNA, protein or fixed for immunohistochemistry at various time points.

Intracerebral prion inoculation. Mice were intracerebrally inoculated with 30 µl of brain homogenate diluted in PBS with 5% BSA and containing 3 × 5 log LD50 U of RML6. Scrapie was diagnosed according to clinical criteria (ataxia, kyphosis, priapism, and hind leg paresis). Mice were sacrificed on the day of onset of terminal clinical signs of scrapie.
**Osmotic pump implantation.** *Tga20+/+;TK−* and *tga20+/+;TK−* mice were intracerebrally inoculated with RML6 (3 × 5 log LD50, U, 30 µl). At 38 dpi, mice were anesthetized using iso-flurane and an intraventricular catheter was implanted in the right lateral ventricle. The primed pump loaded with GCV (50 mg ml−1) or PBS was placed subcutaneously and connected to the catheter. Mice received pre- and postoperative care by injection of opioids (Temgesic; 2 µg g−1 bodyweight), NSAIDs (Finazyne; 5 µg g−1 bodyweight), and glucosocotic solution and were treated with antibiotics (Borgal; 0.1% in drinking water). Mice were kept for 2 d on a 37°C heating pad to improve surgical recovery. At terminal disease, mice were sacrificed by CO2 inhalation, and brain, spinal cord, and cardiac blood were immediately collected. To monitor possible hematological changes, blood was analyzed by manually counting total RBCs and WBCs after lysis of RBCs. Hematocrit (the ratio of the packed blood volume over the total volume) was determined by spinning blood in heparin tubes, and then the hemoglobin concentration was determined using Drabkin’s reagent (Drabkin and Austin, 1935).

**Quantitative real-time PCR (qRT-PCR).** Total RNA from brain or cultured slices was extracted using TRIzol (Invitrogen) according to the manufacturer’s instruction. The quality of RNA was analyzed by Bioanalyzer 2100 (Agilent Technologies). RNAs with RNA integrity number >6 were used for cDNA synthesis. cDNA were synthesized from ∼1 µg total RNA using QuantiTect Reverse Transcription kit (QIAGEN) according to the manufacturer's instruction. qRT-PCR was performed using the SYBR Green PCR Master Mix (Roche) on a ViiA7 Real-Time PCR system (Applied Biosystems).

The following primer pairs were used: GAPDH sense 5′-CCACCCCAAGGAGACCT-3′; antisense, 5′-GAAATTGTGAGGAATGCT-3′; TNF sense, 5′-CATTCTCTCTCAAATGCAGTGAAC-3′; antisense, 5′-TGGGAGTAGACAAAGTTACAACCC-3′. IL-1β sense, 5′-CAACCAAGTGATTCTCCATG-3′; antisense, 5′-GATCCACACTCTCCAGCTGCA-3′. RANTES sense, 5′-ATGCCCGATTCTCCACAGGACC-3′; antisense, 5′-TTTTCCCTACCTCTCCACTG-3′. MCP-1 sense, 5′-TTAGCTTCTGAATTTACGGGT-3′. IL-6 sense, 5′-TTACAAAACCTGAGTCCGAAACCA-3′; antisense, 5′-GCAATTGGCTTACATTACGGGT-3′. Expression levels were normalized using GAPDH.

**Western blot analysis.** To detect PrPSc in COCS, cultures were washed twice in PBS and the tissue was scraped off the membrane using 10–15 µl PBS per slice and homogenized by trituration using a 30-G syringe, followed by 2 × 30-s pulses in an ultrasound bath. Protein concentration was determined using the bicinchoninic acid assay (Thermo Fisher Scientific). Samples containing the same amount of total protein were digested with PK (20 µg protein in 20 µl, digested with 25 µg ml−1 PK) in digestion buffer (0.5% wt/vol sodium deoxycho-
late and 0.5% vol/vol Nonidet P-40 in PBS) for 30 min at 37°C. PK digestion was stopped by adding loading buffer (NuPAGE; Invitrogen) and boiling the samples at 95°C for 5 min. Proteins were separated on a 12% Bis–Tris polyacrylamide gel (NuPAGE; Invitrogen) and blotted onto a nitrocellulose membrane. Membranes were blocked with 5% wt/vol Topblock (LuBioScience) in Tris-buffered saline supplemented with Tween (150 mM NaCl, 10 mM Tris HCl, and 0.05% vol/vol Tween 20) and incubated with POM1 mouse IgG1 antibody to PrPSc (anti-PrPSc; 200 ng ml−1) in 1% Topblock. Secondary antibodies used was horseradish peroxidase (HRP)–conjugated rabbit anti–mouse IgG1 (1:10,000; Zymed) and the blots were developed using SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific) using the LAS-3000 system (FujiFilm). To detect PrPSc in the *IL34+/−* and *IL34−/−* (WT) brains, one hemisphere from each brain was homogenized with buffer PBS containing 0.5% Nonidet P-40 and 0.5% CHAPS/SDSO. Total protein concentration was determined using the bicinchoninic acid assay (Thermo Fisher Scientific). Approximately 8 µg proteins were loaded and separated on a 12% Bis–Tris polyacrylamide gel (NuPAGE; Invitrogen) and then blotted onto a nitrocellulose membrane. Membranes were blocked with 5% wt/vol Topblock (LuBioScience) in PBS supplemented with 0.05% Tween 20 (vol/vol) and incubated with primary antibodies POM1 in 1% Topblock (200 ng ml−1) overnight. After washing, the membranes were incubated with secondary antibody HRP-conjugated rabbit anti–mouse IgG1 (1:10,000; Zymed). Blots were developed using Luminata Crescendo Western HRP substrate (EMD Millipore) and visualized using the Stella system (model 3200; Raytest). To avoid variation in loading, the same blots were stripped and incubated with an anti-actin antibody (1:10,000; EMD Millipore). The PrPSc signals were normalized to actin as a loading control. To detect PrPSc in prion infected *IL34+/−* and *IL34−/−* (WT) brains, prion-infected forebrains were homogenized in sterile 0.32 M sucrose in PBS. Total protein concentration was determined using the bicinchoninic acid assay (Thermo Fisher Scientific). Samples were adjusted to 20 µg protein in 20 µl and digested with 25 µg ml−1 PK in digestion buffer (PBS containing 0.5% [wt/vol] sodium deoxycholate and 0.5% [vol/vol] Nonidet P-40) for 30 min at 37°C. PK digestion was stopped by adding loading buffer (Invitrogen) and boiling samples at 95°C for 5 min. Proteins were then separated on a 12% Bis–Tris polyacrylamide gel (NuPAGE; Invitrogen) and blotted onto a nitrocellulose membrane. POM1 and HRP-conjugated rabbit anti–mouse IgG1 were used as primary and secondary antibodies, respectively. Blots were developed using Luminata Crescendo Western HRP substrate (Millipore) and visualized using the LAS–3000 system (FujiFilm). To detect Iba-1 and GFAP in prion-infected brains by Western blot, 20 µg of total brain protein were loaded and anti–Iba-1 antibody (1:1,000; Wako) and anti–GFAP antibody (D1F4Q) XP Rabbit mAb (1:3,000; Cell Signaling Technology) were used. Actin was used as loading control.
Immunohistochemistry. For immunohistochemistry of COCS, the organotypic slices were fixed in 4% wt/vol PFA overnight at 4°C. Membrane inserts were washed and incubated for 1 h in blocking buffer (0.05% [vol/vol] Triton X-100 and 3% vol/vol goat serum dissolved in PBS), and then incubated for 3 d at 4°C with primary antibodies diluted in blocking buffer. Slices were stained with mouse anti-NeuN, NeuN conjugated secondary antibody (3 µg ml−1; Molecular Probes), and then counterstained with DAPI (1 µg ml−1). For quantification of NeuN immunoreactivity, images were recorded using a 4× lens on an Olympus BX61 fluorescence microscope equipped with a cooled black and white CCD camera. For quantification purposes all pictures were acquired at identical exposure times and the area of immunoreactivity was determined with image analysis software analySIS version 5.0, using identical grayscale threshold settings for identifying positive pixels. For quantification of microglia density, a 63× oil lens was used to record micrographs on a Leica SP5 confocal laser-scanning microscope.

For immunohistochemistry of prion-infected brains, formalin-fixed tissues were treated with concentrated formic acid for 60 min to inactivate prion infectivity, and then embedded in paraffin. Paraffin sections (2 µm) of brains were stained with hematoxylin and eosin (H&E). After deparaffinization through graded alcohols, antibodies GFAP (1:300; DAKO) for astrocytes were applied and visualized using standard methods, Iba-1 (1:1,000; Wako) was used for highlighting activated microglial cells. Stainings were visualized using an IVIEW DAB Detection kit (Ventana), with a hematoxylin counterstain applied subsequently. For the histological detection of partially PK-resistant prion protein deposition, deparaffinized sections were pretreated with formaldehyde for 30 min and with 98% formic acid for 6 min, and then washed in distilled water for 30 min. Sections were incubated in Ventana buffer and stains were performed on a NEXEX immunohistochemistry robot (Ventana) using an IVIEW DAB Detection kit (Ventana). After incubation with protease 1 (Ventana) for 16 min, sections were incubated with anti-PrP SAF-84 (SPI bio; A03208; 1:200) for 32 min. Sections were counterstained with hematoxylin. Sections were imaged using a Zeiss Axioptot light microscope. Quantification of Iba-1 and GFAP staining was performed on acquired images, where regions of interest were drawn, and the percentage of brown Iba-1 and GFAP staining over the total area was quantified using in-house–developed software. The operator was blind to the genotype and treatment of the analyzed tissues.

Scoring of microglia depletion. To score microglia depletion in prion-infected CD11b-HSVTK mice, hemibrains were fixed in formalin and thin sagittal brain sections were stained for Iba-1 (Wako). Brain sections were scanned at 40× magnification using a Hamamatsu tissue scanner (Nanozoomer), then microglia were counted automatically using the Cavalieri estimator method with a counting grid size of 34.5 × 34.5 µm as described elsewhere (Long et al., 1998). Staining artifacts were manually excluded and ambiguous features were counted both as a positive and a negative event. Representative areas in the superior frontal cortex and central cerebellar folia were counted in two 50-µm step sections for each mouse and normalized to the result for the tga20+/+;TK+ RML6 group.

Statistical analysis. Results are presented as the mean of replicas ± SEM. We used one-way ANOVA with Tukey’s post-test for multicomparison comparison for comparison of all columns to a control column for statistical analysis of experiments involving the comparison of three or more samples. Unpaired Student’s t test was used for comparing two samples. For in vivo experiments, all groups were compared by Log-rank (Mantel-Cox) test. P-values <0.05 were considered statistically significant.

Online supplemental material. Fig. S1 describes hematological analyses of peripheral blood in PBS or GCV-treated tga20+/CD11b-HSVTK mice inoculated with RML or NBH. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.201510000/DC1.

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REFERENCES

Aguzzi, A., and J. Fabig. 2012. Prion propagation, toxicity and degradation. Nat. Neurosci. 15:936–939. http://dx.doi.org/10.1038/nn.3120
Aguzzi, A., B.A. Barres, and M.L. Bennett. 2013a. Microglia: sapper, saboteur, or something else? Science. 339:156–161. http://dx.doi.org/10.1126/science.1227901
Aguzzi, A., M. Nuvolone, and C. Zhu. 2013b. The immunobiology of prion diseases. Nat. Rev. Immunol. 13:888–902. http://dx.doi.org/10.1038/nri3553
Aguzzi, A., J. Kramich, and N.J. Krautler. 2014. Follicular dendritic cells: origin, phenotype, and function in health and disease. Trends Immunol. 35:105–113. http://dx.doi.org/10.1016/j.it.2013.11.001
Alliot, F., I. Godin, and B. Pesac. 1999. Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. Brain
Hanisch, U.K., and H. Kettenmann. 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat. Neurosci. 10:1387–1394. http://dx.doi.org/10.1038/nn1997

Heppner, F.L., M. Greter, D. Marino, J. Falig, G. Raivich, N. Hövelmeyer, A. Waisman, T. Rülicke, M. Prinz, J. Pirrler, et al. 2005. Experimental autoimmune encephalomyelitis repressed by microglial paralysis. Nat. Med. 11:146–152. http://dx.doi.org/10.1038/nm1177

Herrmann, U.S., T. Sonati, J. Falig, R.R. Reimann, P. Dametto, T. O’Connor, B. Li, A. Lau, S. Hornemann, S. Sorce, et al. 2015. Prion infections and anti-PrP antibodies trigger converging neurotoxic pathways. PLoS Pathog. 11:e1004662. http://dx.doi.org/10.1371/journal.ppat.1004662

Hughes, M.M., R.H. Field, V.H. Perry, C.L. Murray, and C. Cunningham. 2010. Microglia in the degenerating brain are capable of phagocytosis of beads and of apoptotic cells, but do not efficiently remove PrPSc, even upon LPS stimulation. Glia. 58:2017–2030. http://dx.doi.org/10.1002/glia.21070

Jeffery, M., C.M. Goodin, M.E. Bruce, P.A. McBride, and C. Farquhar. 1994. Morphogenesis of amyloid plaques in 87V murine scrapie. Neutrophop. Appl. Neurol. 20:535–542. http://dx.doi.org/10.1111/j.1365-2990.1994.tb01007.x

Kierdorf, K., D. Erny, T. Goldmann, V. Sander, C. Schulz, E.G. Periquero, P. Wieghefer, A. Heintz, P. Riemke, C. Hölscher, et al. 2013. Microglia emerge from erythromyeloid precursors via Pu.1- and IfN-β-dependent pathways. Nat. Neurosci. 16:273–280. http://dx.doi.org/10.1038/nn.3318

Klein, T.R., D. Kirsch, R. Kaufmann, and D. Riesner. 1998. Prion rods contain small amounts of two host sphingolipids as revealed by thin-layer chromatography and mass spectrometry. Biol. Chem. 379:655–666. http://dx.doi.org/10.1515/bchm.1998.379.6.655

Kranich, N.J., N.J. Krautler, J. Falig, B. Ballmer, S. Li, G. Hutter, P. Schwarz, R. Moos, C. Julius, G. Miele, and A. Aguzzi. 2010. Engagement of cerebral apoptotic bodies controls the course of prion disease in a mouse strain-dependent manner. J. Exp. Med. 207:2271–2281. http://dx.doi.org/10.1084/jem.20092401

Long, J.M., A.N. Kalezha, N.J. Muth, J.M. Hengemihle, M. Jucker, M.E. Callhoun, D.K. Ingram, and P.R. Mouton. 1998. Stereological estimation of total microglia number in mouse hippocampus. J. Neurosci. Methods. 84:101–108. http://dx.doi.org/10.1016/S0165-2478(00)003346-3

McHugh, D., T. O’Connor, J. Bremer, and A. Aguzzi. 2012. ZygFISH: a simple, rapid and reliable zygosity assay for transgenic mice. PLoS One. 7:e37881. http://dx.doi.org/10.1371/journal.pone.0037881

Mildner, A., B. Schlevogt, K. Kierdorf, C. Böttcher, D. Erny, M.P. Kummer, M. Quinn, W. Brück, I. Bechmann, M.T. Heneka, et al. 2011. Distinct and non-redundant roles of microglia and myeloid subsets in mouse models of Alzheimer’s disease. J. Neurosci. 31:11159–11171. http://dx.doi.org/10.1523/JNEUROSCI.2609-10.2011

Nimmerjahn, A., F. Kirchhoff, and F. Helmchen. 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science. 308:1318–1318. http://dx.doi.org/10.1126/science.1110647

Paolicelli, R.C., M. Bolasco, G. Bolasco, F. Pagani, L. Maggi, M. Scianni, P. Panzanelli, M. Giustetto, T.A. Ferreira, E. Guiducci, L. Dumas, et al. 2011. Synaptic pruning by microglia is necessary for normal brain development. Science. 333:1456–1456. http://dx.doi.org/10.1126/science.1202529

Parkhurst, C.N., G. Yang, I. Ninan, J.N. Savas, J.R. Yates III, J.J. Lafilea, B.L. Hempstead, D.R. Littman, and W.B. Gan. 2013. Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. Cell. 155:1596–1609. http://dx.doi.org/10.1016/j.cell.2013.11.030

Rogers, J.T., J.M. Morganti, A.D. Bachettem, C.E. Hudson, M.M. Peters, B.A. Grimmig, E.J. Weeber, P.C. Bickford, and C. Gennari. 2011. CX3CR1 deficiency leads to impairment of hippocampal cognitive function and...
synaptic plasticity. *J. Neurosci.* 31:16241–16250. http://dx.doi.org/10.1523/JNEUROSCI.3667-11.2011

Rouvinski, A., S. Karmely, M. Kounin, S. Moussa, M.D. Goldberg, G. Warburg, R. Lyakhovetsky, D. Papy-Garcia, J. Kutsche, C. Korth, et al. 2014. Live imaging of prions reveals nascent PrPSc in cell-surface, raft-associated amyloid strings and webs. *J. Cell Biol.* 204:423–441. http://dx.doi.org/10.1083/jcb.201308028

Schafer, D.P., E.K. Lehrman, A.G. Kautzman, R. Koyama, A.R. Mardinly, R. Yamasaki, R.M. Ransohoff, M.E. Greenberg, B.A. Barres, and B. Stevens. 2012. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron.* 74:691–705. http://dx.doi.org/10.1016/j.neuron.2012.03.026

Schulz, C., E. Gomez Perdiguero, L. Chorro, H. Szabo-Rogers, N. Cagnard, K. Kierdorf, M. Prinz, B. Wu, S.E. Jacobsen, J.W. Pollard, et al. 2012. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science.* 336:86–90. http://dx.doi.org/10.1126/science.1219179

Sonati, T., R.R. Reimann, J. Falsig, P.K. Baral, T. O’Connor, S. Hornemann, S. Yaganoglu, B. Li, U.S. Herrmann, B. Wieland, et al. 2013. The toxicity of antiprion antibodies is mediated by the flexible tail of the prion protein. *Nature.* 501:102–106. http://dx.doi.org/10.1038/nature12402

Wang, Y., K.J. Szretter, W. Verma, S. Gilfillan, C. Rossini, M. Cella, A.D. Barrow, M.S. Diamond, and M. Colonna. 2012. IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia. *Nat. Immunol.* 13:753–760. http://dx.doi.org/10.1038/ni.2360

Williams, A., P.J. Lucassen, D. Ritchie, and M. Bruce. 1997. PrP deposition, microglial activation, and neuronal apoptosis in murine scrapie. *Exp. Neurol.* 144:433–438. http://dx.doi.org/10.1006/exnr.1997.6424

Zhan, Y., R.C. Paulicelli, F. Sforazzini, L. Weinhard, G. Bolasco, F. Pagani, A.L. Vysotski, A. Bifone, A. Gozzi, D. Rapozzino, and C.T. Gross. 2014. Deficient neuron-microglia signaling results in impaired functional brain connectivity and social behavior. *Nat. Neurosci.* 17:400–406. http://dx.doi.org/10.1038/nn.3641