Knockout of fractalkine receptor Cx3cr1 does not alter disease or microglial activation in prion-infected mice

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Microglial activation is a hallmark of the neuroimmunological response to Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis and prion disease. The CX3C chemokine axis consists of fractalkine (CX3CL1) and its receptor (CX3CR1); these are expressed by neurons and microglia respectively, and are known to modulate microglial activation. In prion-infected mice, both Cx3cr1 and Cx3cl1 are altered, suggesting a role in disease. To investigate the influence of CX3C axis signalling on prion disease, we infected Cx3cr1 knockout (Cx3cr1-KO) and control mice with scrapie strains 22L and RML. Deletion of Cx3cr1 had no effect on development of clinical signs or disease incubation period. In addition, comparison of brain tissue from Cx3cr1-KO and control mice revealed no significant differences in cytokine levels, spongiosis, deposition of disease-associated prion protein or microglial activation. Thus, microglial activation during prion infection did not require CX3C axis signalling.
Health/National Institute of Allergy and Infectious Diseases (NIH/NIAID) Exchange Program. Cx3cr1-KO mice, designated B6.129-Cx3cr1<sup>tm12sel</sup> (line 4167), had been backcrossed 10 times to C57BL/6Nai and then five times to C57BL/6NTac (C57) mice. To ensure deletion of Cx3cr1 in knockout mice, PCR assays were performed using primers and conditions as described previously (data not shown) (Combadière et al., 2003).

Mice were housed at Rocky Mountain Laboratories (RML) in an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility compliant with current guidelines. Experimentation followed NIH-RML Animal Care and Use Committee approved protocol 2008-48.

Cx3cr1-KO mice and C57 control mice were infected with two strains of mouse-adapted scrapie: 22L and RML. Briefly, mice were inoculated intracranially with 50 μl 1% (v/v) dilution of brain homogenate pools from scrapie-infected, terminally ill C57 mice. Inocula contained 1.3 × 10<sup>6</sup>LD<sub>50</sub> (infectious dose for 50% of the test population) for strain 22L and 4.0 × 10<sup>6</sup>LD<sub>50</sub> for strain RML, as described previously (Striebel et al., 2011). Beginning at 80 days post-infection (p.i.), researchers were blinded, and mice were weighed and scored for six scrapie-associated clinical parameters, including nesting behaviour, body condition, hind-limb weakness, kyphosis, somnolence and gait changes (Fig. 1a–d). Data were collected twice weekly for all parameters, using the following methodology.

Somnolence was assessed first, prior to other stimulation. Scoring was as follows: 0, mouse awoke rapidly and began to move about cage; 1, mouse was slow to move, but became active and remained active during the observation period; 2, mouse was slow to move and preferred to become still soon after arousal; 3, mouse was extremely sleepy and not easily roused.

Hind-limb weakness was assessed by testing the ability of the mouse to resist a backward force applied gently to the tail whilst the mouse was resting on a flat, textured surface. Mice were scored using an arbitrary scale: 0, strong resistance; 1, moderate resistance; 2, mild resistance; 3, no resistance.

Kyphosis was scored by visual observation and direct palpation: 0, none; 1, mild, only appreciated when palpated; 2, moderate, visible and palpable; 3, severely arched, easily visible.

Gait abnormalities, i.e. circling, ataxia, tippy-toed gait, hyperacticity and wobble, were assessed by observation of mice travelling in their home cage (on bedding) and over an adsorbent laboratory diaper. Scoring was based on presence and degree of any of the above listed abnormalities: 0, normal; 1, subtle abnormalities; 2, moderate abnormalities; 3, severe abnormalities.

Body condition was scored on a scale from 0 to 3: 0, normal, ideal body mass; 1, mild wasting; 2, moderate wasting; 3, advanced wasting. Body condition scoring was adapted/modified from a system developed by Ullman-Culleré & Foltz (1999). In order to correspond with the other parameters in our system, score values were inverted compared with conventional body condition scoring.

Nesting was scored as described previously (Cunningham et al., 2003). Briefly, a cotton square (nestlet) was added to each cage. Scoring was as follows: 1, untouched (90% intact) nestlet; 2, partially (50–90% intact) torn nestlet; 3, mostly (>90%) torn nestlet, but no defined nest site; 4, identifiable nest without walls; 5, nest resembles a crater with walls.

The clinical end-point for each mouse was defined as the time point when a mouse progressed to a score of 3 in two or more of the five clinical parameters and a score of 2 in two other parameters. Nesting scores were not used to determine the end-point. Mice were euthanized when they reached the clinical end-point.

Scoring of nesting gave the earliest reliable determination of disease at 105–110 days p.i. (Fig. 1a). Subsequently, weight loss was noted in 22L-infected mice starting at 120 days p.i. and in RML-infected mice at 140 days p.i. (Fig. 1b). For both 22L and RML strains, nearly identical incubation periods were seen in survival curves comparing Cx3cr1-KO and C57 controls (Fig. 1c). Furthermore, measurements of body condition, hind-limb weakness, kyphosis, somnolence and gait were all predictive of disease onset, and began to increase in infected mice at 116 days p.i. (Fig. 1d). While changes in all of these parameters were significant between infected and uninfected mice, there were not significant differences between infected C57 and Cx3cr1-KO mice. Thus, knockout of Cx3cr1 had no detectable effect on scrapie-associated clinical signs or incubation period. Interestingly, weight loss was significantly earlier and greater after 22L infection compared with RML (P<0.01) (Fig. 1b). To the best of our knowledge this is a feature not previously reported to distinguish these two strains.

As deletion of Cx3cr1 has been shown to elicit phenotypes which vary with experimental model (Wolf et al., 2013), we also assessed clinical phenotypes for other indicators of disease severity and microglial activation, i.e. accumulation of disease-associated prion protein (PrP<sup>Sc</sup>), degree of spongiform degeneration, and changes in glial density and morphology. Comparison of Cx3cr1-KO and C57 control mice indicated no significant differences in brain deposition of PrP<sup>Sc</sup> as detected by immunoblot (Fig. 2a), immunohistochemistry (Fig. 2b) or location and degree of spongiform degeneration (Fig. 2b). Surprisingly, a comparison of anti-IBA1 immunohistochemical staining in Cx3cr1-KO and C57 mice revealed nearly identical morphologies and levels of activated microglia, characterized by increased cytoplasm and retracted processes (Fig. 2b). Similarly, anti-glia fibrillary acidic protein (GFAP) staining suggested equivalent levels of astrogliosis in both mouse strains (Fig. 2b). Although slight variation in regional distribution of microgliosis and astrogliosis occurred between individual mice (as shown in Fig. 2b), this
Fig. 1. Effect of Cx3cr1 knockout on development of scrapie (‘Sc’)–associated clinical signs, weight change and incubation period. (a) Comparison of nesting behaviour scores (5, near perfect nest; 1, no nest). (b) Comparison of percentage weight change in 22L- and RML-infected Cx3cr1-KO mice and C57 mice beginning at preclinical times. Uninfected Cx3cr1-KO mice and C57 mice are shown for comparison. Knockout of Cx3cr1 had no significant effect on weight change. (c) Survival curves showing Cx3cr1-KO versus C57 control mice for 22L and RML scrapie strains. Data are percentage of surviving animals versus days p.i. Mean ± SD incubation period for each strain is set inside each graph. Statistical analysis by Mantel–Cox log-rank analysis indicated no significant differences. (d) Graphs show comparison of five scrapie-associated parameters (a score of 0 was considered normal and a score of 3 was the most advanced stage consistent with a humane point of euthanasia; see Methods for details of scoring). For all graphs, except (c), data are mean ± SD. Numbers of uninfected mice were: C57, n = 4 and Cx3cr1-KO, n = 4. For scrapie-infected mice, respective numbers were (22L, RML): C57, n = 11, 12 and Cx3cr1-KO, n = 10, 12. Statistical differences were evaluated by two-way ANOVA (Prism software; GraphPad).
variation was associated with deposition of PrP\textsuperscript{Sc} and not with knockout of Cx3cr1. Slight variations in regional PrP\textsuperscript{Sc} deposition between infected mice is typical. Immunohistochemical and immunoblot analyses were performed as described previously (Striebel \textit{et al.}, 2011; Rangel \textit{et al.}, 2014).

In order to further characterize the potential effect of Cx3cr1 knockout on prion disease and microglial activation, we used a multiplex immunoassay (Bio-Rad) to compare levels of 23 cytokines in brains of scrapie-infected Cx3cr1-KO and C57 mice taken at the clinical end-point. In other models of

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\textbf{Fig. 2.} PrP\textsuperscript{Sc} immunoblot and neuropathology in 22L-infected mice. (a) Immunoblots of brain homogenates from clinical 22L-infected C57 mice (n = 3) and Cx3cr1-KO mice (n = 3) and one uninfected C57 mouse. Equivalent loads of brain homogenate were treated for 1 h with 50 mg proteinase K ml\textsuperscript{-1}, run on SDS-PAGE gels, blotted and probed with anti-PrP antibody D13, and exposed with chemiluminescent reagents. Lanes 1, 2 and 3 are similar to lanes 5, 6 and 7, suggesting equivalent amounts of PrP\textsuperscript{Sc} deposition in Cx3cr1-KO and C57 mice. (b) Histopathological analysis of brain from clinical 22L-infected C57 and Cx3cr1-KO mice. An uninfected Cx3cr1-KO age-matched mouse is shown as a control. These images are representative of six C57 mice and five Cx3cr1-KO mice analysed. The top row shows comparative staining of whole-brain sagittal sections for PrP\textsuperscript{Sc} using anti-PrP antibody D13 (brown). The inset images show close-up views of PrP\textsuperscript{Sc} deposition in the thalamus. The second row shows vacuolation in the thalamus using haematoxylin and eosin (H&E) staining. The third row compares brain sagittal sections stained with anti-IBA1 antibody (red), which highlights microglial morphology and distribution. Inset images show close-ups of the anti-IBA1-positive microglia in the thalamus. Note the large cell bodies with short, thickened processes, indicative of microglial activation, in the infected mice. In contrast, microglia in the uninfected mouse have small cell bodies with long, thin, highly ramified processes. The bottom row compares astrogliosis in brain sagittal sections by staining with anti-GFAP antibody (red). Images are for 22L only; analysis of RML-infected brain gave similar results (data not shown).
Fig. 3. Elevated cytokine levels in the brain of scrapie-infected mice at clinical time point. Using multiplex immunoassays, protein levels of 23 cytokines [eotaxin, granulocyte colony stimulating factor, granulocyte/macrophage colony stimulating factor, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17A, KC, CCL2, CCL3, CCL4,
CCL5 and TNF-α] were measured in brain homogenates from scrapie-infected mice taken at the clinical end-point. Levels of 10 cytokines that were elevated in 22L and RML-infected C57 and Cx3cr1-KO mice compared with mock are shown here. Levels of cytokines in scrapie-infected Cx3cr1-KO mice were not significantly different from those of scrapie-infected C57 mice, except for CCL2 levels for scrapie strain 22L. Each dot represents one mouse and data are presented as mean ± SD. Statistical differences were evaluated by one-way ANOVA (Prism software; GraphPad).

neurodegeneration, such as Alzheimer’s disease, Parkinson’s disease and ALS, knockout of Cx3cr1 produced significant differences in microglial activation and cytokine profile in brain (Limatola & Ransohoff, 2014; Lee et al., 2010; Cho et al., 2011). In our experiments, elevated levels of 10 of the 23 cytokines tested were detected in both RML- and 22L-inoculated Cx3cr1-KO and C57 mice compared with mock controls (Fig. 3). However, there were no significant differences between Cx3cr1-KO and C57 mice in the levels of any of these 10 elevated cytokines, except for CCL2, which was significantly increased in 22L-infected Cx3cr1-KO mice. Interestingly, deletion of Cx3cr1 did not affect levels of IL-1α, IL-1β, IL-10, IL-12(p40), TNF-α or IL-6 (data not shown), which are known to be expressed by activated microglia (Fig. 3) (Cardona et al., 2006; Park et al., 2007; Sajjo & Glass, 2011; Smith et al., 2012; Tribouillard-Tanvier et al., 2012).

In this study, Cx3cr1 deletion did not alter the onset, duration or pathology of disease caused by two distinct murine scrapie strains (22L and RML). Surprisingly, Cx3cr1 knockout did not affect the activation of microglia as assessed by morphological alteration and by cytokine expression. Whilst Cx3cr1 knockout experiments in Parkinson’s disease, ALS and Alzheimer’s disease’s models have indicated a role for CX3C signalling (Cardona et al., 2006; Lee et al., 2010; Cho et al., 2011), our experiments suggest that CX3CR1 is not essential to the microglial response in prion disease. As others have proposed, some redundancy may exist in the signalling mechanisms that regulate microglia (Biber et al., 2014), thus other molecules such as CD200/CD200R and/or TREM2 may play an augmented role upon deletion of Cx3cr1 in prion disease models.

After infection with two scrapie strains, we observed a non-significant difference of 0–4 days in survival of Cx3cr1-KO versus C57 control mice, whereas in the similar study conducted by Grizenkova et al. (2014) using three scrapie strains, there was a statistically significant difference of 6–10 days between Cx3cr1-KO and control mice. One explanation for these differing conclusions may be that the two studies used unique, independently generated Cx3cr1-KO mouse strains, which differ in genetic background and in the genetic constructs used in their creation. The Cx3cr1-KO mice (B6.129-Cx3cr1<sup>tm1Zm</sup>)) used in our study were generated by insertion of a neomycin cassette into the Cx3cr1 gene in 129/Sv mouse embryonic stem cells and subsequent back-crossing to a C57BL/6 background (Combadère et al., 2003). Whereas Cx3cr1-KO mice used by Grizenkova et al. (2014) (Cx3cr1/GFP, C.129P2-Cx3cr1<sup>tm2Jrwe</sup>) were created by insertion of an EGFP gene into the Cx3cr1 locus of 129 Ola embryonic stem cells and then back-crossed to a BALB/c background (Jung et al., 2000). Although both mouse lines have undetectable levels of Cx3cr1 transcripts, the differences in genetic background (C57BL/6 versus BALB/c) could still affect the disease incubation period. Indeed, in a single experiment, Tamgiyne et al. (2008) noted a 19% reduction in the scrapie incubation period in IL-10<sup>−/−</sup> mice on a C57BL/6 background, but no change in IL-10<sup>−/−</sup> mice on a 129S6 background. Additionally, two studies on the role of intestinal macrophages in ulcerative colitis utilized Cx3cr1-KO mice on different genetic backgrounds (C57BL/6 versus BALB/c) and had opposing results (Kostadinova et al., 2010; Medina-Contreras et al., 2011).

The dissimilar results found by Grizenkova et al. (2014) and us may also be due to differences in experimental protocols, i.e. criteria for assigning clinical end-point, use of blinded observers in end-point determination and in the infectious dose (LD<sub>50</sub>) administered for each scrapie strain. Thus, conclusions on the role of CX3C signalling in prion disease, as in other neurodegenerative models, may vary with experimental system. Taken together, these studies suggest a rather weak effect of Cx3cr1 knockout on prion disease. Moreover, as microglia were activated by scrapie infection of Cx3cr1-KO mice in both studies, a role for microglial activation in prion disease pathogenesis remains a possibility.

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