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Abstract

To reach the brain from peripheral sites, prions must colonize various cell types within the lymphoreticular compartment. However, no prion entry receptors are yet known. Toll-like receptors (TLRs) are pattern-recognition receptors that bind a multitude of pathogens and are therefore candidates as effectors of prion entry. Moreover, injection of unmethylated CpG oligodinucleotides, which stimulate TLR9, has been reported to delay peripherally initiated scrapie. We therefore studied prion infection in MyD88(-/-) mice, which are defective in TLR signalling. Despite subtle defects in splenic microarchitecture, MyD88(-/-) mice challenged intraperitoneally or intracerebrally were fully susceptible to disease and died of scrapie after similar incubation times to those of wild-type mice. Splenic infectivity titres rose to similar levels with the same kinetics, and brains showed similar histopathological changes. TLR signalling therefore does not have any major role in prion pathogenesis, and the protective effect of TLR stimulation is unlikely to result from direct interactions with prions.

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Prion pathogenesis in the absence of Toll-like receptor signalling

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To reach the brain from peripheral sites, prions must colonize various cell types within the lymphoreticular compartment. However, no prion entry receptors are yet known. Toll-like receptors (TLRs) are pattern-recognition receptors that bind a multitude of pathogens and are therefore candidates as effectors of prion entry. Moreover, injection of unmethylated CpG oligodinucleotides, which stimulate TLR9, has been reported to delay peripherally initiated scrapie. We therefore studied prion infection in *MyD88*$^{-/-}$ mice, which are defective in TLR signalling. Despite subtle defects in splenic microarchitecture, *MyD88*$^{-/-}$ mice challenged intraperitoneally or intracerebrally were fully susceptible to disease and died of scrapie after similar incubation times to those of wild-type mice. Splenic infectivity titres rose to similar levels with the same kinetics, and brains showed similar histopathological changes. TLR signalling therefore does not have any major role in prion pathogenesis, and the protective effect of TLR stimulation is unlikely to result from direct interactions with prions.

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INTRODUCTION

Host organisms detect infection by recognizing pathogen-associated molecular patterns that induce innate responses to infections. Toll-like receptors (TLRs) are crucial mediators of such responses. Toll is crucial in antifungal immunity of *Drosophila* (Lemaitre *et al.*, 1996). TLRs are mammalian Toll homologues expressed by immune cells. At least 10 members of the TLR family exist. At present, ligands have been identified for TLR1 to TLR9. Most of these ligands are derived from pathogens and comprise a heterogeneous group, including viruses, bacteria, DNA, RNA, toxins and chemical compounds. TLR9 is required for immune responses triggered by bacterial DNA (Hemmi *et al.*, 2000). MyD88 is a cytoplasmic adapter protein that associates as an obligate functional partner with all members of the TLR and interleukin-1 receptor (IL-1R) family (Muzio *et al.*, 1997; Medzhitov *et al.*, 1998). *MyD88*$^{-/-}$ mice do not show any response to IL-1 family cytokines *in vivo* (Adachi *et al.*, 1998), are resistant to lipopolysaccharide-induced endotoxin shock syndrome (Kawai *et al.*, 1999), and fail to respond to peptidoglycans and lipoproteins (Takeuchi *et al.*, 2000), antiviral compounds (Hemmi *et al.*, 2002), CpG DNA (Hacker *et al.*, 2000) and flagellins (Hayashi *et al.*, 2001).

In prion diseases, the immune system exerts a crucial role during the early extracerebral phase that follows peripheral administration of the agent (Aguzzi *et al.*, 2001). B cells (Klein *et al.*, 1997), follicular dendritic cells (FDCs) (Montrasio *et al.*, 2000) and other haematopoietic and stromal cells in spleen and lymph nodes (Kaeser *et al.*, 2001; Prinz *et al.*, 2002) are involved in the process of peripheral uptake, amplification and probably transport of prion infectivity. However, the receptors used by invading prions to enter host cells are unknown. FDCs trap opsonized antigens on their surface with Fcγ and complement receptors, and mice lacking opsonins or complement receptors are partly protected against the intraperitoneal administration of prions (Klein *et al.*, 2001; Mabbott *et al.*, 2001). However, complement is evidently not the only system involved, because complement-deficient mice succumb to scrapie after peripheral challenge with high doses of scrapie inoculum (Klein *et al.*, 2001).

A recent study has shown that the injection of CpG oligodeoxynucleotides, which stimulate the innate immune system via the TLR9 receptor, provides partial protection against prions administered intraperitoneally (Sethi *et al.*, 2002). The mechanism underlying this spectacular finding might be indirect: stimulation of TLR9-expressing immune cells might trigger a constellation of cytokines and other mediators that eventually results in an environment unfavourable for prion replication. Alternatively, prions might conceivably bind and/or signal through TLRs—a process that could be competed for by 'therapeutic' TLR ligands. Here we investigated a possible direct role of TLR-mediated responses in peripheral and central prion pathogenesis by administering prions peripherally or intracerebrally to mice lacking the cytoplasmic adapter protein MyD88, which is the central transducer of TLR and IL-1R signalling.

RESULTS

Germinal centres and FDCs in *MyD88*$^{-/-}$ mice

To judge the extent to which the absence of MyD88 affects the morphology of germinal centres, we examined spleens with immunohistochemical stains for B cells, FDCs and complement receptor 1 (Fig. 1). In wild-type littermates from *MyD88*$^{+/-}$ intercrosses, which served as control mice, normal shape of the follicles and segregation of distinct B- and T-cell zones were apparent on spleen sections

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stained with the B cell marker B220. In both *MyD88*$^{-/-}$ and wild-type mice the number of germinal centres was similar and ranged between five and eight in an area of 10^6 μm^2.

However, follicles in *MyD88*$^{-/-}$ mice seemed to be smaller and sometimes malformed. Morphometric measurements of more than 50 germinal centres of each genotype stained with B220 revealed that follicles of *MyD88*$^{-/-}$ mice were significantly smaller than those of wild-type mice (mean 307 μm versus 493 μm for the full length of transverse sections, $P < 0.01$). Furthermore, germinal-centre B cells did not form classical B-cell areas within follicles, and marginal-zone B cells, a specific subset of B cells that surrounds the germinal centre in a ring-like fashion, were not visible in the B220 staining in the spleens of *MyD88*$^{-/-}$ mice. This finding was demonstrated with immunostains for complement receptor 1 (CD35), which is expressed in marginal-zone B cells and in FDCs. Indeed, CD35-positive marginal-zone B cells surrounding the germinal centres were absent from *MyD88*$^{-/-}$ mice.

FDCs within germinal centres accumulate the disease-associated prion protein (PrP^{Sc}) early during prion diseases. We therefore stained spleens with monoclonal antibody FDC-M1, a marker of mature FDCs (Kosco *et al.*, 1992) and of tingible-body macrophages (Kosco-Vilbois, 2000) (Fig. 1). CD35-positive FDC networks were also conspicuous in wild-type and *MyD88*$^{-/-}$ mice.

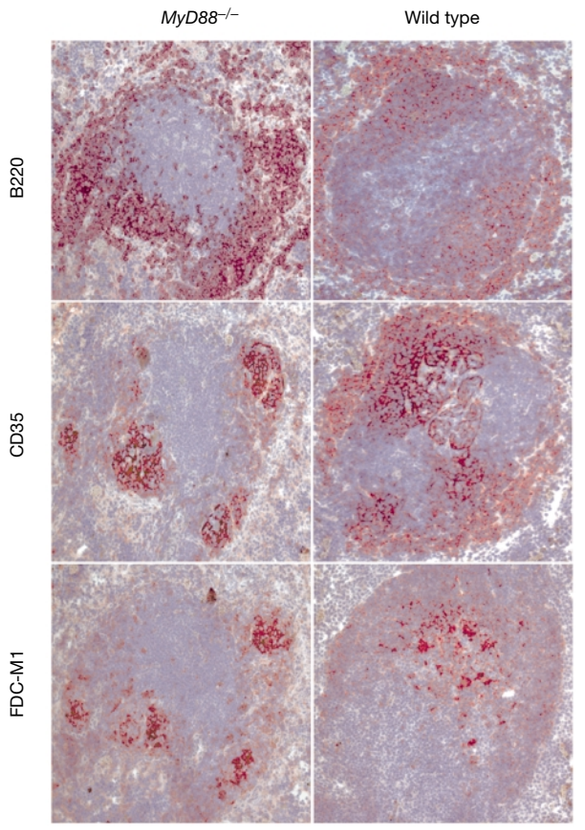

Fig. 1 | Morphological appearance of germinal centres in spleens of *MyD88*$^{-/-}$ mice. Spleen sections of MyD88-deficient mice (left column) and non-mutant control littermates (right column) were stained for B cells (B220), complement receptor 1 (CD35) and FDCs (FDC-M1). Original magnification of all pictures, ×100.

Morphometric measurements of the FDCs surface-stained with FDC-M1 showed that similar areas were occupied by this cell type in *MyD88*$^{-/-}$ and wild-type mice (2489 ± 1372 μm^2 versus 2362 ± 972 μm^2; results expressed as mean ± s.d.). However, FDC networks in *MyD88*$^{-/-}$ mice occasionally seemed oddly-shaped and slightly disorganized when compared with those of wild-type mice (data not shown).

MyD88$^{-/-}$ mice are highly susceptible to scrapie

Mice deficient for MyD88 were challenged with various quantities of prions, and the incubation time to terminal disease was measured. After intracerebral injection with a high dose (3×10^5 LD_{50}) or a low dose (3×10^2 LD_{50}) of Rocky Mountain Laboratory scrapie strain (RML), all treated *MyD88*$^{-/-}$ and wild-type mice reached terminal disease with similar incubation times (Table 1). In both genotypes, terminal illness was correlated with typical clinical signs of scrapie such as ataxia, kyphosis, hind leg paresis and, in males, priapism. These results clearly demonstrate that *MyD88*$^{-/-}$ mice are highly susceptible to cerebral prion infection.

After high-dose intraperitoneal prion challenge (6 $logLD_{50}$), *MyD88*$^{-/-}$ and wild-type mice developed scrapie with similar incubation times of between 197 and 207 days (Table 1), indicative of unaffected peripheral prion pathogenesis and neuroinvasion. The mean incubation time after administration of a 1,000-fold diluted inoculum (3 $logLD_{50}$) to *MyD88*$^{-/-}$ mice was slightly longer (Table 1). Five *MyD88*$^{-/-}$ mice died between 213 and 261 days after injection, which was superimposable on the incubation range of wild-type mice. However, two mice had longer incubation times (270 and 274 days after injection), resulting in a larger standard deviation within this group. A Student's *t*-test yielded $P > 0.05$, indicating that the incubation times in the two groups were not significantly different. Importantly, all of these seven peripherally challenged *MyD88*$^{-/-}$ mice developed scrapie after administration of a low-dose inoculum, indicating that MyD88 is dispensable for peripheral prion pathogenesis.

Immunoblot analysis confirmed PrP^{Sc} accumulation in the brains and spleens of terminally ill *MyD88*$^{-/-}$ mice (Fig. 2). Accordingly, haematoxylin/eosin stains and glial fibrillary acidic protein (GFAP) immunohistochemistry of terminally ill mice lacking MyD88 and of wild-type mice showed similar patterns of vacuolization and of reactive astrogliosis within the hippocampal ribbon (Fig. 3). Brain pathology during prion disease was therefore also unaltered in *MyD88*$^{-/-}$ mice.

Prion replication in *MyD88*$^{-/-}$ mice

We then determined prion infectivity over time in spleens of mice inoculated intraperitoneally (6 $logLD_{50}$). Infectivity was determined by transmission of homogenized tissues into *tga20* indicator mice (Fischer *et al.*, 1996) and comparison of incubation times against a calibration curve. Infectivity in spleens of *MyD88*$^{-/-}$ (Fig. 4, red circles) and wild-type (blue circles) mice after intraperitoneal infection was generally similar in mice of both genotypes. In addition, the time courses of the process by which prion invaded the spleen were clearly similar: a considerable amount of infectivity was already present at 35 days after injection in the spleen of both groups. Further measurements at 90 and 120 days after inoculation confirmed that the levels of prion infectivity in wild-type and *MyD88*$^{-/-}$ mice were always very similar. A lack of MyD88 therefore does not impair the rate and kinetics of lymphoreticular prion invasion after intraperitoneal administration.

Table 1 | High susceptibility to scrapie of *MyD88*$^{-/-}$ mice after peripheral and intracerebral prion challenge

	Intraperitoneal infection						Intracerebral infection					
	6 $logLD_{50}$			3 $logLD_{50}$			3 x 10^5 LD_{50}			3 x 10^2 LD_{50}		
Mouse genotype	Attack rate	Latency (d)	Mean ± s.d. (d)	Attack rate	Latency (d)	Mean ± s.d. (d)	Attack rate	Latency (d)	Mean ± s.d. (d)	Attack rate	Latency (d)	Mean ± s.d. (d)
MyD88$^{-/-}$	6/6	173, 181, 181, 197, 216, 236	197 ± 17	7/7	215, 225, 251, 251, 261, 270, 274	249 ± 24	5/5	135, 135, 156, 163, 163	150 ± 14	4/4	175, 177, 180, 189	180 ± 6
Wild-type *MyD88*$^{+/+}$	5/5	192, 206, 206, 213, 220	207 ± 10	5/5	213, 222, 222, 225, 241	225 ± 10	5/5	135, 135, 135, 156, 163	145 ± 14	6/6	168, 168, 177, 186, 186	177 ± 9
Prnp$^{o/o}$	n.d.	–	–	0/5	>370	>370	n.d.	–	–	0/4	>370	>370

MyD88$^{-/-}$ and wild-type mice developed scrapie after similar incubation periods after administration of a high or a low intracerebral dose of prions. A high peritoneal prion challenge (6 $logLD_{50}$) also elicited scrapie with a similar latency, whereas after intraperitoneal challenges with a lower dose (3 $logLD_{50}$) *MyD88*$^{-/-}$ mice developed terminal disease with a slightly longer incubation period than wild-type mice. Latency, latency of disease in individual mice. *Prnp*$^{o/o}$, mice deficient for PrP^c.

DISCUSSION

The innate immune system is the first line of defence against infection. The most important sensors for the recognition of these invading microorganisms are the TLRs. Activation of the innate immune system by CpG oligodeoxynucleotides has been shown to prevent prion disease in mice (Sethi *et al.*, 2002). This observation is surprising because activation of the immune system was thought to enhance, rather than protect from, peripheral prion disease. Protection might be due to TLR9-mediated immune activation or to direct competition of PrP^{Sc} with TLRs. PrP^{Sc} is highly aggregated and forms ordered molecular arrays (Wille *et al.*, 2002) of congophilic, anisotropic amyloid with predominant β-sheet conformation. These characteristics led us to propose that canonical pattern receptors, such as TLRs, might be involved in prion pathogenesis. However, the results presented here imply that prion replication and neuroinvasion can take place in secondary lymphoreticular organs in the absence of TLR signalling.

After intraperitoneal low-dose challenge with prions, we observed a delay of 24 days in mean incubation of *MyD88*$^{-/-}$ in comparison with wild-type mice of the same genetic background. Statistical analysis did not reveal a significant difference between the two groups. The slight difference might be due to a subtle impairment in germinal-centre architecture of *MyD88*$^{-/-}$ mice: this compartment is of pivotal importance in peripheral prion pathogenesis, and the observed changes might slightly affect prion pathogenesis. All other investigated parameters of pathogenesis, such as amount of PrP^{Sc} in infected brains and spleens, kinetics and amount of infectivity in the spleens, as well as neuropathological changes of end-stage brains, were identical in *MyD88*$^{-/-}$ and wild-type mice.

The cytoplasmic portion of TLRs is similar to that of IL-1R and IL-18R. The signalling pathway via TLRs is therefore homologous to that of the IL-1R family, and both TLRs and IL-1Rs interact with MyD88. *MyD88*$^{-/-}$ mice do not show any response to IL-1 or IL-18 (Adachi *et al.*, 1971). These cytokines might have a significant role in many neurodegenerative diseases (Allan & Rothwell, 2001). Expression of IL-1 was detected in brains of animals that were ill with scrapie (Williams *et al.*, 1994) but another study failed to reproduce this finding (Walsh *et al.*, 2001). The present study indicates that IL-1R signalling is positively needed neither for peripheral prion pathogenesis nor for the chronic neurodegeneration seen in prion disease.

Our findings are not incompatible with the reported prophylactic effect of CpG oligonucleotides in scrapie-inoculated mice (Sethi *et al.*, 2002) but make it unlikely that stimulators of innate immunity compete with prions for a common pathway. Instead, it is more likely that strong stimulation of TLRs exerts its effects indirectly, for example by conjuring an immunological environment generally hostile to prion infection.

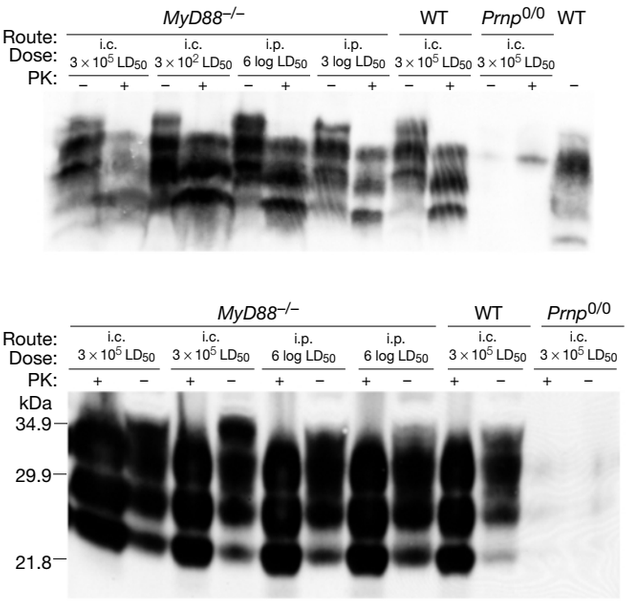

Fig. 2 | Western blot analysis of brains and spleens of infected *MyD88*$^{-/-}$ mice. Western blots of spleen material subjected to electrophoresis in native form (–) or after digestion with proteinase K (PK) (+) are shown. Similar amounts of PK-resistant prion protein (PrP^{Sc}) were detected in the brains and spleens of both *MyD88*$^{-/-}$ and wild-type (WT) mice that had developed terminal scrapie. PrP^{Sc} was detectable in all mice that had been challenged intraperitoneally (i.p.) or intracerebrally (i.c.) with high (6 $logLD_{50}$ or 3 × 10^5 LD_{50}) or low (3 $logLD_{50}$ or 3 × 10^2 LD_{50}) doses of RML. *Prnp*$^{o/o}$, mice deficient for PrP^c.

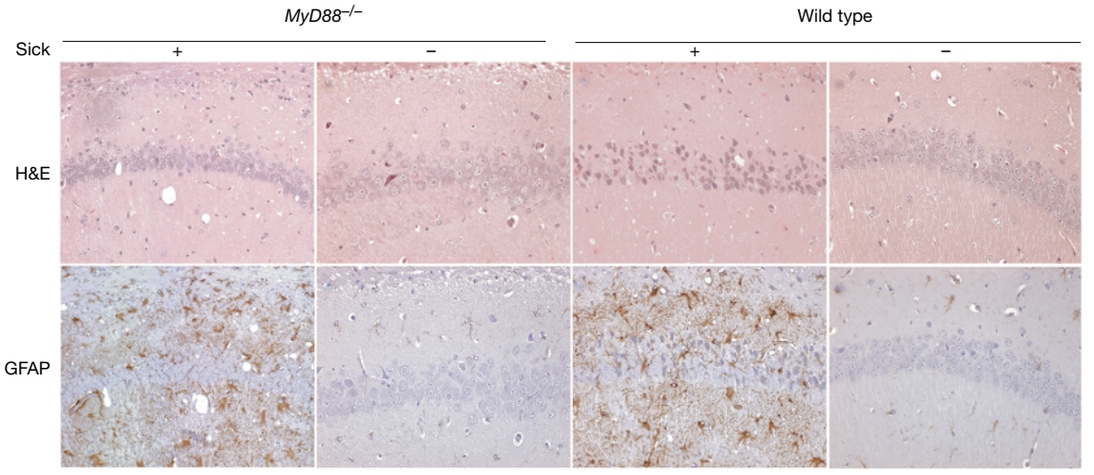

Fig. 3 | Brain pathology of terminally ill mice after intraperitoneal challenge with scrapie. Pronounced astrogliosis (detected with an immunohistochemical stain for GFAP; lower row) was visible in the hippocampal neuronal ribbon of scrapie-infected (terminal disease) *MyD88*$^{-/-}$ and wild-type mice, whereas no gliosis and vacuolization (haematoxylin/eosin stain (H & E); upper row) was found in clinically healthy mice. Original magnification of all micrographs, ×200.

Two caveats still persist before we can conclude that TLRs do not have any role in prion diseases. First, activation of TLR4 signalling was described to occur by an alternative pathway that does not involve MyD88 (Fitzgerald *et al.*, 2001), and might mediate some aspects of prion pathogenesis. Second, scrapie prions might use TLRs for docking to relevant cells but fail to activate MyD88-mediated signalling. This possibility is not totally remote because prion infection does not typically elicit strong adaptive immune reactions.

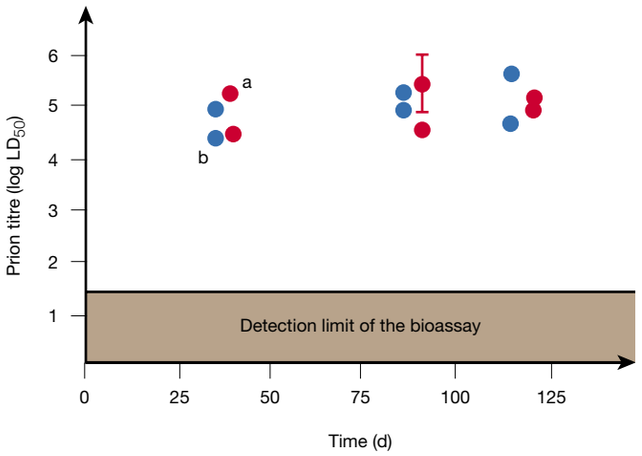

Fig. 4 | Determination of prion infectivity titres in spleens of infected *MyD88*$^{-/-}$ mice. Titres were determined in spleens of *MyD88*$^{-/-}$ mice (red circles) and wild-type mice (blue circles). Mice were treated intraperitoneally with 6 $logLD_{50}$ of scrapie prions as indicated. Standard deviations within groups are drawn only when they exceed ± 0.50 $logLD_{50}$. [a,b] In these transmissions 1 out of 4 *tga20* mice died 24 h after inoculation, most probably as a result of perioperative intracerebral bleeding.

The identification of the recognition receptors that are involved in prion entry, replication and accumulation in host cells therefore continues to be a priority in prion research, because it will help in our understanding of the molecular pathogenesis of prions and might even point to prophylactic or therapeutic strategies against these diseases.

METHODS

Scrapie infections. *MyD88*$^{+/-}$ mice (Adachi *et al.*, 1998) were interbred to obtain homozygous and wild-type C57Bl/6 mice. Animals were maintained under specific pathogen-free conditions and were infected intraperitoneally with brain homogenate in 100 µl PBS containing 6 $logLD_{50}$ or 3 $logLD_{50}$ units of the RML scrapie strain (passage 5). For intracerebral inoculations, 30 µl of inoculum with 3×10^5 LD_{50} or 3×10^2 LD_{50} were administered. Mice were monitored every second day, and scrapie was diagnosed by standard clinical criteria. Mice were killed on the day of onset of terminal clinical signs of scrapie.

Infectivity bioassay with *tga20* indicator mice. Assays were performed on 1% spleen homogenates. Tissues were homogenized in 0.32 M sucrose with a micro-homogenizer, diluted in PBS and 5% BSA, then passed several times through 18-gauge and 22-gauge needles. When the solution seemed homogenous, it was centrifuged for 5 min at 500*g*. Supernatants (30 µl) were inoculated intracerebrally into groups of four *tga20* mice (Fischer *et al.*, 1996). The relationship $y = 11.45 - 0.088x$ (y, log LD_{50} per milliliter of homogenate; x, incubation time in days to terminal disease) was calculated by linear regression (Prusiner *et al.*, 1982; Kaeser *et al.*, 2001).

Western blot analysis. Tissue homogenates were adjusted to 8 mg ml^{-1} protein, then treated with proteinase K (20 µg ml^{-1}, 30 min, 37 °C). Total protein (50 µg) was subjected to SDS–12% polyacrylamide-gel electrophoresis. Proteins were transferred to nitrocellulose by semi-dry blotting. Membranes were blocked with Tris-buffered saline containing 0.1% Tween 20, pH 8, plus 5% non-fat milk, incubated with monoclonal antibody ICSM18 (Hill & Collinge, 2002) and detected by enhanced chemiluminescence (ECL; Amersham).

Histology and immunohistochemistry. Paraffin-embedded and frozen sections from brain and spleen were stained with haematoxylin/eosin. Immunostaining for the astrocytic marker glial fibrillary acidic protein (GFAP) was performed with a rabbit antiserum against GFAP (1:300 dilution; DAKO) and detected with biotinylated pig anti-rabbit serum (1:250 dilution; DAKO) and diaminobenzidine (Sigma). The follicular dendritic cell marker FDC-M1 (clone 4C11; 1:300 dilution), the complement marker CD35 (8C12; Pharmingen) and the pan-B cell marker anti-CD45RO/B220 (RA3-6B2; Pharmingen) were used as described (Karrer *et al.*, 2000). Morphometric analysis was performed with Analysis Pro 3.1 software (Soft Imaging System).

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