Complement facilitates early prion pathogenesis

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New-variant Creutzfeldt–Jakob disease and scrapie are typically initiated by extracerebral exposure to the causative agent, and exhibit early prion replication in lymphoid organs[1,2]. In mouse scrapie, depletion of B-lymphocytes prevents neuropathogenesis after intraperitoneal inoculation[3,4], probably due to impaired lymphotoxin-dependent maturation of follicular dendritic cells[5] (FDCs), which are a major extracerebral prion reservoir[6]. FDCs trap immune complexes with Fc-γ receptors and C3d/C4b-opsonized antigens with CD21/CD35 complement receptors. We examined whether these mechanisms participate in peripheral prion pathogenesis. Depletion of circulating immunoglobulins or of individual Fc-γ receptors had no effect on scrapie pathogenesis if B-cell maturation was unaffected. However, mice deficient in C3, C1q, Bf/C2, combinations thereof[7,8] or complement receptors[9] were partially or fully protected against spongiform encephalopathy upon intraperitoneal exposure to limiting amounts of prions. Splenic accumulation of prion infectivity and PrP^{Sc} was delayed, indicating that activation of specific complement components is involved in the initial trapping of prions in lymphoreticular organs early after infection.

Upon intracerebral injection into susceptible species, prions are capable of inducing a progressive, invariably lethal disease of the central nervous system. However, in bovine spongiform encephalopathy, new-variant Creutzfeldt-Jakob disease (nvCJD), and most cases of iatrogenic CJD, the portal of entry of prions is extracerebral, and the immune system is pivotal for neuroinvasion. Disease-associated prion protein (PrP^{Sc}) accumulates in FDCs (ref. 6), which function as antibody- and complement-dependent antigen traps. Here we investigated whether the mechanisms by which FDCs trap antigens are relevant to the pathogenesis of prion infections. These trapping mechanisms consist essentially of capture of immune complexes by Fc-γ receptors, and binding of opsonized antigens (linked covalently to C3d and C4b complement adducts) to the CD21/CD35 complement receptors.

In order to assess the relative importance of circulating immunoglobulins to the pathogenesis of prion diseases, we compared PrP^{Sc} accumulation and prion replication in spleens and brains, as well as incubation time of the disease, in: 1) μMT mice that lack secreted immunoglobulins, B-cell receptors and mature B-cells[10]; 2) agammaglobulinemic *tg*141*m* mice (see Methods) that express a single monoclonal transgenic membrane-bound μ heavy chain; 3) *tg*141*m*+*s* mice (see Methods) expressing the same transgenic μ-immunoglobulin heavy chain as membrane-bound and secreted moiety; and 4) syngeneic wild-type control mice.

As described before[3], deletion of all μ-immunoglobulin heavy chains, which fully prevents B-cell differentiation in μMT mice, blocked both splenic prion accumulation and neuroinvasion (Table 1). Instead, reintroduction into μMT mice of a transgenic μ-chain directed against mouse mammary tumor virus (MMTV) sufficed to fully restore prion lymphoinvasion and neuroinvasion irrespective of whether this heavy chain allowed for secretion of immunoglobulins as in *tg*141*m*+*s* mice, or only production of membrane-bound immunoglobulins as in *tg*141*m* mice (Table 1).

Because the above strategy resulted in B-cells with a fixed heavy chain and with a repertoire of light chains, we investigated whether these surprising findings might be explained by spurious cross-reactivity with PrP^{C} of the skewed immunoglobulin repertoire in *tg*141*m* and *tg*141*m*+*s* mice. However, FACS and western-blot analysis performed as described[3] did not support this interpretation (data not shown). We therefore conclude that B-lymphocytes, but not circulating immunoglobulins, are crucial to prion replication in lymphoid organs and to neuroinvasion.

We further investigated whether circulating complexes of PrP^{Sc} with immunoglobulins might be involved in pathogenesis by inoculating mice carrying deletions of the Fc receptor γ-chain[11], Fc-γRII (ref. 12) and Fc-γRIII (ref. 13) receptors. All of these mice developed clinical scrapie upon intraperitoneal inoculation. Attack rates and kinetics (Table 1) as well as histopathological findings (Fig. 1) were identical to those of wild-type mice of the same genetic background. Accumulation of prions in spleen was also similar to that of wild-type mice

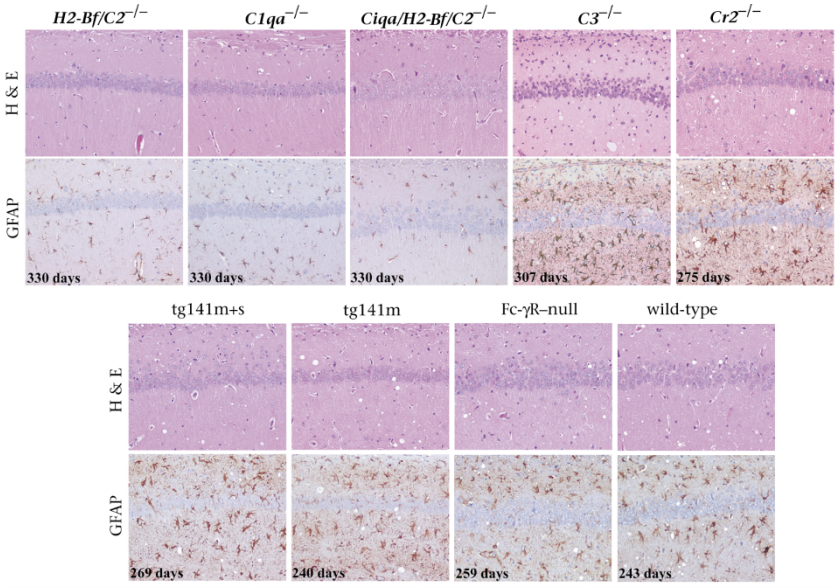

Fig. 1 Scrapie pathogenesis in mice with defects in immunoglobulin production and/or in the complement system. Histological analysis of the hippocampal pyramidal cell ribbon upon intraperitoneal challenge with prions failed to reveal any pathological changes in *C1qa*$^{-/-}$, *H2-Bf/C2*$^{-/-}$ and *C1qa/H2-Bf/C2*$^{-/-}$ mice. Mice of all other genotypes had diffuse microvacuolation of brain tissue (upper row), and activation of astrocytes with strong expression of GFAP (lower row). Days after inoculation are indicated. ×200 Magnification, .

(Fig. 2*a*). These findings make it unlikely that Fc-γ receptors are limiting for prion pathogenesis.

A second mechanism exploited by FDCs for antigen trapping involves covalent linking of proteolytic fragments of the complement components C3 and C4 (refs. 14–16). The CD21/CD35 complement receptors on FDCs bind C3b, iC3b, C3d and C4b through short consensus repeats in their extracellular domain. We therefore investigated the relevance of the complement system to the targeting of FDCs by prions using mice lacking the C3 or Bf/C2 complement components[8], as well as *Cr2*$^{-/-}$ mice lacking CD21/CD35 complement receptors and *C1qa*$^{-/-}$ mice[7].

Deletion of C3, or of its receptor CD21/CD35, delayed neuroinvasion after intraperitoneal inoculation by approximately 40 days upon administration of a limiting dose of prions (Table 1). Similar to wild-type mice, histopathological analysis of brains from terminally sick mice revealed vacuolation, astrogliosis, activation of microglia and neuronal loss (Fig. 1).

Deletion of C1q (alone or combined with *H2-Bf/C2*$^{-/-}$) had more profound effects on all parameters of pathogenesis. When a low dose of prions was administered, prion infectivity (Fig. 2*a*) and PrP^{Sc} (Fig. 2*b*) were undetectable in spleens of *C1qa*$^{-/-}$ and of *C1qa/H2-Bf/C2*$^{-/-}$ mice 38 days after inoculation, whereas titers of 4.5–5 log LD_{50} (Fig. 2*a*) and robust protease-resistant PrP^{Sc} signals (Fig. 2*b*) were detectable at the same time point in spleens of wild-type mice. At later time points, or if a larger inoculum (1×10^6 ID_{50}) was administered, splenic prion titers and PrP^{Sc} levels rose to levels similar to those of wild-type mice, indicating that the splenic compartment (and presumably FDCs therein) possesses an intrinsic affinity for prion accumulation that can override the requirement for complement when exposed to saturating amounts of prions.

CD21/CD35 has two important roles in humoral immunity. On B-cells it provides a costimulatory signal for ligation with the B-cell receptor (BCR) and lowers the threshold of BCR sig-

Table 1 Latency of scrapie in mice

		intracerebral inoculation				intraperitoneal inoculation			
		3×10^5 IU		300 IU		1×10^6 IU		1×10^3 IU	
Host genotype	Strain	Attack rate	days ± s.d.	Attack rate	days ± s.d.	Attack rate	days ± s.d.	Attack rate	days ± s.d.
Fc-γR$^{-/-}$	C57BL/6	5/5	166 ± 10	6/6	189 ± 11	10/10	201 ± 7	13/13	245 ± 11
Fc-γRII$^{-/-}$	129Sv × C57BL/6	4/4	130 ± 2	5/5	152 ± 7	8/8	181 ± 18	12/12	236 ± 23
Fc-γRIII$^{-/-}$	129Sv		ND	6/6	148 ± 9	9/9	180 ± 9	11/11	220 ± 7
Tg141m + s × μMT	C57BL/6μMT	2/2	147,147	5/5	181 ± 9	10/10	219 ± 14	8/8	252 ± 19
Tg141m × μMT	C57BL/6μMT		ND	2/2	161,182	6/6	213 ± 10	6/6	242 ± 18
μMT	C57BL/6		ND	8/8	181 ± 6[a]	0/8	> 534[a]		ND
Cr2$^{-/-}$	129SV × C57BL/6	3/3	122 ± 9	4/4	160 ± 5	9/9	209 ± 10	13/15	280 ± 15
C3$^{-/-}$	C57BL/6	3/3	154 ± 12	3/3	178 ± 4	8/8	193 ± 13	5/5	276 ± 19
C1qa$^{-/-}$	C57BL/6	5/5	132 + 2	6/6	185 ±12	10/10	193 ± 5	0/15	>541[b]
H2-Bf/C2$^{-/-}$	C57BL/6	2/2	123, 134	5/5	182 ± 20	10/10	197 ± 4	0/11	>541[c]
C1qa/H2-Bf/C2$^{-/-}$	C57BL/6	2/2	125, 127	4/4	169 ± 12	8/8	217 ± 21	0/9	>541[d]
Wild-type	129Sv	2/2	134/134	4/4	163 ± 2	3/3	187 ± 3	4/4	231 ± 6
Wild-type	C57BL/6	5/5	142 ± 1	5/5	155 ± 3	6/6	196 ± 13	6/6	240 ± 5
Wild-type	129Sv × C57BL/6	3/3	132 ± 2	4/4	170 ± 7	4/4	199 ± 5	5/5	234 ± 6

Mice with defects in Fc-γ receptors, immunoglobulin genes and components of the complement system. Although intracerebral inoculation produced disease in all genotypes investigated, the occurrence of scrapie after intraperitoneal exposure to limiting amounts of prions was delayed in *C3*$^{-/-}$ and in *Cr2*$^{-/-}$ mice, and abolished in *C1qa*$^{-/-}$, *H2-Bf/C2*$^{-/-}$ and *C1qa/H2-Bf/C2*$^{-/-}$ mice. IU, infectious units.[a], data previously reported[3]. [b], 10 asymptomatic mice were killed for analysis of spleens and brains at 268–330 d after inoculation. No histopathological signs of scrapie were detected in brain sections (data not shown). [c], 3 asymptomatic mice were killed for analysis of spleens and brains at 270–304 d after inoculation. No histopathological signs of scrapie were detected (data not shown). One additional asymptomatic mouse was killed at 330 d, its brain was homogenized and transmitted to *tga20* transgenic mice intracerebrally (see Methods). No prion disease was found in the latter mice (incubation time > 140 d). [d], 7 asymptomatic mice were killed for analysis of spleens and brains at 272–386 d after inoculation. No histopathological signs of scrapie were detected (data not shown).

naling. On FDCs, it acts to bind complexes of antigen coupled to C3d and C4b: *Cr2*$^{-/-}$ mice display smaller follicular dendritic networks than wild-type mice, and a corresponding reduction in serum levels of the switched immunoglobulin isotypes[17]. It is therefore possible that in *C3*$^{-/-}$ and *Cr2*$^{-/-}$ mice, a decreased number or impaired maturation of FDCs, rather than the actual absence of C3d or its receptor, might be responsible for decreased sensitivity to prions. When challenged with large inocula or at late time points, *Cr2*$^{-/-}$ mice, which have germinal-center histoarchitecture similiar to that of *C3*$^{-/-}$ mice, developed splenic prion titers (Fig. 2*a*) and PrP^{Sc} accumulation (Fig.2*b*) similiar to those of wild-type mice. These findings exclude that the genetic manipulations described here affected the physiology of FDCs to an extent that would impair the capacity of spleens to accumulate prions. Instead, the lack of detectable prion infectivity 38 days after inoculation indicates that CD21/CD35 is involved in prion capturing by FDCs in the earliest phase of the disease and becomes dispensable at a later phase.

As expression of PrP^{C} is rate-limiting for scrapie development[18], a lower level of PrP^{C} on FDCs might be responsible for the delay in incubation periods. However, immunofluorescence analysis of spleens of complement-deficient mice did not show modulation of PrP^{C} expression in FDCs by complement system components (Fig. 3). Despite some variability between the different complement deficient genotypes in the arborization of the follicular dendritic networks and in the number of FDCs, FDCs and PrP immunostains were superimposable. Therefore, deficiencies of complement system components in the mice of the genotypes analyzed did not impact on the expression of PrP^{C} by follicular dendritic cells. This indicates that variations in total PrP^{C} content of complement-deficient spleens (Fig. 2*b*) occurred in non-FDC compartments.

Given the dual role of CD21/CD35, the antiprion effect of its absence might be due to its effect on B-cell signal transduction or on prion trapping on FDCs. Complementation studies[19] showed that CD21/CD35 expression by B-lymphocytes is essential in the formation of a normal secondary response. The susceptibility (Table 1) of *tg*141*m* and *tg*141*m*+*s* transgenic mice to peripheral prion inoculation indicates that a fully developed humoral response might not be needed for peripheral prion pathogenesis, and indicates that expression of CD21/CD35 on FDCs, rather than on B-lymphocytes, is important for prion pathogenesis.

The absence of C1q, alone or in combination with the deletion of Bf/C2, prevented disease completely after inoculation with limiting prion inocula. C1q is a component of the classical pathway of the complement system and can be activated directly by binding to pathogens such as HIV (ref. 20). As the first component in the classic pathway, C1q is necessary for activation of C4 which binds to antigen complexes and mediates uptake in FDCs. Alternatively, C1q may mediate uptake directly via CD35 as proposed for the human receptor[21]. These mechanisms may also be operational in prion diseases.

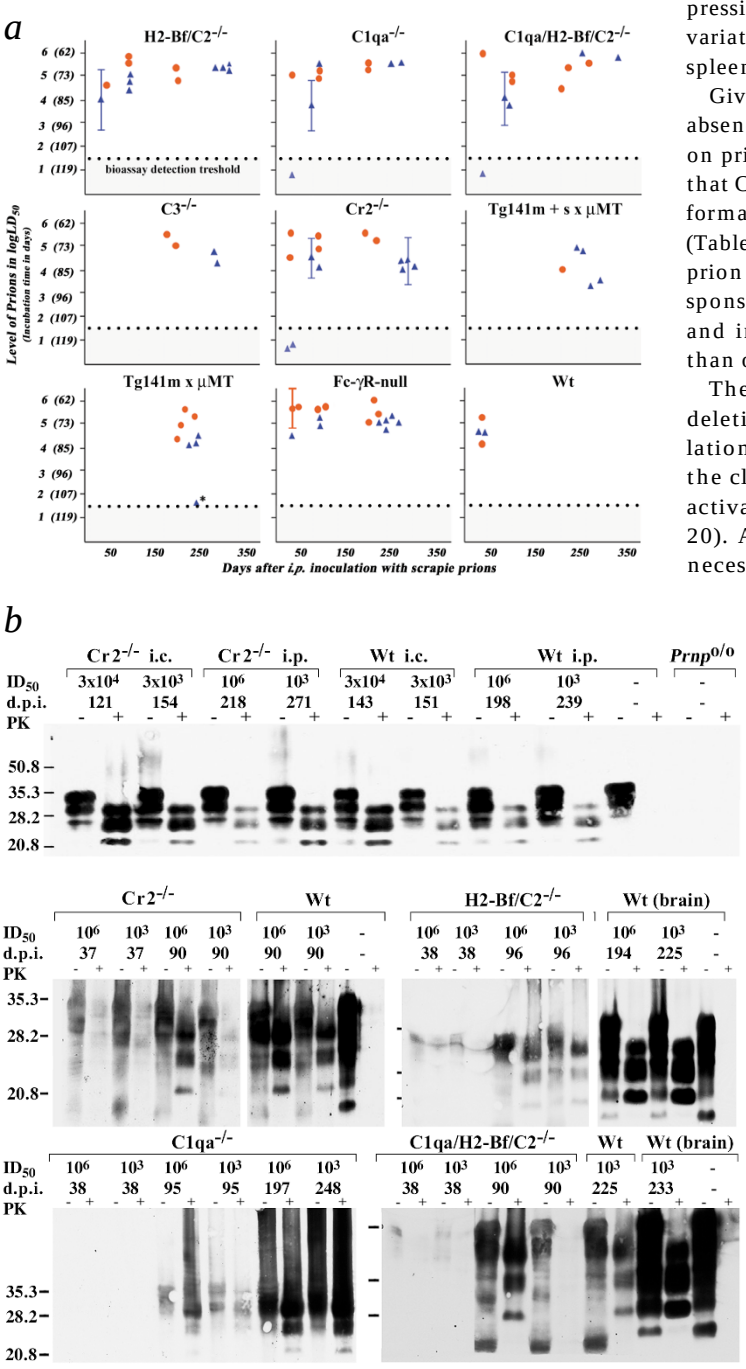

Fig. 2 Prion accumulation in complement-deficient mice. ***a***, Determination of prion infectivity titers. In all cases, 30 µl of spleen homogenates were injected intracerebrally (i.c.) into *tga20* indicator mice (see Methods). ●, results of spleen titrations upon intraperitoneal (i.p.) inoculation with saturating amounts (1×10^6). ▲ ,inoculation with limiting amounts (1×10^3) of scrapie prions. Symbols underneath the dotted line represent prion titers below detection threshold (indicator mice did not develop scrapie). When exposed to saturating levels of prion infectivity, mice of all genotypes accumulated high infectivity titers (4–6 log LD_{50}/g). Exposure to limiting amounts induced high titers at early time points in spleens of wild-type and of Fc-γR–null mice, whereas no infectivity was detectable in *C1qa*$^{-/-}$, *C1qa/H2-Bf/C2*$^{-/-}$ and *Cr2*$^{-/-}$ mice (titers at 35 d.p.i. were not determined in *C3*$^{-/-}$ mice). Within-group standard deviations (n = 4) are indicated only if exceeding ± 0.75 log LD_{50}. [a], 1/4 indicator mice died d.p.i. ***b***, Western-blot analysis of prion protein in spleen and (where indicated) brains of mice lacking complement factors at indicated d.p.i. with indicated amounts of prions. Whereas strong protease-resistant signals of 27–30 kD were visible in all spleens at late time points (≥ 180 d.p.i.), very little or no PrP (refs. 27–30) was present in spleens of *Cr2*$^{-/-}$, *C1qa*$^{-/-}$, *H2-Bf/C2*$^{-/-}$ and *C1qa/H2-Bf/C2*$^{-/-}$ mice 38 d.p.i. with 1×10^3 or 1×10^6 IU of scrapie prions. IU, infectious units.

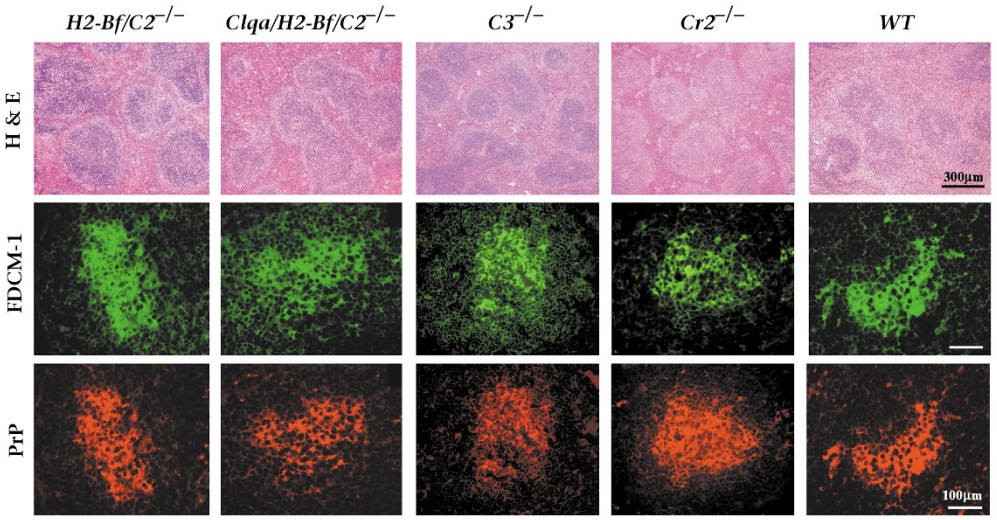

Fig. 3 Immunofluorescence analysis of PrP^C expression in spleen follicles of mice with complement and complement receptor defects. Spleens sections were stained with H&E and photographed at low magnification (upper row). Single follicles were double-immunostained with antibody FDC-M1 against follicular dendritic cells (green) and with antibody XN (red) against PrP. Scale bars: 300 μm (upper row); 100 μm (middle and lower rows).

Our data indicate that activation of the complement cascade and/or events mediated by the CD21/CD35 complement receptor on FDCs are involved in peripheral prion pathogenesis. These complement components exert their action in extracerebral compartments, as mice of all genotypes reported here developed scrapie similarly to wild-type mice upon intracerebral administration of high or low prion inocula (Table 1).

The effects might be indirect; signaling mediated by the binding of C3/C4 breakdown products to the Cr2 receptor may conceivably trigger a specific state of differentiation or activation of FDCs that allows them to accumulate prions—although the role of complement receptors on FDCs is thought to be passive binding rather than signaling. Alternatively, direct opsonization of the infectious agent may enhance its accessibility to germinal centers by facilitating docking to FDCs. If so, C4b may opsonize prions similarly to C3d and therefore partially restore pathogenesis in $C3^{-/-}$ mice which accumulate prion in spleens similarly to wild-type mice, at least when assayed at more than 180 days after inoculation.

Very large prion inocula ($\geq 1 \times 10^6$ infectious units) appear to override the requirement for a functional complement receptor in prion pathogenesis. This is similar to systemic viral infections and coreceptor-dependent retention within the follicular compartment, the necessity of which can be overridden by very high affinity antigens[22] or adjuvants[23]. Additional retention mechanisms for prions might therefore exist in FDCs, which are not complement-dependent, or depend on complement receptors that were not investigated here. The latter possibility would be congruent with the finding that deletion of *Cr2* alone did not confer the same degree of protection—at low infective doses of prions—as deletion of *C1qa* and *H2-Bf/C2*, either alone or in combination. Thus interaction of prion-complement fragment complexes with FDC may involve other receptors in addition to Cr2. A possible candidate is the CR3 complement receptor, also termed CD11b or Mac-1.

Invasion of lymphoid organs by prions occurs almost immediately after inoculation, and significant prion titers can be detected in spleen as early as four days after inoculation. Lymphoinvasion most likely plays an important role in the pathogenesis of nvCJD: prion accumulation in tonsils of nvCJD patients can be detected with 100% sensitivity and specificity[1,24]. We suggest that manipulation of the complement cascade, by inhibiting trapping of antigens in the marginal zone, for example, or by reducing their opsonization, might add to the growing arsenal of interventional options for post-exposure prophylaxis against prion diseases after peripheral exposure to the pathogenic agent.

Methods

Generation and analysis of *tg*141*m* and *tg*141*m*+*s* mice. Gene segments coding for the MMTV envelope-specific and weakly neutralizing Ig heavy-chain V region originated from the B-cell hybridoma 141 (I.X., unpublished data). The VH segment containing the sequence encoding the leader, the rearranged VDJ and a splice donor sequence was amplified with primers containing an *Nco*I site at the 5′ end, and splice donor sites at the 3′ end. The primers used were: 5′-GGGGATATCCACCATGGCTGTCTTGGGGCTGCTCTTCT-3′ and 5′-AGAAGGCCATTCTTACCAGAGG-3′. The fragment was subcloned into genomic expression vectors yielding either the secreted and membrane-bound form (*tg*141*m*+*s*) or the membrane-bound form only (*tg*141*m*) of IgM^a (refs. 25–27). To prepare DNA for microinjection, the μ–heavy-chain transgene was excised using restriction endonucleases *Aat*II and *Xho*I, and injected into male pronuclei of fertilized oocytes of F_1(BALB/c × C57BL/6) mice. Transgene integration and expression were screened by PCR and Southern-blot analysis of tail DNA and by FACS analysis of blood lymphocytes. Transgenic B cells expressed physiological levels of IgM on the cell surface. Mice were crossed and backcrossed to C57BL/6 μMT mice[10]. Serum Ig concentrations were measured by ELISA: microtiter plates were coated with rabbit IgM or IgG_1 against mouse (Zymed, San Francisco, California), and incubated with various dilutions (1:5,000 to 1:50,000) of serum derived from *tg*141*m* or from *tg*141*m*+*s* mice. Signals were visualized with horseradish peroxidase-coupled rabbit IgG/IgM/IgA against mouse (Zymed) and ABTS. *tg*141*m*+*s* μMT mice had IgM^a allotype but did not have any detectable Igs of the IgM^b allotype in the serum. The *tg*141*m* μMT mice expressing only the membrane-bound version of IgM did not have detectable IgM^a nor IgM^b allotype antibodies in the serum.

Inoculation of mice. Mice were inoculated intraperitoneally with 100 μl of brain homogenate containing 3 or 6 log LD_{50} infectious units of Rocky Mountain Laboratory (Hamilton, Montana) (passage 4.1) scrapie prions prepared as described[28]. Mice were killed on the day of onset of terminal clinical signs of scrapie. Titers were determined by incubation time assay[30] using the relationship: y = 11.45 – 0.088x, where y is log LD50 and x is the incubation time in days to terminal disease[31].

Western-blot analysis. Tissue homogenates were adjusted to 5 mg/ml (brain) or 8 mg/ml (spleen) protein and treated with proteinase K (20 μg/ml, 30 min, 37 °C). Fifty μg (brain) or 80 μg (spleen) of total protein of

each sample were electrophoresed through a SDS-PAGE (12%). Proteins were transferred to nitrocellulose by semi-dry blotting. Membranes were blocked with TBST/5% non-fat milk, incubated with antibody 6H4 (brain) or 1B3 (spleen), and developed by enhanced chemiluminescence (Amersham).

Histology, immunocytochemistry and immunofluorescence. 2-μm paraffin or 5–10-μm frozen sections from brain and spleen were stained with hematoxylin/eosin (H&E). Immunostaining for glial fibrillary acidic protein (GFAP) was performed using a rabbit antibody against GFAP (DAKO, Zug, Switzerland), 1:300) and visualized with biotinylated swine serum against rabbit (DAKO, 1:250), avidin-peroxidase (DAKO) and diaminobenzidine (Sigma). The follicular dendritic cell marker FDC-M1 (clone 4C11) was used on frozen sections of spleens (1:300), and visualized by incubation with goat antibody against rat (Milan) and alkaline-phosphatase(AP)-conjugated donkey antibodies against goat with new fuchsin. Two-color immunofluorescence was performed with antibody 4C11 (1:300) and polyclonal antibody against PrP antiserum R340 (ref. 31, 1:200) on frozen acetone fixed spleen sections. For visualization we used secondary fluorescein isothiocyanate-conjugated $F(ab')_2$ fragments of goat immunoglobulins against rat (BioSource International, Camarillo, California), and Alexa 546-conjugated goat Igs against rabbit (Molecular Probes, The Netherlands). For controls, pre-immune sera were used, or primary antibodies were omitted.

Infectivity bioassays. Assays were performed on 1% brain or spleen homogenates[18]. Spleen tissue was homogenized in 320 mM sucrose (1:10) with a microhomogenizer, passed several times through 18-gauge and 22-gauge needles, and diluted 1:10 in PBS/BSA. Once the solution appeared homogeneous, it was spun for 5 min at 500*g*. Supernatants (30 μl) were inoculated intracerebrally into groups of at least four *tga20* mice[29].

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