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Stromal Complement Receptor CD21/35 Facilitates Lymphoid Prion Colonization and Pathogenesis

Mark D. Zabel,2* Mathias Heikenwalder,* Marco Prinz,† Isabelle Arrighi,* Petra Schwarz,* Jan Kranich,* Adriana von Teichman,* Karen M. Haas,‡ Nicolas Zeller,‡ Thomas F. Tedder,‡ John H. Weis,§ and Adriano Aguzzi2*

We have studied the role of CD21/35, which bind derivatives of complement factors C3 and C4, in extraneural prion replication and neuroinvasion. Upon administration of small prion inocula, CD21/35−/− mice experienced lower attack rates and delayed disease over both wild-type (WT) mice and mice with combined C3 and C4 deficiencies. Early after inoculation, CD21/35−/− spleens were devoid of infectivity. Reciprocal adoptive bone marrow transfers between WT and CD21/35−/− mice revealed that protection from prion infection resulted from ablation of stromal, but not hemopoietic, CD21/35. Further adoptive transfer experiments between WT mice and mice devoid of both the cellular prion protein PrPc and CD21/35 showed that splenic retention of inoculum depended on stromal CD21/35 expression. Because both PrPc and CD21/35 are highly expressed on follicular dendritic cells, CD21/35 appears to be involved in targeting prions to follicular dendritic cells and expediting neuroinvasion following peripheral exposure to prions. The Journal of Immunology, 2007, 179: 6144 – 6152.

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases for which no early diagnosis or treatment other than palliation exists. Accumulation of PrPSc, a proteinase K (PK) resistant form of the normal cellular prion protein, PrPc, is common to most instances of TSEs. Interspecies transmission was seen in TSEs both in the field and in the laboratory, and transmission from bovine spongiform encephalopathy-infected cattle to humans has almost certainly occurred (1–5). The infectious agent, termed prion, has been detected in nervous, lymphoid, and muscle tissues (6–12), as well as blood, urine, and saliva (13–20). Chronic wasting disease in cervids and scrapie in sheep appear to be unique among TSEs because of their high transmission efficiency and prevalence (21–24).

PrPSc activates the classical complement pathway (48, 49). Cleavage of PrPSc by complement may mediate FDC trapping of prions. Extracerebral prion accumulation and replication precedes neuroinvasion in many cases of TSEs. The immune system plays an important role in PrPSc neuroinvasion from peripheral sites in murine models of scrapie (25–27). Prion accumulation and replication occur in lymphoid follicles or inflammatory foci containing follicular dendritic cells (FDCs) that express PrPSc (28–32). FDCs are cells of stromal origin that trap immune complexes on their elaborate projections and present them to B cells, with which they closely associate (33–37). They may retain Ag on their cell surfaces for prolonged periods, maximizing presentation to B cells.

FDCs accumulate PrP- immunoreactive material, and depletion of FDCs suppresses lymphoid prion titers. These observations suggest that FDCs replicate prions (30), although this has not been formally proven. Splenic PrPSc accumulation requires B cells that, despite replicating little or no PrPSc themselves, are required for neuroinvasion (38). Because most B cells express little or no PrPSc (39), this requirement presumably relates to B cells supplying FDCs with lymphotroxins necessary for their maturation and maintenance (40–45). The mechanism by which FDCs trap and propagate prions is poorly understood, and it is unknown whether molecules other than PrPSc are involved. Both B cells and FDCs express and interact with components of the complement system shown to be important for prion neuroinvasion and pathogenesis.

The serum complement protein C1q binding immune complexes triggers the classical complement pathway. Mice deficient in C1q exhibit dramatically delayed PrPSc accumulation and onset of terminal disease when inoculated with scrapie prions (46, 47). C1q binds PrPSc in a conformation- and density-dependent manner and PrPSc activates the classical complement pathway (48, 49). Cleavage products of the soluble complement proteins C3 and C4 covalently attach to microbial surfaces and immune complexes, which are then presented as Ags on the surface of B cells and FDCs via the complement receptors CD21/35 (50–52). Pharmacological or genetic ablation of C3 and full-length membrane-bound receptors CD21/35 delays prion pathogenesis (46, 47). These data suggest that complement may mediate FDC trapping of prions.

In this study, we show that complete elimination of the complement proteins that trap Ag on FDCs significantly delays splenic prion
accumulation and terminal prion disease in mice inoculated i.p. with the Rocky Mountain laboratory (RML) strain of prions. Ablation of complement receptors CD21/35 affected prion trapping and disease more profoundly than ablating their ligand sources, C3 and C4, suggesting a role for CD21/35 in peripheral prion pathogenesis independent of their endogenous ligands. To assess the relative importance of CD21/35 on hemopoietic and stromal cell types, we performed reciprocal reconstitution experiments by bone marrow (BM) transplantation. CD21/35 expression exclusively on FDCs in white pulp follicles resulted in prion titers, PrPSc retention, and disease kinetics and severity similar to those of wild-type (WT) mice. CD21/35 expression on hemopoietic cells also significantly affected these parameters, but far less than FDC expression of CD21/35. Therefore, complement-mediated Ag trapping on FDCs is an important mechanism for lymphoid prion accumulation.

Materials and Methods

**Mice**

C57BL/6 × 129sv (B6 129 SF2J), C3−/− and C4−/− (C57BL/6 × 129sv) mice were purchased from The Jackson Laboratory. C3−/− and C4−/− mice were crossed to produce C3/C4−/− mice. TgA20, which overexpress mouse PrP, PrPsc−/− and PrPpro−/− mice have been previously described (53–55). CD21/35−/− and Prnp−/− mice were crossed to produce Prnp−/−CD21/35−/− mice. Breeding and experiments were performed in compliance with the animal experimentation guidelines of the Kanton of Zürich.

**Prion inoculations**

Transgenic and control (C57BL/6 × 129sv) animals were infected i.p. with 100 μl of brain homogenate diluted in 320 mM sucrose containing 3 or 6 log LD50 U of the Rocky Mountain laboratory (RML) scrapie strain passage 5 (RML 5.0), the titer of which was previously assessed by intracerebral (i.c.) inoculation into TgA20 mice and found to be 8.9 log LD50/g of brain tissue. Mice were monitored every other day, and scrapie were diagnosed according to clinical criteria including ataxia, kyphosis, tail rigidity, and hind leg paresis. Mice were sacrificed at the onset of terminal disease.

**Infectivity mouse bioassay (MBA)**

Assays were performed on 1% spleen homogenates. Spleen tissues were homogenized in sterile 320 mM sucrose (1/10) with a RiboLyser (Hybaid), centrifuged for 5 min at 500 × g. Cleared supernatants were diluted 1/10 in sterile 5% BSA in PBS and 30 μl was injected i.c. into each of four TgA20 mice per homogenate (55). Titers were determined using the relationship: ψ = 11.45 × 0.088x, where ψ is log LD50, and x is the incubation time in days to terminal disease (56). Histological and immunohistochemical analyses using H&E, glial fibrillary acidic protein, and SAF84 staining revealed spongiosis, gliosis, and PrP deposition in all scrapie symptomatic mice. PrP signals were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium chloride for 1 h.

**BM reconstitution and FACS analysis**

BM was taken from tibiae and femurs of respective donor groups and ∞107 cells were injected into tail veins of lethally irradiated (1100 rad for 10 min) recipient mice as previously described (60). Six to 8 weeks after reconstitution, 2 × 105 peripheral blood cells from mice of each group were stained at 4°C with 1/100 dilutions of FITC-labeled rat anti-mouse CD21/35 Ab 7G6, PE-labeled rat anti-mouse B220 mAb (BD Pharmingen) or Cy5-labeled mouse anti-mouse PrP mAb (POM-1) in FACS buffer (0.1% BSA, 10 mM EDTA in PBS). RBCs were lysed using FACSlyse solution and the remaining cells analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). Live lymphocytes were gated based on forward scatter and side scatter properties and analyzed for CD21/35, B220, and/or PrP expression using FlowJo software.

**Sodium phosphotungstic acid (NaPTA) precipitation of PrPsc**

Ten percent spleen homogenates were prepared in PBS as described above. Gross cellular debris was removed by centrifugation at 80 × g and 500 μl of supernatant mixed 1:1 with 4% sarkosyl in PBS. Samples were incubated at 37°C for 30 min with 50 μl of NaPTA stock solution (pH 7.4) was added to a final concentration of 0.3% and the sample was incubated at 37°C for 30 min with constant agitation and centrifuged at 13000 g for 30 min at maximum speed in an Eppendorf microcentrifuge. The pellet was resuspended in 30 μl of 0.1% sarkosyl in PBS and digested with 20 μg/ml PK for 30 min at 37°C.

**Immunoblot analysis**

Tissue homogenates were NaPTA precipitated or adjusted to 5 mg/ml protein and 50 μg treated or not with 20 μg/ml PK for 30 min at 37°C. Samples were heated at 95°C for 5 min in SDS-PAGE loading buffer and pipetted into wells of 12% or 4–12% gradient Novex SDS polyacrylamide gels (Invitrogen Life Technologies) and electrophoresed. Proteins were transferred to nitrocellulose membranes (Schleicher-Schuell) by wet blotting, blocked with TBST containing 5% Topblock (Juro) incubated with anti-mouse PrP mAb POM-1 (1/2000) overnight at 4°C. Membranes were washed and incubated with HRP-conjugated rabbit anti-mouse IgG (1:1 h at room temperature. Bands were detected by chemiluminescence (Pierce) and visualized by the VersaDoc imaging system (Bio-Rad). Bands were quantified using QuantityOne software (Bio-Rad).

**Statistical analyses**

One-way ANOVA and Student’s t test were performed where appropriate using the software packages GraphPad Prism and Microsoft Excel.

**Results**

**Absence of CD21/35 or its ligands delays prion disease**

Mice deficient for both C3 and C4 were obtained by serial crosses of C3−/− and C4−/− mice. The resulting C3/C4−/− offspring were

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Table I.  Prion disease progression in complement-deficient mice

<table>
<thead>
<tr>
<th>Host Genotype*</th>
<th>6 Log LD&lt;sub&gt;50&lt;/sub&gt; Incidence&lt;sup&gt;a&lt;/sup&gt; dpi&lt;sup&gt;c&lt;/sup&gt; ± SD</th>
<th>3 Log LD&lt;sub&gt;50&lt;/sub&gt; Incidence dpi ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>8/8 193 ± 10</td>
<td>5/5 276 ± 19&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>C3/4&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>6/6 249 ± 38&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6/6 290 ± 1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD21/35&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>10/10 255 ± 26&lt;sup&gt;e&lt;/sup&gt;</td>
<td>15/20 350 ± 55&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT</td>
<td>8/8 206 ± 8</td>
<td>15/15 238 ± 8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mice were inoculated i.p. with RML 5 prions.
<sup>b</sup> Incidence = Number of terminally sick mice/number of animals inoculated.
<sup>c</sup> dpi, Days postinoculation to terminal disease.
<sup>d</sup> Value of p < 0.05 compared to WT.
<sup>e</sup> Value of p < 0.05 compared to C3<sup>−/−</sup>.
<sup>f</sup> Value of p < 0.01 compared to WT.

inoculated with saturating (6 log LD<sub>50</sub>) or limiting (3 log LD<sub>50</sub>) doses of RML 5 prions i.p. Upon high-dose prion challenge (Table I), C3<sup>−/−</sup> mice contracted terminal prion disease with an incubation time similar to WT controls (193 ± 10 and 206 ± 8 days, respectively, n = 8/group). In contrast, C3/4<sup>−/−</sup> mice contracted prion disease 43 days later than WT controls (249 ± 38 days, n = 6). Upon limiting-dose inoculation, C3<sup>−/−</sup> (276 ± 19, n = 5) and C3/4<sup>−/−</sup> (290 ± 1, n = 6) mice contracted disease 38 and 52 days later, respectively, than WT controls (238 ± 8, n = 15). Taken together, these data indicate an involvement of both C3 and C4 in extraneural prion pathogenesis and neuroinvasion.

We next analyzed the contribution of the CD21/35 receptors. In a previous study, we had used mice that had been originally reported to lack CD21/35 expression (61). However, these mice express a surface-localized, biologically active hypomorphic isoform of CD21/35 (CD21/35<sup>hyp</sup>) at 40% of the level of full-length CD21/35 in WT mice (62). We reported previously that CD21/35<sup>hyp</sup> mice exhibited minimally delayed incubation times after i.p. prion inoculation (46 days) with limiting doses of RML 5 prions (3 log LD<sub>50</sub>) and no delay at saturating doses (46).

Because the above studies used mice that express functional CD21/35 molecules, they may underestimate the contributions of CD21/35 to prion pathogenesis. Therefore, we now used mice completely deficient in CD21/35 expression (54). When inoculated i.p. with limiting doses of prions, 25% of CD21/35<sup>−/−</sup> mice did not contract disease (Table I, n = 20). Those mice that developed scrapie reached the terminal stage of disease (350 ± 55 days, n = 15) 112 days later than congenic WT controls (238 ± 8, n = 8). CD21/35<sup>−/−</sup> mice contracted disease 74 days later than C3<sup>−/−</sup> (276 ± 19, n = 5) and 60 days later than C3/4<sup>−/−</sup> (290 ± 1, n = 6) mice, all of which died from both groups. When inoculated with saturating doses of prions, CD21/35<sup>−/−</sup> mice contracted disease (255 ± 26, n = 10) 49 days later than WT (206 ± 8, n = 8).

We examined terminally sick WT and CD21/35<sup>−/−</sup> mice for characteristic signs of prion neuropathology. Little or no vacuolation, astrogliosis, or PrP<sup>Sc</sup> deposition was evident in sections of hippocampus from asymptomatic CD21/35<sup>−/−</sup> sacrificed 242 days after inoculation (Fig. 1, A–C). In contrast, microvacuolation and astrogliosis were evident in sections from terminally sick WT (Fig. 1, D and E) and CD21/35<sup>−/−</sup> mice (Fig. 1, G and H). Although the pattern of PrP<sup>Sc</sup> deposition was similar, brains of WT mice accumulated more PrP<sup>Sc</sup> (Fig. 1F) than CD21/35<sup>−/−</sup> brains (Fig. 1I).
We quantified the amount of PrPSc in 20 μg of brain homogenate from each group by Western blot (Fig. 1). We detected all three PrP glycoforms, with the expected protease resistance and shift in m.w. of PrPSc after PK digestion. Brain homogenates from terminally sick WT mice contained 15-fold more PrPSc than homogenates from asymptomatic CD21/35−/− mice sacrificed at similar time points, and 4-fold more PrPSc than brains from terminally sick CD21/35−/− mice (Fig. 1K, p < 0.05).

Absence of CD21/35 impaired early splenic prion accumulation
The reduced attack rate or delay in terminal disease of CD21/35−/− mice after i.p. prion challenge could stem from inefficient prion accumulation and replication in the periphery early after infection. We therefore examined prion loads in spleens of WT and CD21/35−/− mice shortly after peripheral prion inoculation. We used the MBA (Fig. 2A) and scrapie cell endpoint assay (SCEPA; Fig. 2B) to determine prion titers of spleens from mice inoculated 15, 30, and 45 days earlier with 3 log LD50 of RML 5.0 prions. Both assays detected prion infectivity in WT spleens at all three time points, but no infectivity at the detection limits for the MBA (1.5 log LD50) and SCEPA (2.4 log LD50) in CD21/35−/− spleens. These data indicate severe impairment of prion accumulation in CD21/35−/− mice soon after infection.

PrPSc colocalized with CD21/35 early after prion infection
Because accumulation of prion infectivity correlated with CD21/35 expression in spleens of mice inoculated i.p. with prions, we investigated whether early accumulation of PK-resistant PrPSc (PrPSc) correlated with CD21/35 expression in splenic follicles. We analyzed WT and CD21/35−/− spleens for PrPSc accumulation by histoblot (59). We detected PrPSc in WT (Fig. 3A), but not

FIGURE 3. PrPSc accumulation correlates with CD21/35 expression at 47 dpi. A, Consecutive sections display splenic follicle (H&E) that contain PrPSc deposits (histoblots) and express both CD21/35 (green) and PrPC (red) in WT mice (IF). B, No PrPSc deposition was detected in splenic follicles expressing PrPC in CD21/35−/− mice. Higher magnifications of the boxed areas are shown below each figure. Scale bars, 200 μm.

FIGURE 4. FACS analysis of PBLs from WT, CD21/35−/−, and BM chimeric mice. PBLs were stained with Abs recognizing the B cell marker B220 and CD21/35, confirming presence or absence of CD21/35 on B cells. Numbers indicate percentage of double-positive cells.
CD21/35−/− (Fig. 3B), spleens at 47 dpi. Accumulation of PrPSc correlated with CD21/35 and PrPSc expression in WT spleens (Fig. 3A, immunofluorescence stain (IF)).

Loss of CD21/35 expression on FDCs delayed prion disease progression

To define the cellular compartment in which CD21/35 is important for prion replication, we prepared reciprocal BM chimeric mice with CD21/35 expression restricted to hemopoietic (WT→CD21/35−/−) mice or stromal (CD21/35−/−→WT mice) compartments (Fig. 4). For control, WT mice were reconstituted with WT BM (WT→WT mice) and CD21/35−/− mice were reconstituted with CD21/35−/− BM (CD21/35−/−→CD21/35−/− mice). We confirmed highly efficient BM engraftment by FACS analysis of PBLs from unmanipulated (WT and CD21/35−/−) and reconstituted mice (Fig. 4). BM from WT mice reconstituted 97% of CD21/35-expressing B cells in irradiated WT (WT→WT, 65%) and CD21/35−/− (WT→CD21/35−/−, 63%) mice compared with WT mice (65%). CD21/35−/− BM reconstitution eliminated 94–97% of CD21/35-expressing B cells in irradiated WT (CD21/35−/−→WT, 5%) mice compared with CD21/35−/− (1%) or CD21/35−/−→CD21/35−/− mice (3%), respectively.

After high- or low-dose prion challenge, control chimeric WT→WT and CD21/35−/−→CD21/35−/− mice displayed differences in incubation times similar to those of unmanipulated WT and CD21/35−/− mice (Table II). At high-dose prion challenge, CD21/35−/−→CD21/35−/− mice contracted terminal disease 61–91 days later (256 and 280, n = 2) than WT→WT mice (189 and 195, n = 2). We observed incomplete attack rates (two of five) and a 106- to 161-day delay of terminal disease at low-dose challenge of CD21/35−/−→CD21/35−/− mice (312 and 367, n = 2) compared with WT→WT mice (206 ± 12, n = 7). At high-dose challenge, WT→CD21/35−/− mice contracted disease 72–78 days later (267 ± 18, n = 8) than WT→WT mice. At low-dose challenge, WT→CD21/35−/− mice contracted disease 128 days later (334 ± 42, n = 8) than WT→WT mice and 80 days later than CD21/35−/−→WT mice (254 ± 22, n = 8). These data confirm the importance of CD21/35 expression on FDCs for facilitating terminal prion disease. Although the delay observed for these mice (334 days), which lack CD21/35 expression on FDCs, is very similar to that observed for control CD21/35−/−→CD21/35−/− mice (312 and 367 days), we observed no decreased incidence (eight of eight) as we did for the controls (two of five). In addition, CD21/35−/−→WT mice, which lack CD21/35 expression on B cells, contracted disease 48 days later (254 days) than WT→WT mice (206 days) at low-dose prion challenge. Delayed disease progression in CD21/35−/−→WT mice was surprising, and may point to a previously unrecognized function for B cell expression of CD21/35 in prion pathogenesis.

FDCs express significant amounts of CD21/35

We attribute the impaired progression of prion disease in WT→CD21/35−/− mice to their lack of CD21/35 expression on

Table II. Prion disease progression in mice differentially expressing CD21/35

<table>
<thead>
<tr>
<th>Donor BM</th>
<th>Host Genotype</th>
<th>CD21 Expression</th>
<th>6 Log LD50 Incidence</th>
<th>3 Log LD50 Incidence</th>
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<tbody>
<tr>
<td>CD21/35−/−</td>
<td>WT</td>
<td>FDCs</td>
<td>4/4 235 ± 35</td>
<td>8/8 254 ± 22</td>
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<td>WT</td>
<td>CD21/35−/−</td>
<td>B cells</td>
<td>8/8 267 ± 18</td>
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<tr>
<td>CD21/35−/−</td>
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<td>none</td>
<td>2/2 256 280</td>
<td>2/5 312 367</td>
</tr>
<tr>
<td>WT</td>
<td>WT</td>
<td>B cells and FDCs</td>
<td>7/7 189 195</td>
<td>7/7 206 ± 12</td>
</tr>
</tbody>
</table>

a BM was isolated from mice of the indicated genotype.

b Hemopoietic systems of lethally irradiated mice of the indicated genotypes were reconstituted with donor BM.

CD21/35 expression was restricted to the indicated cell types for each reconstitution group.

Values of p < 0.05 compared to WT→WT.

Value of p < 0.01 compared to WT→WT.

Value of p < 0.01 compared to CD21/35−/−→WT.

FIGURE 5. FDCs express significant amounts of CD21/35. Immunohistochemical staining of consecutive splenic sections from WT, CD21/35−/−, and CD21/35−/−→WT chimeric mice with Abs recognizing CD21/35 and the FDC marker FDC-M1. B cells and FDCs express CD21/35 in WT (A and B), but not CD21/35−/− (C and D), spleens. Although splenic B cells from CD21/35−/−→WT chimeric mice do not express CD21/35, FDCs from these mice express significant amounts of CD21/35 (E and F) relative to WT spleens. Scale bar, 50 μm. G, Quantification of CD21/35 and FDC-M1 signal intensities in splenic follicles. The ratio of CD21/35:FDC-M1 staining reveals significant CD21/35 expression on B cells and FDCs in WT mice (3.1 ± 1), none in CD21/35−/− mice (0.3 ± 0.3), and CD21/35 expression exclusively on FDCs in CD21/35−/−→WT mice (1.1 ± 0.4).
B220 (data not shown), CD21/35 and the FDC marker FDC-M1 confirmed that WT splenic follicles coexpressed the B cell marker mice to assess CD21/35 expression exclusively by FDCs. IHC from B respectively), whose FDCs lack either PrPC or CD21/35. BM lacking both PrPc and CD21/35 (P3/C and CD21/35 but no PrPSc deposition in splenic follicles lacking CD21/35 in CD21/35−/− → CD21/35−/− (B) and WT→CD21/35−/− (C) mice. Higher magnifications of the boxed areas are shown to the right of each figure. Scale bars, 200 μm.

FDCs. However, because CD21/35-expressing B cells closely associate with FDCs, CD21/35 expression by FDCs has been difficult to ascertain. We analyzed spleens from CD21/35−/− → WT mice to assess CD21/35 expression exclusively by FDCs. IHC confirmed that WT splenic follicles coexpressed the B cell marker B220 (data not shown), CD21/35 and the FDC marker FDC-M1 (Fig. 5, A and B), while CD21/35−/− follicles expressed B220 (data not shown), FDC-M1 but no CD21/35 (Fig. 5, C and D). These data confirm the absence of CD21/35 on CD21/35−/− B cells, the BM progenitors of which we used to reconstitute the hemopoietic compartment in lethally irradiated WT mice (CD21/35−/− → WT). B cells did not express CD21/35, while FDCs expressed substantial amounts of CD21/35 in CD21/35−/− → WT spleens (Fig. 5, E and F). Quantification of the ratio of CD21/35: FDC-M1 signal intensities from IHC in CD21/35−/− → WT splenic follicles (Fig. 5G) confirmed that FDC-M1 staining correlated very closely with CD21/35 (1.1 ± 0.4, n = 27 follicles) in splenic follicles, convincingly demonstrating significant CD21/35 expression by FDCs. The CD21/35:FDC-M1 ratio increased to 3.1 ± 1 in WT follicles (n = 27), most likely due to B cell expression of CD21/35. The CD21/35:FDC-M1 ratio in CD21/35−/− follicles is 0.3 ± 0.3 (n = 29), confirming lack of CD21/35 expression.

Loss of CD21/35 expression on FDCs impairs early splenic PrPSc accumulation and replication

We next investigated whether disease progression correlates with PrPSc deposition and CD21/35 expression in reciprocal BM chimeric mice (Fig. 6). Histoblot analyses revealed significant PrPSc deposition in WT→WT and CD21/35−/− → WT mice at 47 dpi, whose splenic follicles coexpressed PrPc and CD21/35 (Fig. 6, A and D). No PrPSc was detected in CD21/35−/− → CD21/35−/− or WT→CD21/35−/− splenic follicles, which lack CD21/35 expression entirely or on FDCs, respectively (Fig. 6, B and C). Thus, among the BM chimeras, we detected PrPSc only in spleens that express CD21/35 on FDCs, which colocally expressed CD21/35 and PrPc. We detected no PrPSc on FDCs expressing PrPc but not CD21/35.

FIGURE 6. PrPSc accumulation correlates with CD21/35 expression on FDCs at 47 dpi. Consecutive sections display splenic follicles (H&E) that contain PrPSc deposits (histoblots) and express both CD21/35 (green) and PrPc (red) in WT→WT (A) and CD21/35−/− → WT (D) mice (IF), but no PrPSc deposition in splenic follicles lacking CD21/35 in CD21/35−/− → CD21/35−/− (B) and WT→CD21/35−/− (C) mice. Higher magnifications of the boxed areas are shown to the right of each figure. Scale bars, 200 μm.

FIGURE 7. Efficient PrPSc accumulation correlates with CD21/35 expression on FDCs. Western blot analysis of spleens from the indicated reconstitution groups 20 dpi (A and B) or 47 dpi (C and D) with 6 log LD50 RML 5.0 administered i.p. (A and B) PrPSc detection required NaPTA precipitation from 5 mg of spleen from BM chimeric mice (WT→CD21/35−/−, Prnp0/o→CD21/35−/−, CD21/35−/−→Prnp0/o, CD21/35−/−→ WT, and CD21/35−/−→ CD21/35−/−) unless they expressed CD21/35 on both B cells and FDCs (WT→WT, WT→Prnp0/o, and Prnp0/o→ WT mice). C, NaPTA-precipitated PrPSc was not detected 47 dpi in spleens from Prnp0/oCD21/35−/− BM chimeric mice (P−/− C−/− → P−/− C−/− and C−/− → P−/− C−/−) unless they were reconstituted with CD21-1 expressing BM (P−/− → P−/− C−/− and WT−/− → P−/− C−/− mice), indicating CD21/35 capture of PrPSc. D, PrP Sc detection required NaPTA precipitation from spleens of Prnp0/o or CD21/35−/− mice reconstituted with BM lacking both PrPc and CD21/35 (P−/− C−/− → P−/− and P−/− C−/− → C−/− mice, respectively), whose FDCs lack either PrPc or CD21/35. Expression of both CD21/35 and PrPc on FDCs (P−/− C−/− → WT and WT→WT mice) restored efficient capture and replication of PrPSc, which was detected without NaPTA precipitation.
CD21/35 improves PrPSc retention

To examine retention of PrPSc by CD21/35 in more detail, we inoculated high doses of prions i.p. into reciprocal BM chimeric mice with segregated CD21/35 and PrP expression on B cells or FDCs. Twenty days after inoculation, most samples required NaPTA precipitation of PrPSc from 5 mg of spleen for detection (Fig. 7, A and B). Eliminating CD21/35 expression on either B cells or FDCs significantly diminished PrPSc retention (WT→CD21/35−/−, Prnp−/−→CD21/35−/−, WT→CD21/35−/−, CD21/35−/−→Prnp−/−, and CD21/35−/−→CD21/35−/− mice). PrPSc was detected in 50 μg of spleen homogenate without NaPTA precipitation only in samples from mice expressing CD21/35 on both B cells and FDCs (WT→WT, WT→Prnp−/− and Prnp−/−→WT mice).

Spleenic PrPSc detected at 20 dpi is most likely from the initial prion inoculum, the capture of which may be aided by CD21/35. To investigate whether early prion replication might contribute to the detected PrPSc, we inoculated high doses of prions i.p. into BM chimeric mice lacking PrP expression, but retaining CD21/35 expression. We crossed CD21/35−/− and Prnp−/− mice, and performed reciprocal BM reconstitutions with Prnp−/−CD21/35−/−, Prnp−/−CD21/35−/−, or WT mice. We then inoculated these BM chimeras i.p. with 6 log LD50 of RML prions and analyzed their spleens for PrPSc. Because mice lacking PrP cannot replicate prions, any PrPSc detected in their spleen must originate from the original inoculum. At 47 dpi, we detected no PrPSc in spleens from mice lacking both PrP and CD21/35 (Prnp−/−CD21/35−/−→Prnp−/− CD21/35−/−). Spleens lacking PrPSc expression retained PrPSc if CD21/35 was present (Prnp−/−→Prnp−/−CD21/35−/− and Prnp−/−CD21/35−/−→Prnp−/− mouse), especially on FDCs (Fig. 7, C and D). We detected PrPSc in spleens with B cells expressing only CD21/35 (Prnp−/−→Prnp−/−CD21/35−/− mice), but not in spleens with B cells expressing only PrP (CD21/35−/−→Prnp−/−CD21/35−/− mice). This suggests not only that B cells retain PrPSc, but also that they do so more efficiently when they express CD21/35 rather than PrPSc. We detected significantly more PrPSc from spleens with FDCs expressing CD21/35, PrPSc, or both (Fig. 7D). Spleens incapable of prion replication that expressed CD21/35 on FDCs (Prnp−/−CD21/35−/−→Prnp−/− mice) retained PrPSc as efficiently as replication-competent spleens that lacked CD21/35 (Prnp−/−CD21/35−/−→CD21/35−/− mice). In the latter sample, however, we cannot exclude the possibility that prion replication, in addition to retention, by PrPSc-expressing FDCs contributed to the PrPSc content that we detected. Samples from spleens expressing both CD21/35 and PrPSc on FDCs (Prnp−/−CD21/35−/−→WT and WT→WT mice) did not require NaPTA precipitation for detection, strongly implicating FDCs as the primary source of PrPSc accumulation and replication.

Discussion

We investigated the cellular and molecular mechanisms underlying the involvement of FDCs, B cells, the CD21/35 receptors and their ligands in prion accumulation, replication, and disease progression. Simultaneous elimination of C3 and C4 extended the delay to terminal prion disease observed when only C3 was eliminated, suggesting a role for both CD21/35 ligand sources in prion propagation upon i.p. inoculation.

Although mice deficient in both CD21/35 ligand sources exhibit a more significant delay than C3−/− mice (46), they still succumbed to disease significantly earlier than CD21/35−/− mice, a significant number of which did not contract disease at all. These data contrast with data previously reported using mice which had been erroneously reported to lack CD21/35 expression (61). In these mice, a phenomenon known as exon skipping (63) occurred that caused inappropriate splicing of exons 8 and 11, deletion of targeted exons 9 and 10, and creation of an in-frame transcript that produced CD21/35 proteins (CD21/35pro) that are 14 kDa smaller, yet retain domains required for CD19 interaction and ligand binding (62). CD21/35pro mice exhibit modestly delayed incubation times after low-dose i.p. prion inoculation (46). Mice used in the current study possess a bona fide homozygous ablation of the CD21/35 promoter and signal sequence that completely eliminates CD21/35 protein expression (54). After low-dose i.p. prion inoculation, we now found that CD21/35−/− mice exhibited dramatically prolonged incubation and incomplete attack rates when compared with congenic controls. CD21/35−/− mice also presented with significantly delayed prion neuropathology. The role of CD21/35 in prion pathogenesis may therefore be more prominent than previously appreciated.

CD21/35pro and C3−/− mice show similar delays in peripheral prion pathogenesis even though FDCs failed to bind measurable C3 in CD21/35pro mice (61, 64). C4 could compensate for lack of C3 binding on FDCs of CD21/35pro mice. Indeed, C3/4−/− and CD21/35−/− mice exhibited similar delays in prion pathogenesis when inoculated with high doses of prions. However, when inoculated with a lower prion dose, CD21/35−/− mice exhibited decreased incidence and longer delay in disease progression than C3/4−/− mice. Thus, CD21/35 appear capable of enhancing prion disease independent of known endogenous CD21/35 ligands, most likely by enhancing prion retention.

We found substantial prion titers at very early time points in spleens from WT mice inoculated i.p. with prions, but no detectable prion titers in spleens of mice lacking CD21/35. PrPSc correlated with CD21/35 and PrP expression in infectious splenic follicles, presumably on FDCs, which have been convincingly shown to mediate peripheral prion pathogenesis (28–32). We hypothesized that FDCs require CD21/35 expression to maximize prion accumulation and replication. However, FDC expression of CD21/35 has been difficult to evaluate because of their intimate contact with germinal center B cells, which have confirmed CD21/35 expression. We confirmed substantial CD21/35 expression on FDCs by IHC of splenic follicles from BM chimeric mice lacking CD21/35 expression on B cells. These data corroborate FACS data from a recent report documenting CD21/35 expression on purified FDCs from lethally irradiated WT mice (65).

On B cells, CD21/35 function as Ag presentation molecules and as part of an important B cell coreceptor complex with CD19 and CD81 that helps activate B cells. On FDCs, they are believed to act solely as Ag-presentation molecules. To determine its importance on each cell type during a prion infection, we restricted CD21/35 expression to either B cells or FDCs by reconstituting the hematopoietic system of lethally irradiated CD21/35−/− or WT mice with BM from WT or CD21/35−/−, respectively. FDC expression of CD21/35 was required for PrPSc accumulation in splenic follicles early in infection, and resulted in the most expedient terminal prion disease that we observed. From these and previous data, we expected a more prominent role for CD21/35 on FDCs, which is thought to use CD21/35 mainly to trap and display Ag because they lack signaling components of the B cell coreceptor. More surprisingly, eliminating CD21/35 expression on B cells resulted in a significant delay in disease progression when compared with WT mice. Moreover, B cell-restricted CD21/35 expression abrogated the decreased incidence of terminal disease that we observed at low-dose inoculation of prions for mice completely lacking CD21/35. These data point to a previously undiscovered function in prion pathogenesis for this receptor on B cells, the primary role
of which was thought to be to supply lymphotoxins to FDCs because PrPSc expression occurs on only a small subset of B cells (39) and is dispensable to promote disease (43). Circulating B cells may also function as APCs, actively trapping and transporting PrPSc from peripheral sites to lymphoid follicles, a function proposed for other circulating immune cells such as dendritic cells (66, 67) and macrophages (45, 68–70).

Infected assays revealed no detectable prion titers in CD21/35−/− mice even at 15 dpi, suggesting that CD21/35 facilitates prion retention on cells that express it. Using additional BM chimeric mice with segregated CD21/35 and PrPSc expression on B cells or FDCs, we investigated PrPSc retention in more detail. At 20 dpi, we discovered that the most efficient PrPSc trapping required CD21/35 expression on both B cells and FDCs. PrPSc detection in spleens lacking CD21/35 on either cell type required concentrating PrPSc from 100-fold more splenic tissue. We further asked whether the PrPSc that we detected at this very early time point was from residual inocula or nascent PrPSc. We found severe impairment of prion retention in the absence of CD21/35 in spleens incapable of prion replication at 47 dpi. Retention improved dramatically when CD21/35 expression was restored, especially on FDCs. Surprisingly, we even detected inocula in replication-deficient spleens expressing CD21/35 on B cells, but not in spleens expressing PrPSc on B cells but lacking CD21/35. We conclude that CD21/35 retains PrPSc more efficiently than does PrPC. Although CD21/35 improved PrPSc retention on both B cells and FDCs, retention was much more efficient by CD21/35-expressing FDCs. These data confirmed our histoblot experiments, in which FDC retention was much more efficient by CD21/35-expressing FDCs. These data confirmed our histoblot experiments, in which FDC retention was much more efficient by CD21/35-expressing FDCs. These data confirmed our histoblot experiments, in which FDC retention was much more efficient by CD21/35-expressing FDCs. These data confirmed our histoblot experiments, in which FDC retention was much more efficient by CD21/35-expressing FDCs. These data confirmed our histoblot experiments, in which FDC retention was much more efficient by CD21/35-expressing FDCs. These data confirmed our histoblot experiments, in which FDC retention was much more efficient by CD21/35-expressing FDCs. These data confirmed our histoblot experiments, in which FDC retention was much more efficient by CD21/35-expressing FDCs. These data confirmed our histoblot experiments, in which FDC retention was much more efficient by CD21/35-expressing FDCs. These data confirmed our histoblot experiments, in which FDC retention was much more efficient by CD21/35-expressing FDCs. These data confirmed our histoblot experiments, in which FDC retention was much more efficient by CD21/35-expressing FDCs. These data confirmed our histoblot experiments, in which FDC retention was much more efficient by CD21/35-expressing FDCs. These data confirmed our histoblot experiments, in which FDC retention was much more efficient by CD21/35-expressing FDCs.

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