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In most prion diseases, infectivity accumulates in lymphoreticular organs early after infection. Defects in hematopoietic compartments, such as impaired B-cell maturation, or in stromal compartments, such as abrogation of follicular dendritic cells, can delay or prevent lymphoreticular prion colonization. However, the nature of the compartment in which prion replication takes place is controversial, and it is unclear whether this compartment coincides with that expressing the normal prion protein (PrP<sup>c</sup>). Here we studied the distribution of infectivity in splenic fractions of wild-type and fetal liver chimeric mice carrying the gene that encodes PrP<sup>c</sup> (<em>Prnp</em>) solely on hematopoietic or on stromal cells. We fractionated spleens at various times after intraperitoneal challenge with prions and assayed infectivity by bioassay. Upon high-dose challenge, chimeras carrying PrP<sup>c</sup> on hematopoietic cells accumulated prions in stroma and in purified splenocytes. In contrast, after low-dose challenge ablation of <em>Prnp</em> in either compartment prevented splenic accumulation of infectivity, indicating that optimal prion replication requires PrP<sup>c</sup> expression by both stromal and hematopoietic compartments.

Prion diseases are invariably lethal, transmissible neurodegenerative conditions that affect humans and many animal species. The causative infectious agent, termed prion (37), was proposed to be identical with PrP<sup>Sc</sup>, a pathological conformer of the cellular protein PrP<sup>c</sup> encoded by the cellular gene <em>Prnp</em>. While the central nervous system is the only site of histologically discernible damage, PrP<sup>c</sup> is expressed in many other sites, notably including lymphocytes. Intracerebral (i.c.) or peripheral administration of prions to mice causes a rise of infectivity in spleen and in other lymphatic organs long before the development of neurological symptoms and neuropathological changes (21). Moreover, peripheral inoculation routes are likely to initiate most forms of spongiform encephalopathies such as sheep scrapie, bovine spongiform encephalopathy, iatrogenic Creutzfeldt-Jakob disease (CJD) and new-variant CJD. Intraperitoneal (i.p.) inoculation is used extensively to study the pathogenesis of transmissible spongiform encephalopathies because it causes rapid accumulation of infectivity in secondary lymphatic organs (12, 16, 17). The immune system is important for pathogenesis: development of scrapie disease after i.p. inoculation, in contrast to i.c. challenge, is impaired in SCID mice (30) and B-cell-deficient mice (24) and, to a lesser extent, after splenectomy (21). These and many other studies argue for an active role of the lymphoreticular system in the transport of scrapie infectivity from the periphery to the central nervous system.

The question of which compartments within lymphoreticular tissues support prion replication is of immediate relevance to public health: contamination with new-variant CJD prions of germinal centers in lymph node and tonsillar follicles, for example, might call for precautionary measures in handling, usage, and sterilization of surgical instruments. Conversely, infection of germinal center lymphocytes with prions may raise the question of whether these cells carry infectivity into the bloodstream, a question of great importance to transfusion medicine (1).

Here we have investigated the role of various spleen cell subsets in the preclinical phase of mouse scrapie. We demonstrate that—within the potential limitations of experiments with fetal liver chimeras—prion infectivity can be associated with splenic lymphocytes devoid of <em>Prnp</em> and that congruently with previous results (5), chimeras of PrP-deficient hosts with PrP-expressing hematopoietic cells are able to accumulate chronically prions in the spleen for at least 200 days after inoculation. Furthermore, we show that efficient lymphoreticular prion propagation requires PrP<sup>c</sup> in stromal and hematopoietic cells.

**MATERIALS AND METHODS**

**Preparation of the RML standard inoculum.** Rocky Mountain Laboratory (RML; passage 4.1) mouse-adapted scrapie prion inoculum was prepared from brains of terminally sick CD-1 mice (incubation time, 154 ± 12 days) as described previously (10). Brains (11.5 g, total) of terminally sick mice were pooled and homogenized with 1.2- and 0.7-mm syringes in 103.5 ml of sterile 0.32 M sucrose (without heat inactivation or Sarkosyl treatment). This 10% homogenate was defined as the standard RML inoculum. To determine the infectivity titer, serial 10-fold dilutions of our standard inoculum were prepared in sterile phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) and injected i.c. into 4 tg20 indicator mice (15) (30 μl per mouse). The titer of the standard inoculum (7.9 log<sub>10</sub> of 50% lethal dose [LD<sub>50</sub>/ml, corresponding to 8.9 log LD<sub>50</sub>/g of brain tissue] was determined by the 50% endpoint calculation method (42). The relationship <i>y</i> = 11.45 + 0.0886 (x, log LD<sub>50</sub> per milliliter of homogenate; <i>x</i>, incubation time in days to terminal disease) was calculated by linear regression (38). All animal experiments were performed according to the law of the Kanton of Zürich and were approved by the Committee on Animal Experimentation.

**Construction of fetal liver chimeric mice.** Eight-week-old mice were reconstituted with lymphohemopoietic stem cells (LSCs) derived from fetal livers. Timed pregnancies of wild-type and <em>Prnp<sup>−/−</sup></em> mice served to produce mouse embryos. Fetal livers were collected at embryonic day 14.5 to 15.5 in Dulbecco’s modified Eagle’s medium (DME) and dissociated using 1.2- and 0.7-mm syringes. After a...
collected and diluted in DME. FLCs (2 × 10³ to 3 × 10⁶ cells) were injected into the recipient’s tail vein (injection volume, 100 μl). Recipients had been lethally irradiated with 900 rad 24 h earlier. Mice RML inoculum (10⁷ cells) were injected into the recipient’s tail vein (injection volume, 100 μl), depending on the number of total cells. The preparation of total spleen homogenates and splenic cellular and stromal fractions was collected.

Flow cytometric analysis of PBLs of fetal liver cell-reconstituted mice. At various time points (34, 60, 90, 200, and 360 days), groups of two to three animals per experimental setup were inoculated i.p. with 6 or 3 log LD₅₀ of prions. Analyses were done with MoFlo cell sorter (Cytomation). Analyses were done with WinList (Verity Software House, Inc.), and WinMDI 2.8 (Scripps Research Institute; http://facs.scripps.edu) analysis software. PBLs of Prnp⁺/– and of wild-type mice as well as omission of primary antibody and unstained cells served as controls. A total of 66 successfully reconstituted and control mice (Table 1) were inoculated i.p. with 100 μl of RML inoculum containing either 6 or 3 log LD₅₀ of prions.

Preparation of total spleen homogenates and splenic cellular and stromal fractions. To prepare total spleen homogenates, spleen tissue was hand-homogenized in a 1:500 in PBS. Omission of primary antibodies or rabbit preimmune serum served as controls. A total of 66 successfully reconstituted and control mice as recipients and as donors. A total of 66 successfully reconstituted and control mice as recipients and as donors.

**TABLE 1. Prion load of spleens in individual FLC-reconstituted mice**

<table>
<thead>
<tr>
<th>Mice</th>
<th>RML inoculum (log LD₅₀)</th>
<th>Days after inoculation</th>
<th>No. of animals inoculated</th>
<th>Splenic infectivity (log LD₅₀)</th>
<th>No. of indicator animals succumbing to scrapie/no. inoculated (mean incubation time ± SD [days])</th>
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<tbody>
<tr>
<td>Prnp⁺/+ → Prnp⁻/⁻</td>
<td>6</td>
<td>34</td>
<td>2</td>
<td>5.5, &lt;1.5⁺</td>
<td>4/4 (80.8 ± 9.0), 2/8 (133, 135)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>3</td>
<td>3</td>
<td>5.6, 5.0, 4.3</td>
<td>4/4 (80.3 ± 4.5), 4/4 (87.5 ± 9.1), 3/3 (95.7 ± 12.2)</td>
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<tr>
<td></td>
<td>90</td>
<td>3</td>
<td>3</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>200</td>
<td>4</td>
<td>4</td>
<td>5.0, 4.9, 4.0, ND</td>
<td>4/4 (88.8 ± 8.1), 4/4 (89.3 ± 5.9), 4/10 (105.1 ± 9.0), ND</td>
</tr>
<tr>
<td>Prnp⁻/⁻ → Prnp⁺/+</td>
<td>3</td>
<td>34</td>
<td>3</td>
<td>&lt;1.5, &lt;1.5, ND</td>
<td>0/4, 0/3, ND</td>
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<td></td>
<td>60</td>
<td>3</td>
<td>3</td>
<td>&lt;1.5, &lt;1.5, &lt;1.5</td>
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<td>90</td>
<td>2</td>
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<td>Prnp⁻/⁻ → Prnp⁻/⁻</td>
<td>6</td>
<td>34</td>
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<td>&lt;1.5, &lt;1.5, &lt;1.5</td>
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<tr>
<td>Prnp⁻/⁻ → Prnp⁺/+</td>
<td>3</td>
<td>360</td>
<td>3</td>
<td>&lt;1.5, &lt;1.5, &lt;1.5, &lt;1.5</td>
<td>ND, ND</td>
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<tr>
<td>Prnp⁺/+</td>
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<td>360</td>
<td>5</td>
<td>ND</td>
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*If one or more animals survived >180 days, the titer was assumed to be close to the detection limit of the bioassay (<1.5). b ND, not determined. c The development of scrapie disease in these indicator animals is most likely due to residual inoculum of the primary inoculum.
The lymphocyte layer at the interface was recovered, counted, and diluted to the appropriate concentration (2 × 10^7, 5 × 10^7, or 10^8 cells per 30 μl).

**Infectivity bioassays with bga20 indicator animals.** All assays were done with 1% spleen homogenates, spleen cell preparations, stromal homogenates, and WBCs. Whole spleen homogenates (1% w/vol), spleen cells (10^6 cells), stromal fractions (1% w/vol), spleen cell purifications (B cells, T cells, and non-B/T cells; 2 × 10^7 or 10^8 cells per fraction), and WBCs (2 × 10^5 or 10^5 cells per fraction) were inoculated i.c. (30 μl of each) into groups of three or four bga20 indicator mice (15). Animals were monitored for neurological symptoms and killed after development of terminal scrapie. Scrapie disease in indicator mice was confirmed by typical histopathology. The relationship y = 11.45 – 0.088x (y, log LD_{50} per milliliter of homogenate; x, incubation time in days to terminal disease) was used to calculate the level of infectivity (15, 38) within these homogenates. Prion contents are indicated per spleen; spleen weights were calculated according to the determined weight of the stromal fraction, which was considered to be 10% of the weight of a total spleen (13). Spleen cells consist of approximately 80% lymphocytes (30% T cells and 70% B cells) and 20% nonlymphocytic cells (4, 6).

**ELISA for detection of anti-PrP antibodies.** Sera were analyzed by indirect enzyme-linked immunosorbent assay (ELISA). Wells of 384-well plates were coated with 0.105 μg of recombinant PrP_23-235 diluted in 50 μl of PBS overnight at 4°C. After three washes with ELISA buffer (PBS containing 0.1% Tween), the residual binding capacity of the plate was blocked with 100 μl of ELISA buffer containing 5% milk powder for 2 h at room temperature (RT). Extensive washing with ELISA buffer removed the unbound protein. Then the plates were incubated for 2 h at RT with three dilutions of each mouse serum (1:25, 1:75, and 1:225, in 50 μl of ELISA buffer–1% BSA), using three wells for every dilution. Plates were washed four times with ELISA buffer before incubating with the second antibody (horseradish peroxidase-conjugated rabbit anti-mouse IgG-IgA-IgM [H + L], 0.075 μg per well in 50 μl of ELISA buffer–1% BSA for 2 h at RT). The plates were developed with 50 μl of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; 5 mg of ABTS in 50 ml of 0.1 M NaH_2PO_4) [pH 9] at RT). The plates were read with a LAMBDA E ELISA reader (MWG Biotech AG) at 405 nm.

**PCR of splenic fractions.** Analyses were performed postmortem on spleen cells, B cells, T cells, and non-B/T cells. Cells (3 × 10^6) were digested with proteinase K (18 μg/ml) overnight at 55°C in 100 μl of digestion buffer (50 mM KCl, 10 mM Tris HCL [pH 9], 0.4% NP-40, 0.4% Tween 20). Proteinase K was inactivated for 12 min at 75°C, and the samples were centrifuged at 5,000 × g for 12 min. PCR was carried out with primers P3 (ATT CGC AGC GCA TCG CCT TCT ATC GCC) and P3 (ATA ACC GCC TCC CCC AGA AGC CAC GAG) complementary to genomic sequences adjacent to the 3' end of the Prnp reading frame (15). PCR conditions were 94°C for 5 min 30 s, then 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, followed by extension at 72°C for 7 min. Amplification with primer pair P10-3' rec resulted in a 550-bp DNA fragment representing the Prnp* allele; amplification with primer pair P3-3' rec resulted in a 350-bp DNA fragment showing the presence of the Prnp* allele.

**RESULTS**

**Establishment of a standard prion inoculum.** All inoculation experiments described in this study were performed with the RML 4.1 prion inoculum, which was derived by fourfold serial passage in CD-1 mice of a brain homogenate originally donated by S. Prusiner (San Francisco, Calif.). This homogenate originates from the Chandler mouse-adapted scrapie strain. The prion titer of the RML 4.1 inoculum was determined by the endpoint dilution assay. We transmitted serial dilutions (10^{-1} to 10^{-8} in log_{10} steps) of the isolate into bga20 indicator mice (Fig. 1). We calculated the level of infectivity according to the 50% endpoint (42) and found it to be 8.9 log LD_{50}/g of brain tissue. Because the standard inoculum was derived by diluting brain homogenate 1:10 in 0.32 M sucrose, the titer of the inoculum is 7.9 log LD_{50}/ml. The transmission results evidenced a linear relationship between the logarithmic size of inoculum and incubation time over a dilution range of 10^{-2} to 10^{-6} (Fig. 1) according to the following empirically derived formula: y = 11.45 – 0.088x (y, log LD_{50} per milliliter of homogenate; x, incubation time in days to terminal disease) was determined by linear regression (38).

**Expression of PrP* in lymphocyte subsets of fetal liver chimeras.** To produce chimeric mice expressing PrP* on lymphocytes but not on stromal cells such as FDCs, or vice versa, FLCs of the Prnp^0/o or Prnp^{+/+} genotype, were administered intravenously to Prnp^0/o or Prnp^{+/+} mice that had been lethally irradiated. Radiosensitive splenic cells, including FDCs, were expected to display the PrP phenotype of the host (49), whereas cells derived from the incoming LSCs showed the PrP phenotype of the FLC donors. This was studied by flow cytometric analysis of PBLs 6 to 8 weeks after grafting using monoclonal anti-PrP antibody 6H4 (Fig. 2) (28). Reconstitution of Prnp^0/o mice with Prnp^{+/+} FLCs (creating Prnp^{+/+}→Prnp^0/o mice) yielded 35.5% 6H4-positive B lymphocytes and 12.0% 6H4-positive T lymphocytes in peripheral blood, and 6H4 two-color FACS profiles were superimposable to those of wild-type mice. In contrast, wild-type mice reconstituted with PrP deficient FLCs (Prnp^0/o→Prnp^{+/+} mice) exhibited 2.9% 6H4-positive B cells and 0.8% 6H4-positive T cells when the same instrument settings and gates were used (Fig. 2). The latter findings were identical to the staining pattern of Prnp^0/o PBLs and were most probably due to a small amount of nonspecific binding of the 6H4 antibody to lymphocytes.
PrP topography in spleens of scrapie-infected chimeras. Groups of 10 to 15 chimeric mice and corresponding controls (Table 1) were inoculated i.p. with either a saturating dose (1 mg of RML 4.1 brain homogenate diluted in 100 μl 0.32 M sucrose, corresponding to 6 log LD50 as calculated using the data shown in Fig. 1) or a limiting dose (3 log LD50) of RML scrapie prions. At various time points after inoculation (34, 60, 90, 200, and 360 days), two to three mice per group were analyzed. Cryoconserved spleen sections (10 μm in thickness) were stained with antibody FDC-M1 to FDCs (29) and polyclonal serum against PrP and then analyzed by immunofluorescence microscopy. Representative sections were selected for confocal laser scanning microscopy (Fig. 3). In spleens of noninoculated or scrapie-infected wild-type mice (naive or grafted with Prnpo/o FLCs), signals for FDCs and PrP colocalized precisely (Fig. 3, first and fourth rows). FDCs are situated in germinal centers: accordingly, the staining for PrP was confined to these structures. In contrast, Prnpo/o mice grafted with Prnp1/1 FLCs (Prnp1/1→Prnpo/o), which express PrP on LSCs but not on stromal cells, displayed PrP-positive cells both in FDC-M1-positive areas and in FDC-M1-negative compartments that surrounded the germinal centers (Fig. 3, third row). Distribution of prions in spleens of fetal liver chimeric mice. The findings described above indicate that 6H4 immunoreactivity, at least in fetal liver chimeras, is not confined to germinal centers. We therefore set out to determine the nature of 6H4 non-germinal-center cells and assess whether these cells are associated with prion infectivity in addition to PrP immunoreactivity.

Spleens were fractionated into stroma and spleen cells (here defined as cells that can be recovered as a suspension after passing spleens through a mesh). Spleen cells were then separated into B cells, T cells, and a non-B/T-cell fraction. Each fraction was tested for purity by FACS analysis with anti-CD45R (B220) and anti-CD90.2 (Thy1.2) antibodies. In each fraction, purity was >97% of the sorted cell type (Fig. 4A). Cross-contamination of the purified cell fractions (e.g., T cells contaminating the B-cell fraction, or vice versa) could be excluded to a large extent. Whole spleen homogenates and cell subsets were then transmitted i.c. to groups of 3 to 4 tga20 indicator mice. The relationship \( y = 11.45 - 0.088x \) (\( y, \log \text{LD}_{50}/\text{per milliliter of homogenate}; x, \text{incubation time in days to terminal disease} \)) was used to calculate the level of infectivity.
and (8, 38) within these homogenates and, consequently, the distribution of prions within spleens.

In wild-type mice, prions replicate in secondary lymphatic organs and a plateau level of splenic infectivity is reached early after i.p. inoculation (20, 40). In this study, we recovered 5.3 to 6.5 log LD<sub>50</sub> prions per spleen 34 days after inoculation with a saturating (6 log LD<sub>50</sub>) and a limiting (3 log LD<sub>50</sub>) dose (Table 1), indicating that both inoculum sizes were sufficient to establish chronic spleen infection of wild-type mice. In agreement with previous experiments (5) the infectious agent was captured and stored in spleens of Prnp<sup>−/−</sup> mice carrying LSCs from Prnp<sup>−/−</sup> mice (Prnp<sup>−/−</sup>→Prnp<sup>−/−</sup>; Table 1). Prion amounts ranging between 4 and 5.5 log LD<sub>50</sub> per spleen could be measured throughout a time frame of 34 to 200 days after peripheral inoculation with 6 log LD<sub>50</sub>. We observed only one exception: one spleen of a Prnp<sup>−/−</sup>→Prnp<sup>−/−</sup> chimera sacrificed 34 days after scrapie challenge contained very little prion infectivity (Table 1). When prion loads were measured in individual spleen cell fractions, the highest loads were detected in stromal fractions, whereas Prnp<sup>−/−</sup> spleen cells contained only low amounts of prion infectivity (Fig. 4B). The prion load in MACS-sorted splenic B and T cells amounted to only 10- to 100 infectious units and was therefore close to the detection threshold of the assay. In the same experimental group (Prnp<sup>−/−</sup>→Prnp<sup>−/−</sup>), i.p. inoculation with 3 log LD<sub>50</sub> of scrapie prions resulted in noninfectious spleens (Table 1).

In wild-type mice grafted with FLCs derived from Prnp-null (Prnp<sup>−/−</sup>→Prnp<sup>−/−</sup>) mice and inoculated with 6 log LD<sub>50</sub> of primary inoculum, higher splenic infectivity loads than in the reciprocal (Prnp<sup>−/−</sup>→Prnp<sup>−/−</sup>) chimeras were detected at all time points. Infectivity rose to ca. 7 log LD<sub>50</sub> per total spleen 34 days after i.p. challenge and stayed constant throughout the experimental observation time (Table 1). As in Prnp<sup>−/−</sup>→Prnp<sup>−/−</sup> chimeras, in Prnp<sup>−/−</sup>→Prnp<sup>−/−</sup> fetal liver chimeras 3 log LD<sub>50</sub> i.p. failed to produce disease in indicator animals (Table 1). In Prnp<sup>−/−</sup>→Prnp<sup>−/−</sup> chimeras, stroma and PrP-deficient spleen cells contained similar amounts of scrapie prions, as assessed by bioassay with indicator animals (Fig. 4C): infectivity ranged from 4.8 to 5.8 log LD<sub>50</sub> in both fractions. To exclude contamination of spleen cells with components of the stroma which might nonspecifically pollute fractions with prions, we performed PCR analyses of these subsets and transmitted purified B, T, and non-B/T cells to tga20 mice. Whereas B and T cells contained 3.3 and 2.4 log LD<sub>50</sub> 34 days after i.p. inoculation, very low amounts of infectivity could be recovered in the non-B/T cells, as well as in B- and T-cell fractions at later time points (Fig. 4C). PCR analysis of these infectious spleen cells, B cells, T cells, and non-B/T cells confirmed the Prnp<sup>−/−</sup> genotype of these fractions (Fig. 4F). Prions could not be detected in WBCs of any of the reconstituted mice or in WBCs of control mice (Fig. 4B to E).

Prion replication requires Pr<sup>P<sub>e</sub></sup>, and mice deficient in Pr<sup>P<sub>e</sub></sup> are unable to accumulate prion infectivity in the spleen and brain (10, 43). However, scrapie developed in three of four tga20 indicator animals challenged with a whole spleen homogenate derived from a Prnp<sup>−/−</sup> mouse that had been reconstituted with Prnp<sup>−/−</sup> FLCs 34 days after inoculation (Table 1). No infectivity was detected at later time points in any of the mice belonging to the same experimental group. This is in agreement with earlier findings (43) and is most probably explained by the occasional persistence of traces of residual inoculum.

**Detection of anti-PrP antibodies in sera of inoculated mice.**

We searched for anti-PrP antibodies in mice devoid of Pr<sup>P<sub>e</sub></sup> 200 days after inoculation to investigate whether the clearance of the infectious agent might be due to a specific immune response to the RML inoculum. The sera of seven PrP<sup>e</sup>-deficient mice reconstituted with Prnp<sup>−/−</sup> FLCs were assayed. Only one chimera had a titer higher than two times above the background titer. Two additional mice had a titer close to twice the background titer. The remaining four animals did not display detectable anti-PrP antibody titers (data not shown).

**DISCUSSION**

The nature of cells replicating prions in the periphery, and the precise role of Prnp expression in these cells, is still unclear. A likely candidate for a lymphoreticular prion replication site is the FDC (9, 22, 36), but experimental evidence has so far not been conclusive. FDCs are sessile cells located in germinal centers of lymphoid organs which act as antigen traps and support affinity maturation of B lymphocytes. Although their origin remains controversial, there is general agreement that they are not significantly replaced by transfer of FLCs into adult mice (19, 49) but, due to the expression of fibroblast antigens (7), may originate from the fibroblastic reticulum. It has not been possible to recover prions from FDC-deficient

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**FIG. 4.** Distribution of prions in spleens of scrapie-infected mice. (A) FACS analysis of MACS-purified spleen cell fractions originating from scrape-infected mice. MACS sorting resulted in >97% of the sorted cell type; therefore cross-contamination could be excluded to a large extent. Anti-CD45R (B220) antibodies were used to label B cells, and anti-CD90.2 (Thy 1.2) was used for T cells. Gating for lymphocytes by forward and side scattering did not interfere with the readout. (B to E) Infectivity levels in spleen fractions and WBCs of FLC-reconstituted and control mice. Fractions were transmitted i.c. in groups of three to four tga20 indicator mice. Symbols: blue circles, spleen cells; blue triangles, B cells; blue inverted triangles, T cells; blue crosses, non-B/T cells; red crosses, stromal fraction; black rhombi, WBCs. Within-group standard deviations are given. The nature of cells replicating prions in the periphery, and the precise role of Prnp expression in these cells, is still unclear. A likely candidate for a lymphoreticular prion replication site is the FDC (9, 22, 36), but experimental evidence has so far not been conclusive. FDCs are sessile cells located in germinal centers of lymphoid organs which act as antigen traps and support affinity maturation of B lymphocytes. Although their origin remains controversial, there is general agreement that they are not significantly replaced by transfer of FLCs into adult mice (19, 49) but, due to the expression of fibroblast antigens (7), may originate from the fibroblastic reticulum. It has not been possible to recover prions from FDC-deficient

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spleens derived, e.g., from mice deficient in tumor necrosis factor alpha (TNF-α/−/− mice) (33), nor could PrPSc be visualized in spleens of lymphotxin β-deficient (LTβ−/−) mice (35). Notwithstanding the strong experimental evidence that FDCs are involved in prion pathogenesis, there are several findings that point to an additional role of lymphocytes in prion neuroinvasion after peripheral infection. While the presence of B cells is crucial for neuroinvasion after peripheral challenge (24), the main role of B cells in pathogenesis may consist of supporting FDCs by providing lymphotxin signaling. In tumor necrosis factor receptor 1-deficient (TNFRI−/−) mice, which lack mature FDCs in their spleens (31), development of cerebral disease after peripheral challenge with RML scrapie is unaffected (24), and spleens of TNFRI−/− mice contain only traces of infectivity (M. A. Klein, F. Montrasio, M. Prinz, and A. Aguzzi, unpublished results). In addition, it was reported that LTβ−/− mice, which suffer from a similar defect in FDC maturation (26), may be as susceptible to infection with CJD prions as wild-type mice, although pathologic PrP could not be visualized in spleens and lymph nodes (35). However, the latter study may not be fully representative, since no transmission studies of spleens or lymph nodes were reported. Further, in vivo ablation of FDCs by administration of a soluble lymphotxin β receptor, while efficiently preventing the buildup of a splenic prion burden (36), does not fully shelter the brain from neuroinvasion (32, 36).

In view of the uncertainties delineated above, we first assessed whether prions actively replicate, or just accumulate, at peripheral sites after i.p. infection. Regardless of the dose of the primary inoculum administered (6 or 3 log LD50 i.p.), wild-type spleens contained 5 to 7 log LD50 of scrapie infectivity at early time points after peripheral challenge (Table 1), yielding strong evidence for peripheral prion replication. This is in line with the data recorded in transgenic mouse lines showing peripheral replication of a scapie agent in mice (41).

To investigate the significance of the PrP status of immune cells during peripheral pathogenesis of scrapie disease, we constructed reciprocal fetal liver chimeras with wild-type (Prnp+/+) and with a genetically altered mouse line of the Prnp−/− genotype. A limiting dose of prions (3 log LD50) never produced detectable levels of prion infectivity in spleens of animals with compartment-restricted Prnp expression, indicating that these chimeras were not able to effectively replicate or store the infectious agent. We therefore conclude that expression of PrP+ both in LSCs and in a sessile, radioresistant cellular compartment (most likely consisting of FDCs) is needed for efficient splenic capture and replication of prion infectivity.

Spleens with PrP+ expression confined to LSCs are able to chronically accumulate prions over a time course of at least 200 days after i.p. inoculation with a saturating dose of prions (6 log LD50). These findings confirm and extend our previously published experiments in which long-term prion persistence was detected in Prnp−/− mice bearing a Prnp+/+ brain graft and Prnp−/− hematopoietic cells (5). Therefore, PrP+ expression by the host is not absolutely required for splenic prion accumulation. This result is in contrast with the findings reported by Brown et al. (9), who performed similar experiments with ME7 prions, and may point to differences in the cellular tropism of different prion strains.

Astonishingly, the bulk of prion infectivity in Prnp+/+ → Prnp−/− chimeras was located within the stromal compartment, even if the Prnp+/+ genotype was confined to hematopoietic cells. At least two hypotheses could explain this finding: (i) Prnp-deficient FDCs could acquire PrP+ or prions from LSCs, or (ii) the Prnp+/+ → Prnp−/− FLC reconstitution could have induced development of Prnp+ FDCs from hypothetical precursors within FLCs (19, 49), which may acquire and replicate prion infectivity. We consider the second hypothesis to be improbable since previous analyses do not indicate that FDCs can be efficiently reconstituted by FLCs (19, 49). Conversely, after i.p. challenge with a saturating dose of prions (6 log LD50), spleens of wild-type mice grafted with Prnp−/− FLCs contained 6.1 to 7.2 log LD50 of infectivity. The prion loads of spleen cells and stromal fractions were similar, i.e., 5.0 to 6.0 log LD50. Therefore, Prnp-deficient spleen cells (whose pure Prnp−/− genotype was confirmed by PCR analysis) were able to transmit relatively high infectivity titer to indicator animals. When separated B, T, and non-B/T cells of the same chimeras were transmitted, the resulting findings were consistent with this PrP-independent association of infectivity in spleen cells: highly purified B and T lymphocytes of Prnp−/− origin were able to infect indicator animals. Consequently, splenic lymphocytes of mice infected i.p. with large amounts of prions do not require a Prnp+ allele in order to transmit prions to indicator animals.

The mechanism of chronic splenic infection in mice with compartment-restricted expression of Prnp after challenging with a saturating dose of prions remains unclear. It is possible that under these circumstances prions undergo very slow splenic replication and reach an equilibrium with elimination of infectivity. PrP+ is a glycoprophatidylinositol-anchored protein (46), and since these proteins can easily transfer from one prion to another (3, 27), it is conceivable that “painting” of the Prnp−/− compartment by PrP+-positive cells results in acquisition of a PrP+-positive phenotype and, possibly, in restored support of low-level prion replication.

Alternatively, the original inoculum may be chronically stored: other antigens, such as human immunodeficiency virus type 1 virions (44, 45) and human serum albumin (47), are known to be retained on the plasma membrane of FDCs for months or even years (23, 34, 48). The immunological or cellular mechanism of such retention may be independent of PrP+: molecules acting as partners of antigen presentation like complement factors (25) or Fcγ receptors (11) are likely to be involved. In fact, it has been recently reported that disease-specific PrPSc is detectable ultrastructurally on the surface of FDCs, where it colocalizes with complement and Fcγ receptors (18).

The mismatch between the level of infectivity in spleen cells and the sum of the corresponding subpopulations in all experimental groups is most probably due to the extensive washing procedure associated with MACS and could represent a consequence of the interaction between lymphocytes and prions. Therefore, the association of prions with lymphocytes may be rather loose.

After inoculation with 4.5 log LD50 of prions (30 times less than in the present report), infectivity was associated with spleen cells expressing PrP+ but not with Prnp−/− spleen cells (40). The present results indicate that upon a primary i.p. inoculation with 6 log LD50, prions were associated with spleen
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