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Abstract

Variant Creutzfeldt-Jakob disease and scrapie are typically initiated by extracerebral exposure to prions, and exhibit early prion accumulation in germinal centers. Follicular dendritic cells (FDCs), whose development and maintenance in germinal centers depends on tumor necrosis factor (TNF) and lymphotoxin (LT) signaling, are thought to be indispensable for extraneural prion pathogenesis. Here, we administered prions intraperitoneally to mice deficient for TNF and LT signaling components. LT alpha(-/-), LT beta(-/-), LT betaR(-/-), and LT alpha(-/-) x TNFalpha(-/-) mice resisted infection and contained no infectivity in spleens and lymph nodes (when present). However, TNFR1(-/-), TNFR2(-/-), and some TNFalpha(-/-) mice developed scrapie similarly to wild-type mice. High prion titers were detected in lymph nodes, but not spleens, of TNFR1(-/-) and TNF alpha(-/-) mice despite absence of FDCs and germinal centers. Transfer of TNFR1(-/-) fetal liver cells into lethally irradiated Prnp(0/0) mice restored infectivity mainly in lymph nodes. Prion protein (PrP) colocalized with a minority of macrophages in tumor necrosis factor receptor (TNFR) 1(-/-) lymph nodes. Therefore, prion pathogenesis can be restricted to lymphoreticular subcompartments, and mature follicular dendritic cells are dispensable for this process. Macrophage subsets are plausible candidates for lymphoreticular prion pathogenesis and neuroinvasion in the absence of FDCs, and may represent a novel target for postexposure prophylaxis.
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Lymph nodal prion replication and neuroinvasion in mice devoid of follicular dendritic cells

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Variant Creutzfeldt-Jakob disease and scrapie are typically initiated by extracerebral exposure to prions, and exhibit early prion accumulation in germinal centers. Follicular dendritic cells (FDCs), whose development and maintenance in germinal centers depends on tumor necrosis factor (TNF) and lymphotoxin (LT) signaling, are thought to be indispensable for extraneural prion pathogenesis. Here, we administered prions intraperitoneally to mice for TNF and LT signaling components. LTα−/−, LTβ−/−, LTβR−/−, and LTα−/− × TNFα−/− mice resisted infection and contained no infectivity in spleens and lymph nodes (when present). However, TNFR1−/−, TNFR2−/−, and some TNFα−/− mice developed scrapie similarly to wild-type mice. High prion titers were detected in lymph nodes, but not spleens, of TNFR1−/− and TNFα−/− mice despite absence of FDCs and germinal centers. Transfer of TNFR1−/− fetal liver cells into lethally irradiated Prnp0/0 mice restored infectivity mainly in lymph nodes. Prion protein (PrP) colocalized with a minority of macrophages in tumor necrosis factor receptor (TNFR) 1−/− lymph nodes. Therefore, prion pathogenesis can be restricted to lymphoreticular subcompartments, and mature follicular dendritic cells are dispensable for this process. Macrophage subsets are plausible candidates for lymphoreticular prion pathogenesis and neuroinvasion in the absence of FDCs, and may represent a novel target for postexposure prophylaxis.

Prion diseases are lethal, transmissible neurodegenerative conditions. The causative agent (1) was proposed to be identical with prion protein Sc (PrPSc), a pathological conformer of the cellular protein PrPc encoded by the cellular gene Prnp. PrPc is expressed in many sites, notably including secondary lymphoid organs. Peripheral inoculation routes are likely to initiate most forms of spongiform encephalopathies such as scrapie. The Creutzfeldt-Jakob disease (CJD) and variant CJD (vCJD) cases can be attributed to this process. Also, intracerebral (i.c.) or peripheral administration of prions to mice induces a rise of infectivity in spleen and in other lymphoid organs long before the development of neurological symptoms and neuropsychological changes. Intrapерitoneal inoculation has been extensively used to study the pathogenesis of transmissible spongiform encephalopathies because it causes rapid accumulation of infectivity in secondary lymphoid organs (2–4). The question of which compartments within lymphoreticular tissues support prion replication is of relevance to public health: contamination with vCJD prions of germinal centers in lymph nodal and tonsillar follicles, for example, might call for precautionary measures in handling and sterilization of surgical instruments.

Tumor necrosis factor (TNF) and lymphotoxin (LT) α signal through TNF receptor (TNFR) 1, whereas membrane-bound LTα/β heterotrimeric signal through LTβ receptor (LTβR) (5). TNFR1−/− and LTβR−/− signaling is necessary for development and maintenance of secondary lymphoid organs (6–10). LTβR signaling is also required for maturation and maintenance of follicular dendritic cells (FDCs), which are thought to be essential for prion replication and for accumulation of disease-associated PrPSc within secondary lymphoid organs. Inhibition of the LTβ signaling pathway with a soluble receptor, which depletes FDCs (11), abolishes prion replication in spleens and prolongs the latency of scrapie after i.p. challenge (12). B cell-deficient μMT mice (13) are resistant to prions i.p. (14), perhaps because of impaired FDC maturation (12, 15). In addition to FDCs, PrPSc-expressing hematopoietic cells are required for efficient lymphoreticular prion propagation (16, 17).

Here, we studied peripheral prion pathogenesis in mice lacking TNFα, LTα/β, or their receptors. We report that ablation of LTβR signaling prevents all peripheral pathogenesis, whereas ablation of TNFR1 signaling prevents prion pathogenesis in spleen but not in lymph nodes, despite the absence of FDCs.

Materials and Methods

Inoculation of Mice. Mice were inoculated i.p. with 100 μl of brain homogenate containing 3–6 log LD50 units of the Rocky Mountain laboratory (RML) scrapie strain (passage 4.1) prepared as described (18). Mice were monitored every second day, and scrapie was diagnosed according to standard clinical criteria. Mice were killed on the day of onset of terminal clinical signs of scrapie.

Infectivity Bioassays with tga20 Indicator Mice. Assays were done on 1% spleen or lymph node homogenates. Spleens of one individual mouse, or inguinal, mesenteric, and superficial cervical lymph nodes pooled from three animals from each experimental group, were collected. Lymph nodes from every individual mouse were pooled for the experiments with fetal liver cell (FLC)-reconstituted chimeric mice. Tissues were homogenized in PBS/5% BSA with a microhomogenizer and passed several times through 18-gauge and 22-gauge needles. When the suspension appeared homogenous, it was spun for 5 min at 500 × g. Supernatants (30 μl) were inoculated i.c. into groups of four tga20 mice (19). Indicator mice were killed after development of terminal scrapie; the relationship y = 11.45 − 0.088x (y, log LD50/ml of homogenate; x, incubation time in days to terminal disease) was used to calculate infectivity titers (20).

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 micrograms (brain) or 80 μg (spleen) of total protein of each sample were electro-
phoresed through an SDS/PAGE gel (12%). Proteins were transferred to nitrocellulose by semidy blotting. Membranes were blocked with TBST/5% nonfat milk, incubated with antibodies 6H4 (brain) or 1B3 (spleen), and developed by Tris-buffered saline + Tween 20 (0.05%)/5% nonfat milk enhanced chemiluminescence (ECL, Amersham Pharmacia) as described (12).

**Immunohistochemistry and Immunofluorescence.** Ten-micrometer frozen sections of spleens or lymph nodes were immunostained with the follicular dendritic cell marker FDC-M1 (clone 4C11), CD35 (8C12, PharMingen), peanut agglutinin (PNA), or the pan-B cell marker anti-CD45RO/B220 (RA3-6B2, PharMingen) as previously described (21).

Two-color confocal analysis was performed with antisera to PrP and ER-TR9, MOMA-1, or F4/80 on frozen acetone-fixed tissue sections. For visualization we used secondary FITC-conjugated anti-rabbit IgG and ALEXA 546-conjugated F(ab')2 fragments of goat immunoglobulins against rat (BioSource, Camarillo, CA) and ALEXA 546-conjugated goat anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands). For controls, preimmune sera were used, or, if this option was unavailable, primary antibodies were omitted.

**Construction of Fetal Liver Chimeric Mice.** Eight- to ten-week-old mice were reconstituted with lymphohemopoietic stem cells (LSCs) derived from fetal livers. Timed pregnancies of wild-type, TNFR1−/− and Prnp−/− mice served to produce mouse embryos. Fetal livers were collected and diluted in DMEM. FLCs (5 × 103 cells) were injected into tail veins of recipients that had been lethally irradiated (1100 rad) 24 h earlier. Six to seven weeks after grafting, successful reconstitution was assessed by PCR analysis of peripheral blood taken from the retroorbital plexus for the presence or absence of the Prnp or TNFR1 gene locus as described earlier (17).

**Results**

After i.c. inoculation, all treated mice developed clinical symptoms of scrapie with incubation times similar to those of control mice (Table 1). In all genotypes, topography and intensity of spongiosis and gliosis were similar to wild-type mice (not shown), indicating that TNF/LT signaling is irrelevant to cerebral prion pathogenesis. Upon i.p. prion challenge, wild-type mice showed an inverse logarithmic correlation between inoculum size (3–6 log LD50) and incubation time (ref. 20; Table 1). Instead, mice defective in LT signaling proved virtually noninfectible with 3 log LD50 (Table 1). Although these mice occasionally developed clinical disease when challenged with an extremely large inoculum (6 log LD50), latency was frequently longer than in wild-type mice (Table 1). No PrPSc was detected in brains (Fig. 1A) and spleens (Fig. 1C) of clinically healthy LTα−/−, LTβ−/−, LTβR−/−, or LTα/−/TNFRα−/− mice 450 days postinfection (dpi), indicating that defects in the LTα/LTβ pathway prevent establishment of subclinical disease (22).

In contrast, TNFR1−/− mice were almost fully susceptible to all inoculum sizes, albeit with somewhat longer incubation times than wild-type mice (Table 1). TNFα−/− mice showed dose-dependent susceptibility: 6/7 mice developed scrapie upon inoculation with 6 log LD50, whereas 2/7 responded to 3 log LD50 (Table 1). Disease latency in those TNFα−/− mice that developed scrapie after 3–4 log LD50 was vastly prolonged (Table 1). Immunoblot analysis confirmed PrPSc accumulation in brains of terminally sick TNFR1−/− and TNFα−/− mice (Fig. 1B).

All TNFR2−/− mice had intact FDCs and germinal centers (not shown) and developed scrapie (Table 1), PrPSc accumula-
groups are drawn only when exceeding compared with TNFR2 incubation time was over 120 days, titer was assumed to be close to the none of the four indicator mice developed inoculation, most probably because of i.c. bleeding after injection. (j and k) Two separate transmissions, one of the four indicator mice died 24 h after infection by prions below detection limit (none of the four indicator mice developed infection, most probably because of i.c. bleeding after injection). (j

ules electrophoresed natively (A and B) Western blots of spleen homogenates or after digestion with proteinase K (PK; C). Large amounts of PK-resistant prion protein (PrPSc) were detected in the brain of all mice that had developed scrapie (terminal sick), independently of the genotype. Clinical healthy mice deficient in LT signaling showed no PrPSc accumulation, excluding subclinical scrapie (A). (C) Western blots of spleen homogenates electrophoresed natively (–) or after digestion with proteinase K (PK) (+). TNFR1−/− and TNFα−/− mice accumulated lower amounts of PrPSc as compared with TNFR2−/− and wild-type mice. No PrPSc was detected in the spleens of clinical healthy LT-deficient mice. (D) Prion infectivity in lymphoid organs. Mice were inoculated i.p. with 6 log LD50 or 4 log LD50 of scrapie prions as indicated. Standard deviations within groups are drawn only when exceeding ± 0.75 log LD50 (a and b) in each of the two separate transmissions, one of the four tgα20 mice died 24 h after inoculation, most probably because of i.c. bleeding after injection. (j and k) Intercurrent death during incubation time. Symbols on the abscissa indicate prion titers below detection limit (none of the four indicator mice developed scrapie). If one or more indicator mice survived >180 dpi, or the mean incubation time was over 120 days, titer was assumed to be close to the detection threshold of the bioassay. For these samples (labeled with small letters), the numbers of animals succumbing to scrapie of four inoculated mice were as follows: c, 1/4 (111); d, 3/4 (93, 117, 132); e, 2/4 (90, 95); f, 1/4 (126); g, 3/4 (112, 123, 128); h, 4/4 (106, 115, 124, 136); i, 3/4 (89, 102, 119); j, 4/4 (115, 115, 126, 135); m, 1/4 (98); n, 2/4 (74, 78); o, 2/4 (125, 151); p, 2/4 (131, 193); q, 2/4 (119, 125, 152); r, 2/4 (109, 117); s, 1/4 (108); t, 1/4 (71); u, 3/4 (80, 96, 98); and v, 2/4 (98, 98).

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Fig. 1. Western blot analysis of brains and spleens, as well as determination of prion infectivity titers in spleens and lymph nodes of scrapie-challenged TNF- and LT-deficient mice. (A and B) Western blots of brain material electrophoresed natively (–), or after digestion with proteinase K (PK; +). Large amounts of PK-resistant prion protein (PrPSc) were detected in the brain of all mice that had developed scrapie (terminal sick), independently of the genotype. Clinical healthy mice deficient in LT signaling showed no PrPSc accumulation, excluding subclinical scrapie (A). (C) Western blots of spleen homogenates electrophoresed natively (–) or after digestion with proteinase K (PK) (+). TNFR1−/− and TNFα−/− mice accumulated lower amounts of PrPSc as compared with TNFR2−/− and wild-type mice. No PrPSc was detected in the spleens of clinical healthy LT-deficient mice. (D) Prion infectivity in lymphoid organs. Mice were inoculated i.p. with 6 log LD50 or 4 log LD50 of scrapie prions as indicated. Standard deviations within groups are drawn only when exceeding ± 0.75 log LD50 (a and b) in each of the two separate transmissions, one of the four tgα20 mice died 24 h after inoculation, most probably because of i.c. bleeding after injection. (j and k) Intercurrent death during incubation time. Symbols on the abscissa indicate prion titers below detection limit (none of the four indicator mice developed scrapie). If one or more indicator mice survived >180 dpi, or the mean incubation time was over 120 days, titer was assumed to be close to the detection threshold of the bioassay. For these samples (labeled with small letters), the numbers of animals succumbing to scrapie of four inoculated mice were as follows: c, 1/4 (111); d, 3/4 (93, 117, 132); e, 2/4 (90, 95); f, 1/4 (126); g, 3/4 (112, 123, 128); h, 4/4 (106, 115, 124, 136); i, 3/4 (89, 102, 119); j, 4/4 (115, 115, 126, 135); m, 1/4 (98); n, 2/4 (74, 78); o, 2/4 (125, 151); p, 2/4 (131, 193); q, 2/4 (119, 125, 152); r, 2/4 (109, 117); s, 1/4 (108); t, 1/4 (71); u, 3/4 (80, 96, 98); and v, 2/4 (98, 98).
that efficient lymphoreticular prion propagation required PrP c expression in both stromal and hematopoietic compartments, in our hands an extremely consistent finding (16, 17).

Two-color immunofluorescence evidenced strong PrP signals in scrapie-infected TNFR1−/− (Fig. 3A) and TNFa−/− (not shown) lymph nodes in the absence of germinal centers and mature FDCs. In wild-type mice, PrP colocalized mainly with FDC networks, but strong PrP immunoreactivity was also detected in areas devoid of FDC-M1+ cells, suggesting that cells other than FDCs may accumulate PrPsc in lymph nodes (Fig. 3A, arrowheads). Indeed, PrP colocalized with the macrophage marker ER-TR9 and MOMA-1 in wild-type lymph nodes (Fig. 3A). No bright PrP signal was detected in secondary lymphoid tissues of LTβ−/− and Prnp0/0 mice, concordantly with the finding that these tissues lacked prion infectivity.

What is the nature of the compartment responsible for the high infectivity of TNFR1−/− and TNFa−/− lymph nodes? The above results indicate that this compartment does not consist of mature FDCs, but that both hematopoietic and stromal cells are involved. Confocal two-color immunofluorescence analysis revealed intense PrP signals colocalizing with F4/80-, ER-TR9-, and MOMA-1-positive cells (Fig. 3B) in lymph nodes and, less frequently, in spleens of TNFR1−/− and TNFa−/− mice. No colocalization was found with follicular dendritic cell markers (IDC-M1, FDC-M2, and CD35), T cell markers (CD3, CD4, and CD8), B cell markers (B220 and CD35), dendritic cell marker (NLDC-145), natural killer cell marker (Pan-NK), and endothelial cell marker (CD54). As expected, lymphoreticular organs devoid of prion infectivity (LTβ−/− and Prnp0/0) displayed no bright PrP immunoreactivity (Fig. 3 A and B).

**Discussion**

We show here that deletion of TNFR1 or TNFα preserves susceptibility to peripheral prion challenge, whereas deletion of LT signaling components confers high resistance to peripheral prion infection. These differential effects on scrapie susceptibility are surprising, because all defects (except TNFR2−/−) abolish FDCs, which were thought to be crucial for pathogenesis. However, LT/TNF family members play distinct roles in lymphocyte and stromal cell compartments.

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**Table 2. Prion load of spleens and lymph nodes in individual FLC-reconstituted chimeric mice**

<table>
<thead>
<tr>
<th>Chimera</th>
<th>Spleen titrations</th>
<th>Lymph node titrations</th>
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<tbody>
<tr>
<td></td>
<td>Infectivity (log LD50)</td>
<td>Attack rate (mean ± SD, days)</td>
</tr>
<tr>
<td>Wild-type → Prnp0/0</td>
<td>3.5</td>
<td>4/4 (91 ± 6.6)</td>
</tr>
<tr>
<td></td>
<td>3.4</td>
<td>4/4 (93 ± 5.8)</td>
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<tr>
<td></td>
<td>3.6</td>
<td>4/4 (89 ± 5.9)</td>
</tr>
<tr>
<td>Prnp0/0 → wild-type</td>
<td>5.7</td>
<td>4/4 (66 ± 8.4)</td>
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<tr>
<td></td>
<td>4.6</td>
<td>4/4 (78 ± 4.3)</td>
</tr>
<tr>
<td></td>
<td>5.7</td>
<td>3/3 (65 ± 4.0)</td>
</tr>
<tr>
<td>TNFR1−/− → Prnp0/0</td>
<td>&lt;1.5</td>
<td>1/4 (105)</td>
</tr>
<tr>
<td></td>
<td>&lt;1.5</td>
<td>2/4 (113, 152)</td>
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<td>&lt;1.5</td>
<td>0/4</td>
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<td></td>
<td>&lt;1.5</td>
<td>0/4</td>
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<tr>
<td>Prnp0/0 → TNFR1−/−</td>
<td>&lt;1.5</td>
<td>1/4 (85)</td>
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<tr>
<td></td>
<td>&lt;1.5</td>
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<td>&lt;1.5</td>
<td>2/4 (113, 152)</td>
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<td>&lt;1.5</td>
<td>1/4 (97)</td>
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<tr>
<td>Wild-type → wild-type</td>
<td>4.3</td>
<td>4/4 (81 ± 8.1)</td>
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<tr>
<td></td>
<td>4.5</td>
<td>4/4 (80 ± 1.9)</td>
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<tr>
<td>Prnp0/0 → Prnp0/0</td>
<td>&lt;1.5</td>
<td>0/4</td>
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<td></td>
<td>&lt;1.5</td>
<td>0/4</td>
</tr>
<tr>
<td>TNFR1−/− → TNFR1−/−</td>
<td>&lt;1.5</td>
<td>2/4 (87, 89)</td>
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<tr>
<td></td>
<td>&lt;1.5</td>
<td>1/4 (107)</td>
</tr>
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Hematopoietic cells from TNFR1−/− mice are able to confer prion replication capability to Prnp0/0 FLCs-reconstituted TNFR1−/− mice indicate that a stromal component plays an additional role in prion propagation. Attack rate, number of indicator mice succumbing to scrapie/number of mice inoculated with prions.
In addition, we found now that, in the absence of mature FDCs, the PrP signal colocalized with a subset of single red-pulp, metallophilic, and marginal-zone macrophages in TNFR1\(^{-/-}\) lymph nodes. All of the above imply that the cells involved in prion replication and PrP\(^{\text{Sc}}\) accumulation in the lymph nodes of TNFR1\(^{-/-}\) and TNF\(\alpha^{-/-}\) mice are likely to be macrophages, or some subsets thereof. This hypothesis is strengthened by the fact that, even in wild-type lymph nodes, bright PrP signals outside FDC-networks colocalized with a subset of ER-TR9- and MOMA-1-positive cells (Fig. 3A). TNF\(\alpha\) signaling through TNFR1 is required for proper homing of macrophages to the splenic marginal zone, and their absence can cause strong aberrations in macrophage subsets. However, MOMA-1\(^+\) and ER-TR9\(^+\) macrophages were normally distributed in the subcapsular area of TNFR1\(^{-/-}\) and TNF\(\alpha^{-/-}\) lymph nodes (ref. 24, and data not shown), but were strongly disturbed in mesenteric lymph nodes of LT\(\beta^{-/-}\) mice. If the distribution of macrophages is important for peripheral prion pathogenesis, these histoarchitectural differences may account for the differences in splenic vs. lymph nodal prion load of infected TNFR1\(^{-/-}\) and TNF\(\alpha^{-/-}\) mice.

Primary and secondary follicles may be functionally different in spleen vs. mesenteric lymph nodes of TNFR1\(^{-/-}\) mice (25). However, we did not identify morphological differences between splenic and mesenteric germinal centers, and the TNFR1\(^{-/-}\) line used here (26) did not show abnormal germinal center responses after infection with vesicular stomatitis virus (21).

The unexpected finding of high prion titers in inguinal, mesenteric, and cervical lymph nodes of TNF-deficient mice, but not in mesenteric lymph nodes of LT\(\beta^{-/-}\)deficient mice, indicates that prion replication within secondary lymphoid organs is LT\(\beta\)R dependent, yet may occur in the absence of mature FDCs and of functional germinal centers, as revealed by the lack of PNA-positive B cell clusters. Therefore, cell types other than FDCs participate in the process of prion replication/accumulation in lymph nodes and, probably, in spleens. Because marginal zone macrophages might entertain close contacts to immature FDCs in the marginal zone, whose presence was postulated for the TNFR1\(^{-/-}\) mice (24), and also interact with marginal zone B cells, this hematopoietic cell type is certainly a candidate for supportive effects in the process of prion uptake and replication. There is a caveat to this interpretation: immunofluorescence detects prion protein rather than infectivity, and does not differentiate unequivocally between PrP\(^{\text{C}}\) and PrP\(^{\text{Sc}}\). Therefore, further studies will need to focus on whether macrophage ablation, e.g., by using macrophage-specific suicide transgenes, can suppress the infectibility of TNF-deficient lymph nodes.

These findings are at striking variance with reports that LT\(\beta^{-/-}\) mice are fully susceptible to infection with CJD prions (27), and that TNF\(\alpha^{-/-}\) mice peripherally challenged with ME7 prions were largely protected (28). These and other discrepancies have been attributed to the use of different prion strains in these studies. This may well be the case, but the present data indicate that resistance in each mouse strain is dose dependent and can always be overridden (Table 1). Therefore, challenge with one single size of inoculum, as done in other studies, may yield misleading results. Invasion of lymphoid organs by prions occurs very rapidly after peripheral inoculation, and consistently high infectivity titers are detected until terminal disease. Lymphoivasion most likely plays an important role in the pathogenesis of vCJD, because prion infectivity can be detected in tonsils of virtually every vCJD patient (29, 30). After lymphoivasion, neuroinvasion occurs via autonomic nerves (31–35), but the network between germinal centers and nerves is still elusive. By virtue of their mobility, macrophages may represent a plausible candidate for transport of prion infectivity from germinal centers to sympathetic nerve terminals.
How could a possible prion amplification in macrophages be reconciled with their apparent protective role, at least in the very early phase of prion pathogenesis (36)? Maybe the action of macrophages is dose dependent: small inocula may be destroyed by phagocytosis, whereas larger inocula cannot be digested and will be transported or amplified. Alternatively, the absence of TNFR1 may interfere directly with the interaction of macrophages and prions, because ablation of TNF signaling reduces the phagocytic ability of macrophages in several infectious models (37, 38). Be that as it may, the fact that a cell type other than mature FDCs is involved in prion replication and accumulation within secondary lymphoid organs may help developing postexposure prophylaxis strategies aimed at blocking prion neuroinvasion.

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