Oral Prion Infection Requires Normal Numbers of Peyer's Patches but Not of Enteric Lymphocytes

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Prion pathogenesis following oral exposure is thought to involve gut-associated lymphatic tissue, which includes Peyer's patches (PPs) and M cells. Recruitment of activated B lymphocytes to PPs requires $\alpha_4\beta_7$ integrin; PPs of $\beta 7^{-/-}$ mice are normal in number but are atrophic and almost entirely devoid of B cells. Here we report that minimal infectious dose and disease incubation after oral exposure to logarithmic dilutions of prion inoculum were similar in $\beta 7^{-/-}$ and wild-type mice, and PPs of both $\beta 7^{-/-}$ and wild-type mice contained 3–4 log LD₅₀/g prion infectivity ≥ 125 days after challenge. Despite marked reduction of B cells, M cells were present in $\beta 7^{-/-}$ mice. In contrast, mice deficient in both tumor necrosis factor and lymphotoxin- α (TNF $\alpha^{-/-}$ $\times$ LT $\alpha^{-/-}$) or in lymphocytes (RAG-1 $^{-/-}$, μ MT), in which numbers of PPs are reduced in number, were highly resistant to oral challenge, and their intestines were virtually devoid of prion infectivity at all times after challenge. Therefore, lymphoreticular requirements for enteric and for intraperitoneal uptake of prions differ from each other. Although susceptibility to prion infection following oral challenge correlates with the number of PPs, it is remarkably independent of the number of PP-associated lymphocytes. (*Am J Pathol* 2003, 162:1103–1111)

Consumption of food contaminated with bovine spongiform encephalopathy (BSE) is believed to cause variant Creutzfeldt-Jakob disease (vCJD) in humans^{1,2} and ingestion of prions has been implicated in the transmission of other transmissible spongiform encephalopathies (TSE).³ Following experimental intragastric or oral exposure of rodents to scrapie, infectivity and/or disease-associated protease-resistant prion protein (PrP^{Sc}) accumulate rapidly in Peyer's patches (PPs), gut-associated

lymphoid tissues (GALT), and ganglia of the enteric nervous system^{4,5} long before they are detected in the central nervous system (CNS). Similarly, following experimental oral exposure of non-human primates or sheep to BSE, PrP^{Sc} was first detected in lymphoid tissues draining the gastrointestinal tract, long before detection in the CNS.^{6,7}

B lymphocytes play a crucial role in peripheral prion pathogenesis: mice devoid of B lymphocytes do not develop disease after intraperitoneal exposure.⁸ This is possibly because B lymphocytes induce maturation of follicular dendritic cells (FDCs) by providing tumor necrosis factor- α (TNF- α) and lymphotoxin α/β (LT α/β) trimers to lymphoid organs.⁹ Early PrP^{Sc} deposition can be detected in FDCs within B cell follicles in lymphoid tissues of patients with vCJD¹⁰ and in rodents inoculated with scrapie by peripheral routes.¹¹ In mouse spleens, mature FDCs have been shown to be crucial for both prion replication and PrP^{Sc} accumulation,^{12,13} although prion replication in lymph nodes can occur in the absence of mature FDCs.¹⁴ In contrast, the role of intestinal B cells in prion pathogenesis following oral challenge is still unclear.

Intestinal mucosal immunity provides an important level of defense against foreign pathogens. The ability of B and T lymphocytes to be recruited to the site of infection is critical for an effective immune response. This process is mediated by homing receptors on effector cells with cognate ligands at peripheral or mucosal sites.¹⁵ Integrin $\alpha_4\beta_7$ plays an important role in the homing of activated lymphocytes to PPs and to the intestinal lamina propria.¹⁶ Integrin-deficient ($\beta 7^{-/-}$) mice suffer from severely-reduced cellularity of PPs (>90% less B and T cells)¹⁷ and from impaired intestinal immunity in a variety of disease models.^{18–20} With the exception of the GALT, lymphoid organs of $\beta 7^{-/-}$ mice are otherwise normal. These mice are therefore well-suited to dissect the role of mucosa-associated immune tissue, including

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B cells, in the pathogenesis of enterically-initiated prion disease.

In addition, B lymphocytes exert an important organogenic role in the GALT^{21,22} and are likely to be involved in the B cell-dependent development of the follicle-associated epithelium (FAE).²³ However, splenic lymphocytes can acquire prion infectivity,²⁴ and it is unclear whether their role in prion pathogenesis is restricted to the generation and maintenance of FDCs¹³ or whether they may also be involved in prion trafficking.²⁵ To dissect the organogenic effects from trafficking components, we administered prions orally to $\text{TNF}\alpha^{-/-}/\text{LT}\alpha^{-/-}$ mice, which have normal lymphocyte counts but lack the two cytokines $\text{TNF}\alpha$ and $\text{LT}\alpha$,²⁶ to B cell-deficient μMT mice,²⁷ and to $\text{RAG-1}^{-/-}$ mice²⁸ which lack all T and B lymphocytes. While there were no recognizable PPs in $\text{TNF}\alpha^{-/-}/\text{LT}\alpha^{-/-}$ mice, unexpectedly we found that μMT and $\text{RAG-1}^{-/-}$ mice had some FDC-M1-positive cells in their atrophic Peyer's patches, but not in spleen nor in lymph nodes. However, these FDC-like structures were not sufficient for enteric prion replication.

Here we show that prion replication in the GALT and subsequent neuroinvasion was independent of B cells within the mucosa-associated lymphatic tissue and that the remaining M cells are most likely important for this process. $\text{TNF}\alpha^{-/-}/\text{LT}\alpha^{-/-}$, μMT , and $\text{RAG-1}^{-/-}$ mice were highly resistant to oral challenge, and their intestines were virtually devoid of prion infectivity at all times after challenge. Therefore, lymphoreticular requirements for enteric and intraperitoneal uptake of prions differ, and the presence of intramucosal B lymphocytes does not appear to be important for prion pathogenesis following oral challenge.

Materials and Methods

Animals

C57BL/6, Sv129 $\times$ BL/6, μMT ,²⁷ $\text{RAG-1}^{-/-}$,²⁸ and $\text{TNF}\alpha^{-/-}/\text{LT}\alpha^{-/-}$ ²⁶ mice were bred at the Institute of Laboratory Animal Science of the University of Zurich and maintained under specific pathogen-free conditions. Wild-type mice were obtained originally from the Jackson Laboratory, Bar Harbor, ME.

Alkaline Phosphatase Detection

For detection of alkaline phosphatase (AP) activity, fixed whole PPs were studied. Segments of the small intestine containing one PP were excised, opened, and pinned on dental wax. Tissues were fixed for 10 minutes in Baker formalin calcium medium (pH 6) on ice. At this stage, villi were removed using a binocular microscope, and incubated with 125 nmol/L Tris buffer (pH 9.2) at 37°C for 5 minutes and then incubated in Tris buffer containing naphthol AS-BI phosphate (Sigma, Deisenhofen, Germany) with constant shaking for 10 minutes. The reaction was stopped with ice-cold buffer. Goblet cells were detected by incubating tissue briefly in 1% Alcian blue dissolved in 3% acetic acid. Whole-mount FAEs were

then used to enumerate AP-negative M cells as a percentage of the total cell population within the FAE. Statistical significance was determined using the unpaired Student's *t*-test. Values of $P < 0.05$ were considered as significant.

Challenge with Scrapie Prions

Mice were fed with logarithmic dilutions of 10% heat- and sarcosyl-treated brain homogenate prepared from mice infected with the Rocky Mountain Laboratory (RML) scrapie strain (passage 4.1). 8 log LD₅₀ of the prion homogenate RML were administered via gastrolavage directly into the stomach. Smaller amounts (see Table 1) were administered by feeding a mixture of brain homogenate with food pellets. For intraperitoneal inoculations, 6 log LD₅₀ of scrapie inoculum was administered. Mice were monitored on alternate days, and scrapie was diagnosed according to standard clinical criteria.

Infectivity Bioassay

Bioassays were performed on 1% homogenates of spleens or of PBS-perfused small intestines (containing Peyer's patches). Tissues were homogenized in PBS/BSA with a microhomogenizer and passed several times through 18-gauge and 22-gauge needles. Following preparation of a homogeneous suspension, centrifugation was performed at 500 $\times g$ for 5 minutes. Supernatants (30 μl) were inoculated intracerebrally into groups of at least four *tga20* mice.²⁹ The titer of the standard inoculum (7.9 log 10 of 50% lethal dose (LD₅₀)/ml, corresponding to 8.9 log LD₅₀/g of brain tissue) was determined by the 50% endpoint calculation method.³⁰ The relationship $y = 11.45 - 0.088 (y, \log \text{LD}_{50} \times \text{ml}^{-1} \text{ of homogenate}; x, \text{incubation time in days to terminal disease})$ was calculated by linear regression.^{25,31} Indicator mice were sacrificed after development of terminal scrapie and infectivity titers were calculated.

Histology and Immunohistochemistry

Paraffin sections (2 μm) and cryostat sections (10 μm) from brain, spleen and PPs were stained with hematoxylin and eosin. Immunostaining for GFAP was performed using rabbit GFAP antisera (1:300 dilution; DAKO, Carpinteria, CA) and detected with biotinylated swine anti-rabbit serum (1:250 dilution; DAKO), avidin-peroxidase and diaminobenzidine (Sigma). Antibodies reactive against the follicular dendritic cell marker FDC-M1 (clone 4C11; 1:300 dilution) or the pan-B cell marker CD45R/B220 (BD Pharmingen, San Diego, CA) were used for immunohistochemistry of frozen sections as previously described.³²

Western Blot Analysis

Tissue homogenates were adjusted to protein concentrations of 5 mg/ml (brain) or 8 mg/ml (spleen) and treated with proteinase K (20 $\mu\text{g}/\text{ml}$, 30 minutes, 37°C). 50 μg

Figure 1. Number of Peyer's patches in mice of various immunodeficient genotypes. Peyer's patches were counted using stereo microscopical transillumination (magnification, $\times 16$) of the small intestine after filling with PBS. The box and whiskers plot shows range and quartiles. The box extends from the 25th to the 75th percentile, with a line at the median (the 50th percentile). The whiskers extend above and below the box to show the highest and lowest values.

(brain) or 80 μ g (spleen) of total protein of each sample were electrophoresed through a 12% SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes by semi-dry blotting. Membranes were blocked with Tris-buffered saline containing 0.1% Tween 20, pH 8.0 (TBST)/5% nonfat milk, incubated with antibodies 6H4 (for brain protein) or 1B3 (for spleen protein), and detected using alkaline phosphatase-linked anti-mouse (6H4) or anti-rabbit (1B3) sera followed by development by enhanced chemiluminescence (ECL; Amersham, Uppsala, Sweden).

Results

Normal Numbers of PPs in $\beta 7^{-/-}$ Mice

To evaluate the extent to which the absence of $\alpha_4\beta_7$ integrin, TNF/lymphotoxin, and B and T cells affects the presence of PPs, mice were examined anatomically. PPs of wild-type mice of the C57BL/6 and 129Sv $\times$ BL6 genetic background were easily visible to the naked eye, whereas in $\beta 7^{-/-}$, RAG-1^{-/-}, and μ MT mice PPs were undetectable by gross inspection. However, when small intestines were inflated with saline buffer (PBS) and transilluminated under a dissecting microscope (16 $\times$ magnification), numerous small Peyer's patches could be discerned (Figure 1). The number and location (data not shown) of Peyer's patches in $\beta 7^{-/-}$ mice ($n = 6-10$, mean 8.0 ± 1.5) were similar to wild-type mice ($n = 6-11$, mean 7.3 ± 1.5). A comparison with Student's *t*-test, however, yielded a *P* value of >0.05 , indicating that the number of Peyer's patches in the two groups was not significantly different. In contrast, the number of PPs in B cell-deficient μ MT mice were significantly reduced ($0-7$, mean 2.5 ± 2.2 ; $P < 0.01$ if compared to the respective wild-type control). Combined B cell and T cell deficiency in RAG-1^{-/-} mice further reduced the number of PPs ($0-5$, mean 2.0 ± 1.7 ; $P < 0.01$). The absence of TNF- α

and lymphotoxin- α (LT α) completely prevented the formation of visible Peyer's patches ($P < 0.01$).

The size of the M cell-containing subepithelial domes of $\beta 7$ -deficient mice were reduced by at least 70% compared to those of wild-type mice (approximately 250 μ m versus up to 800 μ m for full length of equatorial sections). A similar reduction in the size of FAE was found in B cell-deficient PPs of RAG-1^{-/-} and μ MT mice.

Peyer's Patches of $\beta 7^{-/-}$, RAG-1^{-/-}, and μ MT Mice Are Atrophic but Contain FDCs

To determine whether the absence of $\alpha_4\beta_7$ integrin and lymphocytes alters the cellular composition of secondary lymphoid organs, we studied spleen and PPs by immunohistochemistry using antisera reactive against the B lymphocyte marker B220 and with the FDC marker M1 (Figure 2). The germinal center histology of spleens of $\beta 7^{-/-}$ mice was indistinguishable from wild-type spleens. B cell zones were distinct and contained FDC-M1-positive cells of normal shape and localization.

In wild-type PPs, FDC networks occupied large regions of the germinal centers. Although there was a significant reduction in the number of B cells in $\beta 7^{-/-}$ PPs, some FDCs were still detectable. In contrast, no PPs were observed in TNF- α /LT α -deficient mice, although a small number of B cells were still present in the small intestine of these mice. Lymphoid organ structure of spleens of TNF- α /LT α -deficient mice was severely impaired, without any separation between T and B cell zones, and FDCs were undetectable.

No FDC-M1 positive cells were detected in the spleens of RAG-1^{-/-} and μ MT mice, in agreement with earlier reports.²² Unexpectedly, clusters of cells reactive with FDC-M1 and CD35 (CR1, not shown) were consistently detected in the small atrophic Peyer's patches of RAG-1^{-/-} and μ MT mice. Immunostaining also revealed a number of B220 positive cells in spleens and PPs. The majority of these B220-positive cells are most likely NK cells, because the antibody used (CD45R/B220) reacts with an epitope on the extracellular domain of the CD45 glycoprotein, which is expressed on lytically active subsets of lymphokine-activated killer cells such as NK cells and their progenitors in addition to B lymphocytes.³³ The smaller B220⁺ cell population in the PPs of RAG-1^{-/-} and μ MT mice most likely consist of immature B cells which are still able to produce functional immunoglobulins of the A class even in the absence of surface, membrane-bound IgM or IgD, therefore representing a developmental pro-B cell block in these strains.³⁴

$\beta 7^{-/-}$ Intestinal Epithelium Contains M Cells Despite Depleted Intraepithelial B Cells

M cells of the FAE are believed to be important antigen sampling sites, and can act as entry sites for pathogens. To determine whether the absence or reduction of lymphocytes alters the composition of the FAE, we performed immunostaining of PPs of wild-type, $\beta 7^{-/-}$, RAG-

Figure 2. Morphological appearance of Peyer's patches and spleens in $\beta 7^{-/-}$, $RAG-1^{-/-}$, μMT , $TNF\alpha^{-/-} \times LT\alpha^{-/-}$ and wild-type mice. The atrophic Peyer's patches found in $\beta 7^{-/-}$ mice contained an almost complete absence of B cells (B220) compared to wild-type mice and a reduced FDC network within Peyer's patches. Instead, there was a normal composition of B cell follicles and FDC networks in spleens of $\beta 7^{-/-}$ mice. The microarchitecture of spleen and Peyer's patches in $RAG-1^{-/-}$ and μMT mice was severely impaired. However, B220 staining revealed single positive cells in $RAG-1^{-/-}$ and μMT tissues, these most likely being NK cells (see Results). Surprisingly, positive staining with the FDC marker M1 was observed in the Peyer's patches (but not spleens) of these mice. In the case of $TNF\alpha^{-/-} \times LT\alpha^{-/-}$ mice a portion of the small intestine was stained. Original magnifications, $\times 100$ and $\times 200$ for B220 staining and FDC-M1, respectively.

Figure 3. Presence and distribution of M cells in Peyer's patches in several B cell-deficient mice. **A–D:** Enterocytes with high alkaline phosphatase (AP) activity of wild-type (**A**), $\beta 7^{-/-}$ (**B**), μMT (**C**), and $RAG-1^{-/-}$ (**D**). Peyer's patches were stained red, and mature M cells lacking AP activity appeared white. Goblet cells that also lack AP activity were selectively stained in blue using Alcian blue. Some pink cells observed with this technique were considered to represent cells with a phenotype intermediate between mature enterocytes and M cells. Original magnification of all large figures, $\times 100$. **Insets** represent a high-power view ($\times 400$) of part of the domes. **E:** Quantification of intestinal M cells on whole-mount preparation Peyer's patches stained for AP. Whole mounts were stained as in **A–D** and AP-negative and AP-positive cells were counted. The abundance of M cells (% of the total epithelial cells within the FAE) was enhanced in B cell-reduced/deficient mice. Values are presented as a box and whiskers plot as in Figure 1.

Table 1. Susceptibility of Mice with Different Alterations of the Gut-Associated Lymphoid Tissue to Scrapie

Mouse genotype	Genetic background	Oral infection						i.p. Infection, 6 log LD ₅₀		Intracerebral infection, 3 × 10 ⁵ LD ₅₀	
		8 log LD ₅₀		7 log LD ₅₀		6 log LD ₅₀		Attack rate	Disease latency	Attack rate	Disease latency
		Attack rate	Disease latency (Average ± SD)	Attack rate	Disease latency	Attack rate	Disease latency				
$\beta 7^{-/-}$	129Sv × BL/6	6/6	230 ± 9	2/4	261,272 2 × >510	0/5	5 × >510	6/6	217 ± 4	4/4	148 ± 7
RAG-1 ^{-/-}	C57BL/6	0/5	5 × >510	0/3	3 × >510	ND		0/5	5 × >510	5/5	139 ± 6
μ MT ^{-/-}	C57BL/6	0/6	6 > 510	0/7	7 × >510	ND		0/4	4 × >510	3/3	154 ± 2
TNF $\alpha^{-/-}$ × LT $\alpha^{-/-}$	129Sv × BL/6	0/8	8 × >510	0/5	5 × >510	0/5	5 × >510	2/5	226,277 3 × >510	4/4	144 ± 5
Wild type	C57BL/6	9/10	224 ± 14, 1 × >510	1/4	247, 3 × >510	0/5	5 × >510	5/5	213 ± 8	6/6	147 ± 9
Wild type	129Sv × BL/6	4/4	213 ± 10	2/4	250,256 2 × >510	0/3	3 × >510	4/4	210 ± 12	5/5	151 ± 6
Prnp ^{0/0}	129Sv × BL/6	0/6	>510	ND		ND		ND		0/5	5 × >50

$\beta 7^{-/-}$ and wild-type mice developed scrapie upon very high oral prion challenge (8 log LD₅₀) and i.p. and intracerebral inoculation at similar time points, while lower oral concentrations only partially evoked scrapie. All the other strains were protected to varying degrees. Mean incubation time and standard errors were calculated for mice that developed scrapie. The total observation time is indicated for mice that remained disease-free.

ND, not determined.

1^{-/-} and μ MT with antibodies reactive against specific markers for M cells (Figure 3, A–D). M cells, visualized histochemically by their reduced AP activity,³⁵ were readily detectable in the FAE of wild-type mice (Figure 3A). In $\beta 7^{-/-}$, μ MT, and RAG-1^{-/-} (Figure 3, B, C, and D, respectively), both PPs and AP-negative M cells were apparent. The presence of PPs and AP-negative M cells was further confirmed by scanning electron microscopy, which allowed visualization of the disorganized brush border of these cells (data not shown).

In contrast to PPs of wild-type mice, in which M cells were located at the periphery of the FAE, the distribution of M cells appeared to be random over the entire surface of FAE in PPs of $\beta 7^{-/-}$, RAG-1^{-/-}, and μ MT mice.

We then estimated the relative number of intestinal M cells per total epithelial cells within the FAE by counting AP-negative M cells under 400× magnification by light microscopy (Figure 3E). The relative number of M cells was increased in the FAE of $\beta 7^{-/-}$ (mean: 15.4 ± 4.0 vs. 6.7 ± 1.8 for 129Sv × BL/6 mice, $P < 0.01$), RAG-1^{-/-} (mean: 13.5 ± 2.3 vs. 7.2 ± 2.7 for BL/6, $P < 0.05$) and μ MT (mean: 14.2 ± 2.8 compared to BL/6, $P < 0.05$) tissues. These data indicate that the differentiation program of the FAE, including M cells, is maintained even in the absence of proper B cell numbers.

Unimpaired Scrapie Pathogenesis in $\beta 7$ -Deficient Mice

To determine the contribution of different components of the GALT to the intestinal uptake of orally-delivered prions, mice were challenged with logarithmic dilutions of scrapie inoculum. Attack rate and time to terminal disease were measured. Following high-dose oral infection (8 log LD₅₀) all $\beta 7^{-/-}$ mice, Sv129 × C57BL/6 wild-type mice, and 9 of 10 C57BL/6 wild-type mice developed clinical signs of scrapie with similar incubation times of 213 to 230 days (Table 1). These results indicate that

$\beta 7^{-/-}$ mice are highly susceptible to disease via oral administration of prions. Accordingly, accumulation of PK-resistant PrP^{Sc} was readily detectable in brains and spleens of clinically sick $\beta 7^{-/-}$ and wild-type mice (Figure 4). Moreover, topography and intensity of spongiosis and gliosis were similar in clinically sick $\beta 7^{-/-}$ mice compared to clinically sick wild-type mice (Figure 5). Importantly, the use of lower prion inocula via the oral route significantly decreased the susceptibility to disease in these mice compared to i.p. challenge. A 10-fold dilu-

Figure 4. Western blot analysis of brains and spleens. Presence or absence of PrP^{Sc} was determined by western blots of spleen or brain material electrophoresed natively (–), or after digestion with PK (+). Molecular weights of the proteins were indicated in kilodaltons on the left. Large amounts of PrP^{Sc} were detected in the brains and spleens of either $\beta 7^{-/-}$ or wild-type mice that had developed scrapie (terminal sick) after the time points indicated. PrP^{Sc} was detected only in mice which were challenged orally with high-dose (8 log LD₅₀) RML or i.p. with 6 log LD₅₀ but not in tissues of clinically healthy mice, thereby excluding subclinical disease.

Figure 5. Morphological changes in the brains of mice orally exposed to prions. Pronounced astrogliosis (GFAP) in the hippocampus of scrapie-sick (terminal disease) $\beta 7^{-/-}$ and wild-type mice was obvious, whereas no gliosis and vacuolization (H&E) was observed in clinically healthy RAG-1 $^{-/-}$, μ MT, and TNF $\alpha^{-/-}$ $\times$ LT $\alpha^{-/-}$ mice. Original magnification, $\times 200$.

tion of the scrapie inoculum reduced the scrapie attack rate to 50% in $\beta 7^{-/-}$ and to 50 to 25% in the different wild-type mice. Further dilution of the inoculum did not evoke scrapie in mice of any genotype.

After i.p. prion challenge (6 log LD₅₀) $\beta 7^{-/-}$ and wild-type mice had similar incubation times of 210 to 217 days, indicating normal peripheral prion pathogenesis. As described earlier,^{8,14} B cell-deficient RAG-1 and μ MT mice were completely resistant, and TNF $\alpha^{-/-}$ /LT $\alpha^{-/-}$ mice partially resistant, to prions administered i.p. Wild-type mice can be experimentally infected via the i.p. route with prion inocula of less than 3 intracerebral log LD₅₀, yet the minimal dose for oral infection is 7 intracerebral log LD₅₀ of scrapie inoculum. Therefore, these results confirm previous observations that the oral route of prion infection is relatively inefficient in mice.

In contrast to wild-type or $\beta 7^{-/-}$ animals, mice deficient for LT α and TNF- α and mice lacking mature B cells were virtually resistant to orally-administered prions, even when extremely large inocula were used. This provides evidence that neither small intestines from TNF $\alpha^{-/-}$ $\times$ LT $\alpha^{-/-}$ mice nor atrophic PPs of RAG-1 $^{-/-}$ and μ MT mice are sufficient as entry sites of prions.

Although PPs of the small intestine are potential entry sites for pathogens, draining lymph nodes and the spleen provide lymphoid sites with a mechanism for pathogen clearance. However, RAG-1 $^{-/-}$, μ MT, and TNF $\alpha^{-/-}$ $\times$ LT $\alpha^{-/-}$ mice were highly resistant to i.p. infection with prions (Table 1),⁸ and therefore the phase of peripheral prion replication is also impaired in these mice. Accordingly, PrP^{Sc} was undetectable in brains and spleens of clinically healthy RAG-1 $^{-/-}$, μ MT, and TNF $\alpha^{-/-}$ $\times$ LT $\alpha^{-/-}$ mice at the indicated postinfection time points.

Peyer's Patches of $\beta 7^{-/-}$ but Not RAG-1 $^{-/-}$ and μ MT Mice Are Sites of Peripheral Prion Replication

We then investigated peripheral prion replication in PPs and spleens of $\beta 7^{-/-}$, RAG-1 $^{-/-}$, and μ MT mice following oral challenge. PPs (Figure 6, red symbols) and spleens (Figure 6, blue symbols) were collected at various time points after oral (8 log LD₅₀) or i.p. (6 log LD₅₀)

infection until terminal disease. Infectivity titers were determined by transmission of homogenized tissues to indicator mice. For those TNF $\alpha^{-/-}$ $\times$ LT $\alpha^{-/-}$ mice which lacked all PPs, selected portions of the small intestine were dissected and used for transmission studies.

Infectivity titers in spleens of wild-type (crosses) and $\beta 7^{-/-}$ mice (circles) after oral challenge were generally

Figure 6. Determination of prion infectivity titers in spleens, Peyer's patches, or small intestines of scrapie-challenged mice with different alterations in the GALT. Titers were determined in spleens (blue symbols) and Peyer's patches (red symbols) of wild-type (crosses), $\beta 7^{-/-}$ (circles), RAG-1 $^{-/-}$ (diamonds), μ MT (squares) and TNF $\alpha^{-/-}$ $\times$ LT $\alpha^{-/-}$ (triangles) mice. Since TNF $\alpha^{-/-}$ $\times$ LT $\alpha^{-/-}$ mice lacked PPs, segments of the small intestine were processed for transmission studies to indicator mice. Mice were challenged orally with 8 log LD₅₀ or i.p. with 6 log LD₅₀ of scrapie prions as indicated. Standard deviations within groups are presented only when they exceed ± 0.75 log LD₅₀. Symbols on the abscissa and below the threshold of the prion titer 1.5 log LD₅₀ indicate prion titers below detection limit (none of the four indicator mice developed scrapie). If one or more indicator mice survived >180 dpi, or if the mean incubation time was more than 120 days, titer was assumed to be close to the detection threshold of the bioassay at prion titer of 1.5 log LD₅₀. For these samples (labeled with small letters) the numbers of animals succumbing to scrapie out of four inoculated *tga20* mice and incubation time (in days) to terminal scrapie were as follows: a, 2/4(87,117); b, 2/4(109,111); c, 1/4(92); e, 2/4(117,134); f, 1/4(109), g, 3/4(124,134,151). d: In this transmission experiment one of the four *tga20* mice died 24 hours after inoculation, most likely as a result of intracerebral bleeding following the injection procedure.

determined to be lower than after intraperitoneal challenge. Additionally, the kinetics of prion replication differed in PPs and spleens after oral and i.p. challenge. Following i.p. inoculation, high titers of infectivity were already present at approximately 50 dpi in both genotypes, whereas following oral challenge, infectivity was only detected at 110 days.

With the exception of trace levels of infectivity in spleens and PPs of RAG-1^{-/-} and TNF α ^{-/-} $\times$ LT α ^{-/-} mice at latter time points, we found no evidence for infectivity in any spleen and PP of challenged RAG-1^{-/-} (diamonds), μ MT (squares), and TNF α ^{-/-} $\times$ LT α ^{-/-} (triangles) mice. Therefore, the absence of PPs in mice deficient in TNF- α and LT α prevents gastroenteric invasion of prions.

Even more surprisingly, PPs of RAG-1^{-/-} and μ MT mice were devoid of infectivity despite the presence of FDC-M1-positive cell cluster (Figure 2). As previously mentioned, it is probable that these cells are FDC-like since they are CD35-positive and have also been reported to be dependent on LT β signaling.²¹ However, it is not obvious whether these cells represent functionally inactive FDCs. The data presented here appear to suggest that ablation of mature B cells, which are the primary sources of LT α and LT β , abolishes the induction of FDC-M1-positive cells in the spleen, but not in PPs.

Discussion

The mechanisms associated with the enteric trafficking of prions following oral infection are poorly understood. Here we have investigated the role of various components of the enteric mucosal immune system in the pathogenesis of scrapie on oral administration of prions. The oral route of prion infection is much less efficient than the intraperitoneal route. In the present series of experiments, we were able to infect wild-type and $\alpha_4\beta_7$ ^{-/-} mice with prion inocula of less than 3 log LD₅₀ via the i.p. route. In contrast, the minimal dose for infection via the oral route was 7 log LD₅₀ of scrapie inoculum. This indicates that the intestinal mucosa constitutes an effective barrier for prions. This may be due to potential antiprion effects of mucosal immunity, or because transepithelial prion transport is rate-limiting.

Our data appear to indicate that proper homing of B lymphocytes to the enteric mucosa is not a compulsory prerequisite for enteric prion uptake. This finding is surprising, since B cells are crucially important for peritoneal prion pathogenesis,⁸ and $\alpha_4\beta_7$ ^{-/-} mice with marked reduction of enteric lymphocytes display significant alterations in various models of intestinal inflammation and infection.^{18,19,20}

The GALT consists of highly-organized PPs in the small intestine, and intraepithelial lymphocytes are present throughout the length of the gastrointestinal tract. The intestinal surfaces of PPs are characterized by the presence of "domes," which are regions free of intestinal villi.³⁶ At these domes, M cells are able to tunnel pathogens through the cytoplasm to the basal surface, where

deep invaginations of their membrane allow close contact with lymphocytes and macrophages.³⁷

Surprisingly, we found no evidence of scrapie infectivity in the atrophic PPs of B cell-deficient μ MT and RAG-1^{-/-} mice orally challenged with prions, despite the presence of M cells in these mice.^{21,22} We estimated that the number of M cells in B cell deficient mice was approximately 150 to 840 times less than in wild-type mice.²² It is possible that this reduction in M cell numbers also leads to strong functional impairment, such that sufficient prion replication in B cell-deficient mice may no longer be possible. In view of the presence of FDC-M1-positive cells within PPs of μ MT and RAG-1^{-/-} mice, the absence of detectable prion infectivity in PPs of these mice is also surprising, since mature B cells are believed to be required to support FDC maturation in adult peripheral lymphoid tissues.³⁸ Whether these FDC-M1-positive cells represent mature FDCs, precursors of FDCs, or an unrelated cell type remains to be established. However, it should be noted that we have been able to determine that these cells are also CD35 (CR1) positive (data not shown) and are highly sensitive to LT β R-Ig fusion protein treatment,²¹ indicating that they share several features in common with splenic FDCs. Importantly, we were unable to detect cells reactive for FDC markers in spleens or lymph nodes of μ MT and RAG-1^{-/-} mice (data not shown).

Three major cell types are known to express LT $\alpha\beta$ for the induction of FDCs in adult mice: T, B, and NK cells. NK cells are the only LT $\alpha\beta$ ⁺ cells retained in μ MT and RAG-1^{-/-} mice. It is conceivable that these cells may supply the LT $\alpha\beta$ signals and indeed, LT $\alpha\beta$ ⁺CD4⁺CD3⁻ cell populations have been observed in developing Peyer's patches.³⁹

The relatively high resistance of B cell-deficient mice to oral prion challenge is in agreement with our earlier observations that following i.p. inoculation, RAG-1^{-/-}, RAG-2^{-/-}, and μ MT mice failed to establish prion replication in the spleen and did not develop scrapie for >600 days.⁸ However although accumulation of PrP^{Sc} has been observed in the brains of some B cell-deficient mice,⁴⁰ we found no cases of subclinical disease on oral administration of prions, as assessed by Western blot analysis of brain homogenates.

As described repeatedly,^{8,14,25} B cell-deficient mice were entirely resistant to i.p. administered prions, while TNF/LT deficient mice were partially resistant. These findings differ from those reported by Mabbott et al⁴¹ and suggest that the importance of B lymphocytes in prion pathogenesis might go beyond their role in FDC maintenance.

The data outlined here indicate that the requirements for oral and i.p. pathogenesis differ profoundly. The significant reduction of mucosal lymphocytes seen in β_7 ^{-/-} mice is not limiting for oral prion uptake. FDCs are unlikely to be rate limiting, since there was a marked reduction in number of these cells in sensitive β_7 ^{-/-} mice, and they were not completely abolished in fully protected RAG-1^{-/-} and μ MT mice. Instead, absolute resistance to oral prion infection was achieved here if Peyer's patches or B cells were totally absent.

It is unclear which elements, lacking from RAG-1^{-/-} and μ MT mice, are necessary for pathogenesis following oral prion challenge in β 7^{-/-} mice. Epithelial M cells are certainly plausible candidates, since they efficiently transcytose prion infectivity from the apical to the basolateral compartment in an *in vitro* co-culture system.⁴² M cells are still present in RAG-1^{-/-} mice, but their functionality is dependent on the presence of mature B cells,²² which are still present in small numbers in β 7^{-/-} mice. Since there is a lack of isolated M cell-specific genes, it is not yet possible to address this directly *in vivo*, for example, by using transgenesis and lineage ablation strategies.

The findings reported here indicate that the pathophysiology of prion infection after oral uptake relies on mechanisms and cellular components significantly different from the established requirements for the intraperitoneal route (lymphocytes, FDCs). Clearer understanding of the process involved in mucosal passage of prions will be crucial in dealing effectively with orally-transmitted prion diseases, such as bovine spongiform encephalopathy, vCJD, and chronic wasting disease.

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