Open

M cell-depletion blocks oral prion disease pathogenesis

DS Donaldson[1,6], A Kobayashi[1,2,6], H Ohno[3], H Yagita[4], IR Williams[5] and NA Mabbott[1]

Many prion diseases are orally acquired. Our data show that after oral exposure, early prion replication upon follicular dendritic cells (FDC) in Peyer's patches is obligatory for the efficient spread of disease to the brain (termed neuroinvasion). For prions to replicate on FDC within Peyer's patches after ingestion of a contaminated meal, they must first cross the gut epithelium. However, the mechanism through which prions are conveyed into Peyer's patches is uncertain. Within the follicle-associated epithelium overlying Peyer's patches are microfold cells (M cells), unique epithelial cells specialized for the transcytosis of particles. We show that following M cell-depletion, early prion accumulation upon FDC in Peyer's patches is blocked. Furthermore, in the absence of M cells at the time of oral exposure, neuroinvasion and disease development are likewise blocked. These data suggest M cells are important sites of prion uptake from the gut lumen into Peyer's patches.

INTRODUCTION

Prion diseases (transmissible spongiform encephalopathies) are subacute neurodegenerative diseases that affect both humans and animals. Many prion diseases, including natural sheep scrapie, bovine spongiform encephalopathy, chronic wasting disease in cervids, and variant Creutzfeldt–Jakob disease in humans, are acquired peripherally such as by oral exposure. After exposure, prions first replicate upon follicular dendritic cells (FDC) as they make their journey from the site of infection to the central nervous system (a process termed neuro-invasion).[1–5] FDC are a unique subset of stromal cells resident within primary B-cell follicles and germinal centers of lymphoid tissues.[6] Prion accumulation and replication upon FDC is critical for efficient disease pathogenesis as in their absence, neuroinvasion is impaired.[1–3,7] During prion disease aggregations of PrP^{Sc}, an abnormally folded isoform of the cellular prion protein (PrP^{C}) accumulate in affected tissues. Prion infectivity co-purifies with PrP^{Sc} and is considered to constitute the major, if not sole, component of the infectious agent.[8,9] Host cells must express cellular PrP^{C} to sustain prion infection, and FDC express high levels of PrP^{C} on their surfaces.[7,10,11] From lymphoid tissues, prions appear to invade the central nervous system via the peripheral nervous system[12] although hematogenous spread cannot be entirely excluded.

Gut-associated lymphoid tissue (GALT) comprises chiefly of the appendix, tonsils, Peyer's patches, colonic and cecal patches, and isolated lymphoid follicles. Together with the mesenteric lymph nodes (MLNs), these tissues help protect the host from gastrointestinal infections. However, our studies in mice show that after oral exposure early prion replication upon FDC in Peyer's patches is obligatory for efficient neuroinvasion.[3] For prions to replicate on FDC in Peyer's patches after ingestion of a contaminated meal they must first cross the follicle-associated epithelium (FAE), but the mechanism by which this occurs is uncertain. The uptake of prions by several cell types including microfold cells (M cells), enterocytes, and mononuclear phagocytes has been proposed, but definitive confirmation of a specific uptake mechanism *in vivo* is lacking. The identification of the cells and molecules involved in the trans-epithelial transport of prions may identify important processes that influence disease susceptibility and to which intervention strategies can be developed.

The luminal surface of the intestine limits the access of pathogenic microorganisms to the underlying host tissues, and is protected by a single layer of epithelial cells bound by tight junctions. Located within the FAE of Peyer's patches and occasionally within villus epithelia are M cells, a unique subset of epithelial cells specialized for the transepithelial transport of

macromolecules and particulate antigens.[13,14] M cells enable the host's immune system to sample the intestinal lumen and mount an appropriate immune response. However, some pathogenic microorganisms exploit M cells and use them to gain entry into mucosal tissues.[15] Data from the immunohistological tracing of prion-infected brain homogenate[16,17] or *in vitro* studies of Caco-2 cells[18] suggest that M cells are also plausible sites for the transcytosis of prions across the intestinal epithelium. However, similar studies suggest that this translocation occurs via enterocytes independently of M cells.[19,20] In response to inflammatory stimuli, mononuclear phagocytes within the lamina propria including macrophages and classical dendritic cell (DC) (a distinct population from stromal FDC[6]) can insert dendrites through the tight junctions between enterocytes. These projections enable mononuclear phagocytes to directly sample the luminal contents.[21,22] As our own data show that the temporary depletion of CD11c^{+} mononuclear phagocytes impairs oral prion pathogenesis,[23] these data highlight another potential route which may influence the transepithelial transport of prions during inflammatory conditions in the intestine. Thus, although several cell populations are plausible sites of prion transcytosis across the FAE into Peyer's patches, definitive evidence of their role *in vivo* is lacking.

The tumor necrosis factor (TNF) superfamily member receptor activator of NF-κB ligand (RANKL) is selectively expressed by subepithelial stromal cells beneath the FAE in Peyer's patches.[13] RANKL signals via its receptor RANK (receptor activator of NF-κB), which is expressed by epithelial cells throughout the intestine. RANKL is the critical factor that controls the differentiation of RANK-expressing enterocytes into M cells.[13] Furthermore, M cells are depleted *in vivo* by RANKL neutralization, and are absent in RANK-deficient mice. Data from histological studies suggest M cells acquire prions after oral exposure.[16,17] Here, to determine the influence of M cells in prion uptake from the gut lumen, M cells were depleted in mice by treatment with an anti-RANKL monoclonal antibody (mAb) and the effects on oral prion pathogenesis studied. Our data show that following RANKL neutralization, both the early prion accumulation upon FDC in Peyer's patches and neuroinvasion were blocked. Together, these data suggest that M cells are important sites of prion uptake from the gut lumen into Peyer's patches.

RESULTS

Depletion of M cells in the FAE of Peyer's patches by RANKL neutralization

To study the requirement for M cells in the transcytosis of prions across the FAE i*n vivo*, RANKL neutralization was used to transiently deplete these cells before oral prion exposure.[13] Mice were injected intraperitoneally with the IK22-5 rat anti-mouse RANKL-specific mAb every 2 days for 8 days as described[13] and tissues collected 24 h after the last treatment. A parallel group of mice were treated with an isotype-matched nonspecific rat IgG2a κ mAb as a control (control Ig). Glycoprotein 2 (GP2) is a novel, specific surface marker for M cells.[24,25] As anticipated, the number of GP2^{+} M cells in the FAE of Peyer's patches of

anti-RANKL mAb-treated mice was dramatically and significantly reduced when compared with controls (**Figure 1**; $P < 0.0079$). In contrast, RANKL neutralization did not significantly affect the number of GP2^{−}UEA-1^{+} goblet cells (**Figure 1**; $P = 0.241$).

RANKL neutralization does not affect FDC status

As prion replication upon PrP[C]-expressing FDC within the GALT is important for efficient neuroinvasion from the intestine,[1,3] we next determined the effect of RANKL neutralization on FDC status. FDC in mice characteristically express high levels of complement receptors 1 (CR1/CD35) and 2 (CR2/CD21)[26] and cellular PrP[C].[7,10,11] Immunohistochemical (IHC) analysis suggested there was no observable difference in the status of CD21/CD35-expressing FDC within B-cell follicles in the GALT and spleens of control Ig and anti-RANKL mAb-treated mice (**Figure 2a**). The expression of PrP[C] by FDC was likewise unaffected by these treatments (**Figure 2b**). These data are consistent with the expression of negligible levels of *Tnfrsf11a* (which encodes RANK) by FDC (**Supplementary Figure 1** online). Meta-analysis of mRNA micro-array data from a large collection of data sets ($n = 136$ individual samples) representing different mouse cells and tissues showed high expression of *Tnfrsf11a* by Peyer's patch FAE, osteoclasts, and lymphoid tissue inducer cells, but negligible levels by FDC. An important role for RANKL–RANK signaling in osteoclast function has been described.[27] However, no observable effects of RANKL neutralization on the presence and distribution of F4/80^{+} and CD11c^{+} mononuclear phagocytes within the intestine, GALT, and spleen were observed (**Figure 2c**). These data are consistent with the expression of negligible levels of *Tnfrsf11a* by tissue macrophages and classical DC from Peyer's patches, lymph nodes, and spleen (**Supplementary Figure 1** online). Furthermore, the density and distribution of CD11c^{+} cells was not affected within cryptopatches of RANKL^{−/−} mice.[28] Together, these data confirm that RANKL neutralization depletes M cells within the FAE without apparent influence on the status of FDC or mononuclear phagocytes in the intestine.

M cell-depletion blocks the early accumulation of prions upon FDC in Peyer's patches

Within weeks after oral exposure, ME7 scrapie prions accumulate first upon FDC within the Peyer's patches where they persist at high levels until the terminal stages of disease.[1,3,23] Therefore, we next determined the effect of M cell-depletion on the uptake of prions into Peyer's patches. Mice were treated for 8 days with anti-RANKL mAb to deplete their M cells. A separate group of mice was treated with control Ig. Two days after the last antibody injection, when M cells were significantly depleted in the FAE by RANKL neutralization, mice were orally exposed to ME7 scrapie prions.

In this study, the normal cellular form of the prion protein is referred to as PrP[C] and two distinct terms (PrP[Sc] and PrP[d]) are used to describe the disease-specific, abnormal accumulations of PrP that are characteristically found only in prion-affected tissues. Prion disease-specific PrP accumulations are relatively
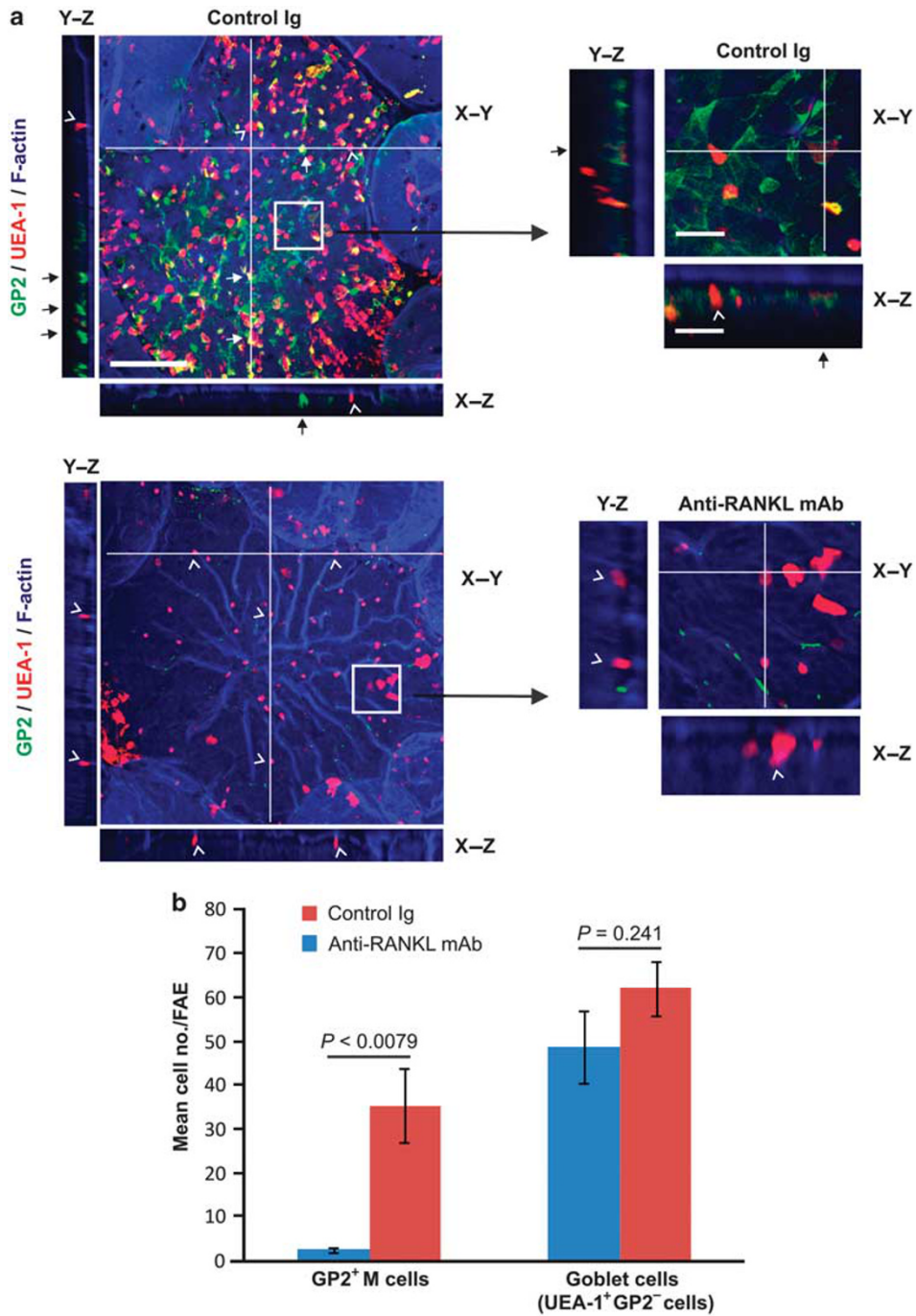
Figure 1 Receptor activator of NF-κB ligand (RANKL) neutralization depletes glycoprotein 2 (GP2+) microfold cells (M cells) within the follicle-associated epithelium (FAE) of Peyer's patches. Whole-mount immunohistochemical analysis of the effect of anti-RANKL monoclonal antibody (mAb) treatment on the presence of GP2+ M cells within the FAE of Peyer's patches. (**a**) Tissues were stained with GP2-specific mAb (green), the lectin UEA-1 (red), and phalloidin (blue) to detect F-actin. The positions of the X–Z and Y–Z projections of the FAE are indicated by the solid line in the X–Y image. Arrows indicate GP2+ M cells with characteristic basolateral pockets. Arrowheads indicate GP2−UEA-1+ goblet cells. The boxed areas in each of the main panels are shown on the right-hand side at higher magnification with their X–Z and Y–Z projections. Scale bars: main panels, 100 μm; boxed areas, 25 μm. (**b**) The number of M cells in the FAE was significantly reduced after anti-RANKL mAb treatment (P=0.0079). Data are representative of 3–5 Peyer's patches from each of four control and four anti-RANKL mAb-treated mice.

resistant to proteinase K (PK) digestion, whereas cellular PrP^C is destroyed. As a consequence, PK-resistant PrP (referred to as PrP^{Sc}) can be used as a biochemical marker for the presence of prions.[8] Unfortunately, the treatment of histological sections with PK destroys the tissue microarchitecture. Therefore, on histological sections we refer to these abnormal disease-specific PrP accumulations as PrP^d. However, to confirm the presence of PrP^{Sc}, adjacent sections were applied to nitrocellulose membrane, treated with PK, and subsequently analyzed by paraffin-embedded tissue (PET) immunoblot analysis.[29]
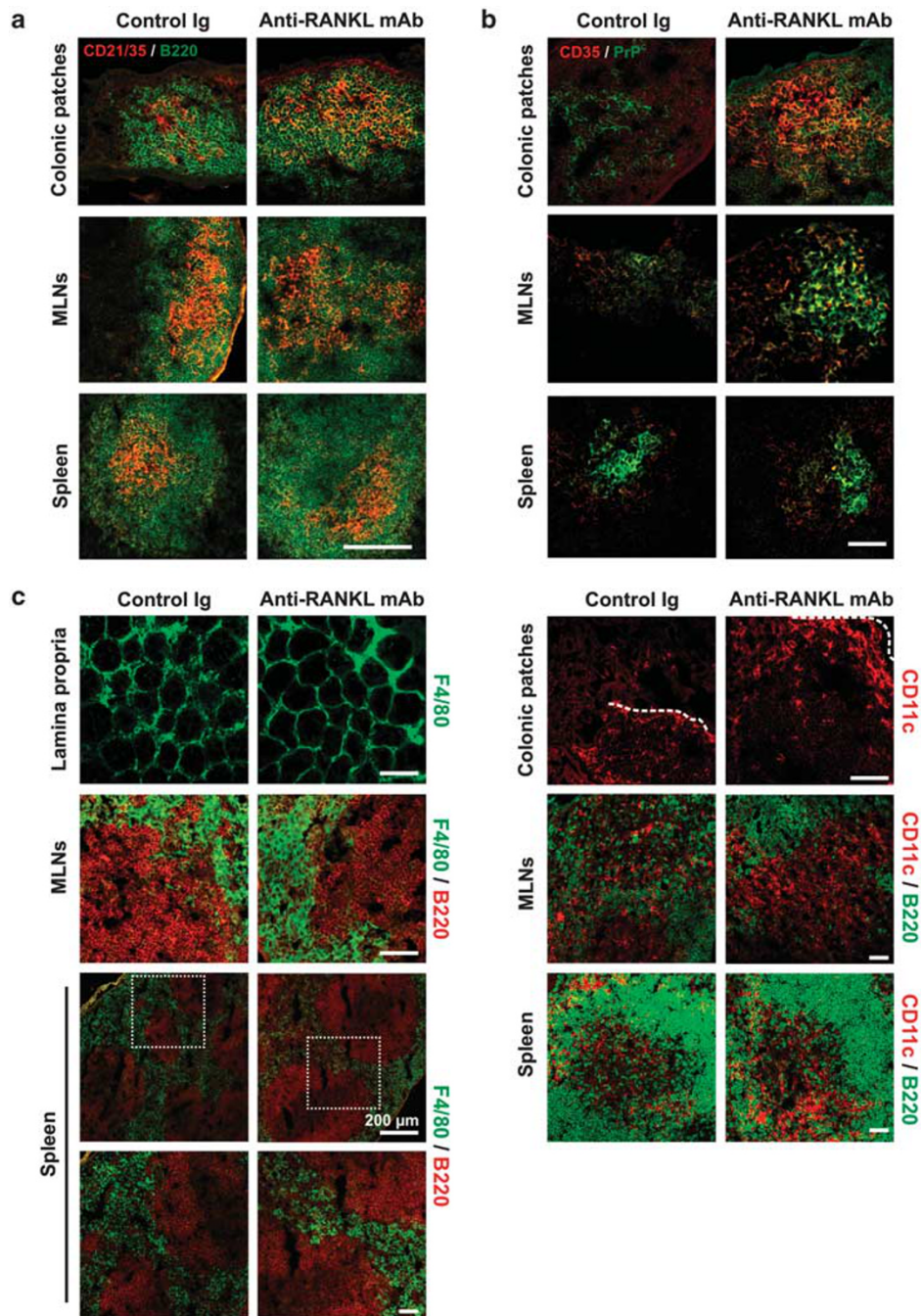
Figure 2 Receptor activator of NF-κB ligand (RANKL) neutralization does not affect the status of follicular dendritic cells (FDC) or mononuclear phagocytes within the gut-associated lymphoid tissue (GALT) and spleen. (**a**) Immunohistochemical (IHC) analysis of the CD21/CD35-expressing FDC networks (red) within B-cell follicles (B220+ cells, green) in the GALT and spleens of anti-RANKL monoclonal antibody (mAb)-treated and control mice suggested no observable effect of treatment on FDC status. (**b**) No observable effect of RANKL neutralization on cellular prion protein (PrP^C) expression (green) by FDC (CD35+ cells; red) was detected by IHC. (**c**) No observable effect of RANKL neutralization on the status of F4/80+ macrophages and CD11c+ cells in the intestine, GALT, and spleen. Dotted line (upper right panels) indicates luminal surface of follicle-associated epithelium. Boxed areas are displayed at higher power in underlying panels. Data are representative of tissues from four mice per group. Scale bars = 50 μm unless stated otherwise. MLNs, mesenteric lymph nodes.

We have repeatedly shown in a series of studies that these PrP^d/PrP^Sc accumulations occur only in prion-infected tissues, and correlate closely with the presence of ME7 scrapie prions.[1,3,10,23]

In Peyer's patches from control mice, heavy PrP^d accumulations, consistent with localization upon FDC within B-cell follicles, were detected at 15 weeks after oral prion exposure

(**Figure 3a**). PET immunoblot of adjacent histological sections confirmed the presence of high levels of PrP^Sc upon FDC in tissues from control mice (**Figure 3b**). At the terminal stage of disease, high levels of PrP^Sc were maintained upon FDC in the Peyer's patches, MLNs, and spleens of control Ig-treated mice (**Figure 3c**). In contrast, in the absence of M cells at the time of
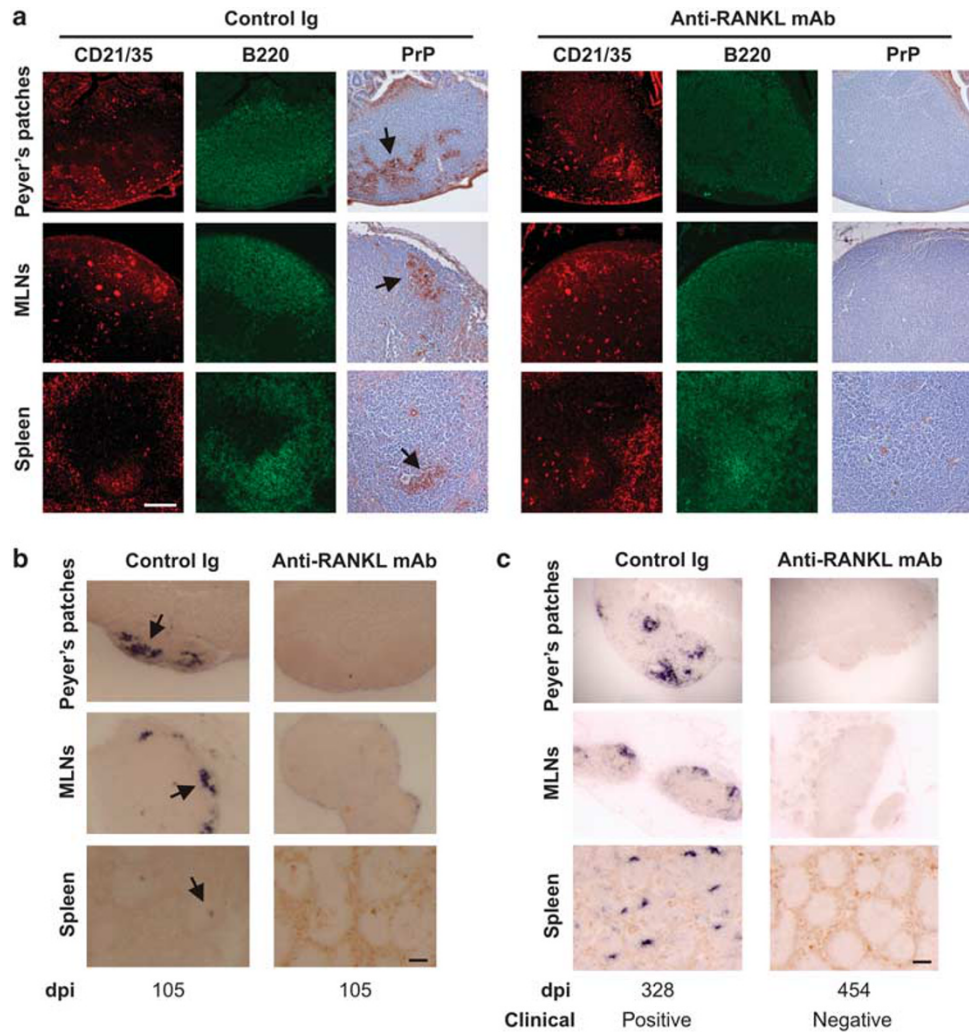
Figure 3 M cell-depletion blocks prion accumulation in the gut-associated lymphoid tissue (GALT) and spleen. Mice were treated with anti-receptor activator of NF-κB ligand (RANKL) monoclonal antibody (mAb) to deplete their microfold cells (M cells) and orally infected with the ME7 scrapie agent. Tissues were collected 105 days post infection (**a** and **b**, $n=4$ mice per group) or at the end stage of disease (**b**), $n=8$ mice per group). (**a**) High levels of prion protein (PrP)[d] (brown, right-hand columns) were detected in association with follicular dendritic cells (FDC) (red, left-hand columns) in the B-cell follicles (green, middle columns) of Peyer's patches, mesenteric lymph nodes (MLNs), and spleens of prion-infected control-Ig-treated mice. (**b** and **c**) Analysis of adjacent sections by paraffin-embedded tissue-immunoblot analysis confirmed the presence of proteinase K-resistant PrP[Sc] (blue/black). In contrast, no PrP[Sc] was detected in any of the Peyer's patches, MLNs, and spleens of anti-RANKL mAb-treated mice, which lacked M cells at the time of oral exposure. Arrows indicate (**a**) PrP[d] and (**b**) PrP[Sc] accumulation upon the same FDC networks. **a**, scale bar = 100 µm. **c**, scale bar = 200 µm. Clinical observation: presence of clinical signs of prion disease at the time of cull. dpi, days post oral prion infection.

prion exposure, PrP[Sc] accumulation within the GALT and spleen was blocked. No PrP[Sc] was observed upon FDC in the Peyer's patches, MLNs, or spleens from anti-RANKL mAb-treated mice collected at 15 weeks after exposure (**Figures 3a** and **b**), or at 454 days after exposure at which time the experiment was stopped (**Figure 3c**). These data clearly demonstrate that in the absence of M cells in the FAE at the time of oral exposure, the uptake of prions into Peyer's patches was blocked.

M cell-depletion blocks prion neuroinvasion from the intestine
We next determined the effect of M cell-depletion on prion disease susceptibility. All control-Ig-treated mice succumbed to clinical prion disease after oral exposure to ME7 scrapie prions with a mean incubation period of 341 days ($n=8$; **Table 1**). In contrast,

the depletion of M cells blocked disease susceptibility as all of the anti-RANKL mAb-treated mice remained free of the clinical signs of prion disease up to at least 454 days after oral exposure at which time the experiment was stopped ($n=8$; **Table 1**).

Characteristic spongiform pathology, astrogliosis, microgliosis, and PrP[Sc] accumulation typically associated with terminal infection with ME7 scrapie prions were detected in the brains of all clinically affected control-Ig-treated mice (**Figure 4a**, upper row). The severity and distribution of the spongiform pathology within the brains of the clinically affected control mice was likewise typical of mice clinically affected with ME7 scrapie prions (**Figure 4b**). In contrast, none of the histopathological characteristics of prion disease were detected within the brains of any of the anti-RANKL mAb-treated mice in which M cells were depleted before oral exposure (**Figure 4**, middle row).

Table 1 Effect of M cell-depletion on susceptibility to oral prion infection

Treatment[a]	Disease incubation periods or survival times (days)[b]	Mean disease incubation period (days±SEM)	Clinical disease[c]	Vacuolar pathology in brain	PrP^Sc in brain	PrP^Sc in GALT and spleen
Control Ig	307, 314, 328, 328, 328, 349, 349, 426	341±13	8/8	8/8	8/8	8/8
Anti-RANKL mAb	8X > *454*		0/8	0/8	0/8	0/8

Abbreviations: GALT, gut-associated lymphoid tissue; mAb, monoclonal antibody; PrP, prion protein; RANKL, receptor activator of NF-κB ligand.
[a]Mice were treated with anti-RANKL mAb for 8 days (or control Ig) and 2 days later orally infected with ME7 scrapie prions.
[b]The notation "NX > *454*" means mice that were free of the clinical and histopathological signs of prion disease up to at least this time after oral exposure.
[c]Incidence=number of animals affected/number of animals tested.

DISCUSSION

These data suggest that M cells are important sites of prion uptake from the gut lumen into Peyer's patches after oral exposure. In order to study the role of M cells in oral prion pathogenesis, a mouse model was used in which M cells within the FAE of Peyer's patches could be depleted through treatment with anti-RANKL mAb. Our data show that coincident with the depletion of M cells by RANKL neutralization, the early accumulation of prions upon FDC in the GALT and spleen was blocked. Furthermore, we show that in the absence of M cells at the time of oral exposure, the spread of prions from the intestinal lumen to the brain and disease susceptibility were also blocked. Although a number of mechanisms have been suggested through which prions may cross the intestinal epithelium after oral exposure, these data suggest that M cells are the main sites of uptake.

The translocation of PrP[d] across the intestinal epithelium in sheep has been studied in an *in vivo* gut loop model.[19] In these studies, scrapie-affected brain homogenate was injected directly into the ligated gut lumen and the dissemination of the inoculum monitored by IHC. In contrast to the data in the current study, these studies suggested that prion uptake across the gut epithelium occurred via enterocytes independently of M cell-mediated transcytosis. However, if enterocytes do significantly contribute to the translocation of prions across the gut epithelium *in vivo*, one would not expect the specific depletion of M cells to completely block oral prion disease susceptibility as shown here. The reasons for the discrepancies between these studies are uncertain. In the sheep studies, no intra-epithelial cell-associated PrP[d] was detected suggesting that the prions may have been carried into the Peyer's patches by M cells at levels below the threshold of IHC detection. In addition, large quantities of prion-infected brain homogenate were injected directly into the lumen of the ligated gut loops. This may have protected the prions from any possible degradative effects of digestive enzymes as they travel along the gastrointestinal tract after oral exposure. As a consequence, the presence of much higher dose of prions may have facilitated their uptake into an alternative compartment to that utilized following exposure to physiologically relevant doses via the oral cavity. To avoid these issues in the current study, we deliberately chose to infect the mice by ingestion of prion-contaminated food pellets to closely model oral pathogenesis via a physiologically relevant and natural route

of exposure. Of course, although a role for RANK stimulation in antigen uptake by enterocytes has not been described, data in the current study cannot entirely exclude the possibility that RANKL neutralization may have affected the uptake of prions by these cells.

The mechanism through which prions may be acquired by M cells is uncertain. The identification of the molecules involved may reveal important targets for the design of treatments to specifically block the uptake of prions from the gut lumen. Prion uptake into M cells could occur nonspecifically, via pinocytosis, or specifically via receptor-mediated endocytosis. For example, the glycoprotein GP2 on the apical plasma membrane of M cells acts as a transcytotic receptor for mucosal antigens.[25] The demonstration that M cells express high levels of PrP^C on their surfaces highlights another plausible molecular mechanism by which prions[14] and other pathogenic microorganisms[30] may be specifically acquired. Tunneling nanotubes have been proposed as an intercellular conduit through which prions may disseminate between cells.[31] The protein M-Sec (encoded by *Tnfaip2*) is expressed at high levels by M cells and mononuclear phagocytes, and functions as a key regulator of tunneling nanotubes formation.[32] Most M cells within the FAE of Peyer's patches appear to have a one-to-one association with mononuclear phagocytes, which extend their dendritic processes into the basolateral pockets of M cells.[33] This tight association raises the suggestion that prions may also be transferred between M cells and mononuclear phagocytes via tunneling nanotubes.

Although RANK–RANKL stimulation is important for the development and function of M cells,[13] osteoclasts,[27] and lymphoid tissue inducer cells,[34] our data show that RANKL neutralization did not appear to affect the status of FDC and mononuclear phagocytes in the GALT. These data are consistent with the restriction of high levels of RANK expression to the epithelium in the intestine[13] and expression of negligible levels of *Tnfrsf11a* by Peyer's patch lymphocytes,[35] FDC, macrophages, and classical DC (including Peyer's patch classical DC) when compared with the FAE, osteoclasts, and lymphoid tissue inducer cells (**Supplementary Figure 1** online). Previous studies have suggested that RANKL can promote the survival of bone marrow-derived DC and their expression of pro-inflammatory cytokines such as IL-12 p40.[36,37] However, these data were derived from the *in vitro* analysis of bone marrow-derived DC, which have a distinct transcriptomic profile from tissue classical
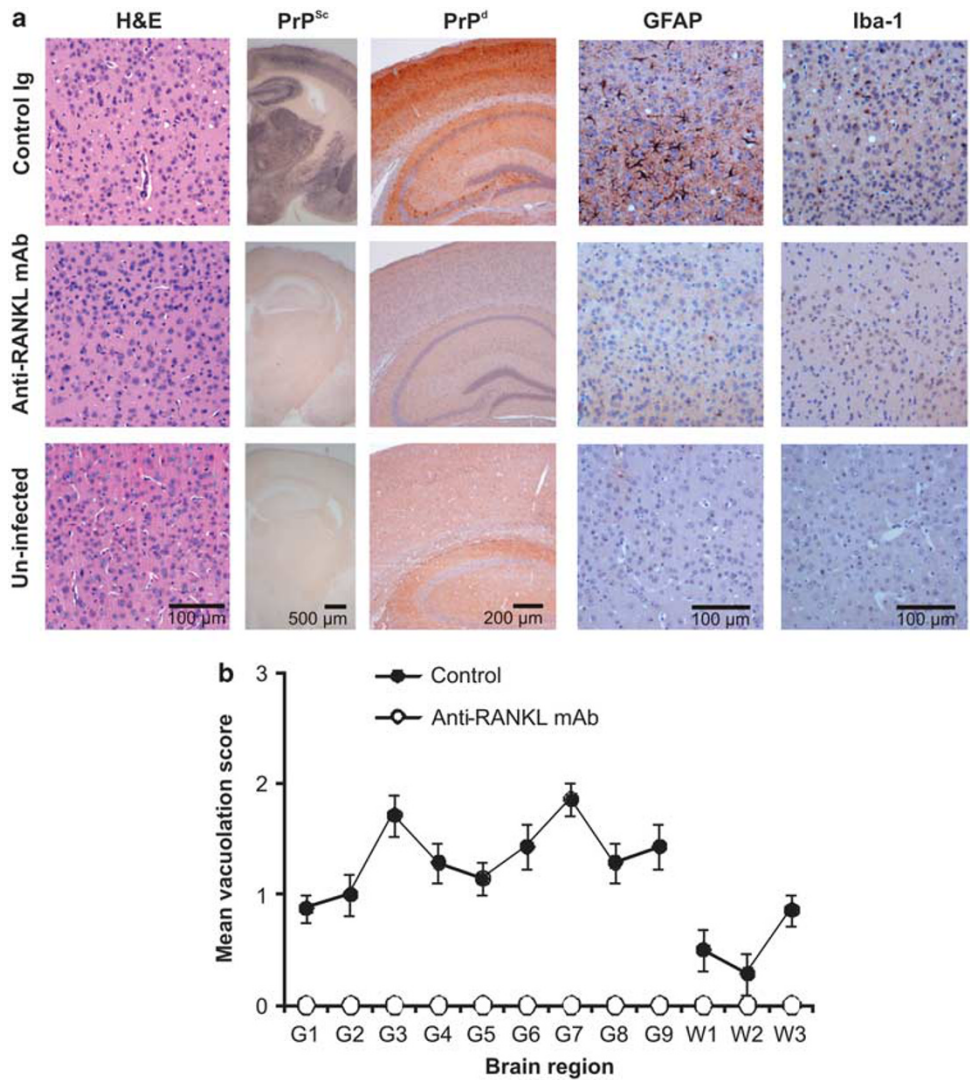
Figure 4 M cell-depletion blocks prion disease susceptibility after oral exposure. Control (control Ig) and M cell-depleted anti-receptor activator of NF-κB ligand (RANKL) monoclonal antibody (mAb)-treated mice were orally infected with ME7 scrapie prions. Brains were collected from clinically scrapie-affected mice and mice that were free of the clinical signs of prion disease at the end of the experiment (454 days after exposure), and the neuropathology within each brain was compared. (**a**) High levels of spongiform pathology (hematoxylin and eosin (H&E), left-hand column), heavy accumulations of disease-specific prion protein (PrP) (brown, middle column), reactive astrocytes expressing glial fibrillary acidic protein (GFAP) (brown, fourth column), and active microglia expressing Iba-1 (brown, right-hand column) were detected in the brains of all clinically scrapie-affected control mice (upper row). Analysis of adjacent sections by paraffin-embedded tissue-immunoblot analysis confirmed the presence of proteinase K-resistant PrP^{Sc} (blue/black, second column). In contrast, none of the M cell-depleted anti-RANKL mAb-treated mice (middle row) developed clinical signs of prion disease during their life spans or displayed histopathological signs of prion disease in their brains. Data are representative of tissues from eight mice per group. (**b**) Pathological assessment of the spongiform change (vacuolation) in brains from terminally scrapie-affected mice and mice that remained free of the signs of disease at the end of the experiment. Vacuolation was scored on a scale of 0–5 in the following gray (G1–G9) and white (W1–W3) matter areas: G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex; W1, inferior and middle cerebellar peduncles; W2, decussation of superior cerebellar peduncles; and W3, cerebellar peduncles. Data are representative of tissues from eight mice per group.

DC.[38] Indeed, unlike bone marrow-derived DC, mucosal DC from Peyer's patches and MLNs express very low levels of RANK on their surfaces and do not exhibit prolonged survival or induce IL-12 p40 expression in response to RANKL treatment.[39] Furthermore, the density and distribution of CD11c^{+} cells is also unaffected in cryptopatches of RANKL$^{-/-}$ mice.[28] Thus, while a potential role for RANK–RANKL stimulation in antigen uptake from the gut lumen cannot be entirely excluded, these data suggest that possible effects of RANKL neutralization

on DC were not the major influence on oral prion pathogenesis. Although an important role for RANK–RANKL stimulation in the regulation of bone remodeling by osteoclasts has been described,[27] a temporary blockade of this activity after treatment with anti-RANKL mAb is highly unlikely to influence the early uptake of prions into Peyer's patches. Taken together, these data, and data from the current study, suggest the major effect of RANKL neutralization on oral prion susceptibility was due to effects on M cell-differentiation.

Data from the current study and elsewhere suggest that after oral exposure, prion neuroinvasion occurs by the following cellular relay. After ingestion of a contaminated meal, prions are initially transcytosed across the FAE from the intestinal lumen by M cells. In the absence of M cells, the intestinal epithelium appears to act an impermeable barrier to the uptake of prions, and as a consequence the prions are most likely degraded within the gut lumen and excreted. Once the prions have crossed the epithelium, they are subsequently acquired by mononuclear phagocytes, which appear to act as 'Trojan horses' and carry them towards the B-cell follicles within the Peyer's patches.[23] The prions are then acquired by FDC, which are considered to amplify the prions above the threshold level required for neuroinvasion.[1,3,7] Following their expansion upon FDC, prions subsequently infect neighboring nerve fibers of the enteric nervous system. Prions are mainly considered to spread to the central nervous system via the peripheral nervous system,[12] (sympathetic and parasympathetic), although the hematogenous route may provide a parallel pathway of neuroinvasion.

Diet (consumption of prion-contaminated food) and prion protein genotype are important factors that affect prion disease susceptibility. The status of lymphoid tissue architecture in the gut also has a role as inflammation may enhance prion uptake or expand their tissue distribution[3,40,41] demonstrated by the fact that bacterial colitis alters oral prion susceptibility.[42] The effects of intestinal inflammatory responses on M cell density may also influence prion pathogenesis. For example, recombinant-RANKL treatment stimulates the widespread expansion of functional M cells enhancing the uptake of antigens and enteric bacteria,[13] and M cell status can also be influenced by the presence of pathogenic bacteria in the gut lumen.[43,44] These data suggest it is plausible that the triggering of M cell-differentiation by concurrent infection with an intestinal pathogen or inflammatory stimuli may also influence oral prion susceptibility.

In conclusion, our data suggest that M cells are important sites of prion uptake from the gut lumen into Peyer's patches. In the absence of M cells at the time of oral exposure, prion accumulation upon FDC in the GALT, neuroinvasion, and disease susceptibility are blocked. Antigen uptake by M cells is an important initial step in the induction of efficient immune responses after oral vaccination. Furthermore, the targeted delivery of vaccine antigens to M cells is an effective means of inducing antigen-specific immune responses.[45] Mucosal immunization has been shown to have significant efficacy against oral prion infection in mice.[46] Therefore, data in the current study suggest M cells represent a novel cellular target for intervention in orally acquired prion infections.

METHODS

Mice. C57BL/6 mice (6–8 weeks old) were used throughout this study. Mice were maintained under specific pathogen free (SPF) conditions. All studies using experimental mice and regulatory licenses were approved by both The Roslin Institute's and University of Edinburgh's Protocols and Ethics Committees. All animal experiments were carried out under the authority of a UK Home Office Project Licence within the terms and conditions of the strict regulations of the UK Home Office 'Animals (scientific procedures) Act 1986'. Where necessary, anesthesia

appropriate for the procedure was administered, and all efforts were made to minimize harm and suffering. Mice were humanely culled using a UK Home Office Schedule One method.

Treatment with anti-RANKL mAb. To neutralize the activity of RANKL *in vivo*, mice were injected intraperitoneally with 250 µg of the IK22-5 rat anti-mouse RANKL mAb[47] every 2 days for 8 days as described.[13] A parallel group of mice were treated with an isotype-matched nonspecific rat mAb, rat IgG2a κ (eBioscience, San Diego, CA) as a control.

Prion exposure and disease monitoring. For oral exposure, mice were fed individual food pellets doused with 50 µl of a 10% (w/v) dilution of scrapie brain homogenate prepared from mice terminally affected with ME7 scrapie prions (containing ~2.5×10^4 intra-cerebral ID_{50} units). To do so, during the dosing period mice were individually housed in bedding- and food-free cages. Water was provided *ad libitum*. A single prion-dosed food pellet was then placed in the cage. The mice were returned to their original cages (with bedding and food *ad libitum*) as soon as the food pellet was observed to have been completely ingested. The use of bedding-free and additional food-free cages ensured easy monitoring of consumption of the prion-contaminated food pellet. Following exposure, mice were coded and assessed blindly for the signs of clinical prion disease and culled at a standard clinical endpoint.[48] Survival times were recorded for mice that did not develop clinical signs of disease. Scrapie diagnosis was confirmed blindly on coded sections by histopathological assessment of vacuolation in the brain. For the construction of lesion profiles, vacuolar changes were scored in nine gray-matter and three white-matter areas of brain as described.[49] Where indicated, some mice were culled at the times indicated post injection with prions and tissues taken for further analysis.

IHC and immunofluorescent analyses. For whole-mount staining, Peyer's patches were dissected from small intestines and fixed with BD Cytofix/Cytoperm (BD Biosciences, Oxford, UK). Tissues were subsequently immunostained with rat anti-mouse GP2 mAb (MBL International, Woburn, MA). Following addition of primary Ab, tissues were stained with Alexa Fluor 488-conjugated anti-rat IgG Ab (Invitrogen, Paisley, UK), rhodamine-conjugated *Ulex europaeus* agglutinin I (UEA-1; Vector Laboratories, Burlingame, CA), and Alexa Fluor 647-conjugated phalloidin (Invitrogen).

Intestines, MLNs, and spleens were removed and snap-frozen at the temperature of liquid nitrogen. Serial frozen sections (10 µm in thickness) were cut on a cryostat and immunostained with the following antibodies: FDC were visualized by staining with mAb 7G6 to detect CR2/CR1 (CD21/CD35) and mAb 8C12 to detect CR1 (CD35; BD Biosciences). Cellular PrP^C was detected using PrP-specific polyclonal antibody (pAb) 1B3.[50] B cells were detected using rat anti-mouse B220 mAb (clone RA3-RB2, Caltag, Towcester, UK). Mononuclear phagocytes were detected using rat anti-mouse F4/80 mAb (clone CI:A31, AbD serotec, Kidlington, UK) and hamster anti-mouse CD11c mAb (clone N418, AbD Serotec). For the detection of disease-specific PrP (PrP^d) in Peyer's patches, MLNs, spleens, and brains, tissues were fixed in periodate-lysine-paraformaldehyde fixative and embedded in paraffin wax. Sections (thickness 6 µm) were deparaffinized, and pre-treated to enhance the detection of PrP^d by hydrated autoclaving (15 min, 121°C, hydration) and subsequent immersion formic acid (98%) for 5 min. Sections were then immunostained with 1B3 PrP-specific pAb. For the detection of astrocytes, brain sections were immunostained with anti-glial fibrillary acidic protein (GFAP; DAKO, Ely, UK). For the detection of microglia, deparaffinized brain sections were first pre-treated with Target Retrieval Solution (DAKO) and subsequently immunostained with anti-ionized calcium-binding adaptor molecule 1 (Iba-1; Wako Chemicals GmbH, Neuss, Germany). Immunolabelling was revealed using HRP-conjugated to the avidin–biotin complex (Novared kit, Vector Laboratories, Peterborough, UK). PET immunoblot analysis was used to confirm

the PrP^d detected by immunohistochemistry, which was PK-resistant PrP^{Sc}.[29] Membranes were subsequently immunostained with 1B3 PrP-specific pAb.

For light microscopy, following the addition of primary antibodies, biotin-conjugated species-specific secondary antibodies (Stratech, Soham, UK) were applied and immunolabelling was revealed using HRP-conjugated to the avidin–biotin complex. Sections were counterstained with hematoxylin to distinguish cell nuclei. For fluorescent microscopy, following the addition of primary antibody, streptavidin-conjugated or species-specific secondary antibodies coupled to Alexa Fluor 488 (green), Alexa Fluor 594 (red), or Alexa Fluor 647 (blue) dyes (Invitrogen) were used. Sections were mounted in fluorescent mounting medium (DAKO) and examined using a Zeiss LSM5 confocal microscope (Zeiss, Welwyn Garden City, UK).

Statistical analyses. Data are presented as mean±s.e. Unless indicated otherwise, significant differences between samples in different groups were sought by Student's t-test. Values of $P < 0.05$ were accepted as significant.

REFERENCES
1. Mabbott, N.A., Young, J., McConnell, I. & Bruce, M.E. Follicular dendritic cell dedifferentiation by treatment with an inhibitor of the lymphotoxin pathway dramatically reduces scrapie susceptibility. *J. Virol.* **77,** 6845–6854 (2003).
2. Prinz, M. *et al.* Oral prion infection requires normal numbers of Peyer's patches but not of enteric lymphocytes. *Am. J. Pathol.* **162,** 1103–1111 (2003).
3. Glaysher, B.R. & Mabbott, N.A. Role of the GALT in scrapie agent neuroinvasion from the intestine. *J. Immunol.* **178,** 3757–3766 (2007).
4. Andreoletti, O. *et al.* Early accumulation of PrP^{Sc} in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie. *J. Gen. Virol.* **81,** 3115–3126 (2000).
5. Sigurdson, C.J. *et al.* Oral transmission and early lymphoid tropism of chronic wasting disease PrP^{res} in mule deer fawns (*Odocoileus hemionus*). *J. Gen. Virol.* **80,** 2757–2764 (1999).
6. Mabbott, N.A. *et al.* Expression of mesenchyme-specific gene signatures by follicular dendritic cells: insights from the meta-analysis of microarray data from multiple mouse cell populations. *Immunology* **133,** 482–498 (2011).
7. McCulloch, L. *et al.* Follicular dendritic cell-specific prion protein (PrP^C) expression alone is sufficient to sustain prion infection in the spleen. *PLoS Pathogens* **7,** e1002402 (2011).
8. Bolton, D.C., McKinley, M.P. & Prusiner, S.B. Identification of a protein that purifies with the scrapie prion. *Science* **218,** 1309–1311 (1982).
9. Legname, G. *et al.* Synthetic mammalian prions. *Science* **305,** 673–676 (2004).
10. Brown, K.L. *et al.* Scrapie replication in lymphoid tissues depends on PrP-expressing follicular dendritic cells. *Nat. Med.* **5,** 1308–1312 (1999).
11. Klein, M.A. *et al.* PrP expression in B lymphocytes is not required for prion neuroinvasion. *Nat. Med.* **4,** 1429–1433 (1998).
12. Glatzel, M., Heppner, F.L., Albers, K.M. & Aguzzi, A. Sympathetic innervation of lymphoreticular organs is rate limiting for prion neuroinvasion. *Neuron* **31,** 25–34 (2001).
13. Knoop, K.A. *et al.* RANKL is necessary and sufficient to initiate development of antigen-sampling M cells in the intestinal epithelium. *J. Immunol.* **183,** 5738–5747 (2009).
14. Nakato, G. *et al.* New approach for M-cell-specific molecules by screening comprehensive transcriptome analysis. *DNA Res.* **16,** 227–235 (2009).
15. Neutra, M.R., Frey, A. & Kraehenbuhl, J.-P. Epithelial M cells: gateways for mucosal infection and immunization. *Cell* **86,** 345–348 (1996).
16. Takakura, I. *et al.* Orally administered prion protein is incorporated by M cells and spreads to lymphoid tissues with macrophages in prion protein knockout mice. *Am. J. Pathol.* **179,** 1301–1309 (2011).
17. Foster, N. & Macpherson, G.G. Murine cecal patch M cells transport infectious prions *in vivo*. *J. Infect. Dis.* **202,** 1916–1919 (2010).
18. Heppner, F.L. *et al.* Transepithelial prion transport by M cells. *Nat. Med.* **7,** 976–977 (2001).
19. Jeffrey, M. *et al.* Transportation of prion protein across the intestinal mucosa of scrapie-susceptible and scrapie-resistant sheep. *J. Pathol.* **209,** 4–14 (2006).
20. Mishra, R.S. *et al.* Protease-resistant human prion protein and ferritin are cotransported across Caco-2 epithelial cells: implications for species barrier in prion uptake from the intestine. *J. Neurosci.* **24,** 11280–11290 (2004).
21. Rescigno, M. *et al.* Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* **2,** 361–367 (2001).
22. Chieppa, M., Rescigno, M., Huang, A.Y.C. & Germain, R.N. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J. Exp. Med.* **203,** 2841–2852 (2006).
23. Raymond, C.R., Aucouturier, P. & Mabbott, N.A. *In vivo* depletion of CD11c$^+$ cells impairs scrapie agent neuroinvasion from the intestine. *J. Immunol.* **179,** 7758–7766 (2007).
24. Terahara, K. *et al.* Comprehensive gene expression profiling of Peyer's patch M cells, villous M-like cells, and intestinal epithelial cells. *J. Immunol.* **180,** 7840–7846 (2008).
25. Hase, K. *et al.* Uptake through glycoprotein 2 of FimH$^+$ bacteria by M cells initiates mucosal immune responses. *Nature* **462,** 226–231 (2009).
26. Zabel, M.D. *et al.* Stromal complement receptor CD21/35 facilitates lymphoid prion colonization and pathogenesis. *J. Immunol.* **179,** 6144–6152 (2007).
27. Kong, Y.Y. *et al.* OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph node organogenesis. *Nature* **397,** 315–323 (1999).
28. Knoop, K.A., Butler, B.R., Kumar, N., Newberry, R.D. & Williams, I.R. Distinct developmental requirements for isolated lymphoid follicle formation in the small and large intestine. *Am. J. Pathol.* **179,** 1861–1871 (2011).
29. Schulz-Schaeffer, W.J. *et al.* The paraffin-embedded tissue blot detects PrP^{sc} early in the incubation time in prion diseases. *Am. J. Pathol.* **156,** 51–56 (2000).
30. Watarai, M. *et al.* Cellular prion protein promotes *Brucella* infection into macrophages. *J. Exp. Med.* **198,** 5–17 (2003).
31. Gousset, K. *et al.* Prions hijack tunnelling nanotubes for intercellular spread. *Nat. Cell. Biol.* **11,** 328 (2009).
32. Hase, K. *et al.* M-Sec promotes membrane nanotube formation by interacting with Ral and the exocyst complex. *Nat. Cell. Biol.* **11,** 1427–1432 (2009).
33. Wang, J., Gusti, V., Saraswati, A. & Lo, D.D. Convergent and divergent development among M cell lineages in mouse mucosal epithelium. *J. Immunol* **187,** 5277–5285 (2011).
34. Kim, D. *et al.* Regulation of peripheral lymph node genesis by the tumour necrosis factor family member TRANCE. *J. Exp. Med.* **192,** 1467–1478 (2000).
35. Ebisawa, M. *et al.* CCR6^{hi}CD11c^{int} B cells promote M-cell differentiation in Peyer's patch. *Int. Immunol.* **23,** 261–269 (2011).
36. Wong, B.R. *et al.* TRANCE (tumour necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J. Exp. Med.* **186,** 2075–2080 (1997).

37. Chino, T., Draves, K.E. & Clark, E.A. Regulation of dendritic cell survival and cytokine production by osteoprotegerin. *J. Leukoc. Biol.* **86,** 933–940 (2009).

38. Mabbott, N.A., Baillie, J.C., Hume, D.A. & Freeman, T.C. Meta-analysis of co-expressed gene signatures in mouse leukocyte populations. *Immunobiology* **215,** 724–736 (2010).

39. Williamson, E., Bilsborough, J.M. & Viney, J.L. Regulation of mucosal dendritic cell function by receptor activator of NF-kB (RANK)/RANKL interactions: impact on tolerance induction. *J. Immunol.* **169,** 3606–3612 (2002).

40. Seeger, H. *et al.* Coincident scrapie infection and nephritis lead to urinary prion excretion. *Science* **310,** 324–326 (2005).

41. Heikenwalder, M. *et al.* Lymphotoxin-dependent prion replication in inflammatory stromal cells of granulomas. *Immunity* **29,** 998–1008 (2008).

42. Sigurdson, C.J. *et al.* Bacterial colitis increases susceptibility to oral prion pathogenesis. *J. Infect. Dis.* **199,** 243–252 (2009).

43. Borghesi, C. & Nicoletti, C. Rapid appearance of M cells after microbial challenge is restricted at the periphery of the follicle-associated epithelium of Peyer's patch. *Lab. Invest.* **79,** 1393–1401 (1999).

44. Meynell, H.M. *et al.* Up-regulation of microsphere transport across the follicle-associated epithelium of Peyer's patch by exposure to *Streptococcus pneumoniae* R36a. *FASEB J.* **13,** 611–619 (1999).

45. Nochi, T. *et al.* A novel M cell-specific carbohydrate-targeted mucosal vaccine effectively induces antigen-specific immune responses. *J. Exp. Med.* **204,** 2789–2796 (2007).

46. Goñi, F. *et al.* Mucosal vaccination delays or prevents prion infection via the oral route. *Neuroscience* **133,** 413–421 (2005).

47. Kamijo, S. *et al.* Amelioration of bone loss in collagen-induced arthritis by neutralizing anti-RANKL monoclonal antibody. *Biochem. Biophys. Res. Commun.* **347,** 124–132 (2006).

48. Fraser, H. & Dickinson, A.G. Agent-strain differences in the distribution and intensity of grey matter vacuolation. *J. Comp. Pathol.* **83,** 29–40 (1973).

49. Fraser, H. & Dickinson, A.G. The sequential development of the brain lesions of scrapie in three strains of mice. *J. Comp. Pathol.* **78,** 301–311 (1968).

50. Farquhar, C.F., Somerville, R.A. & Ritchie, L.A. Post-mortem immunodiagnosis of scrapie and bovine spongiform encephalopathy. *J. Virol. Met.* **24,** 215–222 (1989).