PrP-dependent association of prions with splenic but not circulating lymphocytes of scrapie-infected mice

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An intact immune system, and particularly the presence of mature B lymphocytes, is crucial for mouse scrapie pathogenesis in the brain after peripheral exposure. Prions are accumulated in the lymphoreticular system (LRS), but the identity of the cells containing infectivity and their role in neuroinvasion have not been determined. We show here that although prion infectivity in the spleen is associated with B and T lymphocytes and to a lesser degree with the stroma, no infectivity could be detected in lymphocytes from blood. In wild-type mice, which had been irradiated and reconstituted with PrP-deficient lymphohaematopoietic stem cells and inoculated with scrapie prions, infectivity in the spleen was present in the stroma but not in lymphocytes. Therefore, splenic B and T lymphocytes can either synthesize prions or acquire them from another source, but only when they express PrP.

Keywords: B lymphocytes/follicular dendritic cells/prions/PrP knockout mice/T lymphocytes

Introduction

Intracerebral (i.c.) inoculation of mice with scrapie agent results in a rise of infectivity in the spleen, weeks or months before the brain is affected. However, in the case of non-experimental forms of spongiform encephalopathies, such as sheep scrapie, BSE or iatrogenic forms of Creutzfeldt–Jakob disease (CJD), the agent usually enters from the periphery; intraperitoneal (i.p.) inoculation of mice with scrapie prions is therefore a more realistic model. This procedure results in rapid accumulation of infectious agent in the spleen and other tissues of the lymphoreticular system (LRS) (Eklund *et al.*, 1967; Pattison and Jones, 1968; Fraser and Dickinson, 1970; Mould *et al.*, 1970), following which prions are probably transported along tracts of the peripheral nervous system to enter the brain, presumably via the spinal cord (Kimberlin and Walker, 1980, 1988, 1989; Beekes *et al.*, 1996) or the N.vagus (Beekes *et al.*, 1998). Transport from the periphery to the central nervous system (CNS) depends on elements of the LRS, as evidenced by the fact that development of CNS disease after i.p. inoculation with scrapie is impaired or abolished in mice with various forms of immunodeficiency, such as SCID, RAG^{−/−} or

μMT mice, while i.c. inoculation continues to be fully effective (O'Rourke *et al.*, 1994; Fraser *et al.*, 1996; Lasmezaz *et al.*, 1996; Taylor *et al.*, 1996; Brown *et al.*, 1997; Klein *et al.*, 1997).

Results

Determination of scrapie infectivity in peripheral blood leukocytes and fractionated splenocytes

In a first experiment, we analysed spleens of wild-type (129/Sv-C57BL/6) mice 34 days after i.p. inoculation with RML prions. Spleens were fractionated into pulp and stroma. B and T cells were purified from the pulp fraction by magnetic-activated cell sorting (MACS) followed by complement lysis of B cells in the T-cell fraction and vice versa. Finally, viable cells were isolated by density gradient centrifugation. This three-step procedure led consistently to highly purified T- and B-lymphocyte preparations devoid of detectable cross-contamination, as shown by fluorescence-activated cell sorting (FACS) analysis (Figure 1), in 5–10% yield. In addition, a non-B, non-T cell population was obtained by depleting splenocytes of B and T lymphocytes by MACS; this fraction contained ≤2% T but no detectable B lymphocytes. The cell preparations and the stroma fraction were analysed for infectivity by endpoint titration (Brandner *et al.*, 1996; Fischer *et al.*, 1996) (Table I). Total splenocytes had ~3.5 log LD₅₀ units per 10⁶ cells, and both B and T cells showed infectivity titres within the same order of magnitude, 3.4 and 3.5 log LD₅₀ units per 10⁶ cells, respectively. Strikingly, the non-B, non-T cell population contained only ~1 log LD₅₀ unit per 10⁶ cells (which could be attributed to the ≤2% contamination by T lymphocytes), arguing that prion infectivity in the splenocyte fraction was not due to unspecific contamination with infectivity released from the stromal fraction (Clarke and Kimberlin, 1984). The pulp of a spleen contains 2–4×10⁸ cells, ~80% of which are lymphocytes (Binder *et al.*, 1997). Inasmuch as the purified B and T cells were representative of their class as regards infectivity, ~300×3.5 log LD₅₀ units = 6 log LD₅₀ units of infectivity were associated with the pulp and ~4.3 log LD₅₀ units with the stroma of one spleen (Figure 2). Essentially all infectivity detected in total spleen extracts was accounted for by the fractions. There was ~50 times more infectivity associated with lymphocytes than with stroma. Clarke and Kimberlin (1984) have reported about equal distribution of infectivity between pulp and stroma; however, their data were obtained with mouse and scrapie strains different from ours.

These findings suggested that lymphocytes might be responsible for spreading prions through the organism. We therefore determined the infectivity of peripheral blood leukocytes (PBLs) from the same animals whose spleens had been analysed. We were surprised to find that despite

Fig. 1. Flow cytometric analysis of splenocytes and purified splenocyte fractions. Splenocytes from wild-type mice 34 days after i.p. inoculation with RML scrapie agent were fractionated as described in the Materials and methods, and subjected to FACS analysis. More than 99% of the cells in the purified B cell fraction were positive for the B-cell marker B220 and negative for the T-cell marker CD3. Similarly, >99% of purified cells in the T-cell fraction were positive for CD3 and negative for B220. The same results were obtained whether or not the cells were gated for lymphocytes by forward and side scattering. Ordinate, cell counts; abscissa, logarithm of fluorescence intensity.

Table I. Infectivity in spleen cell fractions and PBLs of scrapie-infected mice^a

Fraction	Cell No. or dilution	Tg94/IRF		<i>Prnp</i> ^{+/+}		<i>Prnp</i> ^{+/+} [FLC- <i>Prnp</i> ^{0/0}]	
		Inc. time	n/No.	Inc. time	n/No.	Inc. time	n/No.
Splenocytes	10 ⁶	n.d.		n.d.		>200	(0/4)
	10 ⁵	88, 90	(2/2)	81, 86	(2/2)	n.d.	
	10 ⁴	106.5 ± 18	(4/4)	89 ± 5	(4/4)		
	10 ³	108, 125	(2/4)	106 ± 7	(4/4)		
	10 ²	>200	(0/4)	>200	(0/4)		
B cells	2×10 ⁵	n.d.		n.d.		>200	(0/4)
	2×10 ⁴	106 ± 10	(4/4)	88 ± 2	(4/4)	n.d.	
	2×10 ³	98, 114	(2/4)	102 ± 14	(4/4)		
	2×10 ²	>200	(0/4)	91	(1/4)		
T cells	10 ⁵	n.d.		n.d.		>200	(0/4)
	10 ⁴	109, 110	(2/4)	94 ± 8	(4/4)	n.d.	
	10 ³	>200	(0/4)	95, 121	(2/2)		
	10 ²	>200	(0/4)	>200	(0/4)		
Non-B/T cells	5×10 ⁵	108	(1/4)	112 ± 14	(3/4)	>200	(0/4)
	5×10 ⁴	n.d.		>200	(0/4)	n.d.	
PBL	10 ⁶	>200	(0/4)	>200	(0/4)	>200	(0/4)
Stroma homogenate	undiluted	69 ± 2	(4/4)	72 ± 3	(4/4)	83 ± 2	(4/4)
	10 ⁻²	80, 94	(2/4)	108 ± 20	(3/4)		n.d.
	10 ⁻⁴	>200	(0/4)	>200	(0/4)		

^aWild-type mice (*Prnp*^{+/+}), transgenic *Prnp*^{0/0} mice overexpressing PrP in spleen (Tg94IRF) and wild-type mice irradiated and reconstituted with FLCs from *Prnp*^{0/0} mice (*Prnp*^{+/+} [FLC-*Prnp*^{0/0}]) were inoculated i.p. with RML prions, spleens were recovered after 34 days and processed as described in the Materials and methods section. The number of cells indicated or a 1% stroma homogenate at the dilution indicated was inoculated intracerebrally into indicator mice and the time to disease (inc. time) was recorded. n/No., number of animals succumbing to scrapie/number inoculated. n.d., not done.

Fig. 2. Distribution of prion infectivity in spleen fractions and peripheral blood leukocytes of scrapie-infected mice. Serial 10-fold dilutions of splenocytes, splenocyte fractions, spleen stroma homogenate or PBLs were inoculated intracerebrally into groups of four indicator mice and the incubation time to terminal scrapie disease was determined. Infectivity titres were calculated by the endpoint titration method (Reed and Muench, 1938) from the data given in Table I, assuming 3×10^8 lymphocytes/spleen, of which 65% were B and 35% T lymphocytes. The detection limit of the infectivity assay corresponds to 100 LD₅₀ units per spleen.

the relatively high infectivity associated with splenic B and T cells, intracerebral inoculation of 10^6 PBLs from buffy coat did not cause disease in any of four indicator mice (Table I). Because ~80% of murine PBLs are lymphocytes (Binder *et al.*, 1997), <1 LD₅₀ unit of infectivity was associated with 8×10^5 lymphocytes; the specific infectivity of peripheral lymphocytes was therefore at least 2000–3000 times less than that of splenic lymphocytes.

We verified the infectivity measurements in a second, similar experiment, using mice transgenic for a cluster consisting of the PrP coding region under the control of a hybrid immunoglobulin heavy chain enhancer/IRF-1 promoter (Yamada *et al.*, 1991). These mice, designated Tg94/IRF, overexpress PrP >1000-fold in spleen and >100-fold in thymus, while PrP in brain is 0.05 of that in wild-type (Raeber *et al.*, 1999). After i.p. inoculation with 10^6 LD₅₀ units of the RML mouse prions, wild-type mice developed scrapie after ~195 days whereas Tg94/IRF mice developed disease after ~450 days, presumably because of the low level expression of PrP in the brain. Between 2 weeks and 6 months after inoculation, splenocytes from Tg94/IRF and wild-type mice had essentially the same titre: ~7 log LD₅₀ U/ml of 10% homogenate. There was no detectable infectivity in brain as compared with 8 log LD₅₀ U/ml of 10% homogenate for wild type. However, 1 year after inoculation, the brain showed a prion titre of ~7 log LD₅₀ U/ml of 10% homogenate (Raeber *et al.*, 1999).

Spleens of Tg94/IRF mice, collected 34 days after i.p. inoculation, were fractionated as before. Again, infectivity was associated with purified B and T cells and stroma, but not with the non-B, non-T cell fraction or with PBLs (<1 LD₅₀ unit per 10^6 cells; Table I; Figure 2).

Association of infectivity with splenic lymphocytes is dependent on PrP expression

We have shown previously that prion replication does not occur in the spleen of *Prnp*^{0/0} mice (Bueler *et al.*, 1993). Moreover, reconstitution of the LRS of *Prnp*^{0/0} mice with PrP-expressing fetal liver cells (FLCs) restored prion accumulation in the spleen (Blattler *et al.*, 1997). To assess whether the association of infectivity with lymphocytes was specific or adventitious, we lethally irradiated *Prnp*^{+/+} mice and reconstituted them with FLCs derived from *Prnp*^{0/0} mice. PCR analysis of splenocytes, PBLs, stroma and tail tissue confirmed that these mice had undergone successful reconstitution, and FACS analysis of lymphocytes demonstrated the *Prnp*^{0/0} origin of these cells (data not shown). Spleens from these mice, 34 days after i.p. inoculation with RML prions, were fractionated and analysed: no infectivity was found in either total splenocytes (<1 LD₅₀ unit per 10^6 cells), or in purified B or T lymphocytes (<1 LD₅₀ unit per 10^5 cells). However, prion titres in the stromal fraction were not significantly different from those in wild-type mice (Table I; Figure 2). Two-colour immunofluorescence analysis of spleen sections stained with follicular dendritic cell marker FDC-M1 and a polyclonal antibody to PrP revealed PrP accumulation in FDCs (Klein *et al.*, 1998). From these results, we conclude that scrapie infectivity in spleens of irradiated mice reconstituted with *PrP*^{0/0} lymphohaemopoietic stem cells is probably associated with FDCs and that splenic lymphocytes devoid of PrP fail to produce or to take up scrapie infectivity.

Discussion

It is remarkable that despite the >1000-fold overexpression of PrP in the spleen of Tg94/IRF mice, the prion titre in this

organ was not higher than that in wild-type mice. Either the level of PrP is not the limiting factor for the formation of prions or else overexpression is not occurring in the cells in which prions are formed. Similarly, at the terminal stage of scrapie, the prion titre in brains of *tga20* mice, which overexpress PrP 5- to 8-fold, is about the same as in wild-type, although the incubation times for the transgenic and wild-type mice are 60 and 160 days, respectively (Fischer *et al.*, 1996).

The experiments described here show that in the spleen of i.p. scrapie-infected wild-type mice, prions are associated with B and T lymphocytes as well as with the stroma, but not with the pulp-derived non-B, non-T cell fraction which consists mainly of monocytes and granulocytes (Binder *et al.*, 1997). Moreover, there was <1 LD₅₀ unit of infectious agent in $\sim 10^6$ lymphocytes recovered from peripheral blood, i.e. the specific infectivity of lymphocytes in PBLs was at least 2000-fold lower than in the spleen. Because a mouse has $\sim 2 \times 10^7$ circulating lymphocytes, there would be <20 LD₅₀ units of cell-associated infectivity, if any, circulating at one time.

Were the prions associated with splenic lymphocytes generated in these cells or were they acquired from other sources? Unspecific contamination is unlikely because the specific infectivity of non-B, non-T cells derived from the pulp was 2000–3000 times less than that of B or T lymphocytes and because B and T lymphocytes devoid of PrP failed to acquire infectivity in a spleen whose stroma did contain infectious agent. We have generated mice expressing PrP exclusively on T cells (Raeber *et al.*, 1996) or on B cells (F. Montrasio, A.J. Raeber and C. Weissmann, unpublished results) and i.p. inoculation of such mice with scrapie agent failed to result in disease or accumulation of infectivity in the spleen. If lymphocytes are at all able to replicate scrapie agent, then perhaps they can do so only in the context of other types of PrP-bearing cells.

Infectivity in the stromal component of peripheral lymphoid organs is thought to reside in radiation-resistant post-mitotic cells (Fraser and Farquhar, 1987; Fraser *et al.*, 1989). A prime candidate is the follicular dendritic cell, because the pathological isoform of the prion protein, PrP^{Sc}, co-localizes with FDCs in mice inoculated with CJD or scrapie agent (Kitamoto *et al.*, 1991; McBride *et al.*, 1992; Muramoto *et al.*, 1992). Because lymphocytes interdigitate with FDCs (Heinen *et al.*, 1995), they might have acquired prions or prion-containing, torn-off membrane fragments from the latter during the isolation procedure. However, when wild-type mice were irradiated and reconstituted with PrP-less FLCs, no infectivity was found on splenic lymphocytes, which means that if this 'transfer' hypothesis is correct, the postulated adhesion of scrapie agent is dependent on the presence of PrP on lymphocytes; PrP would then function as the receptor for the infectious agent. A likely source of prions would be the FDCs.

Why is infectivity found on splenic but not on circulating lymphocytes? Perhaps only distinct subsets of splenic lymphocytes carry infectivity and these do not enter the blood stream, or perhaps prion-carrying splenic lymphocytes are eliminated in the spleen. Alternatively, prions may attach to lymphocytes only during the preparation procedure, albeit through a PrP-dependent interaction.

As regards the role of lymphocytes in the spread of

infectivity from the periphery to the CNS, it would seem that circulating, prion-bearing B cells are not required, not only because they were not detected outside the LRS, but..because reconstitution of irradiated wild-type mice (Blattler *et al.*, 1997) or immunodeficient mice (Klein *et al.*, 1998) with LRS devoid of PrP restored invasion of the CNS by prions following i.p. inoculation.

Materials and methods

Scrapie infection

RML is a mouse-adapted scrapie isolate (Chandler, 1961). It was passaged in Swiss CD-1 mice obtained from Charles River Laboratories. Inocula were 10% (w/v) homogenates of RML-infected CD-1 mouse brains in 0.32 M sucrose. Mice were infected i.p. with 100 μ l of a 10-fold dilution of the inoculum in phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA).

Bone marrow reconstitution

Eight-week-old *Prnp*^{+/+} mice (129/Sv $\times$ C57BL/6) were lethally irradiated and reconstituted with FLCs from E14.5–15.5 *Prnp*^{0/0} (129/Sv $\times$ C57BL/6) embryos as described previously (Blattler *et al.*, 1997). The extent of reconstitution was assessed by FACS and PCR 6–8 weeks after grafting. Inoculation with mouse scrapie prions was carried out 12 weeks after reconstitution.

Preparation of splenocytes and stroma

Spleens were collected from mice 34 days after i.p. inoculation with the RML strain of prions. Splenocytes were obtained by forcing spleens through a fine mesh platinum wire net. Cell suspensions were prepared in PBS with 1% BSA, 5 mM EDTA and 0.01% sodium azide (MACS buffer). The stromal fraction was recovered from the net and a 1% (w/v) homogenate in PBS–5% BSA was prepared by forcing the suspension through an 18 gauge needle. The homogenate was digested with 1 mg/ml collagenase D (Boehringer Mannheim) for 60 min at 37°C and sonicated (Branson Sonifier, maximal output) four times for 1 min.

B and T cell purification

Splenocytes were incubated with anti-mouse B220 or Thy1.2 antibodies conjugated with super-paramagnetic microbeads (Milteny Biotec GmbH, Germany) for 15 min at 4°C and applied to a pre-filled and washed A2 column fixed onto the VARIO MACS (Milteny Biotec GmbH). Unlabelled cells were eluted with MACS buffer using a 23 gauge syringe attached to the column outlet as flow resistor. The column was removed from the magnet and cells were backflushed using a syringe attached to the column outlet. The column was fixed to the magnet and the cell suspension was allowed to enter the column. Unlabelled cells were again rinsed out with MACS buffer. Finally, the column was removed from the magnet and labelled cells were eluted by rinsing the column with MACS buffer.

B and T cell depletion

Splenocytes were incubated with anti-mouse B220 and anti-mouse Thy1.2 antibodies conjugated with super-paramagnetic microbeads for 15 min at 4°C and applied to a CS column fixed onto the VARIO MACS. Unlabelled cells were eluted with MACS buffer as described above. The flow-through fraction was once again loaded onto a CS column and unlabelled cells eluted in MACS buffer.

Complement lysis

MACS-purified B- and T-cell fractions were purified further by complement lysis of B cells in the T-cell fraction and vice versa. Cells were pelleted and resuspended in RPMI-1640 with 25 mM HEPES (pH 7.4) and 0.3% BSA [cytotoxicity medium (CM)] to give $1\text{--}3 \times 10^7$ cells/ml. For B cell depletion, cells were incubated with a 1:200 dilution of rat anti-mouse LR1 antibody (clone LR6.2B6D6.C9, Serotec). For T cell depletion, cells were incubated with a 1:400 dilution of mouse anti-mouse Thy1.2 antibody (clone F7D5, Serotec) at 4°C for 60 min. The cells were resuspended to the original density in CM containing 20% Low-tox-M rabbit complement (Cedarlane, Ontario, Canada) and incubated for 60 min at 37°C. Viable lymphocytes were separated from dead cells and debris by centrifugation over Lympholyte-M as recommended by the manufacturer (Cedarlane, Ontario, Canada).

Isolation of PBLs from whole blood

PBLs were isolated from whole blood by density gradient centrifugation using Lympholyte-M as recommended by the manufacturer (Cedarlane, Ontario, Canada).

FACS analysis

Single-cell suspensions were prepared in PBS, 2% fetal calf serum, 20 mM EDTA, 0.01% sodium azide (FACS buffer). For flow cytometry, cells were stained with saturating concentrations of fluorescein-conjugated antibodies (1 µg/10⁶ cells) for 30 min at 4°C and washed in FACS buffer. Data acquisition and analysis were performed with an EPICS XL (Coulter) flow cytometer. Dead cells were gated out by forward and side scatter properties. Monoclonal antibodies used were fluorescein isothiocyanate (FITC)-conjugated RA3-6B2 (B220) (Gibco-BRL) and FITC-conjugated KT3 (CD3) (Serotec).

Determination of infectivity

Cell fractions were collected by centrifugation for 10 min at 1000 r.p.m. and diluted serially in PBS–5% BSA. Tga20 mice (Fischer *et al.*, 1996) were inoculated i.c. with 30 µl of inoculum into the right parietal lobe, using a 26 gauge hypodermic needle. Prion titres were estimated from the incubation times (Brandner *et al.*, 1996) and/or dilution endpoints (Reed and Muench, 1938).

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References

- Beekes, M., Baldauf, E. and Diringer, H. (1996) Sequential appearance and accumulation of pathognomonic markers in the central nervous system of hamsters orally infected with scrapie. *J. Gen. Virol.*, **77**, 1925–1934.
- Beekes, M., McBride, P.A. and Baldauf, E. (1998) Cerebral targeting indicates vagal spread of infection in hamsters fed with scrapie. *J. Gen. Virol.*, **79**, 601–607.
- Binder, D., Fehr, J., Hengartner, H. and Zinkernagel, R.M. (1997) Virus-induced transient bone marrow aplasia: major role of interferon- α/β during acute infection with the noncytopathic lymphocytic choriomeningitis virus. *J. Exp. Med.*, **185**, 517–530.
- Blattler, T., Brandner, S., Raeber, A.J., Klein, M.A., Voigtlander, T., Weissmann, C. and Aguzzi, A. (1997) PrP-expressing tissue required for transfer of scrapie infectivity from spleen to brain. *Nature*, **389**, 69–73.
- Brandner, S., Isenmann, S., Raeber, A., Fischer, M., Sailer, A., Kobayashi, Y., Marino, S., Weissmann, C. and Aguzzi, A. (1996) Normal host prion protein necessary for scrapie-induced neurotoxicity. *Nature*, **379**, 339–343.
- Brown, K.L., Stewart, K., Bruce, M.E. and Fraser, H. (1997) Severely combined immunodeficient (SCID) mice resist infection with bovine spongiform encephalopathy. *J. Gen. Virol.*, **78**, 2707–2710.
- Bueler, H., Aguzzi, A., Sailer, A., Greiner, R.A., Autenried, P., Aguet, M. and Weissmann, C. (1993) Mice devoid of PrP are resistant to scrapie. *Cell*, **73**, 1339–1347.
- Chandler, R.L. (1961) Encephalopathy in mice produced by inoculation with scrapie brain material. *Lancet*, **1**, 1378–1379.
- Clarke, M.C. and Kimberlin, R.H. (1984) Pathogenesis of mouse scrapie: distribution of agent in the pulp and stroma of infected spleens. *Vet. Microbiol.*, **9**, 215–225.
- Eklund, C.M., Kennedy, R.C. and Hadlow, W.J. (1967) Pathogenesis of scrapie virus infection in the mouse. *J. Infect. Dis.*, **117**, 15–22.
- Fischer, M., Rulicke, T., Raeber, A., Sailer, A., Moser, M., Oesch, B., Brandner, S., Aguzzi, A. and Weissmann, C. (1996) Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *EMBO J.*, **15**, 1255–1264.
- Fraser, H. and Dickinson, A.G. (1970) Pathogenesis of scrapie in the mouse: the role of the spleen. *Nature*, **226**, 462–463.
- Fraser, H. and Farquhar, C.F. (1987) Ionising radiation has no influence on scrapie incubation period in mice. *Vet. Microbiol.*, **13**, 211–223.
- Fraser, H., Farquhar, C.F., McConnell, I. and Davies, D. (1989) The scrapie disease process is unaffected by ionising radiation. *Prog. Clin. Biol. Res.*, **317**, 653–658.
- Fraser, H., Brown, K.L., Stewart, K., McConnell, I., McBride, P. and Williams, A. (1996) Replication of scrapie in spleens of SCID mice follows reconstitution with wild-type mouse bone marrow. *J. Gen. Virol.*, **77**, 1935–1940.
- Heinen, E., Bosseloir, A. and Bouzahtah, F. (1995) Follicular dendritic cells: origin and function. *Curr. Top. Microbiol. Immunol.*, **201**, 15–47.
- Kimberlin, R.H. and Walker, C.A. (1980) Pathogenesis of mouse scrapie: evidence for neural spread of infection to the CNS. *J. Gen. Virol.*, **51**, 183–187.
- Kimberlin, R.H. and Walker, C.A. (1988) Incubation periods in six models of intraperitoneally injected scrapie depend mainly on the dynamics of agent replication within the nervous system and not the lymphoreticular system. *J. Gen. Virol.*, **69**, 2953–2960.
- Kimberlin, R.H. and Walker, C.A. (1989) The role of the spleen in the neuroinvasion of scrapie in mice. *Virus Res.*, **12**, 201–211.
- Kitamoto, T., Muramoto, T., Mohri, S., Dohura, K. and Tateishi, J. (1991) Abnormal isoform of prion protein accumulates in follicular dendritic cells in mice with Creutzfeldt–Jakob disease. *J. Virol.*, **65**, 6292–6295.
- Klein, M.A. *et al.* (1997) A crucial role for B cells in neuroinvasive scrapie. *Nature*, **390**, 687–690.
- Klein, M.A., Frigg, R., Raeber, A.J., Flechsig, E., Hegyi, I., Zinkernagel, R.M., Weissmann, C. and Aguzzi, A. (1998) PrP expression in B lymphocytes is not required for prion neuroinvasion. *Nature Med.*, **4**, 1429–1433.
- Lasmezas, C.I., Cesbron, J.Y., Deslys, J.P., Demaimay, R., Adjou, K.T., Rioux, R., Lemaire, C., Locht, C. and Dormont, D. (1996) Immune system-dependent and -independent replication of the scrapie agent. *J. Virol.*, **70**, 1292–1295.
- McBride, P.A., Eikelenboom, P., Kraal, G., Fraser, H. and Bruce, M.E. (1992) PrP protein is associated with follicular dendritic cells of spleens and lymph nodes in uninfected and scrapie-infected mice. *J. Pathol.*, **168**, 413–418.
- Mould, D.L., Dawson, A.M. and Rennie, J.C. (1970) Very early replication of scrapie in lymphocytic tissue. *Nature*, **228**, 779–780.
- Muramoto, T., Kitamoto, T., Tateishi, J. and Goto, I. (1992) The sequential development of abnormal prion protein accumulation in mice with Creutzfeldt–Jakob disease. *Am. J. Pathol.*, **140**, 1411–1420.
- O'Rourke, K.I., Huff, T.P., Leathers, C.W., Robinson, M.M. and Gorham, J.R. (1994) SCID mouse spleen does not support scrapie agent replication. *J. Gen. Virol.*, **75**, 1511–1514.
- Pattison, I.H. and Jones, K.M. (1968) Detection of the scrapie agent in tissues of normal mice and in tumours of tumour-bearing but otherwise normal mice. *Nature*, **217**, 102–104.
- Raeber, A., Sailer, A., Fischer, M., Rulicke, T., Brandner, S., Aguzzi, A. and Weissmann, C. (1996) Prion propagation in PrP null mice with ectopic PrP expression. *Experientia*, **52**, A44.
- Raeber, A.J., Sailer, A., Hegyi, I., Klein, M.A., Rulicke, T., Fischer, M., Brandner, S., Aguzzi, A. and Weissmann, C. (1999) Ectopic expression of prion protein (PrP) in T lymphocytes or hepatocytes of PrP knockout mice is insufficient to sustain prion replication. *Proc. Natl Acad. Sci. USA*, **96**, 3987–3992.
- Reed, J. and Muench, H. (1938) A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.*, **27**, 493–497.
- Taylor, D.M., McConnell, I. and Fraser, H. (1996) Scrapie infection can be established readily through skin scarification in immunocompetent but not immunodeficient mice. *J. Gen. Virol.*, **77**, 1595–1599.
- Yamada, G. *et al.* (1991) Specific depletion of the B-cell population induced by aberrant expression of human interferon regulatory factor 1 gene in transgenic mice. *Proc. Natl Acad. Sci. USA*, **88**, 532–536.

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