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Research paper

Pathogenesis of scrapie in ARQ/ARQ sheep after subcutaneous infection: Effect of lymphadenectomy and immune cell subset changes in relation to prion protein accumulation

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ABSTRACT

It is well established that the infectious agent of scrapie can replicate in the lymphoreticular system (LRS). However, the effects of removal of LRS target tissues on the pathogenesis of the infection and the accumulation of disease-associated prion protein (PrP^d) in LRS tissues on specific immune cell subsets are poorly understood aspects. To address these questions 16 ARQ/ARQ sheep were subcutaneously inoculated in the drainage area of the prefemoral lymph node with brain homogenate derived from Suffolk sheep naturally infected with scrapie. Fourteen sheep were then subjected to either early (14–17 days post-inoculation [dpi]) or late (175–201 dpi) lymphadenectomy and culled at preclinical or clinical stages of infection. Neither late nor even early lymphadenectomy prevented infection or had any effect on the accumulation of PrP^d in the LRS or CNS suggesting a rapid organic dissemination of the infectious agent after inoculation. Lymph nodes from eight scrapie inoculated sheep selected on the basis of the amount of PrP^d in their LRS tissues (negative, low or high) were examined for six different immune cell markers. The PrP^d negative lymph nodes from two sheep with no evidence of scrapie infection showed lower numbers of cluster of determination (CD) 21 positive cells than PrP^d positive nodes, irrespective of their location (hind leg or head). However, quantitative differences in the expression of this marker were not detected when comparing lymph nodes with low and high levels of PrP^d accumulation, suggesting that proliferation of CD21 positive cells is related to scrapie infection, but not directly linked to the magnitude of PrP^d accumulation. An additional observation of the study was that sheep that were methionin–threonine at codon 112 of the prion protein gene showed lower attack rates than methionine homozygotes (67% and 100%, respectively) and also generally lower levels of PrP^d accumulation in the LRS and brain and increased survival times, suggesting an influence of such polymorphism in the susceptibility to scrapie.

1. Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of infectious neurodegenerative diseases affecting animals and humans (Hur et al., 2002). Long incubation periods are a well documented characteristic of TSEs but tissues can become infectious long before the onset of

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clinical signs (Carp et al., 1994; Hadlow et al., 1982). Recent studies have provided strong evidence to support the protein only hypothesis (Prusiner, 1982) which proposes a misfolded version (PrP^{Sc}) of the normal cellular prion protein (PrP^{c}) as the infectious agent (Chianini et al., 2012; Kim et al., 2010; Legname et al., 2004; Makarava et al., 2010; Wang et al., 2010). This pathological form of the prion protein is also called protease-resistant prion protein (PrP^{res}) and is primarily used when referring to western blot (WB) results, or disease-associated prion protein (PrP^{d}) when referring to immunohistochemistry (IHC).

Scrapie is a TSE that affects sheep and goats and has been reported in Great Britain since the mid eighteenth century (Parry, 1983). The prion protein gene (*Prnp*), which encodes for the cellular protein PrP^{c}, is an important determinant of the susceptibility of sheep to scrapie (Hunter et al., 1997), although there are uncertainties as to why and how polymorphisms of that gene are influential with respect to susceptibility (Baylis and Goldmann, 2004). Sheep of the Suffolk and Romney breeds which are ARQ/ARQ (amino acids at codons 136, 154 and 171, respectively) are generally susceptible to natural and experimental scrapie, but it has been shown that sheep with one *Prnp* allele encoding for threonine (T) at codon 112 have reduced susceptibility compared to sheep homozygous for methionine (M; Ikeda et al., 1995; Laegreid et al., 2008; González et al., 2012).

In sheep, oral exposure to the scrapie agent results in accumulation of disease-associated PrP (PrP^{d}) initially in the lymphoid tissues of the alimentary tract such as the retropharyngeal lymph node and the ileal Peyer's patches (Hadlow et al., 1982; van Keulen et al., 2002). However it is not known whether this initial accumulation is restricted to such tissues or is simultaneous with an early organic dissemination, as other studies suggest (Jeffrey et al., 2006; Dagleish et al., 2010). It is also well known that the subcutaneous route is efficient for transmission of experimental scrapie to mice (Kimberlin and Walker, 1979; Lasmezas et al., 1996) and sheep (Houston et al., 2002). This route provides an opportunity to answer the question of local restriction versus early dissemination of the infectious agent by removal of the drainage lymph node of the area of subcutaneous challenge, even though the pathways (lymph or blood) or cells (if any) involved in the dissemination of the infectious agent from the point of subcutaneous inoculation to lymphoreticular system (LRS) tissues and other organs are not fully understood, particularly in the natural sheep host.

Additionally, little is known about the relevance of the LRS involvement in the pathogenesis of scrapie, and also it is generally believed there is no specific immunological response to infection. However, it has been shown by ultrastructural studies of scrapie infected mice that changes in development and maturation occur in secondary follicles of the spleen and lymph nodes (McGovern et al., 2004). There is also evidence suggesting that several cell types, such as follicular dendritic cells (FDCs), tingible body macrophages (TBM) and B cells, are involved in different aspects of the pathogenesis of scrapie in the LRS (Brown et al., 2009; Eaton et al., 2007, 2009; McGovern et al., 2009; Mabbott, 2012). Moreover, it has been shown that PrP^{c} is present in immature immunocytes and T cells (Kubosaki et al., 2003), although studies on the role of those cells in the pathogenesis of the disease are limited. More recently, plasmacytoid dendritic cells were found to play a key role in sequestering prion titres at an early stage of infection in mice (Castro-Seoane et al., 2012). However, most of those studies have been carried out either on transgenic mice or using flow cytometry, and in both cases the availability of primary antibodies is greater than for IHC in ovine fixed tissues. However, if ovine tissues are fixed using a zinc-salt solution rather than formalin, the possibility to label FDCs, B and T cells is increased either using antibodies specifically raised against ovine epitopes or antibodies that cross reacts with them (González et al., 2001).

The experiment reported here aimed primarily to determine if surgical removal of the lymph node local to the injection point had an effect on the progression of scrapie in sheep and to assess whether the changes in immune system cell subsets were qualitatively and/or quantitatively associated with PrP^{d} accumulation in LRS tissues. The paper also describes the kinetics of PrP^{d} accumulation in LRS and central nervous system (CNS) tissues in relation with polymorphisms at codon 112 of PrP.

2. Materials and methods

2.1. Animals and experimental design

Individual details of the sheep used are provided in Table 1 and illustrated in Fig. 1. Seventeen ARQ/ARQ lambs of Suffolk or Romney breed (where letters indicate amino acid codes at codon 136 [A, alanine], 154 [R, arginine] and 171 [Q, glutamine] of the prion protein) were obtained from a New Zealand-derived, scrapie-free flock (Arthur Rickwood Sheep Unit, AHVLA, Weybridge, UK). At 6 months of age, 16 lambs were injected subcutaneously in the drainage area of the right prefemoral lymph node with 1 ml of a 10% clarified scrapie positive brain pool homogenate (SBH) derived from Suffolk sheep, as described previously (Eaton et al., 2007). One lamb was inoculated with a similarly prepared scrapie negative brain homogenate from a Suffolk sheep for negative control purposes.

To determine the effect of lymphadenectomy, seven lambs inoculated with SBH were subjected to surgical removal of the ipsi- (site of injection) and contra-lateral prefemoral lymph nodes at 13–16 days post inoculation (dpi; early lymphadenectomy [EL]), another seven at 169–201 dpi (late lymphadenectomy [LL]), and two sheep were not subjected to this surgical procedure (positive controls). Animals were monitored for clinical signs of scrapie following a previously reported end-point scoring system (González et al., 2012). All seven sheep of the EL group and four of the LL group were culled at a preclinical stage, between 478 and 529 dpi (together with the negative control sheep), and three animals from the LL group and the two positive control animals were killed when they reached clinical end point (Table 1 and Fig. 1). All animal procedures complied with the Animals (Scientific Procedures) Act 1986 and were approved by the Moredun Research Institute ethics committee.

Table 1
Details of the ARQ/ARQ sheep used in the study.

ID.	Codon 112 genotype	Lymphadnectomy		Clinical status	PM dpi	PrP^d presence or score						CD marker study
		Group	dpi			ENS	Trig	Nod	CMG	CNS	LRS	
J165	MT	EL	16	pre	478	N	N	N	N	2	3.6	Low PrP
J093	MT	EL	16	pre	490	N	N	N	N	0	13.6	Low PrP
J156*	MT	EL	13	pre	529	N	N	N	P	9	46.4	High PrP
J160	MM	EL	17 (ipsi +)	pre	491	N	P	N	P	15	43.2	High PrP
J162*	MM	EL	14	pre	511	P	P	N	P	14	36.2	
8803*	MM	EL	16	pre	513	P	N	N	N	10	25.5	
J187	MM	EL	16	pre	486	P	P	N	P	10	23.9	
J169	MT	LL	175	pre	478	N	N	N	N	0	0	No PrP
J137	MT	LL	194	pre	504	N	N	N	N	0	0	No PrP
J184	MM	LL	185	pre	483	P	N	N	N	3	38.7	
603	MM	LL	199	pre	513	P	N	N	N	10	43.4	
J149	MM	LL	169	clin	743	P	P	P	P	30	52.1	
J117	MM	LL	201 (ipsi +)	clin	763	P	P	na	P	30	50.3	Clinical
J103	MM	LL	184 (contra +)	clin	810	P	P	N	N	29	41.4	Clinical
J147	MT	+ Control	ND	clin	1188	P	P	P	P	30	48.4	
J153	MM	+ Control	ND	clin	578	P	P	N	P	26	41.8	
J182	MT	− Control	16	neg	493	N	N	N	N	0	0	

ID, sheep identification number (in bold those selected for the CD markers study and in the last column their category); Lymphadenectomy: EL, early; LL, late; dpi, days post-infection: the lymph nodes that showed positive PrP^d immunolabelling are indicated as (ipsi +) or (contra +); the rest of the excised lymph nodes were negative. ND, not done. Clinical status at post-mortem (PM): pre, preclinical; clin, clinical; neg, negative. ENS, enteric nervous system; Trig, trigeminal nerve; Nod, nodose ganglion; CMG, cranial mesenteric ganglion; CNS, central nervous system; LRS, lymphoreticular system (the scores do not take into account the prefemoral lymph nodes subjected to lymphadenectomy); N, negative; P, positive; ns, not sampled.

* Lymph was consistently obtained from these animals for 3–4 days after challenge.

2.2. Tissue sampling at post-mortem and IHC procedures

Sheep were killed by intra-venous barbiturate overdose. At post-mortem examination the following tissues were collected, fixed in 10% buffered formalin and processed for IHC detection of PrP^d: central nervous system (CNS): half brain sagittally sliced, and samples of the cervical, thoracic and lumbar segments of the spinal cord. Peripheral nervous system (PNS): trigeminal, nodose and cranial mesenteric ganglia, and enteric nervous system (ENS) of the jejunum, distal ileum and colon. LRS tissues: Peyer's patches in the jejunum and distal ileum, lymphoid follicles in the colon, plus proximal and distal jejunal, medial retropharyngeal, submandibular, popliteal, inguinal and prescapular lymph nodes (LNs), palatine tonsil and spleen. With paired tissues, samples were taken from both sides.

Four μm-thick sections of each of the formalin-fixed tissues above were mounted on glass microscope slides and subjected to antigen retrieval procedures (immersion in formic acid followed by citrate buffer autoclaving) and immunolabelling for PrP^d as described previously (González et al., 2002). Tissues were incubated overnight at 24 °C with a primary monoclonal antibody, R145, which recognises the 222–226 amino acid sequence of ovine PrP (Jeffrey et al., 2006). The IHC procedure was completed by an immunoperoxidase method using diaminobenzidine (DAB) as chromagen and Mayer's haematoxylin for counterstaining.

For identification of immune cell subsets, samples of medial retropharyngeal, submandibular, inguinal and popliteal LNs adjacent to those fixed in 10% buffered formalin, and both ipsi- and contra-lateral to the side of injection, were immersed in zinc salts fixative, processed routinely and embedded in paraffin-wax as described previously (González et al., 2001). Five μm-thick serial sections of those samples were mounted on glass microscope slides and subjected to IHC using a panel of monoclonal antibodies against T cell, B cell and FDC cluster of determination (CD) markers, as detailed in Table 2. After quenching endogenous tissue peroxidase activity and blocking non-specific binding, sections were incubated overnight at 4 °C with the different primary antibodies and visualised using the EnVision™ System-HRP (Dako, Ely, UK), using DAB as the chromagen and Z haematoxylin for counterstaining as

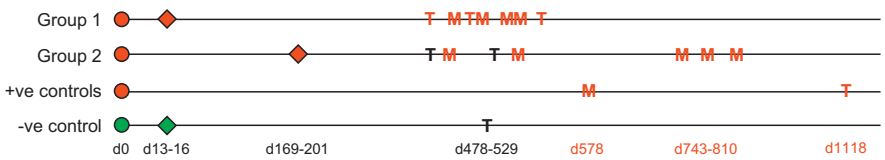

Fig. 1. Schematic representation of the experimental design and outcome overview. Circles represent day of subcutaneous injection (d0) of sheep with scrapie brain pool (red) or normal brain (green). Diamonds represent day post-infection at which lymphadenectomies were performed. Letters represent days post-infection (black, preclinical stage; red, clinical stage) at which post-mortem were carried out in individual sheep: M = methionine homozygotes at codon 112; T = methionine–threonine heterozygotes at codon 112; red letter = scrapie confirmed by IHC; black letter = no evidence of scrapie on IHC examination investigation of full set of tissue samples as described in the text.

Table 2
CD markers, cell types recognised and antibody details.

CD marker	Cell type	Ab clone	Dilution	Source	Reference
CD3	T lymphocytes	F7.2.38	1/200	Dako	(Alibaud et al., 2000)
CD4	T-helper cells	17D	1/400	Basel	(Mackay et al., 1988)
CD8	T-cytotoxic cells	7C2	1/400	MRI	(Naessens et al., 1997)
γδTCR	γδ T cells	86D	1/400	Basel	(Mackay and Hein, 1989)
CD21	FDCs, Mature B cells	CC21	1/400,000	IAH	(Naessens et al., 1997)
$CD79_{\alpha cy}$	B cell lineage	HM57	1/100	Dako	(Mason et al., 1991)

CD, cluster of determination. TCR, T cell receptor. Ab, antibody (all murine IgG1 monoclonal antibodies). Sources are: Dako, Dako UK Ltd., Ely, UK; Basel, Basel Institute for Immunology, Basel, Switzerland; MRI, Moredun Research Institute; IAH, Institute for Animal Health, Compton Laboratory, Newbury, UK.

described previously (González et al., 2001). These examinations were conducted on 8 of the 16 scrapie-inoculated sheep, which were selected on the basis of their PrP^d scores in CNS and LRS tissues which were obtained following the methods described below.

2.3. Scoring and quantification of PrP^d and immune cell subsets

Accumulation of PrP^d in the CNS was scored subjectively from 0 (absence) to 3 (abundant) in intervals of 0.5 in each of the following brain areas: frontal cerebral cortex, corpus striatum, thalamus/hypothalamus, midbrain, cerebellum, rostral medulla oblongata, obex and spinal cord at cervical, thoracic and lumbar segments following criteria previously described and illustrated (González et al., 2005; Sisó et al., 2010a,b). For each animal, the total magnitude of PrP^d in the CNS was expressed as the sum of the scores in the different areas, so that the range for each individual sheep was from 0 to 30. Accumulation of PrP^d in PNS tissues was scored as positive or negative. The degree of involvement of the different LRS tissues was determined by a combination of the percentage of positive lymphoid follicles and the magnitude of PrP^d labelling within them (from 1 = labelling confined to a few TBMs in the light zone, to 5 = intense labelling of TBMs in light and dark zones and also of FDCs), as previously detailed (Martin et al., 2005). This method was not applied to spleen samples, which were subjectively scored from 0 to 3.

The results of the quantification of PrP^d deposition in LRS and CNS tissues were used to select eight sheep: two devoid of PrP^d labelling, two each with low and high PrP^d scores at the preclinical stage of disease, and two with high PrP^d scores at clinical end point (Table 1). For each chosen sheep, the cortices of the eight lymph nodes specified above were examined for immunoreactivity to the different CD markers. This was subjectively scored from 0 to 5 in each of 50 microscopic fields (25 in secondary follicles and 25 in paracortex, both at 400× magnification), where 0 = no positive cells, 1 = few scattered positive cells, 2 = increasing number of positive cells still with a scattered distribution; 3 = moderate number of positive cells occupying 1/3 of the region, 4 = high number of positive cells diffusely distributed, and 5 = highest number of positive cells with a widespread distribution and clear predominance over non-labelling cells. The mean value for each of the cell markers, generated from the single values given for the different microscopic fields examined, was assigned to each area (follicles and paracortex), and the two values were added to give a total score (from 0 to 10) for each lymph node, which was used for statistical analysis.

2.4. PrP genotyping

Prior to undertaking the experiment, only amino acids at codons 136, 154 and 171 of the ovine PrP gene were determined by sequencing with an ABI Prism 377 DNA sequencer according to the manufacturer's instructions (PE Applied Biosystems; Warrington, UK). At the end of the experiment, in view of information regarding the influence of polymorphisms at codon 112 on the susceptibility of Suffolk sheep to scrapie (Ikeda et al., 1995; Laegreid et al., 2008), brain tissue samples from all 17 sheep were sequenced. Chromosomal DNA was extracted from brain tissue using DNeasy blood and tissue kit (QIAGEN) and then used as a template for PCR using HotStarTaq DNA polymerase (QIAGEN) with forward primer 313 (5′-GTCAAGGTGGTAGCCACAGTCAGTG-3′) and reverse primer 316 (5′-GCTCCACCACTCGCTCCATTATCTTG-3′). PCR was performed in a Hybaid MBS 0.2G Thermal Cycler, and included 5 min of initial denaturation at 95 °C, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The amplified product from each reaction was purified (QIAGEN kit) and sequenced in both directions by Eurofins MWG Operon using the same primers. All sequences were compared at the nucleotide level and translated to provide the corresponding amino acid sequence. DNA and amino acid sequences were compared using SeqMan software (DNASTAR, Inc.) to determine the presence of M or T at codon 112.

2.5. Statistical analyses

Where sufficient number of data were available, unpaired *t*-tests (parametric when data were normally distributed or non-parametric [Mann–Whitney] for data not-normally distributed) were used to determine the effect of polymorphisms at codon 112 on the magnitude of PrP^d accumulation in the CNS and LRS for sheep at preclinical and clinical stages of infection. Paired *t*-tests were employed to evaluate differences in magnitudes of PrP^d accumulation between paired LRS tissues (palatine tonsil and LNs with the exception of those draining the gut) ipsi- and contra-lateral to the site of injection. Unpaired *t*-tests were also used to determine the existence of differences between preclinical and clinical cases in terms of magnitude of PrP^d deposition in LRS and CNS compartments, and

paired *t*-tests for the pair-wise comparison of PrP^d accumulation in the different LRS tissues examined.

To determine any positive or negative association between the magnitude of PrP^d deposition and the quantitative expression of the different immune cell markers explored, unpaired *t*-tests (parametric or non-parametric, as specified above) were used to compare the four different sheep groups established according to their PrP^d load and clinical status.

3. Results

3.1. General pathogenetical aspects: distribution of PrP^d and effect of polymorphisms at codon 112

Fourteen of the 16 sheep inoculated subcutaneously with SBH became infected as demonstrated by the IHC detection of PrP^d in one or more of the tissues examined, giving an overall attack rate of 88.2%. The negative control sheep inoculated with Suffolk sheep derived scrapie-negative brain pool homogenate and killed at 493 dpi was devoid of any PrP^d immunolabelling (Table 1 and Fig. 1).

Only one of the five sheep left to develop clinical disease was $M_{112}T$ (M, methionine; T, threonine) at codon 112 (Table 1 and Fig. 1); this sheep was a positive control, not subjected to lymphadenectomy, and was killed when it reached clinical end point after a much more protracted incubation period (1118 dpi) than the other clinical cases (724 ± 101 days [mean ± standard deviation]). However, the magnitude and distribution of PrP^d in that sheep was similar to the other clinical cases (Table 1). Of the 11 sheep culled at a preclinical stage, five were heterozygote ($M_{112}T$) and six were homozygote ($M_{112}M$; Table 1 and Fig. 1). Despite being killed at similar times after inoculation (average 496 and 500 dpi, respectively), two of the heterozygote sheep were completely negative for PrP^d and another two showed low levels of PrP^d in LRS tissues (one of these was negative in the CNS). Statistical analyses showed significantly less accumulation of PrP^d in heterozygote sheep when compared to the homozygote sheep in both LRS (13.2 ± 20.7 and 37.6 ± 7.9, respectively; $P < 0.05$) and CNS (2.2 ± 3.9 and 11.3 ± 4.8, respectively; $P < 0.01$) when data from the two PrP^d negative sheep are included. If the analysis is restricted to the three PrP^d positive heterozygote sheep, the difference in accumulation of PrP^d was not significant in the LRS (22 ± 23.7 and 37.6 ± 7.9 for heterozygote and homozygote sheep, respectively) but remained significant for the CNS (3.7 ± 4.7 and 11.3 ± 4.8, respectively; $P < 0.05$).

Accumulation of PrP^d in the PNS samples examined was variable among the nine scrapie positive sheep culled during the preclinical phase (Table 1): two sheep were negative in all nervous tissues examined (J165, J093), the trigeminal ganglion was positive in three (J160, J162, J187), the cranial mesenteric ganglion in four (J156, J160, J162, J187) and the ENS in five (J162, 8803, J187, J184, 603), while the nodose ganglion was negative in all of them. All sheep showing PrP^d in PNS tissues were positive not just in the LRS but also in the CNS. Among the five clinical cases, four accumulated PrP^d in the cranial mesenteric ganglion (J149, J117, J147, J153) and two in the nodose ganglion (J149, J147), while all were positive in the ENS and trigeminal ganglion (Table 1).

Accumulation of PrP^d in LRS tissues was confirmed in 9 of 11 preclinical and 5 of 5 clinical scrapie cases (Table 1), but with differences in magnitude and extent, which were associated with their sampling points post infection (p.i.) (499 ± 17 dpi vs. 802 ± 197 dpi, for preclinical and clinical cases, respectively; $P = 0.001$). Thus, while all 16 LRS tissues examined in clinically affected sheep showed PrP^d accumulation, three of the preclinical cases showed incomplete involvement of the same LRS tissues. Clinically affected sheep also showed significantly higher levels of PrP^d accumulation in LRS tissues (48.7 ± 6.1 [mean ± SD]) than preclinical cases (32.4 ± 15.6; $P < 0.05$). This quantitative difference was due to three of the preclinical cases showing total values of 3.6, 13.6 (both $M_{112}T$) and 23.9 ($M_{112}M$); if these sheep are excluded from the comparison the differences in LRS PrP^d load became non-significant, despite incubation periods being still significantly different (507 ± 17 vs. 802 ± 197 for six preclinical and five clinical cases, respectively; $P < 0.01$). Fig. 2 represents the magnitude of PrP^d accumulation in the LRS of scrapie infected sheep at different stages of preclinical infection and clinical disease.

Details of PrP^d scores in individual LRS samples of each sheep examined are given in Supplementary Table 1. With the exception of the popliteal LNs of one preclinical sheep, in which the right (ipsi-lateral) LN was negative and the left node positive, PrP^d accumulation of paired LRS tissues was always bilateral and with no significant differences in magnitude neither among preclinical nor among clinical cases (results of statistical analyses not shown but can be inferred from data in Supplementary Table 1). When comparing the different LRS tissues examined (with the exception of the spleen for which the scoring system differed) from scrapie positive sheep, significantly higher levels of PrP^d accumulation were present in those located in the head (palatine tonsil, retropharyngeal and submandibular LNs; combined score 2.8 ± 1.2) compared to the inguinal, pre-scapular and popliteal LNs (superficial LNs, with a combined score of 2.0 ± 1.2 [$P < 0.001$]). The gut-associated LRS tissues (Peyer's patches and distal and proximal jejunal LNs) provided intermediate results (2.5 ± 1.2), which were significantly higher ($P < 0.05$) than the group of superficial LNs (inguinal, pre-scapular and popliteal) but not significantly different to the LNs of the head (palatine tonsil, retropharyngeal and submandibular).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2013.01.005

The total magnitude of PrP^d accumulation in the CNS of scrapie challenged sheep ranged from 0 to 30 (Table 1), and appear to vary depending on several factors. Preclinical scrapie positive cases showed magnitudes significantly lower than clinically affected sheep (8.8 ± 5.9 vs. 29.0 ± 1.7 respectively; $P < 0.001$), and such differences were maintained if the three cases with the lowest PrP^d accumulation in LRS mentioned above were excluded from the comparison. In other words, preclinical ($n = 6$) and clinical ($n = 5$) sheep with no significant differences in accumulation of

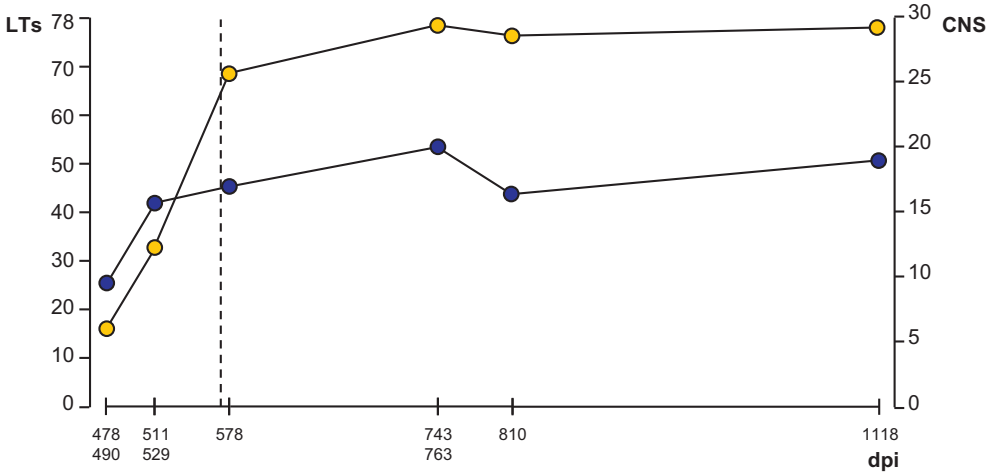

Fig. 2. Schematic representation of the average magnitude of total PrP^d in the lymphoid tissues (LTs, blue circles, against Y1 axis) and in the CNS (gold, against Y2 axis) of preclinical (left of the dotted bar; $n = 9$) and clinical (right of the dotted bar; $n = 5$) scrapie cases, with reference to days post-inoculation (dpi) at which post-mortem examinations were performed.

PrP^d in the LRS tissues showed significant differences in magnitude of accumulation of PrP^d in the CNS (11.2 ± 4.9 vs. 29.0 ± 1.7 respectively; $P < 0.001$) and these were associated with different sampling points d.p.i. as stated above. Within the preclinical scrapie cases, $M_{112}T$ sheep showed PrP^d accumulation in the CNS of magnitudes between 0 and 9, while for $M_{112}M$ sheep the magnitudes were between 3 and 16. These differences were statistically significant ($P < 0.05$) using the Mann–Whitney test. Fig. 2 shows the magnitude of PrP^d accumulation in the CNS of scrapie infected sheep at different stages of preclinical infection and clinical disease.

Regarding the topographical distribution within the CNS, in preclinical cases PrP^d accumulated more often and with highest magnitudes in the medulla oblongata, in the hypothalamus and in the spinal cord examined, whereas in forebrain areas it accumulated the least. PrP^d accumulation in clinical cases was widespread and severe in all areas of the CNS examined.

3.2. Effect of lymphadenectomy on the magnitude and distribution of PrP^d

Among the five sheep which progressed to clinical disease, the one that had not been subjected to lymphadenectomy reached clinical end point at a slightly earlier time point (578 dpi) than the three which had their prefemoral lymph nodes excised at a late time point after challenge (743–810 dpi). As described above, the positive control sheep (not subjected to lymphadenectomy) developed scrapie at a later stage (1118 dpi) but was a $M_{112}T$ heterozygote. All the five clinical cases showed similar amounts of PrP^d in the LRS and CNS samples examined (Table 1), regardless of lymphadenectomy. Among the preclinical cases, similar magnitudes of PrP^d in LRS and CNS were found in sheep killed at similar dpi after being subjected to either EL or LL. The two sheep that were negative for PrP^d, both $M_{112}T$, were LL cases while the three EL cases that were also $M_{112}T$ were positive in LRS tissues (two of them also in the CNS).

Examination of the excised prefemoral LNs to detect PrP^d revealed only one positive (ipsi-lateral) lymph node of 14 collected from the EL group and two positive lymph nodes from two different animals (one ipsi- and one contra-lateral) of 14 collected from the LL group. All three PrP^d positive lymph nodes were from $M_{112}M$ sheep. Cannulation of the afferent lymphatics of the prefemoral lymph nodes, where lymph was consistently obtained before and 3–4 days after subcutaneous injection of scrapie inoculum, was successfully achieved in three out of seven animals. The samples of lymph were not considered sufficient for further studies, however its removal did not affect the progression to disease and the three sheep (identified in Table 1) showed abundant PrP^d deposition in both LRS and CNS samples when killed during the preclinical stage of infection.

3.3. Changes in immune system cell subsets in relation to PrP^d accumulation

As significant differences in PrP^d accumulation were observed between the lymph nodes situated in the head compared to those draining hind leg structures in the two groups of highest PrP^d accumulation (see above), the two areas were analysed separately. However, because the two preclinical sheep with high levels of PrP^d accumulation and the two clinically affected sheep showed almost identical magnitudes PrP^d in the eight lymph nodes examined, the two groups were merged together as a "high PrP^d" group (Table 3).

The results of the quantitative analyses of the expression of the different CD markers between those three sheep groups (negative, low and high PrP^d) in the two areas (head and leg), as explained above, are detailed in Table 3 and illustrated in Figs. 3 and 4. For T-lymphocyte markers CD3, CD4 and CD8, no significant differences were found between the three sheep groups in any of the two areas. For γδTCR positive cells the PrP^d negative group showed significantly lower scores in the LNs of the head (4.6 ± 0.6) than the other two groups ($P < 0.01$), although the highest

Table 3
Immune cell markers reactivity in lymph nodes accumulating different levels of PrP^d.

(a)	Negative	Low	High	Clinical
			3.3 ± 0.2	3.4 ± 0.6
PrP^d	0.0	0.9 ± 1.0	3.4 ± 0.4	
CD3	8.3 ± 0.8	8.7 ± 0.4	8.5 ± 0.5	
CD4	7.7 ± 0.4	8.2 ± 0.5	7.7 ± 0.5	
CD8	6.6 ± 0.6	6.3 ± 0.5	6.2 ± 1.3	
γδTCR	4.6 ± 0.6	6.5 ± 0.9	5.7 ± 0.8	
CD79	8.2 ± 0.7	8.3 ± 0.5	7.8 ± 0.7	
CD21	7.3 ± 0.6	7.9 ± 0.3	8.2 ± 0.5	

(b)	Negative	Low	High	Clinical
			3.1 ± 0.3	2.5 ± 0.8
PrP^d	0.0	0.2 ± 0.3	2.8 ± 0.7	
CD3	8.4 ± 0.8	9.0 ± 0.4	8.7 ± 0.5	
CD4	8.3 ± 0.6	8.0 ± 0.4	8.0 ± 0.5	
CD8	6.6 ± 0.8	6.1 ± 0.6	6.3 ± 1.0	
γδTCR	6.6 ± 0.5	6.7 ± 0.4	6.3 ± 0.6	
CD79	8.2 ± 0.6	8.3 ± 0.5	7.8 ± 0.7	
CD21	7.7 ± 0.7	8.5 ± 0.5	8.3 ± 0.5	

Results expressed as mean ± SD for lymph nodes with no PrP^d (Negative, $n = 8$ [2 nodes × 2 sides – left and right – × 2 sheep]), or with low (Low, $n = 8$) or high (High + Clinical, $n = 16$) PrP^d levels. (a) lymph nodes of the head (medial retropharyngeal and submandibular) (b) lymph nodes of the hind leg (inguinal and popliteal).

expression was in the low PrP^d group (6.5 ± 0.9 vs. 5.7 ± 0.8 in the high PrP^d group; $P < 0.05$). In contrast, no significant differences in the labelling of this marker between sheep groups were found for the lymph nodes draining the hind legs.

No differences in the expression of CD79 were found in any of the comparisons between sheep groups. However, when the high PrP^d group was re-divided into preclinical and clinical cases, the latter showed significantly lower number of CD79 positive cells in the head lymph nodes than any of the other three groups (7.3 ± 0.5 vs. 8.4 ± 0.3, 8.3 ± 0.5 and 8.2 ± 0.7, for high PrP^d, low PrP^d and negative preclinical cases, respectively, with P values of <0.001, <0.01 and <0.05, also respectively; Fig. 3). In the hind leg lymph nodes, clinical cases also showed lower levels of expression of this marker, although differences were not statistically significant. Finally, in the lymph nodes of both the head and hind legs PrP^d negative sheep showed a significantly lower ($P < 0.05$) number of CD21 positive cells compared with the other two groups, low PrP^d and high PrP^d, which displayed similar levels of $CD21^+$ cell immunolabelling (Table 3 and Fig. 3).

4. Discussion

4.1. General aspects of scrapie pathogenesis after experimental subcutaneous injection

According to the results of our study, accumulation of PrP^d in the LRS after subcutaneous injection progresses alongside the course of infection in a non-linear form as it plateaus at around 500 dpi, well before onset of clinical disease in most cases. In agreement with most studies on the pathogenesis of sheep and goat scrapie, either by natural or experimental routes, accumulation of PrP^d in the CNS appears to have a delayed onset in comparison

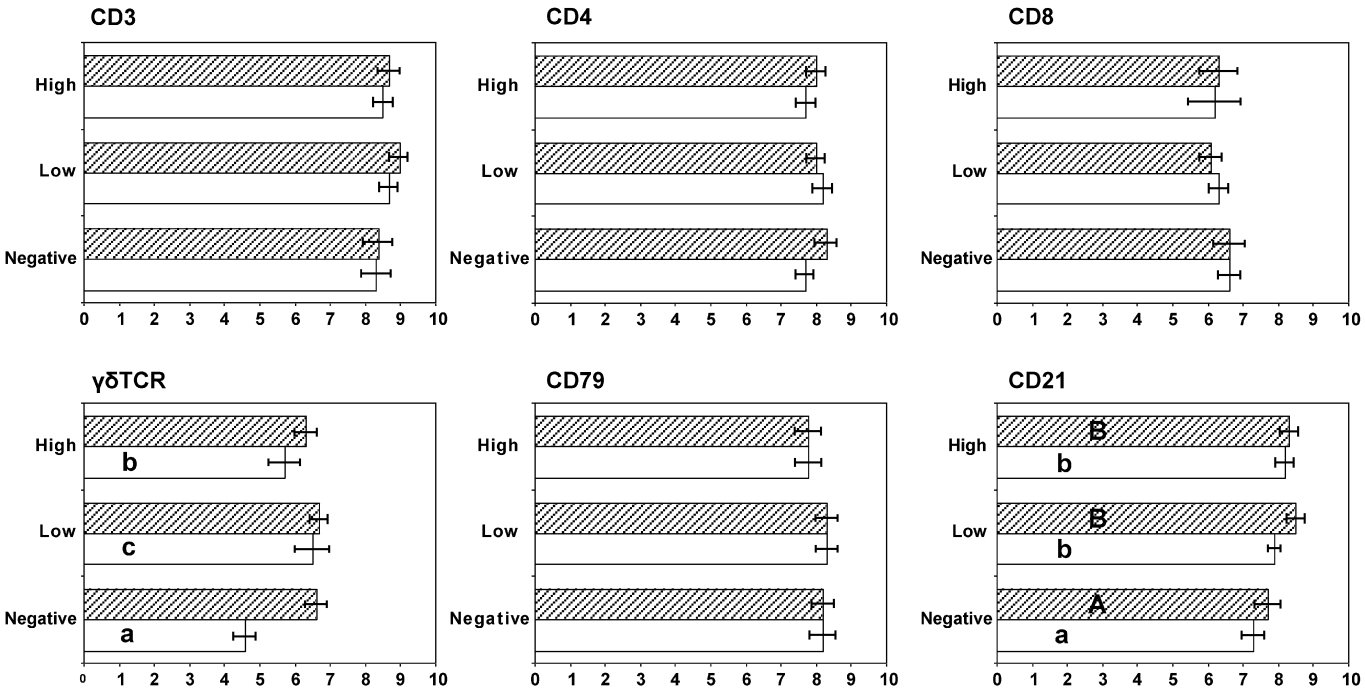

Fig. 3. Labelling of different immune cell markers in lymph nodes of the head (white bars) and hind legs (dashed bars) of sheep accumulating different levels (negative, low and high) of PrP^d in lymphoid tissues. Results expressed as mean values of cell counts for the different markers and standard deviations (error bars). Note that the group of "High" PrP^d includes four sheep (two clinical and two preclinical), as explained in the text. Significant differences in immune cell marker expression between sheep groups are indicated by different letters (for actual values refer to Table 3), so that values with different letters are significantly different.

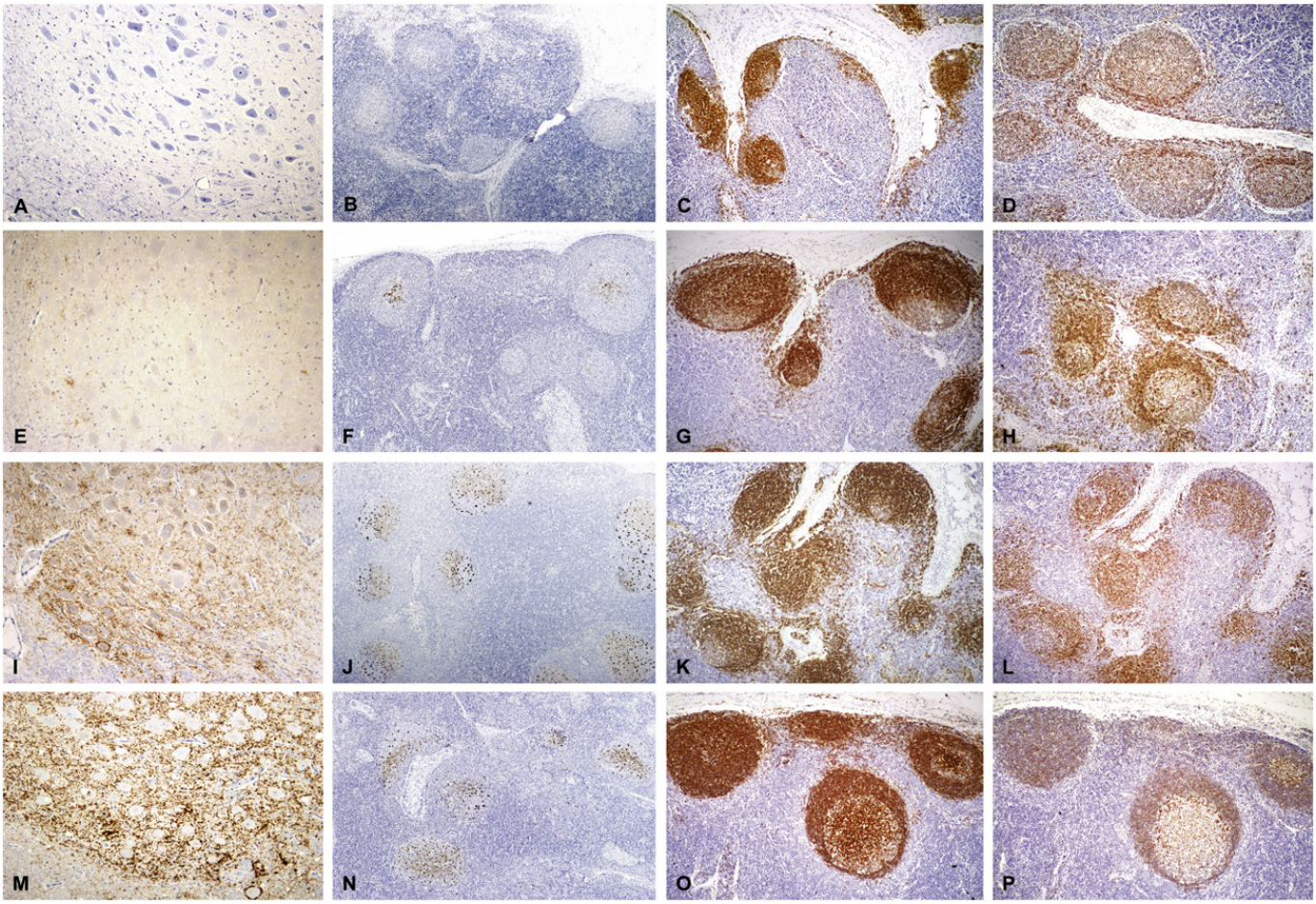

Fig. 4. IHC of CNS and LRS samples from animals representative of different levels of PrP^d immunolabelling and $CD21^+$ and $CD79^+$ positive cells. (A)–(D) (sheep devoid of PrP^d immunolabelling), (E)–(H) (sheep with low magnitude of PrP^d accumulation), (I)–(L) (sheep with high amounts of PrP^d immunolabelling) and (M)–(P) (sheep showing clinical signs of scrapie). PrP^d labelling of obex (A, E, I and M) and retropharyngeal lymph node (B, F, J and N) using anti-PrP^d antibody R145, showing the absence of labelling in scrapie negative sheep and a progressive number of labelled cells in sheep of the low, high and clinical groups. CD21 positive cells in retropharyngeal lymph nodes of sheep from the negative (C), low PrP^d (G) high PrP^d (K) and clinical (O) groups. CD79 positive cells in the retropharyngeal LNs of sheep from the negative (D), low PrP^d (H) high PrP^d (L) and clinical (P) group. Original magnification 40× with the exception of A, E, I and M 100×.

with the LRS and seems to progress during the preclinical and clinical phases, until clinical end point is reached (Andreoletti et al., 2000; van Keulen et al., 2000; González et al., 2010; Tabouret et al., 2010). At this point all affected sheep show similar magnitudes of PrP^d deposition in the brain, although the rate or speed of accumulation appears to differ from sheep to sheep; those differences cannot always be explained by polymorphisms in the *Prnp* genotype of the recipient sheep but have been found to result from an interaction between source and host genetic factors (González et al., 2012).

An early, consistent and high-level accumulation of PrP^d in the palatine tonsil and medial retropharyngeal LN has been repeatedly documented in natural cases of scrapie in sheep of different *Prnp* genotypes (Andreoletti et al., 2000; González et al., 2006; Langeveld et al., 2006; Lacroux et al., 2008) and goats (González et al., 2010) and also in experimental sheep scrapie challenge by the oral route (Heggebo et al., 2003; Ryder et al., 2009). This has been attributed by some authors to an early contact of the infectious agent with the lymphoid tissues associated with the gastrointestinal tract after ingestion (van Keulen et al., 2002; Ryder et al., 2009), which would also explain the early and consistent involvement of the Peyer's patches observed in the same studies. It is therefore surprising that the same two lymphoid structures – medial retropharyngeal LN and palatine tonsil – are those showing the highest magnitudes of PrP^d accumulation after subcutaneous scrapie challenge, particularly at a preclinical stage of infection. One possible explanation is that after early dissemination of the agent in the bloodstream, it reaches the brain through the circumventricular organs (Sisó et al., 2010a,b) and is re-circulated in the interstitial and cerebrospinal fluid reaching the nasal submucosa, or even the nasal cavity itself, through the cribriform plate (Weller et al., 2009). Scrapie agent in the nasal submucosa could then enter afferent lymphatics that drain to the medial retropharyngeal LN, while the agent in the nasopharynx could accumulate in the pharyngeal tonsil or pass to the oropharynx and then arrive in the palatine tonsil; both these tonsils also drain towards the medial retropharyngeal LN. This hypothesis could be sustained by the early and consistent involvement of the circumventricular organs in the preclinical sheep of this study, which has been described in detail previously (Sisó et al., 2009).

Also, the contribution of the haematogenous route in the neuroinvasion after subcutaneous challenge is reinforced by the absence of detectable PrP^d in the ENS and cranial mesenteric ganglion of approximately 50% of preclinical scrapie cases, some of which were positive in the brain (see Table 1).

4.2. Effect of codon 112 polymorphism on susceptibility to and pathogenesis of scrapie

Previous publications have reported the influence of the threonine amino acid at codon 112 of the PrP gene on the susceptibility of ARQ/ARQ sheep to natural scrapie (Ikeda et al., 1995), to sheep experimentally infected by the oral route (Laegreid et al., 2008; González et al., 2012) and to sheep orally and intracerebrally infected with BSE (Saunders et al., 2009). This experiment supports those findings with respect to the subcutaneous route of inoculation, both in terms of attack rates (incomplete in heterozygotes) and incubation period (markedly protracted in the only heterozygote allowed to develop clinical disease). In addition, our results suggest that a delay in accumulation of PrP^d occurs in MT heterozygotes in comparison to MM homozygotes at preclinical stages of infection (478–529 dpi). Thus, two heterozygotes showed little involvement of LRS tissues and just a trace of PrP^d or none at all in brain, and one showed widespread LRS involvement but mild PrP^d accumulation in the CNS. This was in contrast with the findings in homozygotes which, at comparable dpi, showed widespread PrP^d accumulation in LRS tissues and all were positive in the CNS. A possible explanation for this incomplete protective effect can be extrapolated from studies in which VRQ/ARR infected sheep were found to accumulate PrP^{Sc} of the VRQ – and not of the ARR – polymorphism (Jacobs et al., 2011; Morel et al., 2007). Similarly, "*in vitro*" studies have shown that the $M_{112}T$ variant of PrP^c does not easily convert to the PrP^{Sc} isoform (Bossers et al., 2000) therefore it could be postulated that in $M_{112}T$ heterozygotes the only PrP^c that can be converted to PrP^d is the M_{112} one. This could explain the close phenotypic similarity of PrP^d accumulation in homo- and heterozygotes and, because M_{112} PrP^c is less abundant in the latter, the delay of such accumulation in $M_{112}T$ heterozygotes. Moreover, the $M_{112}T$ PrP^c variant could actually interfere with M_{112} protein conversion and accumulation; this phenomenon of allelic interference has been observed in some murine scrapie models (Barron et al., 2003) and in scrapie-ARR/ARQ heterozygotes (Caplazi et al., 2004).

4.3. Effect of lymphadenectomy on the progression of scrapie

Removal of the prefemoral LN, draining lymph from the site of injection, either early or late after inoculation, did not affect the magnitude nor the spread of PrP^d accumulation in the different tissues examined at comparable time points after inoculation. This finding suggests that, after subcutaneous injection of scrapie, the infectious agent rapidly disseminated through the body either because it: (i) gained immediate access to the bloodstream through venous capillaries in the subcutis, or (ii) drained to the regional LN but, rather than being sequestered there, was re-circulated, possibly through the lymph or directly into the blood. The fact that the three sheep from which lymph was collected from the efferent lymphatics from the prefemoral nodes before, during and after subcutaneous inoculation (Table 1) became as efficiently infected (as judged by PrP^d detection) as those from which efferent lymph was not taken does not disprove a re-circulation of the scrapie agent through the efferent lymphatics as these were left in place until the excision of the prefemoral LN several days later. The notion of a low degree of primary infection of the regional lymph node and an early recirculation and dissemination of the scrapie agent is reinforced by the result that only one of four ipsi-lateral nodes in the EL group and only two of four in the LL group from $M_{112}M$ sheep showed PrP^d accumulation, and also by the bilateral PrP^d accumulation in paired lymphoid tissues during the preclinical stage of disease.

4.4. Scrapie-related changes in immune cell subsets

Finally, this study showed little change in the different immune system cell subsets that can be attributed to scrapie infection and/or PrP^d accumulation in LNs. In this respect, we have considered that the two scrapie-inoculated sheep which showed a complete absence of detectable PrP^d at post-mortem (J169 and J137 in Table 1) are the negative controls, not only for lack of PrP^d in the LNs studied, but also for scrapie infection as such.

The lack of appreciable differences in CD3+, CD4+ and CD8+ cells between LNs with and without PrP^d could be explained by the different distribution or lack of co-localization of those cells (paracortex, with the exception of some CD4+ve cells) and PrP^d (follicles) within the nodes. This explanation is consistent with the well documented absence of PrP^d in the thymus of scrapie infected sheep (Andreoletti et al., 2000; Jeffrey et al., 2001), and suggests little or no involvement of T cells in the pathogenesis of the disease. The apparent increase of γδ-positive T lymphocytes in PrP^d positive lymph nodes is difficult to interpret since such increase was particularly noticeable in LNs with low rather than high PrP^d levels and was restricted to those of the head but was not observed in LNs of the hind leg. However, the LNs of the head and particularly the medial retropharyngeal LN, have been repeatedly shown to be the earliest and most consistent lymph nodes to accumulate PrP^d in natural and experimental scrapie (van Keulen et al., 1996; Andreoletti et al., 2000; Ryder et al., 2009; González et al., 2010). Therefore, a scrapie-driven increase of γδ T cell expression in such lymph nodes at early time points (low PrP^d levels) of infection cannot be ruled out.

Using flow cytometry, we have previously reported that subcutaneous injection of scrapie-brain homogenate resulted in a reduction in the numbers of CD21+ cells in the local LN within the first five hours after inoculation (Eaton et al., 2009). However, this situation was reversed with time, so that CD21+ cells were detected in increased numbers in PrP^d-positive lymph nodes at later stages of preclinical infection when compared with PrP^d-negative lymph nodes of ARR/ARR sheep (Eaton et al., 2007). Using IHC, the present study supports the results of the previous

study (Eaton et al., 2007) by showing increased numbers of CD21+ cells early in the incubation period (low PrP^d levels) of scrapie. As lymph node FDCs are not represented in flow cytometry studies, they do not survive the disaggregation process, the similarity of results of both studies points towards mature B cells being those that are actually increased during scrapie infection. This is in agreement with electron microscopy studies on scrapie-infected mouse spleens showing the phenomenon of "emperipolesis", that is, the trapping of mature B cells by FDC processes within the germinal centres of lymphoid follicles (Jeffrey et al., 2000). However, the present study has not revealed a quantitative correlation between the PrP^d levels and B cell increases, so that the latter would more appear to be the result of infection itself rather than of the magnitude of PrP^d in the follicles. If the above hypothesis is correct (increased numbers of CD21+ mature B cells), the lower numbers of CD79+ cells in clinically affected sheep would result from a decrease in immature B cells. Since this change was unapparent in lymph nodes with high PrP^d levels from preclinical cases, it would be more likely associated with other phenomena, either related to clinical disease or to the more advanced age of the sheep. Ageing has been shown to influence the pathogenesis of prion diseases in the LRS of mice and to alter the microarchitecture of the mantle zone (Mabbott, 2012), and the two clinically affected sheep were approximately 10 month older than the preclinical cases.

5. Conclusions

In conclusion, we have found that ARQ/ARQ sheep with a threonine mutation at codon 112 are less susceptible than methionine homozygotes to subcutaneous injection of scrapie and that lymphadenectomy of the local draining lymph node does not have any impact on the pathogenesis of the infection. This suggests an early spread of the scrapie agent throughout the body. Furthermore, clear changes in immune cell population density attributable to scrapie are restricted to increased numbers of $CD21^+$ cells in lymph nodes both close and far from the site of injection; such increases appears to be related to the presence but not to the level of PrP^d in the affected nodes.

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