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Age, scrapie status, PrP genotype and follicular dendritic cells in ovine ileal Peyer's patches

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ABSTRACT

Follicular dendritic cells (FDCs) residing within ileal Peyer's patches (PPs) are of crucial relevance for sheep scrapie early pathogenesis and subsequent scrapie prion neuroinvasion. In this study, ileal PP follicles were significantly more numerous in lambs than in adult Sarda breed sheep, with significant differences being also found in lymphoid follicle area, perimeter and FDC density. Furthermore, PrPd deposition within ileal PPs and host's PrP genotype did not significantly influence these parameters. We conclude that age significantly affects FDC density in ileal PPs from Sarda breed ovines, independently from host's scrapie status and PrP genotype.

Follicular dendritic cells (FDCs) residing in primary B-cell follicles and germinal centres (GCs) of lymphoid tissues are a distinct cell type, non-phagocytic and non-migratory, which is considered to derive from non-haematopoietic stromal precursor cells. There are a number of recent studies addressing FDC phenotype and function, which are leading to a variety of new insights into the biological significance of these cells (Aguzzi and Krautler, 2010).

Sheep scrapie is the "prototype" of transmissible spongiform encephalopathies (TSEs), a group of slowly progressive and fatal neurodegenerative disorders affecting man and animals (Prusiner, 1998). In natural sheep scrapie, the gastro-intestinal tract is the prion entry site, with ileal Peyer's patches (PPs) playing a key role in early infection's pathogenesis. Furthermore, FDCs residing within ileal PPs are crucial for the accumulation of sheep scrapie prions ("disease specific prion protein", PrPd), as neuroinvasion (the long journey from site of infection to central nervous system) is delayed and disease susceptibility is reduced in their absence (Mabbott and MacPherson, 2006).

On the basis of what above, this study was aimed at quali–quantitatively evaluating the FDC network in PPs from the distal ileal tract of Sarda breed ovines, taking into special consideration the following variables: (1) prion protein gene (*PRNP*) polymorphisms (PrP genotypes) modulating susceptibility/resistance (ARQ/ARQ, ARR/ARQ, ARR/ARR) to classical sheep scrapie in Sarda as well as in other ovine breeds (Vaccari et al., 2001; Goldmann, 2008); (2) age, since ileal PPs function as a primary lymphoid tissue undergoing involution at around sexual maturity (Press et al., 2004); (3) immunohistochemical (IHC) evidence or absence of PrPd deposition within ileal PP lymphoid follicles.

In total, the following 30 ovines were investigated, with the same animals having been already included in a previous study (Marruchella et al., 2009): 12 scrapie-free, 2–4-month-old lambs, equally distributed into the 3 aforementioned PrP genotypes; 12 scrapie-free, 2–4-year-old sheep, equally distributed into the 3 aforementioned PrP genotypes; 6 natural scrapie-affected, 3–5-year-old sheep, all of which carrying the susceptible ARQ/ARQ genotype.

Presence or absence of PrPd were preliminarily investigated in all the ovines under study by means of Western Blot (WB) analysis, utilizing a previously published protocol (Ligios et al., 2006). PrPd IHC was subsequently carried out with a mouse monoclonal primary antibody (F99/97.6.1, VMRD, Inc., Pullman, USA), which was utilized at a final dilution of 1:800. A suitable cellular prion protein (PrPc) inactivation protocol was applied to tissue sections (Kovács et al., 2005) before their challenge with the aforementioned primary antibody. Appropriate positive and negative control tissues were also utilized in each IHC run.

We evaluated the FDC network by means of both IHC and indirect immunofluorescence (IIF), using a mouse monoclonal primary antibody (clone CNA-42, DAKO), which was employed at a final dilution of 1:40. Appropriate positive and negative control tissue sections were also utilized in each IHC and IIF run. More in detail, the following parameters were considered: (1) number of lymphoid follicles per section, with data being presented as the mean

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number of follicles per tissue section (± standard deviation, SD); (2) perimeter and area of lymphoid follicles, with data being presented as the mean values (± SD); (3) perimeter and area of germinal centres (GCs), with data being presented as the mean values (± SD); (4) IHC evidence or absence of PrPd deposition within ileal PP lymphoid follicles; (5) FDC distribution pattern; 6) FDC network's extension, with data being obtained by means of IIF microscopy and presented as the mean percentage of the lymphoid follicle (LF) area and of the GC area occupied by CNA-42-immunoreactive (IR) cells (± SD).

Furthermore, in order to properly evaluate the FDC network's density, at least 3 tissue sections and 10 lymphoid follicles were investigated, by means of a 10× microscope objective, for each animal included in the study. Image analysis was carried out by means of *Image J software* (National Institutes of Health, http://rsb.info.nih.gov/ij/).

Statistical analysis was also performed, by means of the Student's *t* test, on the data obtained, with a conventional 1% level being used to define statistical significance ($P \leqslant 0.01$). More in detail, we used "paired *t* tests", that are known to provide more reliable results when comparing "matched pairs of similar units" within a given sample. In our case, each "matched pair" included animals carrying one of the three concerned PrP genotypes (ARQ/ARQ, ARR/ARQ, ARR/ARR), the mean values of which were pairwise compared for the same parameters (ileal PP lymphoid follicle perimeter, LF area, GC perimeter, GC area, FDC network's density in relation to both LF area and GC area) with each of the other two genotypes. Therefore, the mean values obtained from ARQ/ARQ lambs were compared both with ARR/ARQ and with ARR/ARR lambs, while ARR/ARQ lambs were individually compared both with ARQ/ARQ and with ARR/ARR lambs; likewise, the mean values obtained from ARQ/ARQ adults were compared with those both from ARR/ARQ and from ARR/ARR adults, while ARR/ARQ adults were pairwise compared both with ARQ/ARQ and with ARR/ARR adults; furthermore, in order to properly evaluate whether the "scrapie status" (positive *vs* negative) may be associated with any difference(s) in FDC network's extension (and density) between adult, scrapie-affected and scrapie-negative sheep, the mean values from the adult ARQ/ARQ scrapie-negative animals were also compared by the Student's *t* test ("paired *t* test") with those obtained from the adult ARQ/ARQ scrapie-affected ovines under investigation.

The results presented herein, as far as concerns the average values determined for each of the aforementioned parameters (along with their respective ± SD values) in the Sarda breed ovines under study, are shown in Table 1.

In this respect, the average number of ileal PP follicles ranged from 15 in adult sheep to 65 in lambs, with a statistically significant difference ($P \leqslant 0.01$) being also found between adults and lambs with reference to follicle area and perimeter, as well as to germinal centre (GC) area and perimeter. The above parameters did not additionally appear to be affected by the host's PrP genotype and scrapie status, with no significant differences being found between ARQ/ARQ scrapie-negative and ARQ/ARQ scrapie-affected adult ovines (Table 1).

The FDC network, when compared with both lymphoid follicle area and GC area, was significantly ($P \leqslant 0.01$) more dense and widespread in lambs than in adults (Fig. 1, a–d), independently from host's PrP genotype and PrPd deposition (Fig. 2, a–b). More in detail, almost the entire PP follicles' area was populated by a dense FDC network in lambs, with this network extending up to the peri-follicular stroma and up to the dome area underlying the follicle-associated epithelium (FAE). Differently from what above, a much less prominent FDC network was observed in adult sheep, with this network exhibiting a polarized distribution, which mainly involved the GC light zone (Fig. 1, c–d). Intra-follicular microgranulomas, a common finding in ovines, always lacked FDC immunolabeling (Fig. 1, c). As far as the quantitative evaluation of FDC network's extension is concerned, a statistically significant difference ($P \leqslant 0.01$) was also documented between lambs and adults by means of *Image J software* analysis, which was utilized on a given series of suitable IIF-stained microscopic slides in order to calculate the *ratio* between the area occupied by CNA-42-IR labeling, follicle area and GC area. In particular, the FDC network was significantly ($P \leqslant 0.01$) thicker in lambs as compared to both PP follicle area and GC area.

On the basis of the results presented herein, host's PrP genotype and PrPd deposition did not apparently affect the FDC network and distribution within ileal PPs from Sarda breed sheep. This is in agreement with the results of a previous work from our group, in which no apparent influence was found to be exerted on ileal PP innervation by both host's PrP genotype and PrPd deposition (Marruchella et al., 2009). Nevertheless, the inclusion of an additional group of scrapie-infected lambs would have given further strength to the results presented herein, thus allowing a more precise comparison between the "age" and "scrapie status" variables. To this aim, it should be also underlined that such a group of

Table 1
Mean values of ileal Peyer's patch (PP) lymphoid follicles' (LF) perimeter, LF area, germinative centre (GC) perimeter and GC area, along with follicular dendritic cell (FDC) network's density (CNA-42 immuno reactivity) in relation to both LF area and GC area, in ARQ/ARQ, ARR/ARQ and ARR/ARR scrapie-free Sarda breed lambs and adults, as well as in ARQ/ARQ scrapie-affected Sarda breed ovines (SD = standard deviation).

	LF perimeter	LF area	GC perimeter	GC area	FDC density/LF area (%)	FDC density/GC area (%)
Lambs						
ARQ/ARQ	3,724,154 (SD = 465,974)	630,578 (SD = 146,779)	2,738,695 (SD = 496,919)	329,734 (SD = 29,547)	27.8% (SD = 5.3%)	51.84% (SD = 7.3%)
ARR/ARQ	3,426,303 (SD = 491,583)	576,551 (SD = 197,613)	2,466,205 (SD = 555,016)	267,640 (SD = 34,804)	25.8% (SD = 5.9%)	55.5% (SD = 9.4%)
ARR/ARR	3,834,125 (SD = 509,948)	654,122 (SD = 188,670)	3,250,152 (SD = 529,878)	366,807 (SD = 38,741)	29.1% (SD = 6.4%)	51.89% (SD = 8.5%)
Adults						
ARQ/ARQ	743,827 (SD = 95,546)	37,799 (SD = 10,591)	468,781 (SD = 123,505)	15,913 (SD = 4888)	13.1% (SD = 3.84%)	39.3% (SD = 7.7%)
ARR/ARQ	889,218 (SD = 100,796)	53,817 (SD = 15,922)	555,915 (SD = 147,206)	18,610 (SD = 4027)	14.7% (SD = 4.4%)	42.50% (SD = 6.1%)
ARR/ARR	795,368 (SD = 99,872)	45,474 (SD = 13,272)	488,844 (SD = 158,452)	17,978 (SD = 7593)	12.8% (SD = 5.1%)	38.37% (SD = 8.3%)
Scrapie-affected ovines						
ARQ/ARQ	756,542 (SD = 112,944)	40,236 (SD = 12,878)	492,319 (SD = 153,923)	20,689 (SD = 8203)	15.7% (SD = 6.3)	40.6% (SD = 82%)