Replication of small ruminant lentiviruses in aluminum hydroxide-induced granulomas in sheep: a potential new factor for viral dissemination

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Abstract

Aluminum (Al)-based salts are widely used adjuvants in ruminants and other species strengthening the immune response elicited against vaccine antigen/s. However, they can lead to the formation of long-lasting granulomas composed of abundant activated macrophages. Small ruminant lentiviruses (SRLV) are widely distributed macrophage-tropic retroviruses that cause persistent infections in sheep and goats. Infected monocytes/macrophages and dendritic cells establish an inflammatory microenvironment that eventually leads to clinical manifestations. The aim of this work was to study the effect of Al-induced granulomas in the replication and pathogenesis of SRLV. Eleven adult, naturally SRLV-infected sheep showing clinical arthritis were distributed in Vaccine (n=6), Adjuvant-only (n=3) and Control (n=2) groups and inoculated with commercial Al-based vaccines, Al hydroxide adjuvant or phosphate buffered saline, respectively. *In vitro* studies demonstrated viral replication in Al-induced granulomas in 5 out of 10 sheep. Immunohistochemistry (IHC) evinced granular, intracytoplasmic SRLV presence in macrophages within granulomas. Viral sequences obtained from granulomas, blood monocytes and other tissues were highly similar in most animals, suggesting virus circulation among body compartments. However, notable differences between isolated strains in granulomas and other tissues in specific animals were also noted. Interestingly, the B2 subtype was the most commonly found SRLV genotype reaching a wider body distribution than previously described. Recombination events between genotypes B2 and A3 along *gag* region were identified in two sheep. Our results indicate that Al-hydroxide derived granulomas may represent an ideal compartment for SRLV replication, perhaps altering natural SRLV infection by providing a new, suitable target tissue.

44 **Importance**

45 Granulomas are inflammation-derived structures elicited by foreign bodies or certain infections.
46 Aluminum adjuvants included in vaccines induce granulomas in many species. In sheep, these are
47 persistent and consist of activated macrophages. Small Ruminant Lentiviruses (SRLV), which are
48 macrophage-tropic lentivirus, cause a chronic wasting disease affecting animal welfare and
49 production. Here, we studied the occurrence of SRLV in post-vaccination granulomas retrieved from
50 naturally infected ewes after vaccination or inoculation with aluminum only. SRLV infection was
51 confirmed in granulomas by identification of viral proteins, genomic fragments and enzymatic
52 activity. The infecting SRLV strain, previously found exclusively in carpal joints, reached the central
53 nervous system, suggesting that occurrence of SRLV in post-vaccination granulomas may broaden
54 tissue tropism. SRLV recombination was found in inoculated animals, a rare event in sheep
55 lentiviruses. Potentially, virus-host interactions within granulomas may modify viral pathogenesis
56 and lead to more widespread infection.

57

58 **Introduction**

59 Vaccines play a major role in controlling infectious diseases in animals and humans (1). Most
60 vaccines are based on inactivated pathogens or recombinant proteins that are often poorly
61 immunogenic. As a consequence, adjuvants are needed to strengthen the immune response against
62 vaccine antigens (2, 3). Adjuvants in vaccines intended for veterinary use are mainly based on
63 aluminum (Al) compounds and formulated as different salts, mostly Al hydroxide (4). Al content in
64 vaccines and several other factors such as individual genetic background, number of inoculations,
65 and antigen immunogenicity may raise undesired side effects including local inflammation or fever
66 (5). In sheep, post-injection inflammation leading to granuloma formation, is a constant response
67 after the inoculation of Al adjuvant (6). These granulomas consist of Al-loaded activated
68 macrophages and multinucleated giant cells (6) and they have been described in almost all species
69 including mice (7), pigs (8), cows (9), non-human primates (10) and humans (11, 12).

70 Small ruminant lentiviruses (SRLV) are macrophage-tropic retroviruses that cause highly prevalent
71 chronic infections in sheep and goats, linked to important detrimental effects on animal production
72 and breeding stocks trade restrictions (13). SRLV have a defined tropism for the mononuclear-
73 phagocytic system altering cytokine production, MHC-II expression, and maturation state, thus
74 leading to persistent inflammation. Infection is highly restricted in monocytes and classically
75 activated M1-macrophages, but it is productive in M2-like and tissue differentiated macrophages (14,
76 15). SRLV systemically disseminate and mostly affect, in an immune-mediated manner, lungs, brain,
77 mammary gland and joints (16). Four main genotypes (A, B, C and E) and more than 35 subgroups
78 with significant antigenic heterogeneity have been already characterized (17). In the articular form,
79 the main clinical sign is unilateral or bilateral carpal arthritis and in sheep it is mainly caused by
80 genotype B2 strains (18, 19). On the other hand, genotype A strains have been associated with
81 outbreaks of encephalitis (20, 21), and is also related to pneumonia and mastitis. Recombination

between the most widely distributed SRLV genotypes A and B has been reported in goats from Italy (22), Canada (23) and Mexico (24) and only a single description of lentiviral recombination has been reported in sheep (25). Coinfection and recombination are hallmarks of lentivirus infections and may potentially expand their host-range species and promote colonization of different organs (26). After infection through respiratory secretions or colostrum intake, monocytes carrying the integrated provirus may distribute and differentiate into tissue macrophages triggering viral replication and inflammation, contributing to lesion development in target organs (16).

Macrophages in granulomas might represent a replication site for SRLV that could modify viral pathogenesis by altering virus replication and immunological host responses. The aim of this work was to study the potential SRLV presence, replication and recombination in Al-induced granulomas ofnaturally infected sheep.

Material and Methods

Animals and experimental design

Experimental design and procedures were licensed by the Ethical Committee of the University of Zaragoza (PI15/14). Requirements of the Spanish Policy for Animal Protection (RED53/2013) and the European Union Directive 2010/63 on the protection of experimental animals were always fulfilled.

Nine adult (>4 year-old) Rasa Aragonesa ewes at the same production stage naturally infected by SRLV and showing lentivirus-induced bilateral arthritis in carpal joints were selected from three different flocks of Aragón (Spain). No other selection criteria were included at this stage. Upon checking for bacterial growth, only one animal showed *Chlamydia* spp. growth in joints, and it was disregarded for this study. Sheep were lodged at the experimental farm of the University of Zaragoza, and kept for 75 days under appropriate housing, management and diet conditions. Animals were

105 divided into 3 groups and injected with different substances: Vaccine group (n=4; animals V-1, V-2,
106 V-3 and V-4) was inoculated with commercial vaccines, Adjuvant-only group (n=3; animals A-5, A-
107 6, and A-7) received Al hydroxide (Alhydrogel®, CZ Veterinaria) and Control group (n=2; animals
108 C-8 and C-9) was injected with PBS. Vaccination record of these animals prior to the study was
109 unknown.

110 The vaccine inoculation protocol during the experiment followed manufacturer's instructions, i.e.,
111 prime vaccination and boosting at 21-30 days. After the recommended resting period, a second round
112 of vaccination was applied following the same protocol. Commercial vaccines, antigens, inoculations
113 days and Al dosage are detailed in Figure 1. Animals received 8 subcutaneous inoculations
114 distributed in 4 injection dates, i.e., two vaccines were applied at each date, one at each thoracic
115 flank, with 21-days intervals between them. The four injections at each side were symmetrically
116 distributed forming a square in the subcutaneous thoracic tissue. Euthanasia was carried out at 75
117 days post first inoculation (dpi). Al content of each inoculum was measured by inductively coupled
118 plasma mass spectrometry. Vaccine and Adjuvant-only groups received a total of 43.36 mg of Al per
119 animal. Al content in PBS was always below the detection limit of the technique (0.074 µg/mL). A
120 further Vaccine-Extra group (n=2; animals V-10 and V-11) of the same breed and age was
121 established after the end of this first experiment to confirm and complement the molecular results
122 obtained. These two sheep were housed under the same conditions and subjected to the same
123 injection protocol until euthanasia at 40 dpi, therefore receiving four inoculations in total.

124 ***In vivo* studies**

125 General clinical examination and blood sampling were performed at 0, 40 and 75 dpi. Peripheral
126 blood mononuclear cells (PBMCs) were isolated on a Ficoll-Hypaque gradient (δ=1.077;

Lymphoprep Axis-Shield) for DNA extraction from 6 mL EDTA tubes (BD Vacutainer®), and 2 mL of plasma were stored at -20ºC for antibody analysis using ELISA.

Pathology

Tissues were fixed in 10% neutral-buffered formalin for 48-72h. Additionally, selected samples from post-vaccination granulomas were fixed in a Zinc solution for 36h to carry out immunohistochemistry (IHC) against SRLV capsid antigen (27).

Samples were routinely processed for paraffin embedding and 4 µm sections were stained with either standard hematoxylin-eosin (HE), lumogallion or IHC. Lumogallion is a specific staining for Al that was carried out only in granulomas, following a previously-described protocol (6, 28). For IHC, samples were subjected to pre-treatment for antigen retrieval (30 minutes in a solution of 10mM citric acid pH 6 immersed in a water bath at 95ºC), endogenous peroxidase inhibition and non-specific binding sites blockade (20 minutes in normal horse serum diluted 1:200 in PBS). Tissue samples were then incubated overnight at 4ºC with a specific monoclonal antibody against p28 capsid protein of SRLV (CAEP5A1, VMRD, USA), labelled for 30 minutes at room temperature with anti-mouse EnVision HRP System (DAKO, Agilent Technologies) and revealed with 3-3'-diaminobenzidine. The specificity of the technique was controlled by immunolabelling granulomas from SRLV-free animals, obtained from previous studies (6).

Serological tests

Blood plasma was used for SRLV antibody screening using two commercially available ELISA kits: Elitest-MVV (Hyphen-Biomed, France), based on p25 recombinant protein and a transmembrane synthetic peptide both derived from genotype A (29); and Eradikit™ SRLV Screening kit (IN3 diagnostic, Italy), based on a mixture of Gag and Env antigens belonging to three different SRLV

149 viral genotypes (30). Both assays were performed to confirm infection status at the beginning and the
150 end of the experiment.

151 **Virus isolation**

152 For the *in vitro* studies, i) one granuloma per animal of the Vaccine and Adjuvant-only groups, ii) a
153 single granuloma found in control sheep C-8, and iii) the four granulomas found in each animal of
154 the Vaccine-Extra group were collected at necropsy. Therefore, a total of 16 granulomas were
155 analyzed. Granulomas and tissue samples from carpal joints, spleen, mediastinal lymph node, brain
156 and bronchoalveolar lavage (BAL) were kept in PBS with antibiotic/antimycotic mix solution
157 (Sigma) and either processed immediately or stored in RNAlater (ThermoFisher) at -80ºC until used.
158 Samples kept in PBS were cut in small pieces ($1cm^3$) with a scalpel blade and seeded on $25cm^2$ flasks
159 with Roswell Park Memorial Institute (RPMI) complete medium, supplemented with 1% of L-
160 glutamine, 1% of vitamins, 1% antibiotics/antimycotics mix, 1% non-essential amino acids, 10 mM
161 sodium pyruvate, 50 µM β-mercaptoethanol and 10% fetal bovine serum (FBS). Cultures were
162 maintained at 37°C in a humidified atmosphere containing 5% CO_2 and medium was partially
163 replaced every 3–7 days. Once per week, medium was collected and reverse transcriptase (RT)
164 activity was determined using SYBR Green-based PCR-enhanced reverse transcriptase assays (SG-
165 PERT) (31). The presence of cytopathic effect was observed under light microscopy at each culture
166 passage.

167 **Genetic characterization**

168 Genomic DNA from samples was extracted, using E.Z.N.A Blood DNA Mini Kit (Omega bio-tek)
169 from the following sources: i) PBMCs; ii) trypsinized cultured cells from tissue cultures with positive
170 RT activity; iii) tissue samples kept in RNAlater previously homogenized in a Micro-Dismembrator
171 U (Sartorius) using steel beads.

172 SRLV viral DNA detection was carried out by partial amplification of LTR, *gag* and *pol* using PCR
173 with primers and conditions previously described and detailed in Table 1 (32–37). Following PCR,
174 classical amplicons were visualized in 1% agarose gels, purified, and cloned into pGEMT-easy®
175 (Promega) or pJET1.2 blunt vector (ThermoFisher) according to the manufacturer's instructions.
176 Three positive clones were submitted to external Sanger sequencing (Stab Vida). Sequence analysis
177 was carried out by comparison with GenBank deposited sequences using BioEdit, Chromas and
178 SnapGene softwares. Phylogenetic trees were created using the web application (www.phylogeny.fr)
179 and modified using MEGA version 10.0.5 software.

180 SimPlot v.3.5.1 software was used to define genomic regions that display significant percentages of
181 nucleotide sequence identity and provide indications of possible recombination events. Sequences
182 obtained in this study were compared with reference sequences from all described SRLV genotypes.

183 **Results**

184 **SRLV presence in animals**

185 Clinical examination and cell blood count revealed no significant differences between the treatment
186 groups during the inoculation scheme (Kruskal Wallis; $p>0.05$). Infection by SRLV was confirmed in
187 all the animals by both ELISA tests used and reactivity remained positive until the end of the
188 experiment. PCR performed on DNA from PBMCs was less sensitive as a diagnostic tool compared
189 to ELISA tests and identified 5 out of 11 and 6 out of 9 infected animals, at the beginning and the end
190 of the experiment, respectively (Table 2).

191 **Granulomas in animal groups and SRLV presence**

192 At necropsy, 31 subcutaneous granulomas were recovered in the Vaccine group out of 32 vaccine
193 injections (96.7%). In the Adjuvant–only group 19 granulomas were isolated out of 24 inoculations

194 (79.2%). Interestingly, one of the Control animals (C-8) exhibited a single low-sized granuloma,
195 possibly associated with a prior on-farm vaccination. Histologically, granulomas were composed of
196 abundant epithelioid macrophages and occasional multinucleated giant cells admixed with moderate
197 numbers of lymphocytes, plasma cells and fibroblasts immersed in abundant collagenous matrix
198 (Figure 2A). These granulomas inconstantly showed a central necrotic core. Lumogallion staining
199 revealed abundant intracytoplasmic Al-positive deposits within epithelioid macrophages and giant
200 cells in all evaluated granulomas (Figure 2B). Presence of SRLV in post-vaccination granulomas was
201 assessed by IHC. Granular, intracytoplasmic positive immunolabelling was found in macrophages
202 and multinucleated giant cells, whereas no labelling was observed in fibrous capsule, lymphocytes,
203 and plasma cells (Figure 2C and 2D). Granulomas from SRLV negative animals consistently showed
204 no staining.

205 **Virus isolation from tissue samples**

206 A total of 16 granulomas were submitted for tissue culture and monitored for cytopathic effect and
207 RT activity for more than three weeks. Granulomas from sheep V-2, V-3, C-8, three out of four
208 granulomas from V-10, and two out of four granulomas from V-11 showed cytopathic effect with the
209 presence of syncytia and/or positive RT supernatants, thus indicating active SRLV replication in 5
210 out of 10 sheep (Table 2). In spleen or BAL, 6 out of 9 samples showed active SRLV replication
211 evidenced by the appearance of cytopathic effect in tissue culture and RT activity determinations
212 (Table 2).

213 **SRLV genetic characterization**

214 DNA from carpal joint, brain, spleen, mediastinal lymph node, BAL and granulomas was submitted
215 to SRLV genetic characterization. Positive PCR reactions from samples were purified, cloned into
216 shuttle plasmids and sequenced. Genetic regions partially covered by the PCRs (LTR, *gag* and *pol*)

allowed phylogenetic classification of the circulating viruses. PCRs covering the capsid and nucleocapsid clustered sequences from PBMCs mainly within B2 genotypes and, just in one animal (V-11) within A genotypes (Figure 3A). Sequences covering LTR, matrix and capsid depicted exactly the same clustering pattern (Figure 3B). The genotype A sequence obtained from sheep V-11 showed values of about 85% similarity when compared with GenBank reference sequences but was not unequivocally assigned to any previously described subgroup (Table 3).

DNA from granuloma cultures contained sequences from different SRLV genotypes. In general, genotypes present in granulomas resembled those in PBMCs. Capsid and nucleocapsid fragment allocated all the sequences from granulomas as genotype B2 (Figure 3A), while sequences spanning LTR and matrix protein clustered in genotypes B2 and A3 (Figure 3B). Genotype B2 was the most prevalent and actually, it was similar to the previously-described SRLV B2 strain 496 (19). However, genotype A identified in granulomas from animals V-11, A-6 and C-8 (Figure 3A and 3B) were hardly assigned to previously known subtypes, as they showed similarity values of almost 85% in *gag* and *pol* genes compared with the genotypes A1, A2 and A3 (Table 3).

In spleen or BAL, sequences obtained reflected a similar classification compared with PBMCs and granuloma samples, with genotype B2 being present in almost all samples. Remarkably, sequences related to the carpal joint-tropic B2 genotype were also identified in the brain of four sheep (V-2, V-10, V-11 and A-7). Additional genotypes included the unassigned genotype A in spleen from animal V-3 (Figure 4). Viral sequences from granulomas, PBMCs and other tissues where highly similar in animals V-2 and V-10, whereas, they showed notable differences between the isolated strains in animals V-11, A-6 and C-8 (Figures 3 and 4).

Sequences were analyzed with Bootscan and identified recombination events between genotypes A3 and B2 in spleen of sheep C-8 and in PBMCs of sheep A-6 along *gag-pol* (Figure 5A) and LTR-*gag* regions (Figure 5B), respectively.

241 **Discussion**

242 This work describes the presence and replication of SRLV in Al-induced granulomas of SRLV
243 naturally-infected sheep. The occurrence of lentiviruses in iatrogenic granulomas has never been
244 described before, and the study of possible interactions reveals a novel research field. Sheep provides
245 a unique model for understanding this interaction as granuloma formation is a constant feature after
246 the injection of Al-containing vaccines in this species (6), and SRLV is one of the most widespread
247 viral infections in sheep and goats (16). SRLV target macrophages that actively phagocytize Al
248 adjuvants (6, 15). Therefore, SRLV and Al can merge in single animals depending on the SRLV
249 prevalence and the intensity of vaccination programs.

250 Vaccination protocols in sheep farms may remarkably vary between areas or countries depending on
251 the health status and challenges, the farming system, and other environmental and management
252 factors (38, 39). These protocols can be affected by the implementation of compulsory vaccinations
253 against emerging diseases (38), such as the vaccination campaigns for Bluetongue in Spain (40) and
254 other European countries (41) about a decade ago. In the present study, a typical vaccination
255 protocol, using bluetongue virus vaccine was followed to simulate a real-world scenario. Vaccines
256 against bluetongue virus have been associated with increased SRLV seroprevalence in Spanish sheep
257 farms after the compulsory vaccination against the virus (M. Vila, personal communication).
258 However, high SRLV seroconversion rates in the context of this particular vaccination program
259 against bluetongue virus in France have been attributed to false positive ELISA results due to vaccine
260 contamination (42).

261 SRLV infection was confirmed in all animals enrolled in our study, including controls by detecting
262 SRLV antibodies, indicating that infection had been naturally established prior to the experiment.
263 Nearly one granuloma per injection was detected at post-mortem examination, indeed, 96.7% and
264 79.2% of the expected granulomas were recovered in the Vaccine and Adjuvant-only groups,

265 respectively. A similar result was obtained in a previous study (6), in which higher numbers of
266 granulomas were recovered in vaccinated animals compared with sheep injected with Al hydroxide
267 only. Additionally, sheep C-8 (Control group) showed an Al-containing subcutaneous granuloma,
268 likely as a result of previous vaccination, since these granulomas can persist for at least 15 months
269 (6) and the present experiment was performed with SRLV-infected, commercial animals for 75 days
270 only. Therefore, occurrence of granulomas from on-farm vaccinations cannot be totally excluded in
271 animals from the Vaccine or Adjuvant groups.

272 SRLV presence was demonstrated in granulomas by IHC, which showed viral proteins in the
273 cytoplasm of epithelioid macrophages and multinucleated giant cells. The positive signal was
274 observed within intracytoplasmic granules with a pattern very similar to that of Al-containing
275 phagolysosomes (6). This might indicate that the vacuoles induced by Al phagocytosis provide a
276 suitable environment for viral replication. Intracytoplasmic location and granular aspect of positive
277 immunolabelled viral particles have already been described in macrophages of other ovine tissues
278 (43, 44). Similarly, lentivirus replication within granulomas has been found in *Mycobacterium*
279 *tuberculosis* infected macaques and humans (45, 46).

280 Moreover, SRLV presence and replication was detected in cultured macrophages derived from post-
281 vaccination granulomas by PCR, RT-activity and analysis of cytopathic effect in 50% of the sheep (5
282 out of 10) showing subcutaneous granulomas. SRLV most likely reached granulomas via infected
283 circulating monocytes; it is well known that migration of blood monocytes following pro-
284 inflammatory cytokine gradients is the most common mechanism for macrophage accumulation in
285 interstitial tissues (47). Indeed, Al-containing macrophages in post-vaccination granulomas in mice
286 derive from circulating monocytes (48). Additionally, resident macrophages may proliferate and
287 collaborate in granuloma formation, as demonstrated in alveolar macrophages infected with *M.*
288 *tuberculosis* (49).

289 Sequences obtained from circulating PBMCs demonstrated that most of the SRLV amplified
290 belonged to the genotype B2, a genotype strongly related to sheep lentiviral arthritis in the area of the
291 study (18, 19). Sheep V-11 presented sequences similar to the genotype A with similarity values
292 which approximates 85%, around the threshold limit to consider the occurrence of a novel subtype.
293 However, further studies are needed to fully support a new SRLV subtype designation. Sheep V-11,
294 A-6 and C-8 showed coexistence of different quasi-species in the granulomas, the PBMCs and other
295 tissues of the same animal. This phenomenon, known as compartmentalization, can be the result of
296 either genetic variations in a single viral strain along time, or co-infection of a certain individual with
297 several viral strains (22, 50, 51). Most of the virus sequences obtained from granulomas were very
298 similar to those obtained from PBMCs, which is compatible with the aforementioned migration of
299 SRLV-infected circulating monocytes to granulomas.

300 Interesting results were obtained in sheep V-11. In this animal, sequences of the matrix and capsid
301 regions of SRLV isolated in the granuloma were similar to those isolated in PBMCs. However, when
302 capsid and nucleocapsid regions were sequenced, the viral sequences of the granuloma differed from
303 the sequences obtained in PBMCs and were similar to those isolated in spleen, carpal joint and brain.
304 This fact might indicate viral recombination in the granuloma, which was also supported by the
305 presence of recombinant strains in spleen of two further animals, A-6 and C-8. Recombination highly
306 expands virus variability by mixing genetic fragments from different genotypes in double-infected
307 cells. This may have important implications for diagnosis, tissue tropism, host range, generation of
308 escape mutants and SRLV transmission (26, 52). Recombination between genotypes A and B has
309 been occasionally described in goats and rarely in sheep (22–25).

310 These findings suggest that Al-induced granulomas may represent a compartment for SRLV
311 replication and also for cell-to-cell propagation, an evolutionary-conserved mechanism of SRLV to
312 elude immune surveillance (15, 43). SRLV replicate in activated macrophages (15) and granulomas

313 are focal chronic inflammatory reactions mostly composed of activated macrophages. Al hydroxide
314 injection induces a robust inflammation with recruitment of blood monocytes, some of them
315 potentially infected by SRLV (6). Monocyte maturation into activated macrophages will lead to
316 phagocytosis of Al micron-sized aggregates inducing granuloma formation (6, 48) but it would also
317 trigger SRLV replication in infected animals (15). This phenomenon could, at least partially, explain
318 the high SRLV seroprevalence rates observed in Spanish regions, in which nearly all flocks are
319 infected (53), and the application of Al-containing vaccines is intensive (38). This hypothetical
320 association could have deep implications in the sheep industry since increased seroprevalence due to
321 vaccination may bias ongoing SRLV control programs and accreditation schemes, both based on
322 serological identification, hindering breeding stocks trade. Reduced milk and lamb production are
323 linked to SRLV infection (13) and, hence, SRLV replication in Al-derived granulomas might also
324 have a role in clinicopathologic features as well as productive traits. However, further experimental
325 studies are needed to better understand these findings.

326 Additionally, Al-induced granulomas may play a role in SRLV dissemination and tissue tropism due
327 to the translocation of macrophages. It has been demonstrated that Al-loaded macrophages can
328 systemically biodistribute from the granulomas to other body locations (6, 48). Furthermore, Al
329 increase has been detected in the central nervous system (CNS) of experimentally vaccinated mice
330 and sheep (48, 54). The SRLV nervous form is usually caused by genotype A strains (20, 21), which
331 is in contrast with the presence of B2 strains in the brain of four animals in the present study. In
332 previous studies, in sheep of the same breed and at the same regions, natural and experimental
333 infection with a similar B2 strain resulted in clinical affection of carpal joints without presence of this
334 strain in the CNS (55). This may suggest that Al-induced granulomas could favor a wider SRLV
335 tissue tropism since in addition, Al derived from granulomas has been found in the spinal cord of
336 vaccinated sheep (54). SRLV in the spinal cord may be associated with a particular nervous form

characterized by myelitis as the sole lesion (56). Analogous interactions have been described between SIV and *M. tuberculosis*-derived granulomas in infected macaques. SIV induces clinical reactivation and disrupted immune responses against latent *M. tuberculosis* infection by replicating within tuberculous granulomas (45).

Alternative vaccine formulations including biocompatible and biodegradable adjuvants such as microcrystalline tyrosine or calcium phosphate have shown promising results in terms of protection against bacterial pathogens (57, 58). The use of Al-free vaccines in sheep may reduce granuloma formation and accordingly the availability of macrophages for SRLV to infect, while ensuring high protection rates. Further studies are needed to find out whether eventual granulomas after vaccination with alternative vaccines have any effect on SRLV replication.

In conclusion, our results indicate that, in naturally infected sheep, Al-injection site granulomas possibly promote viral replication, body distribution and recombination, therefore, potentially modifying viral tropism and immunological responses. Data presented in this work may open a new research field in which new alternative adjuvants should elicit a protective immune response, avoiding granuloma formation. Finally, it is important to ascertain whether the findings described in this study are exclusive to the SRLV infection in sheep, or rather can be observed in macrophage-tropic infections of other species developing granulomas. In cats, persistent inflammation induced after vaccination may reactivate Feline Leukemia virus infection contributing to emergence of cutaneous lymphomas at injection sites (59). Mechanisms underlying these and likely other interactions, such as HIV and *M. tuberculosis*, may involve virus infection of macrophages within granulomas, and need to be uncovered in order to improve vaccines safety.

Abbreviations

Aluminum (Al)

360 Small ruminant lentiviruses (SRLV)

361 Phosphate buffered saline (PBS)

362 Peripheral blood mononuclear cells (PBMCs)

363 Immunohistochemistry (IHC)

364 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS)

365 Bronchoalveolar lavage (BAL)

366 Fetal bovine serum (FBS)

367 Reverse transcriptase (RT)

368 SYBR Green-based PCR-enhanced reverse transcriptase assays (SG-PERT)

369 Central nervous system (CNS)

370

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RR, LL, DdA, JA, MP and RdM designed the experiment. RdM, JA, ARL, AF, MP and LL were in charge of animal care, treatment administration, clinical analysis and *in vivo* studies. RdM and ARL were in charge of the immunohistochemistry. IE and RR performed serological tests, DNA extraction, PCR studies, cell cultures, viral sequencing, and phylogenetic studies. IE, RdM, RR and LL wrote the manuscript. MP, DdA, LL and RR obtained funds, coordinated and supervised the study. All co-authors reviewed and approved the final version of the manuscript.

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550

551 **Figure Legends**

552 **Figure 1.** Inoculation schedule and vaccines used. **(A)** Inoculations were administered at 0, 21, 42,
553 and 63 days post first inoculation (dpi). Animals were euthanized at 75 dpi. **(B)** Description of the
554 vaccines used. Aluminum (Al) content was established by inductively coupled mass spectrometry
555 and calculated per dose.

556 **Figure 2:** Post-vaccination granuloma. **(A)** Abundant epithelioid macrophages and multinucleated
557 giant cells (asterisk) intermingled with lymphocytes and plasma cells are surrounded by dense
558 fibrous (arrow) tissue and neovessels (arrowhead). Hematoxylin-Eosin stain, 10x. **(B)** Multiple
559 intense yellowish fluorescent aluminum (Al) deposits are located within the cytoplasm of
560 macrophages and giant cells. Lumogallion stain, 40x. **(C)** Positive immunolabelling against Gag
561 proteins of Small ruminant lentiviruses (SRLV) of epithelioid macrophages (asterisk) within the
562 granuloma. Note the negative result in the connective tissue (arrow), 10x. **(D)** Detail of positive
563 macrophages by immunohistochemistry showing granular, intracytoplasmic labelling against SRLV,
564 40x.

565 **Figure 3:** Phylogenetic trees of SRLV sequences isolated from Vaccine (V), Adjuvant-only (A) and
566 Control (C) sheep. **(A)** SRLV capsid and nucleocapsid sequences obtained from peripheral blood
567 mononuclear cells (PBMCs). **(B)** SRLV LTR and matrix sequences in PBMCs.

568 **Figure 4:** Phylogenetic tree of SRLV matrix sequences isolated from carpal joint, brain, spleen,
569 mediastinal lymph node and bronchoalveolar lavage (BAL) obtained from Vaccine (V), Adjuvant-
570 only (A) and Control (C) sheep.

571 **Figure 5:** Recombination analysis of *gag-pol* Small ruminant lentiviruses sequences from sheep C8
572 spleen **(A)** and LTR-*gag* sequences from sheep A6 PBMCs **(B)**. Recombinant sequences were first
573 detected in RDP followed by Bootscan analysis of consensus sequences in Simplot using a window

size of 200, a step size of 20, 1000 replicates, gap striping and the Kimura 2 distance model with the NJ tree model. Breakpoints on the recombinant sequences are indicated by orange vertical lines. Recombination analysis revealed that recombinant SRLV sequences were the result of a recombination between genotype A3 (dark grey) and genotype B2 (black).

579 **Table 1.** Characteristics of primers used in this work for diagnosis and genetic characterization of

580 Small ruminant lentiviruses DNA.

581

Designation	Location	Sequence (5´-3´)	Orientation	Size (nt)	Ref.
LTR OSLO	LTR-GAG	TGACACAGCAAATGTAACCGCAAG GGCATCATGGCTAATACTTCTAA	Fw Rv	1343	(32) (33)
CRAFT OSLO	GAG	TGACAGAAGGAAATTGTYTRTGG GGCATCATGGCTAATACTTCTAA	Fw Rv	490	(34) (33)
CAEV F0 CAEV R2	GAG-POL	AACTGAAACTTCGGGGACGCCTG GGACGGCACCACACGTAKCCC	Fw Rv	1528	(35)
GAG F1 GAG F2 POL R1 POL R2	GAG-POL	TGGTGARKCATAGMTAGAGACATGG CAAACWGTRGCAATGCAGCATGG CATAGGRGGHGCGGACGGCASCA GCGGACGGCASCACACG	Fw Fw Rv Rv	827	(36)
S P28 POLEV1NERV	POL	CATGAAGAGGGGACAAATCAGCA TCCCGAATTTGTTTCTACCC	Fw Rv	921	(37) This study

582

583 **Table 2.** Serological and PCR results from the Small ruminant lentiviruses naturally-infected sheep
584 included in the study including presence of virus in granuloma, bronchoalveolar lavage (BAL) and
585 spleen tissue cultures. Animals from the Vaccine (V), Adjuvant-only (A) and Control (C) groups
586 were analyzed by commercial ELISA and diagnostic PCR targeting *gag* region. The PCR was
587 performed on DNA isolated from PBMCs. Granuloma samples were cultured *in vitro* and analyzed
588 for RT activity by SG-PERT.

589

		ELISA			PCR			RT activity		
Group	Animal	0 dpi	40 dpi	75 dpi	0 dpi	40 dpi	75 dpi	Granuloma	BAL	Spleen
Vaccine	V-1	+	+	+	-	-	-	-	+	+
	V-2	+	+	+	-	-	+	+	+	-
	V-3	+	+	+	-	-	-	+	+	+
	V-4	+	+	+	-	-	+	-	+	-
	V-10	+	+	ND	+	+	ND	+	ND	ND
	V-11	+	+	ND	+	+	ND	+	ND	ND
Adjuvant-only	A-5	+	+	+	-	-	-	-	-	+
	A-6	+	+	+	+	-	+	-	+	+
	A-7	+	+	+	-	-	+	-	+	+
Control	C-8	+	+	+	+	+	+	+	-	+
	C-9	+	+	+	+	-	+	ND	+	-

ND: Not determined
dpi: days post first inoculation

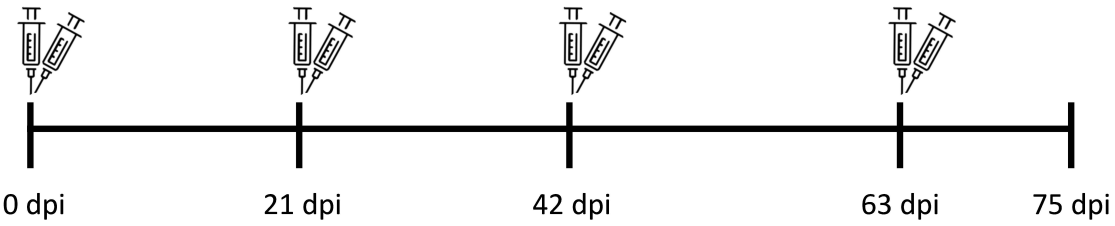
590

591 **Table 3**. Sequence identity matrix among sequences found in animals V-6, V-11 and C-8 and
592 standard GenBank deposited sequences for Small ruminant lentiviruses from the indicated genotypes.
593 Numbers in sequences represent the different clones analyzed. Values in bold highlight the most
594 similar genotypes.

Sequence	Region	Genotype A1	Genotype A2	Genotype A3	Genotype B2	Genotype B1	Genotype E	Genotype C
V-11 PBMCs t0	*gag*	0.776	**0.822**	**0.858**	0.734	0.722	0.650	0.694
V-11 PBMCs t0	*ltr-gag*	0.649	0.667	**0.752**	0.454	0.450	0.510	0.450
V-11 Granuloma	*ltr-gag*	0.707	0.718	**0.815**	0.502	0.488	0.554	0.494
A-6 Granuloma	*gag*	0.727	0.765	0.738	**0.941**	0.830	0.650	0.735
A-6 Granuloma-1	*ltr-gag*	0.710	0.707	**0.790**	0.503	0.486	0.582	0.501
A-6 Granuloma-2	*ltr-gag*	0.711	0.708	**0.791**	0.506	0.489	0.585	0.502
A-6 Granuloma-3	*ltr-gag*	0.710	0.708	**0.792**	0.503	0.486	0.584	0.502
C-8 Granuloma-1	*gag-pol*	**0.809**	**0.826**	**0.847**	0.758	0.753	0.721	0.731
C-8 Granuloma-2	*gag-pol*	**0.808**	**0.826**	**0.847**	0.757	0.752	0.720	0.730
C-8 Granuloma-1	*ltr-gag*	0.711	0.708	**0.791**	0.505	0.489	0.584	0.502
C-8 Granuloma-2	*ltr-gag*	0.711	0.708	**0.791**	0.505	0.489	0.582	0.502
C-8 Spleen-1	*gag-pol*	0.744	0.757	0.755	**0.870**	**0.811**	0.691	0.746
C-8 Spleen-2	*gag-pol*	0.751	0.766	0.755	**0.874**	**0.819**	0.699	0.754
C-8 Spleen-3	*gag-pol*	0.750	0.766	0.752	**0.874**	**0.821**	0.699	0.754

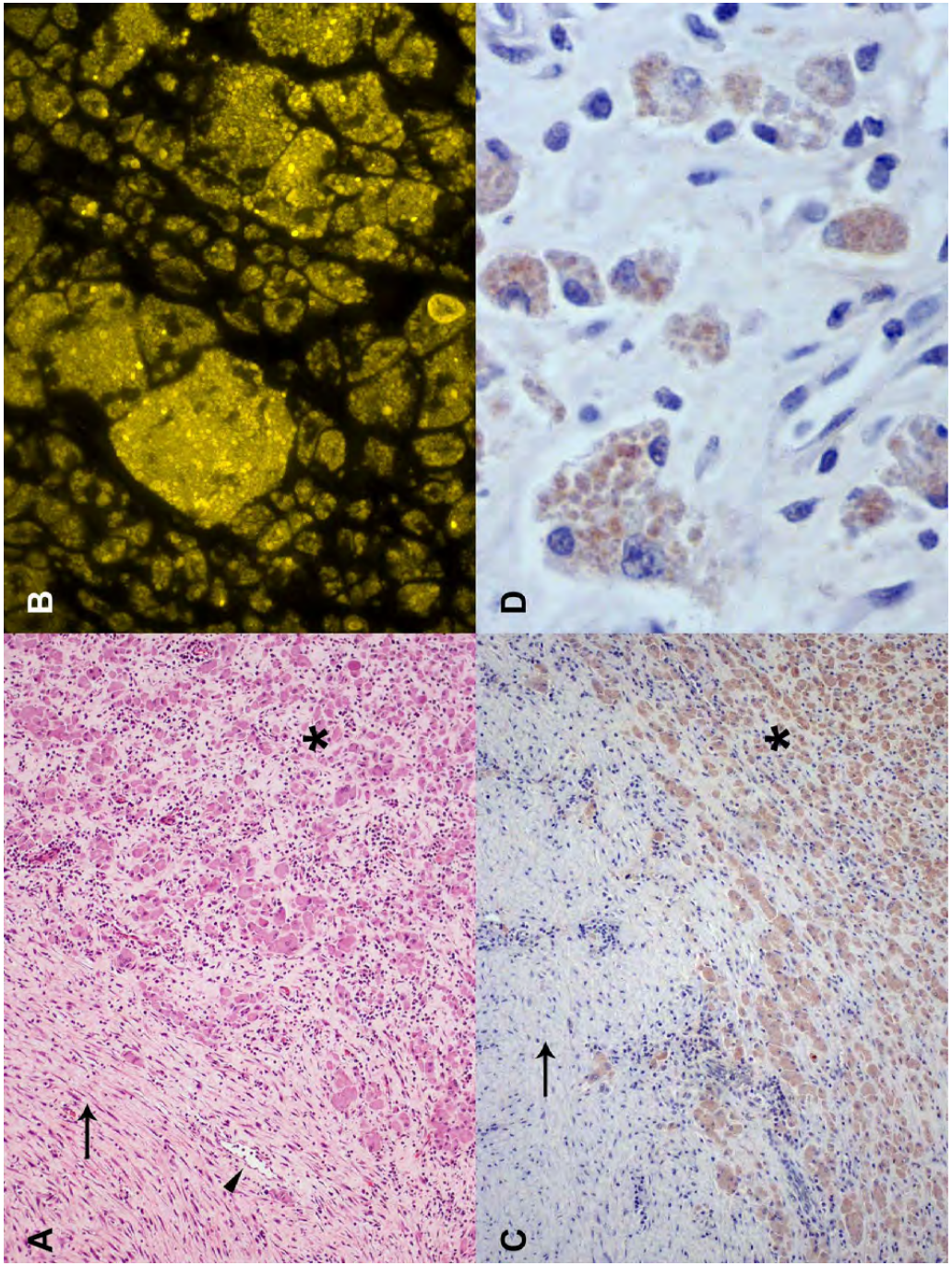
595

A



B

Vaccine number	Commercial name	Antigen/s	Inoculation date	mg of Al per dose
1	Bluevac-1*	Bluetongue virus serotype 1	0 dpi & 21 dpi	4.18
2	Bluevac-BTV8*	Bluetongue virus serotype 8	0 dpi & 21 dpi	4.4
3	Heptavac P Plus*	*Pasteurella multocida* *Mannheimia haemolytica* *Clostridium spp.*	42 dpi & 63 dpi	7.5
4	Ovivac CS*	*Chlamydia abortus* *Salmonella abortus ovis*	42 dpi & 63 dpi	5.6



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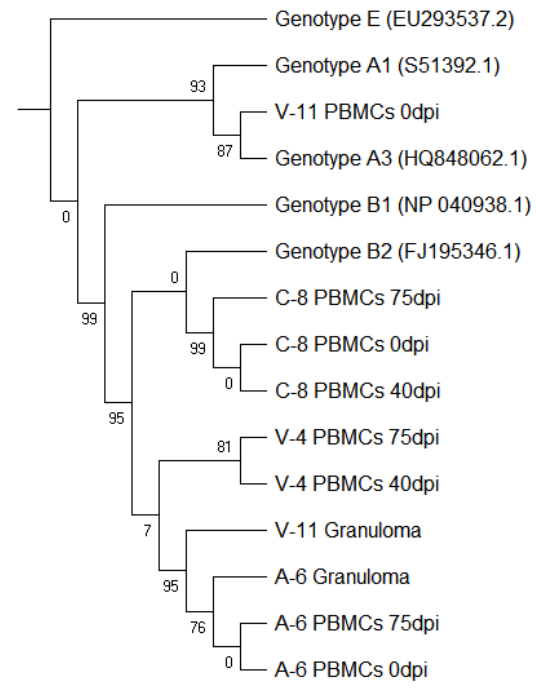

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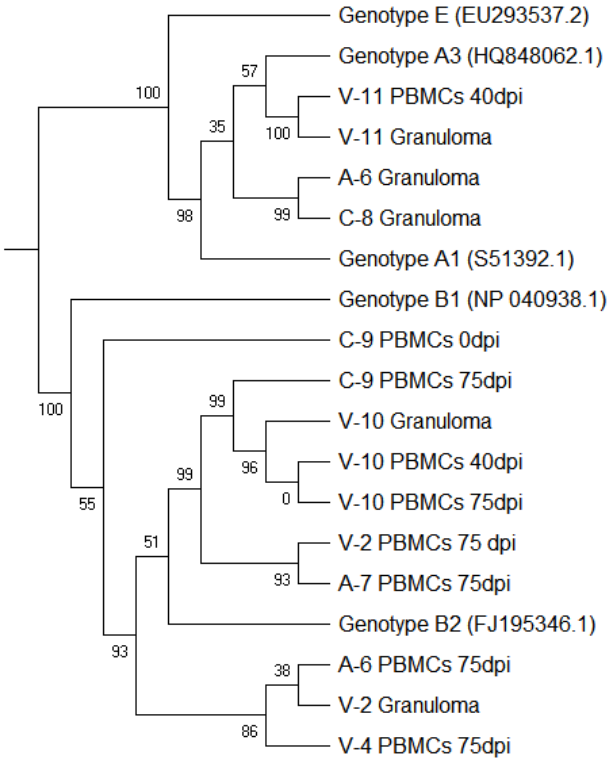

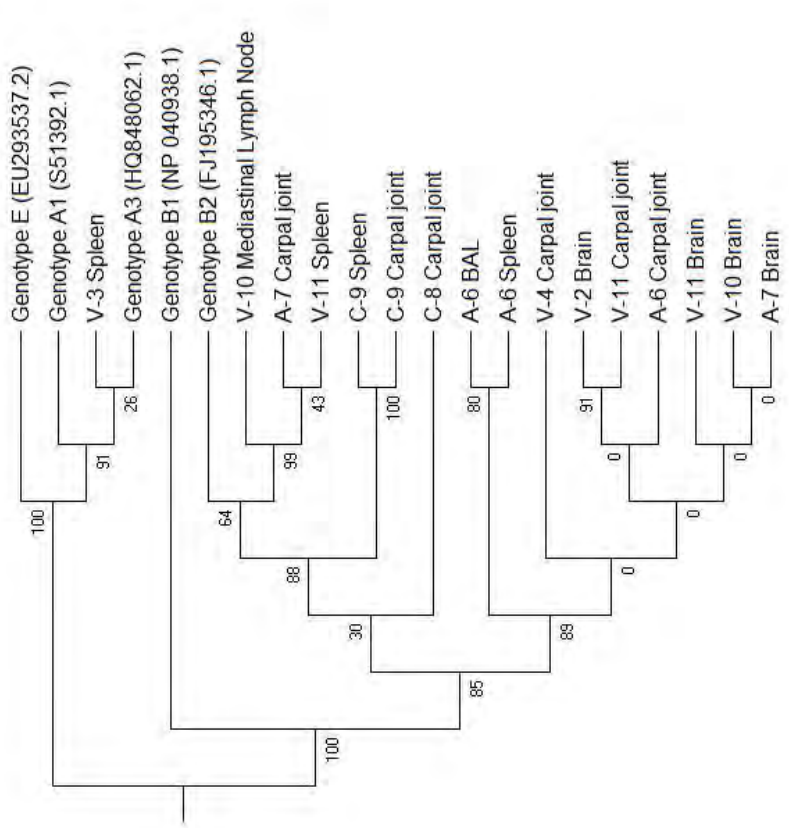

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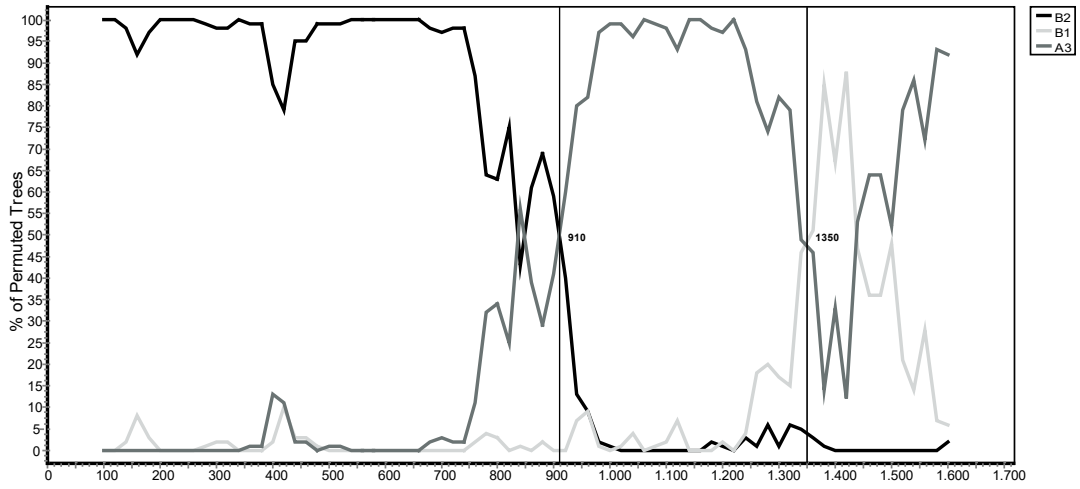

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