Low proviral Small Ruminant Lentivirus load as biomarker of natural restriction in goats

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Highlights

- Seropositive goats carrying high or low proviral load have been found within the same flock.
- Cells from low proviral load animals show a resistance to productive infection in vitro.
- Restriction found in low proviral carriers maybe related to the presence of restriction factors.
- Prophylactic measures based on alternative tools may improve SRLV control.

Abstract

Small ruminant lentiviruses (SRLV) globally affect welfare and production of sheep and goats and are mainly controlled through elimination of infected animals, independently of the viral kinetics within the single animal. Control programs are based on highly sensitive serological tests, however the existence of low antibody responders leads to the permanent presence of seronegative infected animals in the flock, thus perpetuating the infection. On the other hand, long-term non-progressors show a detectable antibody response not indicative of a shedding animal, suggesting immune contention of infection. In this study, we analyse two goat populations within the same herd, harbouring low or high proviral SRLV loads respectively, both showing a robust antibody response. *In vivo* findings were confirmed *in vitro* since fibroblastic cell lines obtained from one high and one low proviral load representative goats, showed respectively a high and a faint production of virus upon infection with reference and field circulating SRLV strains. Differences in virus production were relieved when strain CAEV-Co was used for experimental infection. We analysed LTR promoter activity, proviral load, entry step and production of virus and viral proteins. Intriguingly, proteasomal activity was higher in fibroblasts from low proviral load animals and proteasome inhibition increased viral production in both cell lines, suggesting the implication of active proteasome-dependent restriction factors. Among them, we analysed relative expression and sequences of TRIM5α, APOBEC3 (Z1, Z2, Z3 and Z2-Z3) and BST-2 (Tetherin) and found a global antiviral status in low proviral carriers that may confer protection against viral shedding and disease onset.

Keywords: Small ruminant lentivirus; Proviral load; Long-term non-progressors; TRIM5α; APOBEC3; Tetherin.

INTRODUCTION

SRLV are a heterogeneous group of virus that infect sheep and goats all over the world mainly increasing flock replacement rates and affecting animal health and production. Five genotypes (A-E) have been described so far, including the widely distributed genotype A, prototype of Visna/maedi viruses, and the genotype B including CAEV-like viruses. The remaining genotypes are so far restricted to specific geographic areas.

Transgression of species-specific barrier has been documented in different countries generalizing the idea that SRLV infect sheep and goats (Leroux et al., 2010). An early study in this regard describes the infection of calves with a SRLV strain showing an induced a sustained antibody response, suggesting certain level of virus replication. However, viral isolation was not possible even with different attempts to reactivate viral replication (Morin et al., 2003). Ovine cells from choroid plexus have also shown SRLV low replication likely due to impaired ENV protein production (Chebloune et al., 1996). Intrinsic factors of the innate immune response are responsible of facing heterologous lentiviral infections, thus blocking inter-species transmission and zoonosis. The basic axis of this antiviral pathway is formed by TRIM5, APOBEC3 and BST-2 or tetherin (Malim and Bieniasz, 2012).

Most control programmes against SRLV are based on the identification of seropositive animals and their elimination. Nevertheless, variation in the serological response above and below the detection limit within a single animal and at the flock level could be expected, harming serological diagnosis. New approaches in diagnostic tools include PCR that is able to detect seronegative animals as well as to quantify proviral load. In spite of the control programs implemented in different countries, seroconversions are often described and a clear diagnostic escape is likely occurring mainly when heterologous genotype is involved (Cardinaux et al., 2013; Lacerenza et al., 2006; Nagel-Alne et al., 2014). A direct association between proviral load and the antibody titre could be expected since the higher infected cells the more viral antigen is exposed to the immune system. However, experimental data do not seem to support such an association likely due to the latency exerted by lentiviruses in which low antigen levels

are exposed to the immune system. Several studies have also linked higher proviral load with the presence of clinical signs (Herrmann-Hoesing et al., 2009; Ravazzolo et al., 2006).

In this work we analyse proviral load in a highly infected flock and found a clear division between consistently seropositive animals carrying high proviral load from those with low proviral load. SRLV was easily isolated from high proviral load (HPL) animals whereas low proviral load (LPL) animals did not shed virus. Skin fibroblasts obtained from animals with high and low proviral load infected *in vitro* with SRLV, showed different infection kinetics suggesting the presence of inhibitory factors in LPL animals. A detailed genetic study on main restriction factors against lentiviruses was carried out.

MATERIAL AND METHODS

Animals

Eighty seropositive alpine breed goats were selected from a flock with 100% of seroprevalence, verified by an indirect ELISA based on recombinant matrix-capsid antigen fusion protein from genotypes A and B as described (Lacerenza et al., 2006; Rosati et al., 2004) and by endpoint nested-PCR targeting the *gag* gene (Grego et al., 2007) covering immunodominant epitopes, nucleotides 1070 to 1801 of the CAEV-Cork reference sequence (Genbank accession number M33677). We used home-made ELISA. In each well antigens belonging to A (MVV-like) and B (CAEV-like) antigens were included. In order to be sure about the viral genotype, we also applied a genotyping assay, testing the different antigens in different wells. All test showed a clear reactivity against B (CAEV-like) antigen. To assess the clonal origin of herd–associated SRLV, a number of PCR positive samples were directly sequenced. Chromatograms were manually checked, corrected and sequences were aligned using Clustal X (Jeanmougin et al., 1998). The alignment, including reference sequences, was used to identify the best evolutionary model (ModelTest, (Posada and Crandall, 1998) and to draw a phylogenetic tree using Bayesian approaches (MrBayes). Viral genotype was assigned

comparing obtained nucleotide and deduced amino acid sequences with SRLV of reference, and the clonal origin was investigated evaluating the genetic heterogeneity among samples as well as the tree topology.

Cells and viruses

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation (1.077) from whole blood of selected animals in the basis of the proviral load into high (HPL) and low (LPL) proviral load animals.

Blood derived macrophages (BDM) were obtained from animals 2453H and 1000L by adherence from PBMCs isolated by Ficoll centrifugation. PBMCs were incubated for 2h in 24-well plates, washed three times with PBS and grown in RPMI 1640 complete medium, containing 10% foetal goat serum, 10mM sodium pyruvate, 1% non-essential amino acids, 1% vitamins, 1% antibiotics/antimycotics mix, 1:1000 gentamicin, 1% L-glutamine and 50 μM 2-mercaptoethanol (Sigma) until used after 9 to 12 days as described (Juganaru et al., 2011).

Skin fibroblasts cultures (SF) were established from skin biopsies of selected seropositive animals with high and low proviral load, namely SF7583H and SF1000L, respectively. Cells were maintained in DMEM medium (GIBCO) supplemented with 10% foetal bovine serum (FBS), 2% L-Glutamine, 1% antibiotics/antimycotics solution and 1:1000 gentamicin (Sigma).

Synovial membrane cells (GSM) were established from tissue explantation of SRLV-free caprine foetus obtained by caesarean method. Cells were grown in DMEM medium supplemented with 10% FBS, 2% L-Glutamine, 1% antibiotics/antimycotics solution and 1:1000 gentamicin (Sigma).

Human embryonic kidney (HEK 293-T) cells were cultured in DMEM supplemented with 10% FBS, 1% L-Glutamine, 1% antibiotics/antimicotics and 1:1000 gentamicin (Sigma).

SRLV field strain 2423H was isolated by co-culture of BDM of an HPL infected animal with synovial membrane cells. Virus was titrated onto GSM cells by the Reed-Müench method, as described previously (Reed and Muench, 1938).

Reference strain CAEV-Co belonging to the genotype B1 (Saltarelli et al., 1990) was used for *in vitro* infection experiments as specified in the single experiment.

LTR from strain CAEV-To 1/89 (Grego et al., 2002) was employed as reference in the LTR promoter activity assay.

Proviral load quantification

DNA was extracted from PBMCs and skin fibroblasts using DNA Blood Mini Kit (Qiagen) and quantified by spectrophotometry (Nanodrop). Real time PCRs were performed in an Applied Biosystems 7500 sequence detector system, with primers and probes specifically designed for the circulating CAEV strains (qGAG_Fw: GGAGCATTAACAGTGGATCAAATTATG, qGAG_Rv: TGATCTTAATGCTGATATTACCCATTG, probe: 6FAM-CACAAGCTAACATGGATCAGGCAAGACAAATATGCCTA-TAM). Ten-fold serial dilutions of a plasmid (pDRIVE, Qiagen) containing the *gag* region were prepared to generate a standard curve (Cycle threshold vs copy number) from which copy number values were extrapolated. Plasmid copy number ranged from 10^6 to 1. Results were expressed as provirus copy number/ 50 ng of DNA.

RT activity

Cell lines (SF7583H and SF1000L) were infected with 1 $TCID_{50}$/cell of the CAEV-Cork or the field strain isolated from the studied herd (2423H), and retrotranscriptase activity was measured in clarified supernatants at different time points (0,4,7,9,11 and 14 days post-infection) with HS-Lenti RT activity kit (Cavidi) following manufacturer's instructions.

LTR promoter activity

A reporter gene construction containing the U3 region of genotype B encompassing the whole transcription factors binding sites, and the chloramphenicol acetyltransferase (CAT) gene has been previously generated and named U3BpCAT (Juganaru et al., 2011). Briefly, LTR region of the CAEV-To 1/89 strain (Grego et al., 2002) was amplified by PCR and cloned into pCAT-Basic vector (Promega). Empty pCATBasic (lacking promoter region) and pCATControl (with the SV40 promoter) were used as negative and positive controls respectively.

These constructions were used for transfection of SF with 0.25 μg of DNA per well using Jet Prime (Polyplus) at a ratio of 1:2 (μg DNA: μl jet Prime reagent). Cells were lysed 48 h after transfection and promoter activity in SF cells was assessed by quantification of CAT production by a CAT ELISA kit (Roche), following manufacturer protocol. The promoter expression levels were compared as the percentage of O.D. value (405 nm) compared to SV40 promoter activity of pCAT control and normalized to the basal activity to pCAT Basic values

Antibodies and Immunostaining

Skin fibroblasts plated in 24 well-plates were infected with CAEV-Co at a MOI of 1. Cells were washed with PBS and fixed with methanol-acetone (1:1) for 10 minutes 25 days post-infection. Following 3xPBS washes, 1h incubation with the diluted primary antibody was carried out. Three anti-p25CA monoclonal antibodies (5A1, 10A1 and 8B1; VMRD) were diluted 1:1000 in PBS with 1.25% casein. After 4 washes (PBS with Casein 1.25%), cells were incubated with HRP-conjugated goat anti-mouse antibody (1:1000) for 1h. Immunostaining was visualized under optical microscopy adding Diaminobenzidine (DAB)-H_20_2.

Production of pseudotyped-virus and entry assays

Pseudotyped virions were generated by co-transfection of HEK 293-T cells with two types of plasmids as described previously (Juganaru et al., 2011): the pCAEV-AP

plasmid (encoding alkaline phosphatase and the CAEV backbone), kindly provided by Dr. Isidro Hötzel (Hotzel and Cheevers, 2002); and the pCMV plasmid containing the envelope gene (pCMV-*env*) of CAEV-Co strain (Crespo et al., 2013). HEK 293-T cell culture supernatants containing the pseudotyped viruses were collected 48 h post-transfection, clarified and used in 10-fold dilutions to infect skin fibroblastic cell lines, SF7583H and SF1000L. After 48h, cells were stained using the alkaline phosphate substrate BCIP/NBT staining protocol (Miller Lab [http://labs.fhcrc.org/miller/index.html]) and the results were expressed as focus-forming units per ml (FFU/ml). Experiments were repeated twice.

Proteasome activity assays

Proteasomal chymotrypsin-like activity was assessed in cell extracts by monitoring the production of AMC from the fluorogenic peptide Suc-LLVY-AMC
(Bachem, Bubendorf, Switzerland), as described (Cerruti et al., 2007). Briefly, cells were incubated in ice-cold extraction buffer (50 mM Tris–HCl pH 7.5, 1 mM dithiothreitol, 250 mM sucrose, 5 mM $MgCl_2$, 0.5 mM EDTA, and 2 mM ATP, 0.025% digitonin) for 5 minutes and centrifuged at 15,000 rpm (20,000g) for 30 minutes at 4°C. The protein concentration in supernatants was determined using the QUICK START Bradford Dye Reagent 1x (Bio-Rad Laboratories, Hercules, CA) using a standard curve constructed with bovine serum albumin (BSA). Suc-LLVY-AMC was used at a final concentration of 100 μM in 20 mM Tris-HCl, pH 7.5, 1 mM ATP, 2 mM MgCl2, and 0.2% BSA. Reactions were started by adding an aliquot of cellular extract, and the fluorescence of released AMC (excitation, 380 nm; emission, 460 nm) was monitored continuously at 37°C with a Cary Eclipse spectrofluorometer (Varian Medical Systems, Palo Alto, CA, USA). Background activity (caused by non-proteasomal degradation) was determined by addition of 2 μM epoxomicin. Assays were calibrated using standard solutions of the free fluorophore, and the reaction velocities were calculated from the slopes of the initial

linear portions of the curves. Substrate consumption at the end of incubation never exceeded 1%.

Proteasome inhibition assays (post-entry and late restriction)

For the evaluation of the post-entry restriction activity of proteasome-dependent antiretroviral intrinsic factors, 2.5 $x10^5$ SF cells were seeded per well in 24 well plates and treated for 1 h with a cocktail of irreversible proteasome inhibitors (PI) (Kisselev and Goldberg, 2001) (5μM of Velcade, Bortezomib or PS-341, 5 μM of β-lactone, and 2 μM of epoxomicin final concentration in the well) that was previously reported to effectively block proteins degradation in human cell lines (Cenci et al., 2012). Cells were then infected with 1 MOI of the isolated strain 2423H and proviral DNA was measured 16 h after infection by qPCR as described above. Data represent provirus copy numbers (mean ± standard error) per 50 ng of total DNA. Three independent experiments were performed.

Proteasome inhibition assays were also carried out to evaluate a possible late restriction activity involving proteasome. SF cells $2.5x10^5$ per well were seeded in 24 well plates and infected with 1 MOI of SRLV strain 2423H. Cells were washed 2 h post infection and cultures replaced with fresh medium. PI cocktail was added at different time points post-infection (48, 72 and 96h) and both cell lines and clarified supernatants collected 16h after PI treatment at each time point. Viral DNA in cultured cells and RT activity in supernatants were measured by qPCR and RT activity assay respectively as described above.

Expression of innate immune restriction factors

RNA was extracted from SF cell cultures using QIAamp Viral RNA Mini Kit (Qiagen) and cDNA was obtained with QuantiTect-Reverse-Transcription Kit (Qiagen) following manufacturer´s protocol. Expression of the different restriction factors was assessed by

RT-PCR amplification in an ABI Prism 7900 Sequence Detector (Applied Biosystems, Foster City, CA) using SybrGreen Master Mix (Takara).

For TRIM5α specific primers forward (qPCR3T5Fw 5' TTCCTAGACTATGAGGCTTGCTCTGT 3') and reverse (qPCR3T5Rv 5' TTCTGAGGAAAGGAACATGAAGAGA 3'), designed within the PRYSPRY region were used as described (Jauregui et al., 2012). Primers qPCRT5Fw 5´GTTGGCTGTGTGAGCGATCTC and qPCRT5Rv 5´ TTTCTGGTACTGTGG TGCATTCTC were used to amplify RING-B-BOX (Sakuma et al., 2010).

APOBEC3 genes (A3Z1, A3Z2, A3Z2Z3, A3Z3) and Tetherin expression was quantified using primers previously described (Crespo et al., 2013; Sanjose et al., 2016).

Endogenous β-actin expression was measured as housekeeping gene. β-actin Ct values were subtracted from Ct values of each restriction factor, in order to obtain the $2^{(-\Delta Ct)}$ used for comparisons.

Cloning and sequencing

TRIM5α and Tetherin PCR products were run in 1.5% agarose gels, purified with StrataPrep PCR purification kit (Agilent Technologies) and cloned into pJET1.2/blunt vector (Thermo Scientific). Electrocompetent XL1-Blue cells were transformed and grown overnight in LB agar plates supplemented with ampicillin (100 ng/mL). Positive clones were sequenced (Secugen, Madrid, Spain) and sequences analysed using BLAST and Bioedit.

Statistical analyses

To compare average measures between both cells lines Mann-Whitney tests were adopted. For comparison within cell types Wilcoxon tests were performed.

RESULTS

Animal classification

All selected animals (n=80) were seropositive by ELISA and positive to PCR confirming the infection status. Eighteen PCR positive reactions were sequenced and aligned with reference SRLV genotype/subtypes revealing a cluster of clonal sequence belonging to genotype B, subtype B1 (Fig. 1). The alignment strongly supported the common origin of viral sequences in samples, given the very high similarity among sequenced viruses, considering both nucleotide and amino acid sequences (98.91% and 99.38% respectively). Moreover, similarity was absolute along the immunodominant regions. Proviral load values obtained by real time PCR showed a highly skewed distribution, reporting the presence of a small number of animals with a very high proviral load. Based on median values distribution (268 copies/50ng), animals were classified into high proviral load (mean proviral load: 835.80 copies/50ng) or low proviral load (mean proviral load: 96.54 copies/ 50ng) with almost 1 log of difference between the two groups (Wilcoxon rank sum test $p<0.001$, Fig. 2). Skin fibroblastic cell lines were obtained from skin biopsies of one high and one low proviral load animals, named SF7583H and SF1000L, respectively.

Virus isolation

Virus isolation by co-culture with synovial membrane cells was achieved in 5 days when using BDM from HPL animals and a representative strain, namely 2423H grouped into the genotype B1, was used in infection experiments as well as reference strain CAEV-Co. However, virus isolation from the LPL animals failed, obtaining negative results in the RT activity and the absence of syncytia in marker cells (synovial membrane cells). On the other hand, our attempts to obtain non-infected BDM cultures from high proviral load animals were unsuccessful, hampering *ex-vivo* study on macrophages.

In vitro infection

Skin fibroblasts permissiveness to SRLV infection from HPL and LPL individuals was evaluated by RT activity, LTR promoter activity, immunocytochemistry, cytopathic effect

assessment and entry assay determinations after infection with field (herd specific) 2423H and reference CAEV-Co strains. RT activity values (Fig. 3) showed a high and rapid viral growth curve in SF7583H while virus production was almost null in SF1000L, suggesting some kind of cellular restriction against viral production in SF1000L, which does not occur in SF7583H. While this restriction was evident in the case of strain 2423H, differences were smaller when infecting with CAEV-Co strain. However, syncytia formation, the typical SRLV cytopathic effect, was evident in both viruses and cell lines being syncytia smaller and containing fewer nuclei in the SF1000L (data not shown).

Presence of transcription factors in the fibroblastic cell lines

Since a reduced permissiveness to infection of the SF1000L could be explained by a deficient production of transcriptional factors in these cells, an ELISA CAT was performed using LTR promoter constructions (U3BCAT and controls) aiming at quantifying LTR transcriptional activity in the two cell lines under study (Fig. 4). High levels of CAT production were found in both cell lines when using the construction including LTR from CAEV-To 1/89, being even higher than the observed with the construction bearing the SV40 promoter region. Data revealed no statistical differences between the promoter activities of the two cell lines (U-Mann-Whitney test, $p=0.234$).

Viral protein production

To evaluate the production of viral proteins, an immunocytochemistry assay using serum raised against viral p25 was performed (Fig 5). Twenty five days post infection with strain 2423H and CAEV-Co, staining was clearly positive in SF7583-H while very low in SF1000-L indicating a poor production of the capsid protein. As mentioned, cytopathic effect was observed in both cell lines, which pointed out the suspicion of envelope protein production in both cell cultures. Staining with anti-Env antibodies from a commercial ELISA kit always showed negative results suggesting low specific recognition of both the circulating 2423H and CAEV-Co strains.

Entry assay

Entry assays carried out with CAEV virions pseudotyped with the CAEV-Co envelope protein confirmed a reduced permissiveness to infection in SF1000L (5×10^2 FFU/ml) cell line compared with SF7583H (1.2×10^3 FFU/ml) which points for a possible entry restriction in the SF1000L cells (Two tailed - Wilcoxon test $p=0.09$).

Proteasome inhibition

Most of restriction factors of the innate immune response involve the proteolytic ubiquitin-proteasome system in their restriction pathway, therefore, proteasome inhibition is expected to rescue infection. In this regard, of interest is the observation that basal proteasomal activity is higher in fibroblasts from LPL than from HPL animals (Fig.6; Mann-Whitney $p<0.05$), which might indicate a possible role of proteasome-dependent restriction factors in the low proviral load goat.

To investigate more in detail proteasome implication, Proteasome inhibition assays were performed evaluating both post-entry and late restriction activities. Preliminary *in vitro* experiments showed the effectiveness of the proteasome inhibitors cocktail used in completely blunting proteasomal activity following its *in vivo* administration to both fibroblast cell lines (Fig. 6), in accord with previous results in human lines (Cenci et al., 2012).

To evaluate proteasome involvement on post-entry restriction, cells were treated with the PI cocktail previous to infection, and then infected as routinely, showing an increased proviral load determined by qPCR compared to non-PI treated in SF7583H (Wilcoxon $p=0.001$) but not SF1000L cells ($p= 0.22$) (Fig.7a).

Analysis of the proteasome involvement in late restriction to infection, i.e., infecting before blocking proteasome activity showed a different profile in the two cell lines (Fig. 7b). SF7583H infected and treated with PI cocktail, showed an increased proviral load by five-fold when compared with non-treated cells at 48 and 72h post infection.

On the other hand, SF1000L cultures treated with PI 72h post infection showed an increase of ten-fold in comparison with non-treated cells. This increase was not observed at 48 (Fig.7b).

Differences found at the proviral load level were roughly confirmed at the viral production level. RT activity determinations early after infection showed as expected, absorbance values above 0.7 in SF7583H and lower than 0.3 in SF1000L. Although non-significant, differences tend to be higher in SF7583H cells (Fig.7c).

Trim5α

Increased proviral load and RT activity after proteasome inhibition could be the result of suppressing the TRIM5α-driven proteasomal degradation (Chan et al., 2012). Therefore we first evaluated the TRIM5 mRNA relative expression levels in both cell lines but no statistical differences were detected (p=0.276). Primers were designed within PRYSPRY domain in order to quantify TRIM5α. Additionally, in an attempt to further discriminate expression levels, we designed primers within the RING-BBox2 domain, present in other TRIM5 proteins. Expression was in this case 38.73 times higher in SF1000L cells than in SF7583H.

TRIM5α complete gene was sequenced in both cell lines in order to evaluate the presence of polymorphisms potentially responsible for a different antiretroviral activity in SF1000L. Sequences from SF7583H showed a Q189K mutation compared with SF1000L sequences (Fig. 8).

APOBEC3 expression

Primers designed for ovine and caprine APOBEC3 genes (A3Z1, Z2, Z3 and Z2-Z3) successfully amplified A3Z2, A3Z3 and A3Z2-Z3 mRNA in both cell lines but AZ1 expression was exclusively detected in the SF1000L cell line. In the SF7583H cell line the expression profile showed abundant expression of A3Z2Z3 (ΔCt=6.51) and A3Z2 (ΔCt=4.95), a faint expression of A3Z3 (ΔCt=15.77) and negative for the A3Z1 expression (ΔCt=25.61).

Comparatively, the only difference found between both cell lines was in A3Z1 expression being more than 3000-times higher in the SF1000L cells (2-ΔΔCt=3456,3; Fig. 9).

Tetherin

Expression of tetherin was also evaluated since the presence of syncytia in the absence of viral production stands for tetherin activity. Expression was detected in both cell lines, however SF1000L showed a 5.3-fold increased expression compared to SF7583H. Amplicons were cloned and sequenced, showing identical sequences in both cell lines.

DISCUSSION

This work explored in age-matched animals the intrinsic restriction of the two extremes of a continuum lentiviral restriction exerted by innate mechanisms in two cell lines from animals with a high or a low proviral load as a "proof of concept".

After initial SRLV infection, latently infected circulating monocytes differentiate into tissue macrophages and modulate their transcription profile to express viral proteins that lead to inflammation, chemotaxis, infiltration and development of associated signs (Cordier et al., 1990). Generally, higher viral loads favour disease development (Herrmann-Hoesing et al., 2009) arguing for a potential restriction in low proviral load carriers also referred as long term non-progressors (Stonos et al., 2014). These non-progressors show a competent serological response in the absence of viral replication likely reducing virus shedding, and finally not representing a real danger for virus dissemination within the flock. However, current control programs based on serological diagnosis and culling of seropositive animals do not account for long-term non-progressors (Cardinaux et al., 2013; Ritchie and Hosie, 2014).

This study provides new insights into SRLV epidemiology by identifying high (HPL) and low proviral load (LPL) individuals within the same herd infected by closely related SRLV strains and provide an underlying mechanism of restriction. Differences in proviral load were not accompanied by differences in humoral response, however could account for

an effective pathway of SRLV restriction. A viral strain was isolated from the circulating viral population in the herd, and *in vitro* studies conducted using skin fibroblasts from a HPL (SF7583H) and a LPL (SF1000L) animal respectively. These two cell lines showed opposite phenotypes when infected *in vitro* with field and also laboratory SRLV strains. SRLV isolation was extremely fast from BDM of the HPL subject, and no isolation was attained using BDMs from LPL. While fibroblasts from the high proviral load goat (SF7583H) produced high amounts of virus accompanied by the presence of syncytia, the cell line obtained from the low proviral load animal (SF1000L) showed modest cellular fusion in the absence of virus production (at least under the RT activity assay detection limit). Thus, *in vitro* infection confirmed *ex vivo* findings being replication highly inhibited in the LPL animal. Interestingly, differences were smaller when infecting with CAEV-Co strain that encodes slightly divergent CA and Env proteins (Gudmundsson et al., 2005; Haflidadottir et al., 2008), likely escaping from the restriction exerted by SF1000L. Primate lentiviruses in which punctual mutations have been introduced in host-interacting proteins such as Gag or Env, have demonstrated escape from the specific binding of restriction factors with specific viral determinants (Veillette et al., 2013).

LTR promoter activity of the isolated strain was similar to the promoter activity exerted by prototypic strains in both fibroblastic cells, thereby excluding the absence of transcription factors from the inhibitory pathway. Thus, transcription factors are able to bind the viral LTR promoter region and initiate the transcription of viral proteins in both cell lines at a similar level. Entry assays indicated a reduced ability (by half) of SRLV virions pseudotyped with CAEV-Co ENV protein to enter into SF1000L fibroblastic cells that could partially explain the reduced proviral load found. Higher differences may have been expected using CAEV-AP virions pseudotyped with field strain ENV, but unfortunately *env* cloning is often accompanied by bacterial toxicity and was not successful (McDonald and Burnett, 2005). Polymorphisms in the cellular receptor/s of SRLV could reduce virus internalization and consequently viral load, as it occurs in

humans with a deletion in the CCR5 receptor of HIV-1 (Larruskain et al., 2013; Naif, 2013). In this regard, polymorphisms in the CCR5 co-receptor associated with reduced susceptibility to SRLV have been described (Larruskain et al., 2013). Recently, a new transmembrane protein TMEM154 with yet unknown function has been proposed as a marker for breeding selection of SRLV resistant sheep (Alshanbari et al., 2014). Whether TMEM154 could be proposed as a SRLV receptor has not been explored. In spite of this apparent restriction due to the reduced entry in cells from LPL animals, this difference does not explain by itself the huge difference in terms of proviral load and viral production.

According to our results, SRLV seropositive animals within the same flock may display totally different susceptibility to viral infection. These observations may also be taken into account when foetus cell lines are established for SRLV *in vitro* studies, since permissiveness of cell lines may be influenced by certain genetic background. Obviously, this indicates the importance of both the genetic background and the immune response elicited upon infection. In this latter point it is worthy of note the latest investigations regarding restriction factors such as TRIM5, APOBEC3, Tetherin or SAMHD1 (Malim and Bieniasz, 2012; Simon et al., 2015). Most of them use proteasomal degradation after docking to viral proteins, finally leading to reduced infectivity. In this regard, the observation that basal proteasomal activity is higher in fibroblasts from LPL than from HPL animals is certainly interesting since it suggests a possible role of proteasome-dependent restriction factors in the low proviral load animal analysed. Further studies analysing a greater number of animals will be required to verify this interesting hypothesis. Moreover, of great interest are our results clearly demonstrating an advantage for viral replication in those cells treated with a proteasome inhibitor cocktail (PI). Although this finding might simply rely on inhibition of the normal turnover of viral proteins, this possibility seems unlikely since most of viral proteins are metabolically stable and inherently resistant to proteasome degradation (Anton and Yewdell, 2014; Qian et al., 2006; Schild and Rammensee, 2000; Yewdell et al., 2001). More likely, on

the contrary, our data suggest the activity of restriction pathways involving proteasome degradation.

Although preliminary, due to the limited number of samples analysed, the observation that differences in viral production were smaller when using CAEV-Co strain for *in vitro* infection of the two cells lines obtained may suggest the co-evolution of the circulating virus together with host factors. In this regard, CA antigen targeted by TRIM5α, allows certain degree of variability while conserving function. Differences were found in relative expression when evaluating RINGB-BOX while no difference in expression levels were observed among the fibroblastic cells obtained in PRYSPRY targeting PCR. This could argue for the presence of truncated proteins lacking PRYSPRY domain or alternatively the presence of other TRIM5 different from TRIM5α. A mutation in position 189 within the coiled-coil domain was found in the more permissive cell line (SF7583H) compared with SF1000L fibroblasts and with related TRIM5 sequences deposited in GenBank. Whether this mutation abrogates TRIM5α function despite its effective expression is unknown. A single mutation at position 333 within PRYSPRY region (supposedly in contact with p25) between Ov1 and Ov2 was likely responsible for the loss of antiviral activity (Jauregui et al., 2012).

APOBEC3 activity could alter both proviral load as well as virus production since uracil-containing viral cDNA would accumulate deleterious mutations (Willems and Gillet, 2015). So far, restrictive properties of APOBECZ2-Z3 and A3Z1 have been described against lentiviruses (Jonsson et al., 2006; LaRue et al., 2008). APOBEC3Z2, Z3 and Z2-Z3 were expressed at similar levels in both cell lines or even less in SF1000L (A3Z2). Skin fibroblasts do not normally express A3Z1 (Glaria I., personal communication) and this was confirmed in the case of SF7583H cells but a significant expression was found in SF1000L, potentially contributing to SRLV restriction. APOBECZ1 expression is important in monocyte restriction to different lentiviruses that is relieved in differentiated macrophages (Peng et al., 2007)(Glaria et al., personal communication). APOBEC3 is excluded from virions in the presence of a Vif protein that was present in the field as well

as in the reference SRLV strains. Therefore and taking into account cytidine deaminase independent mechanism of lentiviral restriction, we did not analyse proviral sequences looking for mutated genomes.

Finally, *in vitro* infection experiments fitted well with the presence of a restrictive tetherin protein in SF1000L. The presence of syncytia indicates a fully functional ENV protein and the absence of RT activity in the supernatants strongly suggest the presence of trapped virions. In addition, capsid protein staining in SF1000L could indicate the presence of intracellular immature virions. Tetherin expression was higher in SF1000L compared with SF7583H cell line and sequences were identical indicating that higher expression favours such restriction. Tetherin is counteracted by Vpu encoded by HIV-1, Nef encoded by SIV or Env protein encoded by HIV-2 (Weinelt and Neil, 2014). Indeed, Vpu antagonism of tetherin protects infected cells from antibody-dependent cell-mediated cytotoxicity (Arias et al., 2014). Whether SRLV or other animals lentivirus encode a Vpu-like protein with the ability of counteracting tetherin is currently unknown. Interestingly, and taking into account findings in the HIV field, such a protein would contain transmembrane domains to interact with tetherin's helix and Rtm protein (Valas et al., 2008) besides Env are the most promising candidates to accomplish this function.

CONCLUSION

The involvement of the innate immune response in the natural control of SRLV infection in goats may help to maintain a low proviral load, limiting viral spread and consequently avoiding disease progression, potentially improving control programmes for endangered breeds or with genetic value.

Furthermore, the identification of restriction factors in small ruminants could generate alternative tools in the control of SRLV epidemics.

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References

Alshanbari, F.A., Mousel, M.R., Reynolds, J.O., Herrmann-Hoesing, L.M., Highland, M.A., Lewis, G.S., White, S.N., 2014. Mutations in Ovis aries TMEM154 are associated with lower small ruminant lentivirus proviral concentration in one sheep flock. Animal genetics 45, 565-571.

Anton, L.C., Yewdell, J.W., 2014. Translating DRiPs: MHC class I immunosurveillance of pathogens and tumors. Journal of leukocyte biology 95, 551-562.

Arias, J.F., Heyer, L.N., von Bredow, B., Weisgrau, K.L., Moldt, B., Burton, D.R., Rakasz, E.G., Evans, D.T., 2014. Tetherin antagonism by Vpu protects HIV-infected cells from antibody-dependent cell-mediated cytotoxicity. Proceedings of the National Academy of Sciences of the United States of America 111, 6425-6430.

Cardinaux, L., Zahno, M.L., Deubelbeiss, M., Zanoni, R., Vogt, H.R., Bertoni, G., 2013. Virological and phylogenetic characterization of attenuated small ruminant lentivirus isolates eluding efficient serological detection. Veterinary microbiology 162, 572-581.

Cenci, S., Oliva, L., Cerruti, F., Milan, E., Bianchi, G., Raule, M., Mezghrani, A., Pasqualetto, E., Sitia, R., Cascio, P., 2012. Pivotal Advance: Protein synthesis modulates responsiveness of differentiating and malignant plasma cells to proteasome inhibitors. Journal of leukocyte biology 92, 921-931.

Cerruti, F., Martano, M., Petterino, C., Bollo, E., Morello, E., Bruno, R., Buracco, P., Cascio, P., 2007. Enhanced expression of interferon-gamma-induced antigen-processing machinery components in a spontaneously occurring cancer. Neoplasia 9, 960-969.

Cordier, G., Cozon, G., Greenland, T., Rocher, F., Guiguen, F., Guerret, S., Brune, J., Mornex, J.F., 1990. In vivo activation of alveolar macrophages in ovine lentivirus infection. Clinical immunology and immunopathology 55, 355-367.

Crespo, H., Bertolotti, L., Juganaru, M., Glaria, I., de Andres, D., Amorena, B., Rosati, S., Reina, R., 2013. Small ruminant macrophage polarization may play a pivotal role on lentiviral infection. Veterinary research 44, 83.

Chan, E., Schaller, T., Eddaoudi, A., Zhan, H., Tan, C.P., Jacobsen, M., Thrasher, A.J., Towers, G.J., Qasim, W., 2012. Lentiviral gene therapy against human

immunodeficiency virus type 1, using a novel human TRIM21-cyclophilin A restriction factor. Human gene therapy 23, 1176-1185.
Chebloune, Y., Sheffer, D., Karr, B.M., Stephens, E., Narayan, O., 1996. Restrictive type of replication of ovine/caprine lentiviruses in ovine fibroblast cell cultures. Virology 222, 21-30.
Grego, E., Bertolotti, L., Quasso, A., Profiti, M., Lacerenza, D., Muz, D., Rosati, S., 2007. Genetic characterization of small ruminant lentivirus in Italian mixed flocks: evidence for a novel genotype circulating in a local goat population. The Journal of general virology 88, 3423-3427.
Grego, E., Profiti, M., Giammarioli, M., Giannino, L., Rutili, D., Woodall, C., Rosati, S., 2002. Genetic heterogeneity of small ruminant lentiviruses involves immunodominant epitope of capsid antigen and affects sensitivity of single-strain-based immunoassay. Clinical and diagnostic laboratory immunology 9, 828-832.
Gudmundsson, B., Jonsson, S.R., Olafsson, O., Agnarsdottir, G., Matthiasdottir, S., Georgsson, G., Torsteinsdottir, S., Svansson, V., Kristbjornsdottir, H.B., Franzdottir, S.R., Andresson, O.S., Andresdottir, V., 2005. Simultaneous mutations in CA and Vif of Maedi-Visna virus cause attenuated replication in macrophages and reduced infectivity in vivo. Journal of virology 79, 15038-15042.
Haflidadottir, B.S., Matthiasdottir, S., Agnarsdottir, G., Torsteinsdottir, S., Petursson, G., Andresson, O.S., Andresdottir, V., 2008. Mutational analysis of a principal neutralization domain of visna/maedi virus envelope glycoprotein. The Journal of general virology 89, 716-721.
Herrmann-Hoesing, L.M., Noh, S.M., White, S.N., Snekvik, K.R., Truscott, T., Knowles, D.P., 2009. Peripheral ovine progressive pneumonia provirus levels correlate with and predict histological tissue lesion severity in naturally infected sheep. Clinical and vaccine immunology : CVI 16, 551-557.
Hotzel, I., Cheevers, W., 2002. Differential receptor usage of small ruminant lentiviruses in ovine and caprine cells: host range but not cytopathic phenotype is determined by receptor usage. Virology 301, 21-31.
Jauregui, P., Crespo, H., Glaria, I., Lujan, L., Contreras, A., Rosati, S., de Andres, D., Amorena, B., Towers, G.J., Reina, R., 2012. Ovine TRIM5alpha can restrict visna/maedi virus. Journal of virology 86, 9504-9509.
Jeanmougin, F., Thompson, J.D., Gouy, M., Higgins, D.G., Gibson, T.J., 1998. Multiple sequence alignment with Clustal X. Trends in biochemical sciences 23, 403-405.
Jonsson, S.R., Hache, G., Stenglein, M.D., Fahrenkrug, S.C., Andresdottir, V., Harris, R.S., 2006. Evolutionarily conserved and non-conserved retrovirus restriction activities of artiodactyl APOBEC3F proteins. Nucleic acids research 34, 5683-5694.
Juganaru, M., Reina, R., Bertolotti, L., Stella, M.C., Profiti, M., Armentano, M., Bollo, E., Amorena, B., Rosati, S., 2011. In vitro properties of small ruminant lentivirus genotype E. Virology 410, 88-95.
Kisselev, A.F., Goldberg, A.L., 2001. Proteasome inhibitors: from research tools to drug candidates. Chemistry & biology 8, 739-758.
Lacerenza, D., Giammarioli, M., Grego, E., Marini, C., Profiti, M., Rutili, D., Rosati, S., 2006. Antibody response in sheep experimentally infected with different small ruminant lentivirus genotypes. Veterinary immunology and immunopathology 112, 264-271.
Larruskain, A., Bernales, I., Lujan, L., de Andres, D., Amorena, B., Jugo, B.M., 2013. Expression analysis of 13 ovine immune response candidate genes in Visna/Maedi disease progression. Comparative immunology, microbiology and infectious diseases 36, 405-413.

LaRue, R.S., Jonsson, S.R., Silverstein, K.A., Lajoie, M., Bertrand, D., El-Mabrouk, N., Hotzel, I., Andresdottir, V., Smith, T.P., Harris, R.S., 2008. The artiodactyl APOBEC3 innate immune repertoire shows evidence for a multi-functional domain organization that existed in the ancestor of placental mammals. BMC molecular biology 9, 104.
Leroux, C., Cruz, J.C., Mornex, J.F., 2010. SRLVs: a genetic continuum of lentiviral species in sheep and goats with cumulative evidence of cross species transmission. Current HIV research 8, 94-100.
Malim, M.H., Bieniasz, P.D., 2012. HIV Restriction Factors and Mechanisms of Evasion. Cold Spring Harbor perspectives in medicine 2, a006940.
McDonald, R., Burnett, V., 2005. Novel single-round PCR and cloning of full-length envelope genes of HIV-1 may yield new insight into biomolecular antibacterial drug development. Journal of virological methods 126, 111-118.
Morin, T., Guiguen, F., Bouzar, B.A., Villet, S., Greenland, T., Grezel, D., Gounel, F., Gallay, K., Garnier, C., Durand, J., Alogninouwa, T., Mselli-Lakhal, L., Mornex, J.F., CheblOune, Y., 2003. Clearance of a productive lentivirus infection in calves experimentally inoculated with caprine arthritis-encephalitis virus. Journal of virology 77, 6430-6437.
Nagel-Alne, G.E., Asheim, L.J., Hardaker, J.B., Solverod, L., Lindheim, D., Valle, P.S., 2014. The Norwegian Healthier Goats programme--a financial cost-benefit analysis. Preventive veterinary medicine 114, 96-105.
Naif, H.M., 2013. Pathogenesis of HIV Infection. Infectious disease reports 5, e6.
Peng, G., Greenwell-Wild, T., Nares, S., Jin, W., Lei, K.J., Rangel, Z.G., Munson, P.J., Wahl, S.M., 2007. Myeloid differentiation and susceptibility to HIV-1 are linked to APOBEC3 expression. Blood 110, 393-400.
Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817-818.
Qian, S.B., Reits, E., Neefjes, J., Deslich, J.M., Bennink, J.R., Yewdell, J.W., 2006. Tight linkage between translation and MHC class I peptide ligand generation implies specialized antigen processing for defective ribosomal products. Journal of immunology 177, 227-233.
Ravazzolo, A.P., Nenci, C., Vogt, H.R., Waldvogel, A., Obexer-Ruff, G., Peterhans, E., Bertoni, G., 2006. Viral load, organ distribution, histopathological lesions, and cytokine mRNA expression in goats infected with a molecular clone of the caprine arthritis encephalitis virus. Virology 350, 116-127.
Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. American Journal of Epidemiology 27, 493-497.
Ritchie, C., Hosie, B., 2014. Concern over maedi visna breakdowns. The Veterinary record 175, 50-51.
Rosati, S., Profiti, M., Lorenzetti, R., Bandecchi, P., Mannelli, A., Ortoffi, M., Tolari, F., Ciabatti, I.M., 2004. Development of recombinant capsid antigen/transmembrane epitope fusion proteins for serological diagnosis of animal lentivirus infections. Journal of virological methods 121, 73-78.
Sakuma, R., Ohmine, S., Ikeda, Y., 2010. Determinants for the rhesus monkey TRIM5alpha-mediated block of the late phase of HIV-1 replication. The Journal of biological chemistry 285, 3784-3793.
Saltarelli, M., Querat, G., Konings, D.A., Vigne, R., Clements, J.E., 1990. Nucleotide sequence and transcriptional analysis of molecular clones of CAEV which generate infectious virus. Virology 179, 347-364.

Sanjose, L., Crespo, H., Blatti-Cardinaux, L., Glaria, I., Martinez-Carrasco, C., Berriatua, E., Amorena, B., De Andres, D., Bertoni, G., Reina, R., 2016. Post-entry blockade of small ruminant lentiviruses by wild ruminants. Veterinary research 47, 1.
Schild, H., Rammensee, H.G., 2000. gp96--the immune system's Swiss army knife. Nature immunology 1, 100-101.
Simon, V., Bloch, N., Landau, N.R., 2015. Intrinsic host restrictions to HIV-1 and mechanisms of viral escape. Nature immunology 16, 546-553.
Stonos, N., Wootton, S.K., Karrow, N., 2014. Immunogenetics of small ruminant lentiviral infections. Viruses 6, 3311-3333.
Valas, S., Rolland, M., Perrin, C., Perrin, G., Mamoun, R.Z., 2008. Characterization of a new 5' splice site within the caprine arthritis encephalitis virus genome: evidence for a novel auxiliary protein. Retrovirology 5, 22.
Veillette, M., Bichel, K., Pawlica, P., Freund, S.M., Plourde, M.B., Pham, Q.T., Reyes-Moreno, C., James, L.C., Berthoux, L., 2013. The V86M mutation in HIV-1 capsid confers resistance to TRIM5alpha by abrogation of cyclophilin A-dependent restriction and enhancement of viral nuclear import. Retrovirology 10, 25.
Weinelt, J., Neil, S.J., 2014. Differential sensitivities of tetherin isoforms to counteraction by primate lentiviruses. Journal of virology 88, 5845-5858.
Willems, L., Gillet, N.A., 2015. APOBEC3 Interference during Replication of Viral Genomes. Viruses 7, 2999-3018.
Yewdell, J.W., Schubert, U., Bennink, J.R., 2001. At the crossroads of cell biology and immunology: DRiPs and other sources of peptide ligands for MHC class I molecules. Journal of cell science 114, 845-851.

Figure Captions

Figure 1 Bayesian tree showing genotype membership of sequences obtained in this study together with Genbank reference sequences. Samples were classified according to SRLV proviral load class (LPL or HPL) obtained from quantitative real time PCR assays. Posterior probability supporting the trees' nodes is shown.

Figure 2 Proviral load distribution among animals with a Low (LPL) or a High (HPL) proviral load. SRLV copy number was obtained by real time PCR.

Figure 3 Skin fibroblastic cell lines permissiveness to SRLV infection. RT activity (Abs 450 nm) was determined in clarified supernatants from SF7583H (black dots) and SF1000L (grey dots) upon infection with strains CAEV-Co and 2423H at 0, 4, 7, 9, 11 and 14 days post-infection. The values are the mean of three replica ± SD.

Figure 4 LTR promoter activity in SF7583H and SF1000L cell lines. Cells were transfected with plasmids U3BpCAT (containing the CAEV-To 1/89 strain U3 region), pCAT- Control (bearing the SV40 promoter) and pCAT-Basic (lacking promoter). CAT expression in cell lysates was measured 48h post-transfection by ELISA CAT assay and the mean of four independent replicas is represented ± SE. Transcriptional activity is represented as percentage (%) referred to pCAT-Control plasmid (100%). Data were normalized to the basal activity of the pCAT-Basic in each cell type.

Figure 5 Immunocytochemistry against the viral p25 capsid protein in SF7583H (a) and SF1000L (b) cells. SRLV infected cells were fixed and incubated with anti-p25CA monoclonal antibody followed by incubation with a HRP conjugated goat anti-mouse secondary antibody. DAB was used as staining substrate.

Figure 6 Proteasome activity measurement and effectiveness of proteasome inhibition cocktails. Proteasome chymotrypsin-like activity was measured in SF7583H and SF1000L cellular extracts as described in Material and Methods. Cells were either treated (PI+; grey bars) or not (control; empty bars) with the proteasome inhibitors cocktail. Data are average of 3 independent measures ± S.E.M. $P < 0.05$ Mann-Whitney test.

Figure 7 Proteasome-dependent restriction. Proviral load (SRLV copies in 50ng of DNA) was measured in: (a) Cells from low (SF1000L) and high (SF7583H) proviral load animals treated with (PI+; grey bars) or without proteasome inhibitors (control, empty bars) and infected (post-entry restriction); (b) SF1000L and SF7583H were infected and then treated with PI for 48 or 72h (late restriction). (c) RT activity (Abs 405 nm) in SF1000L and SF7583H supernatants after SRLV infection and PI treatment for 72 or 96h. Infection was performed with field strain 2423H.

Figure 8 TRIM5α amino acid alignment obtained from SF1000L and SF7583H cell lines. Genbank sequences from goats JQ582845, JQ582846, JQ582848 and JQ582849 and sheep JN835300 (Ov.1) and JN835301 (Ov.2) are also included. The difference between high and low proviral cell lines is highlighted. RING, B-Box2, Coiled-coil and PRY/SPRY domains are outlined.

Figure 9 APOBEC3 (Z1, Z2, Z2-Z3 and Z3) relative expression in low proviral cells (SF1000L) compared with high proviral cells (SF7583H). Values are expressed as $2^{-\Delta\Delta Ct}$ and represented in logarithmic scale.