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Research paper

Immunological parameters in goats experimentally infected with SRLV genotype E, strain *Roccaverano*

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ABSTRACT

Genotype E of small ruminant lentivirus has been recently described in goats from different breeds in Italy. Genotype E infection may differ from known genotypes since deletions of dUTPase and VPR proteins have been confirmed in different independent areas and goat breed, and play a key role on virus replication and pathogenesis. In particular, genotype E *Roccaverano* strain has been described as low pathogenic since does not lead to clinical symptoms in goats. In contrast, classical CAEV infected goats of the same area and breed presented arthritis. In this study, we have used intratracheal and intra-bone marrow routes to establish genotype E persistent infections. Humoral and cellular immune responses elicited in the host against genotype E and genotype B derived antigens were evaluated until 200 days post-inoculation. Compared to genotype B antigen, seroconversion against genotype E GAG P16-25 antigen was detected at 2–3 weeks after inoculation, significantly earlier and at higher titres. Interestingly, antibody avidity did not increase in the course of the experiment neither against P16-25 nor against SU5, both derived from genotype E.

T cell proliferation against P25-GST fusion protein antigens derived from genotype E was firstly detected at 15 days post-inoculation and was maintained throughout time until week 20 post-infection, while T cell proliferation against the genotype B P25 was not produced by the end of the experiment at 20 weeks post-inoculation. The strength of reaction was also higher when using P25 E as stimulator antigen.

In contrast with antibody and T cell proliferation, cytotoxic-T-lymphocyte (CTL) activity in the circulating lymphocytes (effector cells) using blood-derived macrophages (BDM) as target cells, was not strain specific being surprisingly higher against genotype B infected antigen presenting cells (APCs).

This is the first study reporting experimentally induced immunological changes in SRLV genotype E infection and indicates that CTL activity may be the adaptive immune response able to induce protection against heterologous infection.

1. Introduction

Small ruminant lentiviruses (SRLV), including Visna/Maedi virus (VMV) and Caprine Arthritis Encephalitis virus (CAEV), are genetically and antigenically a heterogeneous group of viruses that infect sheep and goats causing chronic inflammation in the lungs, udder, carpal joints and cen-

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tral nervous system. Five genotypes from A to E have been described in different countries (Gjerset et al., 2007; Glaria et al., 2009; Grego et al., 2007; Pisoni et al., 2005; Shah et al., 2004), with an apparent correspondence between genetic background and clinical form. For example, genotype A2 sequences are from neurotropic strains causing Visna (Haflidadottir et al., 2008), genotypes B1 and B2 are mainly associated to arthritis in goats (Pisoni et al., 2005) and sheep (Glaria et al., 2009; Rosati et al., 2004), respectively. *Roccaverano* strain (subtype E1) has been described as a low pathogenic strain within genotype E, since it lacks the entire dUTPase and Vpr-like genes and no clinical symptoms including arthritis have been recorded in animals after years of natural infection (Reina et al., 2009a). Furthermore, artificial deletions of these genes in a CAEV-Co backbone resulted in delayed viral replication, accumulation of G to A mutations and decreased proviral load and lesion development in the inoculated animals (Harmache et al., 1996; Turelli et al., 1997). Moreover, in herds with naturally co-infected animals (mainly with subtype B1 and E1) gag-PCR (using universal primers) frequently detected only genotype E sequences, suggesting a higher proviral load of the latter genotype. Lesion development was also unnoticed in these animals, in contrast with those of the same area and breed only infected with B1 subtype (Grego et al., 2007; Reina et al., 2009a). These observations, together with the increasing interest in the last decades for the development of live attenuated viruses able to induce resistance to superinfection, prompted us to characterize in genotype E infections both humoral and cellular early immune responses. In this work we evaluated antibody response, T cell proliferation and cytotoxic-T-lymphocyte (CTL) activity using homologous and heterologous antigens in animals experimentally infected with genotype E. Results demonstrate that after inoculation antibody response and T cell proliferation responses were elicited exclusively against E1 subtype, and not B1 antigen. However, CTL activity against target cells infected with B1 isolate was detected 15 weeks post-inoculation (p.i.), which might be associated with protection to superinfection versus heterologous lentivirus strains.

2. Materials and methods

2.1. Viral strains

Subtype E1 strain *Roccaverano* (prototype of genotype E), recently isolated and sequenced (Reina et al., 2009a) was used in this study for the experimental infection and CTL assays. *CAEV-TO1/89* a subtype B1 strain also characterized in previous studies (Grego et al., 2002) was used for in vitro CTL assay as a conventional CAEV-like strain.

Strain *Roccaverano* was grown and titrated using blood derived macrophages (BDM) and immunocytochemistry as described (Juganaru et al., 2010, unpublished results) since this strain does not efficiently replicate in caprine fibroblastic cells. Virus was stored in aliquots at −80 °C until used. The same viral stock was employed in experimental infection and CTL assays.

2.2. Experimental infection of goats

Animals (breeding stock) from a certified SRLV-free herd of *Roccaverano* breed goats were used in this study. They were purchased as weaned kids and introduced into the experimental facilities at the Faculty of Veterinary Medicine, University of Turin, Grugliasco (CISRA_FMV_UNITO) at least 18 months before the experimental infection. Animals were tested monthly and found consistently negative for SRLV antibodies using genotype A, B and E-derived antigens. In order to determine the optimal infectious dose and the route of inoculation, we conducted a first pilot experimental infection study using 4 animals of 8 months of age, two of which were inoculated intra-tracheally and two by injection into the *trochanteric fossa*. For each route, two different doses were used (10^5 $TCID_{50}$/ml and 10^6 $TCID_{50}$/ml). According to the results obtained, we carried out a second experimental infection study involving 8 animals inoculated intra-tracheally with 2 ml of 2.5×10^5 $TCID_{50}$/ml. Nine goats were used as a negative control group. Animals included in the experimental and control groups had similar age distribution, ranging from 9 to 24 months.

Experiments were carried out in compliance with the relevant national legislation on experimental animals and animal welfare, upon authorization by the competent authority (Italian Ministry of Health-Directorate General Animal Health-Office VI; permit no. 07/2009B).

3. Sampling

3.1. Pilot experiment

Blood samples were collected in K3-EDTA tubes, 15 days prior to infection (−15), and at days 0, 7, 14, 21, 28, 35, 42, 49, 63, 77, 107, 129, 157 and 177 days p.i.

After centrifugation at $800 \times g$ for 20 min, plasma was stored at −20 °C for ELISA analysis. After seroconversion, an additional EDTA-blood sample was obtained in order to isolate peripheral blood mononuclear cells (PBMCs) on a Ficoll gradient ($\delta = 1.077$; Lymphoprep®) for use in proliferation and CTL assays.

3.2. Second experimental infection study

Blood samples from the 8 infected goats were collected in the same manner at regular intervals −15, 7, 14, 21, 28, 35, 42, 49, 63, 77, 91, 104, 118 and 132 dpi. Plasma was stored at −20 °C for ELISA determinations. Additional EDTA-blood samples were obtained before immunization and also 2, 4, 8 and 20 weeks p.i. for T cell proliferation and at 15 weeks p.i. for CTL assays. PBMCs were obtained as described in the pilot experiment.

3.3. Measurement of group-specific antibodies in plasma

Seroconversion was evaluated using an indirect ELISA based on homologous and heterologous matrix and capsid recombinant fusion proteins (P16-25 from genotypes E and B, respectively) obtained as previously described (Reina et al., 2009b). Additionally, type specific antibod-

ies against E antigen were detected by an ELISA using SU5 synthetic peptide as coating antigen corresponding to the 24 amino acids QVRAYTYGVIEMPTGYETPTIRRR from *Roccaverano* strain. Both recombinant and synthetic ELISA procedures have been described in details in previous works (Reina et al., 2009b; Carrozza et al., 2009). Results were expressed as percentage of reactivity of each sample versus positive control serum enclosed in each plate. Samples were considered positive when reactivity was above 40% absorbance of positive control.

3.4. Antibody avidity measurements

Antibody avidity index was determined for both E-derived antigens (P16-25 and SU5) at different time points from 3 to 25 weeks p.i. by testing the stability of the antigen-antibody complexes following an additional washing step with 8 M urea as described (Mordasini et al., 2006). Samples with avidity indexes <30% were considered to be of low avidity.

3.5. T cell proliferation assay

Measurement of T cell proliferation against homologous and heterologous antigens was carried out as described elsewhere (de Andres et al., 2009; Niesalla et al., 2009; Reina et al., 2008). Briefly, PBMCs were plated in 96-well plates at a concentration of 10^5 cells/well and incubated in quadruplicate with recombinant heterologous (genotype B) or autologous (genotype E) GST/P25 fusion protein, or GST (as negative control) at equimolar amounts. Antigens were plated at 25, 12 and 6 μg/ml in 200 μl. After a five-day incubation, cells were labelled with 1 μCi of [^{3}H] thymidine (Amersham) for 5 h; incorporated radioactivity was determined using a Filter Cell Harvester 1540 (Wallac) and a Beta counter. Proliferation was measured as a stimulation index (SI) normalizing incorporated radioactivity in P25 wells with that obtained in the GST wells. The SI was calculated for each antigen using the formula SI = cpm with antigen/cpm with GST protein.

An individual animal was considered to show positive T cell reactivity if the SI was greater than 3 in at least two antigen dilutions.

3.6. CTL assay

Due to a reduced replication of *Roccaverano* strain in fibroblastic cell lines, we used BDM as live viral antigen presenting cells (APC) and as target cells as described (Lee et al., 1994) with minor modifications. Briefly, BDM were cultured on 24-well plates for 10 days in differentiating medium RPMI 10%-goat serum further supplemented with vitamins, 100 U penicillin, 100 μg streptomycin/ml, β-mercaptoethanol (50 μM), 2 mM L-glutamine and non-essential aminoacids (Sigma–Aldrich Company Ltd.), and used from days 11 to 30.

On day 11 BDM were infected with *Roccaverano* strain at a MOI of 1, based on approximate *in situ* cell count. Three days later 2×10^6 autologous PBMCs were added and medium replaced with RPMI-10% FCS and 5 U/ml recombinant human Interleukin 2 (r-Hu IL2; Sigma–Aldrich Company Ltd.). After 7 days, viable lymphocytes were transferred to new BDM infected three days before with *Roccaverano* strain at a MOI of 1 and incubated for additional 7 days. Viable lymphocytes were collected, at the end of the two proliferation steps and added as effector cells to newly autologous or heterologous BDM, each of them separately infected with either *Roccaverano* strain (72 h after infection) or *CAEV-TO1/89* isolate (48 h after infection). Different effector to target ratios were performed. After 16 h, target cells were washed with PBS and lysed in 100 μl lysis buffer (25 mM Hepes, 5 mM EGTA, 1 mM EDTA, 5 mM $MgCl_2$, CHAPS 1%, 5 mM DTT) with protease inhibitors cocktail (Sigma–Aldrich Company Ltd.) and stored at −80 °C. Positive control for caspase activity was performed in replica wells in the presence of 1 μM staurosporine (Sigma–Aldrich Company Ltd.).

To measure the CTL-induced caspase 3 activation within target cells, expression of caspase 3 was assayed by monitoring the production of 7-amino-4-methylcoumarin (AMC) from a specific fluorogenic peptide Ac-DEVD-AMC (BACHEM, Bubendorf, Switzerland) used at a final concentration of 100 μM in 25 mM Hepes pH 7.5, 10 mM DTT, 10% sucrose, 1% CHAPS. Reactions were started by adding an aliquot of sample, and the fluorescence of released AMC (excitation, 380 nm; emission, 460 nm) was monitored continuously at 37 °C with a Carry Eclipse spectrofluorimeter (VARIAN, Palo Alto, CA). Results were expressed as nmol of cleaved peptide/mg × min and specific CTL activity was calculated for each strain by the following formula: infected minus mock infected autologous cells divided by infected minus mock infected heterologous cells. CTL activity cutoff was determined in 9 SRLV free animals, used as negative control, as the mean CTL activity of negative controls plus 3 times standard deviation (SD). In vitro positive control consisted in applying the same protocol using BDM derived from 2 goats naturally infected with a field subtype B1 isolate.

3.7. Statistical analyses

Differences in seroconversion comparing ELISA absorbance values were evaluated using Wilcoxon paired-sample test. T cell proliferation mean S.I. values were compared in immunized and control group animals using Wilcoxon's paired-sample test. In order to use SD in CTL assay cutoff determination, normal distribution of CTL activity values was assessed using Shapiro Wilk's test. All statistical analyses were conducted using R software (R Development Core Team; http://www.R-project.org).

4. Results

4.1. Antibody response

Pilot experimental infection, using two different sites of injection in 4 animals, was assayed during 6 months. Seroconversion was evaluated with a recombinant P16-P25-ELISA specific for genotype E, in order to establish the dose and the efficient injection route causing persistent infection. Time of seroconversion in these 4 goats was independent from the route of infection and slightly delayed in

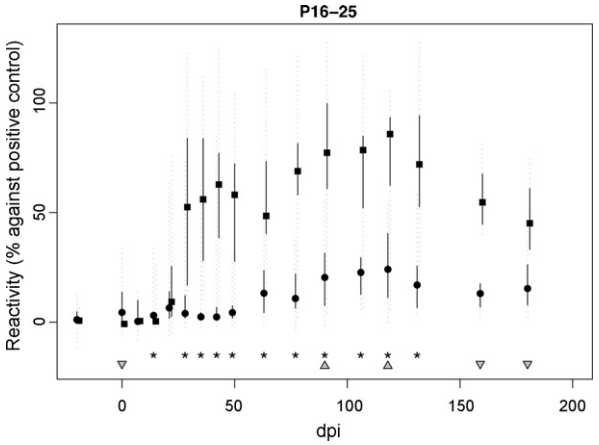

Fig. 1. Seroconversion against B and E P16-25 antigens at different time points post-inoculation (p.i.) with *Roccaverano* strain. Black circles and black squares: median absorbance versus B and E P16-25 antigens. Solid lines: absorbance interquartile ranges. Dotted lines: absorbance ranges. Asterisks: statistically significant differences between B and E absorbance (Wilcoxon's paired-sample test $p < 0.05$). Gray-up triangles: samples only from experimental infection group. Gray-down triangles: samples only from pilot experiment group.

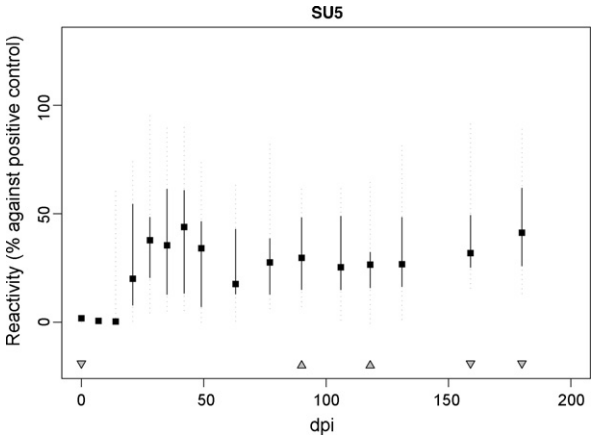

Fig. 2. Seroconversion against E SU5 antigen at different time points p.i. Black squares: median absorbance. Solid lines: absorbance interquartile ranges. Dotted lines: absorbance ranges. Gray-up triangles: samples only from experimental infection group. Gray-down triangles: samples only from pilot experiment group.

animals receiving the lower (10^5 $TCID_{50}$) viral dose. Seroconversion was achieved within 2–3 weeks p.i. using the higher dose (10^6 $TCID_{50}$) while the two animals receiving the lower dose seroconverted within 4 and 14 weeks p.i.

The second experimental infection study ($n = 8$) was carried out using an intermediate dose (5×10^5 $TCID_{50}$) and the intratracheal route, which was more practical and reproducible. All the inoculated subjects seroconverted against homologous P16-25 within 14 and 107 days p.i. (Fig. 1). Absorbance distribution reached a first pick around 50 days p.i. and a second one between 90 and 107 days p.i. leading to a persistent antibody response specifically mounted against genotype E antigen. On the other hand, reactivity against genotype B derived antigen was quite low, reaching in few cases the positivity threshold. Thus, ELISA test based on recombinant P16-25 derived from genotypes B and E clearly indicate that seroconversion against homologous antigen was detected well in advance as regards heterologous antigen (Fig. 1, Wilcoxon's paired-sample test $p < 0.05$ from 15 to 131 days p.i.).

Antibody response against SU5 peptide of genotype E was also consistently detected through time and showed an absorbance distribution comparable to that obtained using the recombinant P16-25 (Fig. 2). All control goats remained negative throughout the experiment to all ELISA tests applied (data not shown).

4.2. Avidity maturation of antibodies to P16-25 and SU5

To explore the possibility of antibody maturation in terms of avidity changes, we performed an alternative ELISA protocol including an additional washing step with urea 8 M using only the 4 immunized goats of the pilot experiment. Considering the cut-off in avidity evaluation (30%), no changes in antibody avidity were observed during the whole experiment neither versus P16-25 (maximum avidity 10.27%) nor against SU5 antigens maximum avidity 5.29%.

4.3. T cell proliferative responses

After seroconversion, cellular immune response was first evaluated in the pilot experiment ($n = 4$) using P25 and P16-25, both antigens derived from genotype E. Reactivity was found in 3 out of 4 animals against both GAG antigens. However, P25 was chosen in subsequent assays as the stimulating antigen, since recombinant protein production was much more efficient than in the case of P16-25 in terms of yield and solubility. In a further step P25 from genotype B was obtained, but in this case reactivity was not found in any of the four animals, although it was high in naturally infected goats with genotype B (data not shown). Proliferations appeared to be higher using the homologous antigen compared with the heterologous one in terms of frequency of positive animals as well as in strength of reaction (SI values). There were no substantial differences among routes of infection although dose was determinant in inducing strong T cell responses. Animals receiving the highest dose appeared to yield increased SI values, although statistical analyses were not conducted due to limited number of animals ($n = 4$).

As expected, basal T cell reactivity (day −15) in animals from the second experimental infection experiment ($n = 8$) was quite low since animals belonged to a long term seronegative flock (Fig. 3) where none of the animals showed a positive proliferation neither against P25E nor against P25B. Positive animals were firstly detected by 2 weeks p.i., and reached a maximum by week 20 with 50% of the animals reacting against P25E. In the whole experiment no animal showed positive proliferation against P25B.

In terms of strength of reaction, P25E induced stronger reactions than P25B from weeks 4 to 20 (Fig. 3), with SI values in positive animals ranging from 3.37 to 53.14 likely showing the normal variation in virus-specific recall responses in this outbred goat population. A significant

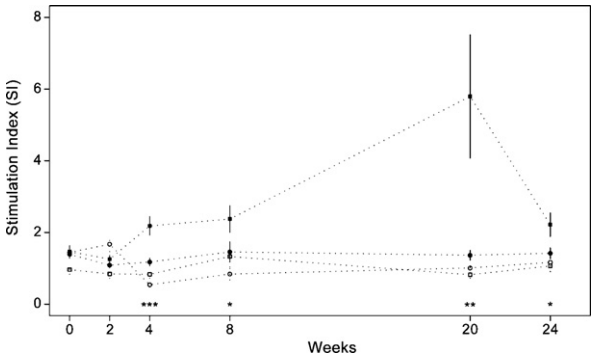

Fig. 3. T cell proliferation responses to GAG antigens derived from genotypes B and E. Results are expressed as the mean stimulation index (SI). Squares: mean reaction against E antigen (white: control group, black: immunized group). Circles: mean reaction against B antigen (white: control group, black: immunized group). Vertical bars: standard error of the mean. Comparison between reaction against E and B antigens in immunized groups: Wilcoxon's test $^{*}p < 0.10$, $^{**}p < 0.05$, $^{***}p < 0.001$.

increase in SI obtained using P25E was observed between 8 and 20 weeks after experimental inoculation (Fig. 3).

4.4. Cytotoxic T cell responses

Next, we determined if lymphocytes from *Roccaverano* infected goats, stimulated in vitro with *Roccaverano* live virus, had a CTL activity against target cells infected with genotype E or B. For this purpose, BDM target cells were tested for caspase 3 activity induced by CTL effectors from infected ($n = 8$) and uninfected ($n = 9$) goats after two stimulation cycles with genotype E virus, and live genotype E or B antigens at week 15 p.i. (Fig. 4).

Based on a cutoff value of 2.73 (mean CTL activity of 9 uninfected goat plus 3×SD, Shapiro–Wilk's normality test $W = 0.96$, $p = 0.7276$), three of the infected goats (37.5%) had a high CTL activity against heterologous strain with an average CTL value of 7.42 in infected group. Surprisingly, no CTL activity was detected against BDM infected with the homologous strain *Roccaverano*. The uninfected group of goats had no CTL activity against the E or B infected cells. The two CAEV-B1 naturally infected goats showed a CTL activity against CAEV-infected BDM, although it was close to the cut-off value (Fig. 4).

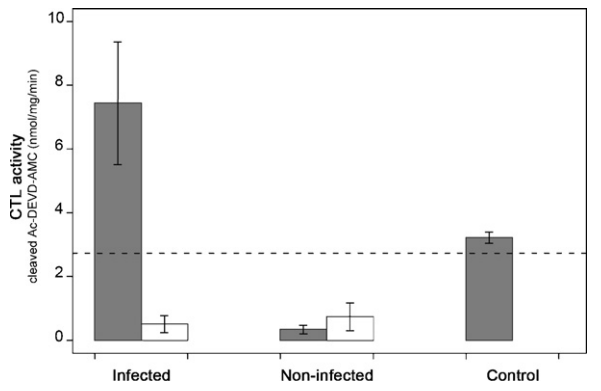

Fig. 4. CTL activity generated 15 weeks post-inoculation (p.i.) with *Roccaverano* strain ($n = 8$), in non-infected goats ($n = 9$) and in the field-strain (subtype B1) infected group ($n = 2$). Amount of caspase 3 cleaved substrate (Ac-DEVD-AMC) per time unit is shown. Effector cells were stimulated with genotype B (gray bars) or genotype E infected BDM (white bars). Vertical bars: standard error of the mean.

5. Discussion

This study describes early immunological changes observed upon experimental infection of goats with low pathogenic SRLV strain *Roccaverano* belonging to genotype E, aiming to provide evidence of a potential cross-protective adaptive response in infected animals.

Several studies have tested different routes of infection for genotypes A and B being the intratracheal the most used infection route (McNeilly et al., 2007, 2008; Torsteinsdottir et al., 2003) with few disadvantages compared to intranasal, conjunctival space, intra pulmonary and endovenous routes (Begara et al., 1996; Niesalla et al., 2008; Torsteinsdottir et al., 2003). In the pilot experiment, we used for the first time the direct inoculation of the infectious dose in the bone marrow, a reservoir of infected cells releasing infected monocytes into the blood for dissemination to target tissues (Gendelman et al., 1985), where actively replicating promonocytes could represent the necessary cellular environment for replication of dUPTase^{-} Vpr^{-} strains since intra-bone marrow inoculation of virus resulted in earlier and stronger seroconversion and T cell proliferations. However, intratracheal inoculation was finally chosen because of the easier management of the animals and consistent results.

Following experimental infection, virus showed an initial burst of replication within a few weeks, followed by an extended period of restricted replication or latency (Brahic et al., 1981; Staskus et al., 1991; Vigne et al., 1987) in which immune response switches from Th1 profile into a Th2 and finally symptoms appeared. Evidence for this switching has been shown in clinically affected animals by the production of IgG1 antibodies, together with a deficient delayed hypersensitivity reaction (Perry et al., 1995; Pyrah and Watt, 1996) and a T cell unresponsiveness abolished partially by IL-2 addition. This T cell anergy has been linked to a deficient antigenic presentation related to costimulatory molecules expression (Reina et al., 2007).

Notably, in this study persistent infection assessed by seroconversion was reached in both, the pilot and the second experimental infection studies, in time intervals comparable with those described for genotypes A and B (Lacerenza et al., 2006; McNeilly et al., 2007) in spite of the restricted replication of *Roccaverano* strain in fibroblastic-like cells in vitro (Juganaru et al., unpublished data).

Seroconversion as detected by P16-25 ELISA, was only observed against the homologous antigen, consistent with previously described results on reactivity to homologous versus heterologous antigens, involving genotypes A and B (Lacerenza et al., 2006) and genotype E under natural conditions (Reina et al., 2009b). This underlines the relevance of using the correct antigen in diagnosis.

Maturation changes in antibody avidity, conformational changes and crossreactivity are strongly related to induced protection (Li et al., 2003; Nilsson et al., 1998). In this study, antibody avidity was neither increased against

P16-25 from 5 to 25 weeks after infection nor against SU5 from 3 to 25 weeks indicating no antibody maturation against main immunodominant epitopes. Antibody responses and protection have not been correlated so far in SRLV immunization-challenge studies. The presence of neutralizing antibodies has been linked to unprotective responses (Gonzalez et al., 2005) and experiments with CAEV-inactivated vaccines, known to elicit mainly humoral response, resulted in increased lesion severity in vaccinated animals following challenge with homologous strain (McGuire et al., 1986; Russo et al., 1993). With this non-established relationship between antibody production and broadened protection, the lack of seroconversion against heterologous antigen, may therefore not necessarily represent an immunological failure in SRLV model.

Although Th1 responses have been linked to protection in lentiviral infections (Kim et al., 1999; Koup et al., 1994) a relationship between these responses and tissue damage related to TNFα production has been described (Lechner et al., 1996). Indeed, SRLV infections in which lesions are immunomediated, have led to cellular responses increasing tissue damage in vaccination-challenge experiments (Reina et al., 2008). However, genotype E infections do not lead to tissue lesions (Reina et al., 2009a). Homologous T cell proliferative responses were normal in 3 out of 4 animals in the pilot experiment and in 5 out of 8 in the second experimental infection study, which would correspond to T cell reactivity found in asymptomatic infected animals (Reina et al., 2007). Interestingly, T cell proliferative responses were entirely directed against homologous antigen and negative reactions were recorded when using genotype B reagents, suggesting a limited role of CD4+ responses in the potential protector role of genotype E infection. This is compatible with the low antibody response to genotype B strain and the antigen relatedness between genotype E and other genotypes.

Cytotoxic T lymphocytes (CTLs) are key components of the cell-mediated immune responses and play an essential role in protection against a variety of pathogens (Turner et al., 2007), including human immunodeficiency virus (HIV) and other lentiviruses (Koup et al., 1994; Letvin, 2007). Thus, CTL activity has become an important parameter for testing the efficacy of candidate vaccines (Deeks and Walker, 2007). SRLV-specific precursor CTL had been detected in the circulating lymphocyte pool of infected sheep (Blacklaws et al., 1994). In this study CTL response was mainly directed against genotype B and not genotype E infection, suggesting that a protective effect (if any) of *Roccaverano* strain against heterologous infections could reside, at least in part, in CTL activity. Recently CTL epitopes have been mapped in the RNase subunit of the *pol* gene of VMV (Wu et al., 2008). Since the corresponding region is rather conserved between *Roccaverano* and CAEV-like strains, with few conservative changes, this result could be expected. Thus, viral epitopes other than P25 could explain the discrepancies in CTL and T cell proliferations observed in this and other studies (Niesalla et al., 2009).

However, the observation that CTL killing of *Roccaverano* infected BDM was not observed at any extent was clearly unexpected. This may be explained by a deficient antigen presentation on the target cells during CTL assay since kinetics of genotype E infection on BDM is slightly protracted in vitro, compared to CAEV isolates (unpublished observation). Taking this into account, we added effector cells to CAEV and *Roccaverano* infected target cells at 48 h and 72 h p.i., respectively. However, this timing (72 h) for the *Roccaverano* infection may have not been optimal to assess CTL activity. Lack of CTL against *Roccaverano* is unlikely due to low antigen load in infected targets, since RT-PCR was positive at 72 h post-infection (not shown). Moreover in the E-infected group, IFN-(was detected in culture supernatants upon PBMC stimulation with p25 (not shown), implying an immune response involving both MHC-I and MHC-II restricted antigen presentation. Alternatively, BDM infected with *Roccaverano* strain may be altered, so that they escape from homologous CTL activity. In any case, special care should be taken when analyzing data on CTL killing assay since there are no studies so far assessing anti-SRLV CTL responses in goats. Whether this CTL response is linked to a diminished viral load and/or to a delayed onset of arthritis, as preliminary observations under natural conditions, is currently being investigated. Besides CTL response, alternative mechanisms inducing protection based on resistance to superinfection due to viral interactions between vaccination and challenge strains (Berry et al., 2008) may be responsible for a potentially protective role of *Roccaverano* strain.

In conclusion, the experimental infection of goats with the low pathogenic *Roccaverano* strain revealed a conventional immune response in terms of route of infection, time and extent of seroconversion and lymphoproliferative responses, which were exclusively directed against homologous antigen. In the absence of antibody avidity maturation, CTL activity was mainly directed towards heterologous SRLV-infected MHC-I-restricted APCs, representing the sole adaptive immune response which could be associated to protection against heterologous strain.

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