

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

3

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17

18 **Abstract**

19 Genotype E of small ruminant lentivirus has been recently described in goats from different breeds  
20 in Italy. Genotype E infection may differ from known genotypes since deletions of dUTPase and  
21 VPR proteins have been confirmed in different independent areas and goat breed, and play a key  
22 role on virus replication and pathogenesis. In particular, genotype E *Roccamare* strain has been  
23 described as low pathogenic since does not lead to clinical symptoms in goats. In contrast, classical  
24 CAEV infected goats of the same area and breed presented arthritis. In this study, we have used  
25 intratracheal and intra-bone marrow routes to establish genotype E persistent infections. Humoral  
26 and cellular immune responses elicited in the host against genotype E and genotype B derived  
27 antigens were evaluated until 200 days post-inoculation. Compared to genotype B antigen,  
28 seroconversion against genotype E GAG p16-25 antigen was detected at 2-3 weeks after  
29 inoculation, significantly earlier and at higher titres. Interestingly, antibody avidity did not increase  
30 in the course of the experiment neither against p16-25 nor against SU5, both derived from genotype  
31 E.

32 T cell proliferation against p25-GST fusion protein antigens derived from genotype E was firstly  
33 detected at 15 days post-inoculation and was maintained throughout time until week 20 post  
34 infection, while T cell proliferation against the genotype B p25 was not produced by the end of the  
35 experiment at 20 weeks post-inoculation. The strength of reaction was also higher when using p25 E  
36 as stimulator antigen. T cell responses against P25 E.

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

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

## 44 **Introduction**

45 Small ruminant lentiviruses (SRLV), including Visna/Maedi virus (VMV) and Caprine Arthritis  
46 Encefalitis virus (CAEV), are genetically and antigenically a heterogeneous group of viruses that  
47 infect sheep and goats causing chronic inflammation in the lungs, udder, carpal joints and central  
48 nervous system. Five genotypes from A to E have been described in different countries (Gjerset et  
49 al., 2007; Glaria et al., 2009; Grego et al., 2007; Pisoni et al., 2005; Shah et al., 2004), with an  
50 apparent correspondence between genetic background and clinical form. For example, genotype A2  
51 sequences are from neurotropic strains causing Visna (Haflidadottir et al., 2008), genotypes B1 and  
52 B2 are mainly associated to arthritis in goats (Pisoni et al., 2005) and sheep (Glaria et al., 2009;  
53 Rosati et al., 2004), respectively. *Roccaverano* strain (subtype E1) has been described as a low  
54 pathogenic strain within genotype E, since it lacks the entire dUTPase and Vpr-like genes and no  
55 clinical symptoms including arthritis have been recorded in animals after years of natural infection  
56 (Reina et al., 2009a). Furthermore, artificial deletions of these genes in a CAEV-Co backbone  
57 resulted in delayed viral replication, accumulation of G to A mutations and decreased proviral load  
58 and lesion development in the inoculated animals (Harmache et al., 1996; Turelli et al., 1997).  
59 Moreover, in herds with naturally co-infected animals (mainly with subtype B1 and E1) gag-PCR  
60 (using universal primers) frequently detected only genotype E sequences, suggesting a higher  
61 proviral load of the latter genotype. Lesion development was also unnoticed in these animals, in  
62 contrast with those of the same area and breed only infected with B1 subtype (Grego et al., 2007;  
63 Reina et al., 2009a). These observations, together with the increasing interest in the last decades for  
64 the development of live attenuated viruses able to induce resistance to superinfection, prompted us  
65 to characterize in genotype E infections both humoral and cellular early immune responses. In this  
66 work we evaluated antibody response, T cell proliferation and cytotoxic-T-lymphocyte (CTL)  
67 activity using homologous and heterologous antigens in animals experimentally infected with  
68 genotype E. Results demonstrate that after inoculation antibody response and T cell proliferation  
69 responses were elicited exclusively against E1 subtype, and not B1 antigen. However, CTL activity

70 against target cells infected with B1 isolate was detected 15 weeks post-inoculation (p.i.), which  
71 might be associated with protection to superinfection versus heterologous lentivirus strains.

72

## 73 **Materials and methods**

### 74 *Viral strains*

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

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

83

### 84 *Experimental infection of goats*

85 Animals (breeding stock) from a certified SRLV-free herd of Roccaverano breed goats were used in  
86 this study. They were purchased as weaned kids and introduced into the experimental facilities at  
87 the Faculty of Veterinary Medicine, University of Turin, Grugliasco (CISRA\_FMV\_UNITO) at  
88 least 18 months before the experimental infection. Animals were tested monthly and found  
89 consistently negative for SRLV antibodies using genotype A, B and E-derived antigens. In order to  
90 determine the optimal infectious dose and the route of inoculation, we conducted a first pilot  
91 experimental infection study using 4 animals of 8 months of age, two of which were inoculated  
92 intra-tracheally and two by injection into the *trochanteric fossa*. For each route, two different doses  
93 were used ( $10^5$  TCID<sub>50</sub>/ml and  $10^6$  TCID<sub>50</sub>/ml). According to the results obtained, we carried out a  
94 second experimental infection study involving 8 animals inoculated intra-tracheally with 2 ml of

95  $2.5 \times 10^5$  TCID<sub>50</sub>/ml. Nine goats were used as a negative control group. Animals included in the  
96 experimental and control groups had similar age distribution, ranging from 9 to 24 months.  
97 Experiments were carried out in compliance with the relevant national legislation on experimental  
98 animals and animal welfare, upon authorization by the competent authority (Italian Ministry of  
99 Health-Directorate General Animal Health-Office VI; permit n. 07/2009B).

100

## 101 *Sampling*

### 102 *Pilot experiment*

103 Blood samples were collected in K3-EDTA tubes, 15 days prior to infection (-15), and at days 0, 7,  
104 14, 21, 28, 35, 42, 49, 63, 77, 107, 129, 157 and 177 days p.i..

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

### 109 *Second experimental infection study*

110 Blood samples from the 8 infected goats were collected in the same manner at regular intervals -15,  
111 7, 14, 21, 28, 35, 42, 49, 63, 77, 91, 104, 118 and 132 dpi. Plasma was stored at -20°C for ELISA  
112 determinations. Additional EDTA-blood samples were obtained before immunization and also 2, 4,  
113 8 and 20 weeks p.i. for T cell proliferation and at 15 weeks p.i. for CTL assays. PBMCs were  
114 obtained as described in the pilot experiment.

115

### 116 *Measurement of group-specific antibodies in plasma*

117 Seroconversion was evaluated using an indirect ELISA based on homologous and heterologous  
118 matrix and capsid recombinant fusion proteins (P16-25 from genotypes E and B, respectively)  
119 obtained as previously described (Reina et al., 2009b). Additionally, type specific antibodies against  
120 E antigen were detected by an ELISA using SU5 synthetic peptide as coating antigen corresponding

121 to the 24 amino acids QVRAYTYGVIEMPTGYETPTIRRR from *Rocccaverano* strain. Both  
122 recombinant and synthetic ELISA procedures have been described in details in previous works  
123 (Reina et al., 2009b; Carrozza et al., 2009). Results were expressed as percentage of reactivity of  
124 each sample vs positive control serum enclosed in each plate. Samples were considered positive  
125 when reactivity was above 40% absorbance of positive control.

126

### 127 *Antibody avidity measurements*

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

132

### 133 *T cell proliferation assay*

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

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

146

147 *CTL assay*

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

154 On day 11 BDM were infected with *Roccoverano* strain at a MOI of 1, based on approximate *in situ*  
155 cell count. Three days later  $2 \times 10^6$  autologous PBMCs were added and medium replaced with  
156 RPMI-10% FCS and 5U/ml recombinant human Interleukin 2 (r-Hu IL2; Sigma–Aldrich Company  
157 Ltd). After 7 days, viable lymphocytes were transferred to new BDM infected three days before  
158 with *Roccoverano* strain at a MOI of 1 and incubated for additional 7 days. Viable lymphocytes  
159 were collected, at the end of the two proliferation steps and added as effector cells to newly  
160 autologous or heterologous BDM, each of them separately infected with either *Roccoverano* strain  
161 (72 h after infection) or *CAEV-TO1/89* isolate (48 h after infection). Different effector to target  
162 ratios were performed. After 16 h, target cells were washed with PBS and lysed in 100 µl lysis  
163 buffer (25mM Hepes, 5 mM EGTA, 1mM EDTA, 5mM MgCl<sub>2</sub>, CHAPS 1%, 5mM DTT) with  
164 protease inhibitors cocktail (Sigma–Aldrich Company Ltd) and stored at -80°C. Positive control for  
165 caspase activity was performed in replica wells in the presence of 1 µM staurosporine (Sigma–  
166 Aldrich Company Ltd).

167 To measure the CTL-induced caspase 3 activation within target cells, expression of caspase 3 was  
168 assayed by monitoring the production of 7-amino-4-methylcoumarin (AMC) from a specific  
169 fluorogenic peptide Ac-DEVD-AMC (BACHEM, Bubendorf, Switzerland) used at a final  
170 concentration of 100 µM in 25 mM Hepes pH 7.5, 10 mM DTT, 10% sucrose, 1% CHAPS.  
171 Reactions were started by adding an aliquot of sample, and the fluorescence of released AMC  
172 (excitation, 380 nm; emission, 460 nm) was monitored continuously at 37°C with a Carry Eclipse

173 spectrofluorimeter (VARIAN, Palo Alto, CA). Results were expressed as nmol of cleaved  
174 peptide/mg\*min and specific CTL activity was calculated for each strain by the following formula:  
175 infected minus mock infected autologous cells divided by infected minus mock infected  
176 heterologous cells. CTL activity cutoff was determined in 9 SRLV free animals, used as negative  
177 control, as the mean CTL activity of negative controls plus 3 times standard deviation (SD). In vitro  
178 positive control consisted in applying the same protocol using BDM derived from 2 goats naturally  
179 infected with a field subtype B1 isolate.

180

### 181 *Statistical analyses*

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

188

## 189 **Results**

### 190 *Antibody response*

191 Pilot experimental infection, using two different sites of injection in 4 animals, was assayed during  
192 6 months. Seroconversion was evaluated with a recombinant P16-P25-ELISA specific for genotype  
193 E, in order to establish the dose and the efficient injection route causing persistent infection. Time  
194 of seroconversion in these 4 goats was independent from the route of infection and slightly delayed  
195 in animals receiving the lower ( $10^5$  TCID<sub>50</sub>) viral dose. Seroconversion was achieved within 2-3  
196 weeks p.i. using the higher dose ( $10^6$  TCID<sub>50</sub>) while the two animals receiving the lower dose  
197 seroconverted within 4 and 14 weeks p.i..

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

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

#### 212

#### 213 *Avidity maturation of antibodies to P16-25 and SU5*

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

#### 219

#### 220 *T cell proliferative responses*

221 After seroconversion, cellular immune response was first evaluated in the pilot experiment (n=4)  
222 using P25 and P16-25, both antigens derived from genotype E. Reactivity was found in 3 out of 4  
223 animals against both GAG antigens. However, P25 was chosen in subsequent assays as the

224 stimulating antigen, since recombinant protein production was much more efficient than in the case  
225 of P16-25 in terms of yield and solubility. In a further step P25 from genotype B was obtained, but  
226 in this case reactivity was not found in any of the four animals, although it was high in naturally  
227 infected goats with genotype B (data not shown). Proliferations appeared to be higher using the  
228 homologous antigen compared with the heterologous one in terms of frequency of positive animals  
229 as well as in strength of reaction (SI values). There were no substantial differences among routes of  
230 infection although dose was determinant in inducing strong T cell responses. Animals receiving the  
231 highest dose appeared to yield increased SI values, although statistical analyses were not conducted  
232 due to limited number of animals (n=4).

233 As expected, basal T cell reactivity (day -15) in animals from the second experimental infection  
234 experiment (n=8) was quite low since animals belonged to a long term seronegative flock (Fig 3)  
235 where none of the animals showed a positive proliferation neither against P25E nor against P25B.  
236 Positive animals were firstly detected by 2 weeks p.i., and reached a maximum by week 20 with  
237 50% of the animals reacting against P25E. In the whole experiment no animal showed positive  
238 proliferation against P25B.

239 In terms of strength of reaction, P25E induced stronger reactions than P25B from weeks 4 to 20  
240 (Fig 3), with SI values in positive animals ranging from 3.37 to 53.14 likely showing the normal  
241 variation in virus-specific recall responses in this outbred goat population. A significant increase in  
242 SI obtained using P25E was observed between 8 and 20 weeks after experimental inoculation (Fig.  
243 3).

244

#### 245 *Cytotoxic T cell responses*

246 Next, we determined if lymphocytes from *Roccaverano* infected goats, stimulated in vitro with  
247 *Roccaverano* live virus, had a CTL activity against target cells infected with genotype E or B. For  
248 this purpose, BDM target cells were tested for caspase 3 activity induced by CTL effectors from

249 infected (n=8) and uninfected (n=9) goats after two stimulation cycles with genotype E virus, and  
250 live genotype E or B antigens at week 15 p.i. (Fig 4).

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

258

## 259 Discussion

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

263 Several studies have tested different routes of infection for genotypes A and B being the  
264 intratracheal the most used infection route (McNeilly et al., 2007; Torsteinsdottir et al., 2003;  
265 McNeilly et al., 2008) with few disadvantages compared to intranasal, conjunctival space, intra  
266 pulmonary and endovenous routes (Begara et al., 1996; Niesalla et al., 2008; Torsteinsdottir et al.,  
267 2003). In the pilot experiment, we used for the first time the direct inoculation of the infectious dose  
268 in the bone marrow, a reservoir of infected cells releasing infected monocytes into the blood for  
269 dissemination to target tissues (Gendelman et al., 1985), where actively replicating promonocytes  
270 could represent the necessary cellular environment for replication of dUPTase<sup>-</sup> Vpr<sup>-</sup> strains since  
271 intra-bone marrow inoculation of virus resulted in earlier and stronger seroconversion and T cell  
272 proliferations. However, intratracheal inoculation was finally chosen because of the easier  
273 management of the animals and consistent results.

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

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

287 Seroconversion as detected by p16-25 ELISA, was only observed against the homologous antigen,  
288 consistent with previously described results on reactivity to homologous vs heterologous antigens,  
289 involving genotypes A and B (Lacerenza et al., 2006) and genotype E under natural conditions  
290 (Reina et al., 2009b). This underlines the relevance of using the correct antigen in diagnosis.

291 Maturation changes in antibody avidity, conformational changes and crossreactivity are strongly  
292 related to induced protection (Li et al., 2003; Nilsson et al., 1998). In this study, antibody avidity  
293 was neither increased against p16-25 from 5 to 25 weeks after infection nor against SU5 from 3 to  
294 25 weeks indicating no antibody maturation against main immunodominant epitopes. Antibody  
295 responses and protection have not been correlated so far in SRLV immunization-challenge studies.  
296 The presence of neutralizing antibodies has been linked to unprotective responses (Gonzalez et al.,  
297 2005) and experiments with CAEV-inactivated vaccines, known to elicit mainly humoral response,  
298 resulted in increased lesion severity in vaccinated animals following challenge with homologous  
299 strain (McGuire et al., 1986; Russo et al., 1993). With this non-established relationship between

300 antibody production and broadened protection, the lack of seroconversion against heterologous  
301 antigen, may therefore not necessarily represent an immunological failure in SRLV model.

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

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

326 epitopes other than P25 could explain the discrepancies in CTL and T cell proliferations observed in  
327 this and other studies (Niesalla et al., 2009).

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

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

351 adaptive immune response which could be associated to protection against heterologous strain.

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354

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493  
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495

496 **Figure captions**

497 **Figure 1.** Seroconversion against B and E P16-25 antigens at different time points post-inoculation  
498 (p.i.) with *Rocaverano* strain. Black circles and black squares:

499 median absorbance versus B and E P16-25 antigens. Solid lines: absorbance inter quartile ranges.

500 Dotted lines: absorbance ranges. Asterisks: Statistically significant differences between B and E

501 absorbance. (Wilcoxon's paired-sample test  $p < 0.05$ ). Gray-up triangles: samples only from

502 experimental infection group. Gray-down triangles: samples only from pilot experiment group.

503

504 **Figure 2.** Seroconversion against E SU5 antigen at different time points p.i.. Black squares: median

505 absorbance. Solid lines: absorbance inter quartile ranges. Dotted lines: absorbance ranges. Gray-up

506 triangles: samples only from experimental infection group. Gray-down triangles: samples only from

507 pilot experiment group.

508

509 **Figure 3.** T cell proliferation responses to GAG antigens derived from genotypes B and E. Results

510 are expressed as the mean stimulation index (SI). Squares: mean reaction against E antigen (white:

511 control group, black: immunized group). Circles: mean reaction against B antigen (white: control

512 group, black: immunized group). Vertical bars: standard error of the mean. Comparison between

513 reaction against E and B antigens in immunized groups: \* Wilcoxon's test  $p < 0.10$ , \*\*  $p < 0.05$ ,

514 \*\*\*  $p < 0.001$ .

515

516 **Figure 4.** CTL activity generated 15 weeks post-inoculation (p.i.) with *Rocaverano* strain (n=8),

517 in non-infected goats (n=9) and in the field-strain (subtype B1) infected group (n=2). Amount of

518 caspase 3 cleaved substrate (Ac-DEVD-AMC) per time unit is shown. Effector cells were

519 stimulated with genotype B (grey bars) or genotype E infected BDM (white bars). Vertical bars:

520 standard error of the mean.

### P16-25

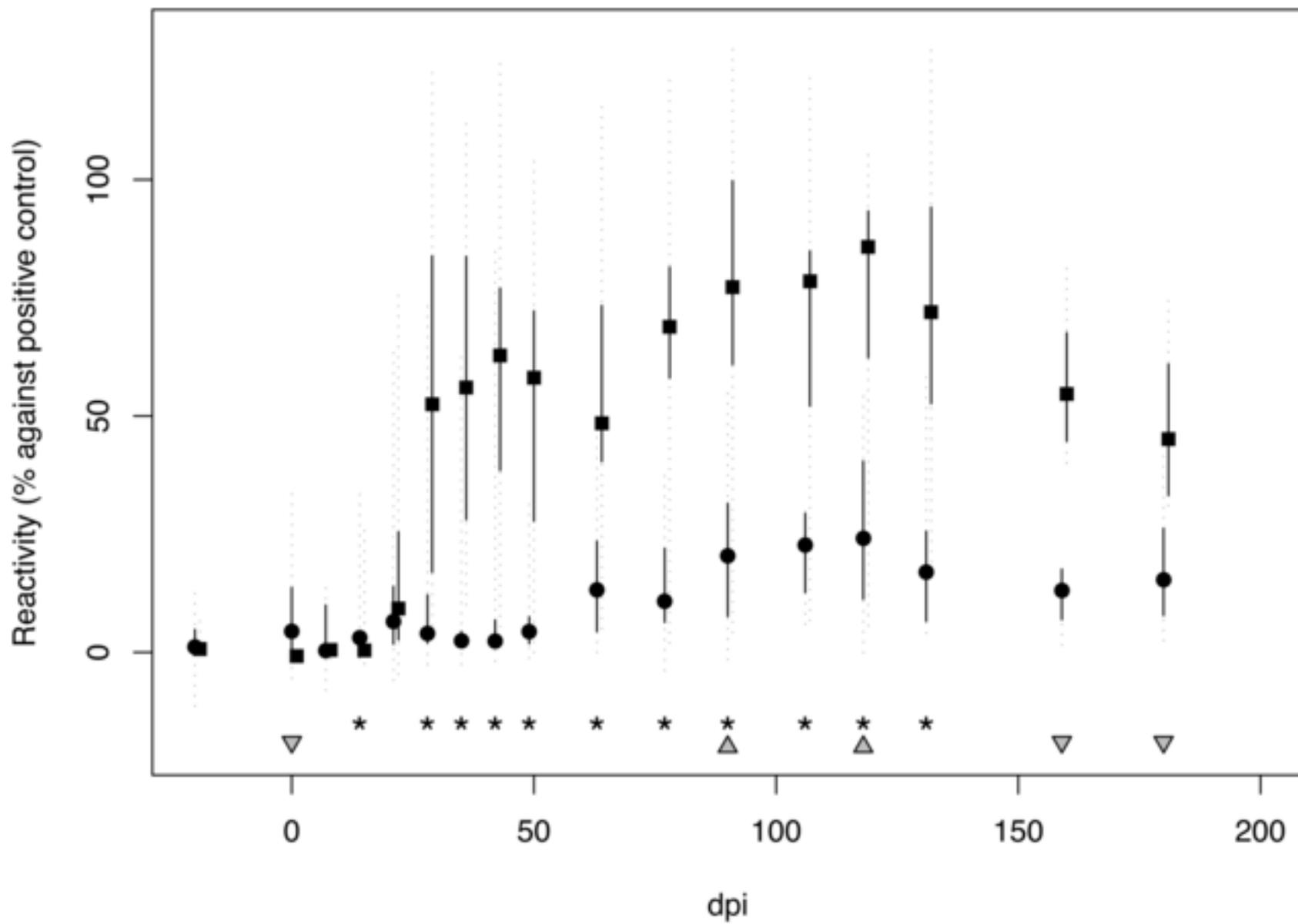


Figure 2

arXiv

### SU5

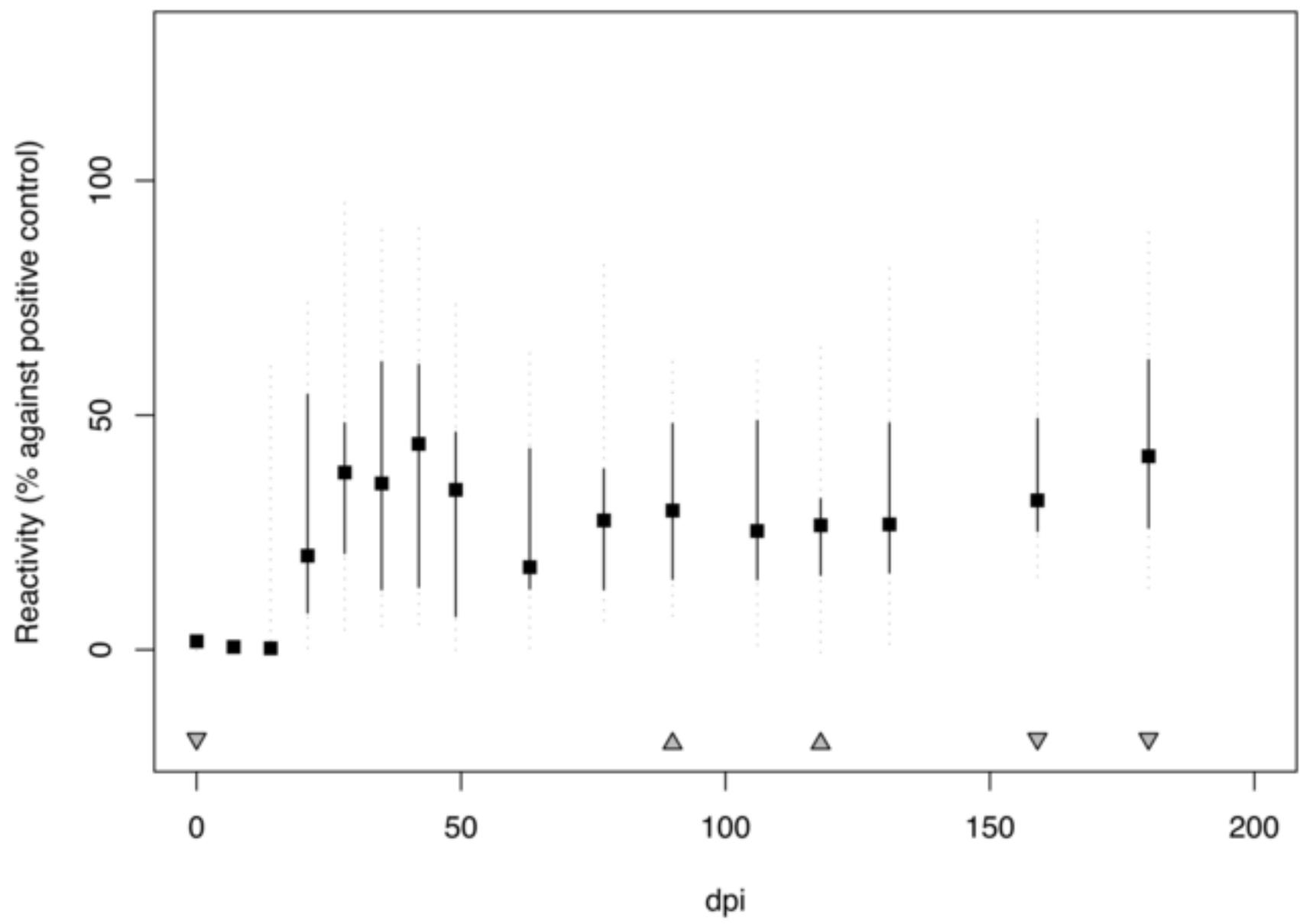


Figure 3

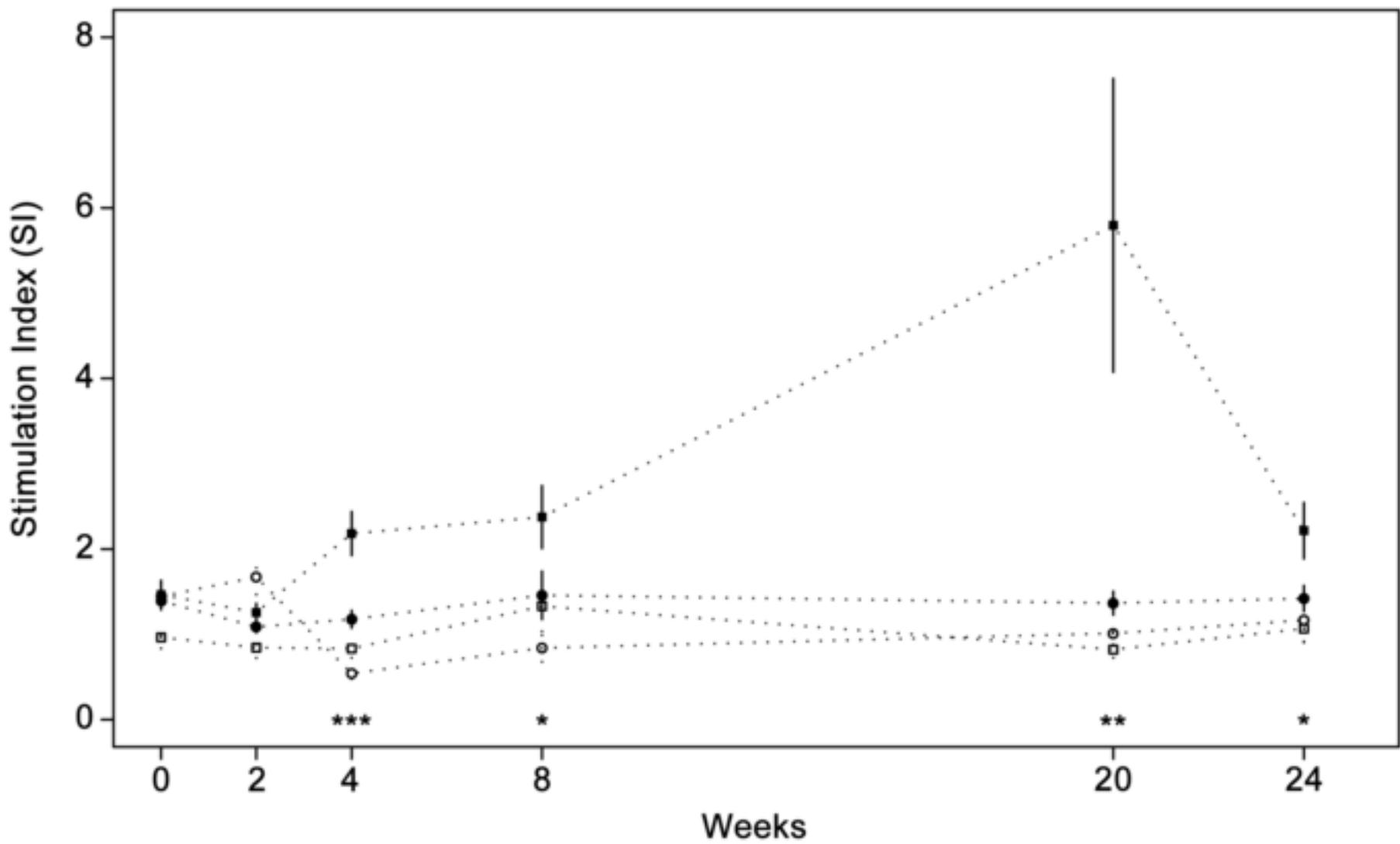


Figure 4

