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22 **Abstract**

23 Myxoma virus (MXV) causes the systemic disease myxomatosis in the European rabbit.  
24 Despite many *in vitro* studies on the function of MXV immunomodulatory proteins and  
25 detailed molecular knowledge of virus, little is known about the dynamics of interaction of the  
26 virus with the integrated host-immune system during infection. In this study changes in  
27 haematological profile, changes in lymphocyte subset distribution and nonspecific  
28 proliferation activity of lymphocytes from different lymphoid compartments on the 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup>,  
29 9<sup>th</sup> and 11<sup>th</sup> day after experimental infection of rabbits with MXV strain Lausanne was  
30 characterised. The relationship between alterations of immune parameters and dynamic of  
31 virus dissemination through the body was investigated. Haematological changes included  
32 moderate leucopenia with significant lymphopenia, neutrophilia, monocytosis and  
33 eosinopenia. A decrease of T cells including CD4<sup>+</sup> and CD8<sup>+</sup> and increase of CD79 $\alpha$ <sup>+</sup> were  
34 observed in draining popliteal lymph node 4 days after virus inoculation. From day 6,  
35 comparable changes were seen in collateral popliteal lymph node, spleen and peripheral  
36 blood. From day 9, the mentioned lymphocyte subsets tended to reach their original state in  
37 all of these lymphocyte compartments except draining popliteal lymph node. In thymus, MXV  
38 infection affected mainly CD4<sup>+</sup>CD8<sup>+</sup> double positive thymocytes. On the other hand,  
39 proliferation activity of lymphocytes determined by the proliferation assay with plant-derived  
40 mitogens was significantly reduced from day 4 or 6 and remained reduced until the end of  
41 experiment in all observed lymphoid organs. Presence of MXV in respective lymphoid  
42 compartments preceded changes in lymphocyte subset distribution or lymphocyte activity.

43

44

45 **Keywords:** myxomatosis; lymphocyte subsets; lagomorphs; flow cytometry

## 46 1. Introduction

47 Myxoma virus (MXV) causes the systemic disease known as myxomatosis in the  
48 European rabbit (*Oryctolagus cuniculus*). This virus is a member of the family *Poxviridae*,  
49 *Leporipoxvirus* genus. It was first described by Sanarelli in 1896 in South America as the  
50 causative agent of a lethal infection in the imported European rabbits (Kerr and Best, 1998).  
51 Subsequent study showed that the natural host of MXV in South America is the jungle rabbit  
52 or tapeti (*Sylvilagus brasiliensis*). A related strain of MXV is found in the North American  
53 brush rabbit (*Sylvilagus bachmani*). In its evolutionary *Sylvilagus* hosts, this virus causes only  
54 a benign infection, characterised by a cutaneous fibroma restricted to the site of inoculation;  
55 there is no systemic spread in the immunocompetent adult rabbits. However, in European  
56 rabbits, MXV infection causes rapid systemic and lethal infection with mortality rates of  
57 almost 100%. The third related virus, termed “rabbit fibroma virus” (Shope fibroma virus)  
58 occurs in the eastern cottontail (*Sylvilagus floridanus*). This virus does not cause a serious  
59 disease in adult European rabbits, but provides cross-protection instead and is used as a  
60 vaccine against myxomatosis (Fenner and Fantini, 1999).

61 MXV might well have remained an interesting but relatively unimportant occasional  
62 disease of veterinary significance if it had not been used in 1950 as a biological control of  
63 European rabbit infestation in Australia, when the Standard Laboratory Strain (SLS), also  
64 known as the Moses strain was released into wild. In 1952, a similar strain known as  
65 Lausanne was illegally released into Europe (Fenner and Fantini, 1999). The virus is  
66 passively transmitted from the skin lesions of infected rabbits by biting arthropods, such as  
67 mosquitoes or fleas. The pathogenesis of myxomatosis in laboratory rabbits was described  
68 by Fenner and Woodroffe (1953). The virus spreads from the initial replication site in the  
69 skin to the lymph node draining inoculation site. The virus then replicates in the lymph node  
70 to high titres and spreads via infected leukocytes to distal tissues such as spleen, testes,  
71 lungs, mucocutaneous sites, such as the nose and conjunctivae, and skin. The early clinical  
72 signs of typical myxomatosis are swelling of the muzzle and the anogenital region, as well as  
73 blepharoconjunctivitis; the late stage of infection is characterised by the formation of

74 subcutaneous gelatinous swellings called myxomas. Late in the infection, rabbits may  
75 develop acute bacterial infections of the conjunctivae and the upper respiratory tract. The  
76 severity of clinical disease can vary between different strains of rabbits as well as MXV  
77 isolates (Best and Kerr, 2000; Silvers et al., 2006). After the virus spread in Australia, the  
78 subsequent rapid coevolution of both host and virus has been well documented (Fenner and  
79 Fantini, 1999). That resulted in the selection of both attenuated field variants of MXV, and an  
80 increasingly resistant population of rabbits (Kerr and Best, 1998; Best et al., 2000). There is  
81 also another type of myxomatosis called amyxomatosis or atypical myxomatosis which is  
82 characterised by intense respiratory distress and reduction of cutaneous lesions (Marlier and  
83 Vindevogel 1996; Marlier et al., 2000; Psikal et al., 2003).

84 The entire genomes of the South American strain Lausanne (Cameron et al., 1999) and  
85 the North American strain MSW (Labudovic et al., 2004) of MXV as well as the closely  
86 related rabbit fibroma virus (Willer et al., 1999) have been sequenced. Like other poxviruses,  
87 MXV encodes at least twenty-eight proteins capable of downregulating the host innate and  
88 acquired immune responses. These immunomodulatory proteins have been divided into  
89 three groups: viroceptors, which closely resemble cellular receptors and are often secreted  
90 or expressed on the surface of virus infected cells, they functionally bind and inhibit  
91 extracellular host ligands which are intended to induce an inflammatory or antiviral response;  
92 on the contrary, virokines are normally secreted and mimic the host immune system  
93 inhibitors or growth factors; and viromitigators, which have antiapoptotic or host range  
94 functions during viral infection. There are many extensive and excellent reviews on the MXV  
95 immunomodulatory proteins (Barrett et al., 2001; Kerr and McFadden, 2002; Zuniga, 2003;  
96 Stanford et al., 2007). Despite many *in vitro* studies on the function of these proteins and  
97 detailed molecular knowledge of MXV, little is known about the dynamics of interaction of the  
98 virus with the integrated host-immune system during infection. In present study changes in  
99 haematological profile, changes in lymphocyte subsets and mitogen-driven proliferation  
100 activity of lymphocytes from different lymphoid compartments on the respective days after  
101 experimental infection of rabbits with the MXV strain Lausanne were characterised. The

102 relationship between alterations of the immune parameters and dynamic of virus  
103 dissemination through the body was investigated.

104

## 105 **2. Materials and methods**

### 106 **2.1. Animals and experimental design**

107 Outbred New Zealand White SPF European rabbits, strain CrI:KBL, lacking antibodies  
108 against MXV, were obtained from the Charles River Laboratories Germany GmbH, Munich,  
109 DE. Rabbits were housed in the animal care facility with controlled conditions at the  
110 Veterinary Research Institute, Brno, CZ. The rabbits were fed pellets (complete food mixture,  
111 Bonagro, CZ) *ad libitum* and had free access to water. The rooms were kept at a  
112 temperature of 21° C, relative humidity of 40 to 60%, and ventilation of approximately 15 air  
113 changes per hour.

114 Fifteen 20-week-old rabbits (12 males, 8 females) were inoculated intradermally into the  
115 dorsum of the left hind foot with 1000 TCID<sub>50</sub> in a volume of 0.1 ml of the virus working stock.  
116 Rabbits were randomly allocated to groups (2 males and 1 female) for each sampling day  
117 prior infection; they were monitored daily, rectal temperatures and clinical signs were  
118 recorded. Three infected rabbits were euthanised under general anaesthesia (5mg/kg  
119 xylazine and 35 mg/kg ketamine (Rometar 2% and Narkamon 5%, SPOFA Inc., Prague, CZ)  
120 on day 2, 4, 6, 9 and 11 after infection. At each time point, one control rabbit, inoculated with  
121 equal volume of saline, was also euthanised (n = 5). The animals were euthanised under the  
122 agreement of the Branch Commission for Animal Welfare of the Ministry of Agriculture of the  
123 Czech Republic.

124

### 125 **2.2. Virus preparation**

126 The MXV strain Lausanne (catalogue number CAMP V-564, Collection of Animal  
127 Pathogenic Microorganisms, Veterinary Research Institute, Brno, CZ) was propagated three-  
128 times on the RK13 cell line in minimal essential medium with antibiotics (10 U penicillin/ml;  
129 0.1 mg streptomycin/ml and 0.25 µg amphotericine/ml) and 5% fetal bovine serum to prepare

130 the working stock. Strain Lausanne is of grade 1 virulence, with the mortality rate 99% and  
131 the mean survival time up to 13 days (Fenner and Marshall, 1957). Virions were released  
132 from the cells by freeze-thawing the culture three times. The virus titers were determined by  
133 TCID<sub>50</sub> method in RK13 cell monolayer as described elsewhere (Hierhofer and Killington,  
134 1996).

135

### 136 **2.3. Sample collection and cell isolation**

137 After anaesthesia, samples of peripheral blood (PB) were collected from *a. auricularis*  
138 *centralis* to heparinised tubes and to tubes for serum separation. At autopsy, samples of  
139 lymphoid organs - the left popliteal lymph node (draining lymph node), right popliteal node  
140 (contralateral lymph node), spleen, mesenteric lymph nodes and thymus were collected for  
141 immunology tests.

142 The total leukocyte counts in blood and organ samples were determined using the Digicell  
143 500 cell counter (Contraves AG, Zurich, CH). Differential leukocyte counts were calculated  
144 from blood smears stained with May-Grünwald and Giemsa-Romanowski (Penta, Prague,  
145 CZ). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient  
146 centrifugation using a cell separating medium with a density of 1.077 g/ml (Histopaque®-  
147 1077, Sigma-Aldrich, St. Louis, MO, USA).

148 Organ samples were collected into RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA)  
149 medium. To define the total number of cells in each organ, the weight of the tissues was  
150 determined at the beginning of the cell isolation process. Cell suspensions were prepared by  
151 careful teasing the lymphoid tissue using two forceps. All cell suspensions were filtered  
152 through a fine nylon mesh and erythrocytes contamination in lymphoid organ suspensions  
153 was lysed by ammonium chloride. The numbers of the isolated cells were expressed as the  
154 cells per gram of tissue. For flow cytometry, the cells were washed in washing and staining  
155 buffer (WSB - PBS with 0.2% gelatin from cold water fish skin, 0.1 % sodium azide and 0.05  
156 mM EDTA, all reagents from Sigma-Aldrich, St. Louis, MO, USA), resuspended in WSB and  
157 adjusted to a density of  $5 \times 10^6$ /ml. For the lymphocyte proliferation assay, the density of the



158 cell suspension was adjusted to  $1 \times 10^6$ /ml of RPMI 1640 medium supplemented with 10%  
159 precolostral calf serum, 100 000 U/l penicillin and 0.2 g/l streptomycin.

160 For virus detection a piece of all weighed lymphoid organs, mononuclear cell isolated from  
161 PB and the skin from inoculation side, skin from the equivalent side on the hind foot to the  
162 inoculation side (distal skin), eyelid, lung, liver, kidney, testis in males and skin lesions if  
163 appeared were collected into liquid nitrogen and then stored at  $-80^\circ\text{C}$ .

164

#### 165 **2.4. Immunostaining and flow cytometric analysis**

166 The following anti-rabbit mAbs were used as primary immunoreagents for indirect  
167 immunofluorescence staining: anti-CD4 (RTH1A, IgG1) together with anti-CD8 (ISC27A,  
168 IgG2a), anti-panT2 (RTH21A, IgG1; pT), anti-CD45 (ISC18A, IgG2a); (all reagents were from  
169 VMRD Inc., Pullman, WA, USA) and anti-human CD14 (TÜK4, IgG2a, DakoCytomation,  
170 Glostrup, DK; Saalmuller et al., 2005). As the secondary immunoreagent FITC or R-PE  
171 labelled goat anti-mouse conjugates of appropriate subisotypes (Southern Biotechnology  
172 Assoc., Inc., Birmingham, AL, USA) were used. For the quantification of B-lymphocytes, R-  
173 PE conjugated mouse anti-human CD79 $\alpha$  (clone HM57, DakoCytomation, Glostrup, DK;  
174 Jones et al., 1993) was used. Detailed immunostaining procedures had been described  
175 previously (Jeklova et al., 2007a). Percentage values of lymphocyte subsets were also  
176 recalculated to absolute cells numbers per 1 gram of tissue or  $10^6$  per 1 ml of PB cells.

177 Data was acquired on a standard FACSCalibur™ flow cytometer (Becton Dickinson,  
178 Mountain View, CA, USA), operated by the CELLQuest™ software. Gating was based on  
179 forward angle and right angle scatter signals. The common leukocyte antigen CD45 and  
180 CD14 expression was used for the “lymphogate” setup and lymphocyte purity determination  
181 as previously described (Jeklova et al., 2007a). Results obtained for the other surface  
182 markers were recalculated to 100% of CD45 $^+$  and CD14 $^-$  cells in the “lymphogate”.  
183 Percentages of lymphocyte subsets were also recalculated on absolute cells numbers per 1  
184 gram of tissue or  $10^6$  per 1 ml of PB cells.

185

## 186 2.5. Proliferation assay

187 Proliferation activity of lymphocytes was determined based on incorporation of <sup>3</sup>H-  
188 thymidine after *in vitro* lymphocyte stimulation in the whole blood or the organ cell  
189 suspension with non-specific mitogen concanavalin A (10 µg/ml; ConA) as described  
190 previously (Jeklova et al., 2007b). The results were expressed in terms of stimulation indices  
191 (SI), which have been calculated as the ratio of counts per minute (CPM) in stimulated  
192 samples versus CPM in non-stimulated ones.

193

## 194 2.6. Viral genome quantification

195 Total DNA was isolated using Dneasy 96 Blood and Tissue kit (QIAGEN, Hilden, DE). In  
196 order to not exceed the column binding capacity, not more than 5 mg of tissue or 8x10<sup>6</sup> cells  
197 were processed in each sample. Because all the DNA samples isolated were within the  
198 detection range, the DNA dilution was not necessary and was not performed. Primers and a  
199 probe for real-time 5' nuclease assay located in MXV m071L gene (AF170726) were  
200 designed using Primer3 on-line service (Rozen and Skaletsky, 2000): forward  
201 5'GACATTTTAGCCTATGGGAAGAGTATGTA3'; reverse  
202 5'CAAGCAACGTCGTATCGTCCT3'; FAM-BHQ1 labelled probe  
203 5'ACGCCCGTCAAAGATCCGAACA3'. Oligos were synthesized by GeneriBiotech (Hradec  
204 Kralove, CZ). The PCR reaction performed by duplicates on LightCycler LC480 (Roche, CH)  
205 in the total volume of 11 µl consisted of LC480 Probe master mix (Roche, CH), 2.9 µM of  
206 each primer, 0.18 µM of the probe and 1.1 µl of DNA. The cycling conditions were as follows:  
207 initial denaturation at 95 °C for 15 minutes, followed by amplification of the target DNA for 50  
208 cycles (95°C for 20 s and 58°C for 60 s). All PCR runs included appropriate negative controls  
209 to exclude the possibility of DNA contamination and positive controls to ensure high PCR  
210 performance. As positive copy number standard, PCR product cloned into pCR2.1  
211 (Invitrogen, USA) plasmid was used. The amplification efficiency was E=1.971 in the range of  
212 10<sup>1</sup>-10<sup>9</sup> copies of virus genome equivalents (Figure 1). The amounts of viral DNA were  
213 recalculated to 1g of tissue.

214

## 215 **2.7. Immunohistochemistry**

216 Monoclonal antibody anti-MXV was derived from BALB/c mice immunized intraperitoneally  
217 with MXV Lausanne strain. Single-cell preparations of spleens from these mice were fused  
218 with the SP2/0 Ag 14 cell line (Galfre and Milstein, 1981) and the resulting hybridomas were  
219 tested by an ELISA using antigen prepared as is described in following section. The antibody  
220 specificity was verified using the immunoperoxidase assay and it was determined, that the  
221 anti-MXV antibody reacted with different strains of MXV including strain Laussane, Litovel 2  
222 (Psikal et al., 2003), Sanar and Mose. Western blot analysis showed that this antibody is  
223 specifically reactive to four protein of MXV strain Lausanne (11 kDa, 14 kDa, 16 kDa and 25  
224 kDa). These proteins probably constitute a single unit on the virus, and the junction sites of  
225 the components may produce discontinuous epitopes (Barlow et al., 1986) or monoclonal  
226 antibody may react with virus-specific glycosyl-groups or with some other virus-specific  
227 residues (Ichihashi, 1990).

228 Cryostat sections of thymus (5  $\mu$ m thick) were aired and fixed in acetone for 5 min at room  
229 temperature. Before staining, slides were stored at -70 °C in dry conditions. Endogenous  
230 peroxidase activity was inhibited by 3% H<sub>2</sub>O<sub>2</sub> treatment for 15 min at room temperature.  
231 Samples were incubated with the anti-MXV antibodies diluted 1:10 for 60 min at room  
232 temperature. EnVision™ system (DakoCytomation, Glostrup, DK) was used for visualization:  
233 30 min incubation was followed by adding 0.03% DAB (3,3'-diaminobenzidine, Lachema,  
234 Brno, CZ) chromogen in PBS containing 0.02% H<sub>2</sub>O<sub>2</sub> as a substrate for 5 min. The sections  
235 were counterstained with hematoxylin solution. PBS was used as a dilution and rinsing buffer  
236 in all steps. The images of sections were recorded using laboratory imaging software LUCIA  
237 GF on Muntech version 4.51 (Laboratory Imaging, Prague, CZ).

238

## 239 **2.8. Detection of antibody response**

240 In parallel with preparation of the virus working stock for rabbit infection, the same virus  
241 was used for preparation of antigen for the ELISA test. After three time freeze-thawing

242 process, these cells were centrifuged at 2 000 x g for 10 minutes. The pellet was  
243 resuspended in a 100 times smaller volume of 0.5% Igepal CA – 630 solution (Sigma-  
244 Aldrich, St. Louis, MO, USA) in PBS (pH 7.2) than was volume of growing medium and  
245 incubated at 4°C for 1 hour. The detritus of cells was removed by centrifugation as described  
246 above. The wells of microtiter plates (Gama, Dalecin, CZ) were coated with prepared  
247 antigen, diluted 1:2000 in 0.05 M carbonate-bicarbonate buffer, pH 9.6 at 4°C overnight and  
248 subsequently washed three times with PBS, pH 7.2, containing 0.05% Tween 20 (PBS-T).  
249 The non-specific binding was blocked by 2% casein hydrolysate in PBS at room temperature  
250 for 30 minutes. The tested sera were diluted at 1:10 in PBS-T with 0.5% casein hydrolysate  
251 and then the threefold dilution series of these sera were prepared. After 1 hour of incubation  
252 in a wet chamber at 37°C, the wells were washed three times in PBS-T and then 100 µl of  
253 goat anti-rabbit IgM (µ chain specific, 1:15 000) or chicken anti-rabbit IgG (whole molecule,  
254 1:25 000) both horseradish peroxidase conjugates (Rockland, Girbeltsville, PA) were added  
255 into each well. After incubation and washing, 100 µl of TMB Complete substrate and  
256 chromogen solution (Test-line, Brno, CZ) was added into each well. The reaction was  
257 stopped after 15 minutes of incubation by adding 50 µl of 2M sulphuric acid and absorbances  
258 were read at 450 nm using the multichannel spectrometer iEMS Reader (Labsystems,  
259 Helsinki, FI). Negative, weak positive and strong positive controls were run as standards in  
260 duplicates on each plate.

261 The serum samples were evaluated as positive if their optical density (OD) was higher  
262 than 0.2. This value was established by adding the average OD and the standard deviation  
263 was multiplied by 3. The values of the average OD (0.041) and the standard deviation  
264 (0.0523) were established from serum samples obtained from control uninfected animals.

265

## 266 **2.8. Data analysis**

267 Due to a relatively small number of experimental animals, nonparametric one-tail Mann-  
268 Whitney test was used for the comparison of values from respective groups (n = 3) after  
269 infection with the control group (n = 5). Differences with a value of  $P < 0.05$  were considered

270 statistically significant. All calculations were performed with MS-Excel<sup>®</sup> (Microsoft Corp., Inc.,  
271 USA) and Prism<sup>®</sup> (Graph Pad Software, Inc., San Diego, CA, USA) software.

272

### 273 **3. Results**

#### 274 **3.1. Clinical signs and autopsy findings**

275 All rabbits developed clinical signs characteristic for typical myxomatosis. On the second  
276 day after infection, the inoculation site showed symptoms of inflammation - pink colour and  
277 oedema, followed by conjunctivitis on day 4. The first secondary skin lesions (myxomas)  
278 appeared on day 5 on the eyelids and ears of infected rabbits, and on this day, oedema of  
279 anogenital region was also observed. Initially serous eye discharge on day 6 was changed to  
280 mucopurulent discharge from eyes and nose on day 7. On day 7 after MXV infection, the  
281 primary lesion at the inoculation site changed from purple to black colour, one day later  
282 became necrotic and on day 10, oozing serous fluid was observed. From day 8, the eyes  
283 were almost closed because of extensive swelling of the eyelids and respiratory distress was  
284 present. On day 11, rabbits suffered from a severe dyspnoea, showed open mouth  
285 breathing, secondary skin lesions on the eyelids and ears were hard and protuberant and  
286 another myxomas developed throughout the body. Due to a terminal stage of the disease, all  
287 the remaining animals were euthanised on this day.

288 Rectal temperatures were elevated over 40°C on days 3 and 4 after infection in two  
289 rabbits, on days 5 and 6 in three rabbits and from day 7 in all the other experimental animals  
290 and remained elevated until the end of the experiment.

291 When compared with control uninfected rabbits, peripheral blood leukocyte counts were  
292 significantly decreased on day 4 and 6 after infection ( $P<0.05$ ); no changes were detected at  
293 any other point of time. Significant decrease in absolute counts of lymphocytes was recorded  
294 on day 4, 6, 9 and 11 ( $P<0.05$ ). On the other hand, significant neutrophilia and monocytosis  
295 ( $P<0.05$ ) were observed on day 9 and 11.

296 At autopsy draining popliteal lymph nodes and spleens were enlarged by day 4,  
297 contralateral popliteal lymph nodes were enlarged by day 6. From day 9, thymus atrophy was  
298 macroscopically evident.

299

### 300 **3.2. Lymphocyte subset distribution**

301 No significant changes were observed on day 2 in any lymphoid organ. On day 4 after  
302 infection, a significant decrease in percentages of CD4<sup>+</sup> and CD4<sup>+</sup>8<sup>+</sup> lymphocytes (P<0.05)  
303 was noted in left popliteal lymph nodes, which drained the site of virus inoculation, when  
304 compared to control rabbits. No changes were detected in absolute numbers of any  
305 lymphocyte subsets at this time point. On day 6 and 9, a decrease of CD4<sup>+</sup>, CD8<sup>+</sup>, CD4<sup>+</sup>8<sup>+</sup>  
306 and pT<sup>+</sup> cells and subsequent increase of CD79α<sup>+</sup> were observed in both percentages as  
307 well as in absolute numbers (P<0.05) in this compartment (Table 1 and 2). On day 11 after  
308 infection, a significant decrease of relative numbers of CD4<sup>+</sup> and pT<sup>+</sup> cells and contrary, an  
309 increase of CD79α<sup>+</sup> was recorded; however in absolute numbers, only a decrease in CD4<sup>+</sup>8<sup>+</sup>  
310 was observed.

311 Similar changes in distribution of lymphocyte subset were detected in contralateral  
312 popliteal lymph nodes, but changes were delayed when compared to draining lymph nodes.  
313 On day 6, a significant decrease in percentages of CD4<sup>+</sup>, CD8<sup>+</sup> and pT<sup>+</sup> cells and an  
314 increase of CD79α<sup>+</sup> were observed (P<0.05) in this tissue. No significant changes were  
315 detected on day 9; nevertheless on day 11, the decrease in percentages of CD4<sup>+</sup> and pT<sup>+</sup>  
316 cells was significant (P<0.05). In absolute numbers, an increase of CD79α<sup>+</sup> lymphocytes was  
317 detected on day 6 and 11.

318 In PB, significant decrease in absolute numbers of CD4<sup>+</sup> and pT<sup>+</sup> lymphocyte subsets  
319 (P<0.05) was recorded on day 4. Six days after infection, significant decrease of T cell and  
320 all T-cell subpopulations was detected (P<0.05). No significant changes were detected on  
321 day 9; nevertheless on day 11, an increase in percentages of CD4<sup>+</sup> and pT<sup>+</sup> cells and a  
322 decrease in percentages and absolute numbers of CD79α<sup>+</sup> was significant (P<0.05).

323 Significant changes of lymphocyte subset distribution in spleen were noted only in  
324 absolute numbers. On day 6, a decrease of CD4<sup>+</sup>, CD4<sup>+</sup>8<sup>+</sup> and pT<sup>+</sup> lymphocytes , and on day  
325 9 a decrease of CD4<sup>+</sup> and CD79α<sup>+</sup> cells were recorded (P<0.05).

326 In all the above lymphoid compartments, lymphocyte redistribution reached the peak on  
327 day 6. In contrast to that, no significant changes in lymphocyte subset distribution were  
328 observed in mesenteric lymph nodes when compared to uninfected rabbits.

329 The results obtained by flow cytometry analysis of lymphocyte subsets in thymus of MXV  
330 infected rabbits were noteworthy. In relative numbers, a decrease of CD4<sup>+</sup>8<sup>+</sup> and an increase  
331 of CD4<sup>+</sup> lymphocytes were detected on day 9 and 11 (P<0.05). In absolute numbers, a  
332 decrease of CD4<sup>+</sup>8<sup>+</sup> and pT<sup>+</sup> cells (P<0.05) were recorded in those points of time.

333

### 334 **3.3. Proliferation activity of lymphocytes**

335 Proliferation ability of lymphocytes after the plant-derived mitogen stimulation was  
336 characterised based on SI values (Table 3). In the draining popliteal lymph nodes, a  
337 significant decrease of SI values (P<0.05) after ConA stimulation was detected from day 4  
338 until the end of experiment. In the contralateral popliteal lymph nodes and spleen, a  
339 decrease of proliferation ability (P<0.05) was recorded from day 6 and reduced proliferation  
340 ability lasted until the end of experiment. A significant decrease (P<0.05) of SI values in PB  
341 was detected on day 9 and 11 and in mesenteric lymph nodes on day 11. In all the above  
342 mentioned samples, SI values decreased because of a significant reduction of CPM values  
343 when compared to uninfected rabbits. In contrast to CPM values, no significant changes  
344 were detected in control non-stimulated lymphocytes; that reflected that no changes in the  
345 ability of spontaneous proliferation occurred. No changes in proliferation activity of  
346 lymphocytes were detected in thymus.

347

### 348 **3.4. Viral genome quantification**

349 Two days after infection, the copies of MXV genome were detected in skin from the  
350 inoculation site and in lower amounts in the draining popliteal lymph nodes (Table 4). From

351 day 4, viral genome was recorded in PBMC and in all studied lymphoid and parenchymatous  
352 organs except testis where MXV was present by day 6. In thymus, viral DNA from two of  
353 three rabbits on day 4 and in all rabbits from later time points was detected. During the entire  
354 experiment, the highest amounts (over  $10^8$  DNA copy equivalents/g) of viral genome were  
355 detected in skin from the inoculation site, draining popliteal lymph nodes and secondary skin  
356 lesions.

357

### 358 **3.5. Localization of virus infected cells in thymus**

359 Infected cells, labelled with specific anti-MXV monoclonal antibody, were detected in  
360 thymus on day 4. MXV positive cells were localized mainly in the medulla of thymus (Figure  
361 2).

362

### 363 **3.6. Antibody response**

364 The specific IgM antibodies were detectable as early as on day 4 in serum of one from  
365 three rabbits. On day 6 after infection, serum samples from all rabbits were positive for anti-  
366 MXV antibodies of IgM isotype. IgG antibodies were present in serum of one rabbit from day  
367 9; on day 11 this antibody class was detected in sera of all infected rabbits (Figure 3).

368

## 369 **4. Discussion**

370 The aim of our study was to extend knowledge of the dynamics of interaction of the MXV  
371 strain Lausanne with the integrated host-immune system based on characterisation of  
372 changes in the haematological profile, the changes in lymphocyte subset distribution and the  
373 non-specific proliferation activity of lymphocytes from different lymphoid compartments on  
374 the respective days after experimental infection.

375 After experimental infection, all the MXV inoculated rabbits developed very severe signs  
376 of myxomatosis. The presented clinical signs were characteristic for a typical form of  
377 myxomatosis - a red swollen lesion at the inoculation site on about day 2, swelling of the  
378 eyelids and the anogenital region between days 4 - 5 and presence of mucopurulent



379 oculonasal discharge from day 7, that later completely occluded nostrils leading to  
380 respiratory distress and eye closure. From day 5, secondary skin lesions appeared on the  
381 eyelids and ears that later generalized over the body. These rabbits were not kept longer  
382 than for 11 days as the previous experience showed that they would die between day 10 and  
383 13 after inoculation (Fenner and Marshall, 1957; Psikal et al., 2003). Clinical signs and the  
384 course of infection with strain Lausanne is comparable with another grade 1 virulence strain  
385 SLS, with the exception of necrosis of primary skin lesions, which has not been described for  
386 SLS strain (Fenner and Marshall, 1957; Best and Kerr, 2000). In the present study, rectal  
387 temperatures of the infected rabbits were elevated above 40°C from day 3 with no decrease  
388 during the course of infection, in contrast to the infection with SLS strain, where rectal  
389 temperatures were elevated from day 5 and decreased between day 8 and 10 indicating poor  
390 prognosis. After inoculation with Lausanne strain in this study, haematology profile was  
391 characterised by decreased whole leukocyte counts on day 4 and 6 due to significant  
392 lymphopenia; marked neutrophilia on day 9 and 11 did not cause elevation of whole  
393 leukocyte counts as described in the case of infection with SLS strain of MXV (Best and Kerr,  
394 2000).

395 Present knowledge of MXV pathogenesis can be summarized as follows: after inoculation  
396 into skin, the virus replicates in MHC II positive dendritic-like cells of dermis, which are also  
397 probably responsible for transporting virus to the draining lymph node; within 24 hours, virus  
398 replicates in T lymphocytes in paracortical region of the draining lymph node and possibly  
399 also in other cells. Both lymphocytes and MHC II positive cells are believed to be crucial for  
400 virus dissemination to distal sites and subsequently for development of systemic disease and  
401 secondary lesions (Best et al., 2000; Kerr and McFadden, 2002). Replication of MXV leads to  
402 massive reduction of lymphocytes in the draining lymph node mostly as a result of apoptosis;  
403 nevertheless, there is no information if any lymphocyte subset is more susceptible for  
404 apoptosis. In the present study, we recorded significant changes in lymphocyte subset  
405 distribution in all studied lymphocyte compartments except mesenteric lymph nodes. As  
406 expected, marked changes of lymphocyte subsets occurred in left popliteal lymph nodes that

407 predominantly drained the foot inoculation site as was demonstrated by Best and Kerr  
408 (2000). A decrease of absolute numbers of T lymphocytes due to a decrease of all T-cell  
409 subsets ( $CD4^+$ ,  $CD8^+$ ,  $CD4^+8^+$ ) and an increase of B cells ( $CD79\alpha^+$ ) on days 6 and 9 after  
410 infection were detected. However, in relative numbers, a decrease of  $CD4^+$  lymphocytes was  
411 observed as soon as 4 days after infection and lasted until the 11<sup>th</sup> day. Similar results were  
412 recorded in other lymphoid compartments except mesenteric lymph node but to a lesser  
413 extent and changes were delayed when compared with the draining popliteal lymph nodes.  
414 The presented results indicated, that T cells are more influenced by MXV infection than B  
415 cells, and within T lymphocytes, the decrease of numbers is more pronounced in the  $CD4^+$   
416 subpopulation. These results are in agreement with *in vitro* studies of malignant rabbit  
417 fibroma virus (MFV). This leporipoxvirus, a hybrid of myxoma virus and rabbit fibroma virus,  
418 replicates preferentially in mature T lymphocytes of spleen but is able to grow in B  
419 lymphocytes as well (Strayer et al., 1987; Strayer, 1992). There is also *in vitro* determined  
420 ability of MXV strain Lausanne to downregulate CD4 molecules from the surface of T-cell line  
421 (RL-5) up to 24 hours after infection (Barry et al., 1995). The present work is the first *in vivo*  
422 study indicating the affinity of MXV to  $CD4^+$  lymphocyte subset. Molecules  $CD4^+$  on T helper  
423 cells play a pivotal role in T-cell ontogeny and T-cell activation. Downregulation of CD4  
424 molecules on infected T helper cells may be involved in inhibiting the infected cells to survive  
425 and disseminating the virus within the host. The reduction of absolute numbers of  $CD4^+$  cells  
426 is probably because of apoptosis that occurred in high degree in paracortex of the draining  
427 lymph node after MXV infection (Best and Kerr, 2000). The decrease in numbers of T helper  
428  $CD4^+$  cells could lead to inhibition of both: Th1 cell polarized cell-mediated effectors  
429 functions, characterised by elimination of virus infected cells through the actions of  $CD8^+$   
430 cytotoxic T-lymphocytes and Th2 mediated antibody reactions.

431 In this work, cell-mediated immunity was characterised based on non-specific proliferation  
432 ability of lymphocytes from various lymphoid compartments to respond to stimulation with  
433 preferentially  $CD4^+$  T-cell plant-derived mitogen ConA (Watkins et al., 1984). Reduction of  
434 proliferation ability was detected in all compartments studied, in draining popliteal lymph

435 nodes from day 4, in contralateral lymph nodes and spleen from day 6, in PB from day 9 and  
436 in mesenteric lymph nodes on day 11. In contrast to the changes in lymphocyte subsets  
437 which culminate on day 6 and then the tendency of the mentioned lymphocyte subsets to  
438 reach their original state in all these lymphocyte compartments except the draining popliteal  
439 lymph nodes from day 9 was observed, the proliferation ability remained reduced until the  
440 end of the experiment. Decrease of proliferation ability was also recorded in lymphoid organs  
441 of rabbits after experimental inoculation of MFV (Strayer et al., 1983; Skaletsky et al., 1984;  
442 Strayer, 1992). But in contrast to this study after MFV infection, inhibition of ConA  
443 proliferation was less severe on day 11 than on day 7, which indicated partial recovery from  
444 the MFV-induced proliferation suppression (Strayer and Leibowitz, 1986). Exact mechanism  
445 of the proliferation ability inhibition is not known, but influence of viral immunomodulatory  
446 proteins is assumed (Strayer et al., 1988; Strayer, 1992; Barrett et al., 2001).

447 Experimentally infected rabbits were able to produce specific IgM antibodies as early as  
448 from day 4 and IgG antibodies from day 9. Detection of antibodies of IgG isotype indicates  
449 that isotype switching occurred and thus there was likely some Th cell activity. Comparable  
450 results were described by Best and Kerr (2000) after infection of laboratory rabbits with the  
451 SLS strain. Reduction of cell-mediated activity and in contrast to that early production and  
452 isotype switching of antibodies could imply that the response of the remaining CD4<sup>+</sup> T cells  
453 has been predominantly Th2, dominated by IL-4, rather than an effective antiviral Th1,  
454 dominated by interferon- $\gamma$ . Nevertheless, even production of neutralizing antibodies did not  
455 protect rabbits from death after infection with virulent MXV because, as in other poxviruses,  
456 the cell mediated immune response is most critical (Best and Kerr, 2000; Kerr and  
457 McFadden, 2002).

458 The relationship between alterations of the immune parameters and the dynamics of virus  
459 dissemination through the body was investigated. The replication and spread of Lausanne  
460 strain MXV were similar to those described previously in SLS strain (Fenner and Woodroffe,  
461 1953; Best and Kerr, 2000). In the present study, virus was detected in the skin at the  
462 inoculation site and in the lymph node draining this region on two days after inoculation. On

463 day 4, it was detected in the contralateral lymph node, spleen, mesenteric lymph node and  
464 PBMC. On this day, MXV was also present in lung, liver, kidney and in one of three rabbit  
465 also in distal skin in contrast to other studies of MXV strains of grade 1 virulence (Best and  
466 Kerr, 2000; Silvers et al., 2006), where virus was not detected in these organs until day 6. As  
467 previously suggested (Fenner and Woodroffe, 1953; Best and Kerr, 2000), this is compatible  
468 with a spread of virus in infected lymphocytes in the lymph to the blood and therefore to  
469 distal lymphoid tissues and parenchymatous organs. Earlier detection of virus in lung, liver  
470 and kidney was probably due to higher sensitivity of real-time PCR used in the present work;  
471 our detection limit was 10 copies of viral genome. On the other hand, in previous studies,  
472 virus titers were measured by the plaque assay with detection limit 100 pfu/g. The highest  
473 amounts of MXV of viral genome were detected in the draining popliteal lymph nodes, skin  
474 from the inoculation site, and secondary skin lesions, which are critical for virus transmission  
475 by arthropod vectors. Presence of virus in respective lymphoid compartments preceded the  
476 changes in lymphocyte subset distribution or lymphocyte activity.

477 Information related to presence of MXV in thymus is scarce. Silvers et al. (2006) did not  
478 detect virus in thymus of rabbits infected with the North American strain MSW. Moreover *in*  
479 *vivo*, MFV could not be recovered from thymus, and *in vitro*, did not replicate in the resting  
480 thymocytes (Strayer et al., 1987; Strayer, 1992). In light of these facts, detection of viral  
481 genome in thymus from two of three infected rabbits on day 4 and from all rabbits in the later  
482 time points and significant changes in lymphocyte subset distribution in thymus were  
483 unexpected. The CD4<sup>+</sup>CD8<sup>+</sup> double positive thymocytes were most affected; these were  
484 significantly reduced both in absolute as well as relative numbers from day 9. The increase in  
485 relative numbers of CD4<sup>+</sup> cells could be a consequence of the percentage reduction of  
486 CD4<sup>+</sup>CD8<sup>+</sup> cells. The decrease of pT<sup>+</sup> cells in absolute numbers reflected the reduction of  
487 double positive cells. The MXV infected cells were mainly localized in the medulla, where  
488 later events in thymocyte development as negative selection to remove auto-reactive T cells  
489 from the mature repertoire take place (von Boehmer et al., 2003). The reduction in numbers

490 of immature T cells leads to a decreased input of mature T helper as well as T cytotoxic  
491 lymphocytes that could contribute to immunosuppression of rabbits after MXV infection.

492 Based on the results presented here we can conclude that experimental intradermal  
493 infection of rabbits with the MXV strain Lausanne caused a marked immunosuppression.  
494 This immunosuppression was characterised by significant lymphocyte subset changes  
495 particularly due to the CD4<sup>+</sup> T cell reduction and the decrease of proliferation ability of  
496 lymphocytes. Rabbits were able to produce specific antibodies of IgM as well as IgG isotype.  
497 Presence of MXV in respective lymphoid compartments preceded the changes in lymphocyte  
498 subset distribution or lymphocyte activity.

499

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503

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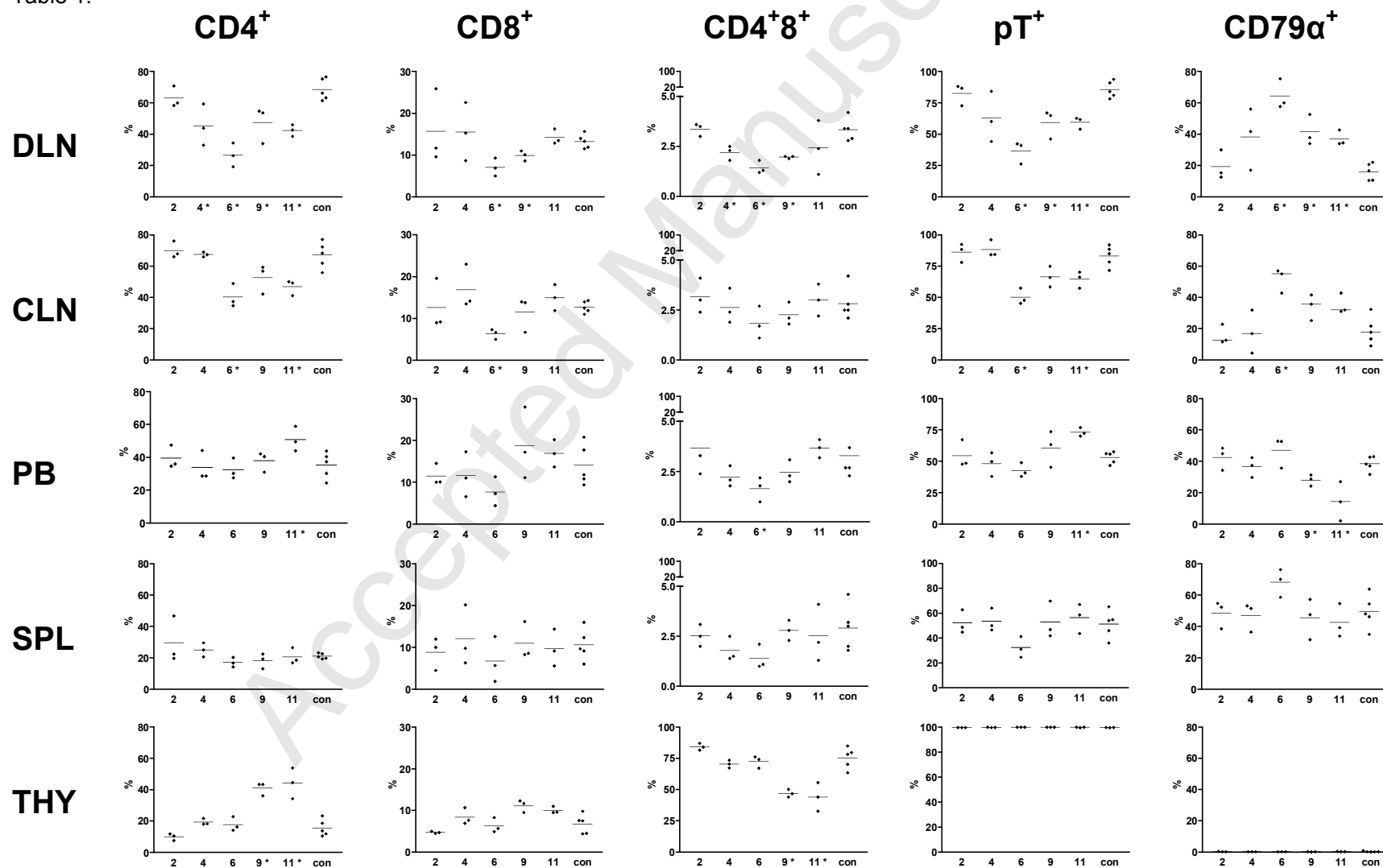
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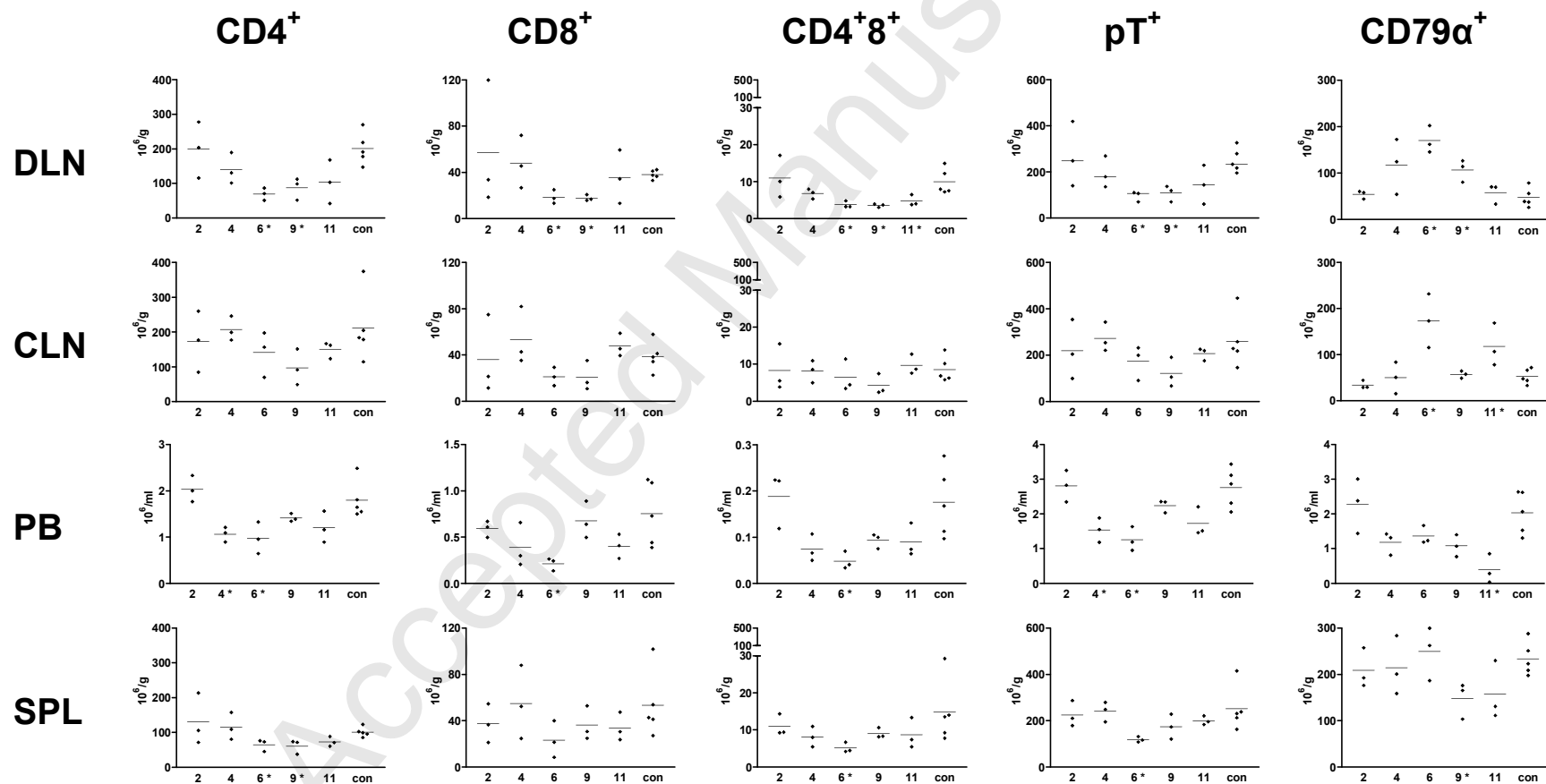
593 Table 1.



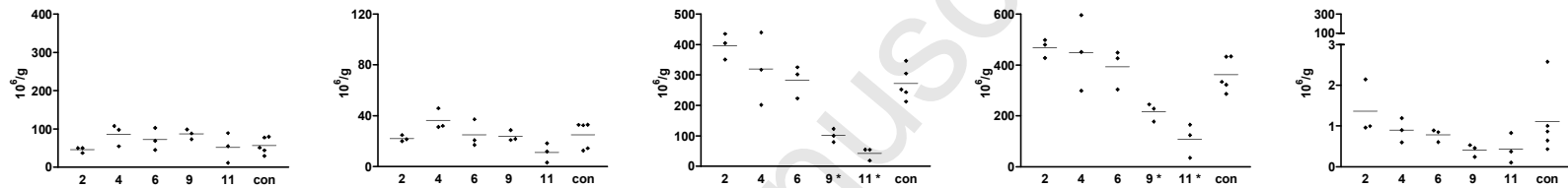
594

595 Changes in percentage values of lymphocyte subsets in draining popliteal lymph nodes (DLN), contralateral popliteal lymph nodes (CLN),  
596 peripheral blood (PB), spleen (SPL) and thymus (THY) of rabbits 1, 2, 6, 9 and 11 days after intradermal inoculation of 1000 TCID<sub>50</sub> MXV strain  
597 Lausanne. The parameters with found significant differences ( $P<0.05$  and  $P<0.01$ ) in comparison with control (con) uninfected rabbits are  
598 marked \* and \*\* respectively.

599 Table 2.



## THY



600 Changes in absolute numbers of lymphocyte subsets in draining popliteal lymph nodes (DLN), contralateral popliteal lymph nodes (CLN),  
 601 peripheral blood (PB), spleen (SPL) and thymus (THY) of rabbits 1, 2, 6, 9 and 11 days after intradermal inoculation of 1000 TCID<sub>50</sub> MXV strain  
 602 Lausanne. The parameters with found significant differences (P<0.05 and P<0.01) in comparison with control (con) uninfected rabbits are  
 603 marked \* and \*\* respectively.

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612 Table 3.  
613

organ		2 day	4 days	6 days	9 days	11 days	control
DLN	CPM-control	79 ± 26	56 ± 25	124 ± 54	202 ± 119	134 ± 14	83 ± 49
	CPM-ConA	17407 ± 4595	277 ± 119*	2963 ± 3408*	4056 ± 1877*	3276 ± 2659*	21892 ± 7734
	SI	224 ± 32	6 ± 34*	20 ± 17*	54 ± 46*	24 ± 20*	313 ± 169
CLN	CPM-control	51 ± 9	97 ± 73	105 ± 33	250 ± 180	136 ± 72	102 ± 28
	CPM-ConA	25951 ± 3501	19546 ± 5948	6643 ± 5127*	10246 ± 8521*	10537 ± 2697*	31139 ± 10755
	SI	525 ± 155	269 ± 170	60 ± 43*	36 ± 31*	85 ± 23*	313 ± 91
PB	CPM-control	175 ± 97	121 ± 82	191 ± 104	37 ± 23	127 ± 86	239 ± 210
	CPM-ConA	29398 ± 11623	19789 ± 5616	8102 ± 6635	520 ± 46*	835 ± 183*	25260 ± 17967
	SI	190 ± 102	200 ± 81	47 ± 33	18 ± 8*	10 ± 8*	144 ± 110
SPL	CPM-control	212 ± 100	295 ± 121	241 ± 116	272 ± 138	325 ± 227	243 ± 111
	CPM-ConA	22012 ± 10259	13235 ± 5474	6183 ± 1818*	2078 ± 1635*	2523 ± 1137*	17870 ± 9271
	SI	127 ± 97	47 ± 22	23 ± 12*	8 ± 4*	10 ± 7*	75 ± 33
MLN	CPM-control	44 ± 20	67 ± 37	44 ± 36	36 ± 7	26 ± 10	45 ± 25
	CPM-ConA	31901 ± 4623	11959 ± 907	6181 ± 3219	4719 ± 3044	1179 ± 1487*	16829 ± 9110
	SI	834 ± 390	248 ± 196	324 ± 182	140 ± 96	44 ± 23*	430 ± 235
THY	CPM-control	35 ± 9	56 ± 50	51 ± 34	40 ± 4	27 ± 15	37 ± 1
	CPM-ConA	6134 ± 4703	2968 ± 1609	1753 ± 1710	4503 ± 1698	632 ± 585	9143 ± 1112
	SI	203 ± 181	101 ± 94	74 ± 50	112 ± 33	39 ± 26	245 ± 174

614 Mitogen-driven proliferation activity of lymphocytes in various lymphoid organs in rabbits 1, 2, 6, 9 and 11 days after intradermal inoculation of  
615 1000 TCID<sub>50</sub> MXV strain Lausanne. Data is expressed as mean ± S.D. Counts per minute of non-stimulated lymphocytes (CPM-control); counts  
616 per minute of lymphocytes stimulated by 10 µg/ml concavalin A mitogen (CPM-ConA); stimulation indexes (SI); draining popliteal lymph nodes  
617 (DLN), contralateral popliteal lymph nodes (CLN), peripheral blood (PB), spleen (SPL), mesenteric lymph nodes (MLN) and thymus (THY). The  
618 parameters with found significant difference (P<0.05 or P<0.01) in comparison with untreated rabbits are marked \* or \*\* respectively.  
619

620 Table 4.  
621

	2	4	6	9	11
PBMC	neg	$2.3 \times 10^3 - 4.7 \times 10^4$	$1.5 \times 10^5 - 6.5 \times 10^5$	$3.2 \times 10^4 - 4.1 \times 10^4$	$2.4 \times 10^4 - 4.3 \times 10^4$
Draining LN	$3.4 \times 10^3 - 2.3 \times 10^5$	$2.1 \times 10^8 - 1.2 \times 10^9$	$1.4 \times 10^9 - 2.3 \times 10^9$	$1.1 \times 10^9 - 1.2 \times 10^9$	$3.9 \times 10^8 - 9.6 \times 10^8$
Contralateral LN	neg	$5.4 \times 10^4 - 5.6 \times 10^5$	$1.5 \times 10^5 - 6.8 \times 10^6$	$1.5 \times 10^7 - 2.1 \times 10^7$	$6.3 \times 10^4 - 6.3 \times 10^6$
Spleen	neg	$1.3 \times 10^5 - 4.1 \times 10^5$	$3.6 \times 10^4 - 5.8 \times 10^7$	$3.5 \times 10^4 - 4.8 \times 10^4$	$2.0 \times 10^4 - 8.4 \times 10^4$
Mesenteric LN	neg	$7.2 \times 10^3 - 8.4 \times 10^4$	$2.7 \times 10^4 - 5.2 \times 10^5$	$1.2 \times 10^4 - 1.2 \times 10^6$	$8.4 \times 10^2 - 7.6 \times 10^4$
Thymus	neg	$5.2 \times 10^3 - 1.5 \times 10^4$ •	$3.7 \times 10^3 - 1.1 \times 10^6$	$5.7 \times 10^4 - 5.5 \times 10^5$	$6.4 \times 10^4 - 5.1 \times 10^7$
Skin (inoculation site)	$2.9 \times 10^5 - 4.8 \times 10^8$	$2.3 \times 10^8 - 7.8 \times 10^8$	$1.8 \times 10^8 - 7.8 \times 10^8$	$6.3 \times 10^8 - 1.1 \times 10^9$	$5.6 \times 10^8 - 2.7 \times 10^9$
Skin (distal)	neg	$3.3 \times 10^2 - 4.4 \times 10^3$ •	$4.4 \times 10^2 - 6.5 \times 10^4$	$2.6 \times 10^5 - 1.7 \times 10^9$	$4.8 \times 10^7 - 5.6 \times 10^8$
Eyelid	neg	$5.9 \times 10^{2*}$	$1.9 \times 10^3 - 2.7 \times 10^7$	$3.2 \times 10^8 - 3.3 \times 10^9$	$3.1 \times 10^8 - 6.9 \times 10^8$
Lung	neg	$1.7 \times 10^4 - 1.0 \times 10^5$	$1.0 \times 10^5 - 4.9 \times 10^6$	$1.1 \times 10^6 - 3.9 \times 10^7$	$6.1 \times 10^5 - 2.5 \times 10^6$
Liver	neg	$3.4 \times 10^3 - 4.1 \times 10^3$	$2.0 \times 10^3 - 7.6 \times 10^4$	$7.9 \times 10^3 - 2.1 \times 10^4$	$7.3 \times 10^3 - 3.9 \times 10^4$
Kidney	neg	$3.8 \times 10^2 - 7.6 \times 10^3$	$1.0 \times 10^4 - 3.4 \times 10^5$	$7.0 \times 10^4 - 1.3 \times 10^5$	$6.0 \times 10^3 - 4.3 \times 10^4$
Testis	neg	neg	$9.4 \times 10^2 - 2.3 \times 10^3$	$2.0 \times 10^7 - 7.4 \times 10^8$	$1.2 \times 10^9 - 2.0 \times 10^9$
Myxomas	-	-	$1.4 \times 10^8 - 1.6 \times 10^9$	$2.1 \times 10^7 - 2.3 \times 10^9$	$6.9 \times 10^8 - 2.1 \times 10^9$

622  
623 Numbers of MXV genome copies in 1g of tissue on day 2, 4, 6, 9 and 11 after inoculation of 1000 TCID<sub>50</sub> MXV strain Lausanne. Organs, in  
624 which was virus detected in one of three rabbit are indicated \*, and organs, in which virus was detected in two of three rabbits are marked •.  
625 Testes were obtained from 2 rabbits because there were two males in each group.  
626  
627  
628  
629

630 **Figure captions:**

631 Figure 1.

632 Standard curve constructed as a relation of CP (crossing point) versus logcopy number of  
633 positive plasmid DNA standards from different dilutions (in the range of  $10^1$ - $10^9$  DNA copy  
634 equivalents) used for viral genome quantification.

635

636 Figure 2.

637 Localization of MXV virus in thymus. Left - immunohistochemical staining of sections from  
638 thymus 6 day after inoculation of 1000 TCID<sub>50</sub> MXV strain Lausanne using specific anti-MXV  
639 mAb. Cortex (C) and medulla (M) are indicated, arrow indicates positive staining of MXV.  
640 Right - immunohistochemical staining of sections from thymus of uninfected rabbit at the  
641 same time after infection using specific anti-MXV mAb. Scale bars represent 100  $\mu$ m.

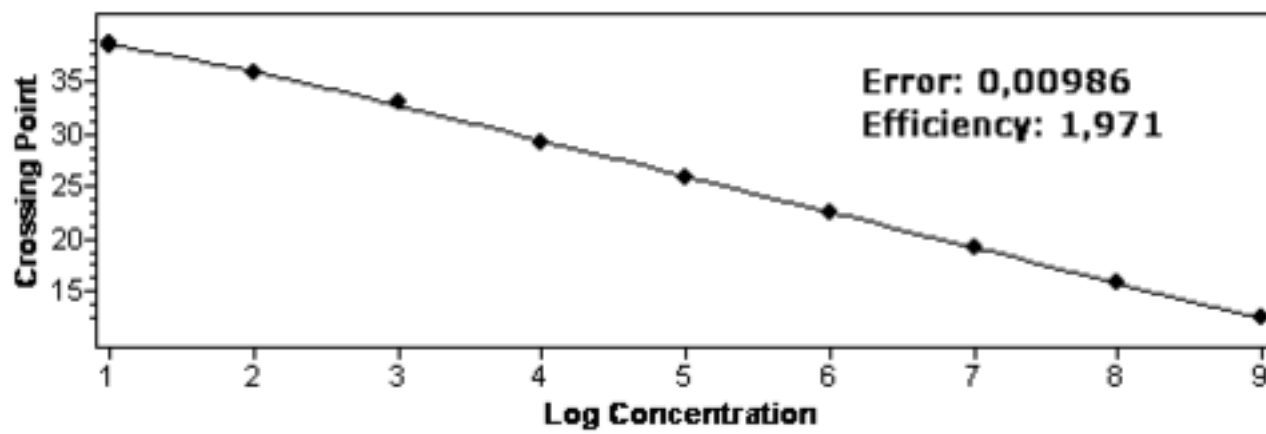
642

643 Figure 3.

644 IgM and IgG antibody response after intradermal inoculation of 1000 TCID<sub>50</sub> MXV strain  
645 Lausanne. Data is shown as mean  $\pm$  SD absorbances of IgM or IgG isotype on day 2, 4, 6, 9  
646 and 11 after infection. White bars represent absorbances of control uninfected animals; solid  
647 bars represent absorbances of MXV infected rabbits.

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**Standard Curve**





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