Intravaginal immunization using a novel antigen-releasing ring device elicits robust vaccine antigen-specific systemic and mucosal humoral immune responses

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1 **Intravaginal immunization using a novel antigen-releasing ring device**

2 **elicits robust vaccine antigen-specific systemic and mucosal humoral**

3 **immune responses**

4

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18 <u>**Short Title**</u>: Immunity generated by intravaginal ring vaccine delivery

19

Abstract

The generation of effective levels of antigen-specific immunity at the mucosal sites of pathogen entry is a key goal for vaccinologists. We explored topical vaginal application as an approach to initiate local antigen-specific immunity, enhance previously existing systemic immunity or re-target responses to the mucosae. To deliver a protein vaccine formulation to the vaginal mucosal surface, we used a novel vaginal ring device comprising a silicone elastomer body into which three freeze-dried, rod-shaped, hydroxypropylmethylcellulose inserts were incorporated. Each rod contained recombinant HIV-1 CN54gp140 protein (167 μg) +/- R848 (167 μg) adjuvant. The inserts were loaded into cavities within each ring such that only the ends of the inserts were initially exposed.

Sheep received a prime-boost vaccination regime comprising intramuscular injection of 100 μg CN54gp140 + 200 μg R848 followed by three successive ring applications of one week duration and separated by one month intervals. Other sheep received only the ring devices without intramuscular priming. Serum and vaginal mucosal fluids were sampled every two weeks and analysed by CN54gp140 ELISA and antigen-specific B cells were measured by flow cytometry at necropsy. Vaccine antigen-specific serum antibody responses were detected in both the intramuscularly-primed and vaginal mucosally-primed groups. Those animals that received only vaginal vaccinations had identical IgG but superior IgA responses. Analysis revealed that all animals exhibited mucosal antigen-specific IgG and IgA with the IgA responses 30-fold greater than systemic levels. Importantly, very high numbers of antigen-specific B cells were detected in local genital draining lymph nodes.

We have elicited local genital antigen-specific immune responses after topical application of an adjuvanted antigen formulation within a novel vaginal ring vaccine release device. This regimen and delivery method elicited high levels of antigen-specific mucosal IgA and large

44   numbers of local antigen-reactive B cells, both likely essential for effective mucosal

45   protection.

Introduction

While it is relatively easy to elicit antigen-specific serum antibodies, it is much more difficult to establish meaningful levels of specific antibodies at mucosal surfaces, the major route of viral invasion. In this study, we sought to determine if mucosal vaccination using topical vaginal application could initiate local antigen-specific immunity, and/or enhance or re-target previously existing systemic immunity to the mucosae. Previous studies in mice have shown that vaccines generating high levels of systemic antigen-specific immunity can lead to the appearance of genital tract mucosal surface responses, derived from the systemic compartment [1-3]. Also in mice, studies have shown that direct mucosal vaccination via the intranasal, sublingual or intravaginal routes can efficiently enhance immune responses at other mucosal surfaces. This observation is generally attributed to common mucosal linkage, but this distribution of mucosal associated responses has been found to be very weak or absent in larger animals, and particularly man [4-7]. In an effort to generate local mucosal immunity, a number of researchers have explored genital tract targeted vaccination to establish regional vaccine specific immunity by injecting genital tract associated lymph nodes or inoculating the vagina mucosae topically. Compared with parenteral immunisations, these mucosal-directed interventions elicited higher levels of both B and T cell vaccine specific immune responses [8, 9].

Polymeric intravaginal rings have a long history of use in providing controlled release of small-molecule therapeutics for hormonal contraception, hormone replacement therapy, HIV prevention and other clinical indications within women's healthcare [10-13]. The application of vaginal ring technology to controlled release of macromolecules, including protein antigens, is considerably more challenging owing to the thermal instability of proteins under the processing conditions commonly used to manufacture rings and the limited permeability

70 of proteins in the polymeric materials. Nonetheless, a small number of papers have been

71 published reporting ring technologies offering sustained/controlled release of macromolecular

72 therapeutic agents [14-16]. We previously reported a silicone elastomer vaginal ring device

73 comprising a one-piece ring body into which various drug-loaded inserts could be placed

74 [15]. The body of this ring device had a similar design to that of the matrix-type dapivirine-

75 releasing vaginal ring being developed for HIV prevention by the International Partnership

76 for Microbicides [17-19], except for the inclusion of three small cavities for placement of the

77 protein drug delivery inserts. With this ring design, it is possible to deliver combinations of

78 macromolecular and small molecule therapeutic agents (e.g. HIV antigen + HIV microbicide

79 combinations) at independently-controlled release rates. In this study, we sought to establish

80 whether this vaginal ring device could be used to deliver recombinant HIV-1 envelope

81 protein CN54gp140 as a vaccine cargo that could be 'seen' by the immune monitoring system

82 of the female sheep genital tract. The sheep vagina and cervix are anatomically and

83 histologically similar and of comparative dimensions to the human female genital tract and

84 have been used to test the delivery and pharmacokinetics of a variety of anti-HIV retrovirals

85 and contraceptives within vaginal ring devices [20-22]. We also examined the role of an

86 adjuvant in eliciting immune responses, its effect on the vaginal vault and detailed the

87 relative antigen-specific humoral immune responses in the mucosal and systemic

88 compartments.

89   **Results**

90

91   *Vaccine Carrier System: Release of Vaccine Antigen*

92   We first assessed the release kinetics of the CN54gp140 vaccine antigen from covered rod-

93   type devices comprising a hydroxypropyl methylcellulose (HPMC) + CN54gp140 ± R848

94   freeze-dried insert contained within a silicone elastomer tube. The recombinant HIV

95   Envelope (Env) protein was released into 2 mL of either PBS (pH 7.2) or a simulated vaginal

96   fluid (SVF; pH 4.2) to model release within the vaginal vault. CN54gp140 protein released

97   into the media was quantified using a specific capture ELISA with a known concentration

98   CN54gp140 standard curve.
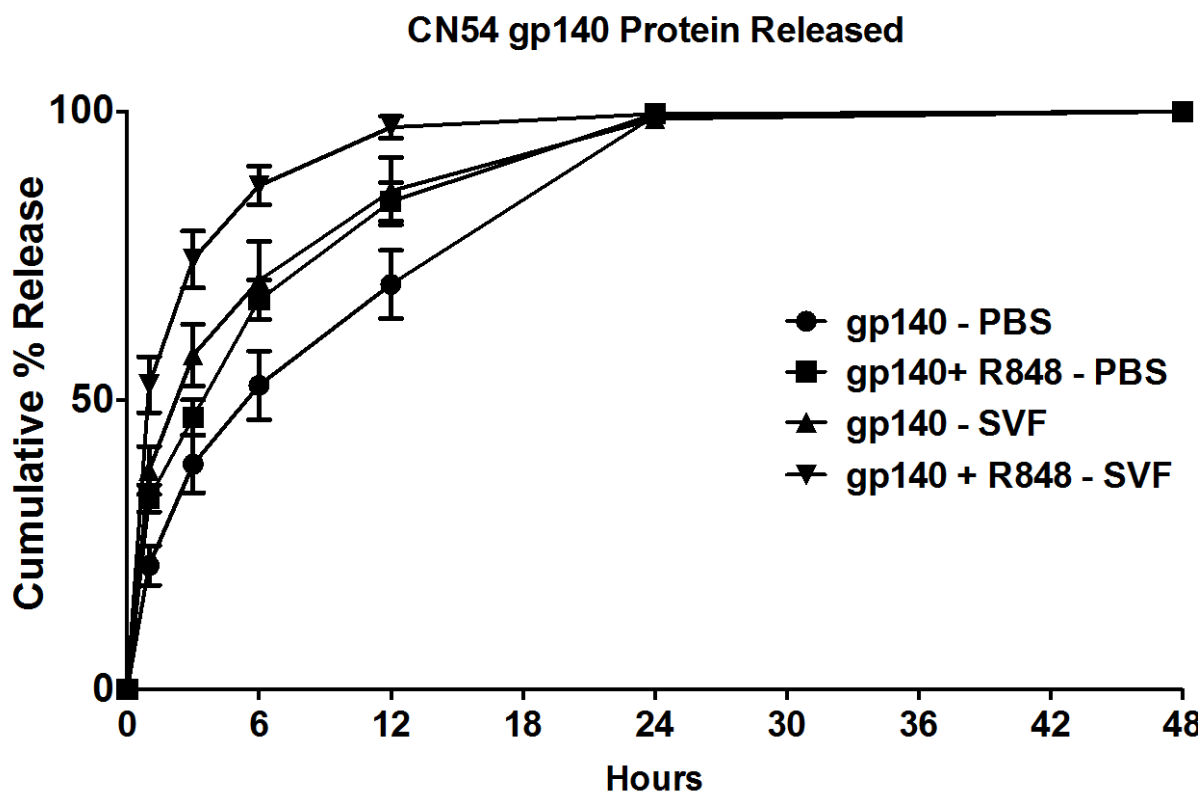
99

100   **Figure 1: CN54gp140 in vitro release profile.** Individual freeze-dried silicone elastomer
101   rods were laced into 2 mL release media (PBS, pH 7.2 or SVF, pH 4.2). Rods contained
102   CN54gp140 alone (167 µg) or CN54gp140 + R848 (167 µg each). The amount of
103   CN54gp140 released into the 2 mL media was measured by quantitative ELISA and plotted

as cumulative % release. Plot symbols represent the mean ± standard deviation of six replicates.

The CN54gp140 protein was released in a sustained manner over a period of 24 h, with >99 % of the total protein being released by all rod formulations in either PBS or SVF at the 24 h sample timepoint (**Fig. 1**). However, there were clear differences in the release kinetic depending on the nature of the release medium and whether the formulation contained R848. Freeze-dried rods released the protein more quickly into the low pH SVF media than PBS and rods containing R848 were more easily reconstituted in solution. When incubated in SVF, CN54gp140 + R848 rods released 88% of the recombinant protein within 6 h and 98% in 12 h whereas in PBS pH 7.2 67% release is achieved in 6 h and 84% in 12 h. Freeze-dried CN54gp140 alone released 71% (6 h) and 86% (12 h) of the recombinant protein when incubated in SVF, and 53% (6 h) and 70% (12 h) when incubated in PBS pH 7.2 (**Fig. 1**).

Mixed route prime-boost combinations with TLR7/8 adjuvantation elicit high levels of CN54gp140-specific humoral immunity.

We initiated a series of vaccinations to assess the ability of silicone elastomer intravaginal ring devices to deliver recombinant antigen directly to the cervicovaginal mucosa and elicit systemic or local vaccine antigen-specific humoral immunity. Sheep received either an intramuscular (IM) priming vaccination of 100 µg CN54gp140 + 200 µg R848 adjuvant followed by three intravaginal ring immunisations at monthly intervals, or intravaginal rings without any prior IM priming vaccination. The intravaginal rings all contained 500 µg recombinant CN54gp140 antigen, matched to previous intravaginal doses used in humans [23] . Two of the four animal groups received intravaginal rings that also contained the TLR7/8 adjuvant R848 (groups 1 and 2) while the other two groups received rings containing CN54gp140 antigen without any R848 adjuvantation (groups 3 and 4; **Table 1**). A final
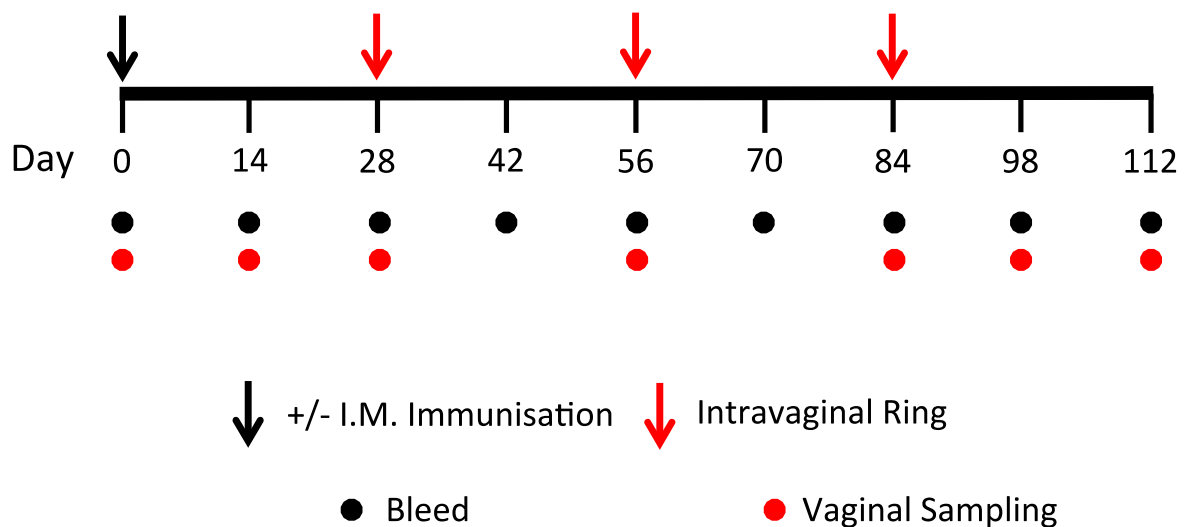
129   control group of sheep received four IM immunisations at monthly intervals (group 5; **Table**

130   **1**). The sheep were bled every two weeks and vaginal samples were taken at regular intervals,

131   according to the immunisation and sampling schedule as shown (**Fig. 2**).

132

133   **Table 1:** Sheep groups and immunization schedule (n = 5). IM – intramuscular
134   administration of 100 µg CN54gp140 + 200 µg R848; IVag – intravaginal administration of
135   500 µg CN54gp140 ± 500 µg R848.

Group	Day of Immunization			
	0	28	56	84
1	IM	IVag / gp140 + R848	IVag / gp140 + R848	IVag / gp140 + R848
2	None	IVag / gp140 + R848	IVag / gp140 + R848	IVag / gp140 + R848
3	IM	IVag / gp140 only	IVag / gp140 only	IVag / gp140 only
4	None	IVag / gp140 only	IVag / gp140 only	IVag / gp140 only
5	IM	IM	IM	IM

136

| Day | 0 | 14 | 28 | 42 | 56 | 70 | 84 | 98 | 112 |

↓ +/- I.M. Immunisation   ↓ Intravaginal Ring

● Bleed   ● Vaginal Sampling

137

138   **Figure 2: Vaccination, bleed and vaginal sampling schedule.** Two-year-old sheep were
139   immunised at monthly intervals using intravaginal rings containing CN54gp140 HIV Env
140   antigen +/- R848 adjuvant. The rings were maintained in place at the cervicovaginal area for
141   1 week before removal. Some sheep received a prior IM vaccination one month before the
142   insertion of the rings. Animals were bled weekly and had vaginal mucosal antibody Weck-
143   Cel® swabs taken at the times indicated.

144

145   Sheep that received an IM prime with adjuvant and were then boosted with an

146   intravaginal ring device containing adjuvanted antigen exhibited strong generation of

147   systemic antigen-specific humoral responses to the HIV Env CN54gp140 vaccine protein,

148   increasing mean blood serum antigen-specific IgG antibody levels from 6.35 µg/mL (day 28

149   after IM prime) to 1375 µg/mL (day 42, two weeks after the IVag ring boost), a 216-fold

150   increase (**Fig. 3a, Table 2**). Indeed, the gp140-Specific IgG responses initially elicited by the

151   R848 adjuvanted IM injection were enhanced after each successive ring application reaching

152   peaks of 2228 µg/mL at day 70, two weeks after the second ring application, and 2683 µg/mL

153   at day 98, two weeks after the third and final ring was inserted. In animals that did not receive

154   any IM prime (group 2 in **Table 1**) the first ring application was able to generate antigen-

155   specific humoral immunity that was detectable in the serum. Interestingly, the second ring

156   application substantially boosted the serum IgG gp140-specific responses by 70-fold, from

157   10.7 µg/mL to 739 µg/mL at the peak response two weeks after insertion of the IVag rings

158   and the third ring application in these IM unprimed animals further boosted the serum

159   antigen-specific IgG response to 3070 µg/mL at two-weeks post application, levels

160   equivalent to those measured in the group 1 IM primed animals (**Fig. 3a, Table 2**). A

161   comparison of the antigen-specific IgG antibody levels between the IM primed (group 1) and

162   unprimed (group 2) animals revealed that the peak levels on day 28 and day 42 were

163   statistically different (**$p = 0.0079$) with there being no difference at any other time.
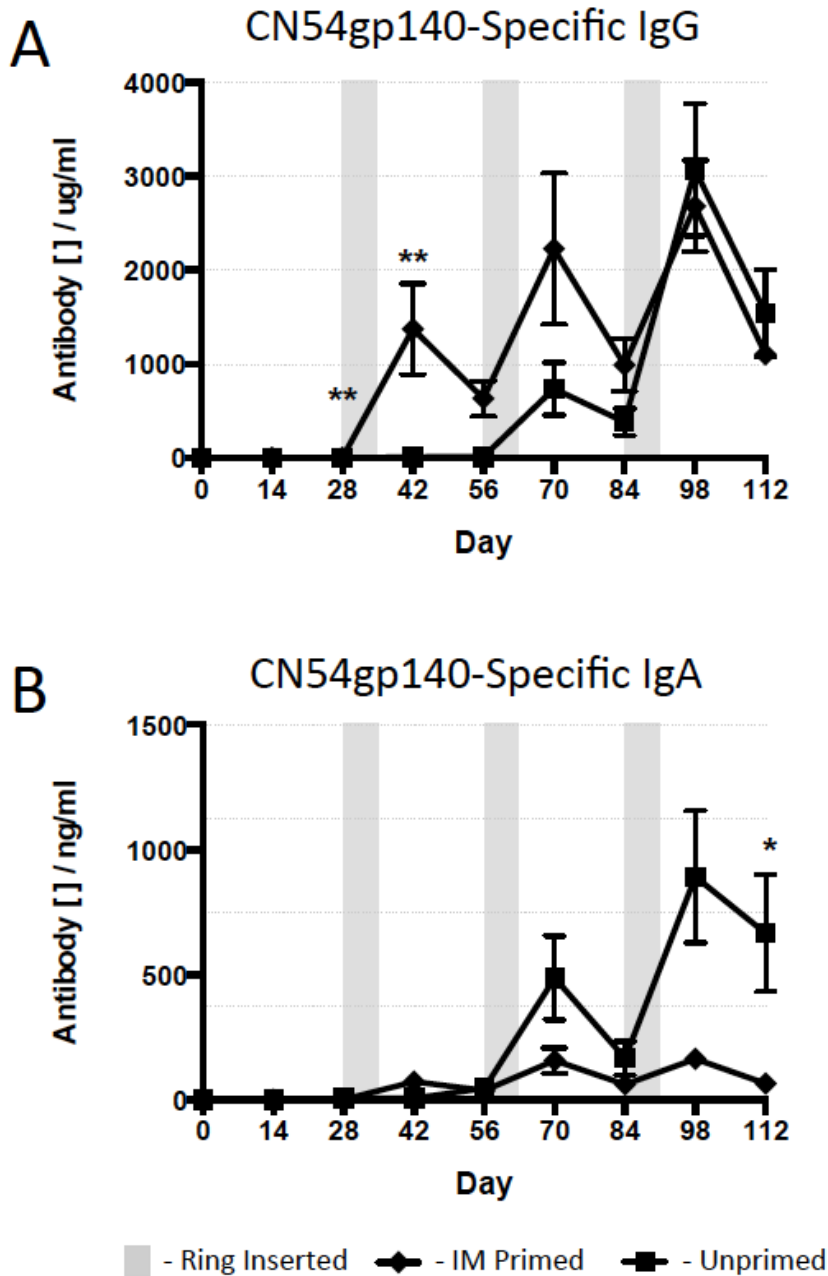
A **CN54gp140-Specific IgG**

B **CN54gp140-Specific IgA**

- Ring Inserted ◆ - IM Primed ■ - Unprimed

164

Figure 3: CN54gp140 antigen-specific antibody serum responses to 500 µg vaccine antigen in the presence of 500 µg R848 adjuvant per vaginal ring. IM Primed – animals received an IM prime at day 0 then three vaginal ring placements, each retained *in situ* for 7 days, at monthly intervals. Unprimed – animals received the vaginal rings without a prior IM vaccination. The shaded areas indicate the 7-day periods during which a ring device was present in the sheep vagina. (A) Vaccine antigen-specific IgG and (B) IgA responses. Data compared with a Mann-Whitney test (n = 5 per group).

172

173 The systemic antigen-specific IgA achieved significantly higher levels in animals that did not

174 have any IM priming immunisation in comparison to those animals that had the potent

175 systemic prime (**Fig. 3b, Table 2**). At the end of the vaccination schedule, following three

176 separate intravaginal ring inoculations, the antigen-specific IgA present in the serum

177 compartment was significantly higher than in those animals that had received an IM prime

178 (*p = 0.0317), although levels were greater than 3 logs lower than that seen for specific IgG.

179 We next examined the antigen-specific immunity at the mucosal surface. We sampled the

180 vaginal mucosae at various times during the schedule and measured gp140-specific IgG and

181 IgA (**Fig. 4**). IM primed gp140-specific mucosal IgG responses were effectively enhanced by

182 intravaginal vaccination with the ring device that contained antigen adjuvanted with R848

183 and antibody levels were boosted by subsequent ring applications. The specific antibody

184 levels were statistically different between the IM primed and the unprimed groups at day 42,

185 2 weeks after and at day 56, one month after the insertion of the first ring (**Fig. 4a**; **p =

186 0.0079). There were no statistical differences at other sampling timepoints, and at the end of

187 the vaccination schedule (after three intravaginal ring inoculations) the CN54gp140 antigen-

188 specific antibody levels were equivalent (**Fig. 4a, Table 2**). Interestingly, antigen-specific

189 mucosal IgA followed the same pattern. Levels were statistically different after the first ring

190 application (day 56), where the IM primed group had higher levels in the vaginal vault, but

191 after the second and then the final ring vaccination the levels were equivalent. Importantly,

192 the levels of mucosal IgA were substantially higher at the mucosal surface compared to the

193 systemic serum compartment. Animals that had not been previously primed with an IM

194 vaccination had 20-fold higher IgA in the vaginal vault while those that had received an IM

195 prime followed by immunisation with the vaginal rings exhibited a marked difference, with

196 the vaginal vault having 400-fold more specific antibody when compared to the serum

197 responses (**Figs. 3b, 4b and Table 2**).

A CN54gp140-Specific IgG

B CN54gp140-Specific IgA
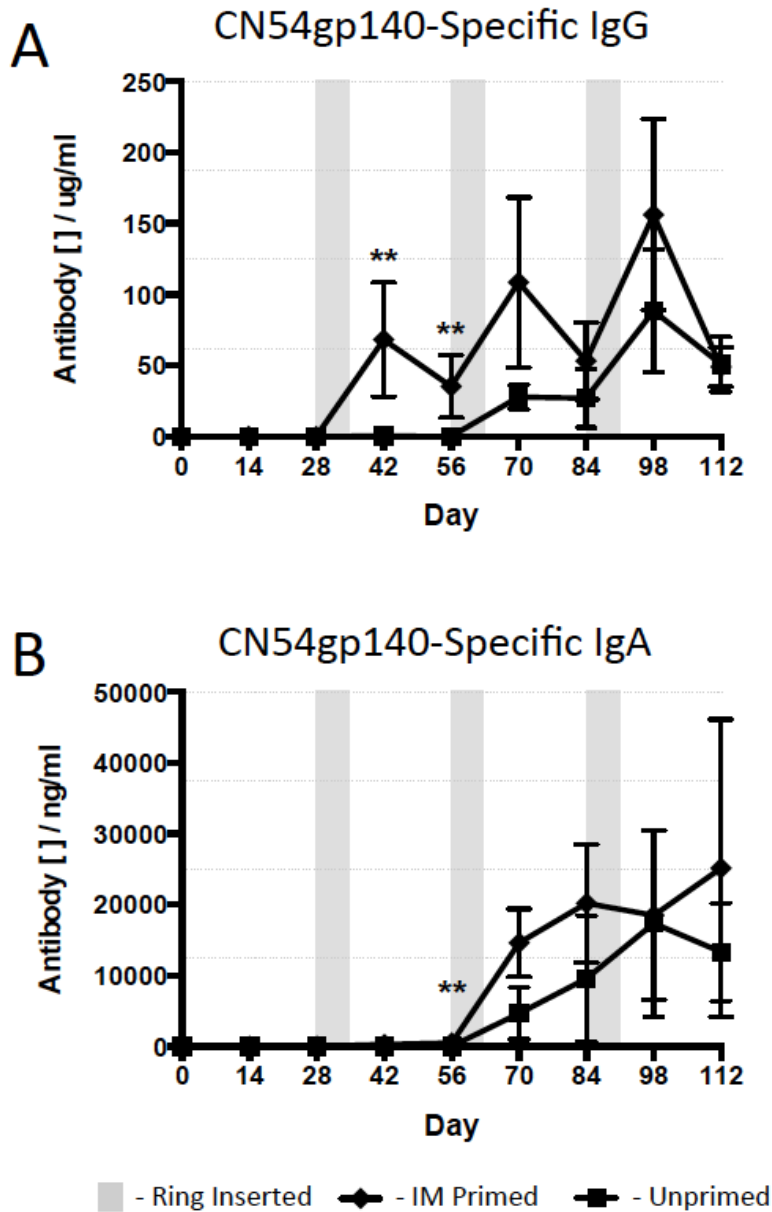
■ - Ring Inserted ◆ - IM Primed ■ - Unprimed

198

199 **Figure 4: CN54gp140 antigen-specific antibody mucosal responses to 500 μg vaccine**
200 **antigen in the presence of 500 μg R848 adjuvant per vaginal ring**. IM Primed – animals
201 received an IM prime at day 0 then three vaginal ring placements, each retained *in situ* for 7
202 days, at monthly intervals. Unprimed – animals received the vaginal rings without a prior IM
203 vaccination. The shaded areas indicate the 7-day period during which a ring device was
204 present in the sheep vagina. (A) Vaccine antigen-specific mucosal IgG and (B) mucosal IgA
205 responses. Data compared with a Mann-Whitney test (n = 5 per group).
206
207

208

209 **Table 2:** Serum and mucosal IgG and IgA levels (μg/mL) 2 weeks after each vaginal ring
210 insertion (Days 42, 70 and 98) and at end of vaccination schedule (Day 112) (+/- SD in
211 parentheses). IM – intramuscular administration of 100 μg CN54gp140 + 200 μg R848; IVag
212 – intravaginal administration of 500 μg CN54gp140 ± 500 μg R848. A dash within a cell
213 indicates that antibody levels were not detected (n = 5 per group).

Sheep group	Immunization schedule*	Antibody response	Sample Day			
			42	70	98	112
1	IM + 3x IVag (gp140+R848)	IgG systemic	1375.9 (482.6)	2228.5 (804.8)	2682.7 (488.2)	1103.9 (112.6)
		IgG mucosal	68.3 (40.2)	108.6 (53.1)	156.1 (67.0)	49.1 (14.1)
		IgA systemic	0.071 (0.024)	0.157 (0.05)	0.163 (0.061)	0.063 (0.029)
		IgA mucosal	0.286 (0.097)	14.6 (4.8)	18.5 (11.9)	25.13 (20.9)
2	3x IVag only (gp140+R848)	IgG systemic	10.65 (7.76)	738.4 (279.5)	3069.6 (706.2)	1543.4 (464.9)
		IgG mucosal	0.37 (0.34)	27.9 (8.6)	88.45 (43.2)	50.9 (19.3)
		IgA systemic	0.005 (0.005)	0.486 (0.167)	0.891 (0.264)	0.666 (0.234)
		IgA mucosal	− (−)	4.64 (3.7)	17.35 (13.19)	13.32 (6.93)
3	IM + 3x IVag (gp140 only)	IgG systemic	1.08 (0.43)	8.63 (5.3)	7.57 (3.8)	4.38 (2.0)
		IgG mucosal	−	−	−	−
		IgA systemic	−	−	−	−
		IgA mucosal	−	−	−	−
4	3x IVag only (gp140 only)	IgG systemic	0.382 (0.22)	0.255 (0.05)	0.35 (0.09)	0.726 (0.53)
		IgG mucosal	−	−	−	−
		IgA systemic	−	−	−	−
		IgA mucosal	−	−	−	−
5	IM only (gp140+R848)	IgG systemic	735.0 (269.9)	1971.6 (568.2)	1272.1 (425.3)	680.1 (189.2)
		IgG mucosal	3.183 (1.09)	8.489 (3.07)	5.992 (2.16)	4.465 (1.3)
		IgA systemic	−	−	−	−
		IgA mucosal	−	−	−	−

214

215 ***Sheep immunised with intravaginal rings without local mucosal R848 adjuvantation fail to***

216 ***elicit significant CN54gp140-specific humoral immunity.***

217 In order to determine the contribution of the R848 TLR7/8 adjuvant to the size and kinetics of

218 the immune response, we next examined the elicitation of antigen-specific serum and

219 mucosal IgG and IgA in animals that were immunised with intravaginal rings that contained

220 antigen but no R848 adjuvant. Sheep received an R848 adjuvanted IM prime and were then

221   boosted with a vaginal ring device containing only gp140 antigen. The systemic CN54gp140-

222   specific IgG levels were very low, though the response was enhanced after the second ring

223   application but not after third. Animals that had not received an IM priming vaccination had

224   statistically higher levels of mucosal IgG than primed animals at day 70 ($p = 0.0159$; two

225   weeks after the 2nd ring insertion), day 84 ($p = 0.0075$; four weeks after the second ring

226   insertion) and at day 98 ($p = 0.0317$; two weeks after the third ring insertion. However, peak

227   systemic responses in these animals that did not receive the R848 adjuvant with the

228   CN54gp140 vaccine in the ring were 300-fold lower than those seen when using rings

229   containing R848 (**Fig 3a**). In these animals no antigen-specific IgA was detected throughout

230   the vaccination schedule (**Fig. 5a, b and Table 2**).

231
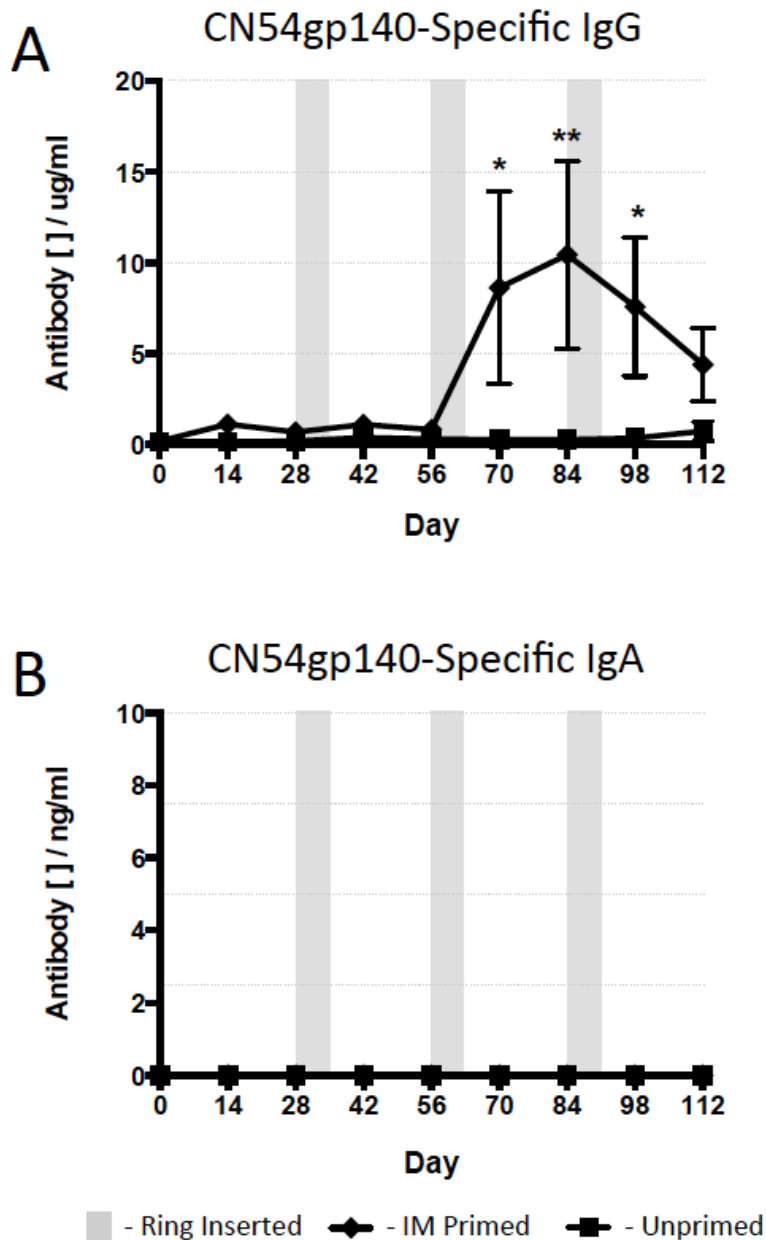
A CN54gp140-Specific IgG

B CN54gp140-Specific IgA

- Ring Inserted   - IM Primed   - Unprimed

232

Figure 5: CN54gp140 antigen-specific antibody serum responses to 500 μg vaccine antigen in the absence of R848 adjuvantation. IM primed – animals received an IM prime at day 0 then three vaginal ring placements, each retained *in situ* for 7 days, at monthly intervals. Unprimed – animals received the vaginal rings without a prior IM vaccination. The shaded areas indicate the 7-day periods when the vaginal ring was present. (A) Vaccine antigen-specific IgG and (B) IgA responses. Data compared with a Mann-Whitney test (n = 5 per group).

242   Animals that did not receive an IM prime mounted only barely detectable antigen-specific

243   IgG responses that were 2000-fold lower than animals that also received no IM prime but

244   were exposed to R848 adjuvanted protein within the intravaginal rings (**Figs. 3 and 5**). There

245   were no detectable antigen-specific IgG or IgA mucosal antibody responses in any of the

246   animals that were vaccinated with intravaginal rings containing antigen without R848

247   adjuvant. At necropsy, there were no antigen-reactive cells in any of the compartments tested

248   (**Table 2**).

249

250   As a comparison, animals that received four IM vaccinations at monthly intervals with R848

251   adjuvanted CN54gp140 protein produced a good systemic IgG response reaching a maximum

252   peak two weeks after the third vaccination with a mean of 1972 µg of CN54gp140 antigen-

253   specific IgG (**Fig. 6**). The subsequent fourth vaccination failed to augment this peak response

254   further. There was no systemic or mucosal antigen-specific IgA detected in these animals

255   during the entire vaccination schedule (**Fig. 6**). Interestingly, systemic responses (IgG and

256   IgA) were lower than those seen in animals receiving mucosal adjuvanted rings (**Table 2**).
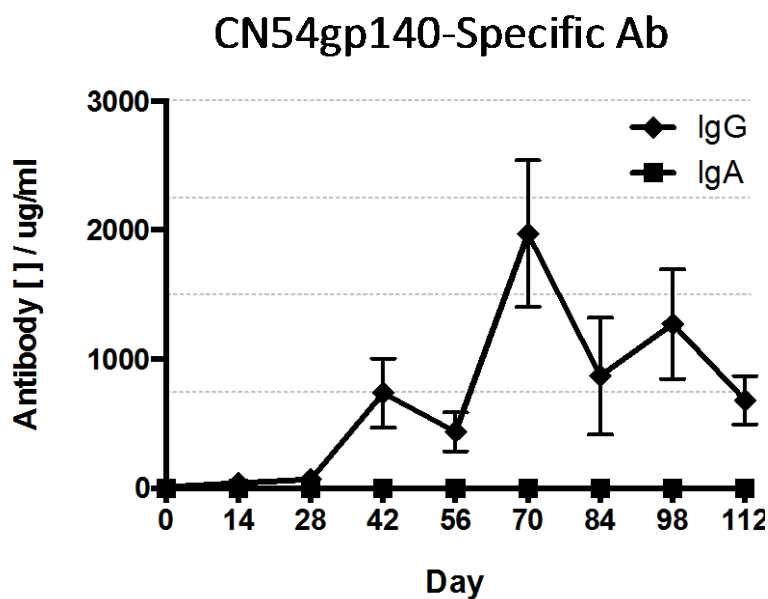
257

258   **Figure 6: CN54gp140 antigen-specific serum antibody responses in animals that**
259   **received only IM vaccinations**. Animals received an IM prime of 100 µg vaccine antigen

260 plus 200 μg R848 adjuvant at day 0 then three IM boost vaccinations with the same vaccine
261 composition at monthly intervals. Vaccine antigen-specific IgG and IgA responses (n = 5 per
262 group).
263
264 Mucosal antigen-specific IgG was detected at low levels in the vaginal mucosal samples and

265 followed the profile of the systemic serum IgG precisely indicating the antibody present was

266 derived from serum exudate (**Fig. 7**). Again, there was no detectable antigen-specific IgA in

267 the vaginal samples taken throughout the vaccination regimen (**Fig. 7**).
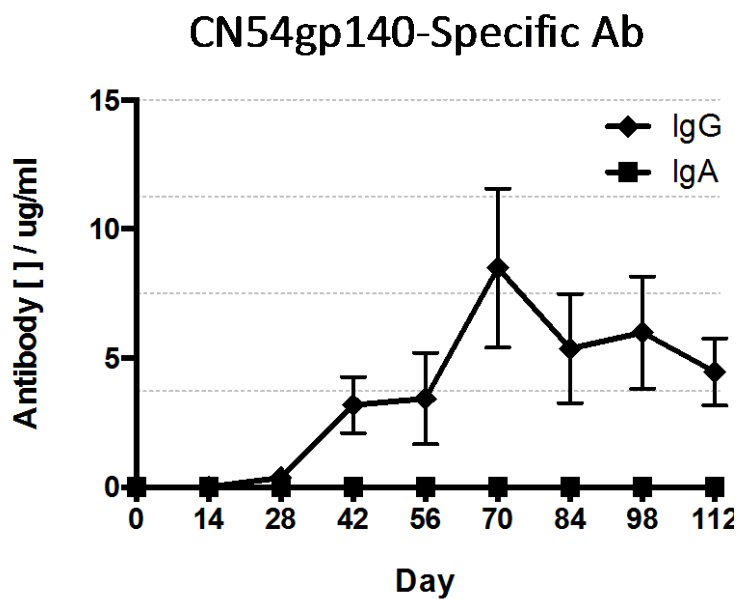
268

269 **Figure 7: CN54gp140 antigen-specific vaginal antibody responses in animals that**
270 **received only IM vaccinations**. Animals received an IM prime of 100 μg vaccine antigen
271 plus 200 μg R848 adjuvant at day 0 then three IM boost vaccinations with the same vaccine
272 composition at monthly intervals. Vaccine antigen-specific mucosal IgG and mucosal IgA
273 responses (n = 5 per group).
274
275

276 *Sheep that received intravaginal rings containing both CN54gp140 and R848*

277 *adjuvantation contained large numbers of antigen-binding cells in the local vaginal*

278 *draining LN*

279 The notable observation that the vaginal vault contained dramatically higher levels of

280 antigen-specific antibody in either unprimed or IM primed animals that received intravaginal

281 rings containing CN54gp140 + R848 led us to hypothesise that this local production may be

282 reflective of local B cell expansion and residency in the lymph nodes draining the female

283 genital tract. At the end of the immunogenicity study, we inserted a new ring (500 μg

284 CN54gp140 + 500 μg R848) into each sheep at day 112 before the animals were necropsied

285 one week later. Our aim was to determine the inflammation caused by the ring after a typical

286 7-day application and also to examine the local lymph nodes immune response at the same

287 time as responses in the systemic compartments. At necropsy, we removed the external iliac

288 lymph nodes, spleen and peripheral blood from each sheep, processed to isolate lymphocytes

289 and then incubated with a fluorochrome-labeled vaccine antigen CN54gp140-Alexa 647 or a

290 control Tetanus Toxoid antigen (TT)-Alexa 647. B lymphocytes expressing an antigen

291 receptor on their surface should bind the labeled antigen and the number and intensity of the

292 bound labeled antigen positive cells was determined using flow cytometry. While the

293 peripheral blood cells showed very low antigen-specific staining and the splenocytes

294 demonstrated slightly more, the level of specific staining in the draining lymph nodes clearly

295 revealed a sizeable population of cells with cell surface receptors that specifically bound the

296 vaccine CN54gp140 antigen in comparison to the control (**Fig. 8**). In the absence of R848 the

297 vaginal ring containing the unadjuvanted CN54gp140 protein did not elicit this population of

298 antigen binding cells in the vaginal draining LN (Supplementary Material S1).
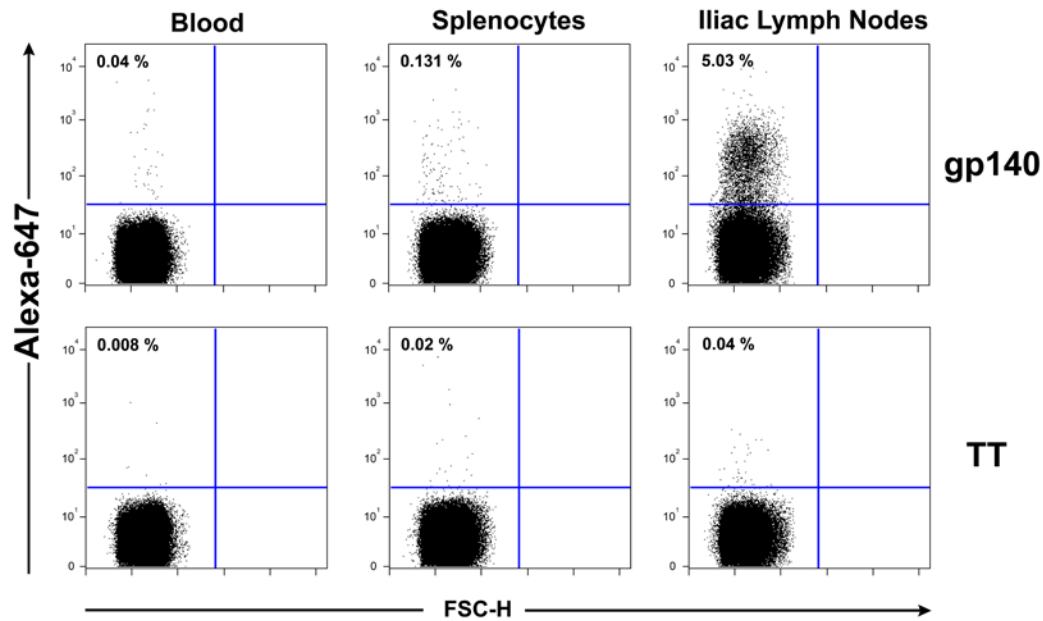
299

Figure 8: Elicitation of Antigen-Specific B cells in vaginal local draining lymph nodes.
Cells were harvested from the peripheral blood, the spleen or the vaginal external iliac lymph
nodes and stained with Alexa-647 conjugated recombinant CN54gp140 protein.

Nine out of the 10 animals that received intravaginal rings containing antigen and R848

adjuvant had large populations of antigen-specific cells in the draining external iliac lymph

nodes (**Table 3**). We statistically compared the levels of antigen-stained cells between the

blood, spleen and the vaginal draining lymph node using an unpaired t tests with Welch's

correction for unequal variances and found that as expected there was no difference between

the levels of specific cells found in the blood and spleen for both groups of animals.

However, group 1 iliac LN contained statistically higher numbers of specific cells than both

the blood ($p = 0.0207$) and the spleen ($p = 0.0225$), and group 2 iliac LN also had higher

numbers though only just achieving statistical differences to the blood and the spleen ($p =$

0.0499).

Table 3: Mean percentage of background control subtracted antigen-specific cells in each
tissue (n=5 per group) (+/- SD in parentheses).

Sheep group	Mean % CN54gp140 specific cells		
	Blood	Spleen	Iliac LN
1	0.05 (0.012)	0.16 (0.078)	2.45 (0.802)
2	0.04 (0.014)	0.08 (0.028)	2.28 (0.839)
3	–	–	–
4	–	–	–

317

318 An important consideration when using intravaginal rings that release antigen and an

319 adjuvant at the mucosal surface is the degree of associated inflammation. Each ring that was

320 removed was examined for the presence of blood and none was found. At necropsy, each

321 vagina was removed and closely examined for redness, tissue pathology and inflammation.

322 The vaginal wall appeared normal with no apparent inflammation; a photograph showing a

323 typical vagina with the ring still in situ is shown (**Supplementary material S2**).

324

325

Discussion

Conventional vaginal ring devices fabricated from hydrophobic polymers provide controlled release of low molecular actives via a molecular permeation mechanism. This involves dissolution of the drug in the surrounding polymer, diffusion of the dissolved drug molecule to the ring surface, and then partitioning into the vaginal fluid. This simple permeation mechanism is generally not useful for release of biomolecules, since their hydrophilic character and high molecular weight result in poor solubility and diffusion through the polymeric material. In the past ten years, the application of ring technology to vaginal administration of HIV microbicide molecules has led to very considerable innovation in vaginal ring design, mostly aimed at providing viable formulation solutions for lead candidate microbicides having a very broad range of physicochemical properties [10]. However, despite these advances, the development of practical ring devices that are simple and inexpensive to manufacture and can offer clinically significant drug release rates remains a challenge [16]. Here, we opted to use a ring technology previously reported for the sustained release of a model protein and a monoclonal antibody [15]. This approach permitted the silicone elastomer ring body and the antigen-loaded freeze-dried rod inserts to be manufactured separately, thereby reducing exposure of the antigen to the high processing temperatures normally associated with ring manufacture and exploiting use of established lyophilization techniques for formulation of the antigen. With this ring design, it is also possible to easily vary the dose of the antigen by reducing or increasing the number of inserts, or to incorporate an antiretroviral microbicide, such as dapivirine, into the ring body to produce a combination microbicide-vaccine formulation.

This current study examined the potential of the vaginal mucosae as a site of initiation and/or enhancement of vaccine antigen-specific immune responses. We compared a direct

351 vaccination to a boost of an existing IM-elicited immune response using the vaginal ring

352 delivery device, with the expectation that direct delivery of antigen to the vaginal mucosae

353 alone might not have the potency to initiate a combined systemic and vaginal antigen-specific

354 immune response. Surprisingly, we discovered that vaginal vaccination alone was as potent

355 (group 2) as IM-prime, vaginal boost (group 1, **Table 2**), with R848 being necessary to

356 induce high levels of antigen-specific systemic and mucosal antibody responses. Importantly

357 vaginal vaccination with R848 was essential to induce localised mucosal responses. Further

358 studies are now required to address the issue of dose response in relation to optimization of

359 antigen:adjuvant ratios. Animals receiving only IM immunisation (group 5) expressed no

360 antigen specific vaginal IgA while IgG levels were $1/10^{th}$ of those seen in groups 1 and 2

361 (**Table 2**). These data are supported by the local expansion of specific B cells in lymph nodes

362 draining the genital tract. In the absence of defined B cell markers for sheep, the nature and

363 phenotype of local responding B and T cell responses will require further study in non-human

364 primate and/or clinical studies.

365 The lack of immune response to non-adjuvanted vaginal vaccination alone and very

366 limited response following an intramuscular prime (groups 3 and 4, **Table 2**) reflects human

367 clinical studies [24, 25]. Very few clinical studies have assessed response to adjuvanted

368 vaginal vaccination. In a previous human study using CN54gp140 and HSP70 as an adjuvant,

369 IVag administration failed to induce detectable systemic or mucosal antibody responses but

370 did induce adaptive CD4 and CD8 T-cell proliferative responses [9]. The difference in

371 response may reflect difference in the adjuvant potential of HSP70 and R848, the latter

372 associated with promoting mucosal IgA [6]. Indeed, two additional clinical studies of vaginal

373 immunisation performed using recombinant cholera toxin subunit B (CTB), were shown to

374 elicit vaginal and systemic antibodies [23, 26, 27]. This may reflect the "self-adjuvanting"

375 properties of CTB, known to be an effective mucosal adjuvant for other proteins [28]. None

376 of these studies assessed responses to vaccine delivered by vaginal rings that may offer

377 additional advantage through direct localized delivery over topical administration of an

378 aqueous formulation.

379   Both our data and previous observations in humans suggest that the inclusion of a

380 mucosal adjuvant is likely necessary to establish effective and long-lasting mucosal immune

381 responses. However, the downsides of using an inflammatory mediator in genital mucosal

382 tissue are clear – the recruitment and activation of CD4 T cells has the potential to increase

383 mucosal targets for HIV-1. Also, inflammation may compromise the integrity of the mucosal

384 barrier increasing risk of infection not only by HIV but also other sexually transmitted

385 infections (STIs), although an increase in mucosal expression of interferons by adjuvants may

386 protect against pathogen infection [29, 30]. Nevertheless, this potential inflammation is likely

387 to be short-lived. In this respect, the fact that no localised signs of inflammation were visually

388 apparent at the end of the study is encouraging. A major advantage of the ring device is that a

389 topical antiretroviral, such as the lead candidate microbicide dapivirine, can readily be co-

390 delivered to the precise site of any local inflammation, protecting the mucosae during the

391 vaccination window. In addition, the adjuvant quantity or adjuvant combinations can be

392 tailored to maximise the immune response while preventing unnecessary bystander

393 inflammation [2, 31, 32].

394   In summary, the ability to generate these vaccine-reactive B cells with local

395 cervicovaginal adjuvanted vaccine administration using a ring release delivery system is a

396 significant and important observation, with potential application to the future delivery

397 modalities for any vaccine against pathogens that enter through genitourinary mucosae.

398

399 **Conclusions**

400   In this present study we have explored an intravaginal ring device for administration of a

401   vaccine antigen at a cervicovaginal mucosal surface. We demonstrated that in the presence of

402   a potent adjuvant a local mucosal antibody response can be generated with high levels of

403   vaccine antigen-specific antibody and also that a population of vaccine antigen-reactive cells

404   are established within the local draining lymph nodes, ready to respond to any re-infection

405   with the same or similar pathogen. In this study, we used HIV Env gp140 as a model antigen

406   but our findings are likely to be applicable to any mucosal vaccine candidate and relevant to a

407   number of sexually transmitted diseases.

408

Materials and Methods

Ethics Statement

The animal studies were approved by the Ethical Review Board of St. George's, University of London where the experiments were carried out and work was performed in strict compliance with project and personal animal experimentation licences granted by the UK government in accordance with the Animals in Scientific Procedures Act (1986). Animals received minimal handling and their physical condition was monitored at least twice daily. All procedures were performed under isoflurane anaesthesia when appropriate, and all efforts were made to minimize suffering. There was a detailed protocol in place, as per requirement of the humane endpoints described in the animal licence, for early euthanasia in the event of onset of illness or significant deterioration in condition. For sheep the humane endpoints included; loss of appetite sufficient to lead to weight loss - the animals were monitored for weight weekly, loss of movement, sedentary state, calls of distress indicating pain or discomfort, bruising at site of blood withdrawal, excessive or uncontrolled bleeding from site of blood withdrawal, incontinence, breathing difficulty, infection or necrosis at site of sampling (leg vein and vaginal). The presence of one of these indicators led to an assessment by a veterinary surgeon and further welfare of the animals was directed by them. In the case of an emergency if an animal became seriously ill or injured at any point when they were on the designated premises then the animal would be first stunned by captive bolt and then killed by exsanguination before the animal regained consciousness (a non-schedule 1 method). If it were possible to handle the animal without causing it further stress and/or injury to it or staff then a schedule 1 method would be used. The captive bolt would be administered by a person licensed to use a captive bolt. One animal became ill and stopped eating during the

433 experiment, the animal was monitored by the onsite vet, but started losing weight and

434 exhibited a deteriorating condition. It was determined that the licence endpoint was likely to

435 be reached and so to prevent this occurrence and any potential suffering by the animal, it was

436 culled by the schedule 1 method of overdose of anaesthetic. Death was confirmed by

437 cessation of blood flow. All other animals enjoyed excellent health for the duration of the

438 experiment. At the end of the experiment all animals were culled using a schedule 1 method

439 and death confirmed before necropsy. Food and water were supplied ad libitum.

440

441 ***Recombinant proteins and R848***

442 HIV gp140, a trimeric gp140 clade C envelope (gp120 plus the external domain (ED) of

443 gp41) and designated CN54gp140, was produced as a recombinant product in CHO cells and

444 the protein manufactured to GMP specification by Polymun Scientific (Vienna, Austria). The

445 identity of the product was confirmed by mass spectrometric analysis of tryptic fragments by

446 the Medical Biomics Centre at St. George's, University of London. The trimeric product was

447 stable, and has been extensively tested to validate stability even when kept at room

448 temperature (D. Katinger - personal communication) and has previously been reported to be

449 immunogenic. Water soluble resiquimond (R848), a low molecular weight imidazoquinoline

450 compound was obtained from Axxora (NY, USA). MED-6382 silicone elastomer was

451 obtained from Nusil Technology, USA. Tetrapropoxysilane (TPOS), stannous-2-

452 ethylhexanaote (catalyst), hydroxypropyl methyl cellulose (HPMC, 6cps), were obtained

453 from Sigma Aldrich.

454

455 ***Preparation of rings***

456 Human sized silicone elastomer vaginal rings, containing three cavities spaced equidistantly

457 around the ring, were prepared by elevated temperature reaction injection moulding (T=80

458 °C, 2 min) of MED-6382 silicone elastomer mix on a laboratory-scale ring-making machine

459 fitted with specially-designed injection moulds (**Fig. 9a+b**). Briefly, silicone elastomer MED-

460 6382 was thoroughly mixed with 2.5% w/w TPOS using an overhead stirrer. 30 g of this

461 mixture was mixed with 0.5% w/w of stannous-2-ethylhexanoate (catalyst) using a DAC 150

462 FVZ-K Speedmixer™ (3000 rpm, 30 s) and injected into the moulds, mounted on a custom,

463 laboratory scale, electrically-heated injection moulding machine. The dimensions of the ring

464 were: outer diameter: 5.8 cm, inner diameter: 4.3 cm and cross-sectional diameter: 0.76 cm.
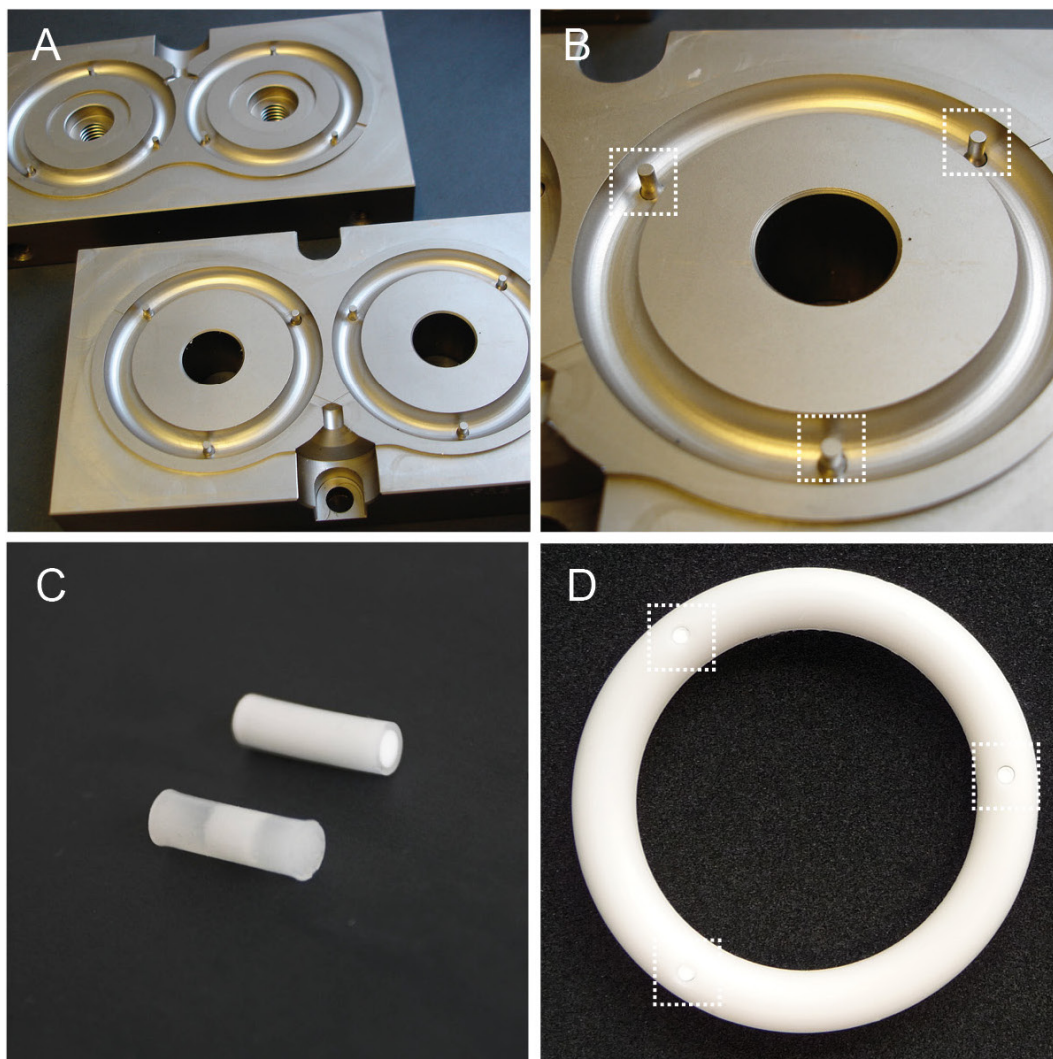
465

466 **Figure 9: A and B – Design of injection molds for fabrication of rod-insert vaginal ring**
467 **devices**. In B, the dashed boxes highlight the pins that create the cavities in the ring device. C
468 – Examples of freeze-dried rod inserts prepared in PVC tubing. The upper rod insert is shown
469 immediately after manufacture; the lower rod insert shows ingression of release medium. D –
470 A silicone elastomer vaginal ring body into which three freeze-dried rod containing gp140
471 have been inserted.

472 *Preparation of rod-inserts*

473 Resiqimod (27.7 mg), hydroxypropyl methyl cellulose (799.8 mg), gp140 solution (2700 μL),

474 and sterile water (473 μL) were added sequentially to a 10 mL Speedmixer™ container and

475 mixed (60 s, 3000 rpm). The gel mixture was hydrated overnight at 2-8 °C, followed by

476 further mixing. The gel was injected into pre-cut sections of medical-grade PVC tubing

477 (Nalgene® metric, 7.0 mm length, 2.0 mm internal diameter, 4.0 mm outer diameter) using a

478 1 mL disposable plastic syringe fitted with a modified micropipette tip. Gel-filled tubes were

479 placed on a stainless steel tray and freeze-dried (AdVantage freeze drier, VirTis, USA). The

480 freeze drying involved ramping to -60 °C and holding for 2 h, followed by primary drying at -

481 30 °C for 15 h and finally ramping to +20 °C over 60 min and holding for 10 h [15]. After

482 freeze-drying, the rods (figure 9c) were removed from the tubes and weighed. A single rod

483 was inserted into each ring cavity (figure 9d). The rings were packaged in pre-labelled semi-

484 permeable paper-plastic ring pouches and heat-sealed using a PacSeal® impulse heat sealer.

485 Each ring device carried a total of gp140 (500 μg) and R848 (500 μg). Similar rods not

486 containing resiqimod were also prepared by omitting its addition during preparation.

487

488 *Release of CN54gp140 protein from silicone elastomer encapsulated rod*

489 Individual silicone elastomer rods containing CN54gp140 protein or CN54gp140 protein plus

490 R848 were individually placed into 15mL polypropylene tubes with 2 mL PBS pH 7.2 or 2

491 mL simulated vaginal fluid (SVF) pH4.2 [33]. Each rod contained 167 μg CN54gp140 and

492   167 µg R848 with n=6 repeats for each rod formulation type and buffer, tubes were incubated

493   in an orbital shaker at 37 °C, 100 rpm. The total 2 mL release media was removed with

494   complete media replacement at the sampling timepoints 1, 3, 6, 12, 24, 48, 72, 96, 120, 144,

495   168 h. CN54gp140 was quantified by a quantitative direct capture ELISA. Briefly, maxisorp

496   high binding 96-well plates were coated overnight with 10 µg/mL sheep anti-gp120 HIV

497   polyclonal antibody D7234 (Aalto Bio Reagents, Ireland), blocked with 1% BSA, 0.05%

498   Tween-20, washed and then incubated with the sample before detection with the anti-gp41

499   HIV human 5F3 antibody followed by anti-human IgG HRP and final development with 50

500   µL/well of KPL SureBlue TMB substrate (Insight Biotechnology, UK) and stopped after 5

501   min by adding 50 µL/well 1 M H_2SO_4, and the absorbance read at 450 nm on a VersaMax

502   spectrophotometer using SoftmaxPro software. All incubations were for 1 h at 37 °C and

503   plates washed between each step.

504

505   *Sheep, immunisation and sampling*

506   Female Welsh Beulah Speckled Face sheep were used in all experiments. All ewes had

507   previously been used for breeding and were between 2 and 3 years of age. Sheep received an

508   intramuscular injection of 100 µg HIV CN54gp140 + 200 µg R848 followed by three

509   successive ring applications, each of one week duration and separated by one month intervals

510   to allow development of somatically hypermutated antigen-reactive B cells in the draining

511   lymph nodes. Each IM dose was administered in a total volume of 500 µL. Other sheep

512   received only the ring devices without IM priming. The ring inserts contained a total of 500

513   µg CN54gp140 recombinant protein per 3 rods, with some rods also containing 500 µg R848

514   per 3 rods. 3 rods were inserted into each ring. Rings were inserted into the vagina using a

515   medium sized speculum to open the vaginal canal and were placed around the cervical

516   opening. Serum and vaginal mucosal fluids were sampled every two weeks and analysed by

517   CN54gp140 ELISA. Antigen-specific cellular responses were determined at necropsy. The

518   studies utilizing the model antigen HIV Env CN54gp140 used five animals per group.

519   Blood drawn from the leg vein was allowed to clot at room temperature for 30 min then

520   centrifuged at $500 \times g$ for 10 min and the serum removed and stored in aliquots at $-80\ {}^0C$.

521   Vaginal sampling was performed by first inserting a medium-long sized speculum into the

522   vagina to allow access to the vaginal wall and 2 x Weck-Cel® (Beaver-Visitec International,

523   USA) cellulose sponge spears, pre-wet with 30 μL antibody extraction buffer (2 x PBS +

524   Protease Inhibitor cocktail) were placed into the vagina and the speculum removed. The

525   sponges were allowed to absorb fluid within the vagina for 5 min before removal by hand and

526   the tips cut off while being placed into the top chamber of a Spin-X column. A further 300 μl

527   of antibody extraction buffer was added into the top chamber of the Spin-X column with the

528   two Weck-Cel® cellulose spears and allowed to incubate at RT for 10 min. The Spin-X®

529   column was then centrifuged at 12,000 g for 15 min to remove large debris and isolate the

530   fluid containing the high salt eluted antibody which was then frozen at $-80\ °C$.

531

532   *Antigen-specific antibody semi-quantitative ELISA*

533   Serum and mucosal antigen-specific binding antibodies against HIV CN54gp140

534   recombinant protein were measured using a standardized ELISA. Maxisorp® high binding 96-

535   well plates were coated with 50 μL recombinant proteins at 5 μg/mL in PBS overnight at

536   4°C. The standard IgG or IgA immunoglobulins were coated onto the Maxisorp® plates

537   overnight at 4°C. Coated plates were washed three times in PBS-T before blocking with 200

538   μL PBS-T containing 1% bovine serum albumin for 1 hr at 37 °C. After further washing, sera

539   diluted 1/100, 1/1,000 and 1/10,000 or mucosal samples diluted 1/10, 1/50 and 1/250 were

540   added to the antigen coated wells at 50 μL/well and incubated for 1 hr at 37°C. Plates were

541   washed four times before the addition of 100 μL of a 1/500 dilution of rabbit anti-sheep IgG-

542   HRP or a 1/1,100 dilution of rabbit anti-sheep IgA secondary antibody and incubated for 1

543   hour at 37°C. The plates were washed four times and developed with 50 μL/well of KPL

544   SureBlue TMB substrate (Insight Biotechnology, UK). The IgA isotype, required a secondary

545   antibody incubation step using goat anti-rabbit Ig biotin and a subsequent streptavidin-HRP

546   (R&D systems) amplification step prior to TMB development. The reaction was stopped after

547   5 min by adding 50 μL/well 1 M H_2SO_4, and the absorbance read at 450 nm on a

548   VersaMax™ spectrophotometer using SoftmaxPro software.

549

550   *Flow Cytometry*

551   Cells were harvested from the animals at necropsy. External and internal iliac lymph nodes,

552   the spleen and peripheral blood were harvested and lymphocytes isolated. Single cell

553   suspensions of lymphocytes derived from each of these tissues were incubated with an aqua

554   viability dye, washed 1x with 10 volumes PBS/0.5% FBS and then incubated with 1 ug/mL

555   Alexa 647-conjugated CN54gp140 HIV Env protein or the control 1 μg/mL Alexa 647-

556   conjugated TT protein for 30 min at room temperature, washed 2x with 10 volumes

557   PBS/0.5% FBS and then resuspended to a single cell suspension in 250 μL PBS before

558   fixation with paraformaldehyde, final concentration of 1.5%. Dead cells were gated out using

559   the live/dead cell aqua dye and the Alexa-647 positive staining assessed on live cells.

560   *Statistical Analysis*

561 The mean serum, mucosal antibody and antigen-specific flow binding data were compared

562 using two-tailed Mann Whitney non-parametric tests or an unpaired t-test with Welch's

563 correction if the data variance was not equal.

564 **Acknowledgements.**

571

572

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694

695

Figure Legends

Figure 1: CN54gp140 in vitro release profile. Individual freeze-dried silicone elastomer rods were laced into 2 mL release media (PBS, pH 7.2 or SVF, pH 4.2). Rods contained CN54gp140 alone (167 µg) or CN54gp140 + R848 (167 µg each). The amount of CN54gp140 released into the 2 mL media was measured by quantitative ELISA and plotted as cumulative % release. Plot symbols represent the mean ± standard deviation of six replicates.

Figure 2: Vaccination, bleed and vaginal sampling schedule. Two-year-old sheep were immunised at monthly intervals using intravaginal rings containing CN54gp140 HIV Env antigen +/- R848 adjuvant. The rings were maintained in place at the cervicovaginal area for 1 week before removal. Some sheep received a prior IM vaccination one month before the insertion of the rings. Animals were bled weekly and had vaginal mucosal antibody Weck-Cel® swabs taken at the times indicated.

Figure 3: CN54gp140 antigen-specific antibody serum responses to 500 µg vaccine antigen in the presence of 500 µg R848 adjuvant per vaginal ring. IM Primed – animals received an IM prime at day 0 then three vaginal ring placements, each retained *in situ* for 7 days, at monthly intervals. Unprimed – animals received the vaginal rings without a prior IM vaccination. The shaded areas indicate the 7-day periods during which a ring device was present in the sheep vagina. (A) Vaccine antigen-specific IgG and (B) IgA responses. Data compared with a Mann-Whitney test (n = 5 per group).

Figure 4: CN54gp140 antigen-specific antibody mucosal responses to 500 µg vaccine antigen in the presence of 500 µg R848 adjuvant per vaginal ring. IM Primed – animals received an IM prime at day 0 then three vaginal ring placements, each retained *in situ* for 7 days, at monthly intervals. Unprimed – animals received the vaginal rings without a prior IM vaccination. The shaded areas indicate the 7-day period during which a ring device was present in the sheep vagina. (A) Vaccine antigen-specific mucosal IgG and (B) mucosal IgA responses. Data compared with a Mann-Whitney test (n = 5 per group).

Figure 5: CN54gp140 antigen-specific antibody serum responses to 500 µg vaccine antigen in the absence of R848 adjuvantation. IM primed – animals received an IM prime at day 0 then three vaginal ring placements, each retained *in situ* for 7 days, at monthly intervals. Unprimed – animals received the vaginal rings without a prior IM vaccination. The

shaded areas indicate the 7-day periods when the vaginal ring was present. (A) Vaccine antigen-specific IgG and (B) IgA responses. Data compared with a Mann-Whitney test (n = 5 per group).

Figure 6: CN54gp140 antigen-specific serum antibody responses in animals that received only IM vaccinations. Animals received an IM prime of 100 μg vaccine antigen plus 200 μg R848 adjuvant at day 0 then three IM boost vaccinations with the same vaccine composition at monthly intervals. Vaccine antigen-specific IgG and IgA responses (n = 5 per group),

Figure 7: CN54gp140 antigen-specific vaginal antibody responses in animals that received only IM vaccinations. Animals received an IM prime of 100 μg vaccine antigen plus 200 μg R848 adjuvant at day 0 then three IM boost vaccinations with the same vaccine composition at monthly intervals. Vaccine antigen-specific mucosal IgG and mucosal IgA responses (n = 5 per group).

Figure 8: Elicitation of Antigen-Specific B cells in vaginal local draining lymph nodes. Cells were harvested from the peripheral blood, the spleen or the vaginal external iliac lymph nodes and stained with Alexa-647 conjugated recombinant CN54gp140 protein.

Figure 9: A and B – Design of injection molds for fabrication of rod-insert vaginal ring devices. In B, the dashed boxes highlight the pins that create the cavities in the ring device. C – Examples of freeze-dried rod inserts prepared in PVC tubing. The upper rod insert is shown immediately after manufacture; the lower rod insert shows ingression of release medium. D – A silicone elastomer vaginal ring body into which three freeze-dried rod containing gp140 have been inserted.

Tables

Table 1: Sheep groups and immunization schedule (n = 5). IM – intramuscular administration of 100 µg CN54gp140 + 200 µg R848; IVag – intravaginal administration of 500 µg CN54gp140 ± 500 µg R848.

Table 2: Serum and mucosal IgG and IgA levels (µg/mL) 2 weeks after each vaginal ring insertion (Days 42, 70 and 98) and at end of vaccination schedule (Day 112). (+/- SD in parentheses). IM – intramuscular administration of 100 µg CN54gp140 + 200 µg R848; IVag – intravaginal administration of 500 µg CN54gp140 ± 500 µg R848. A dash within a cell indicates that antibody levels were not detected.

Table 3: Mean percentage of background control subtracted antigen-specific cells in each tissue (n=5 per group) (+/- SD in parentheses).

Supplementary Material

Supplementary Figure 1: Elicitation of Antigen-Specific B cells in vaginal local draining lymph nodes of animals that received intravaginal rings containing CN54gp140 only. Cells were harvested from the peripheral blood, the spleen or the vaginal external iliac lymph nodes and stained with Alexa-647 conjugated recombinant CN54gp140 protein.
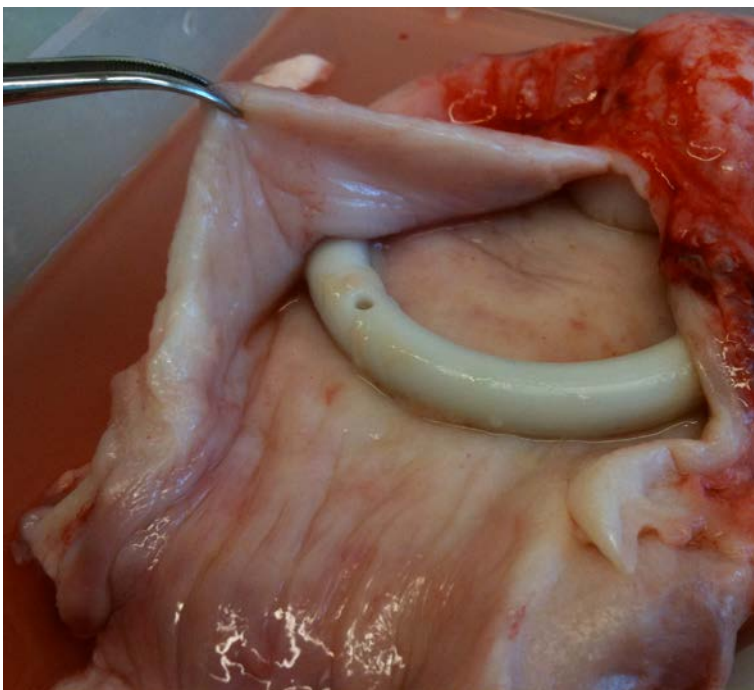
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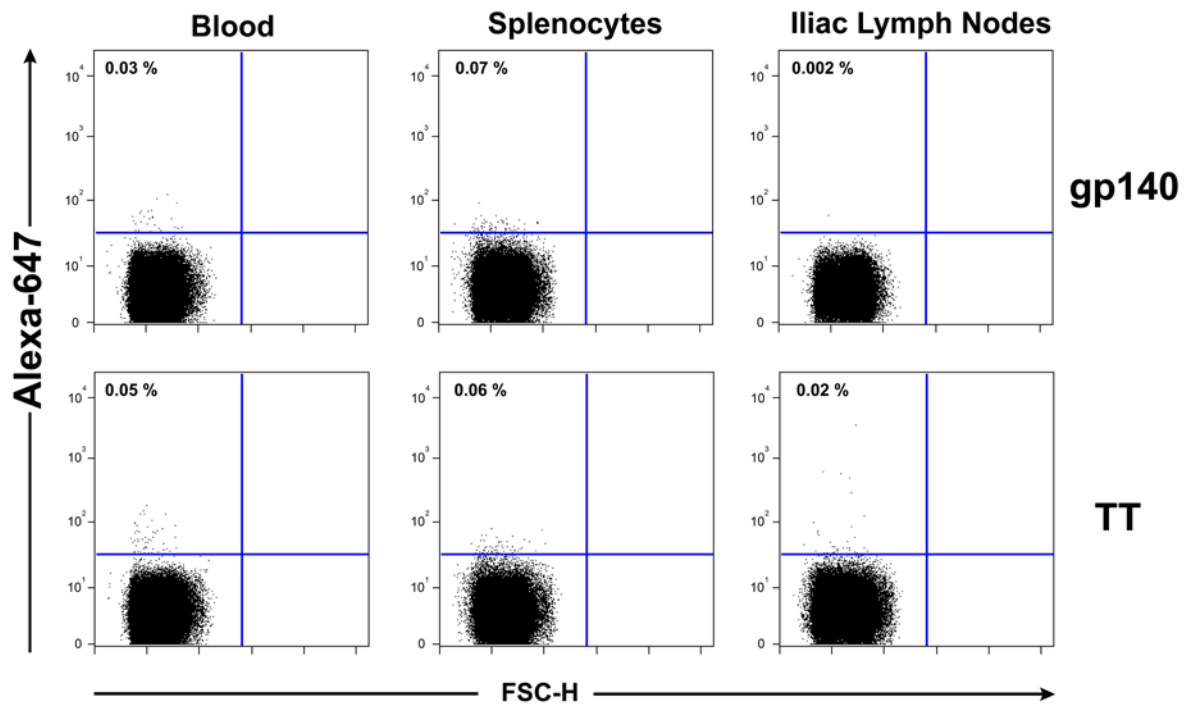
Supplementary Figure 2: Excised sheep vagina at necropsy. A vaginal ring antigen-release device *in situ* within a vagina removed from a culled sheep. No inflammation or infection is apparent on the vaginal vault wall. Note that the lyophilized rod that originally contained the vaccine formulation is no longer present in the visible ring cavity.

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