

1 **Pregnancy alters innate immune responses to Zika virus infection in the genital**  
2 **tract**

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14 administration, supervision, visualization, and writing.

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## 20 **Abstract**

21           Recent outbreaks of Zika virus (ZIKV) have been associated with birth defects,  
22 including microcephaly and neurological impairment. However, the mechanisms which  
23 confer increased susceptibility to ZIKV during pregnancy remain unclear. We  
24 hypothesized that poor outcomes from ZIKV infection during pregnancy are due in part  
25 to pregnancy-induced alteration of innate immune cell frequencies and cytokine  
26 expression. To examine the impact of pregnancy on innate immune responses, we  
27 inoculated pregnant and non-pregnant female C57BL/6 mice with  $5 \times 10^5$  FFU of ZIKV  
28 intravaginally. Innate immune cell frequencies and cytokine expression were measured  
29 by flow cytometry at day 3 post infection. Compared to non-pregnant mice, pregnant  
30 mice exhibited higher frequencies of uterine macrophages (CD68+) and tolerogenic  
31 dendritic cells (CD11c+ CD103+ and CD11c+ CD11b+). Additionally, ZIKV-infected  
32 pregnant mice had lower frequencies of CD45+ IL-12+ and CD11b+ IL-12+ cells in the  
33 uterus and spleen. These data show that pregnancy results in an altered innate immune  
34 response to ZIKV infection in the genital tract of mice and that pregnancy-associated  
35 immune modulation may play an important role in the severity of acute ZIKV infection.

## 36 **Importance**

37           Pregnant females longer duration that viremia following infection with Zika virus  
38 but the mechanism of this is not established. Innate immune cellular responses are  
39 important for controlling virus infection and are important for development and  
40 maintenance of pregnancy. Thus, the acute immune response to Zika virus during  
41 pregnancy may be altered so that the pregnancy can be maintained. To examine this  
42 interaction, we utilized a mouse model of Zika virus infection during pregnancy using

43 intravaginal inoculation. We found that following Zika virus infection, pregnant mice  
44 exhibited increased expression of tolerant or non-inflammatory dendritic cells.  
45 Additionally, we found that pregnant mice have significantly depressed ability to secrete  
46 the cytokine IL-12 from innate immune cells in the uterus and the spleen while  
47 maintaining MHCII expression. These findings show that pregnancy-induced changes in  
48 the innate immune cells are biased towards tolerance and can result in decreased  
49 antigen-dependent stimulation of immune responses.

## 50 **Introduction**

51           Zika virus (ZIKV) is a neurotropic flavivirus originally isolated from a febrile  
52 rhesus macaque in the Zika Forest of Uganda (1). Although ZIKV was first identified in  
53 humans in 1952, only sporadic infections occurred in humans until 2007, when the first  
54 major outbreak was reported on Yap Island (2). Since its emergence, infections have  
55 been reported in Africa, Asia, the Pacific Islands, and the Americas. ZIKV is transmitted  
56 to humans predominantly through bites from *Aedes* mosquitos, but infections after  
57 sexual contact and blood transfusions have also been reported (3, 4). The majority of  
58 infected individuals are asymptomatic, with some infections causing mild symptoms  
59 such as fever, rash, conjunctivitis, muscle and joint pain, malaise, and headache (5).  
60 However, ZIKV infections in French Polynesia in 2013 and 2014 were linked to an  
61 increase in Guillain-Barré syndrome in adults (6). In 2015, a ZIKV outbreak in Brazil was  
62 associated with a marked increase in microcephaly in infants born to acutely infected  
63 mothers (7-9). Other reports also show that ZIKV infection in fetuses may cause a  
64 spectrum of disease from severe microcephaly to more subtle brain and developmental  
65 abnormalities together referred to as “congenital ZIKV syndrome” (8, 10-12). Multiple  
66 studies have provided additional evidence of vertical transmission of ZIKV from infected  
67 pregnant mothers to the fetus (9, 13). Vertical transmission of ZIKV often occurs  
68 following periods of prolonged maternal viremia, and this is supported by data from both  
69 human studies and nonhuman primate models of congenital ZIKV infection (14, 15).

70           The mechanisms underlying the increased severity of ZIKV infection during  
71 pregnancy remain understudied. During pregnancy, women are at increased risk for  
72 infection and increased severity of infection with ZIKV and several other pathogens,

73 including listeria, cytomegalovirus (CMV), herpes simplex virus (HSV), influenza virus,  
74 and HIV (16-21). In general, successful pregnancy relies on tolerance of the maternal  
75 immune system towards the semi-allogeneic fetus, which is often referred to as  
76 immunotolerance. This results in changes at multiple levels of the maternal immune  
77 system. For example, human natural killer (NK) cells lose their cytotoxic abilities and  
78 instead take on a supportive role during pregnancy (22). Additionally, the decidua  
79 contains an abundance of regulatory T cells (Tregs) during early pregnancy, which  
80 maintain tolerance, prevent inflammation, and promote implantation of the embryo (23-  
81 25). Many of these changes are linked to the induction of pregnancy hormones. Several  
82 studies have suggested that human chorionic gonadotropin (HCG) plays a role in the  
83 recruitment of Tregs to the maternal-fetal interface and promotes the generation of  
84 tolerogenic dendritic cells (DCs) (26, 27). These pregnancy-induced immune changes  
85 impact susceptibility to several pathogens. For example, changes in the immune  
86 response during pregnancy result in progesterone-dependent increased susceptibility to  
87 HSV2 in the genital tract of mice, resulting in lower HSV2-specific IgG and IgA  
88 responses in the genital tract following infection (28). Additionally, influenza infection in  
89 pregnant ferrets results in decreased total CD8+ T-cells and decreased H1N1-specific  
90 B-cell responses compared to non-pregnant ferrets (29).

91         While different stages of pregnancy clearly modulate adaptive immune  
92 responses, less is known about pregnancy-induced innate immune responses during  
93 viral infection. In a pregnant mouse model of influenza infection, Cox-2, PGE2, and  
94 PGF2 $\alpha$  were increased, resulting in remodeling of the placental architecture, preterm  
95 labor, impaired fetal growth, and increased fetal and maternal mortality and morbidity

96 (18). In another study of late stage pregnancy, viral infection of the placenta triggered  
97 an inflammatory response, including the secretion of IL-1, IL-6, IL-8, and TNF $\alpha$ , and  
98 fetal abnormalities in the absence of direct fetal infection (30, 31). A study of ZIKV-  
99 infected mothers reported the presence of interferon gamma-inducible protein-10 (IP-  
100 10), IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), vascular endothelial  
101 growth factor (VEGF), and granulocyte-colony stimulating factor (G-CSF) in the amniotic  
102 fluid of mothers whose infants were born with microcephaly (32). Another study found  
103 that IP-10, CCL5, IL-9, interferon gamma (IFN $\gamma$ ), IL-7, IL-5, and IL-1ra were upregulated  
104 in the plasma of acutely infected individuals compared to healthy donors (33). In the  
105 recovery phase, IL-12p70 and basic fibroblast growth factor (FGF) were found to be  
106 upregulated (33). However, it is unclear how the immune response to ZIKV is impacted  
107 by pregnancy, especially in early pregnancy.

108         Few studies have examined the effect of pregnancy on innate immune responses  
109 in the genital tract at the early stages of pregnancy prior to placental formation. Previous  
110 studies have shown that ZIKV infections in the first trimester of pregnancy confer a  
111 greater risk of microcephaly compared to second and third trimester infections (34).  
112 Since the severity of ZIKV congenital disease increases with infection during early  
113 stages of pregnancy, we examined pregnancy-associated changes in the innate  
114 immune response during early pregnancy. In order to evaluate the innate immune  
115 cellular response in the genital tract, we utilized an immune competent murine model of  
116 ZIKV intravaginal inoculation which has been described previously (35). Following  
117 intravaginal inoculation of ZIKV at embryonic day 4.5 (E4.5), we found that pregnant  
118 mice exhibited increased frequencies of tolerogenic DCs (CD11c<sup>+</sup> CD103<sup>+</sup>, CD11c<sup>+</sup>

119 CD11b+) in the uterus and a higher frequency of uterine macrophages (CD68+)  
120 compared to ZIKV-inoculated non-pregnant mice. Additionally, ZIKV-infected pregnant  
121 mice exhibited lower frequencies of CD45+ IL-12+ cells and CD11b+ IL-12+ in the  
122 uterus and spleen. Taken together, these results suggest that pregnancy alters the local  
123 innate immune response to ZIKV infection, which may decrease immune control of  
124 acute viral infection.

125

## 126 **Results**

### 127 *Intravaginal ZIKV infection in C57BL/6 mice*

128 To generate a mouse model of ZIKV infection during pregnancy, 8-week-old,  
129 female C57BL/6 mice were injected with 2.5 international units (iu) pregnant mare  
130 serum gonadotropin (PMSG) by intraperitoneal (ip) inoculation, ip injected with 2.5 iu  
131 human chorionic gonadotropin (HCG) 48 hours later, and finally mated with male mice  
132 overnight (16 hours). The males and females were separated the following morning  
133 (E0.5). At E4.5, the female mice were inoculated with  $5 \times 10^5$  FFU of ZIKV (PRVABC59)  
134 or mock inoculum. Intravaginal washes and tissue harvests for analysis of virus and  
135 immune parameters, respectively, were performed at specific time points post-infection  
136 (**Fig. 1A**). Pregnancy rates ranged from 30-80% with this approach, allowing for  
137 prospective cohort analysis of both pregnant and non-pregnant mice that were treated  
138 at the same time with the same hormones prior to ZIKV infection. At 48 hours post-  
139 infection, ZIKV PCR of vaginal wash fluid revealed evidence of ZIKV RNA in the genital  
140 tract of both pregnant and non-pregnant mice (**Fig. 1B**). The levels of ZIKV RNA did not

141 differ between the pregnant and non-pregnant mice. ZIKV PCR from vaginal washes at  
142 days 1, 2, and 3 post-infection revealed peak values at day 2 post-infection, and ZIKV  
143 RNA was not detected in fetal tissues at day 3 or 6 post-infection (data not shown). The  
144 weights of the mice did not change significantly during infection (data not shown), and  
145 the numbers of fetuses in both the mock and ZIKV-infected pregnant mice were similar  
146 **(Fig. 1C)**.

147

#### 148 *Pregnancy-induced changes in splenic innate cellular response during ZIKV infection*

149 Nex, we analyzed the innate cellular immune responses in the pregnant and non-  
150 pregnant mice at 3 days post-infection, immediately following peak ZIKV infection of the  
151 genital tract. Following mock and ZIKV intravaginal inoculation, non-pregnant and  
152 pregnant mice were euthanized at day 3 post-infection (E7.5), and spleen and uterine  
153 tissue were analyzed by flow cytometry. We measured the frequencies of cells  
154 expressing several markers of innate immune cells, including CD68 (macrophages),  
155 CD11b (expressed on monocytes, macrophages, and DCs), CD11c (expressed on DCs,  
156 monocytes, macrophages, and granulocytes), Ly6C (expressed on macrophages,  
157 monocytes, and neutrophils), and CD103 (expressed on certain DC subsets). In the  
158 splenic tissue, ZIKV infection during pregnancy resulted in a significantly increased  
159 frequency of CD45+ CD68+ macrophages compared to ZIKV-infected non-pregnant  
160 mice **(Fig. 2A, p= 0.0095, ANOVA=0.0107)**. There was no significant difference in the  
161 frequency of CD45+ CD68+ macrophages between the mock-inoculated pregnant and  
162 non-pregnant mice (p= 0.5849, ANOVA=0.5849). Mock and ZIKV-infected pregnant and  
163 non-pregnant mice exhibited similar frequencies of CD45+ CD11b+ cells **(Fig. 2B; p=**



164 0.984 mock non-pregnant vs mock pregnant,  $p= 0.9992$  ZIKV non-pregnant vs ZIKV  
165 pregnant; ANOVA= 0.7906) and CD45+ CD103+ cells (**Fig. 2E**;  $p= 0.8707$  mock non-  
166 pregnant vs mock pregnant,  $p= 0.9937$  ZIKV non-pregnant vs ZIKV pregnant; ANOVA=  
167 0.8585) in the spleen. However, ZIKV-infected pregnant mice exhibited increased  
168 frequencies of CD45+ CD11c cells in the spleen compared to infected non-pregnant  
169 mice (**Fig. 2C**,  $p= 0.0109$ , ANOVA=0.0104). Additionally, ZIKV infection during  
170 pregnancy resulted in a significantly decreased frequency of CD45+ Ly6C+ cell  
171 populations compared to ZIKV-infected, non-pregnant mice (**Fig. 2D**,  $p= 0.042$ ,  
172 ANOVA=0.0319). In comparison, mock-inoculated pregnant and non-pregnant mice  
173 exhibited no significant changes in the frequency of splenic CD45+ Ly6C+ cells ( $p=$   
174 0.4801). In mice, Ly6C expression in CD11b+ monocytes distinguishes pro-  
175 inflammatory monocytes from anti-inflammatory patrolling monocytes which participate  
176 in tissue repair, with the pro-inflammatory group having high expression of Ly6C (Ly6C  
177 hi) and the anti-inflammatory group having low expression of Ly6C (Ly6C lo). Pregnant  
178 and non-pregnant infected and non-infected mice had similar frequencies of CD11b+  
179 Ly6C hi (ANOVA= 0.2511) and Ly6C lo cells, although there was a significant difference  
180 in Ly6C lo frequencies between the pregnant mock and non-pregnant infected mice  
181 (**Fig. 2G**,  $p= 0.0122$ , ANOVA=0.0147). These data show that Ly6C expression on  
182 CD11b+ monocytes is decreased in the spleen during pregnancy despite acute viral  
183 infection.

#### 184 *Pregnant mice have higher frequencies of CD68+ macrophages in uterine tissue*

185 Next, we evaluated innate immune cell frequencies in the uterus at day 3 post  
186 infection. Following infection, both ZIKV-infected and mock-infected pregnant mice

187 exhibited increased frequencies of uterine CD45+ CD68+ macrophages compared to  
188 non-pregnant mice (**Fig. 3A**;  $p=0.0055$  mock non-pregnant vs. mock pregnant,  $p=$   
189  $0.0004$ , ZIKV non-pregnant vs. ZIKV pregnant; ANOVA  $<0.0001$ ). Despite the changes  
190 in CD68+ cell frequencies, we found no significant changes in the frequencies of CD45+  
191 CD11b+ (**Fig. 3B**, ANOVA=  $0.057$ ), CD45+ CD11c+ (**Fig. 3C**, ANOVA=  $0.7392$ ), CD45+  
192 Ly6C+ (**Fig. 3D**, ANOVA=  $0.1022$ ), CD11b+ Ly6C hi (**Fig. 3E**, ANOVA=  $0.1915$ ), or  
193 CD11b+ Ly6C lo (**Fig. 3F**, ANOVA=  $0.0794$ ) cell populations when comparing mock and  
194 ZIKV-infected non-pregnant and pregnant mice.

195

#### 196 *Pregnant mice have higher frequencies of tolerogenic DCs*

197 Since infiltrating macrophages didn't express pregnancy-associated changes in  
198 activation in the uterine tissue following acute viral infection, we evaluated dendritic cells  
199 (DCs) for evidence of pregnancy-induced changes in activation during acute viral  
200 infection. DCs are important antigen presenting cells which coordinate the innate  
201 immune response and support the development of adaptive immune responses.  
202 Several lines of evidence suggest that uterine dendritic cells take on a tolerogenic  
203 phenotype during pregnancy (36, 37). Tolerogenic DCs are potent secretors of anti-  
204 inflammatory mediators such as IL-10 and weak producers of pro-inflammatory  
205 cytokines including IL-12 and TNF $\alpha$  (38, 39). Two types of tolerogenic DCs are present  
206 in the murine uterus: those positive for CD103 (CD11c+ CD103+) and those double-  
207 positive for CD11c and CD11b (CD11b+ CD11c+) (40). Despite what is known about  
208 these cells during pregnancy, little is known about DC activation during acute viral  
209 infection of the genital tract. Therefore, we evaluated uterine and splenic tissue for

210 changes in tolerogenic DC populations after ZIKV infection. At 3 days post-infection,  
211 ZIKV-inoculated pregnant mice exhibited significantly increased frequencies of uterine  
212 CD45+ CD11b+ CD11c+ cells compared to non-pregnant mice (**Fig. 4A**,  $p=0.0004$ ,  
213 ANOVA= 0.0001). Similarly, ZIKV-infected pregnant mice had a significantly greater  
214 frequency of splenic CD11b+ CD11c+ cells compared to mock-inoculated mice (**Fig.**  
215 **4B**;  $p=0.195$  mock non-pregnant vs. ZIKV pregnant,  $p=0.015$  mock pregnant vs. ZIKV  
216 pregnant; ANOVA=0.0074). Within the uterine CD11b+ CD11c+ population, ZIKV-  
217 inoculated pregnant mice also exhibited a significant increase in MHCII expression (**Fig.**  
218 **4A**,  $p=0.0081$ , ANOVA= 0.0039). Despite the change in MHCII expression, both the  
219 mock and ZIKV-inoculated pregnant mice exhibited markedly decreased CD11b+  
220 CD11c+ MHCII+ CD86+ cell frequencies (**Fig. 4C**;  $p=0.017$  mock non-pregnant vs.  
221 mock pregnant,  $p=0.0007$  ZIKV non-pregnant vs. ZIKV pregnant; ANOVA  $<0.0001$ ).  
222 CD86 expression in the splenic CD11b+ CD11c+ population did not differ between  
223 groups (ANOVA,  $p=0.2215$ ). While frequencies of CD11b+ CD11c+ MHCII+ cells  
224 increased with pregnancy, we found that frequency of IL10 expression decreased  
225 during pregnancy, although not significantly during ZIKV infection (**Fig. 4D**,  $p= 0.0311$   
226 mock non-pregnant vs. pregnant,  $p=0.306$  ZIKV non-pregnant vs. pregnant; ANOVA=  
227 0.016). These data show that pregnancy induces expression of tolerogenic DCs (CD45+  
228 CD11b+CD11c+) that express increased MHCII with ZIKV infection during pregnancy  
229 while decreasing expression of CD86. Moreover, IL-10 expression is significantly  
230 decreased during this early stage of pregnancy in mock infected animals, but, IL-10 is  
231 not significantly suppressed in pregnant mice during ZIKV infection. These data show

232 that during pregnancy, ZIKV infection induces increased expression of tolerogenic  
233 signals (decreased CD86 and less suppression of IL-10) on CD11b+CD11c cells.

234         Similar to CD11b+ CD11c+ cells, we found that ZIKV-inoculated, pregnant mice  
235 exhibited a significant increase in frequency of uterine CD11c+ CD103+ cells (**Fig. 5A**,  
236  $p=0.0004$  ZIKV non-pregnant vs. ZIKV pregnant, ANOVA= 0.0001). ZIKV infection in  
237 non-pregnant mice resulted in a suppression of this tolerogenic cell population but  
238 pregnant mice still exhibited high levels of CD11c+ CD103+ cells despite ZIKV infection.  
239 Within the CD11c+ CD103+ population, there was no significant change in the CD86+  
240 (ANOVA=0.2347) or IL-10+ (ANOVA=0.7101) subpopulations between treatment  
241 groups (**Fig. 5B&C**). In the spleen, there were no significant differences in the  
242 frequencies of CD11c+ CD103+ (ANOVA=0.3143) or CD11c+ CD103+ CD86+  
243 (ANOVA=0.895) cells between groups (**Fig. 5D**). These data show that local factors  
244 likely mediate maintenance of tolerogenic dendritic cell phenotypes during pregnancy  
245 despite acute viral infection.

246

247 *Pregnant mice exhibit decreased IL-12 responses to ZIKV infection in the uterus*

248         Next, we evaluated the expression of pro-inflammatory cytokines in the uterine  
249 innate immune cells. IL-12 promotes the differentiation of T cells into Th1 cells and  
250 activates NK cells, and it is upregulated during certain viral infections (41, 42).  
251 Additionally, multiple studies have shown that IL-12 levels are increased in the blood  
252 and endometrial tissue of in women with recurrent pregnancy loss, suggesting that IL-12  
253 may be detrimental during pregnancy (43, 44). In uterine tissue at day 3 post-infection,

254 we found that the frequency of CD45+ cells producing IL-12 was decreased following  
255 ZIKV infection in pregnant compared to non-pregnant mice (**Fig. 6A**,  $p=0.0081$ ,  
256 ANOVA= 0.007). This difference was not seen when non-pregnant and pregnant mock-  
257 infected mice were compared ( $p=0.9535$ ). Next, we further analyzed IL-12 expression in  
258 several immune cell subtypes. We found that IL-12+ CD11b+ (**Fig. 6B**,  $p=0.007$ ,  
259 ANOVA= 0.0059), IL-12+ CD68+ (**Fig. 6C**,  $p=0.0018$ , ANOVA= 0.0011), and IL-12+  
260 Ly6C+ (**Fig. 6E**,  $p=0.004$ , ANOVA= 0.0028) cells were significantly decreased in ZIKV-  
261 infected, pregnant mice compared to ZIKV-infected non-pregnant mice. CD45+ CD103+  
262 cells exhibited a trend towards decreased IL-12 expression during pregnancy in both  
263 mock and ZIKV-infected mice when compared to non-pregnant mice (ANOVA= 0.006)  
264 (**Fig. 6D**). There was no significant difference in CD11c+ IL-12+ cells between groups  
265 (data not shown, ANOVA=0.1943).

266 Additionally, we measured the frequencies of IL-6+ cells within each of these  
267 populations, as it has been reported that IL-6 is upregulated during ZIKV infection (45).  
268 We found no significant differences in the frequencies of IL-6+ CD45+, IL-6+ CD68+, IL-  
269 6+ CD11b+, IL-6+ CD11c+, IL-6+ Ly6C+, or IL-6+ CD103+ cells between groups (**Fig.**  
270 **6F&G**). These data show that pregnancy results in decreases in IL-12-expressing cells  
271 during ZIKV infection, chiefly IL-12+ monocytes and macrophages.

272

273 *Pregnant mice exhibit decreased IL-12 responses to ZIKV infection in the spleen*

274 Our murine model utilized a localized intravaginal infection, and we found  
275 evidence of pregnancy-associated modulation of IL-12 expression in subsets of CD45+

276 cells in the uterus following infection. Next, we evaluated the spleen for similar changes  
277 in IL-12 expression. We found a significant decrease in splenic CD45+ IL-12+ cells in  
278 pregnant ZIKV-infected mice compared to non-pregnant ZIKV-infected mice (**Fig. 7A**,  
279  $p=0.0323$ , ANOVA=0.0288). In the subset analysis, we found that frequencies of IL-12+  
280 cells were significantly decreased within the CD68+ (**Fig. 7B**,  $p=0.0441$ ,  
281 ANOVA=0.0241) and CD11b+ (**Fig. 7C**,  $p=0.0093$ , ANOVA=0.0107) populations in  
282 ZIKV-infected pregnant mice compared to ZIKV-infected non-pregnant mice. Similar to  
283 the uterus, inhibition of IL-12 expression appeared to be specific to monocytes and  
284 macrophages, as CD11c+ (**Fig. 7D**, ANOVA=0.2629) and CD103+ (**Fig. 7F**,  
285 ANOVA=0.9808) cells did not exhibit significant changes in IL-12 expression between  
286 treatment groups. The frequency of Ly6C+ IL-12+ cells trended toward a decrease in  
287 the ZIKV-infected pregnant mice compared to the other groups, but this change did not  
288 reach significance when the groups were compared to each other (**Fig. 7E**,  
289 ANOVA=0.0478). Similar to the results seen in the uterus, IL-6 expression did not differ  
290 significantly in any of the cell subsets tested (**Fig. 7G&H**).

291

## 292 **Discussion**

293 Our data are the first to evaluate acute, innate immune cellular responses in the  
294 genital tract of immune competent mice to Zika virus infection during early pregnancy.  
295 The data show that early stages of pregnancy results in inhibition of innate cellular  
296 activation and maintenance of tolerogenic immune cell changes despite acute viral  
297 infection in the genital tract. We show that pregnant mice exhibited decreased  
298 CD45+Ly6C+ cells in the spleen following acute viral infection. We also found that

299 pregnancy induces expression of tolerogenic DCs (CD45+ CD11b+CD11c+) that  
300 express increased MHCII with ZIKV infection during pregnancy while decreasing  
301 expression of CD86. Moreover, IL-10 expression is significantly decreased during this  
302 early stage of pregnancy in mock infected animals likely representing the importance of  
303 some inflammatory responses to develop early pregnancy. However, during acute viral  
304 infection, IL-10 is not significantly suppressed in pregnant mice during ZIKV infection.  
305 This implies that ZIKV infection induces increased expression of tolerogenic signals  
306 (decreased CD86 and less suppression of IL-10) on CD11b+CD11c cells during  
307 pregnancy. These findings indicate that pregnancy inhibits virus-induced acute  
308 inflammation during early implantation likely as a mechanism to protect the developing  
309 fetus.

310 We also found that some of the inhibitory cell phenotypes were specific to the  
311 uterine tissue. For example, during acute ZIKV infection, non-pregnant mice significantly  
312 suppress immunotolerant CD11c+CD103+ cells to support acute inflammation for the  
313 infection; however, pregnant mice exhibit a significant increase in CD11c+CD103+ cells  
314 in the uterus. These changes were not seen in the spleen implying that the expression  
315 of CD11c+CD103+ cells in the uterus is regionally regulated to support the developing  
316 pregnancy.

317 In contrast, some of the inhibitory cell phenotypes were found in both the uterus  
318 and the spleen. We found that CD45+IL-12 responses to acute viral infection were  
319 significantly decreased in both the uterus and spleen of pregnant mice compared to  
320 non-pregnant mice. In the uterus, decreased IL-12 production was largely due to  
321 CD11b+, CD68+, and Ly6C+ cells, and in the spleen decreased IL-12 production was

322 largely due to CD11b+ cells. These data show for the first time, that both systemic and  
323 regional responses during pregnancy modulate the acute, innate immune cellular  
324 response to acute viral infection.

325         In our mouse model, implantation occurs at E4 (46), and we inoculated mice  
326 intravaginally with ZIKV at this early stage of pregnancy. At this timepoint, embryo  
327 implantation is driving changes in immune cell infiltrates, which support the  
328 development of the pregnancy. Approximately 70% of decidual leukocytes are natural  
329 killer (NK) cells, 20-25% are macrophages, 1.7% are DCs, and approximately 3-7% are  
330 T cells (47, 48). The presence and modulation of each individual cell type is important to  
331 support decidual development and promote tolerance of the haploidentical fetus.  
332 However, the impact of the intricate immune modulation during pregnancy is not well  
333 examined during acute viral infection in the genital tract.

334         Macrophages are involved in remodeling of the spiral arteries during early  
335 pregnancy, a process which is crucial in establishing blood flow to the placenta (49, 50).  
336 Macrophages are broadly classified into two subtypes: classically activated M1  
337 macrophages, which are considered pro-inflammatory, and alternatively activated M2  
338 macrophages, which have anti-inflammatory properties and are involved in tissue repair  
339 (51-53). We found that pregnant mice exhibited higher frequencies of uterine CD68+  
340 macrophages with lower expression of IL-12 upon ZIKV infection. Previous studies have  
341 shown that several M2 markers, including CD206, CCL18, CD163, IL-10, and mannose  
342 receptor c type (MRC)-1 are expressed on decidual macrophages (54-56). Human  
343 placental macrophages, or Hofbauer cells, are targets of ZIKV and promote  
344 dissemination of the virus. Infected Hofbauer cells produce pro-inflammatory cytokines,



345 including MCP-1, IL-6, IP-10, and type I interferons (57). It is unclear how pregnancy-  
346 induced immunotolerance would impact responses of Hofbauer cells, and future studies  
347 should examine the interaction between maternal and placental immune regulation  
348 during acute viral infection.

349 CD11c+ DCs are crucial for early placentation and regulate tissue remodeling  
350 and angiogenesis (58). While the inflammatory activity of decidual DCs is important to  
351 support early implantation, they are altered by the local environment, resulting in loss of  
352 migration of uterine DCs to the lymph nodes (59). However, the specific changes in  
353 activation and cytokine production in DCs during acute infection have not yet been  
354 characterized. Tolerogenic DCs, including the CD11b+ CD11c+ and CD11c+ CD103+  
355 populations, promote immunotolerance toward the fetus during pregnancy and secrete  
356 anti-inflammatory mediators including IL-10 (36-39). We found that both subtypes were  
357 upregulated in pregnant mice, regardless of infection. Interestingly, we found that there  
358 were fewer CD11b+ CD11c+ IL-10+ cells in the uteruses of mock-inoculated pregnant  
359 mice than mock-inoculated non-pregnant mice. However, in the ZIKV-infected pregnant  
360 mice did not decrease IL-10 expression or CD11c+ CD103+ cells. These results  
361 suggest that pregnancy signals maintain immunotolerant signaling and cell types  
362 despite acute viral infection in these tissues. Additionally, pregnant mice exhibited fewer  
363 CD86+ CD11b+ CD11c+ cells in the uterus, which is indicative of less mature,  
364 unactivated DCs. Taken together, these results indicate that pregnancy-associated  
365 tolerogenic DCs are not significantly suppressed by ZIKV infection. This likely impacts  
366 the induction of downstream immune responses to the virus. Further studies are needed

367 to determine the effects of the pregnancy-induced tolerogenic immune environment on  
368 the anti-viral adaptive immune responses.

369 Our data also show that uterine monocytes, macrophages, and DCs are deficient  
370 in IL-12 production upon challenge with ZIKV during pregnancy. Since IL-12 is an  
371 important activator of NK cell responses(60), suppression of IL-12-induced activation of  
372 uterine NK cells is likely important to prevent NK activation and increased risk to the  
373 pregnancy. Our data show that virus-induced production of IL-12 by CD45+ cells is  
374 significantly reduced in pregnant mice compared to non-pregnant mice. This may be an  
375 important mechanism by which the localized immune response in the decidua is  
376 modulated to protect the pregnancy while still mounting an immune response to viral  
377 infection that is efficacious but not deleterious to the developing fetus. These findings  
378 should be evaluated as a potential biomarker of pregnancy loss during acute infection,  
379 as these markers may provide prognostic value for pregnancy loss and complications.

380 The findings of this study may be broadly applicable to other acute infections  
381 during pregnancy, and further studies are needed to evaluate pregnancy-induced  
382 immune modulation during acute infection and vaccination. Additionally, our data show  
383 that activation of important antigen presenting cells are modulated during pregnancy..  
384 These findings have important implications for vaccine studies during pregnancy as  
385 well, since dendritic cells and other antigen presenting cells are vital for development of  
386 the adaptive immune response that defines vaccine outcomes. In conclusion, our  
387 results show that pregnancy-induced immunotolerance impacts the acute innate cellular  
388 response to ZIKV infection and inhibits important features of the acute anti-viral immune  
389 response. Further studies are also needed to examine the impact of pregnancy-induced

390 modulation of acute anti-viral immune responses on the adaptive immune response, in  
391 pregnancy outcomes during acute infection, and in vaccine outcomes during pregnancy.

392

## 393 **Materials and Methods**

### 394 *Ethics Statement*

395 All animal research was approved by the University of Colorado and Denver VAMC  
396 local Institutional Animal Care and Use Committees. Approval number 1098v3. All laws  
397 and regulations regarding animal care and euthanasia were followed according to  
398 guidelines from the PHS/NIH/OLAW policy, Animal Care Policy (USDA), and the AVMA  
399 guidelines on euthanasia.

### 400 *Virus propagation and cell culture*

401 Vero cells (ATCC, Manassas, VA) and C6/36 cells (ATCC) were cultured at 37°C  
402 and 5% CO<sub>2</sub> in complete Minimal Essential Media (MEM) supplemented with 10% fetal  
403 bovine serum (FBS, HyClone, Thermo Fisher Scientific, Waltham, MA). ZIKV strain  
404 PRVABC59 (GenBank: KU501215) was provided by the Centers for Disease Control  
405 (CDC, Atlanta, GA). ZIKV stocks were propagated in Vero cells at passage 4 and C6/36  
406 cells at passage 1, and cell culture supernatants were harvested at 6 days post  
407 infection. Virus stocks were titrated in Vero cells using a focus forming assay (FFA) and  
408 were aliquoted and stored at -80°C.

### 409 *Mice*

410 Six-week-old C57BL/6J (stock no. 000664) male and female mice were  
411 purchased from Jackson Laboratory (Bar Harbor, ME). The mice were housed in a  
412 pathogen-free animal facility at the University of Colorado Anschutz Medical Campus  
413 (Aurora, CO) and maintained on a 12:12 light/dark cycle at 21-24°C. Eight-week-old  
414 female mice were mated with male mice ranging from 8-20-weeks-old. Each mating pair  
415 was housed separately.

#### 416 *Hormone Treatment*

417 To increase the likelihood of pregnancy in the mice, female mice were treated  
418 with exogenous gonadotropins to increase ovulation (61). Two days before mating,  
419 female mice were injected intraperitoneally with 2.5 iu of Pregnant Mare Serum  
420 Gonadotropin (bioWORLD, Dublin, OH). 48 hours later, they were intraperitoneally  
421 injected with 2.5 iu human chorionic gonadotropin (HCG, Sigma Aldrich, St. Louis, MO)  
422 and immediately mated with male mice overnight (16 hours). The males and females  
423 were separated the following morning (E0.5).

#### 424 *Zika virus infection*

425 On day E4.5, the eight-week-old pregnant and non-pregnant female mice were  
426 randomly assigned to either the mock or ZIKV infection groups. The mice were  
427 anaesthetized with isoflurane (McKesson Corporation, Irving, TX) and infected  
428 intravaginally with 5x10<sup>5</sup> FFU of PRVABC59 ZIKV in 15 µL of HBSS (Gibco, Thermo  
429 Fisher). Mock-infected mice received 15 µL of HBSS intravaginally. Spleen and uterine  
430 tissues were harvested for flow cytometry at 3 days post infection (E7.5).

#### 431 *Vaginal lavages*

432 Vaginal lavages were performed 48 hours post infection. Mice were  
433 anaesthetized with isoflurane, and 50  $\mu$ L of sterile phosphate buffered saline (PBS,  
434 Corning, Corning, NY) was inserted into the urogenital tract using a micropipette. The  
435 liquid was expelled slowly into the urogenital tract and then drawn back up and mixed  
436 with 200  $\mu$ L of sterile PBS supplemented with 1% FBS. The samples were vortexed for  
437 30 seconds and then aliquoted and stored at -80°C.

#### 438 *RNA extraction and qPCR*

439 ZIKV RNA was isolated from the vaginal lavage samples using the E.Z.N.A. Viral  
440 RNA Kit (Omega Bio-tek, Norcross, GA) according to the manufacturer's instructions.  
441 The primer and probe set Zika1087/1108FAM/1163c (IDT, Coralville, Iowa) was used to  
442 detect viral RNA. Real-time qPCR was performed using the Luna Universal Probe  
443 qPCR Master Mix (New England Biolabs, Ipswich, MA) with amplification on the Biorad  
444 CFX96 Real Time PCR Detection system, both per the manufacturer's instructions. The  
445 sensitivity of this assay was evaluated by testing known dilutions of an RNA transcript  
446 copy of the ZIKV P1 plasmid. Concentration of viral RNA (copies/microliter) was  
447 calculated using the standard curve generated by the CFX96 instrument.

#### 448 *Tissue processing*

449 Spleen tissues were processed into single-cell suspensions by mechanical  
450 dissociation; tissues were crushed through a 70  $\mu$ m cell strainer (CELLTREAT,  
451 Pepperell, MA) using disposable plastic pestles (CELLTREAT). Red blood cells (RBC)  
452 were removed by incubating the cell suspensions in 5 mL 1X RBC Lysis Buffer  
453 (eBioscience, Thermo Fisher) for 5 minutes at room temperature. The cells were then

454 washed in 30 mL of R10 media (RPMI with L-glutamine (Corning) + 10% FBS + 1%  
455 Penicillin/Streptomycin (Corning) + 1% HEPES (Gibco) + 1% Sodium Pyruvate (Gibco)  
456 + 1% MEM Non-essential amino acids (MEM-NEAA, Gibco)), vortexed, and centrifuged  
457 at 500 rcf.

458 Uterine tissues were enzymatically digested using Liberase TL (Roche, Basel,  
459 Switzerland) at a final concentration of 160 µg/mL in HBSS (Gibco, Thermo Fisher).  
460 First, each tissue was suspended in 500 µL of cold liberase + HBSS in a 1.5 mL  
461 Eppendorf tube and mechanically dissociated using small surgical scissors. Next,  
462 another 500 µL of Liberase + HBSS was added to each tissue, and the samples were  
463 incubated at 37°C for 35 minutes with occasional vortexing. Samples were kept on ice  
464 in between steps. After incubation at 37°C, the dissociated tissues were filtered through  
465 100 µm cell strainers (CELLTREAT).

466 After preparation of single-cell suspensions, the samples were centrifuged at 500  
467 rcf for 5 minutes, counted using Trypan blue (Corning), and resuspended at a  
468 concentration of  $1 \times 10^6$  cells/mL in R10 media. The cells were then aliquoted into FACS  
469 tubes ( $0.25\text{-}1 \times 10^6$  cells/tube) with strainer caps (BD Biosciences, San Jose, CA).

#### 470 *Flow cytometry*

471 The following antibodies were used for extracellular flow cytometry: anti-mouse  
472 CD45 BV650 (clone 30-F11, Biolegend, San Diego, CA), anti-mouse/human CD11b  
473 APC-Cy7 (clone M1/70, Biolegend), anti-mouse CD11c PE-eFluor 610 (clone N418,  
474 eBioscience), anti-mouse I-A/I-E (MHCII) FITC (clone M5/114.15.2, Biolegend), anti-  
475 mouse CD103 BV711 (clone 2E7, Biolegend), anti-mouse Ly6C BV785 (clone HK1.4,

476 Biolegend), anti-mouse CD24 PerCP-eFluor 710 (clone M1/69, eBioscience), anti-  
477 mouse CD86 PE-Cy7 (clone GL-1, Tonbo Biosciences, San Diego, CA), and anti-  
478 mouse F4/80 BUV395 (clone T45-2342, BD Biosciences). The following antibodies  
479 were used for intracellular flow cytometry: anti-mouse CD68 PE-Cy7 (clone FA-11,  
480 Biolegend), anti-mouse CD3 BUV395 (clone 145-2C11, BD Biosciences), anti-mouse  
481 CD3 BV785 (clone 17A2, Biolegend), anti-mouse IL-12 (p40/p70) PE (clone C15.6, BD  
482 Biosciences), anti-mouse IL-6 APC (clone MP5-20F3, BD Biosciences), and anti-mouse  
483 IL-10 APC (clone JES5-16E3, Biolegend). Ghost Violet 510 dye (Tonbo Biosciences)  
484 was used to assess viability.

485         Single cell suspensions were washed in PBS, centrifuged at 500 rcf for 5  
486 minutes, and briefly vortexed. Next, 10  $\mu$ L of viability dye (0.1  $\mu$ L dye + 10  $\mu$ L FACS  
487 buffer (1% FBS in PBS) per sample) was added to each sample, vortexed, and  
488 incubated at room temperature for 10 minutes. Next, 50  $\mu$ L of extracellular antibodies  
489 prepared in FACS buffer were added to each sample, vortexed, and incubated at 4°C  
490 for 25 minutes. 210  $\mu$ L of Cytofix/Cytoperm solution (BD Biosciences) per sample was  
491 then added to permeabilize the cells, followed by vortexing and incubation for 20  
492 minutes at 4°C. The cells were then washed in 1 mL of 1x Perm/Wash buffer (BD) or  
493 Flow Cytometry Perm Buffer (Tonbo Biosciences) twice, centrifuged at 700 rcf for 5  
494 minutes, and vortexed. Next, 50  $\mu$ L of intracellular antibodies in Perm/Wash or Perm  
495 buffer were added to each sample, vortexed, and incubated at 4°C of 45 minutes. The  
496 samples were then washed once more in Perm/Wash or Perm buffer, centrifuged at 700  
497 rcf for 5 minutes, vortexed, and finally fixed in 1% paraformaldehyde (Thermo Fisher).

498           The data was acquired on a LSRII flow cytometer (BD) using voltages  
499 standardized according to previously published methods (62). FlowJo software (FlowJo,  
500 LLC, Ashland, Oregon) was used to analyze the data. The gating strategies

#### 501 *Statistics*

502           All statistical analysis was performed in Prism 7 software (GraphPad, San Diego,  
503 CA). One-way ANOVA and Tukey's multiple comparison tests were used to compare  
504 cell frequencies between pregnant and non-pregnant mock and ZIKV-infected mice. P  
505 values, F values, and degrees of freedom for each parameter measured are shown. T  
506 tests were used when only two groups were compared. All the data are presented as  
507 mean  $\pm$  standard deviation.  $P < 0.05$  was considered statistically significant. All data  
508 shown represent 2 experiments of 19-20 mice each (n=39-40 mice total for each  
509 parameter measured).

510

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691

## 692 **Figure Legends:**

693 **FIG 1. Intravaginal ZIKV infection in C57BL/6 mice. A)** Experimental methodology:  
694 female C57BL/6 mice were treated with gonadotropins, mated, and then infected  
695 intravaginally with ZIKV at embryonic day 4.5 (E4.5). Spleen and uterine tissues were  
696 harvested at day 3 post infection (E7.5). **B)** Vaginal lavages were performed 48 hours  
697 post infection, and ZIKV RNA was detected by qPCR. N=25. **C)** Fetuses were dissected

698 from intact uteruses from ZIKV-infected (red triangles) and mock-infected (blue circles)  
699 pregnant mice. N=25. Error bars represent the mean  $\pm$  standard deviation.

700

701 **FIG 2. Changes to the peripheral immune response in the spleen during**  
702 **pregnancy and ZIKV infection.** Flow cytometry was performed on the splenic immune  
703 cells of ZIKV-infected (red triangles) and mock-infected (blue circles) pregnant and non-  
704 pregnant mice, and frequencies of CD68+ (A), CD11b+ (B), CD11c+ (C), Ly6C+ (D),  
705 CD103+ (E), CD11b+ Ly6C high (hi) (F), and CD11b+ Ly6C low (lo) (G) cells were  
706 measured. Representative pseudocolor plots showing gating for CD11b are shown in  
707 (A). \* $p < 0.05$ , \*\* $p < 0.01$ ; one-way ANOVA and Tukey's multiple comparisons test. N=39.  
708 Error bars represent the mean  $\pm$  standard deviation.

709

710 **FIG 3. Pregnant mice have higher frequencies of uterine CD68+ macrophages.**  
711 Flow cytometry was performed on the uterine immune cells of ZIKV-infected (red  
712 triangles) and mock-infected (blue circles) pregnant and non-pregnant mice.  
713 Frequencies of CD68+ (A), CD11b+ (B), CD11c+ (C), Ly6C+ (D), CD11b+ Ly6C hi (E),  
714 and CD11b+ Ly6C lo (F) cells were measured. Left panel, A) representative  
715 pseudocolor plots showing gating for CD68+ cells. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; one-way  
716 ANOVA and Tukey's multiple comparisons test. N=40 for (B), N=39 for all other panels.  
717 Error bars represent the mean  $\pm$  standard deviation.

718 **FIG 4. Pregnant mice have higher frequencies of CD11b+ CD11c+ tolerogenic**  
719 **dendritic cells in the uterus.** Flow cytometry was performed on the uterine immune

720 cells of ZIKV-infected (red triangles) and mock-infected (blue circles) pregnant and non-  
721 pregnant mice. **A)** Frequencies of CD11b+ CD11c+ cells and CD11b+ CD11c+ MHCII+  
722 cells in the uterus and **(B)** frequencies of CD11b+ CD11c+ cells in the spleen. **C)**  
723 CD86+ CD11b+ CD11c+ MHCII+ cells, and **D)** IL-10+ CD11b+ CD11c+ MHCII+ cells  
724 were measured. Representative flow cytometry plots are also shown in A (CD11b+  
725 CD11c+ cells within the CD45+ population) and B (CD86+ cells within the CD11b+  
726 CD11c+ MHCII+ population). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; one-way ANOVA and  
727 Tukey's multiple comparisons test. N=40. Error bars represent the mean  $\pm$  standard  
728 deviation.

729

730 **FIG 5. Pregnant mice have higher frequencies of CD11c+ CD103+ tolerogenic**  
731 **dendritic cells in the uterus.** Flow cytometry was performed on the uterine immune  
732 cells of ZIKV-infected (red triangles) and mock-infected (blue circles) pregnant and non-  
733 pregnant mice. Frequencies of **A)** CD11c+ CD103+ cells, **B)** CD11c+ CD103+ CD86+  
734 cells, and **C)** CD11c+ CD103+ IL-10+ cells were measured in the uterus. **D)**  
735 Frequencies of CD11c+CD103+ cells in the spleen. A representative flow cytometry plot  
736 (CD11c+ CD103+ cells within the CD45+ population) is shown in (A). \* $p < 0.05$ , \*\* $p < 0.01$ ,  
737 \*\*\* $p < 0.001$ ; one-way ANOVA and Tukey's multiple comparisons test. N=40. Error bars  
738 represent the mean  $\pm$  standard deviation.

739

740 **FIG 6. Pregnant mice have lessened IL-12 responses in the uterus during Zika**  
741 **virus infection.** Flow cytometry was performed on the uterine immune cells of ZIKV-

742 infected (red triangles) and mock-infected (blue circles) pregnant and non-pregnant  
743 mice. Frequencies of IL-12-expressing cells were measured within the uterine CD45+  
744 (A), CD11b+ (B), CD68+ (C), CD103+ (D), and Ly6C+ (E) populations. (F&G)  
745 Frequencies of IL-6-expressing cells were measured within the uterine CD45+, CD68+,  
746 CD11b+, CD11c+, Ly6C+, and CD103+ populations. N=39. Representative flow  
747 cytometry plots depicting IL-12+ cells within the CD45+ population are shown in (A).  
748 \*p<0.05, \*\*p<0.01; one-way ANOVA and Tukey's multiple comparisons test. N=39. Error  
749 bars represent the mean  $\pm$  standard deviation.

750

751 **FIG 7. Pregnant mice exhibit decreased IL-12 responses in the spleen during Zika**  
752 **virus infection.** Flow cytometry was performed on the splenic immune cells of ZIKV-  
753 infected (red triangles) and mock-infected (blue circles) pregnant and non-pregnant  
754 mice. Frequencies of IL-12-expressing cells were measured within the splenic CD45+  
755 (A), CD68+ (B), CD11b+ (C), CD11c+ (D), Ly6C+ (E), and CD103+ (F) populations.  
756 (G&H) Frequencies of IL-6-expressing cells were measured within the uterine CD45+,  
757 CD68+, CD11b+, CD11c+, Ly6C+, and CD103+ populations. N=39. \*p<0.05, \*\*p<0.01;  
758 one-way ANOVA and Tukey's multiple comparisons test. N=39. Error bars represent the  
759 mean  $\pm$  standard deviation.

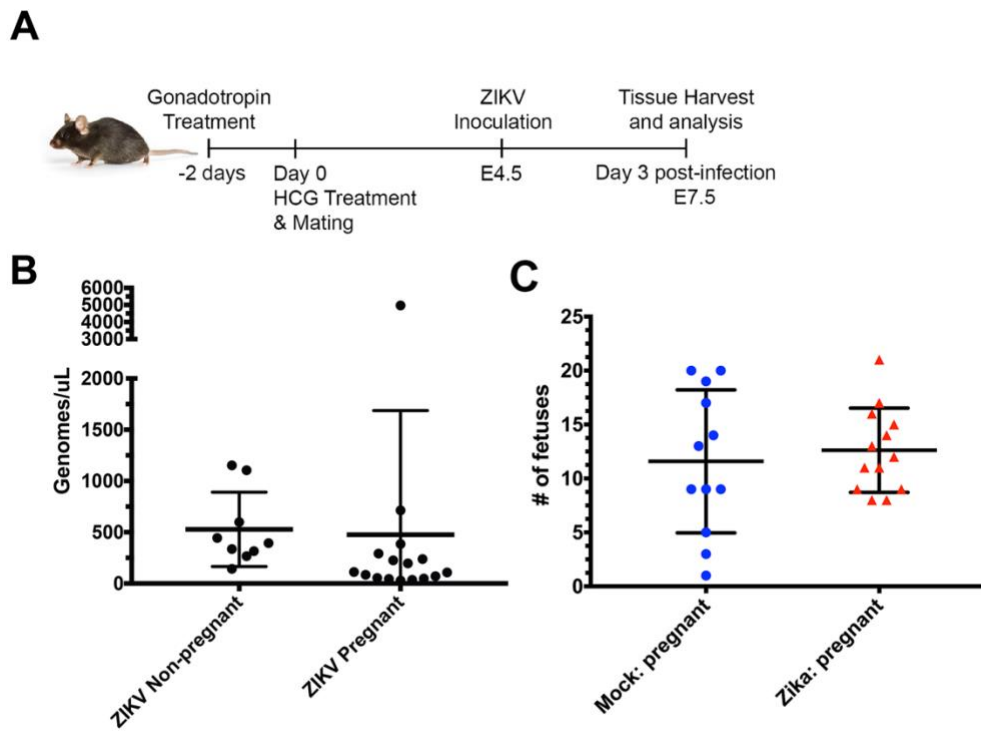
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764 Figure 1



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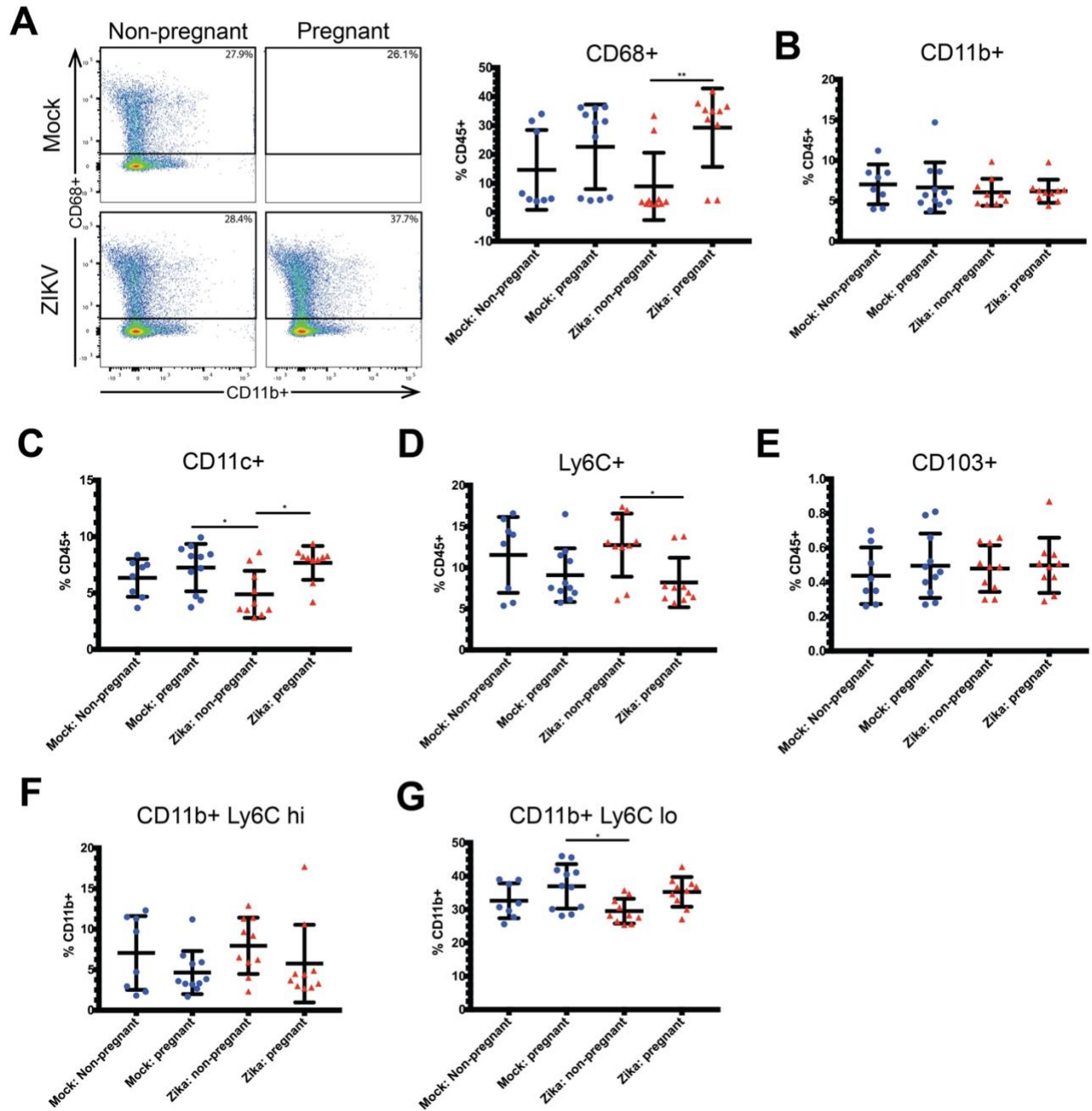
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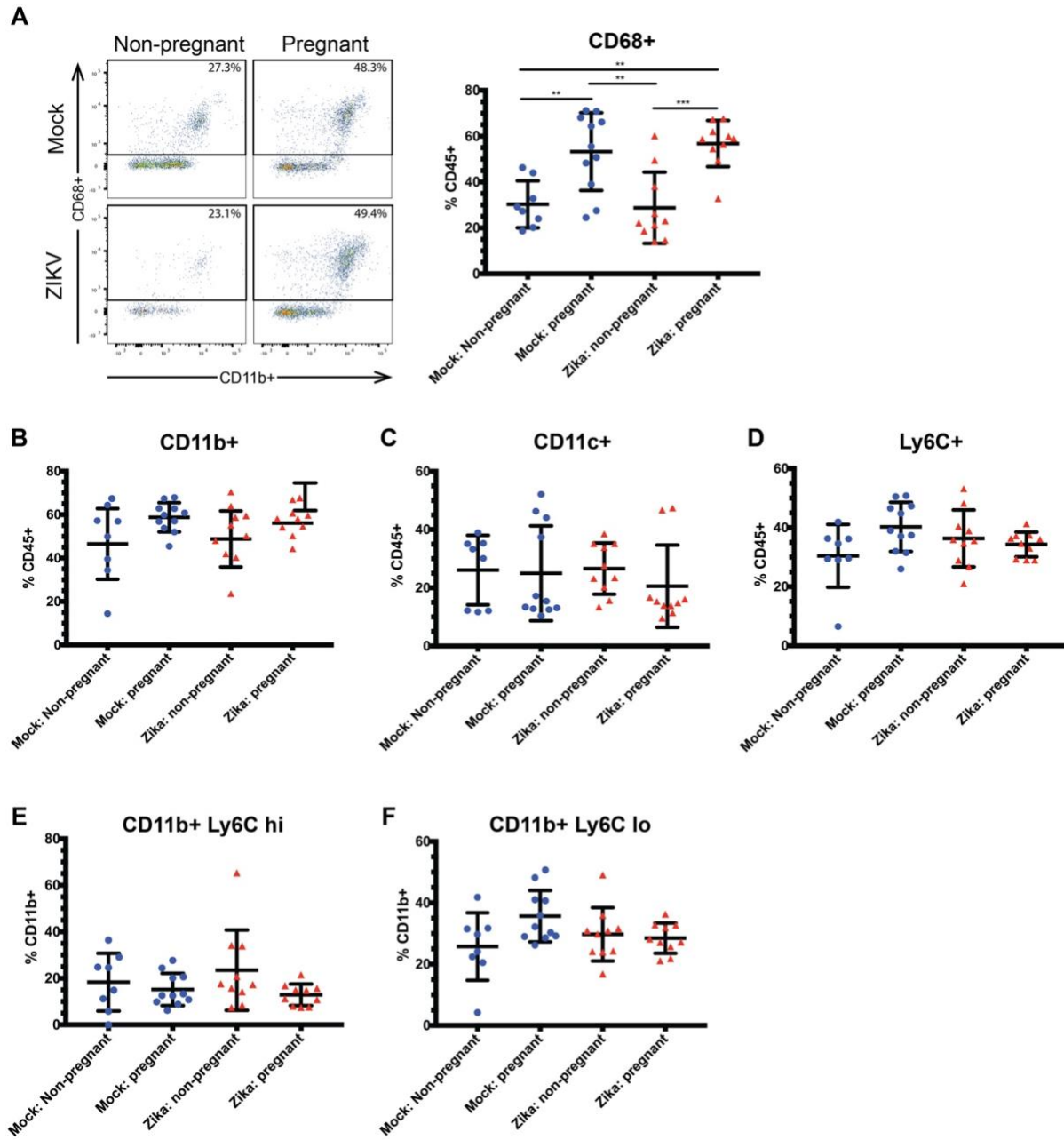
774 **Figure 2**



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777 **Figure 3**



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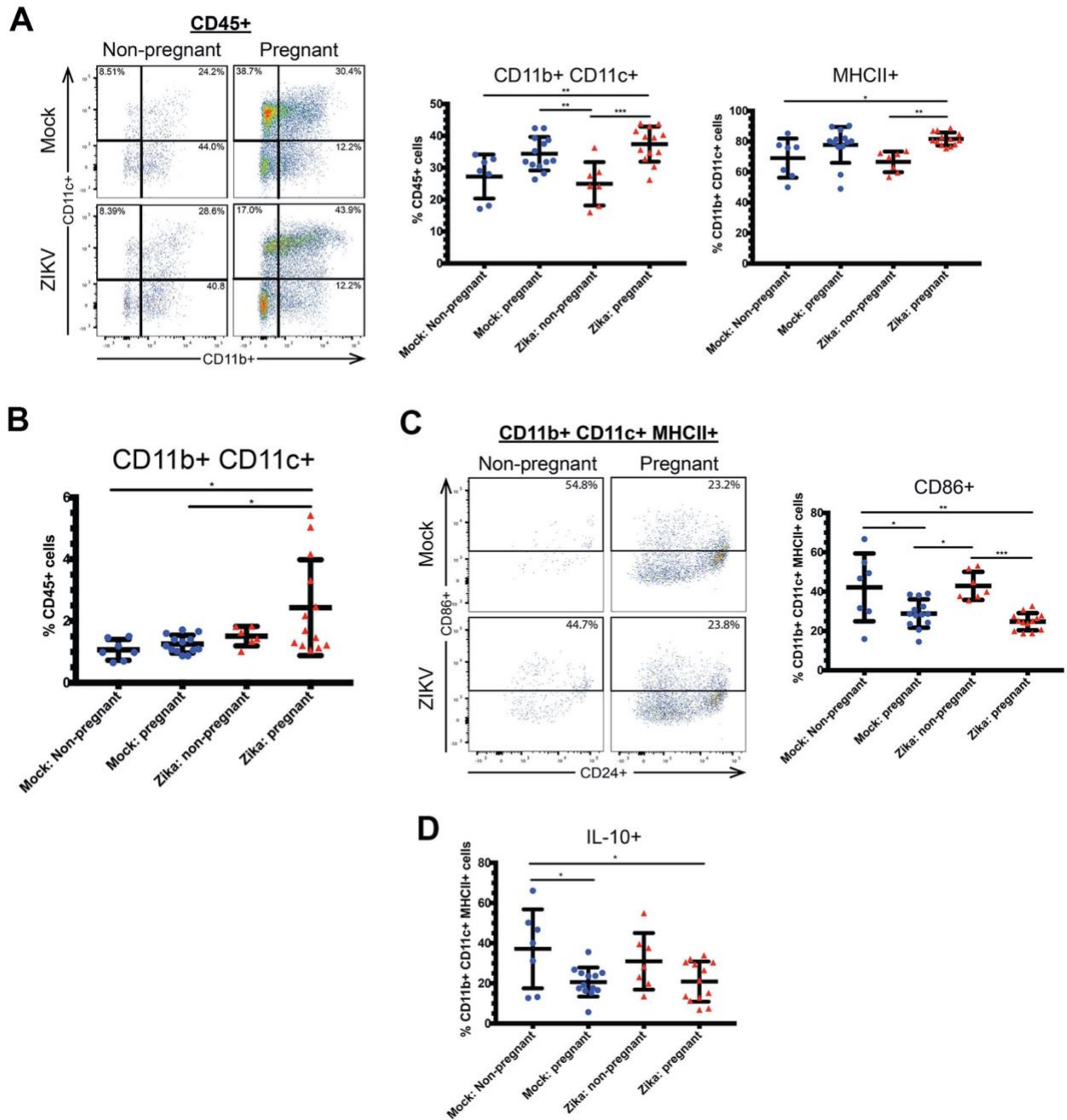
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782 **Figure 4**



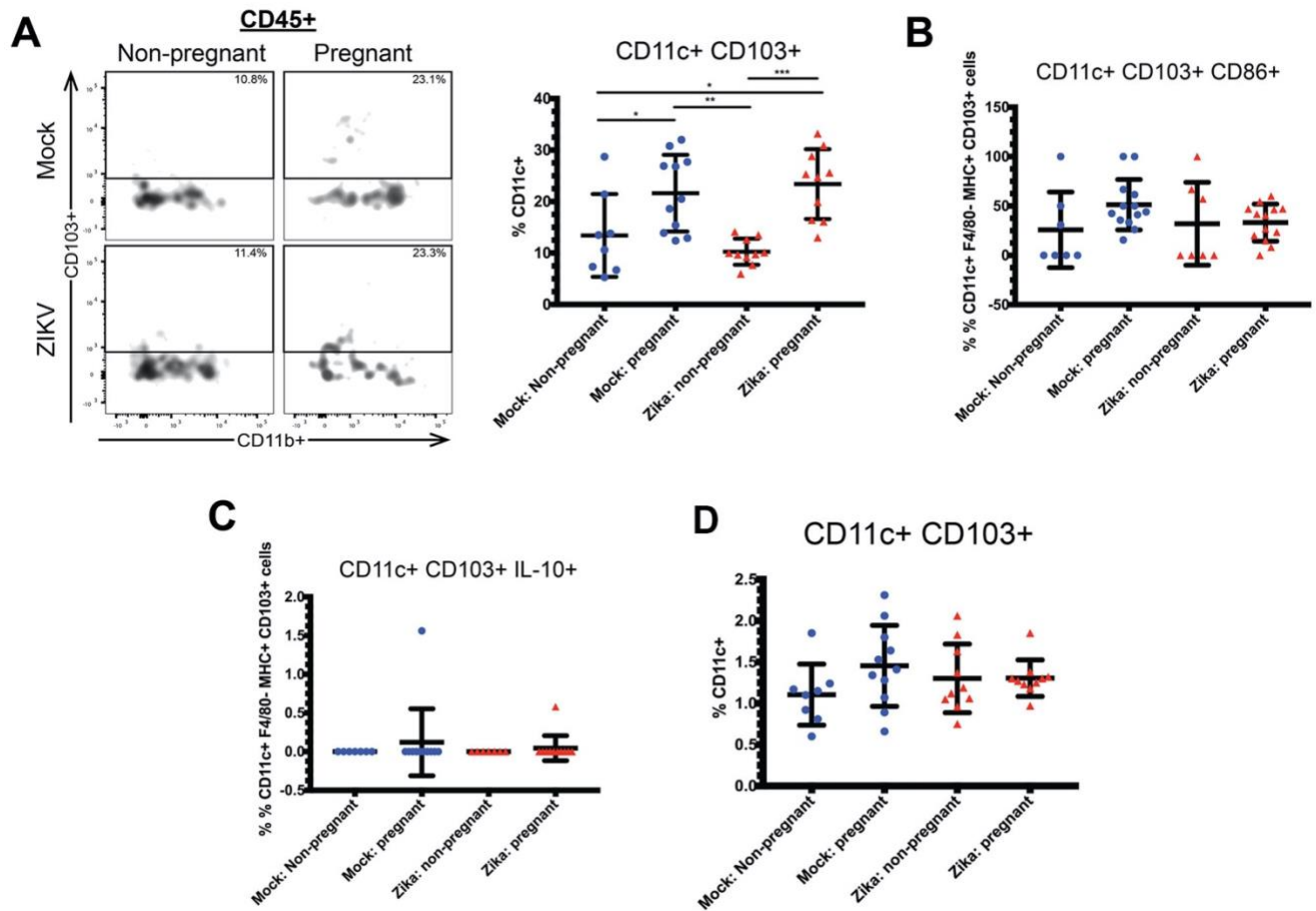
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786 **Figure 5**



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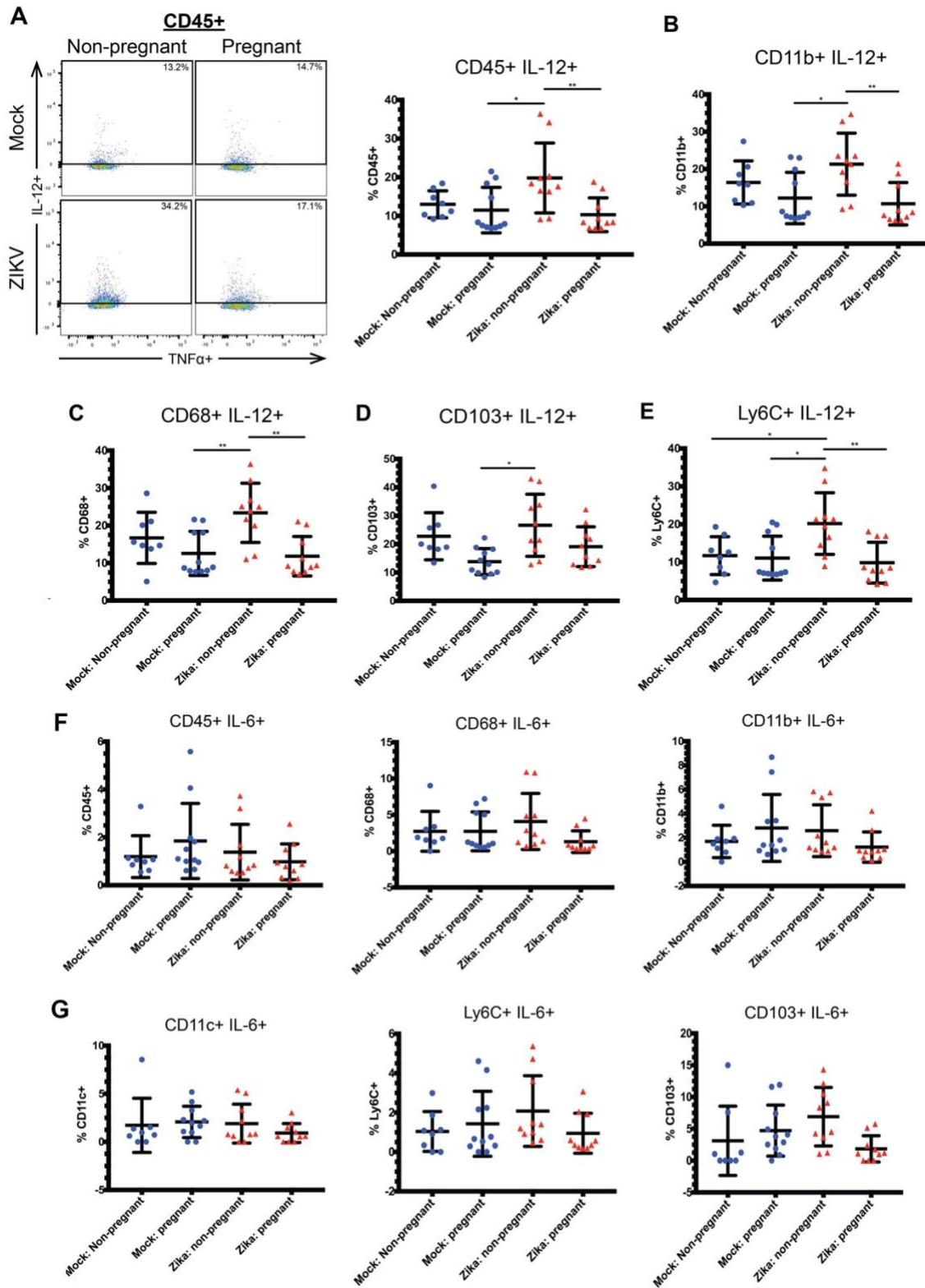
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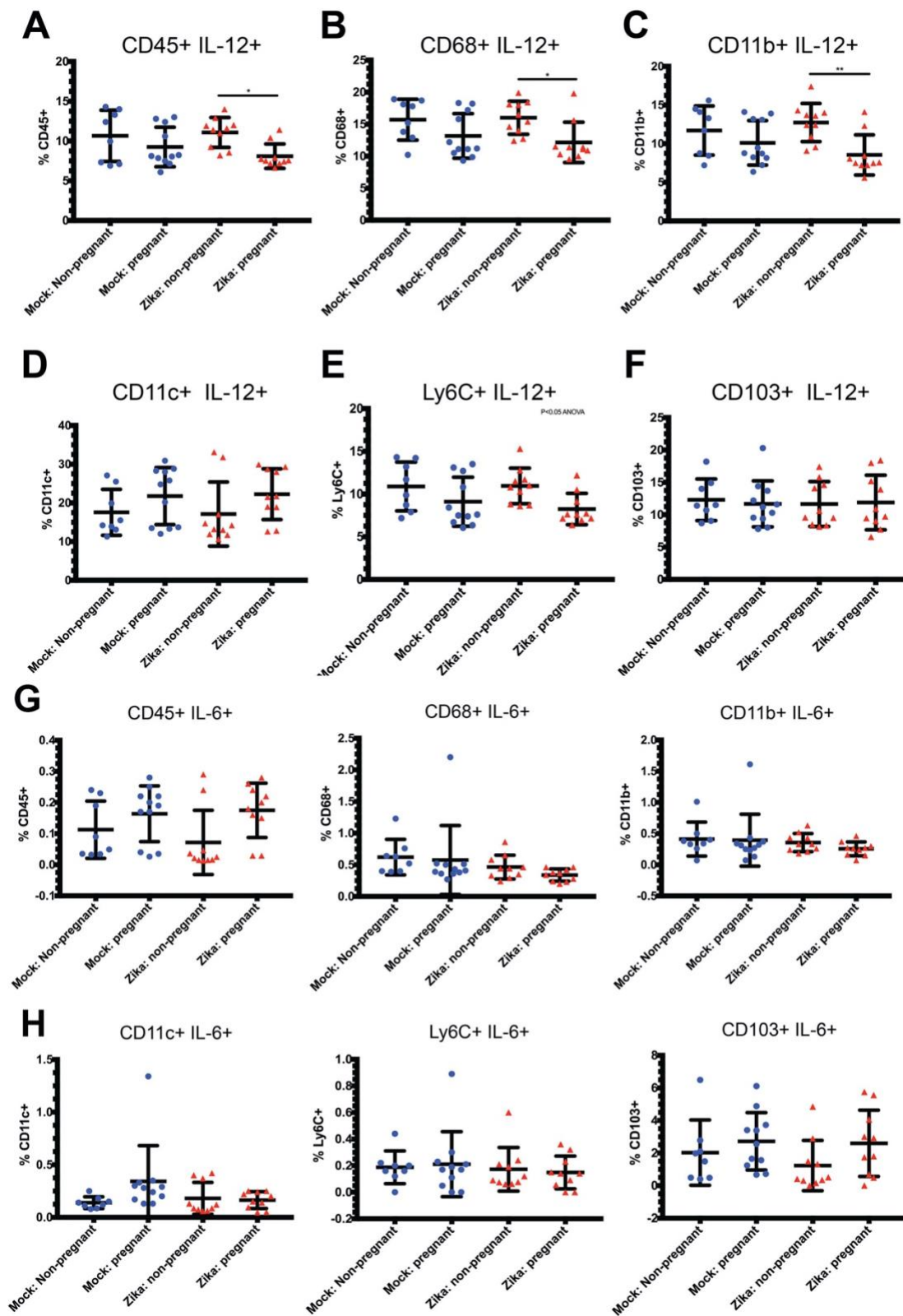
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795 **Figure 6**



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797 **Figure 7**



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