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Prolonged high-intensity exercise induces fluctuating immune responses to herpes simplex virus infection via glucocorticoids

（長時間高強度の運動はグルココルチコイドを介して単純ヘルペスウイルス感染症に対して変動性免疫応答を誘導する）

足立　晃正

主論文

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1 **Prolonged high-intensity exercise induces fluctuating immune responses to herpes**
2 **simplex virus infection via glucocorticoids**
3
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44 **Conflict of interest statement**

45 The authors have declared that no conflict of interest exists.

46

47 **Keywords**: prolonged high-intensity exercise, HSV-2, glucocorticoids, corticosterone,

48 CXCR4, CXCL12, plasmacytoid dendritic cell, fluctuation, viral infection

49

50 **Capsule summary**

51 We describe that prolonged high-intensity exercise leads to impaired or enhanced

52 immune responses against HSV-2 infection depending on the timing between infection

53 and exercise.

54

55 **Key messages**

56 - Prolonged high-intensity exercise 17 hours after HSV-2 infection induces impaired
57 viral clearance via glucocorticoid-induced pDC homing to the bone marrow.

58 - Prolonged high-intensity exercise 8 hours after HSV-2 infection induces enhanced
59 viral clearance via glucocorticoid-induced the number of pDC in the blood.

60

61 **Abbreviations**

62 HSV, herpes simplex virus

63 pDC, plasmacytoid dendritic cell

64 IFN, interferon

65 CXCR4, C-X-C chemokine receptor type 4

66 CXCL12, C-X-C motif chemokine 12

67 NK, natural killer

68 TLR, toll like receptor

69 LC-MS/MS, liquid chromatography tandem mass spectrometry

70 HIF, hypoxia inducible factor

71 GR, glucocorticoid receptor

72 GFP, green fluorescence protein

73

74

75 **Abstract**

76 **Background**: Epidemiological studies have yielded conflicting results regarding the
77 influence of a single bout of prolonged high-intensity exercise on viral infection.
78 **Objective**: Here, we show that prolonged high-intensity exercise induces either
79 exacerbation or amelioration of herpes simplex virus type 2 (HSV-2) infection depending
80 on the interval between viral exposure and exercise.
81 **Methods**: Mice were intravaginally infected with HSV-2 and exposed to run on the
82 treadmill.
83 **Results**: Prolonged high-intensity exercise 17 h after infection impaired the clearance of
84 HSV-2, while exercise 8 h after infection enhanced the clearance of HSV-2. These
85 impaired or enhanced immune responses were related to a transient decrease or increase
86 in the number of blood-circulating plasmacytoid dendritic cells (pDCs). Exercise-induced
87 glucocorticoids transiently decreased the number of circulating pDCs by facilitating their
88 homing to the bone marrow via the CXCL12-CXCR4 axis, which led to their subsequent
89 increase in the blood.
90 **Conclusion**: Thus, a single bout of prolonged high-intensity exercise can be either
91 deleterious or beneficial to antiviral immunity.

92

93

94 **Introduction**

95 Epidemiological studies indicate that individuals who perform regular, moderate exercise
96 have a reduced risk of infectious diseases and cancers[1,2]. However, the effect of a single
97 bout of exercise on immune function remains hotly debated. It was previously reported
98 that a single bout of prolonged high-intensity exercise, such as a marathon, may increase
99 the number of episodes of upper respiratory tract infection or herpes simplex virus (HSV)
100 infection[3–5]. Consistently, a single bout of prolonged high-intensity exercise in mice
101 induced high morbidity and mortality in response to several viral infections[6–8], suggesting
102 that there is an association between a single bout of prolonged high-intensity exercise and
103 immune system impairment against viral infection. The involvement of anti-
104 inflammatory molecules, such as glucocorticoids, catecholamines, and interleukin-10,
105 has been suspected[9]. On the other hand, some studies showed that prolonged high-
106 intensity exercise improves immune surveillance, enhancing antibacterial and antiviral
107 immunity[10,11]. Thus, the influence of a single bout of prolonged high-intensity exercise
108 on the immune system remains controversial, and the cause of these discrepancies
109 remains unclear.

110 These conflicting results might stem from the complexity of immune cell
111 dynamics during and after prolonged high-intensity exercise. In humans, the number of
112 circulating natural killer (NK) cells, myeloid dendritic cells (mDCs), neutrophils,
113 monocytes and T cells transiently increased during the exercise. After the exercise, the
114 number of circulating plasmacytoid dendritic cells (pDCs), NK cells and T cells decreased,
115 while circulating neutrophils and monocytes increased[12–18]. Functional impairments, such
116 as the downregulation of Toll-like receptor (TLR) expression on immune cells, have also
117 been reported[15,19]. Redistribution of T cells in the bone marrow, Peyer's patches and the

118 lungs has been observed after a single bout of prolonged high-intensity exercise in a
119 mouse study[10]. However, to date, there have been no studies that address the association
120 between these transient changes in blood immune cells and subsequent immune responses
121 to viruses. In addition, the underlying molecular mechanisms that cause transient changes
122 in the number of blood cells are poorly understood.

123 Here, we explored the impact of a single bout of prolonged high-intensity
124 exercise on the immune system and the underlying mechanisms using a mouse model of
125 HSV type 2 (HSV-2) intravaginal infection. Prolonged high-intensity exercise either
126 exacerbated or ameliorated clinical symptoms of HSV-2 infection depending on the
127 interval between viral exposure and exercise. The number of circulating pDCs, which
128 play important roles in protection against HSV-2, transiently decreased immediately after
129 prolonged high-intensity exercise and increased 6-12 h after exercise. Circulating pDCs
130 were selectively homing to the bone marrow via the CXCL12-CXCR4 pathway during
131 exercise in a glucocorticoid-dependent manner. The blockade of glucocorticoid signaling
132 inhibited the homing to the bone marrow of circulating pDCs and abrogated the effects
133 of exercise on immune responses to HSV-2. Our results demonstrate that glucocorticoids
134 play a novel role in coordinating the CXCL12-CXCR4 axis and antiviral immune
135 regulation in response to a single bout of prolonged high-intensity exercise.
136

Methods

Animals. C57BL/6 (B6) female mice were purchased from Oriental Bio Services (Kyoto, Japan) and B6. Tg-(CAG-GFP) mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). B6.Cg-Tg(Itgax-cre)1-1Reiz/J (*Cd11c*-Cre) mice, B6.129-*Hif1α*^{tm3Rsjo}/J (*Hif1a*$^{fl/fl}$) mice, R26-CAG-tdTomato mice, and EIIa^{Cre} mice were purchased from Jackson Laboratories (Bar Harbor, ME), and B6. 129-Cxcl12^{tm2Tng} (*Cxcl12*-GFP) and B6.129-*Nr3c1*^{tm2Gsc}(*Nr3c1*$^{fl/fl}$) mice were generated as previously described[54–57]. All mice were maintained under specific pathogen-free conditions at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. All experiments were performed on 7- to 12-week-old female mice. All procedures were carried out under isoflurane anesthesia. Mice were housed in groups with controlled humidity, temperature, and light conditions (14-hr light/10-hr dark cycle; lights on at 7:00 a.m. and off at 9:00 p.m.). Mice were acclimated to our laboratory environment for at least 2 weeks before experiments were performed. Food and water were available *ad libitum*.

Exercise protocol. The prolonged high-intensity exercise protocol and short periods of moderate-intensity exercise protocol were performed as previously described[20,22] with some modifications. Briefly, mice were acclimated to a custom-made treadmill for 10 min at 5-7 m/min and exposed to the environment of the laboratory room for 1 h for three consecutive days. Exercise was started during the last hour of the dark cycle. The treadmill grade was set at 0%. In the prolonged high-intensity exercise group, the speed of the treadmill was initially set to 11.5 m/min and was increased by 1-1.5 m/min every 25 min. The maximum speed was 19 m/min, and the total duration of exercise was 3 h. When the speed reached over 12 m/min or 17 m/min, the intensity corresponded to

161 approximately 76% or 84% of maximal oxygen uptake (VO_2max), respectively; 50-80%
162 of VO_2max was considered to be moderate-intensity exercise, and over 80% of VO_2max
163 was considered to be high-intensity exercise[58–60]. In the short periods of moderate-
164 intensity exercise group, the speed of the treadmill was initially set to 11.5 m/min, and
165 the total duration of exercise was 30 min. Electric shock was never used in the exercise
166 session because mice readily responded to a gentle tap that encouraged them to maintain
167 the pace of the treadmill. Mice in the no-exercise group were placed in cages near the
168 treadmill, and no food or water was provided during the exercise session.
169
170 *Viral infection and cells*. HSV-2 wild-type strain 186 and Vero cells were previously
171 described[61,62]. HSV-2 stocks were prepared in Vero cells and quantified by a plaque-
172 forming assay. For HSV-2 infection, mice were pretreated by the subcutaneous injection
173 of the neck ruff with 1.67 mg of medroxyprogesterone acetate (A.N.B. Laboratories,
174 Bangkok, Thailand) per mouse in a volume of 100 μL. Five days later, mice were
175 anesthetized with isoflurane (Wako, Osaka, Japan). The vagina was washed with 100 μL
176 of phosphate-buffered saline (PBS), and mice were intravaginally infected with 3.5×10^2
177 plaque-forming units (PFU) of HSV-2 strain 186 in 10 μL volumes suspended in 199V.
178 Disease severity was assessed daily until 14 d post infection using a clinical scoring
179 system that was previously described[63]. The growth mediums used were Dulbecco's
180 modified Eagle's medium (Sigma-Aldrich, St Louis, MO) supplemented with 5% heat-
181 inactivated fetal calf serum (FCS) (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, and
182 100 μg/mL streptomycin (Wako); and 199V medium (430 mL of sterilized distilled water
183 (dH_2O) supplemented with 50 mL of 199 medium (Sigma-Aldrich), 5 mL of 5% (wt/vol)
184 $NaHCO_3$ (Nacalai, Kyoto, Japan), 5 mL of 1% FCS (Invitrogen), 100 U/mL of penicillin

185 and 100 μg/mL streptomycin with L-glutamine (Wako)).

186

187 *Viral titer measurement in the vagina*. Vaginal washes were collected for 2 consecutive
188 days after infection with HSV-2 by washing with 100 μL of 199V. Viral titers were
189 obtained titrating vaginal wash samples with 199V (400 μL) on a Vero cell monolayer in
190 12-well plates for 1 h at 37°C with 5% CO_2. After aspirating all medium, 1 mL of 199O
191 medium (199V and γ-globulins from human blood (Sigma-Aldrich)) was added, and
192 plates were incubated for 48 h at 37°C with 5% CO_2.

193

194 *Plasmacytoid dendritic cell depletion*. To deplete pDCs, mice were intraperitoneally
195 injected with 200 μg PDCA-1 Ab (clone #927; Bioxell, Lebanon, NH) three days and one
196 day prior to HSV-2 intravaginal infection. As a control, mice were intraperitoneally
197 injected with same amounts of isotype rat IgG2b Ab (clone#LTF-2; Bioxell). At 24 h and
198 48 h after injection, blood was collected by puncture with an 18G needle on the
199 submandibular area and confirmed pDC depletion by flow cytometry.

200

201 *Antibodies and flow cytometry*. Single-cell suspensions of vaginal tracts were obtained as
202 previously described[64] with some modifications. Briefly, the vagina was separated from
203 the urethra and cervix, cut into small pieces and incubated in 4 mg/mL Dispase II (Roche,
204 Basel, Switzerland), 0.425 mg/mL collagenase D (Roche), 30 μg/mL DNase I (Sigma-
205 Aldrich) and 100 U/mL hyaluronidase (Sigma-Aldrich) for 45 min at 37°C. After
206 perfusion, the lungs, liver, and gastrocnemius muscles were cut into small pieces. Lungs
207 were digested with 150 U/mL collagenase type IV (Worthington Biochemical, Lakewood,
208 NJ) and 150 μg/mL DNase I at 37°C for 55 min. Livers were digested with 0.05%

209 collagenase/Dispase (Roche) at 37°C for 30 min. Gastrocnemius muscles were digested
210 with 250 U/mL collagenase type II (Worthington Biochemical) and 150 μg/mL DNase I
211 at 37°C for 55 min. Cells were further digested with 0.01 M EDTA at 37°C for 5 min and
212 were filtered through a 70-μm cell strainer. Leukocytes of liver and muscles were
213 separated by centrifugation with a 33% Percoll solution (GE Healthcare, Fairfield, Conn).
214 Bone marrow cell suspensions, which were flushed from femurs and tibias, lymph nodes,
215 and spleens, were digested with 500 U/mL collagenase type II (Worthington Biochemical)
216 and 100 μg/mL DNase I at 37°C for 25 min, further digested with 0.01 M EDTA at 37°C
217 for 5 min and filtered through a 40-μm cell strainer. Peripheral blood was collected by
218 puncture with an 18G needle on the submandibular area. Nonspecific antibody binding
219 was blocked with an anti-CD16/32 antibody (BD Biosciences, San Jose, Calif) at 4°C for
220 10 min, and then cells were stained for surface antigens. For the intracellular staining of
221 IFN-α, cells were fixed and permeabilized with fixation and permeabilization solution
222 (BD Biosciences). After lysing red blood cells, cells were stained with antibodies and
223 fixable viability dye (eBioscience, San Diego, CA) for 30 min at 4°C in Brilliant Stain
224 Buffer (BD Biosciences) for surface antigens. Fluorescent dye- or biotin-conjugated
225 antibodies against the following proteins were purchased from BD Bioscience,
226 eBioscience, BioLegend (San Diego, CA), R&D Systems (Minneapolis, MN), Miltenyi
227 Biotec (Bergisch Gladbach, Germany), and PBL Assay Science (Piscataway, NJ): B220
228 (RA3-6B2), CD3 (17A2), Ly6G (1A8), Ly6C (HK1.4), CXCR3 (CXCR3-173), CXCR4
229 (L276F12), CCR2 (475301), CCR5 (HM-CCR5), CCR7 (4B12), CCR9 (CW-1.2),
230 CD11a/CD18 (H155-78), CD11b (M1/70), CD11c (N418), CD19 (6D5), CD45 (30-F11),
231 CD49d (R1-2), CD115 (AF598), CD127 (A7R34), CD317 (927), ChemR23 (477806), I-
232 A/I-E (M5/114.15.2), NK1.1 (PK136), Siglec-H (551), TLR9 (M9.D6), and IFN-α

233 (RMMA-1). Each cell is identified as following panel; pDC; $CD3^{-}CD19^{-}CD11b^{-}$
234 $B220^{+}PDCA1^{+}$, NK cell; $CD3^{-}NK1.1^{+}$, neutrophil; $CD11b^{+}Ly6G^{+}$, inflammatory
235 monocytes $CD11b^{+}Ly6C^{high}$, cDC; $CD11c^{+}I\text{-}A/I\text{-}E^{high}$. Multiparameter analyses were
236 performed on an LSRFortessa cytometer (LSR II; BD Biosciences) and were analyzed
237 using FlowJo software (Tree Star, Ashland, OR).
238
239 *Drug administration*. Drugs were administered either intraperitoneally (AMD3100,
240 RU486, eplerenone, 6-OHDA, and corticosterone) or subcutaneously (SR59230A and
241 propranolol). All drugs were purchased from Sigma-Aldrich. AMD3100 was dissolved in
242 PBS and administered 30 min before exercise. RU486 (30mg/kg) was dissolved in
243 ethanol/sesame oil (1:10 vol/vol) and administered 1 h before exercise. Corticosterone
244 (10mg/kg) was dissolved in an ethanol/sesame oil solution (1:10 vol/vol) and
245 administered 3 h before harvesting the blood and bone marrow. SR59230A (5mg/kg),
246 propranolol (10mg/kg) and eplerenone (1mg/kg) were dissolved in DMSO and further
247 diluted with PBS. 6-OHDA was dissolved in 0.9% NaCl and 10^{-7} M ascorbic acid and
248 administered as previously described [65]. All dosing was determined by previously
249 established protocols[66–71].
250
251 *Adoptive cell transfer*. For adoptive transfer, the spleen and bone marrow were extracted
252 from B6-Tg (CAG-EGFP) mice or $EIIa^{Cre}$-R26-CAG-tdTomato mice. The tissues were
253 digested with 500 U/mL collagenase type II and 100 μg/mL DNase I at 37°C for 25 min
254 and further digested with 0.01 M EDTA at 37°C for 5 min. After lysing red blood cells,
255 cells were resuspended in PBS, and 4×10^{7} cells were intravenously injected into each
256 mouse 17 h before exercise.

257

258 *Immunohistochemistry*. To prepare bone marrow sections, mice were sacrificed
259 immediately after exercise and perfused with 4 mL of 4% paraformaldehyde for 15 min.
260 Then, femurs were harvested and fixed in equivalent amounts of 4% paraformaldehyde
261 and 20% sucrose for 1 h. Fixed samples were embedded in SCEM medium (Section-Lab,
262 Hiroshima, Japan) and frozen in cooled hexane. Sections (5 μm) of undecalcified femoral
263 bone were generated by Kawamoto's film method (Cryofilm transfer kit; Section-Lab)
264 and stained with anti-PDCA1 antibody. Confocal microscopy was performed with an
265 A1RMP (Nikon, Tokyo, Japan). The distance from transferred tdTomato$^+$PDCA1$^+$ cells
266 to the nearest GFP$^+$ cells in the bone marrow of *Cxcl12*-GFP mice was analyzed with
267 NIS-Elements AR analysis 4.50.00 64 bit for Windows (Nikon) and ImageJ version 1.52p
268 (NIH, Bethesda, USA).

269

270 *Enzyme-linked immunosorbent assay (ELISA) for corticosterone, noradrenaline and*
271 *CXCL12*. Plasma corticosterone was analyzed by a competitive enzyme immunoassay
272 (Enzo Lifesciences, Farmingdale, NY), plasma noradrenaline was analyzed by a
273 sandwich ELISA (IBL International, Hamburg, Germany), and CXCL12 in extracellular
274 fluid was collected by flushing femurs and tibias with 1 mL of PBS and was analyzed by
275 a sandwich ELISA (R&D systems). All procedures were performed according to the
276 manufacturer's protocol for each ELISA kit.

277

278 *LC-MS/MS analysis*. Steroid hormones were measured with LC-MS/MS, as previously
279 described [72] with some modifications. Briefly, LC-MS/MS was performed with a 1260
280 high-performance liquid chromatographer (Agilent Technologies, Santa Clara, CA) and

281 6460 triple quadrupole tandem mass spectrometer (Agilent Technologies). The analytical
282 column was a Capcell core C18 (Osaka Soda Co. Ltd., Osaka, Japan). Acetic acid/water
283 (0.1:100, vol/vol) and acetic acid/acetonitrile/methanol (0.1:50:50, vol/vol/vol) were used
284 as mobile phases A and B, respectively. The flow rate was set at 0.4 mL/min. Gradient
285 elution was performed by increasing B from 50% to 100% over 12 min. The selected
286 reaction monitoring (SRM) mode was set for MS/MS detection. All SRM transitions were
287 optimized with the infusion analysis of each standard solution. All source parameters
288 were optimized with the flow injection analysis of standard solutions.
289
290 *In vitro stimulation of bone marrow cells*. Single-cell suspensions from the bone marrow
291 were incubated at 4×10^6 cells/well in 96-well plates in RPMI (Invitrogen) supplemented
292 with 10% fetal calf serum (Invitrogen), 1% sodium pyruvate, 1% nonessential amino
293 acids (Invitrogen), 50 μM 2-mercaptoethanol (Nacalai), 100 U/mL penicillin, and 100
294 μg/mL streptomycin (Wako). For intracellular IFN-α staining, cells were cultured at 37°C
295 in the presence of 0.2 μg/mL granulocyte-macrophage colony-stimulating factor (GM-
296 CSF; Peprotech, Rocky Hill, NJ) and were stimulated with CpG-ODN 2395 (InvivoGen,
297 San Diego, CA) (5 μM) for 9 h. GolgiStop (1 μL/mL) (BD Biosciences) and brefeldin A
298 (1 μL/mL) (Sigma) were added for the last 6 h of the incubation. Subsequently, cells were
299 harvested and stained with antibodies against surface markers. Fixation and
300 permeabilization were performed with Cytofix/Cytoperm solution (BD Bioscience),
301 followed by intracellular staining with FITC-conjugated anti-IFN-α (PBL Assay Science).
302
303 *Chemotaxis assay*. Whole blood cells were lysed and incubated for 3 h with corticosterone
304 (Sigma) or vehicle. Then, cells were placed in the upper chamber of an uncoated transwell

305 with 5-μm pores (Corning, Corning, NY) for 1 h. CXCL12 (10 ng/mL; R&D Systems) or
306 medium was placed in the lower chamber. Cells in the lower chamber were analyzed with
307 flow cytometry. Cells were cultured in phenol red-free RPMI with 10% hormone-depleted
308 FCS.
309
310 *Quantitative RT-PCR analysis*. Total RNA was isolated with TRIzol reagent (Invitrogen)
311 and RNeasy kits (Qiagen, Hilden, Germany). cDNA was reverse transcribed from total
312 RNA samples by using a Prime Script RT reagent kit (Takara Bio, Kusatsu, Japan).
313 Quantitative RT-PCR was performed by monitoring double-stranded DNA synthesis
314 during various PCR cycles with SYBR Green I (Roche) and a LightCycler real-time PCR
315 apparatus (Roche) according to the manufacturer's instructions. All primers were
316 obtained from Greiner Japan (Tokyo, Japan). The primer sequences were as follows:
317 *Gapdh*, 5'-AGGTCGGTGTGAACGGATTTG-3' and 5'-
318 GGGGTCGTTGATGGCAACA-3'; *Cxcl9*, 5'-GGCACGATCCACTACAAATCC-3'
319 and 5'-GGTTTGATCTCCGTTCTTCAGT-3'; *Cxcl10*, 5'-CCAAGTGCTGCCGTC
320 ATTTTC-3' and 5'-GGCTCGCAGGGATGATTTCAA-3'; *Cxcl11*, 5'-GGCTTCCT
321 TATGTTCAAACAGGG-3' and 5'-GCCGTTACTCGGGTAAATTACA-3'; *Ccl3*, 5'-
322 TGAAACCAGCAGCCTTTGCTC-3' and 5'-AGGCATTCAGTTCCAGGTCAGTG-
323 3'; *Ccl4*, 5'-CCATGAAGCTCTGCGTGTCTG-3' and 5'-
324 GGCTTGGAGCAAAGACTGCTG-3'; *Ccl5*, 5'-AGATCTCTGCAGCTGCCCTCA-3'
325 and 5'-GGAGCACTTGCTGCTGGTGTAG-3'; and pan *Ifna*, 5'-
326 CCTGAGAGAGAAGAAACACAGCC-3' and 5'-TCTGCTCTGACCACYTCCCAG-
327 3'. For each sample, the gene expression of duplicate test reactions and a control reaction

328 lacking reverse transcriptase were analyzed, and the results were normalized to those of
329 *Gapdh* mRNA.
330
331 *Statistics*. In the present study, we hypothesized that the clinical scores at day 14 and viral
332 titer at day 1 and 2 might show substantial differences between CONTROL and
333 PROLONGED groups under the respective situations with vehicle only or antagonists
334 (AMD3100 or RU486). Also, we hypothesized that the clinical scores at day 14 and viral
335 titer at day 1 and 2 might show substantial differences between CONTROL and
336 PROLONGED groups in $Nr3c1^{fl/fl}$ and $Nr3c1^{fl/fl}Cd11c^{-}$Cre^{+} mice, respectively. Mann-
337 Whitney test was performed at day 14 for clinical scores and at day 1 and day 2 for viral
338 titers. For the analysis of survival rates, the log-rank test was performed between those
339 groups. For the analysis of cell counts, expression levels of chemokine receptors,
340 distances from GFP-positive cells to tdTomato-positive cells, levels of hormones and
341 chemokines, we hypothesized that those values might show substantial differences
342 between CONTROL and PROLONGED groups under the respective situations in each
343 mouse genotype or drug-treated group. Mann-Whitney test was employed to compare
344 continuous values between two groups. For nonparametric pairwise multiple comparison
345 after Kruskal-Wallis test, Dunn's test with Holm adjustment was performed. For the
346 analysis of the interaction effect of mouse genotype or drugs on the prolonged high-
347 intensity exercise, the aligned rank transform (ART) procedure using analysis of variance
348 (ANOVA) was performed to deal with non-normal distributed data[73]. All statistical
349 analyses were performed with GraphPad Prism software ver. 7.0 for Windows and Stata
350 SE ver. 14.2 (StataCorp LP, College Station, TX). *P*-values less than 0.05 were considered
351 statistically significant. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, and n.s., not

352 significant.

353

354 *Study approval*. All mouse protocols were approved by the Institutional Animal Care and

355 Use Committee of the Kyoto University Graduate School of Medicine, and all efforts

356 were made to minimize stress.

357

358 **Results**

359 **Prolonged high-intensity exercise induces impaired or enhanced immunity against**
360 **HSV-2 infection depending on the interval between infection and exercise**

361 To examine how a single bout of prolonged high-intensity exercise influences the immune
362 system, we used a well-established mouse model of intravaginal HSV-2 infection in
363 combination with prolonged high-intensity exercise on a treadmill that was based on
364 previous reports with some modifications[20]. Because it has been demonstrated that HSV-
365 2 expansion in the vagina peaks approximately 18 h after infection[21], mice were first
366 intravaginally infected with HSV-2 (3.5×10^2 PFU), and then, 17 h later, the mice ran for
367 3 h at high speed (19 m/min) on a customized treadmill (late exposure to prolonged high-
368 intensity exercise) (for details, see Material and Methods). Then, we evaluated the clinical
369 scores and survival rates for the following two weeks and viral titers in the vagina for the
370 following two days (Fig. 1A). With these conditions, mice in the prolonged high-intensity
371 exercise group exhibited higher clinical scores and lower survival rates than those in the
372 control no-exercise group (Fig. 1B). Consistently, the HSV-2 viral load in the vagina was
373 significantly higher in the prolonged high-intensity exercise group than in the control no-
374 exercise group (Fig. 1B). To examine whether the impaired immune response was
375 specifically induced by prolonged high-intensity exercise, we examined the effect of short
376 periods of moderate-intensity exercise (running for 30 min at a moderate speed (11.5
377 m/min))[22] on HSV-2 infection. In this condition, no significant difference was observed
378 in the clinical symptoms of HSV-2 infection between the exercise group and the control
379 no-exercise group (Fig. 1C).

380 Since it has been reported that the number of circulating immune cells changes
381 dynamically after exercise on an hour basis[16], it is possible that the immune response may

382 be fluctuated by a small difference in interval from infection. To examine this possibility,
383 mice were infected with HSV-2 and ran at an earlier time point (8 h after infection (early
384 exposure to prolonged high-intensity exercise)). Then, the clinical symptoms of HSV-2
385 infection were evaluated. In this protocol, mice in the prolonged high-intensity exercise
386 group exhibited lower clinical scores and viral titers than those in the control no-exercise
387 group (Fig. 1D). During the exercise, a slight weight loss was observed, however, no
388 significant differences were observed between early exposure group and late exposure
389 group (see Fig. S1A in the Online Repository). Collectively, these results suggest that a
390 single bout of prolonged high-intensity exercise may either impair or enhance antiviral
391 immunity against intravaginal HSV-2 infection depending on the interval between
392 infection and exercise.

393

394 **Late exposure to prolonged high-intensity exercise impairs pDC infiltration in the**
395 **vagina after HSV-2 infection**

396 We next examined the mechanism of impaired immunity induced by late exposure to
397 prolonged high-intensity exercise. Various immune cells, such as pDCs, NK cells,
398 inflammatory monocytes and neutrophils, to infected sites is reported to contribute to
399 protection against HSV-2 intravaginal infection[23–26]. In particular, pDCs are believed to
400 play a crucial role in the initial prevention of viral spread by producing interferon (IFN)-
401 α[23,27,28], although some studies reported that pDCs are dispensable in HSV-2 infection[29].
402 Therefore, we first evaluated the influence of late exposure to prolonged high-intensity
403 exercise on the infiltration of those immune cells in the vagina 24 h after infection. While
404 the accumulation of inflammatory monocytes and neutrophils was comparable between
405 the prolonged high-intensity exercise group and the control no-exercise group, the

406 accumulation of pDCs and NK cells after HSV-2 infection was significantly lower in the
407 prolonged high-intensity exercise group than in the control no-exercise group (Fig. 2A).
408 Consistently, the mRNA expression level of *Ifna* in the vagina was lower in the prolonged
409 high-intensity exercise group than in the control no-exercise group (Fig. 2B). Prolonged
410 high-intensity exercise did not affect the IFN-α-producing ability or the expression level
411 of TLR9, which is critical to produce IFN-α, in pDCs (see Fig. S1B and S1C in the Online
412 Repository). These results suggest that late exposure to prolonged high-intensity exercise
413 impairs anti-viral immunity by reducing the infiltration of pDCs.

414 To confirm the contribution of pDCs in our model, we depleted pDCs using anti-
415 PDCA1 antibody[30] and examined its effects on clinical symptoms and viral titer after
416 HSV-2 infection. Administration of the anti-PDCA1 antibody significantly reduced the
417 number of circulating pDCs, but did not affect the number of NK cells, neutrophils,
418 monocytes and conventional DCs (cDCs) (see Fig. S1D in the Online Repository). pDC-
419 depleted mice showed exacerbated clinical symptoms to a similar extent to those of mice
420 with prolonged high-intensity exercise, as well as increased viral titer (Fig. 2C). These
421 results indicate that pDCs are crucial for anti-viral immunity in our model, and support
422 our hypothesis that exercise-induced exacerbation of HSV-2 infection was through the
423 regulation of pDC infiltration to the vagina.

424 It has been reported that prolonged high-intensity exercise induces transient
425 lymphocytopenia after exercise in both humans and mice[12–18], which has been suggested
426 as a possible cause of impaired antiviral immunity. Thus, we next measured the number
427 of circulating pDCs after prolonged high-intensity exercise in our model. In line with
428 previous reports, the number of pDCs significantly decreased immediately after
429 prolonged high-intensity exercise (Fig. 2D). On the other hand, short periods of moderate-

430 intensity exercise did not induce a decrease in pDCs (Fig. 2E). These results suggest that
431 a reduction in infiltrating pDCs into the vagina, which could significantly impair antiviral
432 immunity against HSV-2 infection, might occur due to the decrease in circulating pDCs
433 induced by late exposure to prolonged high-intensity exercise.
434

435 **pDCs home to the bone marrow during the prolonged high-intensity exercise**

436 Because a previous study suggested that circulating T cells are redistributed to several
437 tissues after prolonged high-intensity exercise[10], we hypothesized that the decrease in
438 circulating pDCs immediately after prolonged high-intensity exercise was caused by
439 redistribution from the blood to other organs during the exercise. To test this hypothesis,
440 we intravenously transferred bulk green fluorescent protein (GFP)-labeled splenocytes
441 and bone marrow cells and examined the number of GFP-positive pDCs in other organs,
442 such as the bone marrow, lymph nodes, spleen, lungs, liver, muscle and vagina, with or
443 without prolonged high-intensity exercise. The number of transferred GFP-positive pDCs
444 in the lymph node, spleen, liver, lung, muscle, and vagina were comparable between the
445 prolonged high-intensity exercise group and the control no-exercise group. On the other
446 hand, the number of GFP-positive pDCs in the bone marrow was significantly higher in
447 the prolonged high-intensity exercise group than in the control no-exercise group (Fig.
448 3A and S1), although the increase in the bone marrow was also observed in other immune
449 cells (see Fig. S1E in the Online Repository). In the blood, the number of transferred
450 GFP-positive pDCs significantly decreased in the prolonged high-intensity exercise
451 group (see Fig. S1F in the Online Repository).

452 Next, we verified the possibility of the recruit failure from the blood to the vagina.
453 Because both CXCR3 and CCR5 are involved in the infiltration of pDCs in the

454 mucocutaneous lesion after HSV-2 infection[23,31,32], we examined the effect of prolonged
455 high-intensity exercise on CXCR3 and CCR5 expression on circulating pDCs and the
456 mRNA expression of the ligands for these chemokine receptors (*Cxcl9*, *Cxcl10*, and
457 *Cxcl11* for CXCR3 and *Ccl3*, *Ccl4* and *Ccl5* for CCR5) in the vagina. Both the expression
458 levels of chemokine receptors and chemokines were unaffected by prolonged high-
459 intensity exercise (see Fig. S2 in the Online Repository). These results suggest that
460 impaired infiltration of pDCs into the vagina after late exposure to prolonged high-
461 intensity exercise was not associated with recruit failure from the blood to the vagina, but
462 associated with decrease of circulating pDCs which homed to the bone marrow during
463 the exercise.
464

465 **pDC homing to the bone marrow during the prolonged high-intensity exercise is**
466 **mediated by the CXCL12-CXCR4 axis**

467 We next investigated the mechanisms through which prolonged high-intensity exercise
468 induced the homing of pDCs to the bone marrow. It has been reported that the chemokine
469 receptors (CXCR4, CCR2, CCR7, CCR9, and ChemR23) and adhesion molecules (LFA1,
470 VLA4, and CD62L) may be responsible for pDC homing from the blood to lymphoid
471 organs or inflamed tissues[32–37]. Therefore, we examined the expression levels of these
472 candidates on circulating pDCs and found that the expression of CXCR4 was upregulated
473 by prolonged high-intensity exercise (Fig. 3B). The expression levels of other candidate
474 chemokine receptors were not upregulated (see Fig. S3A in the Online Repository). On
475 the other hand, the production of CXCL12, the ligand of CXCR4, in the bone marrow
476 was not changed by prolonged high-intensity exercise (see Fig. S3B in the Online
477 Repository). These findings suggest that the promotion of pDC homing to the bone

478 marrow during the prolonged high-intensity exercise might be mediated by the
479 upregulation of CXCR4 expression. In line with this hypothesis, we observed that
480 transferred tdTomato-positive, PDCA-1-positive pDCs localized closer to CXCL12-
481 producing cells in the bone marrow after prolonged high-intensity exercise (Fig. 3C).

482 To examine whether the CXCL12-CXCR4 axis was involved in the homing of
483 circulating pDCs to the bone marrow during the prolonged high-intensity exercise, we
484 administered either vehicle or AMD3100, a CXCR4 antagonist, to mice before prolonged
485 high-intensity exercise and examined the number of pDCs in the blood and bone marrow.
486 As reported previously[38], the administration of AMD3100 inhibited the homing of pDCs
487 to the bone marrow and increased the number of circulating pDCs under the steady states
488 (Fig. 3D). In addition, AMD3100 abrogated the decrease in circulating pDCs induced by
489 prolonged high-intensity exercise (Fig. 3D). Consistently, an increase in the number of
490 pDCs in the bone marrow was not observed in the prolonged high-intensity exercise group
491 treated with AMD3100 (Fig. 3D). AMD3100 did not affect the cellular contents in the
492 vagina (see Fig. S3C in the Online Repository). Collectively, these results suggest that
493 the CXCL12-CXCR4 axis plays a crucial role in the pDC homing to the bone marrow
494 during the prolonged high-intensity exercise.

495

496 **Blockade of CXCR4 signaling abolishes the exacerbation of HSV-2 intravaginal**
497 **infection induced by late exposure to prolonged high-intensity exercise**

498 We then examined whether circulating pDC homing to the bone marrow via the CXCL12-
499 CXCR4 axis is involved in the exacerbation of HSV-2 infection that is induced by late
500 exposure to prolonged high-intensity exercise. To examine this question, we administered
501 either vehicle or AMD3100 to mice in the presence or absence of prolonged high-intensity

502 exercise and evaluated the clinical phenotype of intravaginal HSV-2 infection. The
503 exacerbation of HSV-2 infection (clinical scores, survival rates, and viral titers in the
504 vagina) by late exposure to prolonged high-intensity exercise was attenuated by
505 AMD3100 treatment (Fig. 3E). These results strongly suggest that circulating pDC
506 homing to the bone marrow through the CXCL12-CXCR4 axis mediates the exacerbation
507 of HSV-2 infection induced by late exposure to prolonged high-intensity exercise.

508

509 **Glucocorticoids upregulate the expression of CXCR4 on pDCs**

510 We further explored the underlying mechanisms by which prolonged high-intensity
511 exercise upregulates CXCR4 expression on pDCs. Prolonged high-intensity exercise is
512 known to induce hypoxemia[39]. Hypoxia inducible factor-1α (HIF-1α) is known to
513 regulate the expression level of CXCR4[40]; therefore, we evaluated whether HIF-1α was
514 involved in the induction of CXCR4 expression on pDCs by prolonged high-intensity
515 exercise. We generated *Hif1a*$^{fl/fl}$*Cd11c*-Cre^{+}, in which CD11c^{+} cells, including pDCs[41],
516 specifically lack HIF-1α. In *Hif1a*$^{fl/fl}$*Cd11c*-Cre^{+} mice, CXCR4 expression on pDCs was
517 upregulated to a level similar to that of *Hif1a*$^{fl/fl}$ mice during prolonged high-intensity
518 exercise (see Fig. S4 in the Online Repository). This result suggests that HIF-1α is not
519 involved in the upregulation of CXCR4 expression on pDCs in our model.

520 Next, we focused on hormonal factors in the blood that are considered to be
521 involved in CXCR4 expression, including noradrenaline, glucocorticoids, progesterone,
522 estrogen, and aldosterone[42–46], by performing liquid chromatography tandem mass
523 spectrometry (LC-MS/MS) analysis of steroid hormones and enzyme-linked
524 immunosorbent assays for noradrenaline using blood samples taken immediately after
525 prolonged high-intensity exercise. We found that the blood levels of noradrenaline,

526 corticosterone, and aldosterone were significantly higher in the exercise group (see Fig.
527 S5A in the Online Repository). Then, we tested the effect of inhibitors for each candidate
528 on CXCR4 expression on pDCs. The blockade of adrenergic receptor and aldosterone-
529 mineralocorticoid receptor signaling did not affect the exercise-induced upregulation of
530 CXCR4 expression on pDCs in the blood (see Fig. S5B in the Online Repository). On the
531 other hand, the administration of RU486, a glucocorticoid receptor (GR) antagonist,
532 abrogated the exercise-induced upregulation of CXCR4 expression on pDCs in the blood
533 (Fig. 4A). Furthermore, the treatment of pDCs with corticosterone upregulated the
534 expression of CXCR4 *in vivo* and *in vitro* and facilitated the migration of pDCs toward
535 CXCL12 in a dose-dependent manner *in vitro* (Fig. 4B, 4C, and see Fig. S6A in the Online
536 Repository). Upregulation of CXCR4 expression by the administration of corticosterone
537 *in vivo* was also observed in cDCs and monocytes, but not in NK cells and neutrophils
538 (see Fig. S6B in the Online Repository). The serum corticosterone level peaked
539 approximately 3 h after the start of prolonged high-intensity exercise (see Fig. S6C in the
540 Online Repository). On the other hand, in mice with short periods of moderate-intensity
541 exercise, an increase in the serum corticosterone level was not observed (see Fig. S6D in
542 the Online Repository).

543 These results indicate that the upregulation of CXCR4 expression on pDCs
544 during prolonged high-intensity exercise was mediated by glucocorticoids.

545

546 **Blockade of glucocorticoid signaling abrogates the effects of exercise on pDC homing**
547 **and impaired antiviral immunity induced by prolonged high-intensity exercise**

548 We next examined the effects of RU486 on the homing of circulating pDCs from the
549 blood to the bone marrow. The administration of RU486 abolished the fluctuation of

550 pDCs in the blood and bone marrow in response to prolonged high-intensity exercise (see
551 Fig. S7A in the Online Repository). RU486 also abrogated the exacerbation of HSV-2
552 intravaginal infection induced by prolonged high-intensity exercise (see Fig. S7B in the
553 Online Repository). Conversely, the administration of corticosterone decreased the
554 number of pDCs in the blood and tended to increase the number of pDCs in the bone
555 marrow (see Fig. S7C in the Online Repository). Consistently, the administration of
556 corticosterone exacerbated HSV-2 intravaginal infection (see Fig. S7D in the Online
557 Repository).

558 To further confirm the effects of glucocorticoids on pDCs, we generated CD11c-
559 expressing cell-specific GR-deficient (*Nr3c1*$^{fl/fl}$*Cd11c*-Cre^{+}) mice. In *Nr3c1*$^{fl/fl}$*Cd11c*-
560 Cre^{+} mice, the exercise-induced upregulation of CXCR4 expression on pDCs and the
561 fluctuation of pDCs in the blood and bone marrow were abrogated (Fig. 5A and 5B).
562 Furthermore, *Nr3c1*$^{fl/fl}$*Cd11c*-Cre^{+} mice did not exhibit the exacerbation of HSV-2
563 intravaginal infection in response to late exposure to prolonged high-intensity exercise
564 (Fig. 5C).

565 CD11c is expressed by other cells, such as mDCs, but the above findings indicate
566 that glucocorticoids mediate the exacerbation of HSV-2 infection induced by late
567 exposure to prolonged high-intensity exercise by upregulating CXCR4 expression on
568 pDCs and facilitating pDC homing to the bone marrow.

569

570 **Glucocorticoids are involved in enhanced antiviral immunity induced by early**
571 **exposure to prolonged high-intensity exercise by an increase in circulating pDCs**

572 Finally, we examined the underlying mechanism of enhanced immune responses against
573 HSV-2 infection induced by early exposure to prolonged high-intensity exercise. We

574 monitored the number of circulating pDCs after their decrease in the blood subsequent to
575 prolonged high-intensity exercise. We found that following the transient decrease after
576 exercise, the number of circulating pDCs increased transiently around 6-12 h later (Fig.
577 6A). The inhibition of bone marrow homing by a RU486 abrogated their subsequent
578 increase in the blood (Fig. 6B); therefore, we consider that this increase seemed to be
579 dependent on their prior homing to the bone marrow after exercise in response to
580 glucocorticoid activity. Furthermore, the administration of RU486 abrogated the
581 enhanced antiviral immune responses induced by early exposure to prolonged high-
582 intensity exercise (Fig. 6C). These results suggest that a single bout of prolonged high-
583 intensity exercise increases the number of circulating pDCs via glucocorticoids following
584 the transient decrease of circulating pDCs, and enhances antiviral immunity.
585
586

587 **Discussion**

588 Here, we demonstrated that a single bout of prolonged high-intensity exercise either
589 impaired or enhanced antiviral immunity to HSV-2 intravaginal infection in mice
590 depending on the interval between infection and exercise. Glucocorticoids induced by
591 prolonged high-intensity exercise facilitated the homing of circulating pDCs to the bone
592 marrow through the CXCL12-CXCR4 axis, which caused a transient decrease of
593 circulating pDCs after the exercise. Following this transient decrease, the number of
594 circulating pDCs increased 6-12 h later. These transient decreases and increases of
595 circulating pDCs were associated with impaired and enhanced antiviral immunity,
596 respectively (Fig. 7). Our results provide novel insights into the mechanistic link between
597 a single bout of prolonged high-intensity exercise and the immune system.

598 We showed that GR signaling was involved in both the impairment and
599 enhancement of antiviral immunity against HSV-2 intravaginal infection. Glucocorticoids
600 are generally considered to cause immunosuppression; however, glucocorticoids may
601 function as immunostimulants in some contexts, as we have previously discovered that
602 glucocorticoids drive the diurnal accumulation of T cells in lymphoid organs to enhance
603 adaptive immune responses[47]. Previous reports also showed that glucocorticoids exhibit
604 biphasic dose-response effects in an animal model of delayed-type hypersensitivity and
605 in macrophages activated by lipopolysaccharide and IFN-γ[48,49]. Thus, the effects of
606 glucocorticoids on the immune system may be bidirectional and fluctuate in a context
607 dependent manner. That may explain why the contradictory results have been reported
608 regarding the influence of prolonged high-intensity exercise on the viral infection rate.

609 There are several limitations and unsolved issues in our study. First, we used
610 *Cd11c*-Cre$^+$ mice to examine the involvement of GR signaling in pDCs *in vivo*. CD11c is

611 expressed on other cells such as NK cells and mDCs, which are considered to be
612 important in the defense against HSV-2 infection[24,50]. Thus, our results using *Cd11c*-Cre^{+}
613 mice may not necessarily specific to the effects of GR signaling in pDCs. However,
614 antiviral functions of NK cells are induced by pDCs-derived IFN-α[27,28]. In addition,
615 mDCs exert their antiviral functions at much later time points (48 h after the HSV-2
616 intravaginal infection)[50,51] than our analysis timing (24 h after the infection). Furthermore,
617 mice depleted with pDCs showed similar clinical scores and viral titers to those of mice
618 with prolonged high-intensity exercise. Therefore, although we cannot exclude the
619 possibility of the contribution of GR signaling in other cells, we consider that GR
620 signaling in pDCs is, at least mainly, involved in inducing the fluctuating immune
621 responses in our model. Second, the mechanisms increasing the number of circulating
622 pDCs following the transient decrease after the exercise remain unclear. Because the
623 blockade of the pDC homing to the bone marrow by a GR antagonist diminished the
624 increase, prior homing of pDCs to the bone marrow seems to be important for the increase.
625 Homing of pDCs to the bone marrow may induce the release of progenitor cells from the
626 bone marrow into the peripheral blood, as reported in neutrophils[52]. Third, the
627 significance of pDC homing to the bone marrow during the prolonged high-intensity
628 exercise remains unclear. One hypothesis is that the bone marrow may protect pDCs from
629 the stress of prolonged high-intensity exercise. In line with this, it has recently been
630 reported that memory T cells accumulate in the bone marrow during stress from dietary
631 restriction because T cells in the bone marrow express high levels of the antiapoptotic
632 factor BCL-2, which protects them from apoptosis[53]. Thus, the positive and negative
633 effects of prolonged high-intensity exercise on antiviral immunity, as seen in our model,
634 may be secondary phenomena.

635 Together, our findings provide an important clue to elucidate the long-
636 controversial questions about the influence of a single bout of prolonged high-intensity
637 exercise on antiviral immune response. Regulation of glucocorticoid may be utilized for
638 the control of our immune responses after such strenuous exercise, although the
639 applicability of our findings in mice to humans, and in other infectious diseases such as
640 respiratory infection, are important subjects for future analysis. Furthermore,
641 understanding the biological meaning of the unique immune cell dynamics from the blood
642 to the bone marrow during the prolonged high-intensity exercise may lead to an
643 elucidation of a novel mechanism protecting us from various stress.
644

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655

656 **Author contributions**

657 A.A. designed and performed experiments and wrote the paper. G.E., T.D., Y.Y., T.N.,
658 S.N., A.O., A.K. discussed the data and wrote the paper. M.M. and N.M. performed LC-
659 MS/MS analysis. N.K. and Y.K. discussed the data and lectured the protocol of virus
660 experiments. T.O. provided *Cd11c*-Cre mice, discussed the data and wrote the paper. T.N.
661 provided *Cxcl12*-GFP mice, discussed the data, and wrote the paper. K.I. provided
662 *Nr3c1*$^{fl/fl}$ mice, discussed the data, and wrote the paper. T.H. and K.K. conceptualized and
663 supervised studies, designed some experiments, discussed the data, and wrote the paper.

664

665

666

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898

899 **Figure legends**

900 **Figure 1. Prolonged high-intensity exercise induces impaired or enhanced immunity**
901 **against HSV-2 infection depending on the interval between infection and exercise.**
902 (A) Experimental design. Mice were intravaginally infected with HSV-2 and ran on a
903 treadmill 8 or 17 h after infection. Then, the indicated experiments were performed.
904 Created with BioRender.com. (B-D) Mean clinical scores, survival rates and viral titers
905 in vaginal wash. (B) Prolonged high-intensity exercise (PROLONGED) was performed
906 17 h after HSV-2 intravaginal infection (CONTROL: n=21; PROLONGED: n=22), (C)
907 short periods of moderate-intensity exercise (SHORT) was performed 17 h after HSV-2
908 intravaginal infection (n=22 per group), and (D) prolonged high-intensity exercise was
909 performed 8 h after HSV-2 intravaginal infection (n=26 per group). The results were
910 pooled from three independent experiments. Error bars represent the mean ± the standard
911 error of the mean (SEM). *P<0.05, **P<0.01, and n.s., not significant (Mann-Whitney
912 test for clinical scores and viral titers and log rank test for survival rates (B-D)).
913
914 **Figure 2. Late exposure to prolonged high-intensity exercise impairs pDC**
915 **infiltration in the vagina after HSV-2 infection.**
916 (A) Flow cytometric analysis of the number of indicated cells in the vagina 24 h after
917 HSV-2 infection. MOCK: mock-infected with Vero cell lysate. (B) Quantitative PCR
918 analysis of *Ifna* mRNA expression in the vagina 24 h after HSV-2 infection. (C) Mean
919 clinical scores, survival rates and viral titers in vaginal wash. (C) Mice were injected with
920 either isotype antibodies or anti-PDCA-1 antibodies and prolonged high-intensity
921 exercise was performed 17 h after HSV-2 intravaginal infection (Isotype CONTROL:
922 n=23; Isotype PROLONGED: n=21; Ab CONTROL: n=21; Ab PROLONGED: n=18).

923 (D) Representative flow cytometric panels of pDCs and the number of pDCs in the blood
924 immediately after late exposure to prolonged high-intensity exercise. (E) Flow cytometric
925 analysis of the number of pDCs in the blood immediately after late exposure to short
926 periods of moderate-intensity exercise. Data are representative of three independent
927 experiments (A, D, E) and pooled from two to three independent experiments (B, C).
928 Error bars represent the mean ± SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and n.s., not
929 significant (Dunn's test with Holm adjustment after Kruskal-Wallis test (A, B) or Mann-
930 Whitney test (C, D)).
931

932 **Figure 3. Exacerbation of HSV-2 infection induced by prolonged high-intensity**
933 **exercise is mediated through pDC homing to the bone marrow via the CXCL12-**
934 **CXCR4 axis**
935 (A) The number of transferred pDCs in the bone marrow immediately after late exposure
936 to prolonged high-intensity exercise. (B) A representative histogram (left panel) and the
937 mean fluorescence intensity (MFI) (right panel) of CXCR4 expression on pDCs in the
938 blood 2 h after the start of prolonged high-intensity exercise. (C) Immunohistochemical
939 analysis of pDCs in the bone marrow (left panel) and the mean distance from the
940 transferred tdTomato^{+}PDCA1^{+} cells to the nearest GFP^{+} cells in the bone marrow of
941 *Cxcl12*-GFP mice (right panel). Scale bar = 50 μm. (D) The number of transferred pDCs
942 immediately after prolonged high-intensity exercise. (E) Mean clinical scores, viral titers
943 in vaginal washes, and survival rates in mice that did or did not receive AMD3100
944 (CONTROL Vehicle: n=22; PROLONGED Vehicle: n=21; CONTROL AMD3100: n=22;
945 PROLONGED AMD3100: n=21). Error bars represent the mean ± SD (A, B, D) and
946 mean ± SEM (E). The results were representative of two to three independent experiments

947 (A-D) or were pooled from three independent experiments (E). *P<0.05, **P<0.01,
948 ***P<0.001, and n.s., not significant (Mann-Whitney test (A-E)).
949

950 **Figure 4. GR signaling regulates the expression of CXCR4 on pDCs.**

951 (A) Flow cytometric analysis of CXCR4 expression on pDCs in the blood 2 h after the
952 start of prolonged high-intensity exercise (PROLONGED) with or without RU486 (30
953 mg/kg). Representative histograms (left panel) and MFI of CXCR4 expression (right
954 panel) are shown. (B) Representative histograms (left panel) and MFI (right panel) of
955 CXCR4 expression on pDCs in the blood 3 h after the administration of vehicle or
956 corticosterone (10 mg/kg), as analyzed by flow cytometry. (C) Transwell migration assay.
957 Whole blood cells were lysed and pretreated with vehicle or corticosterone at the
958 indicated concentration for 3 h and were placed in the upper chamber of the transwell for
959 1 h. The number of pDCs in the lower chamber containing CXCL12 (10 ng/mL) was
960 evaluated by flow cytometry. Each dot represents a single mouse. Error bars represent the
961 mean ± SD. The results are representative of two to three independent experiments.
962 *P<0.05, **P<0.01, ***P<0.001, and n.s., not significant (Mann-Whitney test (A-C) and
963 the aligned rank transform (ART) procedure using analysis of variance (ANOVA) for the
964 interaction effect (A)).
965

966 **Figure 5. Loss of GR signaling in CD11c-expressing cells abrogates the effects of late**
967 **exposure to prolonged high-intensity exercise on HSV-2 infection.**

968 (A) Representative histograms (left panel) and MFI (right panel) of CXCR4 expression
969 on pDCs in the blood 2 h after the start of prolonged high-intensity exercise
970 (PROLONGED) in $Nr3c1^{fl/fl}$*Cd11c*-Cre^{+} mice and $Nr3c1^{fl/fl}$ mice. (B) The number of

971 pDCs in the blood and in the bone marrow in *Nr3c1*$^{fl/fl}$*Cd11c*-Cre^{+} mice and *Nr3c1*$^{fl/fl}$
972 mice immediately after prolonged high-intensity exercise. Each dot represents a single
973 mouse. (C) Mean clinical scores, viral titers in vaginal washes, and survival rates in the
974 intravaginal HSV-2 infection model with *Nr3c1*$^{fl/fl}$ mice and *Nr3c1*$^{fl/fl}$*Cd11c*-Cre^{+} mice
975 (CONTROL *Nr3c1*$^{fl/fl}$ mice: n=27; PROLONGED *Nr3c1*$^{fl/fl}$ mice: n=25; CONTROL
976 *Nr3c1*$^{fl/fl}$*Cd11c*-Cre^{+} mice: n=27; PROLONGED *Nr3c1*$^{fl/fl}$*Cd11c*-Cre^{+} mice: n=27). The
977 results are representative of three independent experiments (A, B) or were pooled from
978 three independent experiments (C). Error bars represent the mean ± SD (A, B) and the
979 mean ± SEM (C). **P<0.01, ***P<0.001, ****P<0.0001, and n.s., not significant (Mann-
980 Whitney test (A-C), ART procedure using ANOVA for the interaction effect (A) and log
981 rank test for survival rates (C)).
982
983 **Figure 6. Glucocorticoids are involved in enhanced antiviral immunity induced by**
984 **early exposure to prolonged high-intensity exercise by an increase in circulating**
985 **pDCs**
986 (A) Flow cytometric analysis of the number of pDCs in the blood before (-3 h) and after
987 prolonged high-intensity exercise at the indicated time points (n=5). (B) Flow cytometric
988 analysis of the number of pDCs in the blood 12 h after the start of exercise. Mice were
989 administered vehicle or RU486 (30 mg/kg) 1 h before exercise. (C) Mean clinical scores,
990 survival rates, and viral titers in vaginal washes. Mice were administered vehicle or
991 RU486 (30 mg/kg) 1 h before prolonged high-intensity exercise (PROLONGED)
992 (CONTROL Vehicle: n=16; PROLONGED Vehicle: n=14; CONTROL RU486: n=16;
993 PROLONGED RU486: n=14). Each dot represents a single mouse (A, B). Error bars
994 represent the mean ± SD (A, B) and the mean ± SEM (C). The results are representative

995 of two independent experiments (A) or were pooled from two to three (B, C) independent
996 experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, and n.s., not significant
997 (Mann-Whitney test (A-C) and log rank test for survival rates (C)).
998
999 **Figure 7. A graphical summary.**
1000 Prolonged high-intensity exercise increases the level of glucocorticoids in the blood.
1001 Glucocorticoids upregulate CXCR4 expression on pDCs in the blood, which facilitates
1002 their homing to the bone marrow and their decrease in the blood. The decrease in pDCs
1003 in the blood leads to the impaired infiltration of pDCs to infected sites (vagina), causing
1004 the exacerbation of HSV-2 infection. The glucocorticoid-induced pDC homing to the
1005 bone marrow, on the other hand, drives the recovery of pDCs in the blood approximately
1006 6-12 h after exercise, leading to enhanced immune responses against HSV-2 infection.
1007 Created with BioRender.com.
1008

1009 **Legend to Supplementary Figures**

1010 **Figure S1. Interferon production and Toll-like receptor (TLR) 9 expression in pDCs**
1011 **and the number of pDCs in the blood, spleen, liver, vagina, muscle, lymph node, and**
1012 **lung after prolonged high-intensity exercise.**

1013 (A) Weight loss during the exercise in early and late exposure group (n=15). (B) Flow
1014 cytometric analysis of intracellular IFN-α in pDCs. (C) Representative histogram (left
1015 panel) and MFI (right panel) of TLR9 expression in pDCs in the blood immediately after
1016 prolonged high-intensity exercise. (D) Flow cytometric analysis of the number of pDCs,
1017 cDCs, NK cells, neutrophils and monocytes in the blood (day 0) of mice. (E) Flow
1018 cytometric analysis of the number of the number of transferred NK cells, cDCs,
1019 neutrophils and monocytes in the bone marrow immediately after late exposure to
1020 prolonged high-intensity exercise. (F) Flow cytometric analysis of the number of
1021 transferred pDCs in the indicated tissues immediately after prolonged high-intensity
1022 exercise. Each dot represents a single mouse (B-F). The results were pool of three
1023 independent experiments (A) or were representative of two to three independent
1024 experiments (B-F). Error bars represent the mean ± standard deviation (SD) (A-F).
1025 *P<0.05, **P<0.01, ***P<0.001, and n.s., not significant (Mann-Whitney test (A-F)).

1026

1027 **Figure S2. The expression levels of CCR5, CXCR3 in the blood and their ligands in**

1028 **the vagina after prolonged high-intensity exercise.**

1029 (A) Representative histograms (upper panels) and MFI (lower panels) of CCR5 and

1030 CXCR3 on pDCs in the blood, as analyzed by flow cytometry. (B) Quantitative PCR

1031 analysis of *Ccl3, Ccl4, Ccl5, Cxcl9, Cxcl10*, and *Cxcl11* in the vagina 24 h after HSV-2

1032 infection. Each dot represents a single mouse. Error bars represent the mean ± SD. Data

1033 are representative of two to three independent experiments. Mann-Whitney test (A) and

1034 Dunn's test with Holm adjustment after Kruskal-Wallis test (B).

1035

1036 **Figure S3. The expression of chemokine receptors and adhesion molecules on pDCs**

1037 **after prolonged high-intensity exercise and the concentration of CXCL12 in the bone**

1038 **marrow.**

1039 (A) Flow cytometric analysis of the expression levels of CCR2, CCR7, CCR9, ChemR23,

1040 LFA1, VLA4, and CD62L on pDCs in the blood immediately after prolonged high-

1041 intensity exercise. MFIs are shown. (B) The concentration of CXCL12 in the bone

1042 marrow. Immediately after prolonged high-intensity exercise, femurs and tibias were

1043 flushed with PBS, and the concentration of CXCL12 in extracellular fluids was analyzed

1044 by ELISA. (C) Flow cytometric analysis of the number of pDCs, NK cells, cDCs,

1045 neutrophils and inflammatory monocytes in the vagina 24 h after mock infection in mice

1046 treated with vehicle or AMD3100 17 h after mock infection. Each dot represents a single

1047 mouse. Error bars represent the mean ± SD. These results are representative of two to

1048 three independent experiments. n.s., not significant (Mann-Whitney test).

1049

1050 **Figure S4. HIF-1α is not involved in the upregulation of CXCR4 expression on pDCs**

1051 **after prolonged high-intensity exercise.**

1052 Flow cytometric analysis of CXCR4 expression on pDCs in the blood 2 h after the start

1053 of prolonged high-intensity exercise (PROLONGED) in *Hif1a*$^{fl/fl}$ or *Hif1a*$^{fl/fl}$-*Cd11c*-Cre^{+}

1054 mice. Each dot represents a single mouse. Error bars represent the mean ± SD. Data are

1055 representative of three independent experiments. *$P<0.05$, and n.s., not significant

1056 (Mann-Whitney test and ART procedure using ANOVA for the interaction effect).

1057

1058 **Figure S5. Blood levels of noradrenaline and steroid hormones, and the effects of**

1059 **antagonists for adrenergic receptor and mineralocorticoid receptor on CXCR4**

1060 **expression on pDCs after prolonged high-intensity exercise.**

1061 (A) Immediately after prolonged high-intensity exercise, whole blood was harvested, and

1062 serum or plasma was isolated by centrifugation. Then, serum was subjected to LC-

1063 MS/MS analysis to analyze steroid hormones, and plasma was subjected to ELISA to

1064 analyze noradrenaline. (B) Flow cytometric analysis of CXCR4 expression on pDCs in

1065 the blood 2 h after the start of prolonged high-intensity exercise. Mice were administered

1066 either vehicle, SR59230A (β3-adrenergic receptor (AR) antagonist) (5 mg/kg),

1067 propranolol (β1- and β2-AR antagonist) (10 mg/kg), 6-OHDA (sympathectomy), or

1068 eplerenone (mineralocorticoid receptor antagonist) (1 mg/kg). Each dot represents a

1069 single mouse. Error bars represent the mean ± SD. *P<0.05, **P<0.01, ***P<0.001,

1070 ****P<0.0001, and n.s., not significant (Mann-Whitney test (A, B), and ART procedure

1071 using ANOVA for the interaction effect (B).

1072

1073 **Figure S6. Upregulation of CXCR4 expression on pDCs by corticosterone, and blood**

1074 **levels of corticosterone over time during and after prolonged high-intensity exercise**

1075 **or short periods of moderate-intensity exercise.**

1076 (A) Representative histogram and flow cytometric panels of CXCR4 expression on pDCs

1077 treated with corticosterone. Whole blood cells from naïve wild-type mice were lysed and

1078 incubated with vehicle or corticosterone for 3 h at the indicated concentration. The cells

1079 were then subjected to flow cytometric analysis. (B) MFI of CXCR4 expression on NK

1080 cells, cDCs, neutrophils and monocytes in the blood 3 h after the administration of vehicle

1081 or corticosterone (10 mg/kg), as analyzed by flow cytometry. Each dot represents a single

1082 mouse. (C) Time course of the blood level of corticosterone in mice after prolonged high-

1083 intensity exercise (PROLONGED) (n=4-5 per group). The time after the start of exercise

1084 is shown. (D) Time course of the blood level of corticosterone in mice with short periods

1085 of moderate-intensity exercise (SHORT) (n=4-5 per group). Time after the start of

1086 exercise is shown. Data are representative of two-three independent experiments. Error

1087 bars represent the mean ± SD. *P<0.05 and **P<0.01 (Mann-Whitney test).

1088

1089 **Figure S7. GR signaling mediates the effects of late exposure to prolonged high-**

1090 **intensity exercise on the clinical symptoms of intravaginal HSV-2 infection.**

1091 (A) Flow cytometric analysis of the number of transferred pDCs immediately after

1092 prolonged high-intensity exercise. (B) Mean clinical scores, viral titers in vaginal washes,

1093 and survival rates in the intravaginal HSV-2 infection model in mice administered with

1094 vehicle or RU486 (n=21 per group). (C) Flow cytometric analysis of the number of

1095 transferred pDCs in the blood and in the bone marrow 3 h after the administration of

1096 vehicle or corticosterone (10 mg/kg). (D) Mean clinical scores, viral titers in vaginal

1097 washes, and survival rates in mice treated with vehicle or corticosterone (10 mg/kg) 17

1098 and 20 h after HSV-2 infection (VEHICLE: n=28; CORTICOSTERONE: n=26). Each

1099 dot represents a single mouse (A, C). Error bars represent the mean ± SD (A, C) and the

1100 mean ± SEM (B, D). These results are representative of two independent experiments (A,

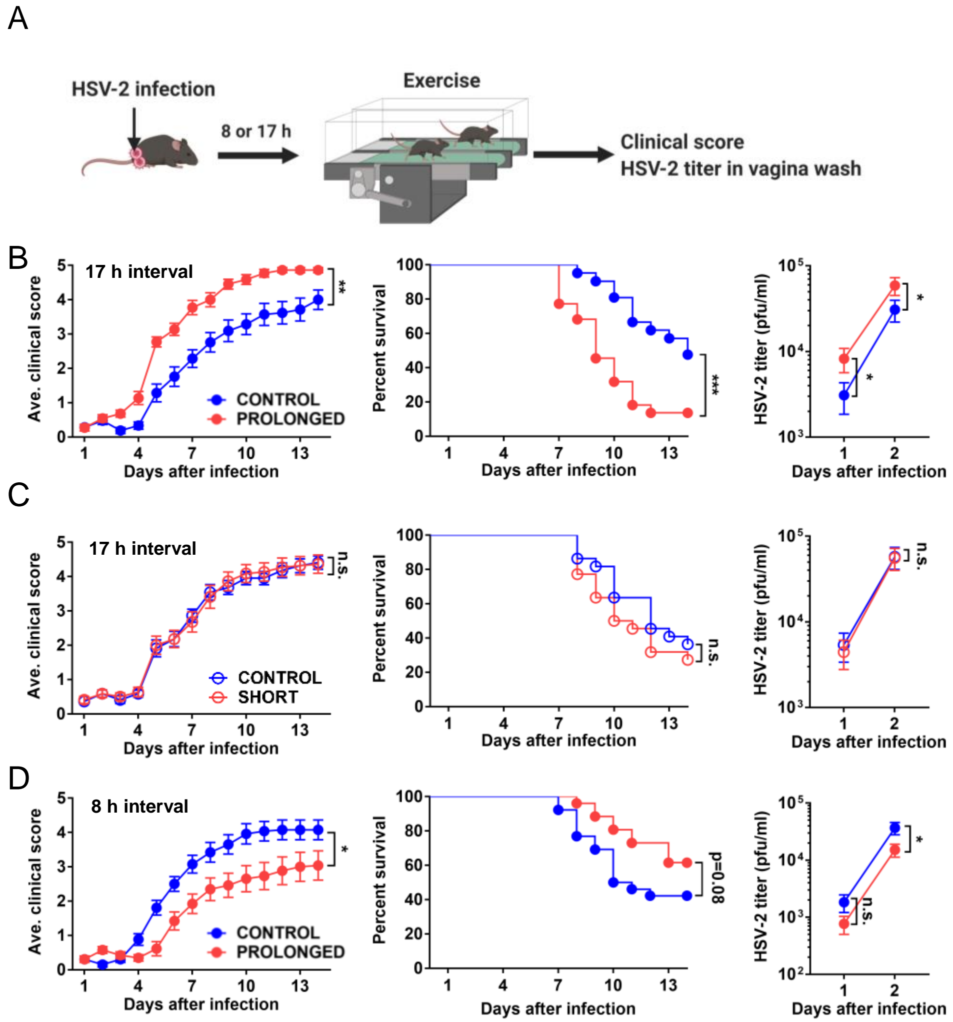
1101 C) or were pooled from three independent experiments (B, D). *P<0.05, **P<0.01, and

1102 n.s., not significant (Mann-Whitney test (A-D) and log rank test for survival rates (B, D)).

1103

1104

Figure 1



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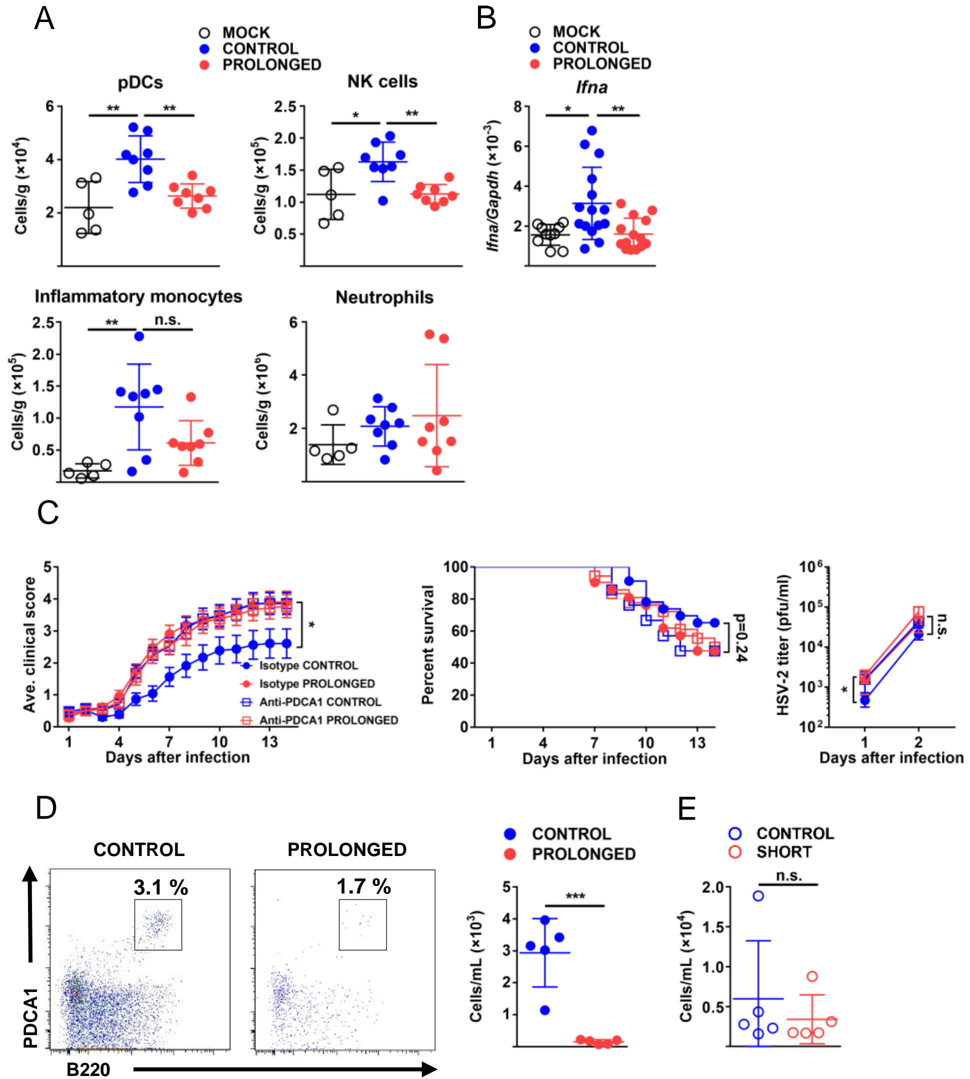

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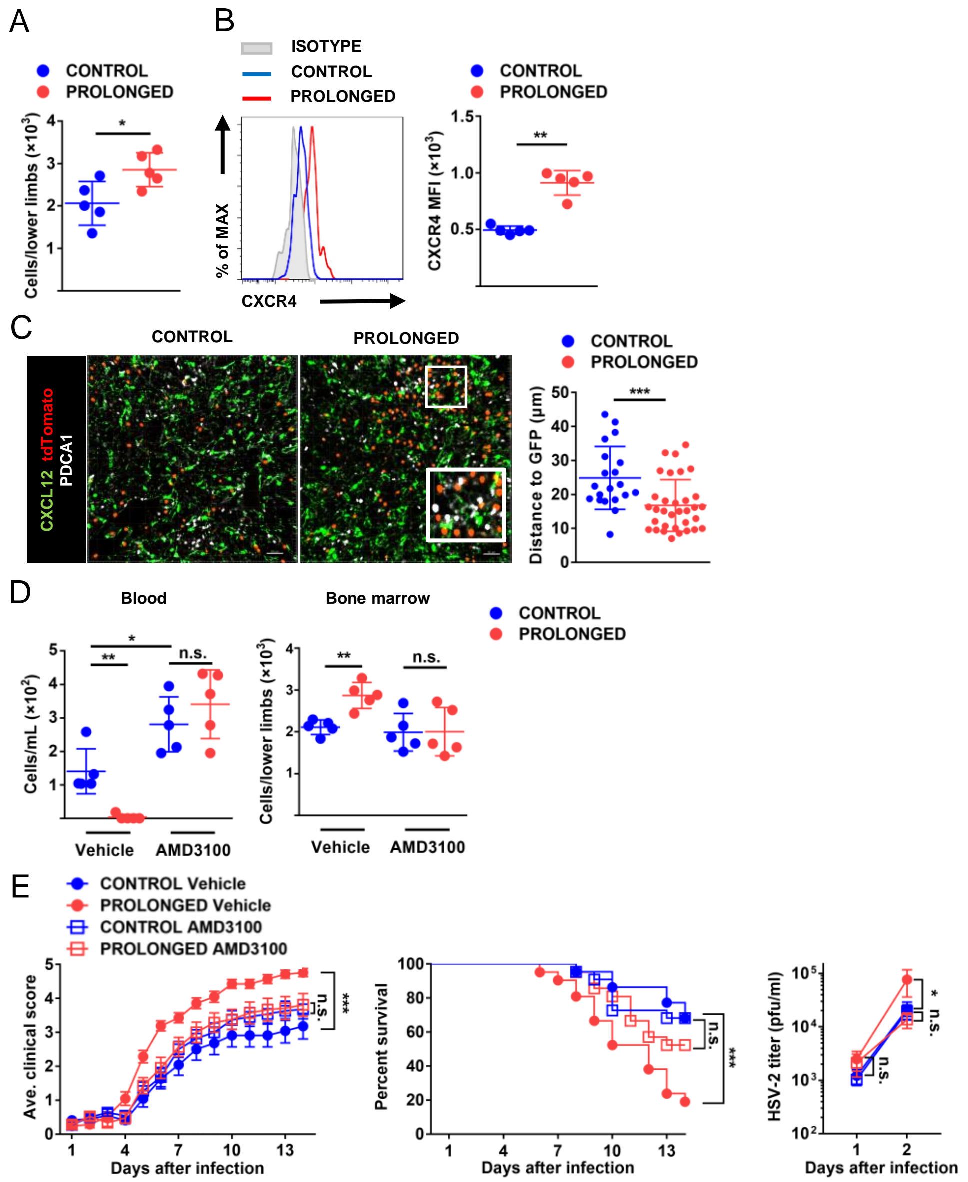

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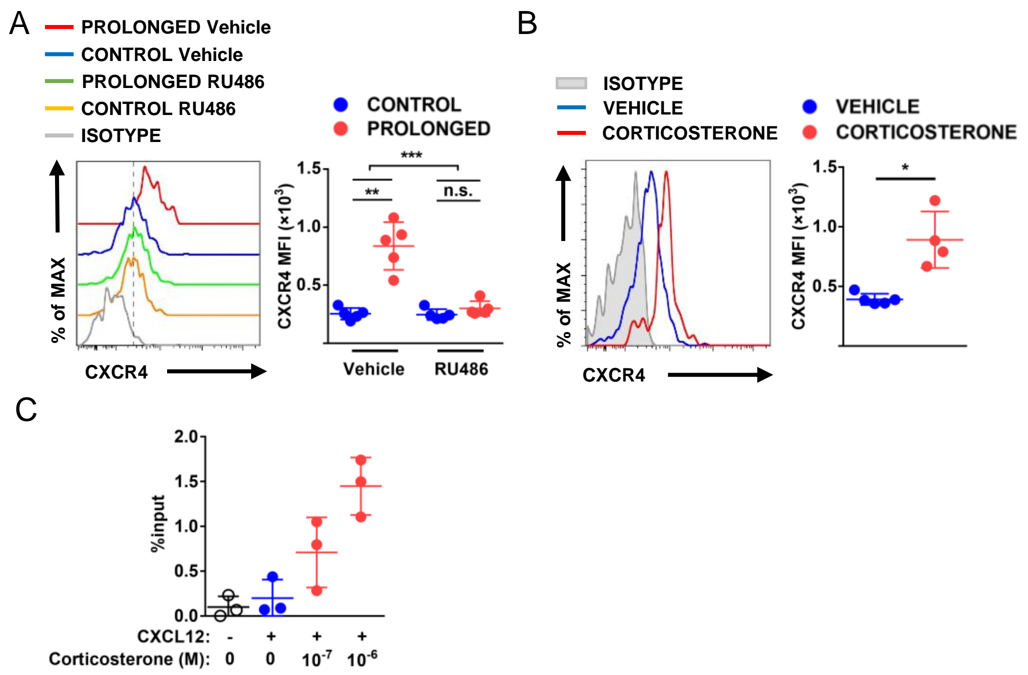

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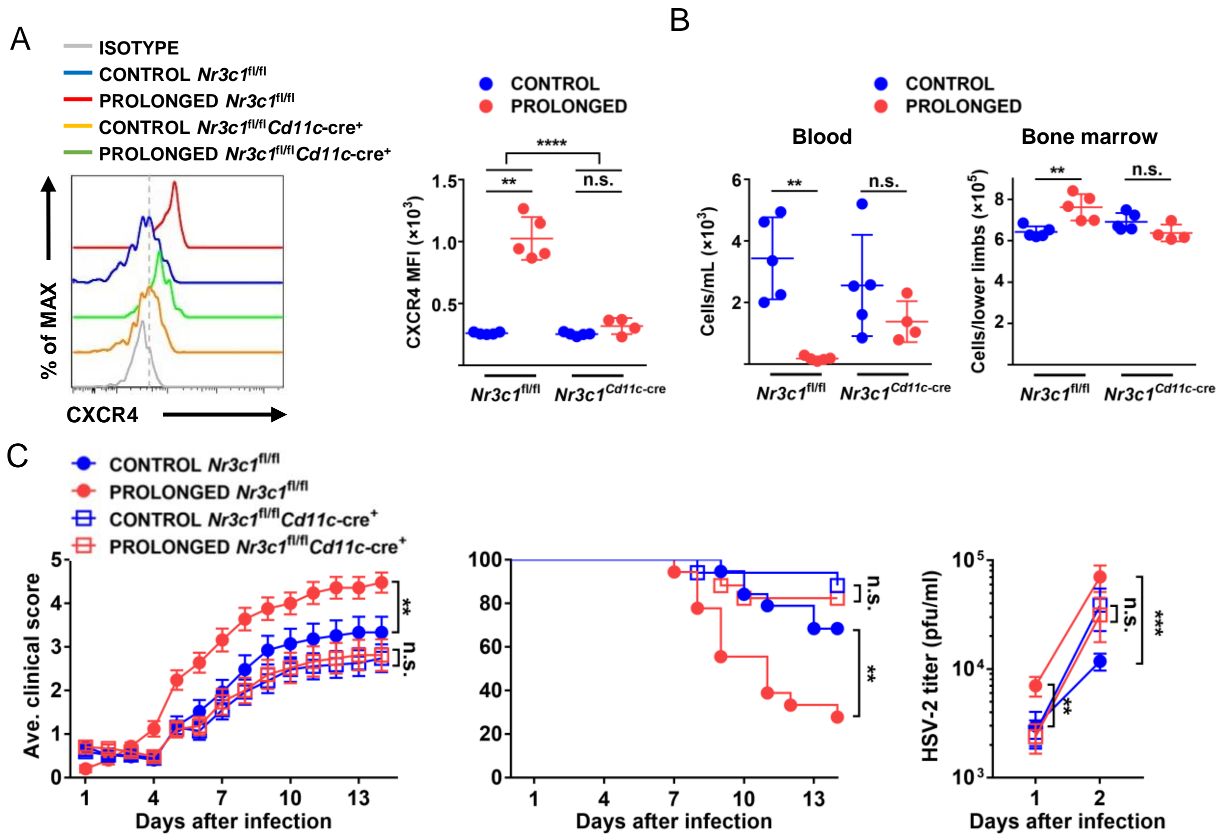

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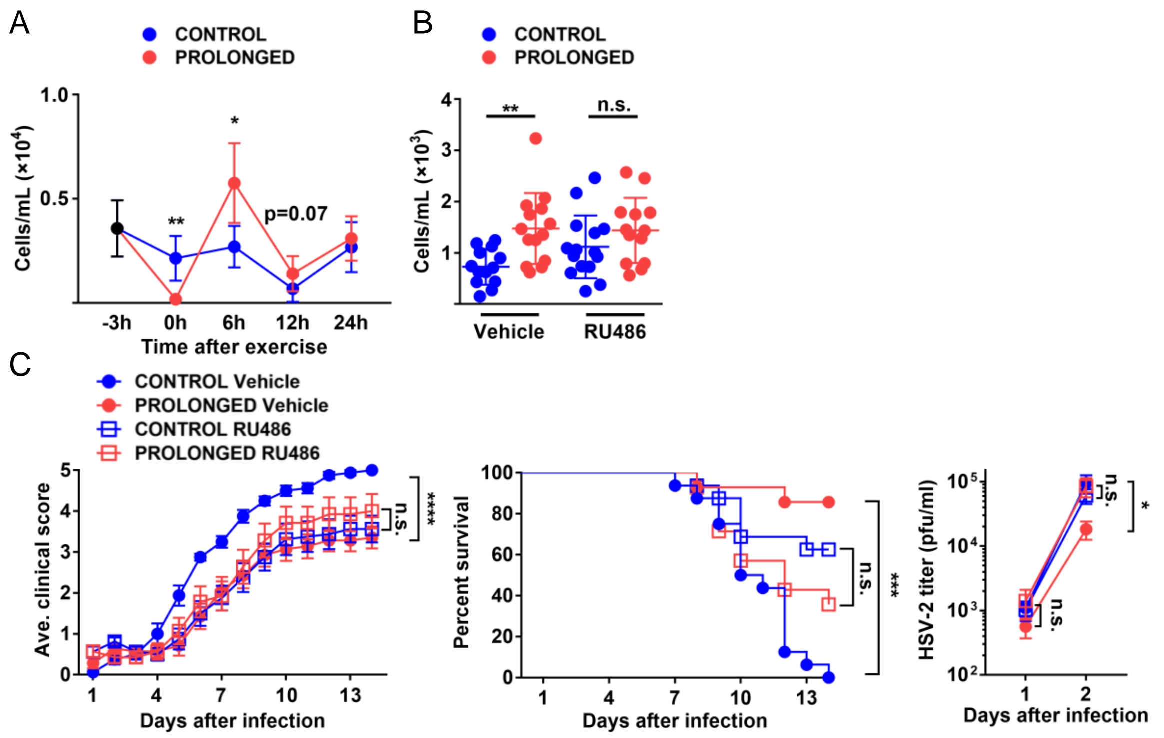


Figure 7

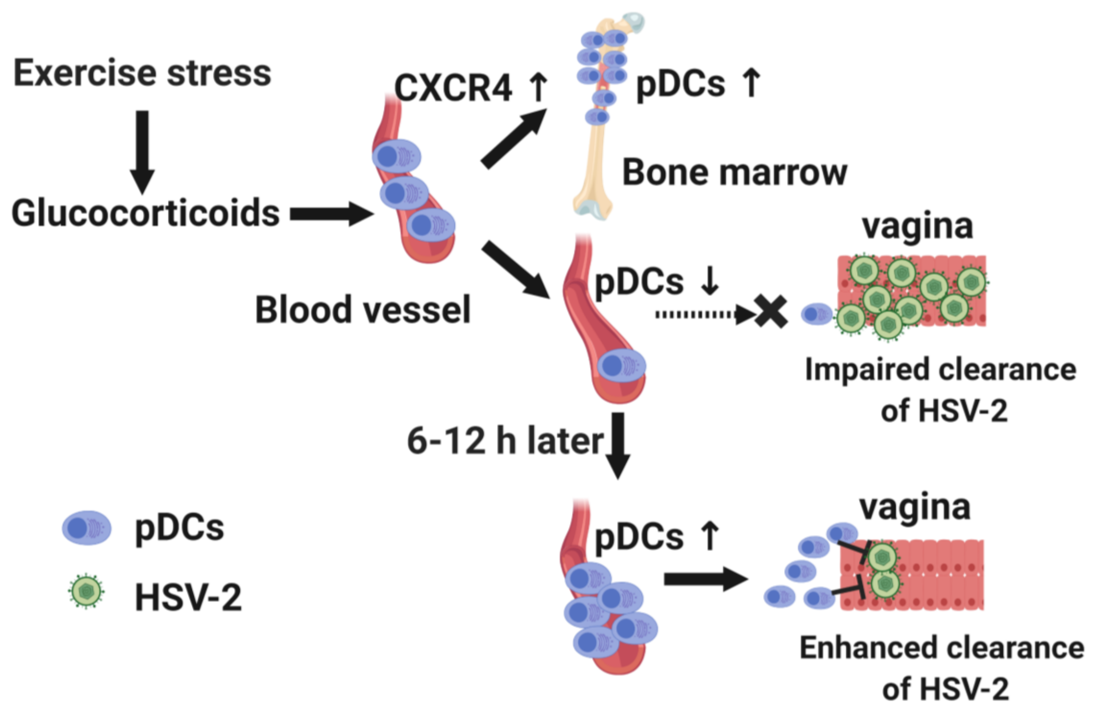


Figure S1

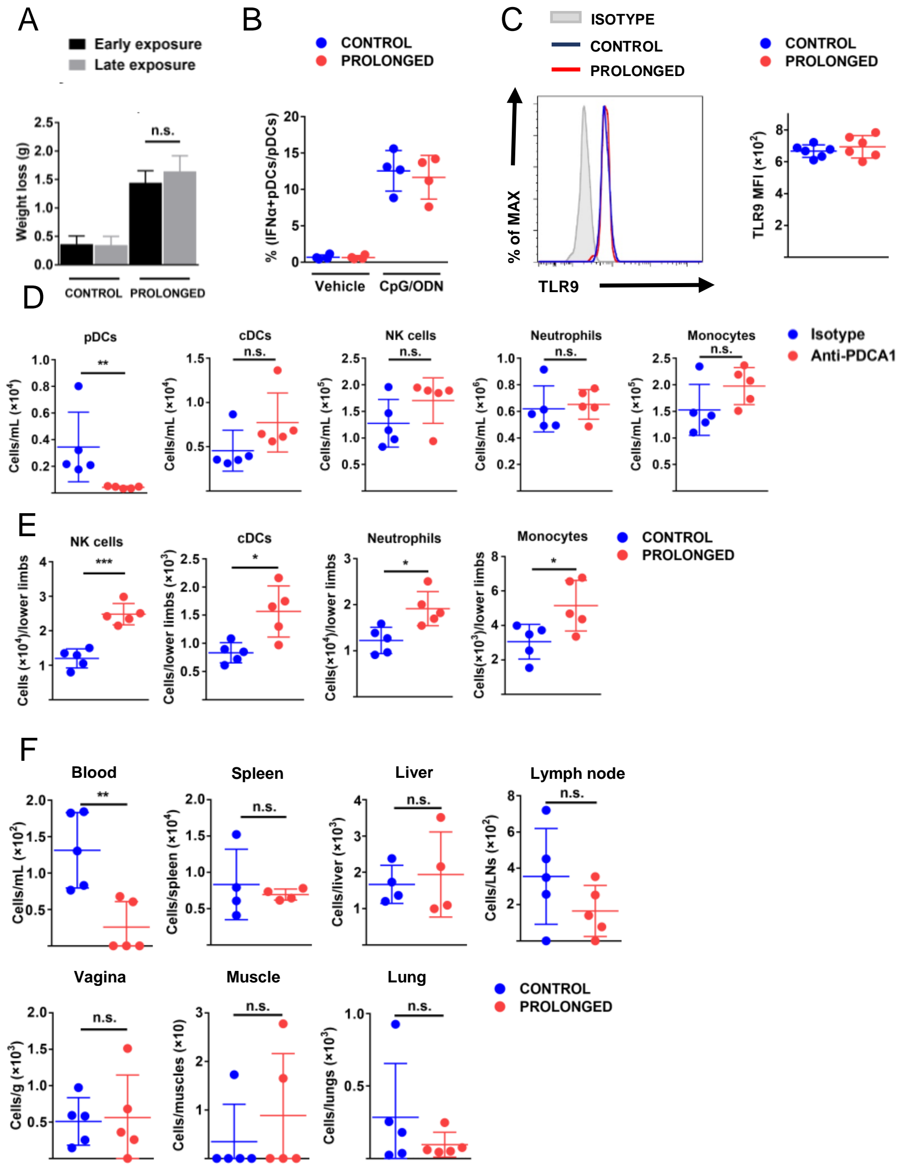


Figure S2

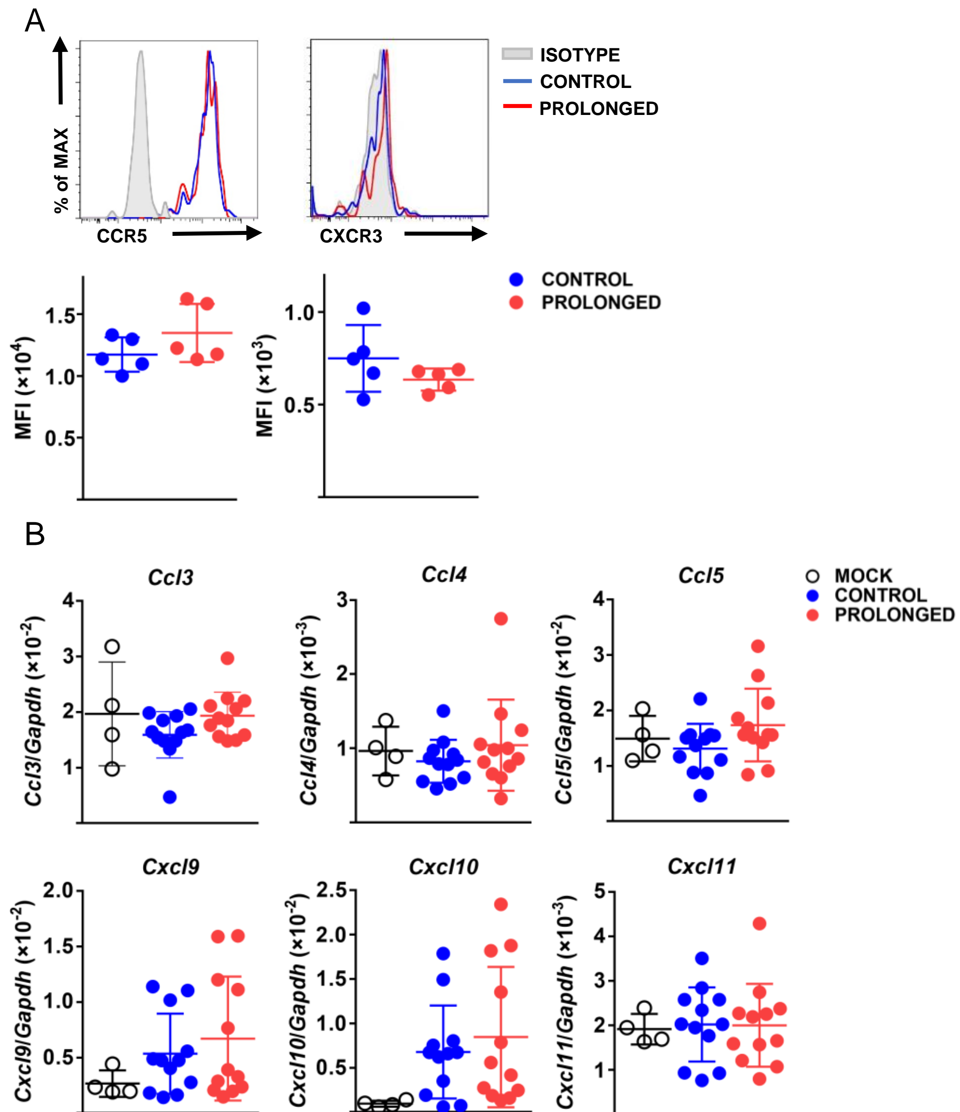


Figure S3

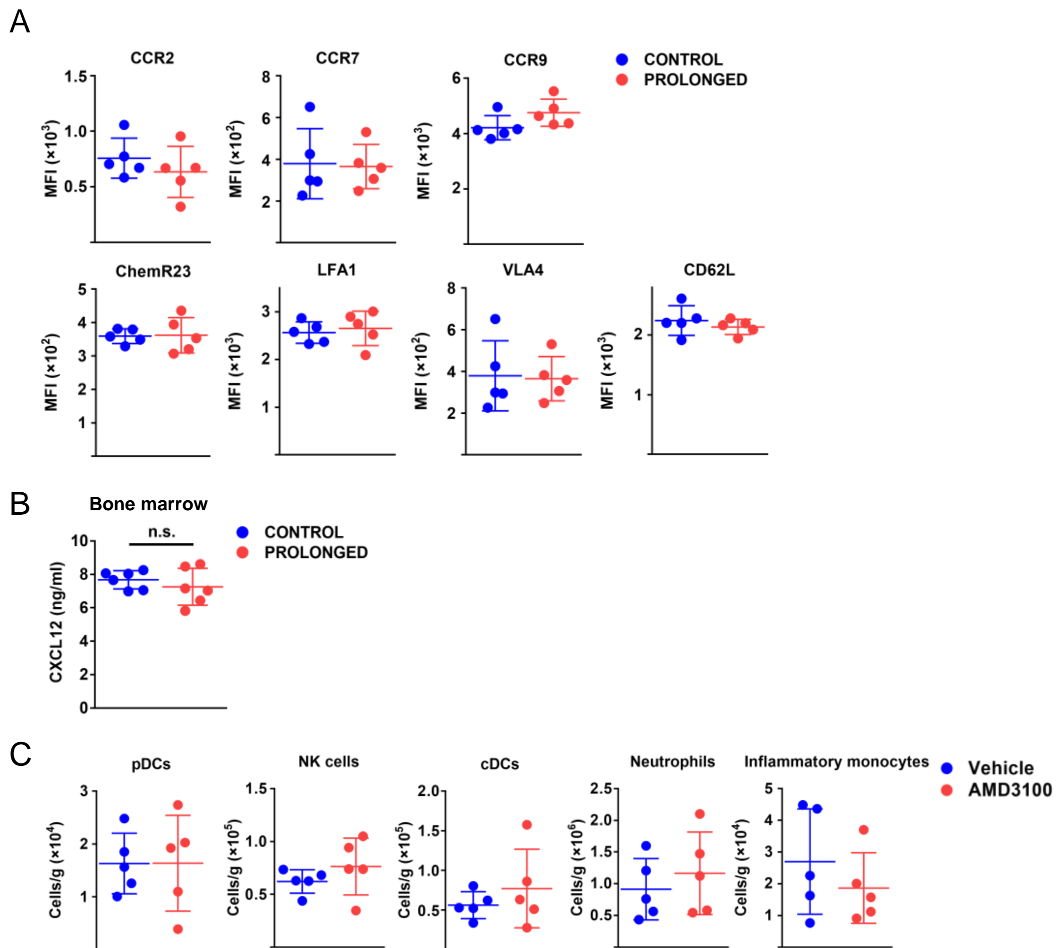


Figure S4

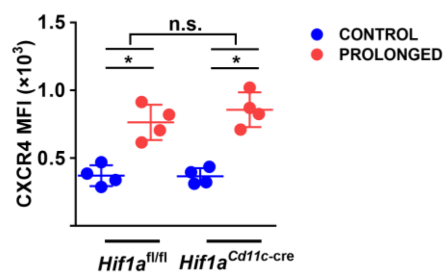
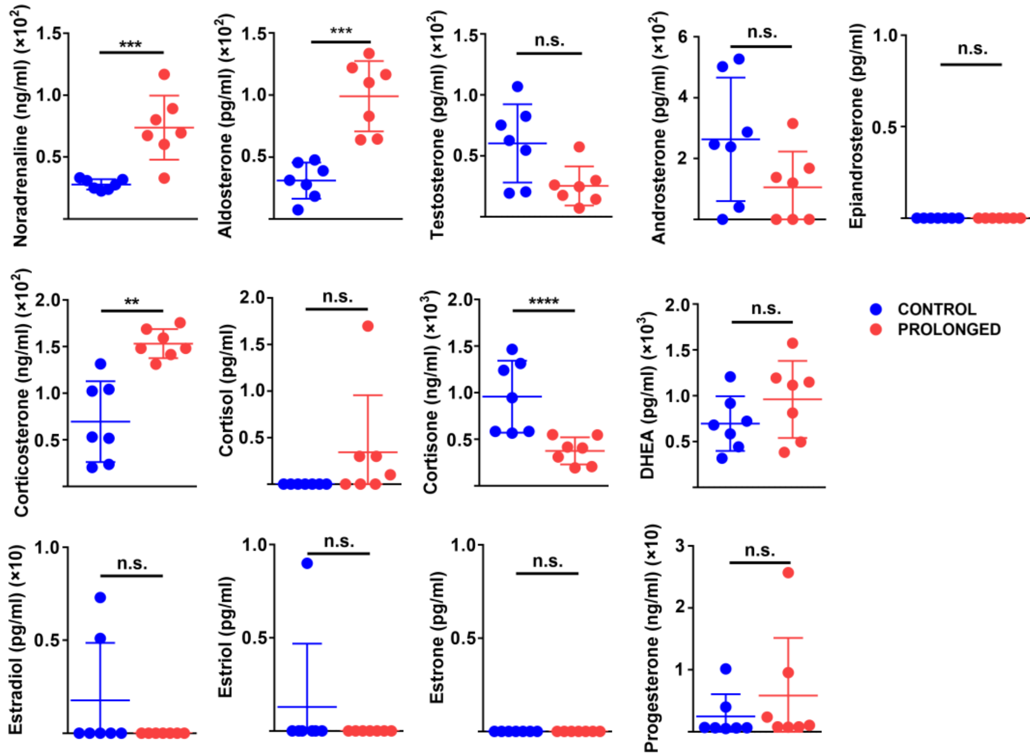


Figure S5



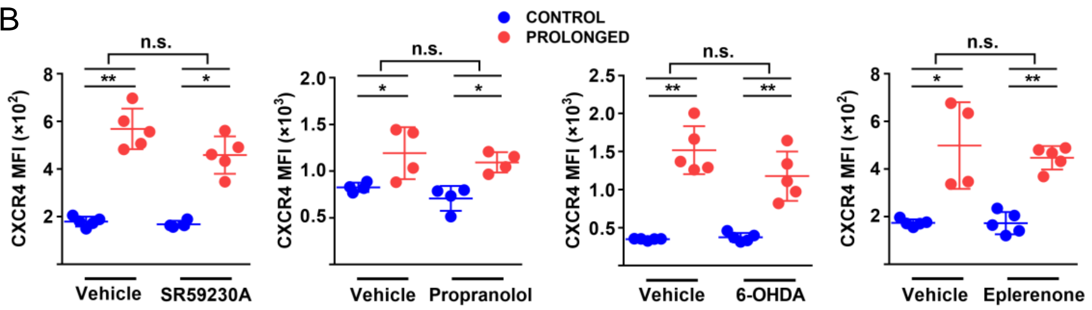


Figure S6

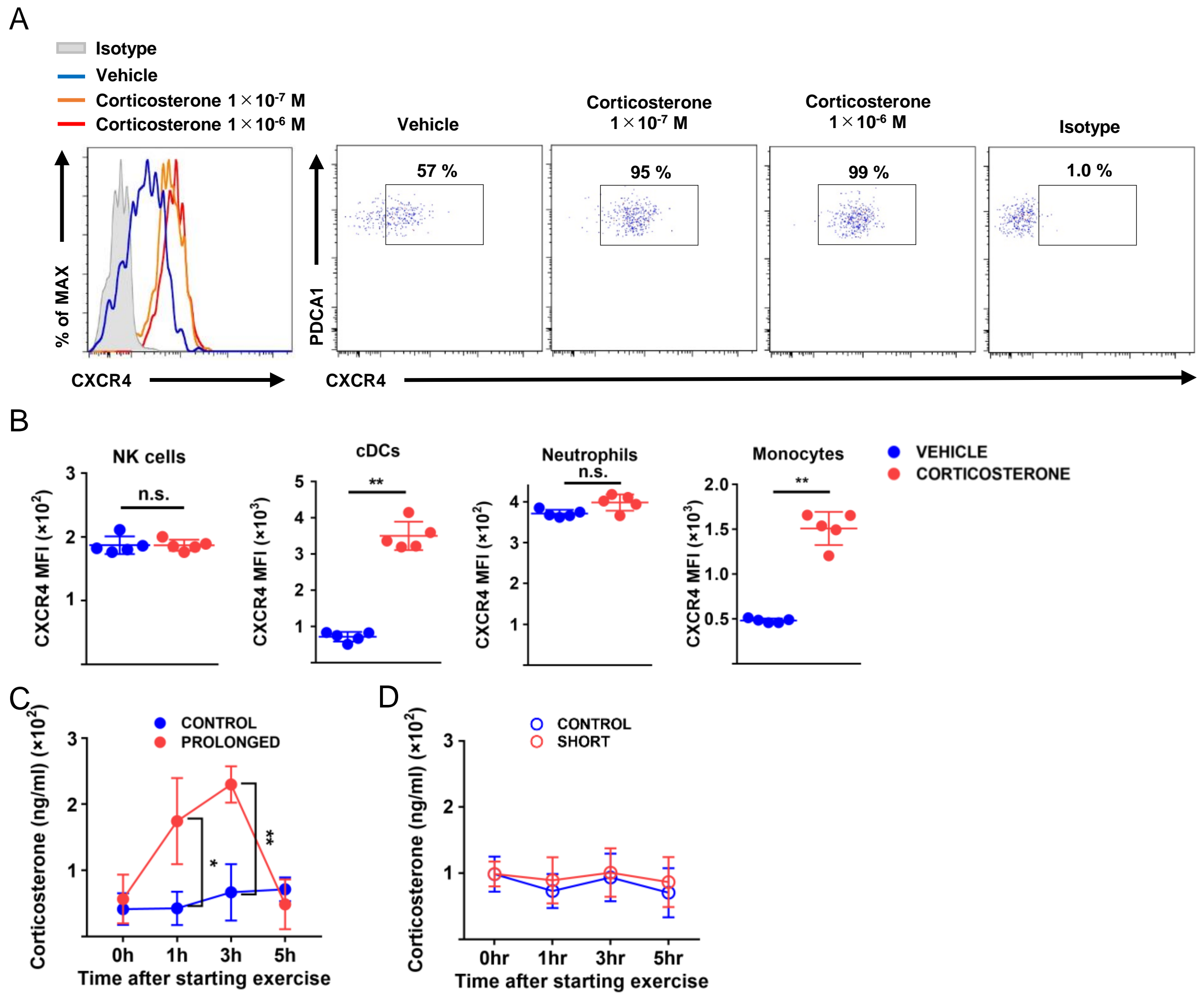


Figure S7