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（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）

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

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Conflict of interest statement

The authors have declared that no conflict of interest exists.

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Capsule summary

We describe that prolonged high-intensity exercise leads to impaired or enhanced immune responses against HSV-2 infection depending on the timing between infection and exercise.

Key messages

- Prolonged high-intensity exercise 17 hours after HSV-2 infection induces impaired viral clearance via glucocorticoid-induced pDC homing to the bone marrow.
- Prolonged high-intensity exercise 8 hours after HSV-2 infection induces enhanced viral clearance via glucocorticoid-induced the number of pDC in the blood.

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

Abstract

Background: Epidemiological studies have yielded conflicting results regarding the influence of a single bout of prolonged high-intensity exercise on viral infection.

Objective: Here, we show that prolonged high-intensity exercise induces either exacerbation or amelioration of herpes simplex virus type 2 (HSV-2) infection depending on the interval between viral exposure and exercise.

Methods: Mice were intravaginally infected with HSV-2 and exposed to run on the treadmill.

Results: Prolonged high-intensity exercise 17 h after infection impaired the clearance of HSV-2, while exercise 8 h after infection enhanced the clearance of HSV-2. These impaired or enhanced immune responses were related to a transient decrease or increase in the number of blood-circulating plasmacytoid dendritic cells (pDCs). Exercise-induced glucocorticoids transiently decreased the number of circulating pDCs by facilitating their homing to the bone marrow via the CXCL12-CXCR4 axis, which led to their subsequent increase in the blood.

Conclusion: Thus, a single bout of prolonged high-intensity exercise can be either deleterious or beneficial to antiviral immunity.

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 infection³⁻⁵. Consistently, a single bout of prolonged high-intensity exercise in mice
101 induced high morbidity and mortality in response to several viral infections⁶⁻⁸, 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 suspected⁹. 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 increased¹²⁻¹⁸. 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

lungs has been observed after a single bout of prolonged high-intensity exercise in a mouse study¹⁰. However, to date, there have been no studies that address the association between these transient changes in blood immune cells and subsequent immune responses to viruses. In addition, the underlying molecular mechanisms that cause transient changes in the number of blood cells are poorly understood.

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

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-*Hif1a*^{tm3Rsj0/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 described⁵⁴⁻⁵⁷. 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

approximately 76% or 84% of maximal oxygen uptake (VO_2max), respectively; 50-80% of VO_2max was considered to be moderate-intensity exercise, and over 80% of VO_2max was considered to be high-intensity exercise⁵⁸⁻⁶⁰. In the short periods of moderate-intensity exercise group, the speed of the treadmill was initially set to 11.5 m/min, and the total duration of exercise was 30 min. Electric shock was never used in the exercise session because mice readily responded to a gentle tap that encouraged them to maintain the pace of the treadmill. Mice in the no-exercise group were placed in cages near the treadmill, and no food or water was provided during the exercise session.

Viral infection and cells. HSV-2 wild-type strain 186 and Vero cells were previously described^{61,62}. HSV-2 stocks were prepared in Vero cells and quantified by a plaque-forming assay. For HSV-2 infection, mice were pretreated by the subcutaneous injection of the neck ruff with 1.67 mg of medroxyprogesterone acetate (A.N.B. Laboratories, Bangkok, Thailand) per mouse in a volume of 100 μL . Five days later, mice were anesthetized with isoflurane (Wako, Osaka, Japan). The vagina was washed with 100 μL of phosphate-buffered saline (PBS), and mice were intravaginally infected with 3.5×10^2 plaque-forming units (PFU) of HSV-2 strain 186 in 10 μL volumes suspended in 199V. Disease severity was assessed daily until 14 d post infection using a clinical scoring system that was previously described⁶³. The growth mediums used were Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO) supplemented with 5% heat-inactivated fetal calf serum (FCS) (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin (Wako); and 199V medium (430 mL of sterilized distilled water (dH_2O) supplemented with 50 mL of 199 medium (Sigma-Aldrich), 5 mL of 5% (wt/vol) NaHCO_3 (Nacalai, Kyoto, Japan), 5 mL of 1% FCS (Invitrogen), 100 U/mL of penicillin

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

Viral titer measurement in the vagina. Vaginal washes were collected for 2 consecutive days after infection with HSV-2 by washing with 100 µL of 199V. Viral titers were obtained titrating vaginal wash samples with 199V (400 µL) on a Vero cell monolayer in 12-well plates for 1 h at 37°C with 5% CO₂. After aspirating all medium, 1 mL of 1990 medium (199V and γ-globulins from human blood (Sigma-Aldrich)) was added, and plates were incubated for 48 h at 37°C with 5% CO₂.

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

Antibodies and flow cytometry. Single-cell suspensions of vaginal tracts were obtained as previously described⁶⁴ with some modifications. Briefly, the vagina was separated from the urethra and cervix, cut into small pieces and incubated in 4 mg/mL Dispase II (Roche, Basel, Switzerland), 0.425 mg/mL collagenase D (Roche), 30 µg/mL DNase I (Sigma-Aldrich) and 100 U/mL hyaluronidase (Sigma-Aldrich) for 45 min at 37°C. After perfusion, the lungs, liver, and gastrocnemius muscles were cut into small pieces. Lungs were digested with 150 U/mL collagenase type IV (Worthington Biochemical, Lakewood, NJ) and 150 µg/mL DNase I at 37°C for 55 min. Livers were digested with 0.05%

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

(RMMA-1). Each cell is identified as following panel; pDC; CD3⁻CD19⁻CD11b⁻
B220⁺PDCA1⁺, NK cell; CD3⁻NK1.1⁺, neutrophil; CD11b⁺Ly6G⁺, inflammatory
monocytes CD11b⁺Ly6C^{high}, cDC; CD11c⁺I-A/I-E^{high}. Multiparameter analyses were
performed on an LSRFortessa cytometer (LSR II; BD Biosciences) and were analyzed
using FlowJo software (Tree Star, Ashland, OR).

Drug administration. Drugs were administered either intraperitoneally (AMD3100,
RU486, eplerenone, 6-OHDA, and corticosterone) or subcutaneously (SR59230A and
propranolol). All drugs were purchased from Sigma-Aldrich. AMD3100 was dissolved in
PBS and administered 30 min before exercise. RU486 (30mg/kg) was dissolved in
ethanol/sesame oil (1:10 vol/vol) and administered 1 h before exercise. Corticosterone
(10mg/kg) was dissolved in an ethanol/sesame oil solution (1:10 vol/vol) and
administered 3 h before harvesting the blood and bone marrow. SR59230A (5mg/kg),
propranolol (10mg/kg) and eplerenone (1mg/kg) were dissolved in DMSO and further
diluted with PBS. 6-OHDA was dissolved in 0.9% NaCl and 10⁻⁷ M ascorbic acid and
administered as previously described⁶⁵. All dosing was determined by previously
established protocols⁶⁶⁻⁷¹.

Adoptive cell transfer. For adoptive transfer, the spleen and bone marrow were extracted
from B6-Tg (CAG-EGFP) mice or EIIa^{Cre}-R26-CAG-tdTomato mice. The tissues were
digested with 500 U/mL collagenase type II and 100 µg/mL DNase I at 37°C for 25 min
and further digested with 0.01 M EDTA at 37°C for 5 min. After lysing red blood cells,
cells were resuspended in PBS, and 4×10⁷ cells were intravenously injected into each
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 μ m) 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

6460 triple quadrupole tandem mass spectrometer (Agilent Technologies). The analytical column was a Capcell core C18 (Osaka Soda Co. Ltd., Osaka, Japan). Acetic acid/water (0.1:100, vol/vol) and acetic acid/acetonitrile/methanol (0.1:50:50, vol/vol/vol) were used as mobile phases A and B, respectively. The flow rate was set at 0.4 mL/min. Gradient elution was performed by increasing B from 50% to 100% over 12 min. The selected reaction monitoring (SRM) mode was set for MS/MS detection. All SRM transitions were optimized with the infusion analysis of each standard solution. All source parameters were optimized with the flow injection analysis of standard solutions.

In vitro stimulation of bone marrow cells. Single-cell suspensions from the bone marrow were incubated at 4×10^6 cells/well in 96-well plates in RPMI (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 1% sodium pyruvate, 1% nonessential amino acids (Invitrogen), 50 μ M 2-mercaptoethanol (Nacalai), 100 U/mL penicillin, and 100 μ g/mL streptomycin (Wako). For intracellular IFN- α staining, cells were cultured at 37°C in the presence of 0.2 μ g/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Peprotech, Rocky Hill, NJ) and were stimulated with CpG-ODN 2395 (InvivoGen, San Diego, CA) (5 μ M) for 9 h. GolgiStop (1 μ L/mL) (BD Biosciences) and brefeldin A (1 μ L/mL) (Sigma) were added for the last 6 h of the incubation. Subsequently, cells were harvested and stained with antibodies against surface markers. Fixation and permeabilization were performed with Cytofix/Cytoperm solution (BD Bioscience), followed by intracellular staining with FITC-conjugated anti-IFN- α (PBL Assay Science).

Chemotaxis assay. Whole blood cells were lysed and incubated for 3 h with corticosterone (Sigma) or vehicle. Then, cells were placed in the upper chamber of an uncoated transwell

with 5- μ m pores (Corning, Corning, NY) for 1 h. CXCL12 (10 ng/mL; R&D Systems) or medium was placed in the lower chamber. Cells in the lower chamber were analyzed with flow cytometry. Cells were cultured in phenol red-free RPMI with 10% hormone-depleted FCS.

Quantitative RT-PCR analysis. Total RNA was isolated with TRIzol reagent (Invitrogen) and RNeasy kits (Qiagen, Hilden, Germany). cDNA was reverse transcribed from total RNA samples by using a Prime Script RT reagent kit (Takara Bio, Kusatsu, Japan). Quantitative RT-PCR was performed by monitoring double-stranded DNA synthesis during various PCR cycles with SYBR Green I (Roche) and a LightCycler real-time PCR apparatus (Roche) according to the manufacturer's instructions. All primers were obtained from Greiner Japan (Tokyo, Japan). The primer sequences were as follows:

Gapdh, 5'-AGGTCGGTGTGAACGGATTTG-3' and 5'-GGGGTCGTTGATGGCAACA-3'; *Cxcl9*, 5'-GGCACGATCCACTACAAATCC-3' and 5'-GGTTTGATCTCCGTTCTTCAGT-3'; *Cxcl10*, 5'-CCAAGTGCTGCCGTCATTTTC-3' and 5'-GGCTCGCAGGGATGATTTCAA-3'; *Cxcl11*, 5'-GGCTTCCTTATGTTCAAACAGGG-3' and 5'-GCCGTTACTCGGGTAAATTACA-3'; *Ccl3*, 5'-TGAAACCAGCAGCCTTTGCTC-3' and 5'-AGGCATTTCAGTTCCAGGTCAGTG-3'; *Ccl4*, 5'-CCATGAAGCTCTGCGTGTCTG-3' and 5'-GGCTTGAGCAAAGACTGCTG-3'; *Ccl5*, 5'-AGATCTCTGCAGCTGCCCTCA-3' and 5'-GGAGCACTTGCTGCTGGTGTAG-3'; and pan *Ifna*, 5'-CCTGAGAGAGAAGAAACACAGCC-3' and 5'-TCTGCTCTGACCACYTCCCAG-3'. For each sample, the gene expression of duplicate test reactions and a control reaction

lacking reverse transcriptase were analyzed, and the results were normalized to those of *Gapdh* mRNA.

Statistics. In the present study, we hypothesized that the clinical scores at day 14 and viral titer at day 1 and 2 might show substantial differences between CONTROL and PROLONGED groups under the respective situations with vehicle only or antagonists (AMD3100 or RU486). Also, we hypothesized that the clinical scores at day 14 and viral titer at day 1 and 2 might show substantial differences between CONTROL and PROLONGED groups in *Nr3c1*^{fl/fl} and *Nr3c1*^{fl/fl}*Cd11c*-Cre⁺ mice, respectively. Mann-Whitney test was performed at day 14 for clinical scores and at day 1 and day 2 for viral titers. For the analysis of survival rates, the log-rank test was performed between those groups. For the analysis of cell counts, expression levels of chemokine receptors, distances from GFP-positive cells to tdTomato-positive cells, levels of hormones and chemokines, we hypothesized that those values might show substantial differences between CONTROL and PROLONGED groups under the respective situations in each mouse genotype or drug-treated group. Mann-Whitney test was employed to compare continuous values between two groups. For nonparametric pairwise multiple comparison after Kruskal-Wallis test, Dunn's test with Holm adjustment was performed. For the analysis of the interaction effect of mouse genotype or drugs on the prolonged high-intensity exercise, the aligned rank transform (ART) procedure using analysis of variance (ANOVA) was performed to deal with non-normal distributed data⁷³. All statistical analyses were performed with GraphPad Prism software ver. 7.0 for Windows and Stata SE ver. 14.2 (StataCorp LP, College Station, TX). *P*-values less than 0.05 were considered 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

Results

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

To examine how a single bout of prolonged high-intensity exercise influences the immune system, we used a well-established mouse model of intravaginal HSV-2 infection in combination with prolonged high-intensity exercise on a treadmill that was based on previous reports with some modifications²⁰. Because it has been demonstrated that HSV-2 expansion in the vagina peaks approximately 18 h after infection²¹, mice were first intravaginally infected with HSV-2 (3.5×10^2 PFU), and then, 17 h later, the mice ran for 3 h at high speed (19 m/min) on a customized treadmill (late exposure to prolonged high-intensity exercise) (for details, see Material and Methods). Then, we evaluated the clinical scores and survival rates for the following two weeks and viral titers in the vagina for the following two days (Fig. 1A). With these conditions, mice in the prolonged high-intensity exercise group exhibited higher clinical scores and lower survival rates than those in the control no-exercise group (Fig. 1B). Consistently, the HSV-2 viral load in the vagina was significantly higher in the prolonged high-intensity exercise group than in the control no-exercise group (Fig. 1B). To examine whether the impaired immune response was specifically induced by prolonged high-intensity exercise, we examined the effect of short periods of moderate-intensity exercise (running for 30 min at a moderate speed (11.5 m/min))²² on HSV-2 infection. In this condition, no significant difference was observed in the clinical symptoms of HSV-2 infection between the exercise group and the control no-exercise group (Fig. 1C).

Since it has been reported that the number of circulating immune cells changes dynamically after exercise on an hour basis¹⁶, it is possible that the immune response may

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

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

We next examined the mechanism of impaired immunity induced by late exposure to prolonged high-intensity exercise. Various immune cells, such as pDCs, NK cells, inflammatory monocytes and neutrophils, to infected sites is reported to contribute to protection against HSV-2 intravaginal infection^{23–26}. In particular, pDCs are believed to play a crucial role in the initial prevention of viral spread by producing interferon (IFN)- α ^{23,27,28}, although some studies reported that pDCs are dispensable in HSV-2 infection²⁹. Therefore, we first evaluated the influence of late exposure to prolonged high-intensity exercise on the infiltration of those immune cells in the vagina 24 h after infection. While the accumulation of inflammatory monocytes and neutrophils was comparable between the prolonged high-intensity exercise group and the control no-exercise group, the

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

To confirm the contribution of pDCs in our model, we depleted pDCs using anti-PDCA1 antibody³⁰ and examined its effects on clinical symptoms and viral titer after HSV-2 infection. Administration of the anti-PDCA1 antibody significantly reduced the number of circulating pDCs, but did not affect the number of NK cells, neutrophils, monocytes and conventional DCs (cDCs) (see Fig. S1D in the Online Repository). pDC-depleted mice showed exacerbated clinical symptoms to a similar extent to those of mice with prolonged high-intensity exercise, as well as increased viral titer (Fig. 2C). These results indicate that pDCs are crucial for anti-viral immunity in our model, and support our hypothesis that exercise-induced exacerbation of HSV-2 infection was through the regulation of pDC infiltration to the vagina.

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

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

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

Because a previous study suggested that circulating T cells are redistributed to several tissues after prolonged high-intensity exercise¹⁰, we hypothesized that the decrease in circulating pDCs immediately after prolonged high-intensity exercise was caused by redistribution from the blood to other organs during the exercise. To test this hypothesis, we intravenously transferred bulk green fluorescent protein (GFP)-labeled splenocytes and bone marrow cells and examined the number of GFP-positive pDCs in other organs, such as the bone marrow, lymph nodes, spleen, lungs, liver, muscle and vagina, with or without prolonged high-intensity exercise. The number of transferred GFP-positive pDCs in the lymph node, spleen, liver, lung, muscle, and vagina were comparable between the prolonged high-intensity exercise group and the control no-exercise group. On the other hand, the number of GFP-positive pDCs in the bone marrow was significantly higher in the prolonged high-intensity exercise group than in the control no-exercise group (Fig. 3A and S1), although the increase in the bone marrow was also observed in other immune cells (see Fig. S1E in the Online Repository). In the blood, the number of transferred GFP-positive pDCs significantly decreased in the prolonged high-intensity exercise group (see Fig. S1F in the Online Repository).

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

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

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

We next investigated the mechanisms through which prolonged high-intensity exercise induced the homing of pDCs to the bone marrow. It has been reported that the chemokine receptors (CXCR4, CCR2, CCR7, CCR9, and ChemR23) and adhesion molecules (LFA1, VLA4, and CD62L) may be responsible for pDC homing from the blood to lymphoid organs or inflamed tissues³²⁻³⁷. Therefore, we examined the expression levels of these candidates on circulating pDCs and found that the expression of CXCR4 was upregulated by prolonged high-intensity exercise (Fig. 3B). The expression levels of other candidate chemokine receptors were not upregulated (see Fig. S3A in the Online Repository). On the other hand, the production of CXCL12, the ligand of CXCR4, in the bone marrow was not changed by prolonged high-intensity exercise (see Fig. S3B in the Online Repository). These findings suggest that the promotion of pDC homing to the bone

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

To examine whether the CXCL12-CXCR4 axis was involved in the homing of circulating pDCs to the bone marrow during the prolonged high-intensity exercise, we administered either vehicle or AMD3100, a CXCR4 antagonist, to mice before prolonged high-intensity exercise and examined the number of pDCs in the blood and bone marrow. As reported previously³⁸, the administration of AMD3100 inhibited the homing of pDCs to the bone marrow and increased the number of circulating pDCs under the steady states (Fig. 3D). In addition, AMD3100 abrogated the decrease in circulating pDCs induced by prolonged high-intensity exercise (Fig. 3D). Consistently, an increase in the number of pDCs in the bone marrow was not observed in the prolonged high-intensity exercise group treated with AMD3100 (Fig. 3D). AMD3100 did not affect the cellular contents in the vagina (see Fig. S3C in the Online Repository). Collectively, these results suggest that the CXCL12-CXCR4 axis plays a crucial role in the pDC homing to the bone marrow during the prolonged high-intensity exercise.

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

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

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

Glucocorticoids upregulate the expression of CXCR4 on pDCs

We further explored the underlying mechanisms by which prolonged high-intensity exercise upregulates CXCR4 expression on pDCs. Prolonged high-intensity exercise is known to induce hypoxemia³⁹. Hypoxia inducible factor-1 α (HIF-1 α) is known to regulate the expression level of CXCR4⁴⁰; therefore, we evaluated whether HIF-1 α was involved in the induction of CXCR4 expression on pDCs by prolonged high-intensity exercise. We generated *Hif1a*^{fl/fl}*Cd11c*-Cre⁺, in which CD11c⁺ cells, including pDCs⁴¹, specifically lack HIF-1 α . In *Hif1a*^{fl/fl}*Cd11c*-Cre⁺ mice, CXCR4 expression on pDCs was upregulated to a level similar to that of *Hif1a*^{fl/fl} mice during prolonged high-intensity exercise (see Fig. S4 in the Online Repository). This result suggests that HIF-1 α is not involved in the upregulation of CXCR4 expression on pDCs in our model.

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

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

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

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

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

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

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

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

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

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

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

Discussion

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

We showed that GR signaling was involved in both the impairment and enhancement of antiviral immunity against HSV-2 intravaginal infection. Glucocorticoids are generally considered to cause immunosuppression; however, glucocorticoids may function as immunostimulants in some contexts, as we have previously discovered that glucocorticoids drive the diurnal accumulation of T cells in lymphoid organs to enhance adaptive immune responses⁴⁷. Previous reports also showed that glucocorticoids exhibit biphasic dose-response effects in an animal model of delayed-type hypersensitivity and in macrophages activated by lipopolysaccharide and IFN- γ ^{48,49}. Thus, the effects of glucocorticoids on the immune system may be bidirectional and fluctuate in a context dependent manner. That may explain why the contradictory results have been reported regarding the influence of prolonged high-intensity exercise on the viral infection rate.

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

expressed on other cells such as NK cells and mDCs, which are considered to be important in the defense against HSV-2 infection^{24,50}. Thus, our results using *Cd11c-Cre*⁺ mice may not necessarily be specific to the effects of GR signaling in pDCs. However, antiviral functions of NK cells are induced by pDCs-derived IFN- α ^{27,28}. In addition, mDCs exert their antiviral functions at much later time points (48 h after the HSV-2 intravaginal infection)^{50,51} than our analysis timing (24 h after the infection). Furthermore, mice depleted with pDCs showed similar clinical scores and viral titers to those of mice with prolonged high-intensity exercise. Therefore, although we cannot exclude the possibility of the contribution of GR signaling in other cells, we consider that GR signaling in pDCs is, at least mainly, involved in inducing the fluctuating immune responses in our model. Second, the mechanisms increasing the number of circulating pDCs following the transient decrease after the exercise remain unclear. Because the blockade of the pDC homing to the bone marrow by a GR antagonist diminished the increase, prior homing of pDCs to the bone marrow seems to be important for the increase. Homing of pDCs to the bone marrow may induce the release of progenitor cells from the bone marrow into the peripheral blood, as reported in neutrophils⁵². Third, the significance of pDC homing to the bone marrow during the prolonged high-intensity exercise remains unclear. One hypothesis is that the bone marrow may protect pDCs from the stress of prolonged high-intensity exercise. In line with this, it has recently been reported that memory T cells accumulate in the bone marrow during stress from dietary restriction because T cells in the bone marrow express high levels of the antiapoptotic factor BCL-2, which protects them from apoptosis⁵³. Thus, the positive and negative effects of prolonged high-intensity exercise on antiviral immunity, as seen in our model, 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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Author contributions

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

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Figure legends

Figure 1. Prolonged high-intensity exercise induces impaired or enhanced immunity against HSV-2 infection depending on the interval between infection and exercise.

(A) Experimental design. Mice were intravaginally infected with HSV-2 and ran on a treadmill 8 or 17 h after infection. Then, the indicated experiments were performed. Created with BioRender.com. (B-D) Mean clinical scores, survival rates and viral titers in vaginal wash. (B) Prolonged high-intensity exercise (PROLONGED) was performed 17 h after HSV-2 intravaginal infection (CONTROL: n=21; PROLONGED: n=22), (C) short periods of moderate-intensity exercise (SHORT) was performed 17 h after HSV-2 intravaginal infection (n=22 per group), and (D) prolonged high-intensity exercise was performed 8 h after HSV-2 intravaginal infection (n=26 per group). The results were pooled from three independent experiments. Error bars represent the mean $\pm$ the standard error of the mean (SEM). *P<0.05, **P<0.01, and n.s., not significant (Mann-Whitney test for clinical scores and viral titers and log rank test for survival rates (B-D)).

Figure 2. Late exposure to prolonged high-intensity exercise impairs pDC infiltration in the vagina after HSV-2 infection.

(A) Flow cytometric analysis of the number of indicated cells in the vagina 24 h after HSV-2 infection. MOCK: mock-infected with Vero cell lysate. (B) Quantitative PCR analysis of *Ifna* mRNA expression in the vagina 24 h after HSV-2 infection. (C) Mean clinical scores, survival rates and viral titers in vaginal wash. (C) Mice were injected with either isotype antibodies or anti-PDCA-1 antibodies and prolonged high-intensity exercise was performed 17 h after HSV-2 intravaginal infection (Isotype CONTROL: n=23; Isotype PROLONGED: n=21; Ab CONTROL: n=21; Ab PROLONGED: n=18).

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

Figure 3. Exacerbation of HSV-2 infection induced by prolonged high-intensity exercise is mediated through pDC homing to the bone marrow via the CXCL12-CXCR4 axis

(A) The number of transferred pDCs in the bone marrow immediately after late exposure to prolonged high-intensity exercise. (B) A representative histogram (left panel) and the mean fluorescence intensity (MFI) (right panel) of CXCR4 expression on pDCs in the blood 2 h after the start of prolonged high-intensity exercise. (C) Immunohistochemical analysis of pDCs in the bone marrow (left panel) and the mean distance from the transferred tdTomato⁺PDCA1⁺ cells to the nearest GFP⁺ cells in the bone marrow of *Cxcl12*-GFP mice (right panel). Scale bar = 50 μ m. (D) The number of transferred pDCs immediately after prolonged high-intensity exercise. (E) Mean clinical scores, viral titers in vaginal washes, and survival rates in mice that did or did not receive AMD3100 (CONTROL Vehicle: n=22; PROLONGED Vehicle: n=21; CONTROL AMD3100: n=22; PROLONGED AMD3100: n=21). Error bars represent the mean $\pm$ SD (A, B, D) and mean $\pm$ SEM (E). The results were representative of two to three independent experiments

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

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

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

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

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

pDCs in the blood and in the bone marrow in *Nr3c1^{fl/fl}Cd11c-Cre⁺* mice and *Nr3c1^{fl/fl}* mice immediately after prolonged high-intensity exercise. Each dot represents a single mouse. (C) Mean clinical scores, viral titers in vaginal washes, and survival rates in the intravaginal HSV-2 infection model with *Nr3c1^{fl/fl}* mice and *Nr3c1^{fl/fl}Cd11c-Cre⁺* mice (CONTROL *Nr3c1^{fl/fl}* mice: n=27; PROLONGED *Nr3c1^{fl/fl}* mice: n=25; CONTROL *Nr3c1^{fl/fl}Cd11c-Cre⁺* mice: n=27; PROLONGED *Nr3c1^{fl/fl}Cd11c-Cre⁺* mice: n=27). The results are representative of three independent experiments (A, B) or were pooled from three independent experiments (C). Error bars represent the mean $\pm$ SD (A, B) and the mean $\pm$ SEM (C). **P<0.01, ***P<0.001, ****P<0.0001, and n.s., not significant (Mann-Whitney test (A-C), ART procedure using ANOVA for the interaction effect (A) and log rank test for survival rates (C)).

Figure 6. Glucocorticoids are involved in enhanced antiviral immunity induced by early exposure to prolonged high-intensity exercise by an increase in circulating pDCs

(A) Flow cytometric analysis of the number of pDCs in the blood before (-3 h) and after prolonged high-intensity exercise at the indicated time points (n=5). (B) Flow cytometric analysis of the number of pDCs in the blood 12 h after the start of exercise. Mice were administered vehicle or RU486 (30 mg/kg) 1 h before exercise. (C) Mean clinical scores, survival rates, and viral titers in vaginal washes. Mice were administered vehicle or RU486 (30 mg/kg) 1 h before prolonged high-intensity exercise (PROLONGED) (CONTROL Vehicle: n=16; PROLONGED Vehicle: n=14; CONTROL RU486: n=16; PROLONGED RU486: n=14). Each dot represents a single mouse (A, B). Error bars represent the mean $\pm$ SD (A, B) and the mean $\pm$ SEM (C). The results are representative

of two independent experiments (A) or were pooled from two to three (B, C) independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, and n.s., not significant (Mann-Whitney test (A-C) and log rank test for survival rates (C)).

Figure 7. A graphical summary.

Prolonged high-intensity exercise increases the level of glucocorticoids in the blood. Glucocorticoids upregulate CXCR4 expression on pDCs in the blood, which facilitates their homing to the bone marrow and their decrease in the blood. The decrease in pDCs in the blood leads to the impaired infiltration of pDCs to infected sites (vagina), causing the exacerbation of HSV-2 infection. The glucocorticoid-induced pDC homing to the bone marrow, on the other hand, drives the recovery of pDCs in the blood approximately 6-12 h after exercise, leading to enhanced immune responses against HSV-2 infection. Created with BioRender.com.

Legend to Supplementary Figures

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

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

Figure S2. The expression levels of CCR5, CXCR3 in the blood and their ligands in the vagina after prolonged high-intensity exercise.

(A) Representative histograms (upper panels) and MFI (lower panels) of CCR5 and CXCR3 on pDCs in the blood, as analyzed by flow cytometry. (B) Quantitative PCR analysis of *Ccl3*, *Ccl4*, *Ccl5*, *Cxcl9*, *Cxcl10*, and *Cxcl11* in the vagina 24 h after HSV-2 infection. Each dot represents a single mouse. Error bars represent the mean $\pm$ SD. Data are representative of two to three independent experiments. Mann-Whitney test (A) and Dunn's test with Holm adjustment after Kruskal-Wallis test (B).

Figure S3. The expression of chemokine receptors and adhesion molecules on pDCs after prolonged high-intensity exercise and the concentration of CXCL12 in the bone marrow.

(A) Flow cytometric analysis of the expression levels of CCR2, CCR7, CCR9, ChemR23, LFA1, VLA4, and CD62L on pDCs in the blood immediately after prolonged high-intensity exercise. MFIs are shown. (B) The concentration of CXCL12 in the bone marrow. Immediately after prolonged high-intensity exercise, femurs and tibias were flushed with PBS, and the concentration of CXCL12 in extracellular fluids was analyzed by ELISA. (C) Flow cytometric analysis of the number of pDCs, NK cells, cDCs,

neutrophils and inflammatory monocytes in the vagina 24 h after mock infection in mice treated with vehicle or AMD3100 17 h after mock infection. Each dot represents a single mouse. Error bars represent the mean $\pm$ SD. These results are representative of two to three independent experiments. n.s., not significant (Mann-Whitney test).

Figure S4. HIF-1 α is not involved in the upregulation of CXCR4 expression on pDCs after prolonged high-intensity exercise.

Flow cytometric analysis of CXCR4 expression on pDCs in the blood 2 h after the start of prolonged high-intensity exercise (PROLONGED) in *Hif1a*^{fl/fl} or *Hif1a*^{fl/fl}-*Cd11c*-Cre⁺ mice. Each dot represents a single mouse. Error bars represent the mean $\pm$ SD. Data are representative of three independent experiments. *P<0.05, and n.s., not significant (Mann-Whitney test and ART procedure using ANOVA for the interaction effect).

Figure S5. Blood levels of noradrenaline and steroid hormones, and the effects of antagonists for adrenergic receptor and mineralocorticoid receptor on CXCR4 expression on pDCs after prolonged high-intensity exercise.

(A) Immediately after prolonged high-intensity exercise, whole blood was harvested, and serum or plasma was isolated by centrifugation. Then, serum was subjected to LC-

MS/MS analysis to analyze steroid hormones, and plasma was subjected to ELISA to analyze noradrenaline. (B) Flow cytometric analysis of CXCR4 expression on pDCs in the blood 2 h after the start of prolonged high-intensity exercise. Mice were administered either vehicle, SR59230A (β 3-adrenergic receptor (AR) antagonist) (5 mg/kg), propranolol (β 1- and β 2-AR antagonist) (10 mg/kg), 6-OHDA (sympathectomy), or eplerenone (mineralocorticoid receptor antagonist) (1 mg/kg). Each dot represents a single mouse. Error bars represent the mean $\pm$ SD. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001, and n.s., not significant (Mann-Whitney test (A, B), and ART procedure using ANOVA for the interaction effect (B)).

Figure S6. Upregulation of CXCR4 expression on pDCs by corticosterone, and blood levels of corticosterone over time during and after prolonged high-intensity exercise or short periods of moderate-intensity exercise.

(A) Representative histogram and flow cytometric panels of CXCR4 expression on pDCs treated with corticosterone. Whole blood cells from naïve wild-type mice were lysed and incubated with vehicle or corticosterone for 3 h at the indicated concentration. The cells were then subjected to flow cytometric analysis. (B) MFI of CXCR4 expression on NK cells, cDCs, neutrophils and monocytes in the blood 3 h after the administration of vehicle

or corticosterone (10 mg/kg), as analyzed by flow cytometry. Each dot represents a single mouse. (C) Time course of the blood level of corticosterone in mice after prolonged high-intensity exercise (PROLONGED) (n=4-5 per group). The time after the start of exercise is shown. (D) Time course of the blood level of corticosterone in mice with short periods of moderate-intensity exercise (SHORT) (n=4-5 per group). Time after the start of exercise is shown. Data are representative of two-three independent experiments. Error bars represent the mean $\pm$ SD. *P<0.05 and **P<0.01 (Mann-Whitney test).

Figure S7. GR signaling mediates the effects of late exposure to prolonged high-intensity exercise on the clinical symptoms of intravaginal HSV-2 infection.

(A) Flow cytometric analysis of the number of transferred pDCs immediately after prolonged high-intensity exercise. (B) Mean clinical scores, viral titers in vaginal washes, and survival rates in the intravaginal HSV-2 infection model in mice administered with vehicle or RU486 (n=21 per group). (C) Flow cytometric analysis of the number of transferred pDCs in the blood and in the bone marrow 3 h after the administration of vehicle or corticosterone (10 mg/kg). (D) Mean clinical scores, viral titers in vaginal washes, and survival rates in mice treated with vehicle or corticosterone (10 mg/kg) 17 and 20 h after HSV-2 infection (VEHICLE: n=28; CORTICOSTERONE: n=26). Each

dot represents a single mouse (A, C). Error bars represent the mean $\pm$ SD (A, C) and the mean $\pm$ SEM (B, D). These results are representative of two independent experiments (A, C) or were pooled from three independent experiments (B, D). *P<0.05, **P<0.01, and n.s., not significant (Mann-Whitney test (A-D) and log rank test for survival rates (B, D)).

Figure 1

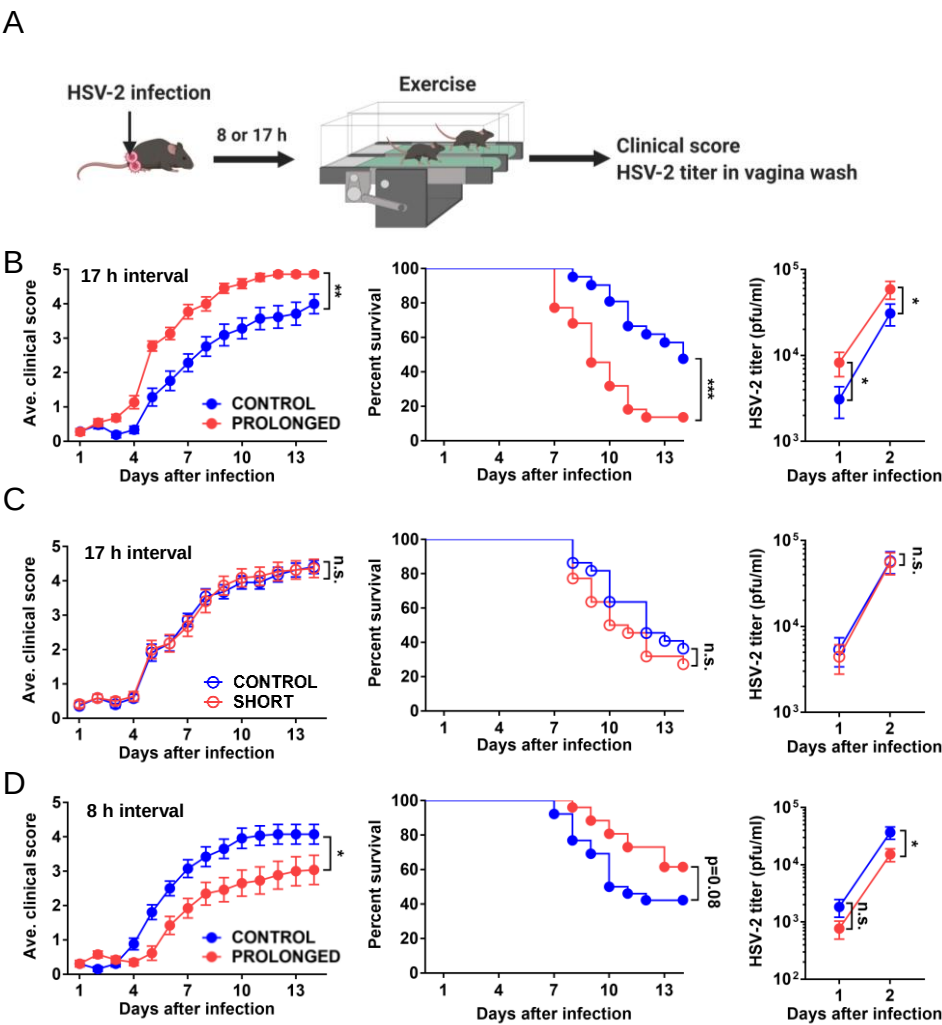


Figure 2

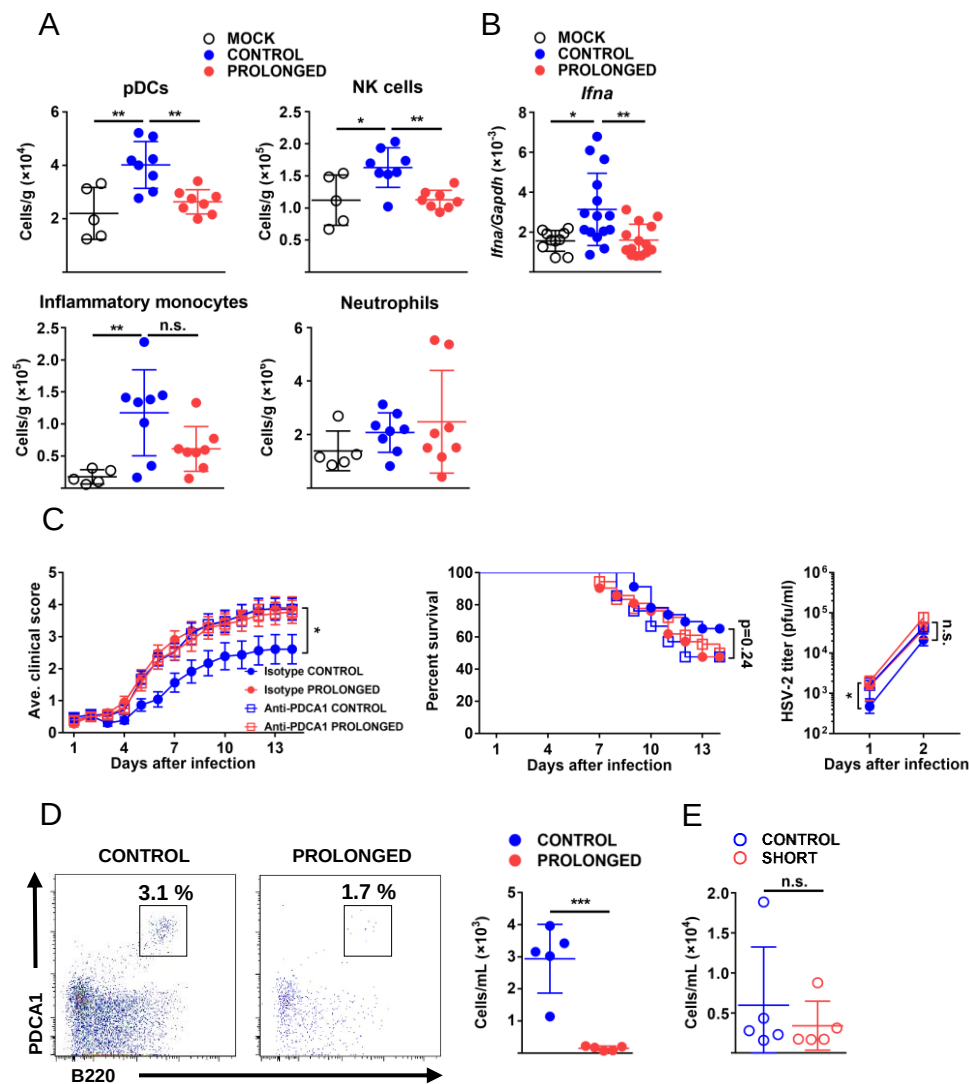


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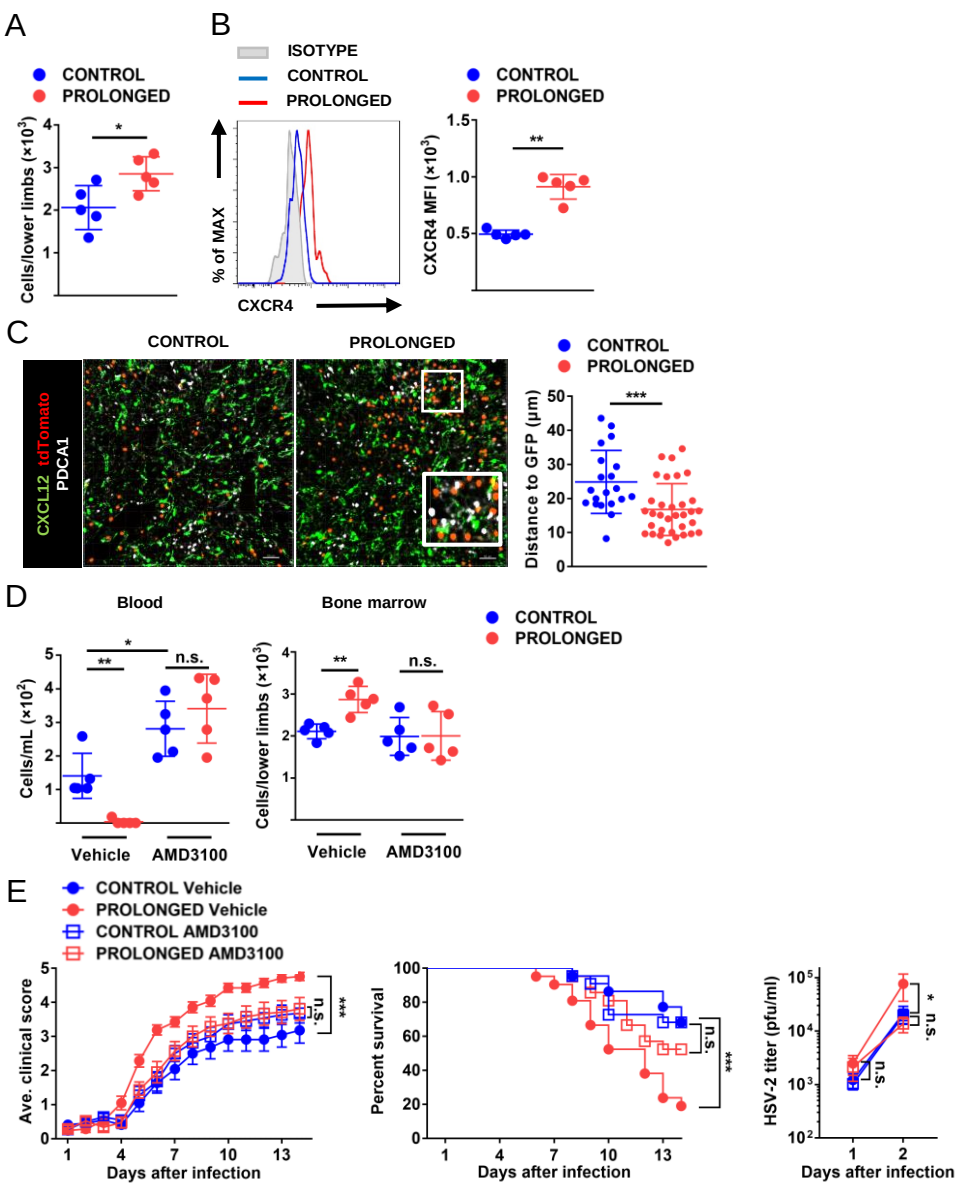


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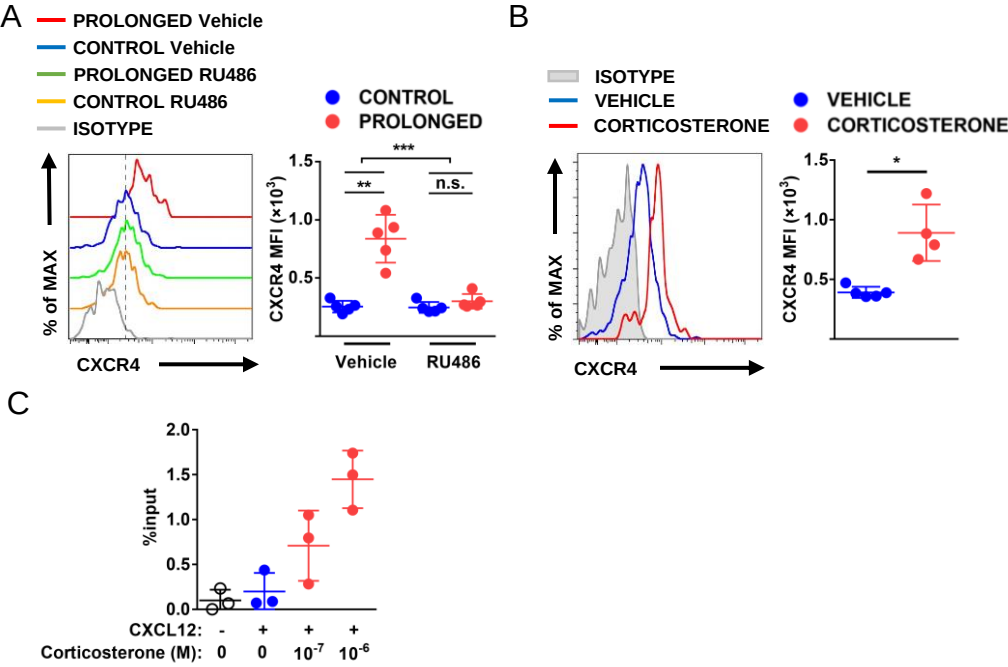


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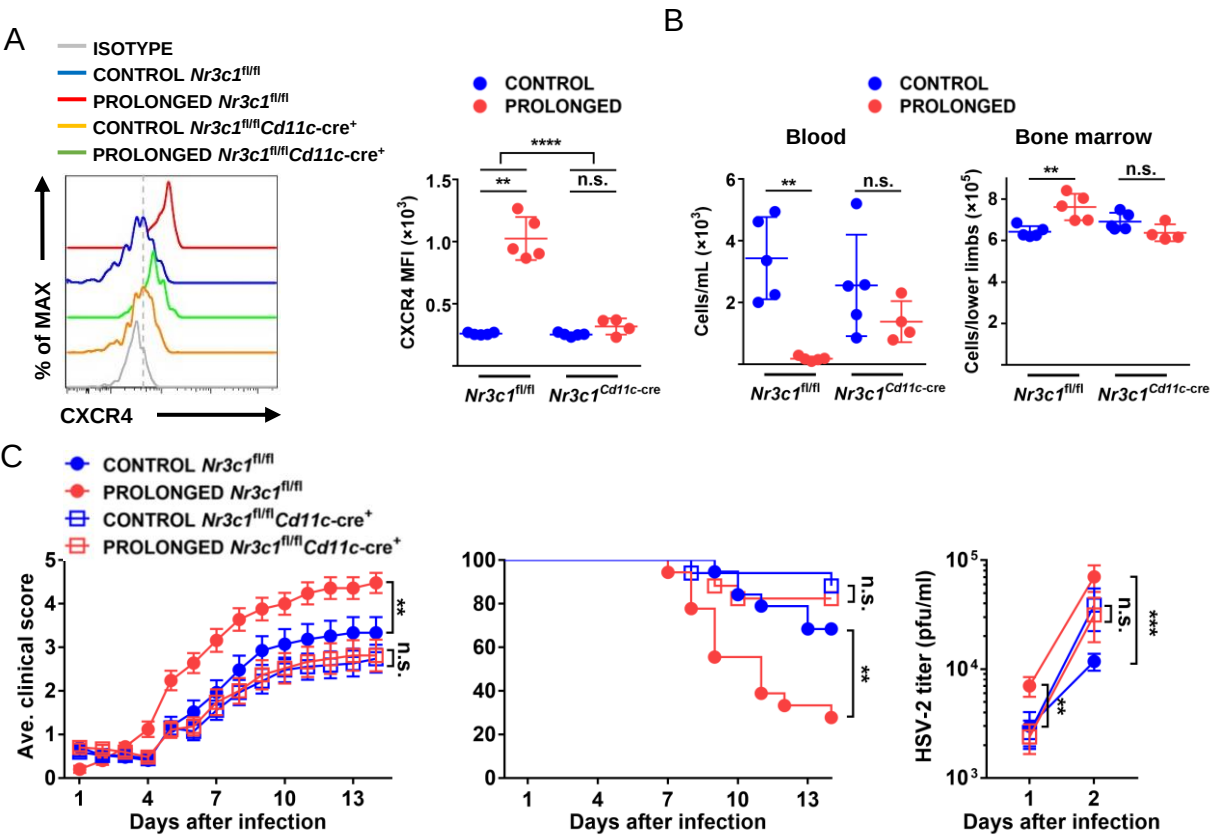


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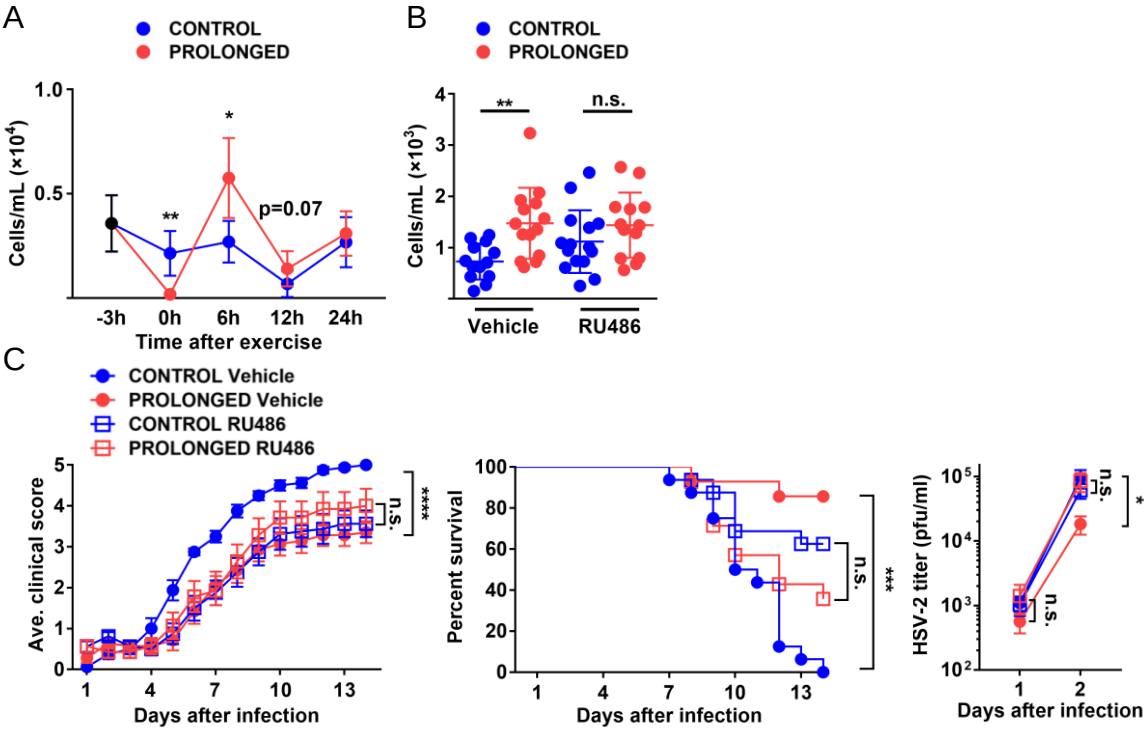


Figure 7

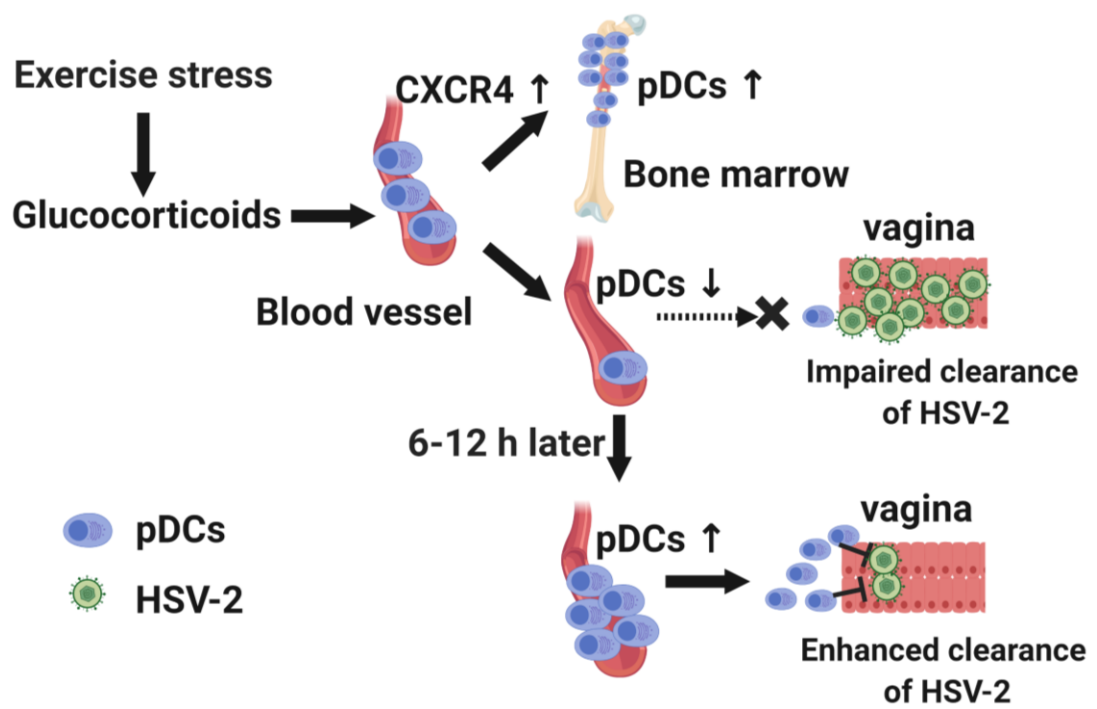


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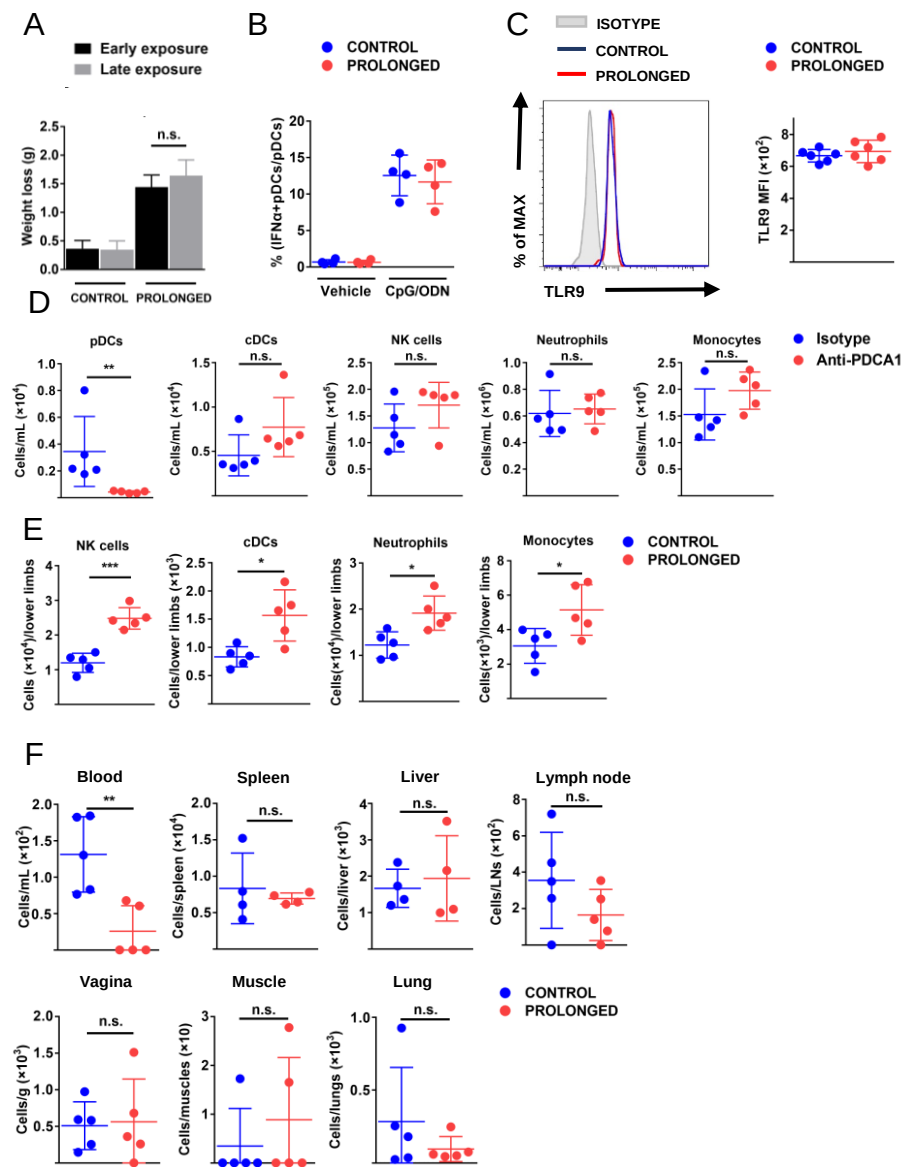


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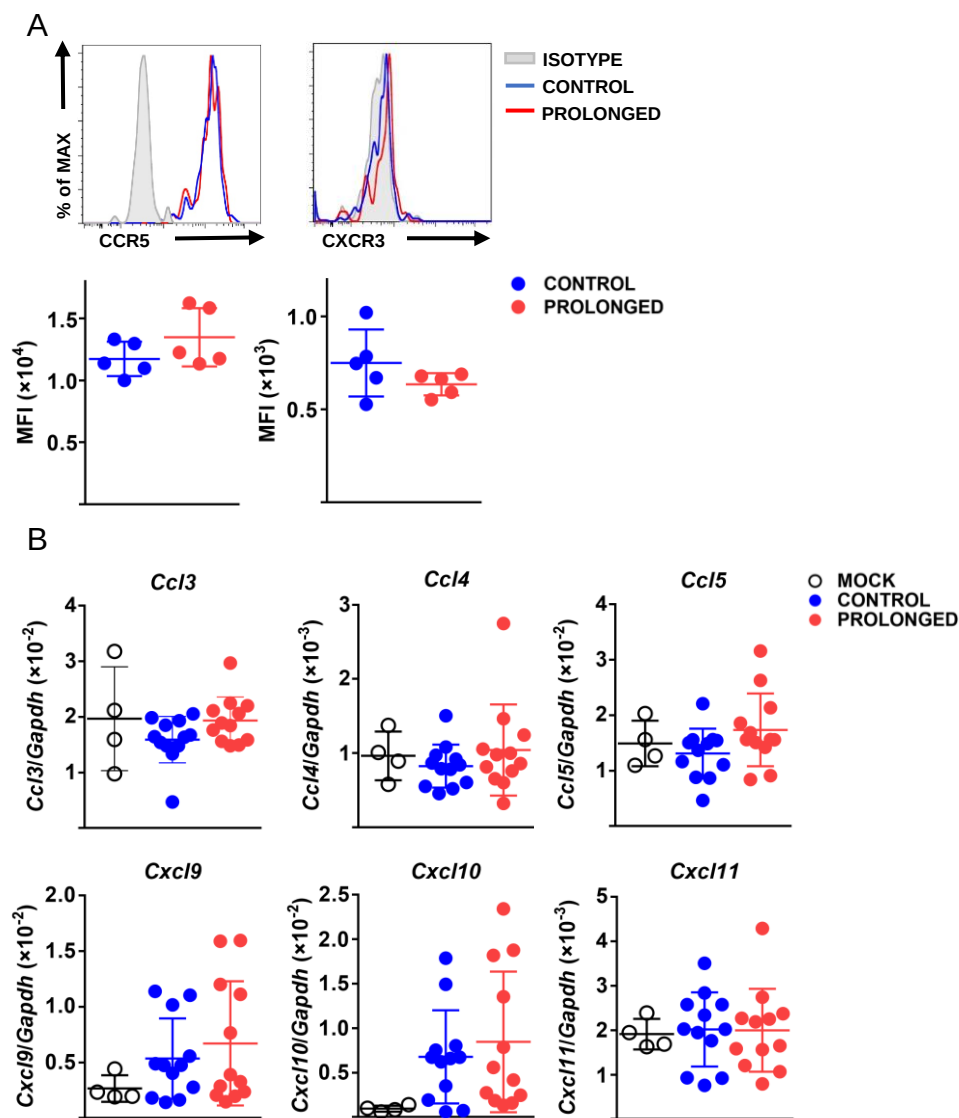


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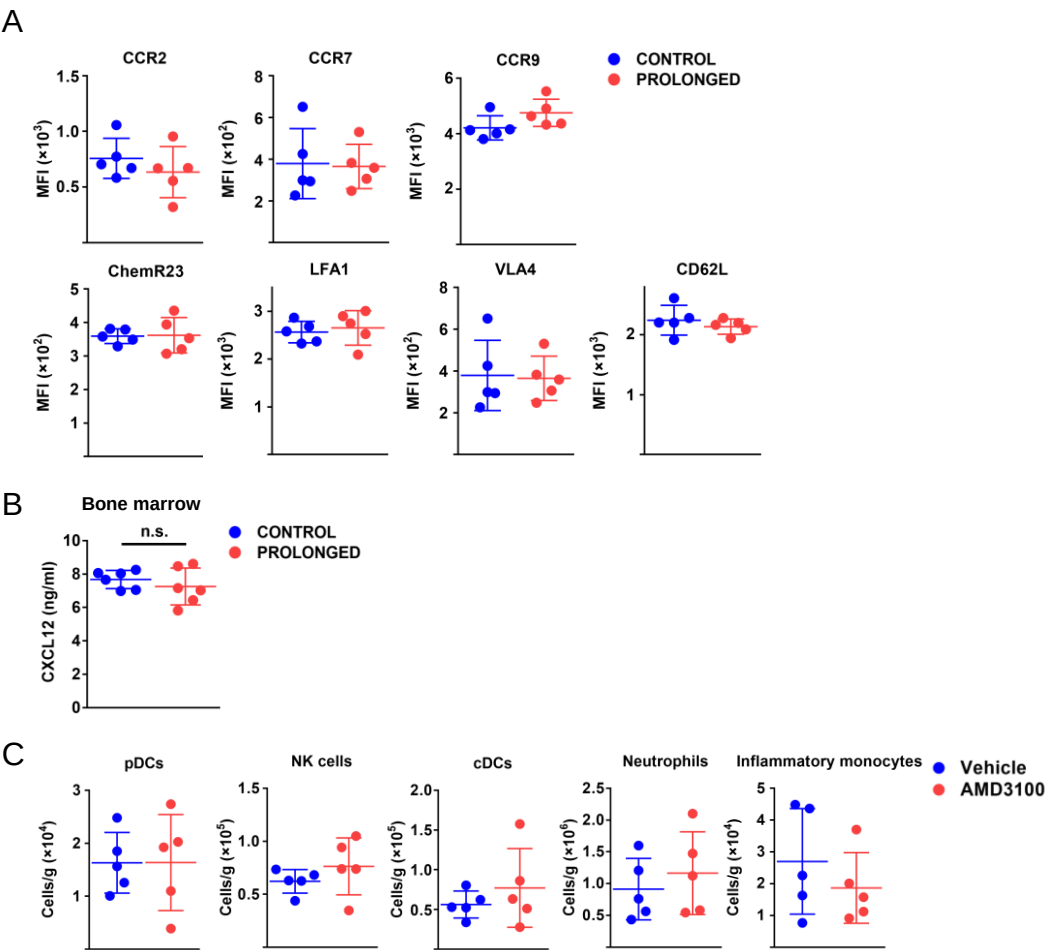


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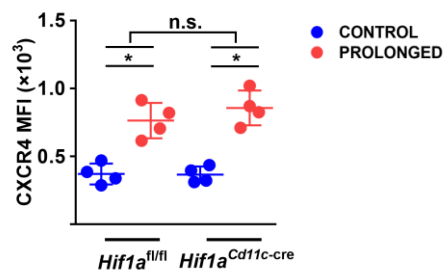
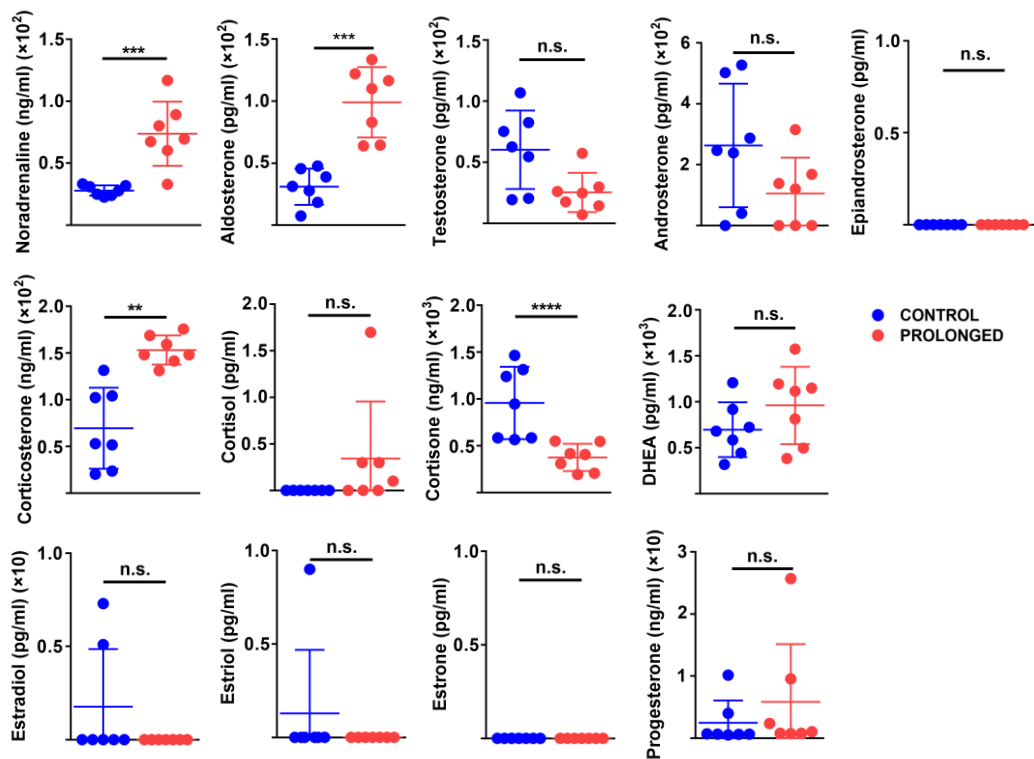


Figure S5

A



B

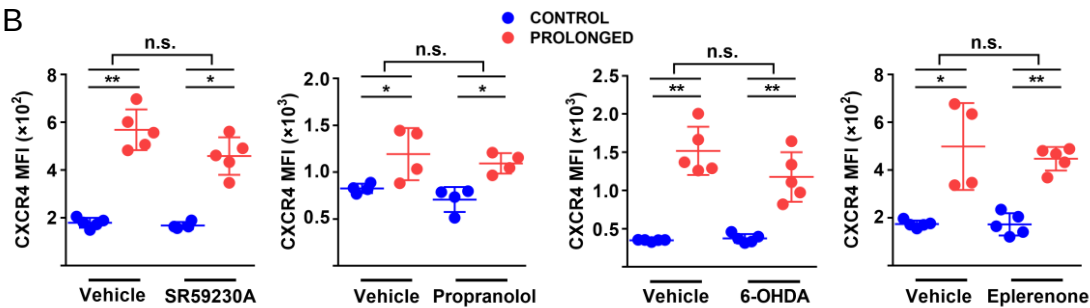


Figure S6

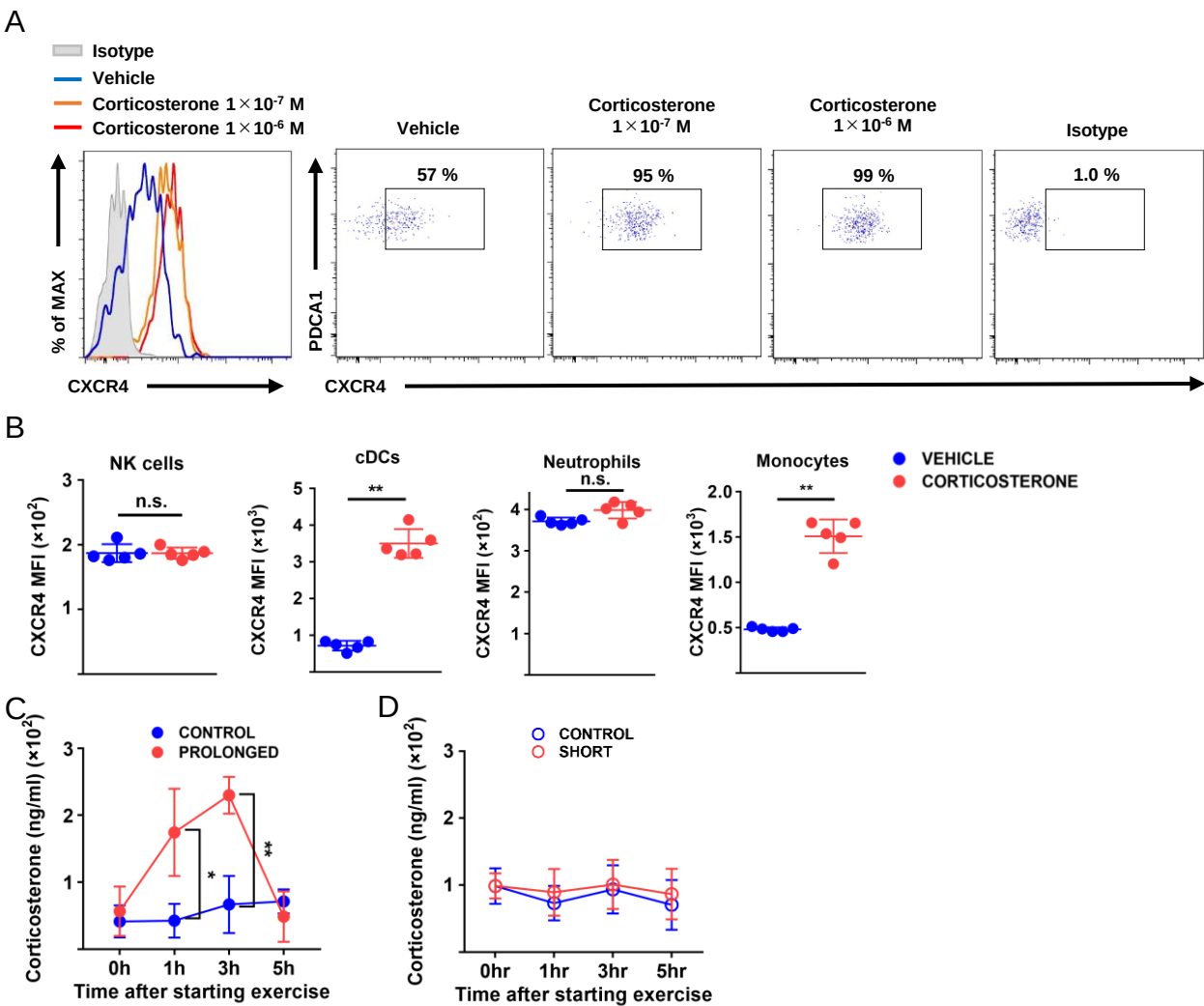


Figure S7

