

Supplementary Information for

Altered regulatory T cell parameters in women using oral contraception

Lachlan M. Moldenhauer^{1†}, Min Jin^{1,2†}, Jasmine Wilson¹, Ella S. Green¹, David J. Sharkey¹, Thomas Bristow¹, M. Louise Hull³, Gus A. Dekker^{3,4}, Sarah A. Robertson¹

¹Robinson Research Institute and School of Biomedicine, University of Adelaide, Adelaide, Australia 5005.

²Center for Reproductive Medicine, The Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China 310009

³Robinson Research Institute and Adelaide Medical School, University of Adelaide, Adelaide, Australia 5005

⁴Division of Women's Health, Lyell McEwin Hospital, Elizabeth Vale, Australia 5112

[†]Equal contributors

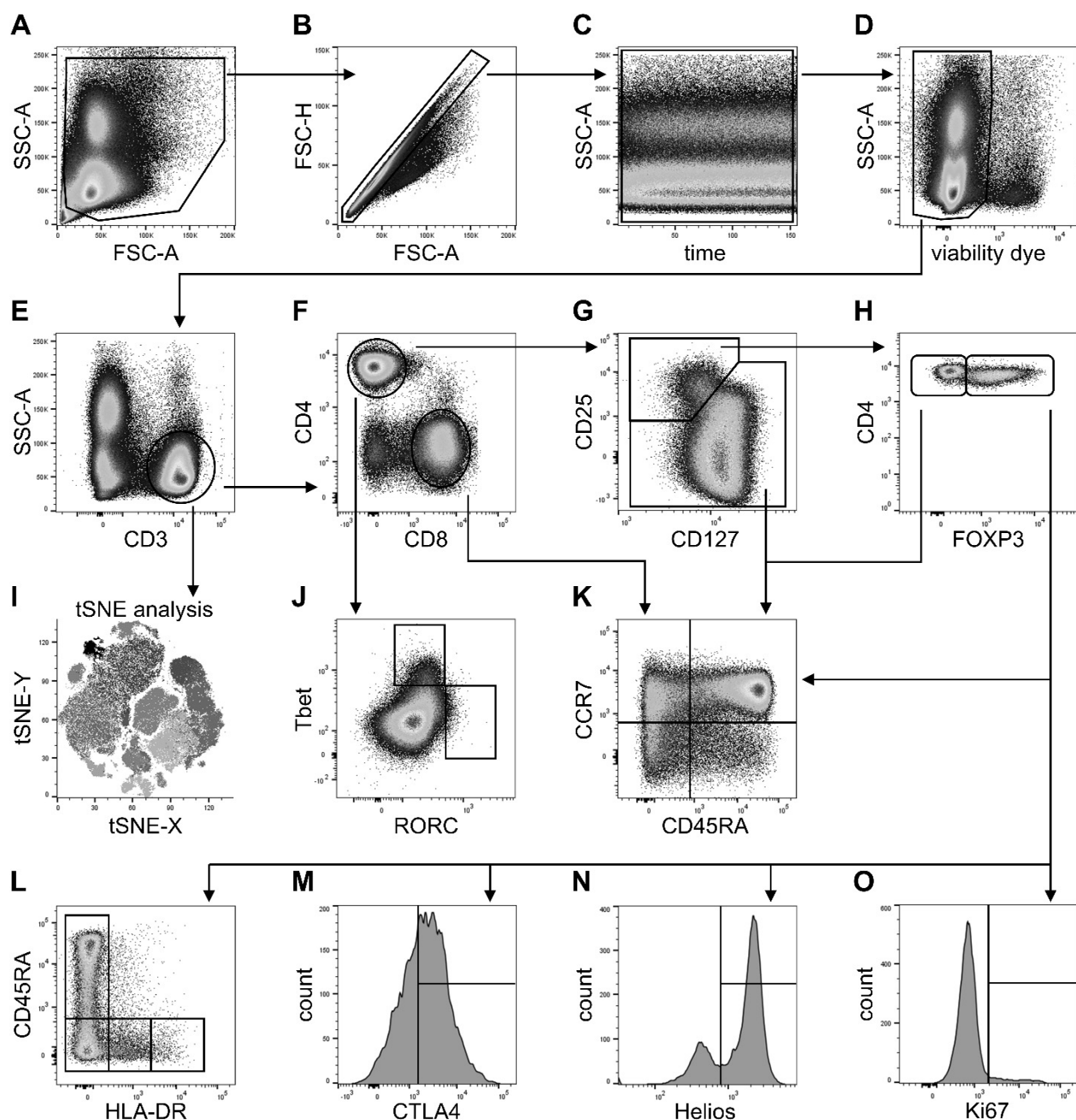
Corresponding author:

Sarah A Robertson, PhD, Robinson Research Institute, Adelaide School of Medicine, University of Adelaide, Adelaide Health and Medical Sciences Building, North Terrace, Adelaide SA 5005, Australia, T: +61 8 8313 4094, E-mail: sarah.robertson@adelaide.edu.au

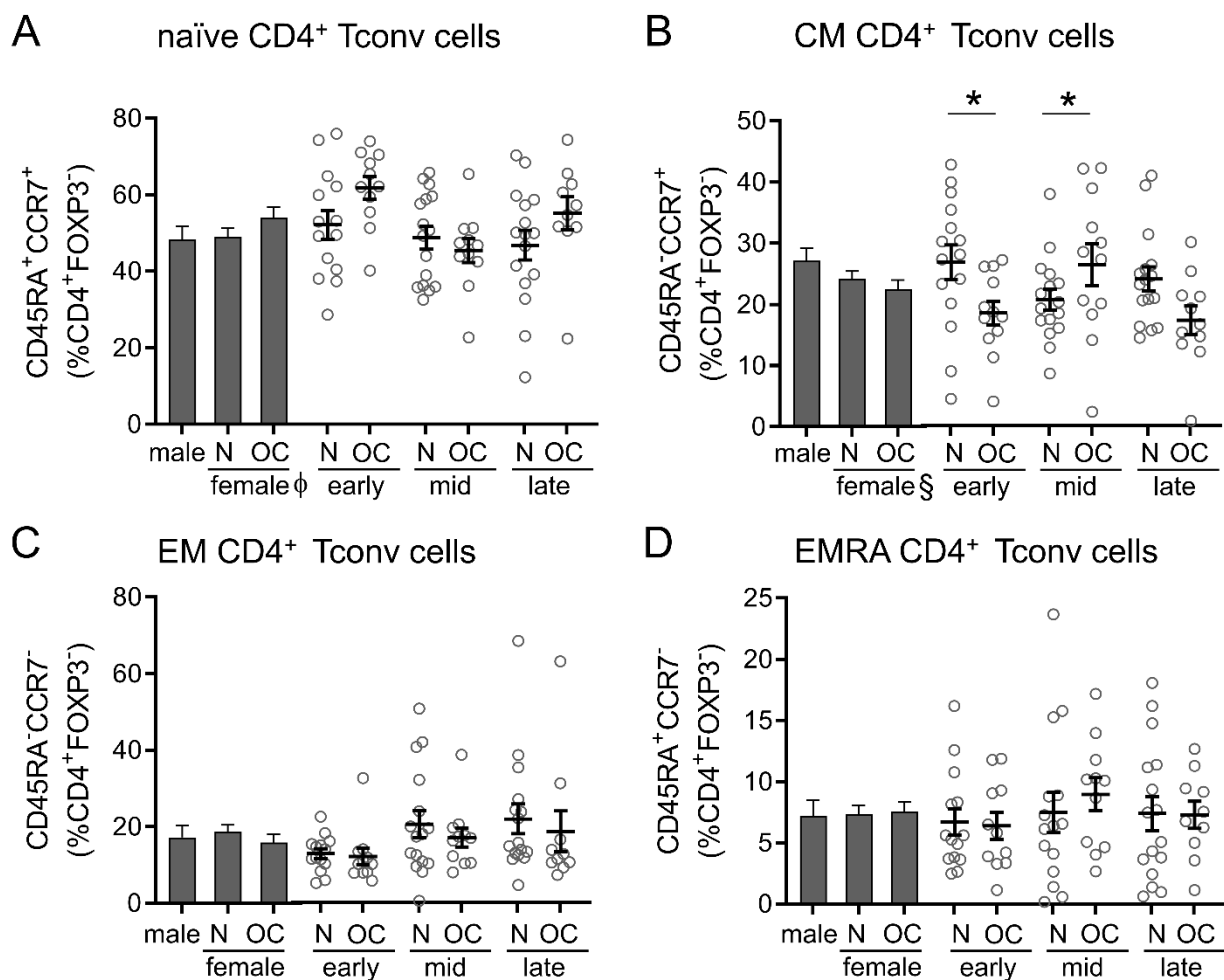
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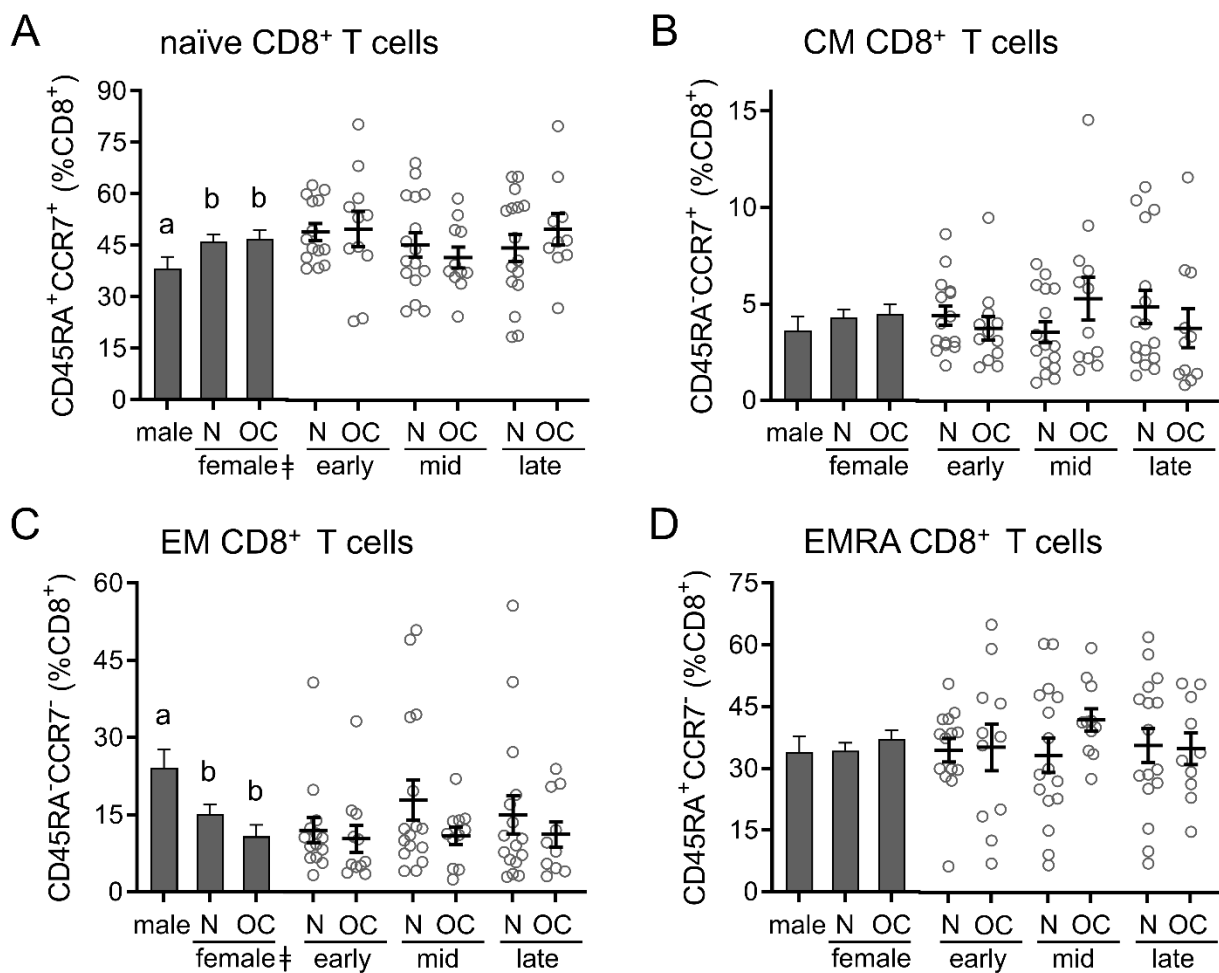
Supplemental Figure S1: Flow cytometry gating strategy. Gates were established to include immune cells and exclude debris (A) and doublets (B). Cells were applied to a time gate (C) before viable cells were identified by a lack of fixable viability dye staining (D). Viable CD3⁺ cells were gated (E) and either assessed by tSNE analysis (I) or applied to a CD4 v CD8 plot to identify CD4⁺ T cells and CD8⁺ T cells for conventional analysis (F). Treg cells were defined from the CD4⁺ T cells as CD25⁺CD127^{-lo} (G) and FOXP3⁺ (H). Treg cells were then assessed for their memory and naïve status by CCR7 and CD45RA expression (K). Treg cell suppressive phenotype was analysed by assessing the proportion of Treg cells that were HLA-DR⁺CD45RA⁺, HLA-DR⁺CD45RA⁻, HLA-DR^{lo}CD45RA⁻ and HLA-DR^{hi}CD45RA⁻ (L). Within the Treg cell population the suppressive markers CTLA4 (M) and Helios (N) were measured, in addition to the proliferation marker Ki67 (O). CD4⁺ T cells that were not Treg cells (gated in (G) as not CD25⁺CD127^{-lo} and gated in (H) as FOXP3⁻) were assessed for Tbet and RORC expression as markers of Th1 and Th17 cells respectively (J). CD4⁺ T cells that were not Treg cells and CD8⁺ T cells were categorised into different memory and naïve phenotypes by CCR7 and CD45RA expression (K).



Supplemental Figure S2. The effect of oral contraception on CD45RA/CCR7 memory phenotype marker expression in CD4⁺ Tconv cells. Peripheral blood drawn at early, mid, and late stages of the menstrual cycle in naturally cycling women (N) and women using oral contraception (OC), and from men, was analyzed by flow cytometry to assess CD45RA and CCR7 expression in CD4⁺FOXP3⁻ Tconv cells. Panels show the number of CD45RA⁺CCR7⁻ cells (**A**), the number of CD45RA⁻CCR7⁻ Treg cells (**B**), the number of CD45RA⁻CCR7⁺ Treg cells (**C**), and the number of CD45RA⁺CCR7⁺ Treg cells (**D**). Data is shown by group as estimated marginal means (left side, bar graphs) calculated by linear mixed model analysis (Model 1, all participants). Individual data points are also shown for each woman at each cycle phase in the N and OC groups (right side) with mean±SEM indicated. The effects of participant group, female sex, OC use, and time of cycle were assessed by linear mixed model analysis and pairwise comparisons, n=10-17 participants / group.

§significant effect of cycle stage (Model 2); §significant effect of OC x cycle phase interaction (Model 2);

*significant effect of OC at specific cycle stages (Model 2)(all $P < 0.05$).



Supplemental Figure S3. The effect of oral contraception on CD45RA/CCR7 memory phenotype marker expression in CD8⁺ T cells. Peripheral blood drawn at early, mid, and late stages of the menstrual cycle in naturally cycling women (N) and women using oral contraception (OC), and from men, was analyzed by flow cytometry to assess CD45RA and CCR7 expression in CD8⁺ T cells. Panels show the number of CD45RA⁺CCR7⁻ cells (A), the number of CD45RA⁺CCR7⁻ Treg cells (B), the number of CD45RA⁺CCR7⁺ Treg cells (C), and the number of CD45RA⁺CCR7⁺ Treg cells (D). Data is shown by group as estimated marginal means (left side, bar graphs) calculated by linear mixed model analysis (Model 1, all participants). Individual data points are also shown for each woman at each cycle phase in the N and OC groups (right side) with mean±SEM indicated. The effects of participant group, female sex, OC use, and time of cycle were assessed by linear mixed model analysis and pairwise comparisons, n=10-17 participants / group. ^{a,b,c} different superscripts indicate differences between participant group (Model 1); †significant effect of sex (Model 1)(all $P < 0.05$).

Supplemental Table S1: Combined oral contraceptive formulation used by study participants.

Brand Name	Estrogen	Progestin	Mono/Triphasic	N*
Brenda-35 / Estelle-35 ED	35 µg ethinylestradiol	2mg cyproterone acetate	Monophasic	4
Levlen ED	30 µg ethinylestradiol	150 µg levonorgestrel	Monophasic	2
Microgynon 20 ED	20 µg ethinylestradiol	100 µg levonorgestrel	Monophasic	3
Minulet ED	30 µg ethinylestradiol	75 µg gestodene	Monophasic	2
Yaz	20 µg ethinylestradiol	3 mg drospirenone	Monophasic	2
Yasmin	20 µg ethinylestradiol	3 mg drospirenone	Monophasic	2
Logynon ED	30-40 µg ethinylestradiol	50-125 µg levonorgestrel	Triphasic	3

* N = the number of participants using each oral contraceptive formulation.

Supplemental Table S2: Antibody Research Resource Identifier numbers for reagents.

Reagent	Supplier	Catalogue #	Antibody ID
anti-human CD3 antibody conjugated to APC-H7 (clone SK7)	BD Biosciences	560176	AB_1645475
anti-human CD4 antibody conjugated to BUV496 (clone SK3)	BD Biosciences	612936	AB_2870220
anti-human CD8 antibody conjugated to BUV737 (clone SK1)	BD Biosciences	564629	AB_2744464
anti-human CD25 antibody conjugated to BUV786 (clone 2-A3)	BD Biosciences	741035	AB_2740652
anti-human CD127 antibody conjugated to PE-Cy7 (clone HIL-7R-M21)	BD Biosciences	560822	AB_2033938
anti-human FOXP3 antibody conjugated to PE-CF594 (clone 236A/E7)	BD Biosciences	563955	AB_2738507
anti-human CCR7 (CD197) antibody conjugated to BUV395 (clone 3D12)	BD Biosciences	740267	AB_2740009
anti-human CD45RA antibody conjugated to BB515 (clone HI100)	BD Biosciences	564552	AB_2738841
anti-human HLA-DR antibody conjugated to BV510 (clone G46-6)	BD Biosciences	563083	AB_2737994
anti-human Helios antibody conjugated to AlexaFluor 647 (clone 22F6)	BD Biosciences	563951	AB_2738506
anti-human CTLA4 (CD152) antibody conjugated to PE-Cy5 (clone BNI3)	BD Biosciences	561717	AB_10893816
anti-human Ki67 antibody conjugated to APC-700 (clone SolA15)	eBiosciences	56-5698-82	AB_2637480
anti-human ROR γ t antibody conjugated to PE (clone Q21-559)	BD Biosciences	563081	AB_2686896
anti-human Tbet antibody conjugated to BV421 (clone O4-46)	BD Biosciences	563318	AB_2687543
Human Fc Block (clone Fc1.3216)	BD Biosciences	564220	AB_2869554
Progesterone Radioimmunoassay	Beckman Coulter	IM1188	AB_2920867
Estradiol ELISA Kit	LDN GmbH	E-2000	AB_2916329