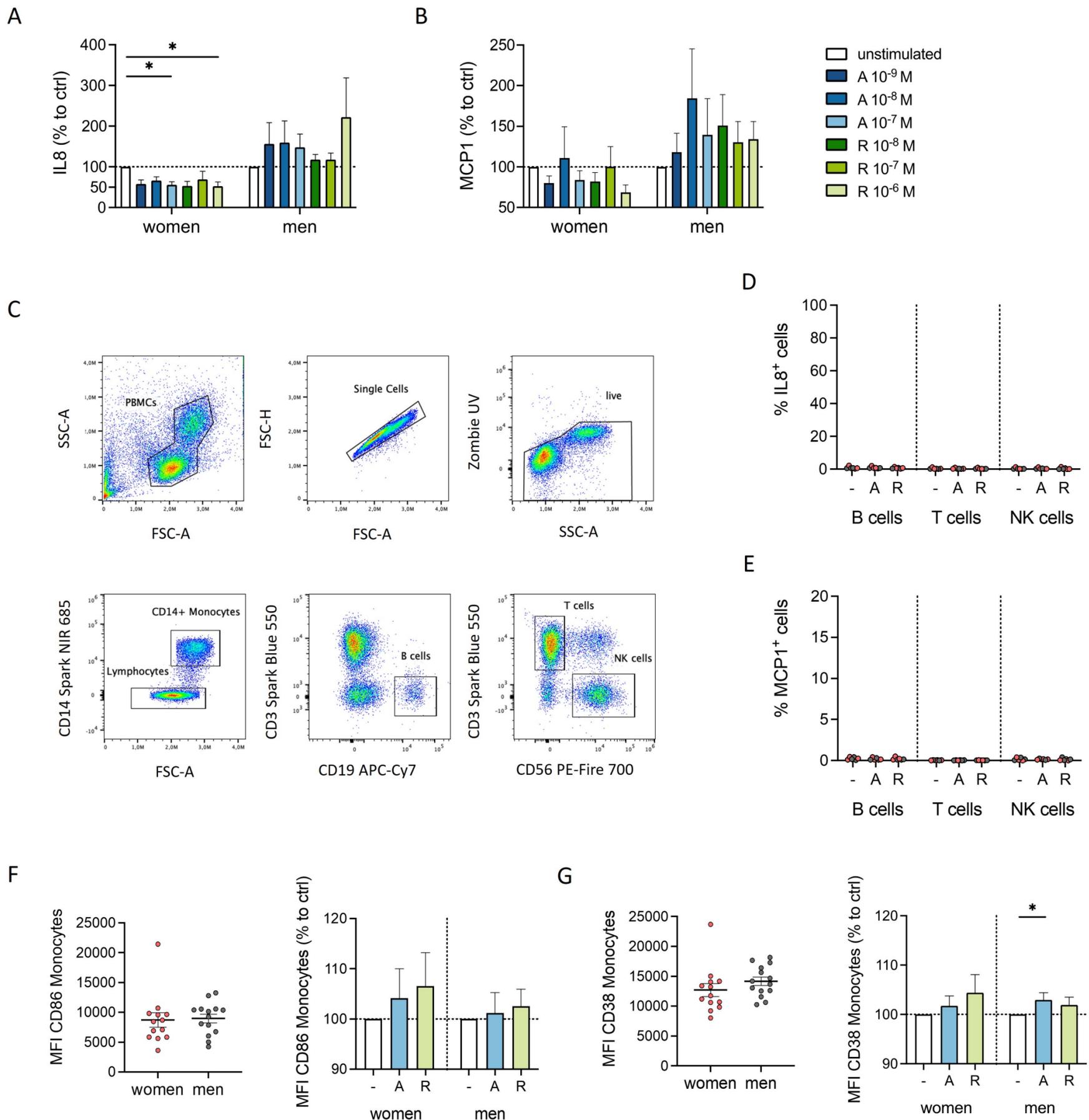# Supplementary Figures:
Acute stimulation of PBMCs drives switch from dopamine-induced anti- to proinflammatory phenotype of monocytes only in women

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Supplementary Figure 1: Sex-specific effects of DR stimulation on immunological responses of monocytes under physiological conditions. A, B) Secreted IL8 (A) and MCP1 (B) in supernatant from PBMCs from women and men after *in vitro* stimulation of PBMCs with A68930 (10^{-7}, 10^{-8}, 10^{-9} M) and Ropinirole (10^{-6}, 10^{-7}, 10^{-8} M) for 24 h normalized to unstimulated control measured via ELISA; data of A 10^{-7} M and R 10^{-6} M are the same as in Figure 1 A and D; n=11-13 each group. **C**) Representative scheme of gating strategy for mixed PBMCs obtained by flow cytometry. **D, E**) Percentage of IL8+ (D) and MCP1+ (E) B cells, monocytes, T cells and NK cells after 24 h in cell culture without stimulation measured via flow cytometry; n=5 each subtype. **F, G**) Expression of CD86 (F) and CD38 (G) on monocytes from women and men after 24 h in cell culture of mixed PBMCs without stimulation (left) and after stimulation with A68930 (10^{-7} M) and Ropinirole (10^{-6} M) for 24 h normalized to unstimulated control (right) measured via flow cytometry; n=13-14 each group. One-Way ANOVA or mixed-effects analysis with Geisser-Greenhouse correction and Dunnett multiple comparisons test was used for statistical testing of DR stimulation using three different concentrations of A68930 and Ropinirole. Mann-Whitney test was used for testing statistical significance between unpaired data of women and men. For comparison of paired data including unstimulated vs. stimulated samples, Wilcoxon test was used.; *p ≤ 0.05.
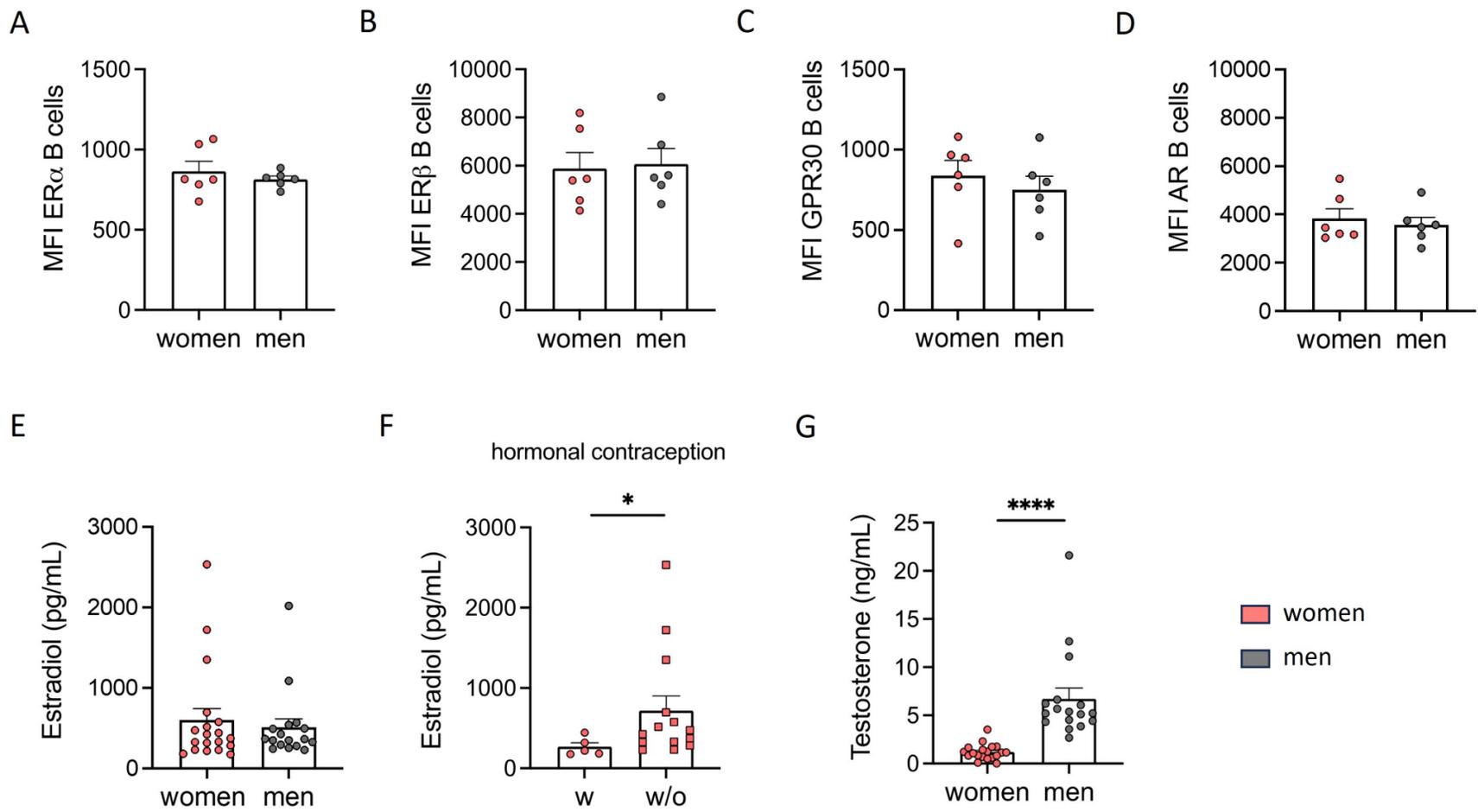
Supplementary Figure 2: Women and men exhibit similar estrogen levels and sex hormone receptor expression on B cells, while testosterone is higher in men. A-D) Basal expression of ERα (A), ERβ (B), GPR30 (C) and AR (D) on B cells from women and men measured via flow cytometry; n=6 each group. **E, G**) Basal estrogen (E) and testosterone (G) level in plasma from women (pink) and men (grey) measured via ELISA; n=17-19 each group. **F**) Basal estrogen level in plasma from women with (w, circles) and without (w/o, squares) hormonal contraception measured via ELISA; n=5-14 each group. Mann-Whitney test was used for testing statistical significance between data of women and men or women with and without hormonal contraception; *p ≤ 0.05, ****p ≤ 0.0001.
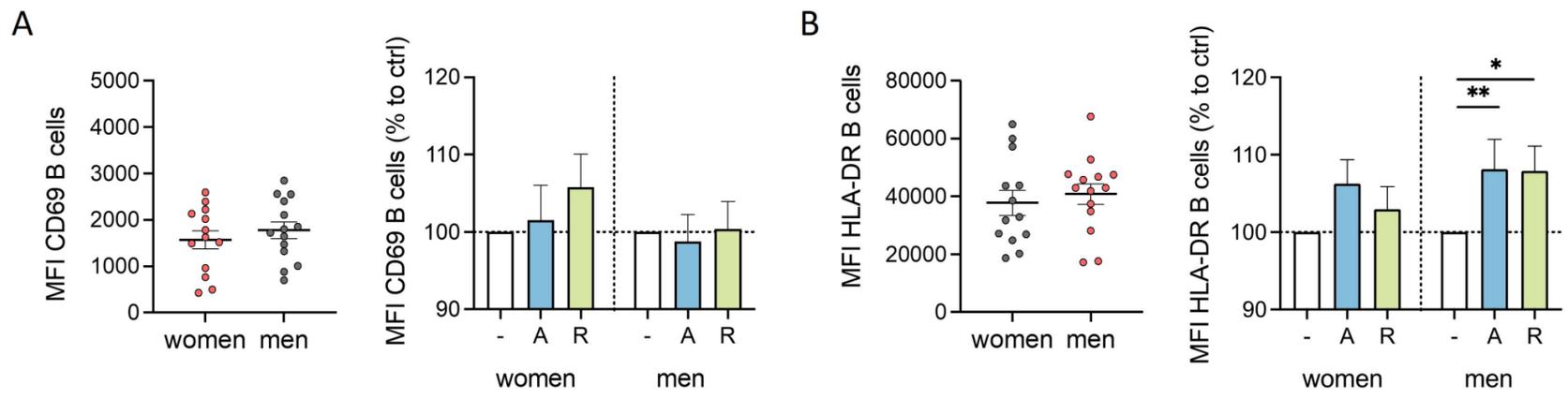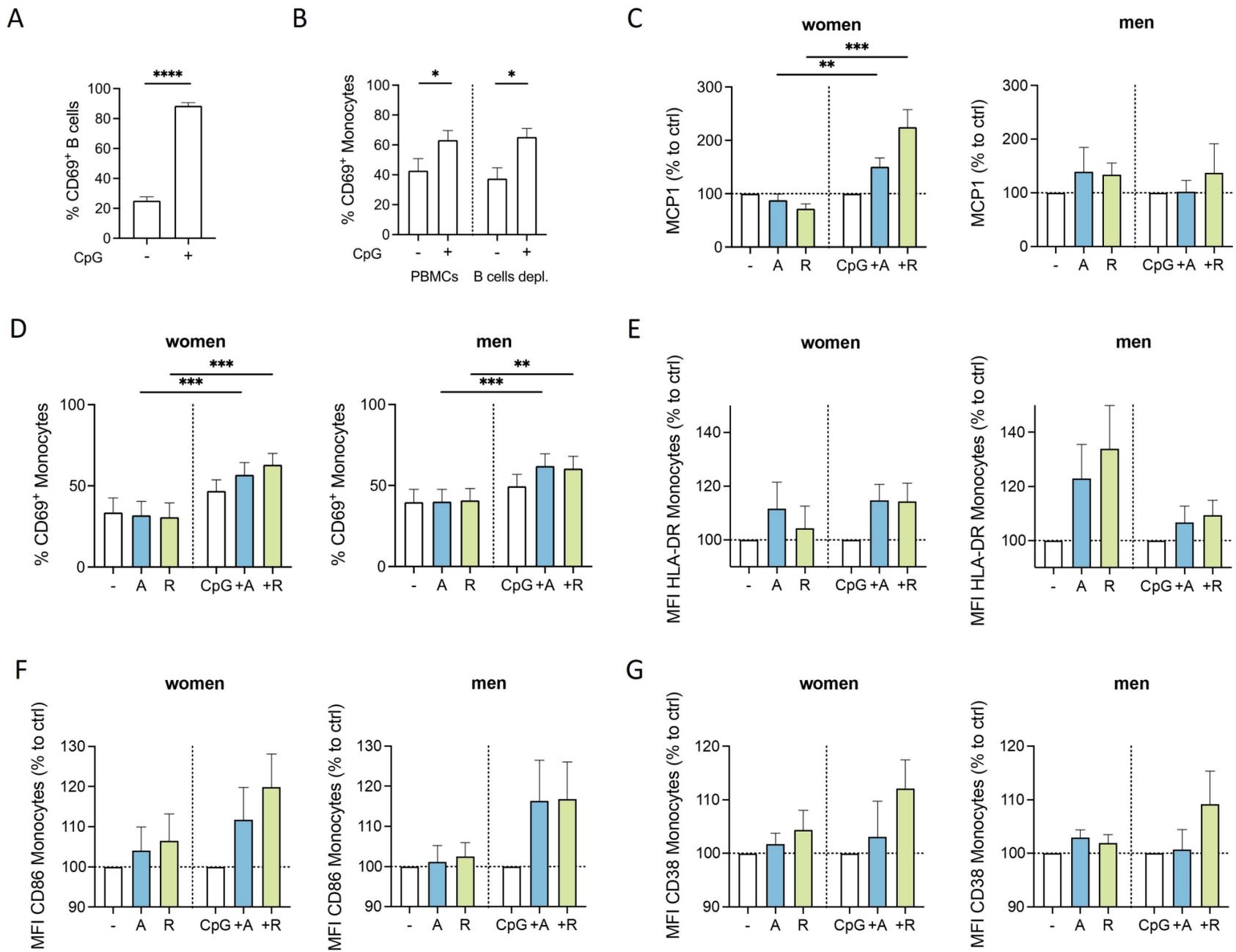
Supplementary Figure 3: Effects of DR stimulation on B cells from women and men. A, B) Expression of CD69 (A) and HLA-DR (B) on B cells from women and men after 24 h in cell culture of mixed PBMCs without stimulation (left) and after stimulation with A68930 (10^{-7} M) and Ropinirole (10^{-6} M) for 24 h normalized to unstimulated control (right) measured via flow cytometry; n=13-14 each group. Mann-Whitney test was used for testing statistical significance between unpaired data of women and men. For comparison of paired data including unstimulated vs. stimulated samples, Wilcoxon test was used.; *p ≤ 0.05, **p ≤ 0.01.
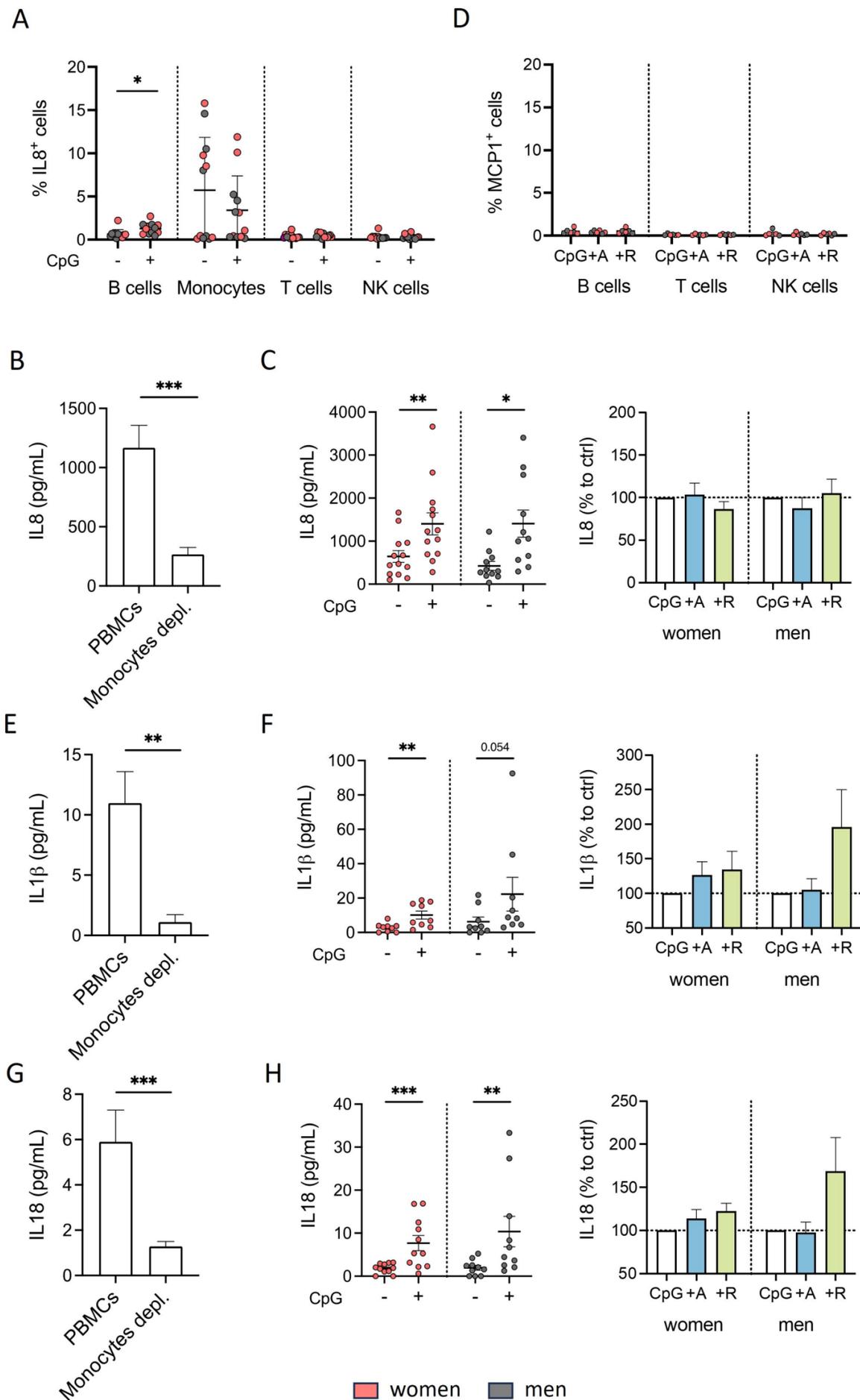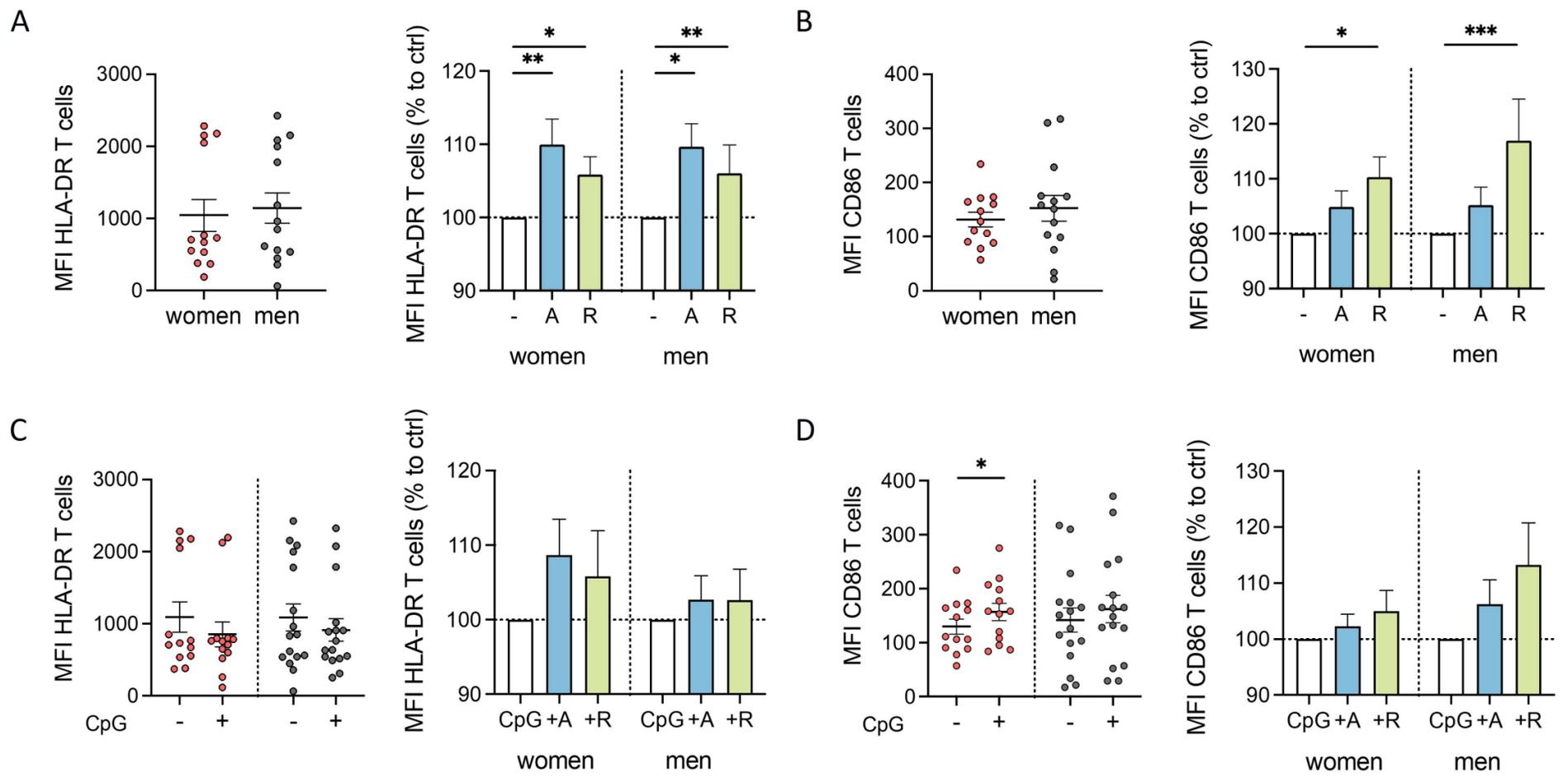
Supplementary Figure 4: Effect of DR stimulation on secretion of other cytokines of mixed PBMCs. A) Percentage of IL8$^+$ B cells, monocytes, T cells and NK cells after 24 h in cell culture of mixed PBMCs with or without CpG (0.195 μM) stimulation measured via flow cytometry; n=12 each group. **B)** Secreted IL8 in supernatant from mixed PBMCs and Monocyte-depleted PBMCs after 24h of CpG (0.195 μM) stimulation measured via ELISA; n=11 each condition. **C)** Secreted IL8 in supernatant from PBMCs from women and men after 24 h in cell culture with or without CpG (0.195 μM) stimulation (left) and secreted MCP1 after *in vitro* stimulation of PBMCs with CpG (0.195 μM) + A68930 (10^{-7} M) and CpG (0.195 μM) + Ropinirole (10^{-6} M) for 24 h normalized to CpG control (right) measured via ELISA; n=11-13 each group. **D)** Percentage of MCP1$^+$ B cells, T cells and NK cells after *in vitro* stimulation of PBMCs with CpG (0.195 μM) + A68930 (10^{-7} M) and CpG (0.195 μM) + Ropinirole (10^{-6} M) for 24 h measured via flow cytometry; n=6 each group. **E, G)** Secreted IL1β (E) and IL18 (G) in supernatant from mixed PBMCs and Monocyte-depleted PBMCs after 24 h of CpG (0.195 μM) stimulation measured via Legendplex; n=9-11 each condition. **F, H)** Secreted IL1β (F) and IL18 (H) in supernatant from PBMCs from women and men after 24 h in cell culture with or without CpG (0.195 μM) stimulation (left) and secreted IL1β (H) and IL18 (J) after *in vitro* stimulation of PBMCs with CpG (0.195 μM) + A68930 (10^{-7} M) and CpG (0.195 μM) + Ropinirole (10^{-6} M) for 24 h normalized to CpG control (right) measured via Legendplex; n=9-10 each group. For comparison of paired data including unstimulated vs. stimulated samples as well as PBMCs vs. monocytes depleted, Wilcoxon test was used. Mann-Whitney test was used for testing statistical significance between unpaired data of women and men; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Supplementary Figure 5: Comparison of Effects after DR stimulation with and without an inflammatory stimulus. A) Percentage of CD69+ B cells after 24 h in cell culture of mixed PBMCs with or without CpG (0.195 μM) stimulation measured via flow cytometry; n=30 each condition. **B)** Percentage of CD69+ monocytes after 24 h in cell culture of mixed PBMCs and B cell-depleted PBMCs with or without CpG (0.195 μM) stimulation measured via flow cytometry; n=14 each condition. **C)** Secreted MCP1 in supernatant from mixed PBMCs from women (left) and men (right) after *in vitro* stimulation of PBMCs with A68930 (10^{-7} M), Ropinirole (10^{-6} M), CpG (0.195 μM) + A68930 (10^{-7} M) and CpG (0.195 μM) + Ropinirole (10^{-6} M) for 24 h normalized to unstimulated or CpG control, respectively, measured via ELISA; n=11 each condition. **D-G)** Percentage of CD69+ monocytes (G) and expression of HLA-DR (E), CD86 (F) and CD38 (G) on monocytes from women (left) and men (right) after *in vitro* stimulation of PBMCs with A68930 (10^{-7} M), Ropinirole (10^{-6} M), CpG (0.195 μM) + A68930 (10^{-7} M) and CpG (0.195 μM) + Ropinirole (10^{-6} M) for 24 h normalized to unstimulated or CpG control, respectively, measured via flow cytometry; n=14 each condition. For comparison of paired data including unstimulated vs. stimulated samples as well as PBMCs vs. B cells depleted, Wilcoxon test was used. Mann-Whitney test was used for testing statistical significance between unpaired data of women and men; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

Supplementary Figure 6: Increase in HLA-DR on T cells after DR stimulation is independent of sex and presence of an acute inflammatory stimulus.
A, B) Expression of HLA-DR (A) and CD86 (B) on T cells from women and men after 24 h in cell culture of mixed PBMCs without stimulation (left) and after stimulation with A68930 (10^{-7} M) and Ropinirole (10^{-6} M) for 24 h normalized to unstimulated control (right) measured via flow cytometry; n=13-14 each group. **C, D)** Expression of HLA-DR (C) and CD86 (D) on T cells from women and men after 24 h in cell culture of mixed PBMCs with or without CpG (0.195 μM) stimulation (left) and after stimulation with CpG (0.195 μM) + A68930 (10^{-7} M) and CpG (0.195 μM) + Ropinirole (10^{-6} M) for 24 h normalized to CpG control (right) measured via flow cytometry; n=11-12 each group. Mann-Whitney test was used for testing statistical significance between unpaired data of women and men. For comparison of paired data including unstimulated vs. stimulated samples, Wilcoxon test was used; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Depletion of CD19⁺ B cells and CD14⁺ monocytes

CD19⁺ B cells or CD14⁺ monocytes were depleted from complete PBMCs using the following protocol. After thawing, PBMCs were washed and resuspended in depletion buffer (DPBS (Gibco, #14190-094), containing 2 mM EDTA (Carl Roth, #8043.2) and 2% FBS, sterile filtered). 5 µL FBS, 2.5 µL purified anti-human CD19 antibody (Biolegend, #302202) or purified anti-human CD14 antibody (Biolegend, #399202) and 2.5 µL Mouse IgM (BD Pharmingen™, #555583) per 1 Mio PBMCs were added to the cell suspension. Samples were mixed under rotation for 20 min at 4 °C. For bead preparation, 6.25 µL Dynabeads™ Pan Mouse IgG (Invitrogen, #11041) in 0.125 mL of depletion buffer per 1 Mio PBMCs were magnetically separated for 1 min and washed by exchanging the buffer. After incubation, PBMCs were washed with 2.5 mL of depletion buffer per 1 Mio cells, centrifuged and resuspended in 0.125 mL depletion buffer per 1 Mio cells. After adding 0.125 mL of beads, the mixture was mixed by rotation for 15 min at RT. 5 mL of depletion buffer was added to the tube before placed into the Dynal MPC®-15 magnet (Dynal Biotech, #120.29) for 3 min. The cells that were not attached to the beads were collected. The beads were resuspended a second time in depletion buffer and separated from the rest of the cells using the magnet for 3 min. The obtained depleted PBMC fractions were placed again into the magnet for separation. Counting of the cells with CASY counter followed. The efficacy of depletion was analyzed by staining for CD19 or CD14 via flow cytometry, and samples were used only if the remaining proportion of the depleted subtype was less than 1%.

Flow cytometry staining of dopamine receptors, sex hormone receptors and activation markers including Annexin V

After PBMCs were thawed and counted, a cell suspension with a concentration of 2 Mio cells per mL of PBS was prepared. When cultured PBMCs or depleted fractions were stained after stimulation, cells were centrifuged, and the supernatant stored at -80°C for following cytokine/chemokine analysis. 200,000 cells were placed into a 96-well V-bottom plate for each staining condition and washed once using PBS. To stain for dead cells, they were incubated with 100 µl Zombie UV™ Fixable Viability Dye (1:1000, Biolegend, #423108) in PBS for 20 min at 4°C in the dark, followed by two wash steps with PBS. PBS containing 2% Albumin Fraction V (Carl Roth, #0163.4) was added to each condition to block unspecific binding sites for 20 min at 4°C in the dark. Staining of extracellular markers followed using respective antibodies in 50 µl staining buffer (PBS supplemented with 2% FBS) for 20 min at 4°C. Dopamine receptor panel: Brilliant Violet 510™ anti-human IgD (1:200, Biolegend, #348220), Spark Blue™ 550 anti-human CD3 (1:400, Biolegend, #344851), PerCP anti-human CD4 (1:200, Biolegend, #317432), Brilliant Violet 711™ anti-human CD8 (1:200, Biolegend, #344734), PE/Fire™ 700 anti-human CD56 (1:200, Biolegend, #392428), PE/Cyanine7 anti-human CD27 (1:200, Biolegend, #356412), Brilliant Violet 650™ anti-human CD14 (1:400, Biolegend, #301836), APC/Cyanine7 anti-human CD19, APC/Fire™ 810 anti-human CD38, Dopamine Receptor D1 Antibody, PE conjugated (1:50, Bioss, #10610R-PE), DRD3 Polyclonal Antibody, Cy5 conjugated (1:200, Bioss, #bs-1743R-Cy5), Human Dopamine D5R/DRD5 Alexa Fluor® 405-conjugated Antibody (1:100, R&D, #FAB82861P). Activation marker panel: Brilliant Violet 510™ anti-human IgD, Spark Blue™ 550 anti-human CD3, PE/Fire™ 700 anti-human CD56, PE/Cyanine7 anti-human CD27, Spark NIR™ 685 anti-human CD14 (1:800, Biolegend, #399209), APC/Cyanine7 anti-human CD19, APC/Fire™ 810 anti-human CD38, Brilliant Violet 421™ anti-human CD71 (1:400, Biolegend, #334122), Brilliant Violet 605™ anti-human HLA-DR (1:200, Biolegend, #307640), Brilliant Violet 711™ anti-human CD69 (1:200, Biolegend, #310943), PE/Dazzle™ 594 anti-human CD21 (1:400, Biolegend, #354922), APC anti-human CD86 (1:400, Biolegend, #374207). Sex hormone receptor panel: Spark Blue™ 550 anti-human CD3, PE/Fire™ 700 anti-human CD56, Brilliant Violet 650™ anti-human CD14, APC/Cyanine7 anti-human CD19, GPR30 antibody (FITC) (1:50, Biorbyt, #orb15689). After two washing steps, cells previously stained with activation marker panel were incubated in 50 µl FITC Annexin V (1:1600, Biolegend, #640906) in Annexin V Binding Buffer (Biolegend, #422201) for 15 min at RT in the dark. Cells that were previously stained with dopamine or sex hormone receptor panel were incubated with 50 µl staining buffer containing 2% paraformaldehyde (Aldrich, #16005) per condition for 10 min at RT in the dark and washed again. For permeabilization, 50 µl FACS™ Permeabilizing Solution 2 (diluted 1:10 with dH₂O, BD, #340973) was added to each well and incubated for 10 min at RT in the dark. Washing and blocking of unspecific binding sites followed before respective antibodies for intracellular staining were added to the cells. Dopamine receptor panel: D2DR/Dopamine D2 Receptor Antibody Alexa Fluor 488® (1:100, Santa Cruz, #sc-5303 AF488), D4DR Antibody Alexa Fluor® 594 (1:100, Santa Cruz, #sc-136169 AF594). Sex hormone receptor panel: Human/Mouse/Rat Androgen R/NR3C4 Alexa Fluor® 405-conjugated Antibody (1:50, R&D, #FAB5876V), Estrogen Receptor alpha Antibody Alexa Fluor® 546 (1:100, Santa Cruz, #sc-8002 AF546), Estrogen Receptor beta Antibody Alexa Fluor® 647 (1:100, Santa Cruz, #sc-53494 AF647), Progesterone Receptor Antibody Alexa Fluor® 594 (1:200, Santa Cruz, #sc-166169 AF594). After washing twice, cells were measured with the Cytek® Aurora (5 Lasers) flow cytometer.

Intracellular cytokine measurement via flow cytometry

After thawing and stimulation of PBMCs, Brefeldin A (1:1000, Sigma Aldrich, #B7651-5MG) was added to the culture directly with applied stimulation reagents or 18 h after stimulation. Staining of dead cells, blocking of unspecific binding sites and staining of extracellular markers using following panel was conducted as described in 2.7. Cytokine panel: Brilliant Violet 510™ anti-human IgD, Spark Blue™ 550 anti-human CD3, PE/Fire™ 700 anti-human CD56, PE/Cyanine7 anti-human CD27, Brilliant Violet 650™ anti-human CD14, APC/Cyanine7 anti-human CD19 (1:400, Biolegend, #302218), APC/Fire™ 810 anti-human CD38 (1:200, Biolegend, #356643) and Brilliant Violet 711™ anti-human CD24 (1:200, Biolegend, #311135).

Afterwards, PBMCs were incubated with 2% paraformaldehyde, FACS™ Permeabilizing Solution 2, 2% Albumin Fraction V as described in 2.7. To stain intracellular cytokines following antibodies were added to the cells in 50 µl staining buffer for 20 min at RT in the dark. Cytokine panel: FITC anti-human IL-1β (1:50, Biolegend, #511705, PE anti-human IL-8 (1:50, Biolegend, #511408), Pacific Blue™ anti-human IL-6 (1:50, Biolegend, #501113), APC anti-human MCP-1 (1:50, Biolegend, #502611), Brilliant Violet 421™ anti-human TNF (1:50, Biolegend, #502931), PE/Dazzle™ 594 anti-human IL-10 (1:50, Biolegend, #506811). After washing samples twice, they were measured at the Cytek® Aurora (5 Lasers) flow cytometer.