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Depot medroxyprogesterone acetate and norethisterone enanthate differentially impact T-cell responses and expression of immunosuppressive markers

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Abstract

Problem: Injectable contraceptive use may impact immune cell responsiveness and susceptibility to infection. We measured responsiveness of T-cells from women before and after initiating depot medroxyprogesterone acetate (DMPA) or norethisterone enanthate (Net-En).

Method of study: Peripheral blood mononuclear cells collected from women aged 18–34 years prior to, at steady state, and nadir concentrations after initiating DMPA (n = 30) or Net-En (n = 36) and from women initiating copper intrauterine device (CU-IUD; n = 32) were stimulated with phorbol myristate acetate and analyzed using flow cytometry. We evaluated percentage change in T-cells expressing programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte associated protein-4 (CTLA-4).

Results: Compared to baseline, there were decreased numbers of CD4+CTLA4+ ($P < .001$) and CD8+CTLA4+ ($P < .01$) T-cells following ex vivo stimulation challenge at steady state DMPA concentrations and no differences at nadir concentrations ($P = .781$ and $P = .463$, respectively). In Net-En users, no differences in CD4+CTLA4+ T-cells at steady state ($P = .087$) and nadir concentrations ($P = .217$) were observed. DMPA users had fewer CD4+PD-1+ ($P < .001$) and CD8+PD-1+ ($P < .001$) T-cells at nadir concentrations. Number of CD4+PD-1+ and CD8+PD-1+ T-cells decreased at steady state concentration ($P = .002$ and $P = .001$, respectively) and at nadir concentrations after Net-En initiation ($P < .001$ and $P < .001$). In CU-IUD users, there were no changes in number of CD4+CTLA4+ ($P = .426$) and CD8+CTLA4+ ($P = .169$) and no changes in CD4+PD-1+ ($P = .083$) and CD8+PD-1+ ($P = .936$) compared to baseline.

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CONFLICT OF INTEREST

The author has no conflict of interests to declare.

Conclusion: Activation of T-cells in response to ex vivo stimulation is suppressed at steady state DMPA concentration and resolves at nadir concentration, suggesting DMPA immunosuppressive effects may be transient.

Keywords

depot medroxyprogesterone acetate; norethisterone enanthate cytotoxic T-lymphocyte associated protein-4; programmed cell death-1

1 | INTRODUCTION

Safe and effective contraceptives significantly reduce maternal morbidity and mortality[1] and thus are essential for women of reproductive age. Depot medroxyprogesterone acetate (DMPA) is one of the most commonly used contraceptives in sub-Saharan Africa, a region where 70% of new HIV infections occur globally.[2] DMPA is a progestin-only injectable contraceptive, administered intramuscularly as 150 mg/mL every 3 months. Norethisterone enanthate (Net-En), another progestin-only injectable is administered as 200 mg/mL at 2 monthly intervals. Both injectables provide effective contraception yet they are different progestins with different binding affinities to steroid receptors. Notably, studies have reported that medroxyprogesterone acetate (MPA), the active ingredient found in DMPA, binds the glucocorticoid receptor with higher affinity compared to Net-En.[3,4] Multiple observational and laboratory studies have also reported an association between DMPA use and increased risk of HIV acquisition and the same has not been reported for Net-En.[5–10] A recent meta-analysis estimates approximately 40% increased risk of HIV acquisition in women using DMPA compared to women not using contraception or using non-hormonal contraception.[11] Evidence for Contraceptive Options and HIV Outcomes (ECHO), the first randomized controlled clinical trial, reported no significant increase in HIV incidence in women using DMPA compared to those on copper T380A intrauterine devices (CU-IUD) and levonorgestrel (LNG).[12] Notably, the ECHO trial was designed to detect a 50% change in HIV incidence and given current published data, the possibility of a smaller increase is plausible, thus interest in understanding biological changes that could mechanistically contribute to such an association remains. Structured studies are needed to investigate the multiple hypothesized biological mechanisms by which DMPA may cause increased susceptibility to HIV infection.

Several published studies suggest that DMPA may suppress both innate and adaptive immune mechanisms,[13] which may be mediated by the known strong affinity of DMPA for the glucocorticoid receptor[4,14] and subsequent signaling for T-cell apoptosis.[4] There are currently no published studies investigating responses of T-cells from healthy reproductive-aged women to ex vivo stimulation before and after dosing of contraception, including DMPA.

Cytotoxic T-lymphocyte associated protein-4 (CTLA-4) also referred to as CD152 and programmed cell death-1 (PD-1) are two critical immune checkpoint inhibitors known to confer central and peripheral immune tolerance. CTLA-4, with ligands CD80 and CD86 on antigen-presenting cells (APCs), is important during initial stages of naïve T-cell activation

and prevents potentially autoreactive T-cells from attacking self-peptides.[15] PD-1 regulates the activity of previously activated T-cells in later stages of immune responses in peripheral tissues. PD-1 is a member of the B7/CD28 group of co-stimulatory receptors and regulates T-cells through binding to its two ligands PD-L1 and PD-L2 effectively inhibiting T-cell proliferation and production of IFN-ϒ and TNF-α, which reduces T-cell survival. PD-1 expression on T-cells is typically associated with cell exhaustion and dysfunctionality after prolonged immune activation.[16]

Cytotoxic T-lymphocyte associated protein-4 and PD-1 both inhibit immune responses and published evidence has shown upregulation of these markers on T-cells exposed to specific anti-inflammatory glucocorticoids, including dexamethasone.[17] We hypothesized that DMPA, a steroid hormone with high affinity for glucocorticoid receptors, may similarly cause an immunosuppressive effect.

We aimed to investigate the expression of CTLA-4 and PD-1 on T-cells from women using injectable contraceptives (DMPA, Net-En) or non-hormonal CU-IUD. Since CTLA-4 is not normally expressed on naïve/resting T-cells and is only upregulated following activation, we also measured CTLA-4 on T-cells as a proxy for activation following ex vivo stimulation. CTLA-4 intracellular stores increase significantly, and the protein relocates to the cell surface upon activation with 50 ng/μL PMA/ionomycin, peaking at 4 hours and expression is sustained for up to 48 hours. A synergistic relationship has been shown between anti-CTLA-4 mAb and anti-CD28 in blocking activated CD4 cells from adhering to CD80 and thus augmenting cell proliferation.[18] We included CD28/49d in the stimulating cocktail to enhance the activation and our processing and analysis was completed well within 48 hours, a period within which surface expression was still optimal.

2 | METHODS

The study was approved by the Medical Research Council of Zimbabwe and the University of Pittsburgh Institutional Review Board.

2.1 | Study participants

This is a sub-study of samples collected as part of a larger prospective cohort of Zimbabwean women who enrolled into the Zim CHIC (Zimbabwe Contraceptive Hormone Induced Changes) study between February 2014 and December 2015. The study was conducted under Clinical Trial Registration: clinicaltrials.gov NCT02038335.[19] The study recruited healthy, HIV uninfected women aged 18–34 who self-selected one of six study-provided contraceptives to initiate and use during 6 months of study participation. This study includes Zim CHIC participants who opted to use DMPA, Net-En, or CU-IUD. Participants were confirmed to be HIV-1 negative and free of active sexually transmitted infections (HSV-2, *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, *Treponema pallidum* and *Trichomonas vaginalis*), and in the follicular phase of their menstrual cycle at enrollment. Participants were required not to have used DMPA for 10 months nor any other hormonal or intrauterine contraception 30 days preceding enrollment. Objective confirmation of self-reported hormone-free status was performed using high-performance liquid chromatography and mass spectrometry as previously reported.[19] Serum progestin measurements were also

performed using mass spectrometry to determine MPA and NET concentrations at study sampling time points for both DMPA and Net-En participants. All enrolled participants were confirmed to be free of non-study exogenous hormones at all follow-up visits.

2.2 | Blood samples

Peripheral blood mononuclear cells (PBMCs) were harvested in BD Cell Preparation Tubes (Becton Dickinson and Company) at enrollment, steady state (defined as 30 days after first injection for DMPA with a mean serum MPA concentration of 1303.5 pg/mL or 90 days post-injection for Net-En with mean serum NET concentration of 1565.5 pg/mL) and nadir (period immediately prior to next clinical dosing at day 180 visit and lower mean serum concentration of 279 and 856 pg/mL for MPA and NET, respectively). PBMCs were cryopreserved in 90% heat-inactivated fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO; Sigma Aldrich) and stored in liquid nitrogen until analysis and then quick thawed by gentle agitation in a 37°C water bath followed by drop-wise addition of pre-warmed culture medium (CM) containing 1-part FBS and 9-part RPMI 1640 with glutamine (Sigma Aldrich). Cells were washed by centrifugation at 500 *g* for 8 minutes and re-suspended to 1×10^6 cells/mL in CM and rested overnight at 37°C in 5% CO_2. Cell viability was determined by staining cells using 0.4% trypan blue and counting on a hemocytometer under microscope.[20] Cell viability after recovery was >95% for all specimens before stimulation and >80% after stimulation procedures.

2.3 | Mitogen stimulation of PBMCs

Peripheral blood mononuclear cells were cultured in PMA/ionomycin (Sigma Aldrich) at a final concentration of 50 ng/mL and CD28/CD49d co-stimulatory antibody. A control tube containing unstimulated PBMCs was included with each participant sample. Golgistop and Golgiplug (BD Biosciences) were added to all tubes followed by incubation at 37°C in 5% CO_2 for 4 hours. Following stimulation, cells were washed, stained using FVS510 which distinguishes live from dead cells through binding to surface and intracellular amines and further surface stained using Becton Dickinson (BD) monoclonal antibodies PE Mouse anti-Human CD69 (555531), PerCP Mouse anti-Human CD3 (345766), BB515 Mouse anti-Human CD4 (564419), and APC-H7 Mouse anti-Human CD8 (560179), fixed in BD Cytofix buffer, permeabilized using BD Cytoperm buffer and then stained with BV421 Mouse anti-CTLA-4 (562743) and APC Mouse anti-PD-1 (558694) monoclonal antibodies at 4°C. The cells were washed and re-suspended in 300 μL staining buffer (BD Biosciences) for flowcytometric analysis.

2.4 | Flow cytometry

Samples were analyzed on a BD FACSCanto II analyzer using FASCDIVA software version 6.1.3. Fluorescence minus one (FMO) and unstimulated controls were used as controls for creating gating templates. Gating and analysis were performed using FACSDiva software. The flow cytometer was calibrated daily using Cytometer Setup and Tracking beads (BD Biosciences). Single-color compensation controls were performed, and application settings were used for consistency of sample analysis. All flow cytometry gating was independently agreed upon by three laboratory scientists for consistency and to minimize bias.

Gates included only singlet lymphocytes defined by a SSC-H vs SSC-W plot. Live cells were gated from single cells and thus only live cells were included in the subsequent CD3+ cells gate. A SSC-A vs CD69+ dot plot was used to define all activated T-cells. Successive gates of CD4+ and CD8+ T-cells were based on activated T-cells (CD3+CD69+) gate. Only cells clearly expressing CD69+ were included to quantify expression of both CTLA-4 and PD-1 was gated on all CD3+ cells. The flow cytometric gating strategy is shown in Figure 1.

2.5 | Sample size considerations and statistical analysis

In the parent Zim CHIC study, on average, 55 000 ± 9500 CD4+ cells were isolated from participant PBMC samples at enrollment. Based on previous studies, expression of CTLA-4 and PD-1 on CD4+ cells in healthy humans was 14% and 10%, respectively.[21,22] Therefore, we estimated approximately 7700 CTLA4+ and 5500 PD-1+ cells in the samples from women in our study. A sample size of 28 women in each contraceptive group (DMPA, Net-En, and CU-IUD) was determined to have 80% power to detect a 20% change in CTLA-4 and PD-1 expression after initiation of DMPA or Net-En, based on a paired Student *t* test evaluated at the 2-sided .05 significance level and assumed common standard deviations of 2800 and 2000, respectively. Demographic characteristics between the three contraceptive groups were compared using Kruskal-Wallis and Fisher's exact tests, where appropriate. Mixed effects linear regression models were used to evaluate changes in CTLA-4 and PD-1 expression at steady state and at nadir concentrations of DMPA or Net-En, and after 180 days of CU-IUD use relative to expression measured at baseline.

3 | RESULTS

Peripheral blood mononuclear cells from 98 participants enrolled in the DMPA (n = 30), Net-En (n = 36), and CU-IUD (n = 32) arms and confirmed to be free of non-study hormones at all visits were included in this analysis. The median age of study participants was 28.0 years. There were no statistically significant differences in baseline demographics across the 3 study arms (Table 1). Results are presented as percentage change in number of cells and median fluorescence intensity (Figure 2 and Tables 2 and 3).

3.1 | Progestin-only injectable contraceptives and T-Cell CTLA-4 expression

As shown in Figure 2A, we observed significantly less responsivity to ex vivo stimulation as measured by decreased number of CD4+CTLA-4+ and CD8+CTLA-4+ cells obtained at steady state DMPA concentration compared to baseline ($P < .001$). Number of CD4+CTLA-4+ and CD8+CTLA-4+ cells was the same as baseline at nadir DMPA concentration ($P = .761$ and $P = .463$, respectively; Figure 2A and Table 2). In the Net-En arm, there was no difference in number of CD4+CTLA-4+ cells at steady state concentrations ($P = .087$) when compared to baseline. Although a statistically significant increase was observed for CD8+CTLA-4+ ($P = .039$) compared to the overall mean observed at baseline, the median paired increase over baseline levels was 0.5% among women in the Net-En arm (Figure 2). There was no significant difference in number of both CD4+CTLA-4+ and CD8+CTLA-4+ cells at nadir concentrations compared to baseline ($P = .217$ and $P = .566$, respectively; Figure 2B and Table 2). No changes were observed in

number of CD4+CTLA-4+ and CD8+CTLA-4+ cells at 6 months post-CU-IUD insertion when compared to baseline ($P = .426$ and $P = .169$, respectively; Figure 2C and Table 2).

3.2 | Progestin-only injectable contraceptives and T-Cell PD-1 expression

There were no significant changes in number of CD4+PD-1+ and CD8+PD-1+ cells at steady state concentration for DMPA ($P = .856$ and $P = .456$, respectively) but there was a significant decrease in number of CD4+PD-1+ and CD8+PD-1+ cells at nadir state ($P < .001$) when compared to baseline (Figure 2A and Table 3). For Net-En users, number of CD4+PD-1+ and CD8+PD-1+ cells decreased significantly at steady state ($P = .002$ and $P = .001$, respectively) and at nadir concentrations ($P < .001$; Figure 2B). In all measurements, there were no changes from baseline in number of CD4+PD-1+ and CD8+PD-1+ T-cells from women using CUIUD ($P = .083$ and $P = .936$, respectively; Figure 2C and Table 3). Tables 2 and 3 summarize median absolute numbers and median fluorescent intensities for CD4+ and CD8+ expressing CTLA-4 and PD-1, respectively.

4 | DISCUSSION

This study evaluated responsivity of T-cells to ex vivo stimulation and change in number of T-cells expressing of immunosuppressive markers that may impact HIV susceptibility in women using DMPA and Net-En, both injectable progestin-only contraceptives. We report that DMPA and Net-En differentially regulate number of CD4+CTLA-4 and CD8+CTLA-4+ lymphocytes with reduced numbers following ex vivo stimulation at steady state serum hormone concentrations for DMPA but not Net-En. The observed suppressed T-cell responsivity in DMPA users resolves at nadir serum hormone concentrations suggesting there could be a window of immune suppression at steady state serum DMPA concentrations during which risk of HIV infection could be heightened.

Our results are consistent with the study by Noguchi et al[23] who prospectively followed 3141 women participating in an HIV prevention study in South Africa and reported that compared to Net-En users, women using DMPA for contraception had a 30% increased risk of HIV acquisition after adjusting for other HIV risk factors. Recently released primary results from ECHO study, a randomized clinical trial conducted across three African sites that included 7829 participants who were randomized to DMPA, CU-IUD, or LNG implant contraception, reported no significant increase in HIV incidence for participants recruited into the DMPA arm when compared to either CU-IUD or LNG after 18 months of follow-up. The ECHO trial was designed to detect 50% increase in HIV incidence for each arm compared to the other, and the authors stated that under the design of the study, an observed 30% increase in HIV incidence would have been found to be statistically significant. Proportional increased risk of up to 30% remains potentially consequential for individual women at high HIV risk making contraceptive choices. Further research investigating biological changes associated with contraceptive initiation and use may be important in understanding the possibility of a window of susceptibility suggested by our data. It is possible that a transient immune suppression from DMPA use could account for intermittent increased HIV risk that may not have been detected in ECHO and that may be important to individual women at highest HIV risk. Unlike other progestins, DMPA is a potent agonist of

the glucocorticoid receptor and could plausibly be immunosuppressive especially at higher concentrations. Maritz et al[24] evaluated direct effects of DMPA and Net-En on HIV-1 replication in PBMCs from healthy donors ex vivo and found that DMPA significantly increased HIV-1 replication by threefold in non-activated PBMCs from female donors, while similar concentrations of Net-En had no effect. These findings support the observed differential impact of the 2 progestin-only injectables on immune cells that is reported in our study. No previous studies have directly evaluated the effect of DMPA on CTLA-4 expressing T-cells, and our data show differences in numbers of CD4+CTLA-4+ and CD8+CTLA-4+ cells from women initiating and using DMPA. DMPA users appear to have a transient decrease in T-cell responsiveness to ex vivo stimulation, consistent with previously published observational and ex vivo studies.

Our findings of reduced T-cell responsivity to ex vivo stimulation at steady state serum DMPA levels are consistent with studies suggesting an association between DMPA use and immune tolerance.[25–29] The transient immune suppression observed at steady state resolved at nadir concentration, which is consistent with findings from ex vivo studies showing a dose-dependent impact of DMPA, with greater effects at high concentrations compared to low concentrations.[3] Previous studies used ex vivo exposure of T-cells to varying concentrations of DMPA. Our work demonstrates that T-cells exposed in vivo to varying concentrations of DMPA and stimulated ex vivo behave similarly, thus corroborating prior findings and suggesting the observed effect is biologically plausible and physiologically relevant.

Xing et al[17] demonstrated that potent glucocorticoid dexamethasone increases the expression of PD-1 on CD4+ and CD8+ lymphocytes during activation following ex vivo exposure of PBMCs to varying concentrations in culture. The observed increase in PD-1 expression was reversed by addition of RU486, suggesting the effect is indeed mediated through the glucocorticoid receptor. Given that DMPA has high affinity for the glucocorticoid receptor, we hypothesized that PD-1 expression at steady state DMPA concentrations would be increased, however we observed no change at steady state concentrations and a decrease in number of CD4+PD-1+ and CD8+PD-1+ T-cells at nadir DMPA concentrations. This observation shows that although DMPA binds the glucocorticoid receptor with high affinity, its effect may not necessarily mirror that of other agents such as dexamethasone that has been reported to increase PD-1 expression on T-cells following activation. The significance of this finding requires further investigation.

Our study had several notable strengths, including analysis of PBMCs from women who had in vivo DMPA and Net-En exposures and objective biological analyses to exclude exposure to other non-study hormones intended to minimize confounding of observed changes. Baseline measurements were also used as comparators thereby minimizing intra-individual variation. This study was limited by the assessment of only two markers from a number that could have been considered to answer this question more comprehensively. Further, we also evaluated Net-En at day 90 and DMPA at day 30, thus cannot rule out that the episodic immune suppression only occurs after initiation in the first cycle and after resolution does not recur. Given agreement in published studies suggesting that Net-En does not modulate immune cells as does DMPA, our observation at steady state serum hormone concentration

suggesting a Net-En protective effect could be a subject for further investigation and may partly explain the differential impact for the two contraceptives. Follow-up research is needed to assess other immune-regulatory impacts of contraceptive progestins and to further investigate biological mechanisms of immune regulation that may impact HIV acquisition risk regardless of how small the level of risk may be.

5 | CONCLUSION

Findings from this study suggest that during use of intramuscular DMPA for contraception there may be periodic transient decreases in T-cell responsivity associated with higher serum progestin concentrations. The observed transient immune suppression suggests susceptibility to infection may be confined to specific time points or serum concentration ranges during the DMPA injection cycle. Net-En does not confer decreased T-cell response to ex vivo challenge and thus may offer an alternative to DMPA where risk of HIV acquisition is significantly high.

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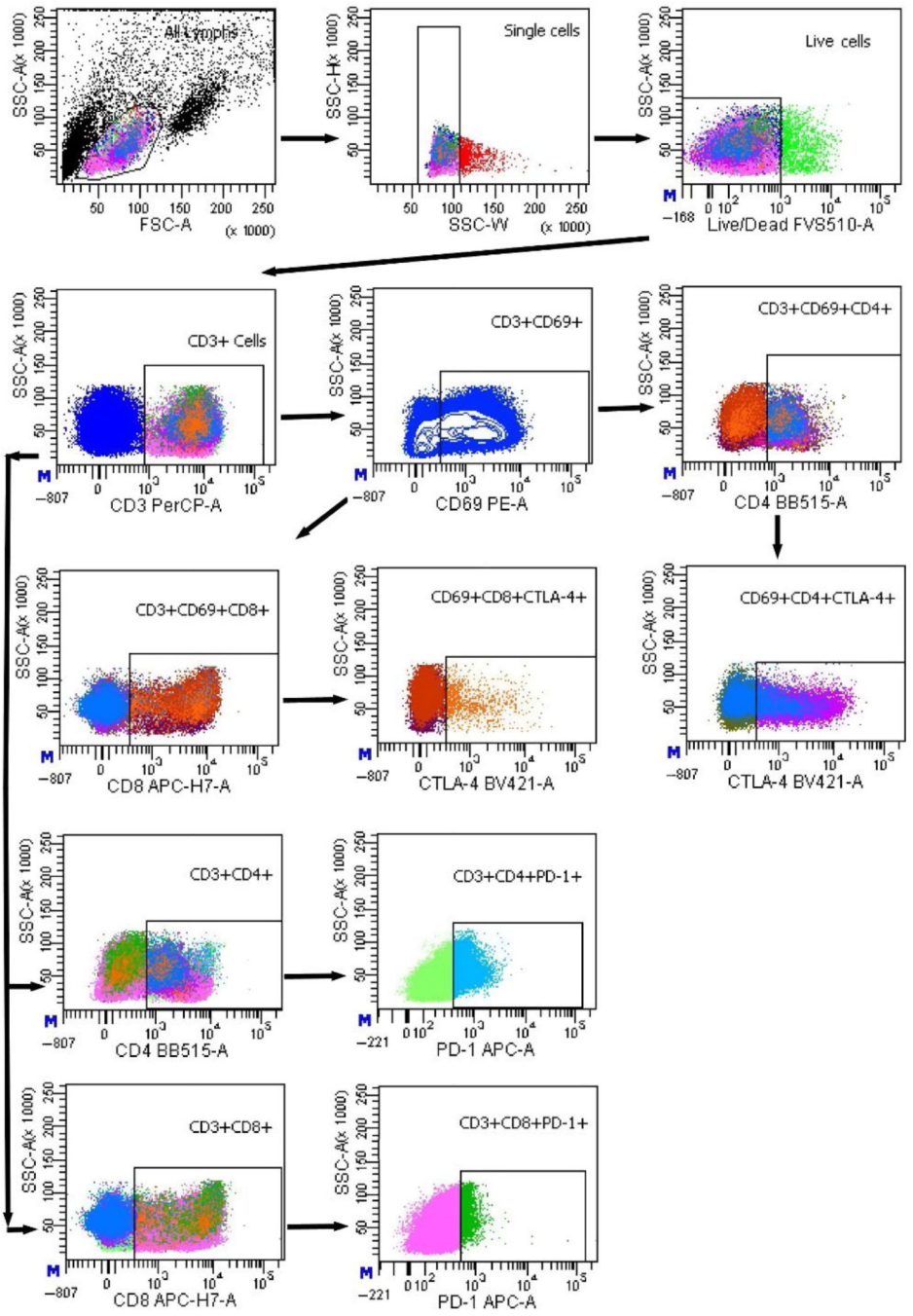

FIGURE 1.

Gating strategy. Representative flow cytometry plots showing gating scheme. PBMCs were isolated from healthy HIV-negative women initiating DMPA, Net-En, or CU-IUD contraception and incubated in 50 ng/μL PMA/ionomycin and Anti-Human CD28/49d antibody cocktail for 4 h. Cells were stained using Becton Dickinson fluorochrome-conjugated antibodies and flowcytometric analysis was performed. The gating strategy was as follows, SSC/FSC (represents the distribution of all cells in the light scatter based on size and intracellular composition, lymphocytes were identified using this scatter plot). SSC-H/

SSC-W plot was used to exclude doublet cells from the analysis. SSC/CD3 PerCP was used to cluster all CD3+ cells and further SSC gates to identify CD3+CD69+ (activated T-cells). CD4+CTLA-4+ and CD8+CTLA-4+ cells were both gated from activated T-cells while CD4+PD-1+ and CD8+PD-1+ cells were derived from the CD3+ gate. Biexponential scaling was used to show cells falling on the axis. CTLA-4, cytotoxic T-lymphocyte associated protein-4; CU-IUD, copper T380A intrauterine device; DMPA, depot medroxyprogesterone acetate; FSC, forward scatter; Net-En, norethisterone enanthate; PBMC, peripheral blood mononuclear cells; PD-1, programmed cell death-1; PMA, phorbol myristate acetate; SSC, side scatter

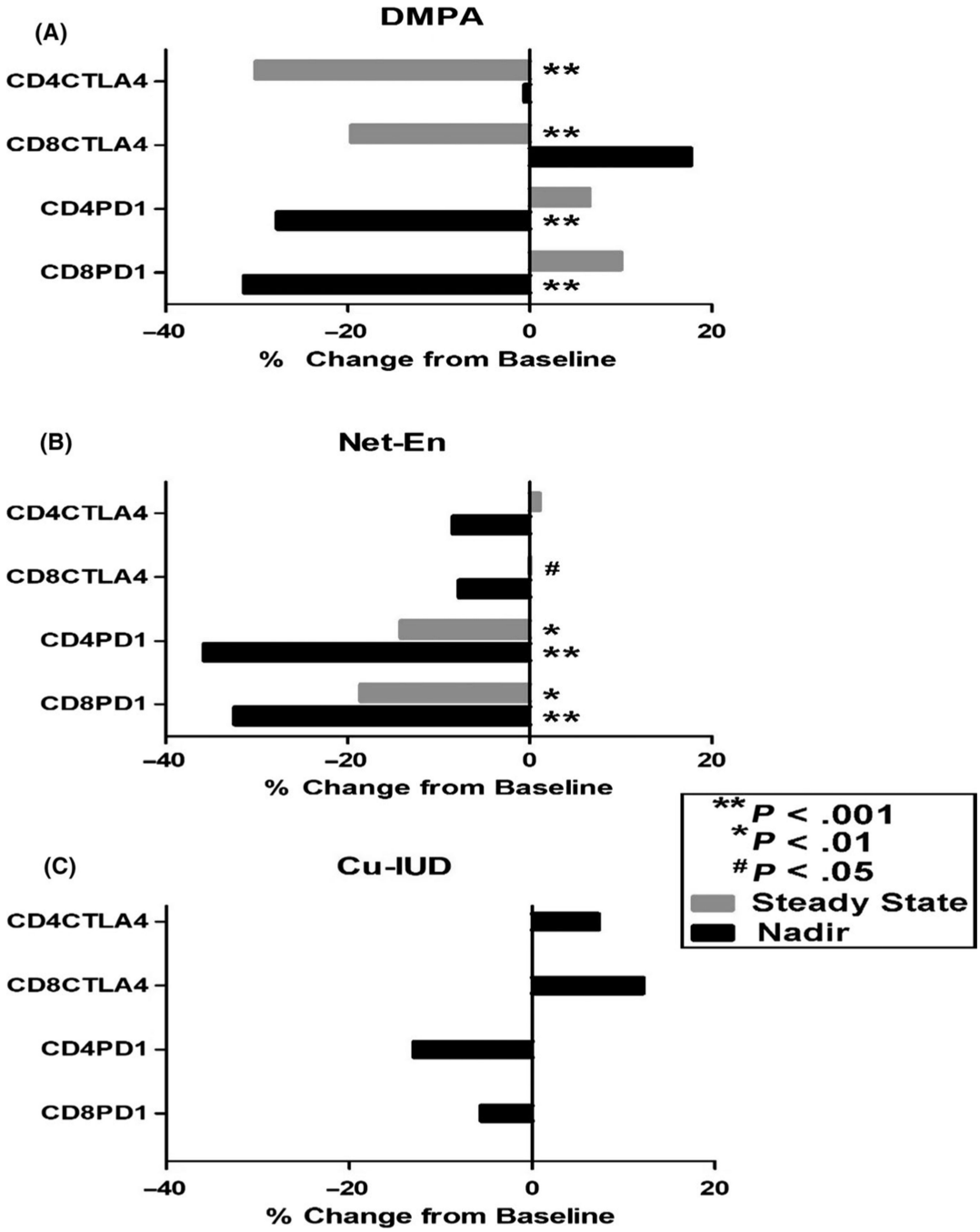

FIGURE 2.

DMPA and not Net-En or CU-IUD reduces number of CTLA-4 expressing CD4+ and CD8+ T-cells following stimulation. PBMCs isolated from women using DMPA, Net-En, and CU-IUD were stimulated ex vivo to assess the inducibility of CTLA-4 and PD-1. For women using DMPA and Net-En, PBMCs were obtained prior to contraceptive initiation (baseline), [s]steady state drug concentration, and [n]nadir drug concentration. For women using CU-IUD, PBMCs were collected at baseline and after 180 d of CU-IUD use. Percent change in T-cells (CD4 and CD8) expressing CTLA-4 and PD-1 were evaluated and *P*-values from mixed

linear models used for comparing baseline to follow-up after initiation are displayed (A-C). *s*—defines 30 d after first injection for DMPA with a mean serum medroxyprogesterone acetate (MPA) concentration of 1303.5 pg/mL or 90 d post-injection for Net-En with mean serum norethisterone (NET) concentration of 1565.5 pg/mL. *n*—defines the period immediately prior to next clinical dosing at day 180 visit and lower mean serum concentration of 279 and 856 pg/mL for MPA and NET, respectively. CTLA-4, cytotoxic T-lymphocyte associated protein-4; CU-IUD, copper T380A intrauterine device; DMPA, depot medroxyprogesterone acetate; Net-En, norethisterone enanthate; PBMC, peripheral blood mononuclear cells; PD-1, programmed cell death-1

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TABLE 1

Demographic characteristics of the study population

Characteristic	DMPA (n = 30)	Net-En (n = 36)	CU-IUD (n = 32)	*P*-value
Age, y	26.5 (23.0, 30.0)	27.5 (23.2, 30.0)	29.0 (26.0, 32.0)	.25 *
Body mass index, kg/m^2	23.3 (21.4, 25.2)	23.2 (21.1, 28.2)	25.8 (22.9, 29.4)	.08 *
Tribe				
Shona	29 (96.7)	34 (94.4)	29 (90.6)	.58 †
Other	1 (3.3)	2(5.6)	3 (9.4)	
Marital status				
Never married	0	1 (2.8)	3 (9.4)	.45 †
Married	26 (86.7)	31 (86.1)	22 (68.8)	
Divorced	3 (10.0)	2 (5.6)	5 (15.6)	
Separated	1 (3.3)	2 (5.6)	2(6.3)	
Partner status				
No current partner	1 (3.3)	0	2 (6.3)	.12 †
Lives with partner	26 (86.7)	31 (86.1)	21 (65.6)	
Does not live with partner	3 (10.0)	5 (13.9)	9 (28.1)	
Education, highest level completed				
Primary	6 (20.0)	2(5.6)	3 (9.4)	.15 †
Secondary	24 (80.0)	33 (91.7)	26 (81.3)	
Tertiary	0	1 (2.8)	3 (9.4)	
Religious belief				
None Christian	2 (6.7)	2(5.6)	3 (9.4)	.68 †
Christian	28 (93.3)	34 (94.4)	29 (90.6)	

Note: Data presented as median (interquartile range) or n (%).

Abbreviations: CU-IUD, copper intrauterine device; DMPA, depot medroxyprogesterone acetate; Net-En, norethisterone enanthate.

* *P*-value from Kruskal-Wallis test.

† *P*-value form Fisher's exact test.

TABLE 2

Median CD4+CTLA-4+ and CD8+CTLA-4+ numbers decreased 30 d post-DMPA injection ($P < .001$)

	Parameter	Baseline	30 d	*P*-value*	180 d	*P*-value*
DMPA	CD4+CTLA-4+	10 564	8101	.001	11 004	.781
	CD8+CTLA-4+	462	401	.001	452	.463
	CD4+CTLA-4+ MFI	8682	1359	.342	6579	.619
	CD8+CTLA-4+ MFI	647	682	.153	654	.467
		Baseline	**90 d**	***P*-value***	**180 d**	***P*-value***
Net-En	CD4+CTLA-4+	12 784	13 716	.087	11 434	.217
	CD8_CTLA-4+	712	680	.039	666	.566
	CD4+CTLA-4+ MFI	1383	1332	.135	1360	.426
	CD8+CTLA-4+ MFI	810	823	.508	836	.311
CU-IUD	CD4+CTLA-4+	12 117			12 800	.426
	CD8_CTLA-4+	584			714	.169
	CD4+CTLA-4+ MFI	1272			1232	.264
	CD8+CTLA-4+ MFI	668			658	.058

Note: Data are displayed as median values.

Abbreviations: CU-IUD, copper intrauterine device; DMPA, depot medroxyprogesterone acetate; MFI, median fluorescence intensity; Net-En. norethisterone enanthate.

**P*-value from mixed effects linear regression that compared CTLA-4 expression at steady state and nadir concentrations of DMPA or Net-En, am CU-IUD use relative to expression measured prior to contraceptive initiation.

TABLE 3

Median CD4+PD-1+ and CD8+PD-1+ numbers decreased following 180 d of DMPA or Net-En use (P < .001)

	Parameter	Baseline	30 d	*P*-value*	180 d	*P*-value*
DMPA	CD4+PD-1+	2158	3740	.858	2112	.001
	CD8_PD-1+	703	1076	.456	446	.001
	CD4+PD-1+ MFI	864	788	.747	748	.361
	CD8+PD-1+ MFI	690	700	.963	685	.254
		Baseline	**90 d**	***P*-value***	**180 d**	***P*-value***
Net-En	CD4+PD-1+	5488	3871	.002	2902	.001
	CD8_PD-1+	1675	1233	.001	1114	.001
	CD4+PD-1+ MFI	778	790	.245	777	.553
	CD8+PD-1+ MFI	822	827	.582	826	.249
CU-IUD	CD4+PD-1+	3470			3387	.083
	CD8+PD-1+	1078			954	.936
	CD4+PD-1+ MFI	800			795	.160
	CD8+PD-1+ MFI	834			836	.020

Abbreviations: CU-IUD, copper intrauterine device; DMPA, depot medroxyprogesterone acetate; MFI, median fluorescence intensity; Net-En, norethisterone enanthate.

**P*-value from mixed effects linear regression that compared PD-1 expression at steady state and nadir concentrations of DMPA or Net-En, and Cu- IUD use relative to expression measured at enrollment.