Changes in genital tract immune cell populations after initiation of intrauterine contraception

Sharon L. ACHILLES, MD, PhD[1,2], **Mitchell D. CREININ, MD**[1,2,3,*], **Mr. Kevin A. STONER, BS**[2], **Beatrice A. CHEN, MD, MPH**[1,2], **Ms. Leslie MEYN, MS**[1,2], and **Sharon L. HILLIER, PhD**[1,2]

[1]Department of Obstetrics, Gynecology, and Reproductive Sciences and Center for Family Planning Research, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213

[2]Magee-Womens Research Institute, Pittsburgh, PA 15213

[3]Department of Epidemiology, University of Pittsburgh Graduate School of Public Health, Pittsburgh, PA 15213

Abstract

Objective—The primary target cells for HIV infection in the genital tract are CD4 T-cells expressing CCR5 on the surface. Alterations in genital tract T-cells expressing CCR5 could impact human immunodeficiency virus (HIV) acquisition risk. We hypothesized that when compared to baseline, use of a hormonal intrauterine device (IUD) would alter HIV target cells (primarily CCR5+ CD4 cells) in the female genital tract more than a non-hormonal IUD.

Study Design—Thirty-four healthy, HIV negative women age 18-40 seeking an IUD for contraception were randomized to receive a levonorgestrel IUD or a copper T380A IUD. A parallel group of 8 control women not needing contraception was also enrolled. Genital tract mucosal immune cell populations collected by cervical cytobrush and endometrial biopsy before and two months after IUD placement were analyzed by flow cytometry. Mean differences in cell number and percent expressing receptors from baseline to follow-up were evaluated using paired Student's t-tests.

Results—Neither IUD altered the number of T-cells within the upper and lower genital tracts. Levonorgestrel IUD users had a decrease in T-cells expressing the HIV co-receptor CCR5 in the endometrium and cervix after two months of use compared with baseline. There was a decrease in

Contact for corresponding author: Sharon L. Achilles, MD, PhD, University of Pittsburgh, Department of Obstetrics, Gynecology and Reproductive Sciences, 300 Halket Street, Pittsburgh, PA 15213, Telephone (412) 641-1403, Facsimile (412) 641-1133, achisx@upmc.edu..
*Dr. Creinin's current affiliation is the Department of Obstetrics and Gynecology, University of California, Davis

activated endometrial T-cells in levonorgestrel IUD users and a decrease in activated cervical T-cells in copper IUD users after two months of IUD use compared with baseline.

Conclusions—Women using IUDs have reduced expression of the CCR5 HIV co-receptor on T-cells in the endometrium and cervix compared to expression prior to IUD placement. These findings suggest that susceptibility to HIV infection would not be increased by IUD use.

Keywords

CCR5; HIV; hormonal contraception; IUD; T-cells

Introduction

Combating the spread of HIV is a major global goal,[1] achievement of which could be accelerated by the eventual development of highly effective dual protection methods that prevent both sexual acquisition of HIV and unwanted pregnancy. There is currently a tension between HIV prevention and family planning because emerging data suggest some hormonal contraceptives, particularly injectable progestins, may increase risk of HIV acquisition and transmission.[2-6] Long acting reversible contraceptives (LARC), which include IUDs and implants, are more effective than combined oral contraceptive pills (COCs) and DMPA and offer a significant reduction or complete elimination of systemic exposure to exogenous hormones compared to these methods. IUDs are the most commonly used reversible contraceptive worldwide[7] and are regaining popularity in the United States; currently approximately 8.5% of American women who use contraception choose an IUD.[8] Observational data from women living in high HIV incidence areas have included few IUD users. Thus, the available data cannot provide reliable estimates of HIV acquisition risk associated with IUD use. [3]

HIV acquisition and sexual transmission are dependent on the immune environment of the female genital tract[9,10] and may be under hormonal regulation. Endogenous sex hormones vary through the menstrual cycle and exogenous hormonal exposure occurs commonly with contraceptive use, which may influence mucosal immune cellular populations.[11]

Mucosal CD4 T lymphocytes in the vagina and cervix are thought to be the primary targets for sexual transmission of HIV to women.[12-16] HIV infects discrete subsets of CD4 T cells, which express phenotypic receptors and co-receptors necessary for HIV to gain intracellular access.[17-20] Antigen-presenting cells, such as mucosal dendritic cells, monocytes and macrophages (CD14+ cells) may help transport HIV from the surface to underlying target cells.[21]

CCR5 is expressed on genital tract T-cells[22-25] and is the predominant target co-receptor for initial HIV infection.[18] CCR5 expression is enhanced by sex hormones as ex-vivo studies that have demonstrated stimulation of CCR5 expression within explanted cervical tissue when incubated in media containing progesterone.[23] In vivo, increased CCR5 expression in the setting of increased progesterone may contribute to the observed increased risk of HIV acquisition during pregnancy.[26-30] However, there are limited data on lymphocyte changes with use of COCs[31] and DMPA;[32-34] there are no data on genital tract immune cell

populations within reproductive tract mucosa of women using other contraceptive methods, including IUDs.

We hypothesized that genital tract immune cell populations, particularly CCR5+ T-cells, would be increased from baseline two months after initiating intrauterine contraception with a levonorgestrel (LNG) IUD containing 52mg LNG more than after initiation of use of a non-hormonal copper IUD (Cu-IUD). We hypothesized that concentrated progestin exposure in the genital tract would recruit HIV target cells to the area. We tested this hypothesis by examining immune cellular populations in upper and lower genital tract samples obtained immediately before and two months after IUD insertion in healthy women randomized to receive a LNG-IUD or Cu-IUD. Because IUDs are placed directly into the uterus, we hypothesized that the greatest impact on genital lymphocytes would be observed in T-cells recovered from endometrial biopsies.

Materials and Methods

We performed a randomized study of women initiating intrauterine contraception plus a parallel control group of women not at risk of pregnancy due to heterosexual abstinence or prior surgical sterilization. The primary objective was to assess the impact of IUD initiation on T-cells in the upper and lower genital tract. The University of Pittsburgh Institutional Review Board approved this study. All participants were enrolled at the Center for Family Planning Research, Magee-Womens Hospital of the University of Pittsburgh Medical Center and signed informed consent before study participation.

Forty-two women, age 18-40 years, were enrolled, including 34 women seeking an IUD for contraception and 8 women not seeking contraception who comprised an observational control group. Eligible women were healthy, HIV negative, non-pregnant and had regular menstrual cycles. All enrolled study participants were free of genital tract infection on screening exam, including rapid testing for *Trichomonas vaginalis* (OSOM, Sekisui Diagnostics, Lexington, MA), yeast vaginitis, symptomatic bacterial vaginosis by Amsel's criteria,[35] and abnormal inflammation (>10 WBC/hpf on wet mount). Women were excluded if within 60 days of enrollment they: 1) used any hormonal or intrauterine contraceptive; 2) were pregnant or breastfeeding; 3) underwent any genital tract procedure (including biopsy); 4) were diagnosed with any genital tract infection; 5) had a new sexual partner. Exclusion criteria included use of DMPA within 10 months of enrollment; use of oral or vaginal antibiotics, oral or vaginal steroids, or any vaginal product except tampons (such as spermicide, microbicide, douche, antifungal, steroid, or hormone) within 30 days of enrollment; having a contraindication to IUD use or an allergy to any component of the IUDs; or having a prior malignancy of the cervix or uterus. Women in the control group had to be not at risk of pregnancy defined as heterosexually abstinent or surgically sterile.

Screening also included urine pregnancy testing, collection of blood to rule out HIV infection and collection of cervical swabs for detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* by nucleic acid amplification testing (NAAT, Gen-Probe, San Diego, CA). One participant was found to be ineligible after enrollment due to chlamydial

infection and a second participant withdrew from the study after IUD insertion; both were replaced to maintain the targeted sample size.

Participants were enrolled immediately after screening if eligible that day, or returned for enrollment on a day when no vaginal bleeding was present. Day of menses at the time of enrollment was recorded. Participants were asked to refrain from any vaginal or anal intercourse for 1 week prior to sample collection at both visits. The 34 women who were seeking an IUD for contraception were randomized 1:1 to receive either a LNG-IUD (Mirena®, Bayer HealthCare Pharmaceuticals, Wayne, NJ) or CopperT380A IUD (ParaGard®, Teva Pharmaceuticals, Sellersville, PA). At the time of randomization, the study investigator opened the next sequentially numbered, opaque, sealed envelope containing the group assignment of LNG-IUD or Cu-IUD. A statistician not involved with the clinical conduct of the study prepared the envelopes using computer-generated random allocations in permutated blocks. The IUD was inserted per standard clinical practice at the enrollment visit immediately following the collection of all study samples. All laboratory personnel were masked to clinical status of participants including randomization to IUD type.

Genital tract samples were collected at enrollment and 8-week follow-up visits. Endocervical specimens were obtained by inserting a cytobrush (Cooper Surgical, Trumbull, CT) into the cervical os, rotating 360°, and placing the cytobrush in 4mL RPMI-1640 medium supplemented with 25mM HEPES, L-glutamine, and 10% fetal bovine serum (tRPMI). The ectocervix and endocervix were cleansed with chlorhexidine solution (Hibiclens, Mölnlycke Health Care, Norcross, GA) and dried with a sterile swab. Endometrial aspiration biopsies (Pipelle®, Cooper Surgical) were obtained with care not to touch the aspirator to the vaginal walls or the ectocervix. Adequacy of the sample was visually assessed by the clinician obtaining the biopsy. Endometrial samples were transported in the aspirator to the laboratory and extruded under sterile conditions. All samples were transported to the laboratory for processing within 30 minutes of collection.

Endometrial biopsies were weighed and then washed 3-4 times with phosphate buffered saline without calcium and magnesium (PBS) (Mediatech, Manassas, VA). The biopsies were minced using sterile scissors and placed in digest buffer containing 20mL RPMI-1640, 1mg/mL collagenase D (Roche Ltd, Nutley, NJ), and 2000U/mL DNase I (0.5μl/ml) (New England Biosciences, Ipswich, MA). Agitation of the tissue in digest buffer was limited to 15 minutes at ~300rpm at 37°C to maintain cell surface marker integrity.[36,37] The transport vial containing the endocervical cytobrush was vortexed and washed with tRPMI to dislodge cells from the cytobrush. Both biopsy and cytobrush collected cells were filtered through a 40μm nylon cell sieve (Becton Dickenson, Franklin Lakes, NJ) to obtain single cell suspensions.

Endometrial biopsies additionally underwent density gradient centrifugation to remove dead cells, red blood cells, and other debris.[36] The digested and filtered endometrial cells were re-suspended in 5mL 36% Histopaque® (Sigma-Aldrich, St. Louis, MO) in RPMI (Mediatech) then layered over 4.5mL undiluted Histopaque® and under 500uL PBS. This tube was centrifuged at 600× g for 30 minutes with no brake; lymphocytes were recovered from the

interface. Recovered cells of both specimen types were then washed by centrifugation in RPMI and re-suspended in 1mL PBS. Using Trypan blue (Sigma-Aldrich) exclusion criteria,[38] viable cell yields were obtained manually with a hemocytometer.

Cell suspensions were adjusted to 1×10^6 cells/mL in PBS and 1ml was stained for viability using LIVE/DEAD® Fixable Aqua Dead Cell Stain (Invitrogen, Carlsbad, CA) and incubated for 25 minutes at room temperature protected from light. Cells were washed once with 1ml of Flow Cytometry Staining Buffer (FACs) (eBioscience, San Diego, CA) by centrifugation for 5 minutes at 400× g, and stained with fluorochrome-conjugated antibodies (BD Biosciences, San Jose, CA) specific for the following cell surface markers: CD45 (FITC), CD3 (PerCP), CD8 (APC-H7), CD4 (PacificBlue), CD195 (CCR5)(APC), CD69 (PE), and CD14 (PE-Cy7). Cells were incubated for 25 minutes at room temperature protected from light, washed with 1mL FACs buffer by centrifugation for 5 minutes at 400× g, and fixed in 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Stained samples were stored at 4°C and flow cytometric analysis was conducted no later than 24 hours after fixation.

Lymphocyte populations were analyzed using a BD™ LSR II flow cytometer (BD Biosciences). To compensate for spectral overlap, single color compensation was applied specific for each flourochrome-conjugate used. T-cell populations were identified using forward and side scatter and fluorescent-minus-one (FMO) controls were utilized to assist in defining gate positions. Two senior laboratory technicians trained in advanced flow cytometry independently reviewed and agreed upon the gating parameters for each sample.

The sample size for this study was calculated based on available published data indicating the mean percent expression of CCR5 on cervical CD4 cells among women not using contraception was 48 +/−4% with a standard deviation of +/−7% for women using COCs.[31] Assuming the standard deviation of the mean change in the percent expression would be no greater than +/−8%, a sample size of 10 would have 90% power to detect at least a 20% difference (set to be greater than one standard deviation for mean change) in the percent expression of CCR5 on CD4 measured prior to and two months after IUD placement, using a paired t-test evaluated at the 0.05 two-sided significance level. The enrollment target was increased to 16 participants per group to account for potential loss to follow-up, post-randomization ineligibility, inadequate specimen quality, and the plan to evaluate additional cell populations.

Data were analyzed using FACS DIVA software v.6.2 (BD Biosciences) and FlowJo software v.10.0.5 (Tree Star Inc., Ashland, OR). The gating strategy for all populations can be seen in Figure 1. Cell numbers from biopsy specimens were normalized per gram of tissue. Cytobrush collected cells were reported as "cells per cytobrush." The cell numbers quantified were $\log_{10}$ transformed for analysis and presentation. Expression of CCR5 and CD69 was reported as the percentage of parent population expressing these cell surface markers. Each participant served as her own control by using the baseline visit as the control normal and assessing change at the two month follow-up. Statistical analysis was performed using SPSS® Statistical software version 20.0 (IBM Corporation, Armonk, NY) and statistical tests were evaluated at the 2-sided 0.05 significance level. Differences in

enrollment characteristics between the groups were assessed using one-way analysis of variance, Kruskal-Wallis, and Fisher's exact tests, where appropriate. Differences in levels of expression from baseline to follow-up were evaluated using paired Student's t-tests.

Results

Between December 2010 and July 2011, 42 women were enrolled in the study (Fig. 2). Demographic information was not significantly different among the groups, including phase of menstrual cycle at the time of enrollment (Table 1). All of the sample data from one participant randomized to the LNG-IUD group were excluded from analysis due to inadequate specimen quality following a tissue handling error. The endometrial biopsies were performed with a single pass for 78/82 (95%) biopsies. The remaining 4 biopsies required two attempts to obtain an adequate sample.

Two months after IUD insertion, there was no statistically significant change from baseline in the number of CD4+ or CD8+ T-cells in the endometrium (Fig. 3) or cervix (data not shown) amongst women using hormonal and non-hormonal IUDs. The number of CD14+ immune cells (macrophages, neutrophils, and dendritic cells) in the endometrium significantly increased two months after Cu-IUD placement ($\log_{10}$ 3.4→4.3, p<0.001); among women receiving a LNG-IUD, the increase was less marked and not statistically significant ($\log_{10}$ 3.8→4.2, p=0.06).

Within the endometrium, the percentage of CD4+ and CD8+ T-cells expressing the CCR5 HIV co-receptor significantly decreased from baseline levels two months after LNG-IUD insertion (66%→34%, p<0.001 and 72%→41%, p<0.005 respectively) (Fig. 4a). CCR5 expression also significantly decreased from baseline levels on endometrial CD8+ T-cells two months following Cu-IUD insertion (70%→43%, p<0.005).

Within the cervix, CCR5 co-receptor expression on endocervical CD4+ T-cells was significantly diminished from baseline levels two months after initiation of an IUD (54%→38%, p<0.05 for LNG-IUD and 55%→35%, p<0.01 for Cu-IUD) (Fig. 4b). There was a decreased, but non-significant, expression of the CCR5 co-receptor on cervical CD8+ T-cells as compared to baseline in women randomized to Cu-IUD (62%→47%, p=0.06).

T-cell activation was assessed by measuring the percentage of T-cells expressing CD69. Endometrial cells from women using the LNG-IUD had significantly decreased CD69 on CD8+ T-cells (81%→68%, p<0.05) as compared to baseline; there was also a non-significant decrease in activation of CD4+ T-cells as compared to baseline (67%→52%, p=0.06) (Fig. 4a). In women who received a Cu-IUD, there was no significant change over two months in the activation of T-cells within the endometrium; however in the cervix there was a significant decrease in the percentage of activated CD4+ and CD8+ T-cells as compared to baseline (66%→47%, p<0.01 and 76%→58%, p<0.05 respectively) (Fig. 4b).

No significant changes were observed over time in any of these parameters among the control women who did not receive an IUD.

Comment

Although there is an incomplete understanding of factors that can increase or decrease CCR5 and CD69 expression on genital T-cells, CCR5 expression appears to increase predominantly with viral[46] and parasitic infections.[47-48] Expression of both CD69[39-40] and CCR5[41-43] on various immune cells appear to be increased with exposure to pro-inflammatory cytokines and chemokines, and such an inflammatory milieu in the genital tract has been prospectively associated with increased HIV acquisition risk.[44-45] Conversely, CD69 and CCR5 expression on lymphocytes may be decreased with exposure to steroids[49-51] and antibiotics.[52] Based on suggestions that injectable progestins may increase susceptibility to HIV infection, we had hypothesized that local progestin delivery with a LNG-IUD would have a greater impact on genital immune cells compared with exposure to a non-hormonal Cu-IUD, altering HIV susceptibility. Surprisingly, there was no change in the number of T-cells, the percentage of T-cells expressing HIV-co-receptor CCR5 was reduced, and the activation state of the T-cells was either reduced or unchanged within the upper and lower genital tracts two months after initiation of either a hormonal or non-hormonal IUD. No statistically significant T-cell changes, including CCR5 and CD69 expression, occurred among women in the parallel control group. Taken together these data suggest IUD use (either hormonal or copper) do not induce a pro-inflammatory milieu in the genital tract and would not increase HIV transmission risk.

We evaluated immune cell populations simultaneously in the upper and lower genital tracts of women initiating IUD use in order to assess a range of sexually exposed mucosa since the primary site(s) of sexual transmission remains unknown. Furthermore, the Cu-IUD has long been purported to create an inflammatory reaction within the endometrium as part of its mechanism of contraceptive action, however we did not find evidence of such. We found no increase in activation of T-cells as measured by CD69 expression in the endometrium of IUD users. We did find a statistically significant increase in macrophages, neutrophils and dendritic cells in the endometrium of Cu-IUD users as measured by CD14, and a similar, but not significant, increase in these cells among the LNG-IUD users. Interestingly, these data suggest differential alterations in immune cellular populations after CU-IUD insertion, with an increase in innate immune cells (CD14+) and a decrease in lymphocyte activation. Importantly, innate immune cells at mucosal surfaces, particularly macrophages and dendritic cells, may also play a significant role in HIV transmission by capturing and transporting HIV virions to susceptible T-cells.

There are few studies to date that have evaluated immune cells from freshly collected upper reproductive tract tissue surrounding IUD use. Studies using *in situ* genital tract immune cells generally use tissue collected at surgery from heterogeneous patients having procedures for a variety of pathologic conditions and with a mean age of greater than 40 years.[53-55] Given evidence that age modifies the relationship between contraceptive use and risk of HIV acquisition with younger women at greater risk,[6] performing biopsies rather than using surgical specimens allows investigation of the endometrial immune cells of younger healthy women. Since most studies evaluating genital tract immune cells to date have been performed with cytobrush collected cervical cells, we also chose to include cytobrush-

collected cells in the present study. More work is needed to better understand the correlation between cells recovered by brush compared with tissue biopsy cells.

In order to minimize the effects of natural variation in cellular populations over time and with respect to sexual practices, we used a pair-wise comparison study design such that women acted as their own controls. The 100% follow-up of study participants in this study contributed to the strength of this analysis. One limitation of the present study is the single follow-up visit and the brief two-month evaluation time. A strength of the present study was the inclusion of a control group of women who were followed in parallel, since this group of women had no statistically significant changes in immune cell populations over time, suggesting that the changes observed among the women initiating IUD use was not due to normal variability of these cell populations over time.

Given the low probability of HIV transmission per sexual exposure to an HIV-infected partner, more research is needed to characterize the HIV-target cells present in the female genital tract and how their numbers, activation status and co-receptor expression relate to HIV susceptibility. Further studies are needed that directly compare genital CD4+ T-cell subsets and antigen presenting cells in women initiating the full range of hormonal and non-hormonal contraceptives, including DMPA, to better understand the range of cellular alterations and to learn which changes, if any, are important determinants of susceptibility to HIV. Furthermore, a better understanding of the endometrial and cervical immune effects of Cu-IUD use, particularly in the context of enrolling Cu-IUD users as non-hormonal contraceptive 'controls' for larger trials designed to understand HIV risk with contraceptive use, are urgently needed.

In conclusion, this study of reproductive-aged women initiating IUD use demonstrated that women using the LNG-IUD had decreased numbers of CD4 cells expressing the HIV co-receptor CCR5, in both the endocervix and the endometrium, compared to baseline suggesting that the numbers of HIV targets in the cervix and endometrium would be decreased following initiation of this hormonal IUD. Women using the Cu-IUD had a similar decrease in CD4 cells expressing the CCR5 receptor in the cervix, suggesting that use of either type of IUD is associated with changes in T-cell populations in the female genital tract that are not suggestive of an increased risk of HIV acquisition. Given that the HIV-target cell populations were largely decreased in the genital tract with IUD use, a hypothesis could be generated that IUD use, particularly LNG-IUD use may be somewhat protective for HIV acquisition. The direct effect of these IUD-induced changes on actual cellular susceptibility either increased, decreased, or unchanged, has yet to be evaluated.

Acknowledgments

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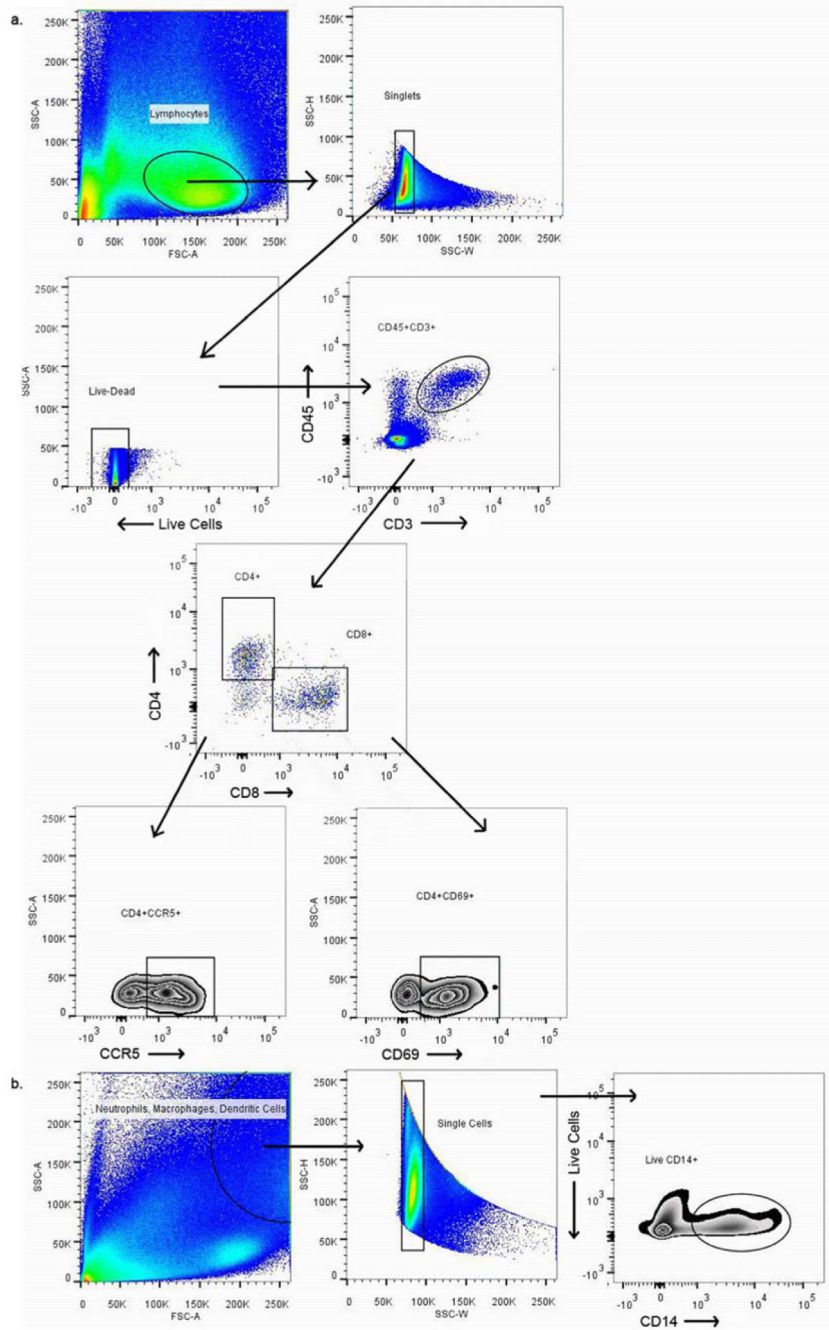
Figure 1. Gating strategy used to identify populations of interest

a.) Live single cells were identified from the lymphocyte population. CD4 and CD8 T-cells were identified from CD45CD3+ populations and CCR5 and CD69 positive cells were identified from both CD4 and CD8 cells. b.) Single cells were identified from the neutrophil, macrophage, dendritic cell populations and live CD14 cells were gated from this population of single cells.
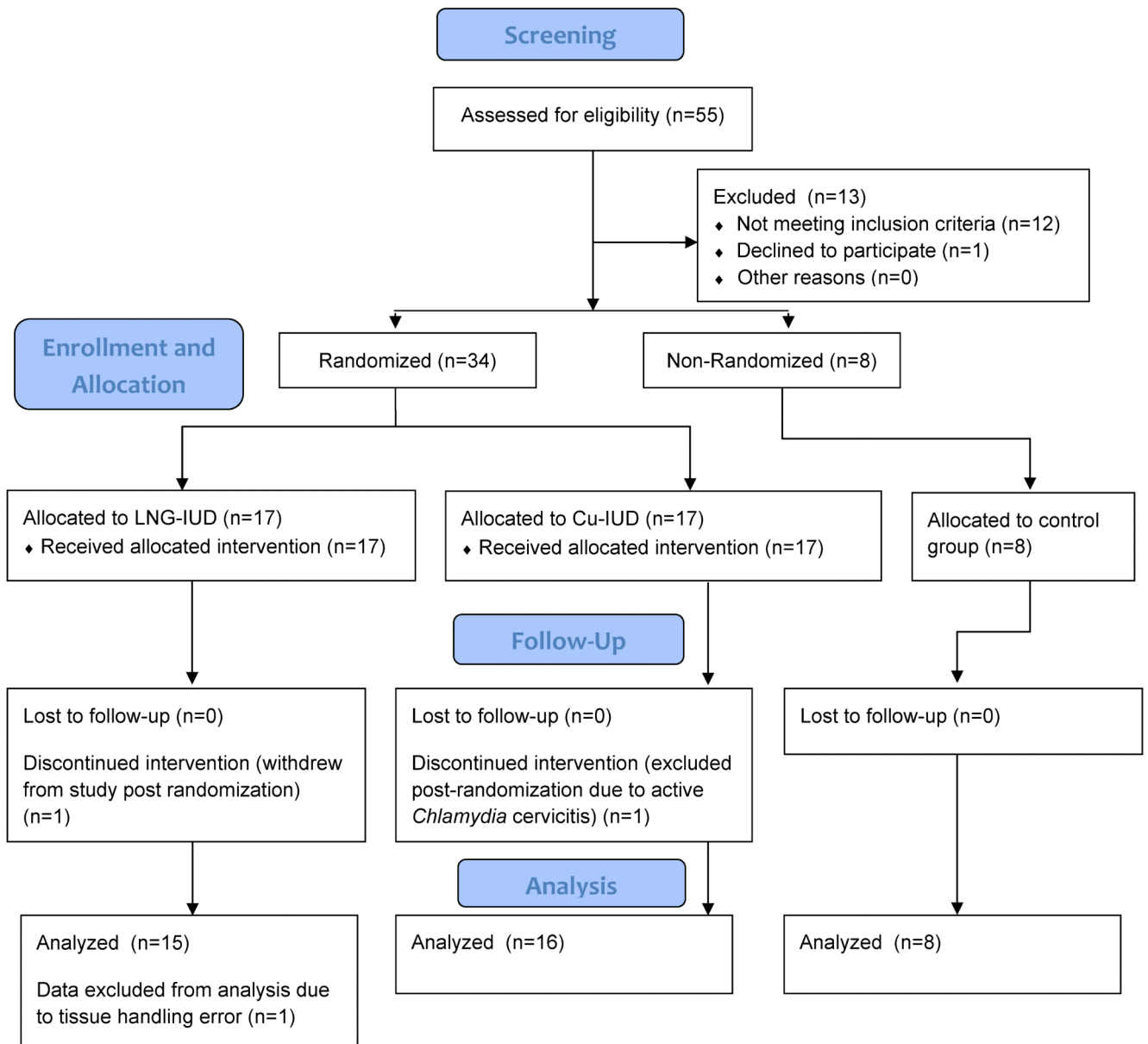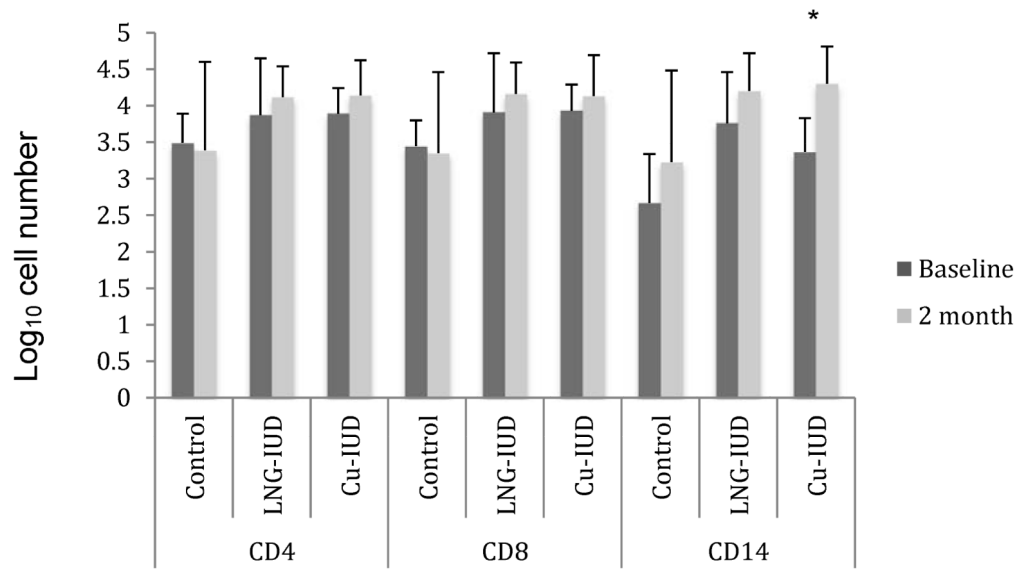
Figure 2. CONSORT flow diagram

Figure 3. Immune Cell Number in Endometrial Biopsy Specimens

The data represent the mean $\log_{10}$ transformed number of cells per gram of tissue weight.
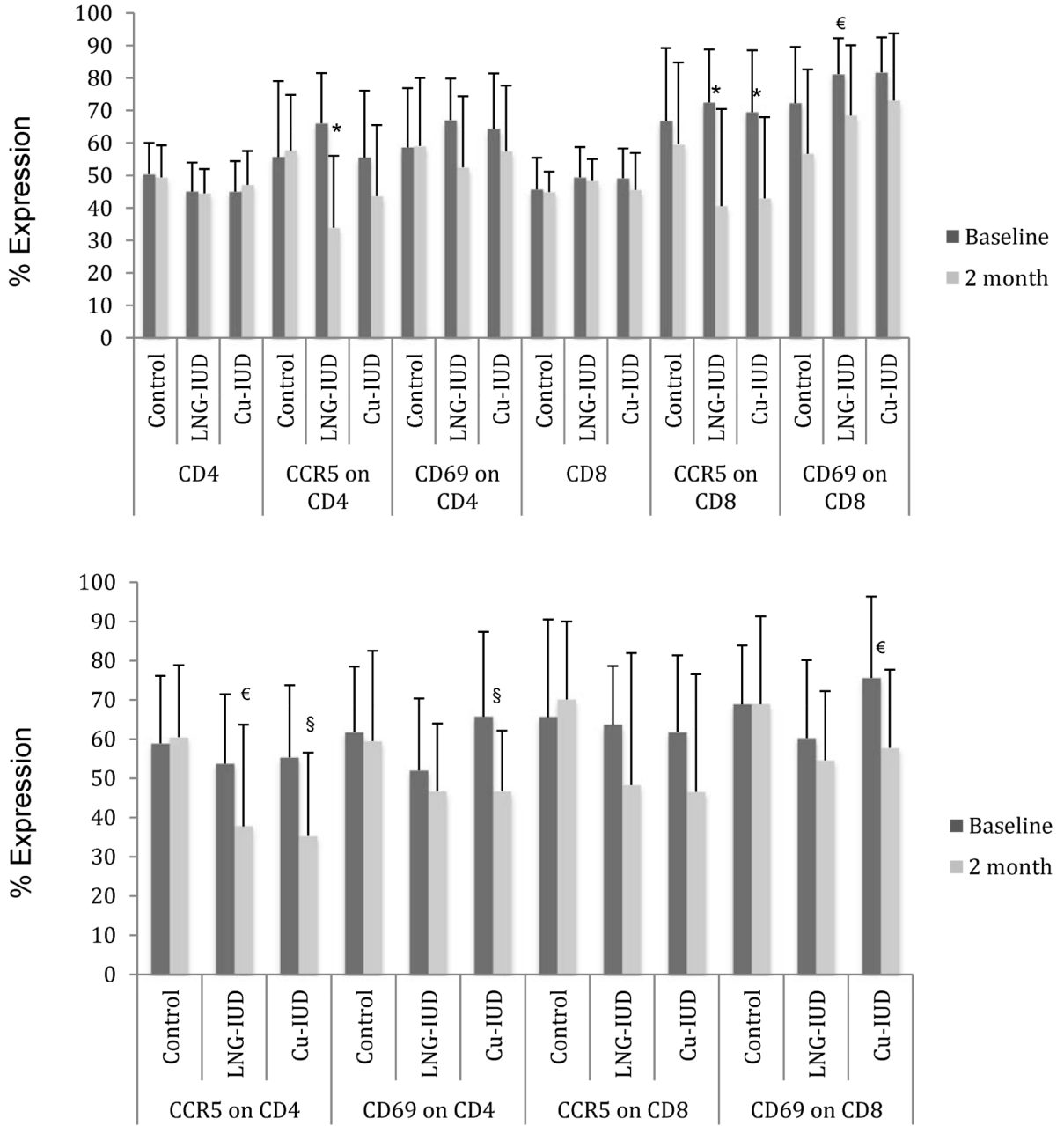
LNG-IUD= levonorgestrel IUD

Cu-IUD= Copper IUD

*p<0.001

Figure 4. Percent of T-cells expressing CCR5 and CD69
LNG-IUD= levonorgestrel IUD
Cu-IUD= Copper IUD
*p<0.005
§p<0.01
€p<0.05

Table 1

Demographics

	Control (N=8)	LNG-IUD (N=15)	Cu-IUD (N=16)	*P-value*
Age, years	27.6 ± 6.0	25.4 ± 6.2	26.9 ± 3.8	0.58[*]
Body Mass Index, kg/m^2	25.8 ± 7.4	27.3 ± 8.3	27.3 ± 7.9	0.88[*]
Gravidity	0 (0, 4)	0 (0, 4)	1 (0, 2)	0.72[†]
Hispanic	1 (12.5%)	1 (6.7%)	0	0.50[†]
Race				0.29[‡]
White	5 (62.5%)	12 (80.0%)	15 (93.8%)	
Black	1 (12.5%)	2 (13.3%)	1 (6.2%)	
Asian	1 (12.5%)	1 (6.7%)	0	
Marital Status				0.82[‡]
Single	7 (87.5%)	14 (93.3%)	13 (81.2%)	
Married	1 (12.5%)	1 (6.7%)	3 (18.8%)	
Education				0.26[‡]
High school graduate/GED	2 (25.0%)	9 (60.0%)	6 (37.5%)	
College graduate	6 (75.0%)	6 (40.0%)	10 (62.5%)	
Insurance				0.69[‡]
None	2 (25.0%)	1 (6.7%)	2 (12.5%)	
Private	5 (62.5%)	13 (86.7%)	12 (75.0%)	
Public	1 (12.5%)	1 (6.7%)	2 (12.5%)	
Menstrual Cycle Day				0.52[‡]
Follicular phase (1-14)	4 (50.0%)	4 (26.7%)	6 (37.5%)	
Luteal phase (15-28)	4 (50.0%)	11 (73.3%)	10 (62.5%)	
Nugent Score 3	5 (62.5%)	13 (86.7%)	11 (68.8%)	0.40[‡]

Data presented as mean ± standard deviation or n (%)

[*] *P-value* from one-way analysis of variance

[†] *P-value* from Kruskal-Wallis test

[‡] *P-value* from Fisher's Exact test