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Menzies, F. M., Oldham, R. S., Waddell, C., Nelson, S. M. and Nibbs, R. J.B. (2020) A comprehensive profile of chemokine gene expression in the tissues of the female reproductive tract in mice. *Immunological Investigations*, 49(3), pp. 264-286. (doi: 10.1080/08820139.2019.1655573)

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Deposited 29 August 2019

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A comprehensive profile of chemokine gene expression in the tissues of the female reproductive tract in mice

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Abstract

Background: Homeostatic leukocyte trafficking into and within the female reproductive tract (FRT) contributes to fertility and reproductive health. It is unclear how this process is regulated in the anatomically distinct reproductive tissues, or whether the genes involved are affected by cyclical changes in reproductive hormones. In tissues such as skin and intestine, mouse studies have defined evolutionarily conserved molecular mechanisms for tissue-specific homing, interstitial positioning, and leukocyte egress. Chemokine family members are invariably involved, with the chemokine expression profile of a tissue regulating leukocyte content.

Methods: Reproductive tissues (ovary, vagina, cervix, uterine horn) of 8 week old virgin female C57BL/6 mice (*n=20*) were collected, and expression of mRNA for leukocyte markers and chemokines conducted by qPCR. Lymphocytic and myeloid cell populations within the uterus, cervix, bone marrow and PALN from virgin C57BL/6 mice was determined by flow cytometric analysis.

Results: Variation in leukocyte content between reproductive tissues is evident, with the uterus and cervix containing complex mixtures of lymphocytes and myeloid cells. Twenty-six chemokine genes are expressed in the FRT, many by several component tissues, some preferentially by one. Most striking are *Xcl1* and *Ccl28*, which are restricted to the uterus. *Ccl20* and genes encoding CXCR2 ligands are primarily transcribed in cervix and vagina. Ovary shows the lowest expression of most chemokine genes, with the notable exception of *Ccl21* and *Ccl27*. We also identify eight chemokines in the vagina whose expression fluctuates substantially across the estrous cycle.

Conclusion: These data reveal complex chemokine networks within the female reproductive tract, and provide a framework for future studies of homeostatic leukocyte trafficking into and within these tissues.

Keywords: chemokine, uterus, estrous cycle

Abbreviations: BM, bone marrow; DC, dendritic cell; DN, double negative; FRT, female reproductive tract; FSC, forward scatter; NK, natural killer; PALN, para-aortic lymph node; SSC, side scatter; Tregs, regulatory T cells

Funding:

R.S.O. was supported by a studentship funded by the Medical Research Council as part of Glasgow University's doctoral training program.

Disclosure Statement:

The Authors declare that there is no conflict of interest

Introduction

Leukocytes are abundant in bone marrow, blood and lymphoid tissues, but these key immune cells are also found in virtually all healthy non-lymphoid tissues under homeostatic conditions. There is constant movement of leukocytes into, within, and out of these tissues, and discrete interstitial niches often contain specialised populations of resident leukocytes. Collectively, these cells maintain peripheral immunological tolerance, detect tissue damage and infection, and initiate the innate and adaptive immune responses that lead to tissue repair and immune protection. In addition, memory T cells generated during an infection are imprinted with specific homing properties that allow them, after homeostasis has been restored, to preferentially home to the tissue where the infection was first detected (Sheridan, 2011). Here they are ideally placed to rapidly respond if the infectious agent is encountered for a second time. Thus, the recruitment, positioning and departure of leukocytes is of fundamental importance to all tissues because they contribute to immunosurveillance, tolerance, tissue repair and immune memory.

Homeostatic leukocyte migration and localisation is largely dependent on leukocytic expression of cell adhesion molecules and chemoattractant receptors. Heptahelical G-protein-coupled receptors for chemokines are particularly important (Bachelerie, 2014; Hughes, 2018). Chemokines are a large family of small, secreted chemoattractants that are subdivided into four groups (CC, CXC, XC and CX_3C) based on the configuration of conserved cysteine residues near their amino-terminus (Zlotnik, 2012). They have been implicated in many processes critical for leukocyte trafficking, including arrest on blood vessel endothelium, transendothelial migration, interstitial motility, retention in specific tissue compartments, and departure from tissue via lymphatic vessels (Hughes, 2018). Some chemokines are constitutively expressed by many non-lymphoid tissues where they contribute to common migratory processes. For instance, CCL21 is widely expressed because it mediates the steady state egress of dendritic

cells (DCs), a process that happens in many tissues (Johnson, 2010; Tal, 2011). Other chemokines show a more restricted pattern of expression and are responsible for recruiting leukocyte populations with critical tissue-specific functions. This has been particularly well studied in the skin and intestine of mice. For example, CCL8 is abundantly produced in resting skin (Islam, 2011), while CCL25 is found almost exclusively in the small intestine (Campbell, 2002). As a consequence, leukocytes expressing CCR8, the CCL8 receptor, have the potential to home to the skin, while those expressing the CCL25 receptor CCR9 can enter the wall of the small intestine. Chemokine receptor expression is used to define the migratory potential of a leukocyte and identify discrete populations of cells with shared functional properties. Similarly, the chemokine expression profile of a tissue gives a clear indication of which molecular pathways are likely to play critical roles in regulating its leukocyte infiltrate. Importantly, while most studies of leukocyte homing have been performed in mice, the pathways that have been characterised to date appear to be conserved in other mammals, including humans (Nomiyama, 2013).

The female reproductive tract (FRT) consists of several anatomically and functionally distinct tissues, each containing discrete microanatomical compartments. These tissues are subject to fluctuations in female sex hormones that drive a cyclical pattern of fertility. In mice this estrous cycle lasts 4-5 days and is split into four main phases: proestrus, estrus (during which ovulation occurs), metestrus and diestrus (Byers, 2012; Bertolin, 2014). Progress through the estrous cycle is accompanied by alterations in the structure and leukocyte content of FRT tissues. In these tissues, as in other tissues, leukocytes will contribute to immunosurveillance, immunological tolerance, tissue repair and post-infection immune memory. They will also initiate the immune and inflammatory responses that are associated with key reproductive processes, such as implantation (Pijnenborg, 2002; Garlanda, 2008; Dekel, 2014), placentation (Spencer, 2014), parturition (Thomson, 1999; Bollapragada, 2009)

and post-partum involution (Shynlova, 2013). Abnormal inflammation in the FRT is associated with gynaecological conditions and reproductive problems in humans, including polycystic ovarian syndrome (Ojeda-Ojeda, 2013; Schmidt, 2014), endometriosis (Maybin, 2011; Laux-Biehlmann, 2015), pre-eclampsia (Spencer, 2014), ovarian cancer (Singh, 2016; Sanguinete, 2017) and preterm labour (Romero, 2006; Blank, 2008; Ito, 2010; Phillips, 2014). Despite the evident physiological and pathological importance of leukocytes in the tissues of the FRT, the mechanisms that control the homeostatic trafficking of these cells into, within, and out of these tissues in mice and humans are largely unknown.

We hypothesised that to regulate constitutive leukocyte trafficking, tissues of the FRT will have overlapping chemokine gene expression profiles that may fluctuate over the course of the estrous cycle. To examine this, we have defined the steady state chemokine gene expression profile of the four major tissues of the mouse FRT, and compared this to equivalent data generated from skin, small intestine and colon, whose homeostatic chemokine profiles have been more widely studied. We have also examined how the expression of each chemokine gene is modulated over the course of the estrous cycle. Remarkably, we find that 26 different chemokine genes are expressed in the FRT under homeostatic conditions. Some are highly restricted to, or preferentially expressed in, one tissue of the FRT, while others show equivalent expression in two or more tissues. We also show that, in the vagina, the estrous cycle modulates the expression of 8 chemokine genes, 7 of which peak during diestrous and/or metestrous. Collectively, these data reveal the complexity of chemokine networks within the FRT, and provide a framework for future investigation of the mechanisms directing homeostatic leukocyte trafficking and localisation in its component tissues.

Methods

Animals and tissue collection

All animal experiments were approved by the University of Glasgow ethical review committee and were performed under the auspices of UK Home Office licences (Home Office Project Licence number 60/3765). All work used 8-week old virgin female C57BL/6 mice maintained in specific pathogen-free conditions at the University of Glasgow's Central Research Facility. Estrous cycle stage (proestrus, estrus, metestrus or diestrus) was determined by vaginal appearance and microscopic examination of the cellularity of vaginal lavage, as previously described (Caligioni, 2009). The ovary, vagina, cervix and mid portions of each uterine horn, along with dorsal skin, small intestine and colon, were collected into RNA*later*® before snap freezing in liquid nitrogen and storing at -80^{o}C. Cycle assessment and tissue collection were performed at the same time each morning to ensure consistency.

RNA isolation and complementary DNA synthesis

Each frozen tissue biopsy was homogenized in 1ml of TRIzol® reagent (Thermo Fisher Scientific, Paisley, UK) using an Omni μH homogenizer (Omni International, Kennesaw, USA). Total RNA was extracted according to the manufacturer's instructions, and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). A260/280 was recorded for each sample as a measure of purity. RNA quality was assessed by gel electrophoresis: all samples showed tight 28S and 18S rRNA bands, indicating that minimal degradation had occurred during the isolation process (data not shown). 5μg of RNA was treated with DNAse-I using the DNA-*free*^{TM} kit (Life Technologies). To synthesise cDNA, the High Capacity Reverse Transcription Kit was used (10μl final reaction volume) (Thermo

Fisher Scientific). Control samples were also prepared in which reverse transcriptase was omitted from the reaction (i.e. –RT control).

Gene expression analysis by quantitative PCR (QPCR)

The expression of genes encoding leukocyte markers was quantified using Taqman® technology on the ABI Prism 7900HT system (Applied Biosystems) and SDS Version 2.3 software. Gene expression assays used are listed in Table 1. Into each well, 1.25µl 20X target or endogenous control assay was added, along with 12.5µl Taqman® Universal Mastermix (Thermo Fisher Scientific), 10.25µl molecular grade water (Sigma Aldrich, St. Louis, USA) and 1µl of the cDNA reaction. Reactions were incubated for 2min at 50°C, 10min at 95°C, followed by 40 cycles of 95°C (15sec) and 60°C (1min). In all experiments, negative controls were performed in which cDNA was replaced with water or with an aliquot of the '–RT control' reaction: these samples consistently failed to generate any PCR product. The formula 2^{-Cq} was used to determine relative expression (Livak, 2001) in comparison to the endogenous control gene, *Gapdh*, and fold change calculated relative to ovary, which was given the arbitrary value of 1. Of those endogenous control genes examined, *Gapdh* gave the most consistent Ct values between samples and tissues (data not shown).

Preparation of single cell suspensions

Uterine horn or cervix was minced with scissors in 7ml or 3ml, respectively, of Hank's Balanced Salt Solution (Sigma) (with Ca^{2+} and Mg^{2+}) containing 0.026 Wunsch Units/ml Liberase TM (Roche) and 50µg/ml DNase I (Roche), and then incubated for 40min at 37°C with gentle continuous shaking. The digested tissues were then mashed through a cell strainer using the plunger of a 2ml syringe. The strainer was then washed with complete RPMI (i.e.

RPMI containing 2mM L-glutamine and 10% heat-inactivated foetal calf serum). Bone marrow (BM) aspirates were prepared by dissecting the femurs from mice, cutting off each end of the bone, and flushing out the cells with 1ml of complete RPMI using a syringe. Single cell suspensions of the para-aortic lymph node (PALN) were prepared by mashing the node through a cell strainer in complete RPMI using the plunger of a 2ml syringe. All samples were centrifuged at 400g for 5min at 4°C, washed once with complete RPMI, resuspended in ice-chilled FACS buffer (PBS containing 1% foetal calf serum, 5mM EDTA and 0.02% sodium azide), and stored at 4°C in preparation for immunostaining.

Immunostaining and flow cytometry

Cells in FACS buffer were incubated in Fc Block (BD Biosciences) for 15mins at 4°C, washed with FACS buffer, and stained for 15mins at 4°C in fluorescently labelled antibodies. After two washes with FACS buffer, cells were incubated for 10mins with Viaprobe (BD Biosciences) (to allow subsequent identification of dead cells) and then passed through a Nitex filter into a FACS tube. Antibodies against the following proteins were used according to the supplier's instructions (fluorophores are indicated in parentheses): CD45 (APC-Cy7), F4/80 (PE-Cy7), CD4 (PE-Cy7), and CD8 (PE) (all from eBiosciences), and CD11b (V450), Ly6C (FITC), CD3 (VioBlue), CD25 (FITC) (all from BD Biosciences). Data were acquired on a MACS Quant flow cytometer (Miltenyi Biotec). Acquisition parameters were set using (i) anti-mouse Ig k/negative control CompBeads (BD Biosciences), (ii) samples stained with a single antibody, and (iii) fluorescence minus one controls (in which one fluorescent antibody in the mixture was omitted). Data were analysed using FlowJo software (Tree Star). During analysis, we excluded cell debris by pre-gating using physical properties (forward scatter (FSC) and side scatter (SSC)), and discounted dead cells by selecting for Viaprobe-negative cells. Cells doublets and higher order aggregates were excluded by plotting FSC area against FSC height. The various

control samples were then used to set gates to identify cells expressing a particular protein, or set of proteins.

Gene expression analysis by Taqman® Gene Expression Array Cards

Custom Taqman® Gene Expression Array Cards (Thermo Fisher Scientific) were designed to include target assay mixes for the endogenous control gene, *Gapdh*, and for all chemokines expressed by C57BL/6 mice that are known to directly regulate leukocyte migration (Table 2). For each sample, we combined cDNA transcribed from 1µg RNA, molecular grade water and Taqman® Universal Mastermix to give a final volume of 100µl and added this to the appropriate well of the array card. All cards contained negative controls in which cDNA was replaced with water or a –RT control sample: these samples consistently failed to generate any PCR product. Cards were centrifuged twice for 1min at 500g, before being placed in the ABI Prism 7900HT system using SDS Version 2.3 software. For analysis, DataAssist^{TM} Version 2.0 was used. In all analyses, the formula 2^{-Cq} was used to determine relative expression (Livak, 2001) in comparison to the *Gapdh*, and fold change calculated relative to ovary, which was given the arbitrary value of 1.

Determination of CCL27 protein concentration by ELISA

Ovary and uterus were removed from C57BL/6 mice, snap frozen in liquid nitrogen and stored at -80ºC. Tissues were weighed and Cell Lytic MT solution (Sigma) containing protease inhibitors (Roche) added (20ml per gram of tissue). Tissues were homogenised and lysates cleared of debris by centrifugation at 13000rpm for 10min at 4ºC. Supernatant was collected and stored at -20ºC. CCL27 concentration was measured using a DuoSet ELISA kit (R&D

systems) according to the manufacturer's instructions and is presented as amount of chemokine (pg) per mg of tissue.

Statistical Analysis

Differences between more than two groups were examined using the Kruskal-Wallis test, followed by a *post hoc* Dunn's test. Comparisons between two groups were determined using the Mann-Whitney Test. $p<0.05$ was considered statistically significant.

Results

The tissues of the FRT differentially express markers of specific leukocyte populations

First, to get an indication of differences in leukocyte content between tissues of the FRT, we compared their expression of genes encoding established markers of T cells (gamma polypeptide of CD3), B cells (CD19), natural killer (NK) cells (CD49b), DCs (CD11c), macrophages (F4/80), neutrophils (Neutrophil Granule Protein (NGP)) and eosinophils (Major Basic Protein (MBP)) in the ovary, uterus, cervix and vagina of virgin C57BL/6 mice (Table 1). Many of these proteins are widely used in flow cytometry and immunohistology to identify specific leukocyte populations in lymphoid and non-lymphoid tissues of mice and to our knowledge, there is no evidence to suggest that they are expressed by non-leukocytic cells in the FRT or elsewhere. Moreover, analysis of data from the Immunological Genome Project (www.immgen.org) confirmed the highly restricted expression of the genes encoding these proteins by the expected leukocyte subset (data not shown). However, it should be noted that macrophages can produce low to intermediate amounts of CD11c (Murray and Wynn 2011) and eosinophils can express F4/80 (Diener *et al.* 2016). With the exception of *Cd19*, all other genes were detectably expressed in the FRT (Figure 1 and data not shown). Moreover, there was clear evidence of variation in their expression between tissues of the FRT. Most notable was the uterus, which compared to other FRT tissues showed highest expression of the genes encoding F4/80, CD11c, MBP or CD3g (Figure 1A-D). The uterus, along with the cervix, also showed highest expression of *Itga2* (which encodes CD49B), the NK cell marker (Figure 1E). Compared to the vagina, the cervix had significantly higher expression of the genes encoding F4/80, CD11c, or MBP (Figure 1A-C), while the neutrophil marker *Ngp* was preferentially expressed by the vagina and cervix (Figure 1F). Thus, the tissues of the FRT vary in their expression of specific markers of major leukocyte subsets suggesting that, as expected, they vary with respect to their resident leukocyte populations.

Uterus and cervix contain diverse populations of lymphocytes and myeloid cells.

To explore the diversity of leukocyte content in the FRT, we performed flow cytometry on single cell suspensions of uterus and cervix (Figure 2). These two tissues showed the highest level, and greatest diversity, of leukocyte marker expression (Figure 1), and, in preliminary analysis, proved to be more amenable than ovary and vagina to analysis by flow cytometry (data not shown). To aid gate setting, and to act as positive controls, leukocyte-rich BM aspirates or single cell suspensions of the PALN (through which FRT lymph drains) were included in experiments examining myeloid cells or $CD3^+$ cells, respectively.

As expected, many BM cells and virtually all PALN cells expressed the pan-leukocyte marker, CD45. A proportion of cells isolated live from cervix and uterus were also $CD45^+$ (Figure 2). A large fraction of these FRT leukocytes carried CD11b, a protein expressed by all myeloid cells (Figure 2A). In BM, the $CD11b^+$ myeloid population can be split according to expression of F4/80 and Ly6C into $F4/80^+$ macrophages/eosinophils, $F4/80^-Ly6C^{hi}$ monocytes, an $F4/80^-$ $Ly6C^{lo}$ population (that contains mostly neutrophils and their precursors), and a small $F4/80^-Ly6C^-$ subset likely to contain other granulocytic cells. Equivalent subsets of $CD11b^+$ myeloid cells were also identifiable in the cervix and uterus, indicating that these tissues contain various populations of cells of the monocyte/macrophage and granulocytic lineages (Figure 2A).

Guided by parallel analysis of PALN, it was also clear that $CD3^+$ leukocytes were present in the uterus and the cervix (Figure 2B). As expected, most $CD3^+$ cells in the PALN were either $CD4^+$ or $CD8^+$, and equivalent populations of cells were also present in the uterus, although their expression of CD4 and CD8 was somewhat lower than that seen in the PALN. As expected, virtually all these $CD4^+$ and $CD8^+$ cells expressed a T cell receptor containing the alpha and beta chains (TCR$\alpha\beta$) (data not shown). As previously reported (Johansson, 2003),

there was also a significant population of $CD4^-CD8^-$ 'double negative' (DN) $CD3^+$ cells in the uterus. In the cervix, $CD4^+CD3^+$ cells were present, but $CD8^+CD3^+$ cells were rare, and most $CD3^+$ cells in this tissue were DN. $CD3^+CD4^+CD25^+$ cells were rare in PALN, uterus and cervix. Cells co-expressing CD3, CD4 and CD25 in mice are typically either regulatory T cells (Tregs) or recently-activated $CD4^+$ T helper cells. However, although previous work has shown that CD25 is almost always co-expressed with the Treg marker FoxP3 in $CD3^+CD4^+$ cells in the non-pregnant mouse uterus (Kallikourdis, 2007), expression of FoxP3 would need to be specifically analysed before the uterine and cervical $CD3^+CD4^+CD25^+$ cells we have detected can be definitively identified as FoxP3+ Tregs. Any FoxP3-negative $CD3^+CD4^+CD25^+$ cells present could be recently-activated $CD4^+$ T helper cells, $FoxP3^-$ Tregs, or another $FoxP3^-$ $CD3^+CD4^+CD25^+$ cell type. Consistent with previous work (Johansson, 2003), the abundant DN $CD3^+$ cells in uterus and cervix, but not those in the PALN, were $CD25^+$ (Figure 2B). Further analysis of the DN $CD3^+$ population in the FRT (data not shown) revealed that it contained some γδ T cells ($TCR\gamma\delta^+$) and NKT cells ($TCR\alpha\beta^+NK1.1^+$), but the majority (>60%) resembled the unconventional, extrathymically-derived, regulatory $TCR\alpha\beta^+$ T cells previously reported to be abundant within the murine FRT (Johansson, 2003).

Collectively, the gene expression and flow cytometry data shown in Figures 1 and 2 indicate that, under homeostatic conditions, the tissues of the FRT carry a diverse repertoire of leukocyte populations. In addition, comparisons of different tissues within the FRT provide evidence of regional specialisation in leukocyte recruitment and/or retention, which was to be expected given the differences in function, microbial exposure and immunological pressure that exist between the discrete components of the FRT.

>70% of chemokine genes are expressed in tissues of the FRT

Chemokines are likely to contribute to leukocyte trafficking into, within, and out of the tissues of the FRT. In order to gain an unbiased and comprehensive overview of constitutive chemokine expression in these tissues, Taqman® Gene Expression Arrays were used to analyse the expression of the 34 chemokine genes in C57BL/6 mice that are known to directly control leukocyte migration (Table 2). This was done using RNA prepared from ovary, uterus, cervix and vagina, and matched samples of skin, colon, and small intestine: these non-reproductive tissues are known to constitutively express several chemokine genes and chemokine-driven tissue-specific homing to these tissues is particularly well-defined, with known involvement of ligands for CCR4, 8, 9 or 10. Expression of *Gapdh*, an endogenous control gene, was used to normalise the data. Transcripts from the *Cxcl9*, *Cxcl10*, *Cxcl13*, *Cxcl15*, *Ccl19*, *Ccl24*, *Ccl25* and *Ccl26* genes were either not present or were barely detectable in any FRT tissue, despite, in most cases, being readily detectable in at least one of the three non-reproductive tissues (e.g. *Ccl25* in the small intestine; *Ccl24* in the skin) (data not shown). These genes were therefore excluded from further analysis. Remarkably, however, the other 26 chemokine genes (>70% of those analysed) were clearly expressed in one or more FRT tissue, particularly the uterus, cervix and vagina. These data are shown in Figures 3-5, and described in more detail below: chemokines have been clustered according to the nature of their expression profile within the FRT. Amongst FRT tissues, the ovary showed the lowest expression of virtually all the chemokines. Thus, for clarity and consistency, the mean expression of each chemokine in this tissue was given an arbitrary relative expression value of 1, and gene expression in all other tissues adjusted accordingly.

In the FRT, the uterus shows highest expression of many chemokines

The predominant chemokine gene expression profile we found was one in which the uterus had higher expression than other FRT tissues (Figures 3 & 4).

Four chemokines – *Ccl1*, *Ccl5*, *Ccl28*, and *Xcl1* – showed significantly higher expression in uterus than the other three FRT tissues (Figure 3). Most notable were *Xcl1* and *Ccl28*, which showed ~20-fold higher expression in the uterus than other FRT tissues. There was also a small (2-3-fold), but statistically significant, increase in *Ccl1* and *Ccl28* expression in vagina compared to cervix.

Six other chemokines (*Ccl2*, *Ccl6*, *Ccl9*, *Ccl12*, *Cxcl14*, and *Cxcl16*) were significantly higher in uterus than ovary and vagina. They also seemed to be more abundant in uterus than cervix, but differences between these two tissues were not statistically significant (Figure 4A). Of these six chemokines, *Ccl6*, *Cxcl14* and *Cxcl16* were expressed at a significantly higher level in cervix than vagina.

Expression of three other chemokines - *Cx3cl1*, *Cxcl12* and *Ccl8* - also appeared to be highest in uterus (Figure 4B). Statistical analyses revealed that uterus expressed more *Cx3cl1* than cervix and vagina, and more *Cxcl12* than vagina. *Ccl8* expression was not significantly different between uterus, cervix and vagina, but these tissues all expressed more *Ccl8* than ovary.

Comparisons to the non-reproductive tissues were also informative (Figures 3 & 4). The uterus expressed *Ccl1* and *Ccl8* to a level equivalent to the skin: CCL1 and CCL8, both ligands for CCR8, are known to control the trafficking of leukocytes in skin (Qu, 2004; Schaerli, 2004; Gombert, 2005; Islam, 2011; McCully, 2015). *Ccl6*, *Ccl28* and *Cx3cl1* were prominently expressed in the intestine, where indispensable functions for these chemokines or their receptors have been defined (Hieshima, 2004; Feng, 2006; Kotarsky, 2010; Hadis, 2011; Medina-Contreras, 2011; Schneider, 2015). *Ccl28* expression in the uterus was similar to that seen in the intestine, while *Ccl6* and *Cx3cl1* were only ~2-fold lower in uterus than intestine. Although there were no striking differences between skin, small intestine and colon with respect to expression of *Ccl2*, *Ccl9*, *Ccl12*, *Xcl1*, *Cxcl12*, *Cxcl14* and *Cxcl16*, it was notable

that these chemokines were all expressed in the uterus to a level that was similar to, or higher than, these non-reproductive tissues. Amongst the seven tissues examined, *Xcl1* could be classified as being the most uterus-specific chemokine. Finally, it is worth noting that, in addition to showing preferential expression in the uterus within the FRT, *Ccl5* was unexpectedly found to be ~10-fold more abundant in the small intestine than the colon.

Thus, of the many chemokine genes expressed in the FRT, 13 show evidence of preferential expression in the uterus. The chemokine expression profile of the cervix bore closest resemblance to the uterus. Several of the chemokines expressed by the uterus have well defined roles in the skin and intestine, and uterine expression levels were comparable to those seen in these non-reproductive tissues.

Identification of chemokines preferentially expressed in ovary, cervix and/or vagina

In addition to the 13 chemokine genes discussed above, another 13 were detectably expressed by the tissues of the FRT (Figure 5A-E). Although many of these were expressed in the uterus, expression by other FRT tissues matched or exceeded uterine expression, and a variety of distinct expression profiles were apparent.

Ccl3, *Ccl4*, *Ccl7* and *Ccl11* were each expressed at an equivalent level in uterus and cervix (Figure 5A). The vagina also expressed *Ccl3* and *Ccl4*, but there was a marked reduction in expression of *Ccl7*, and particularly *Ccl11*, between cervix and vagina. *Ccl17* and *Ccl22*, which encode the only chemokines able to activate the CCR4 receptor, had similar expression profiles within the tissues of the FRT, peaking in the cervix (Figure 5B). Each of these six chemokines was expressed by one or more FRT tissue at a level comparable to, or higher than, skin, colon and small intestine (Figure 5A-B).

Cxcl1, *Cxcl2*, *Cxcl5* and *Cxcl7*, which all encode ligands for the CXCR2 receptor, were also strongly expressed by the cervix (Figure 5C). Transcripts encoding CXCL1 were ~6-fold

more abundant in cervix than uterus and ovary, but this increased to 200-3,000-fold when *Cxcl2*, *Cxcl5* and *Cxcl7* were examined. The vagina also strongly expressed these three chemokines: *Cxcl2* expression was similar between the two tissues, although *Cxcl5* and *Cxcl7* transcripts were ~3-fold lower in vagina than cervix. Transcripts encoding these four chemokines were barely detectable in skin or intestine.

Ccl20, which encodes the only chemokine able to activate the CCR6 receptor, also showed preferential expression by cervix and vagina (Figure 5D). It was the only chemokine for which the vagina appeared to be richest source of expression, although variation in expression between individuals meant that the difference between cervix and vagina failed to achieve statistical significance.

Finally, there were two chemokines for which the ovary was not the lowest expressing tissue of the FRT. These were *Ccl21* and *Ccl27* (Figure 5E). Both were expressed at a similar level in ovary and uterus, but *Ccl21*, and particularly *Ccl27*, were more strongly expressed in ovary than cervix or vagina. The unexpected expression of *Ccl27* in the ovary was examined further by quantifying CCL27 protein by ELISA in lysates of ovarian and uterine tissue (Figure 5F). CCL27 was detected in the uterus, but even more of it was present in ovary lysates with, on average, ~110pg of CCL27 per mg of tissue.

Thus, a number of chemokine genes show broad expression within the FRT, or preferential expression in the cervix and vagina. The ovary typically shows the lowest level of chemokine gene expression, but it does have a relatively high abundance of *Ccl21* and *Ccl27* transcripts and produces appreciable amounts of CCL27 protein.

Changes in chemokine expression in the vagina during the estrous cycle

Next, to explore the impact of the estrous cycle on chemokine gene expression, the data for each individual tissue was split into four groups based on the cycle stage of the mice at the

time of tissue harvest i.e. proestrus, estrus, metestrus or diestrus. This had been determined by microscopic examination of vaginal lavage collected prior to dissection (Caligioni 2009). In uterus, skin, colon and small intestine, we did not detect any statistically significant changes in the expression of any of the chemokine genes over the course of the estrous cycle (data not shown). In the cervix, *Ccl7* was the only chemokine gene whose expression we found to detectably vary across the cycle, with a statistically significant reduction from proestrus to estrus (Figure 6A and data not shown). In the ovary, the few chemokines that showed relatively high expression in this tissue were unaffected by cycle stage, but there was a significant reduction in expression of *Ccl2* and *Ccl4* from proestrus to estrus (Figure 6B and data not shown). In contrast to these other FRT tissues, we readily detected differences in vaginal chemokine expression over the course of the estrous cycle (Figure 6C). *Ccl3*, *Ccl4*, *Ccl22*, *Cxcl1*, *Cxcl2*, *Cxcl5* and *Cxcl7* were all predominantly expressed in metestrus. Expression of many of these genes remained high during diestrus, before plummeting in proestrus and remaining low during estrus. In contrast, but as seen in the cervix, *Ccl7* expression in the vagina dropped as mice moved from proestrus into estrus.

Thus, chemokine gene expression in several FRT tissues can fluctuate over the course of the estrous cycle, but it is in the vagina where these changes are most readily observed.

Discussion

Homeostatic leukocyte trafficking into, within and out of the tissues of the FRT contributes to reproductive health and fertility. We hypothesised that each FRT tissue contains a unique chemokine gene expression profile, components of which might fluctuate with cyclical changes in reproductive hormones. Our data support this hypothesis, and highlight the complexity of chemokine networks in the FRT.

There were clear differences between FRT tissues in their expression of genes encoding markers of specific leukocyte populations. Although these data do not provide an absolute quantitation of leukocyte abundance, they do indicate that there are marked differences in leukocyte content between the tissues of the FRT. Our flow cytometry data further illustrates the complex nature of this leukocyte content. For example, the $CD25^{+}CD3^{+}$ population in uterus and cervix contains up to five distinct cell types and there are also diverse populations of myeloid cells ($CD45^{+}CD11b^{+}$ cells) in these tissues, consistent with a recent report identifying at least nine distinct leukocyte populations in mouse uterus (Diener, 2016). These lymphocytes and myeloid cells, or their precursors, will have been recruited from the blood and then subject to interstitial cues that control localisation, retention and egress. Based on precedents established in non-FRT tissues, chemokines will play a major role in these processes.

We found that 26 chemokine genes are detectably expressed in the FRT of 8-week old virgin C57BL/6 mice housed in specific pathogen-free conditions: it will be of interest to investigate if chemokine expression profiles vary between strains and over time (e.g. prior to sexual maturation, or during and after pregnancy). We found that the uterus is the dominant site of chemokine expression, but most chemokines are also expressed in other FRT tissues. However, *Ccl1*, *Ccl5*, and particularly *Ccl28* and *Xcl1*, appear to be largely restricted to the uterus so may mediate migration specifically to this site. Their function in this context remains

largely unexplored. CCL5 might have indispensable roles in the uterus, although other chemokines expressed in this tissue (CCL3, CCL4, CCL6, CCL7, CCL9, CCL11 and CCL12) can activate one or more of the CCL5 receptors (CCR1, CCR3 and CCR5) so might be capable of doing the same job. Indeed, the complex interactions between these chemokines and their receptors may have evolved to ensure that responses cannot be readily disrupted by pathogen evasion strategies or host genetic variation. However, genetic deficiency in a single chemokine, *Ccl11*, is sufficient to disrupt eosinophil recruitment to the uterus, despite it expressing several other genes, including *Ccl5*, that encode chemokines capable of inducing leukocyte migration through CCR3, the dominant CCL11 receptor on eosinophils. *Ccl11* deficiency also delays the onset of puberty, although estrous cycle and reproduction are not affected (Gouon-Evans, 2001).

CCL1, CCL28 and XCL1, encoded by other chemokine genes expressed primarily by the uterus in the FRT, direct leukocyte trafficking through CCR8, CCR10 and XCR1, respectively. CCR8 and CCR10 are also activated by CCL8 and CCL27, respectively: the genes encoding these chemokines are also expressed in uterus. CCR10 directs IgA-producing antibody-secreting cells (IgA ASCs) to the gut wall driven by the high expression of *Ccl28* in the small intestine and colon (Pan 2000; Kunkel 2003; Lazarus, 2003; Hieshima, 2004; Ogawa, 2004; Feng, 2006). IgA ASCs generated by intranasal or intravaginal immunisation can home to the uterus, but not cervix or vagina, and CCL28 neutralisation attenuates this process (Cha, 2011). *Ccl27* expressed in the uterus might contribute to this process.

CCR10, along with CCR8, might also direct T cell homing to the uterus: they have both been implicated in the trafficking of leukocytes, particularly T cells, into and within the skin of humans and mice during homeostasis and inflammation (Reiss, 2001; Homey, 2002; Soler, 2003; Qu, 2004; Schaerli, 2004; Gombert, 2005; Sigmundsdottir, 2007; Islam, 2011; McCully, 2012; Nagao, 2012; McCully, 2015). Our data show that uterus expresses *Ccl1* and *Ccl8* at a

level equivalent to skin. To our knowledge there have been no studies exploring the function of CCR8 and its ligands in the uterus. This is also the case for XCL1. XCR1 is the only receptor for XCL1 and has no other known ligands. It is highly restricted to a subset of DCs that cross-present antigens to $CD8^+$ T cells to initiate immune responses to viruses or tumours (Dorner, 2009; Crozat, 2010; Crozat, 2011). Mice lacking XCR1 or XCL1 show diminished $CD8^+$ T cell responses to infection (Dorner, 2009). The XCL1/XCR1 axis also maintains intestinal homeostasis by controlling T cell and DC abundance in the tissue (Ohta, 2016). We speculate that XCL1/XCR1 serves a similar homeostatic function in the uterus, and will contribute to anti-viral immune responses in the FRT. Interestingly, male antigens in seminal fluid can be cross-presented to $CD8^+$ T cells (Moldenhauer, 2009) and generate tolerogenic $CD4^+$ Tregs (Robertson, 2009; Guerin, 2011), so XCL1 might play important roles in preparing the uterus for implantation. CCL21 is also likely to be important in this regard. Acting through CCR7, it directs DC egress from tissues into lymphatic vessels (Girard, 2012), after which the DCs travel to lymph nodes where they activate or tolerize naïve T cells.

In contrast to previous studies (Gouon-Evans, 2001; Kallikourdis, 2007; Hickey, 2013b; Yip, 2013), we did not find any statistically significant changes in chemokine mRNA levels in the uterus over the course of the estrous cycle, perhaps because of variation in mouse age, housing conditions or strain (we used C57BL/6 rather than CD-1 (Yip, 2013) or 129/SvEv (Gouon-Evans, 2001)), or because any differences have been masked by our use of the whole tissue, rather than its individual component parts. It is also possible that our study did not have the power to detect small differences in gene expression between phases, which are more likely to be revealed when only two phases of the cycle are compared (Yip, 2013). It is also worth noting that although Diener and colleagues recently reported fluctuations in uterine leukocyte populations across the estrous cycle, these failed to achieve statistical significance (Diener *et al.* 2016) so a major change in chemokine expression across the cycle may not be required.

Ccl21 and *Ccl27* are the only chemokine genes that are expressed at a higher level in ovary than other FRT tissues. The most likely function of CCL21 is to mediate DC migration out of the ovary, but roles for CCL27 are harder to predict. An important first step will be to identify cells in the ovary carrying functional receptors for this protein.

A number of chemokine genes showed highest expression in cervix and/or vagina, including those encoding the CCR4 ligands CCL17 and CCL22, whose expression peaked in the cervix. CCR4 can direct T cell homing to skin (Campbell, 1999): expression of *Ccl17* and *Ccl22* in the cervix was at least twice that of skin. These chemokines may therefore contribute to the recruitment of the various T cell populations present in the cervix. Our finding that *Ccl20* is highly expressed in the vagina is consistent with studies showing CCL20 protein in mouse vaginal secretions (Hicke, 2013a). CCL20, which activates CCR6, has been proposed to drive the recruitment of Langerhans cell precursors into the human vaginal epithelium (Cremel, 2005) and the keratinocyte layer of human skin (Charbonnier, 1999; Le Borgne, 2006). CCL20 in the vaginal fluid of Balb/c mice peaks during diestrus, and its secretion is suppressed by estradiol (Hickey, 2013a). *Ccl20* transcripts were not measured in this study, and, using C57BL/6 mice, we did not find any differences in *Ccl20* expression in the vagina across the estrous cycle. The effect of estradiol on CCL20 secretion may therefore occur post-transcriptionally, although *Ccl20* expression might be regulated differently in Balb/c and C57BL/6 mice. The presence of CCL20 in vaginal secretions is interesting because high concentrations have direct antimicrobial properties (Yang, 2003; Guesdon, 2015). In fact, many other chemokines expressed in the FRT exhibit antimicrobial properties (Wolf, 2012) and could conceivably regulate the vaginal microbiome.

The other chemokine genes preferentially expressed in cervix and vagina encode ligands for CXCR2, a receptor that directs neutrophil migration. Vaginal expression is profoundly influenced by estrous cycle stage, each peaking in metestrus and diestrus, before

dropping dramatically as mice enter proestrus. At metestrus, neutrophils enter the vaginal epithelium and lumen: neutralisation of CXCR2 profoundly inhibits this process (Sonoda, 1998), and neutrophil depletion results in a block at diestrus, suggesting neutrophil recruitment to the FRT is critical for progress through the estrous cycle (Sasaki, 2009). CXCR2, and hormonal regulation of its ligands, is also critical in regulating neutrophil migration into the vaginal wall and lumen during *Candida albicans* infection (Lasarte, 2016) so CXCR2 appears to play major physiological and immunological roles in the vagina.

Expression of other chemokines was also affected by the estrous cycle. In vagina, *Ccl3*, *Ccl4* and *Ccl22* transcripts peaked during diestrus and/or metestrus, while *Ccl7* was suppressed in estrus in both cervix and vagina. *Ccl3*, *Ccl4* and *Ccl7* encode chemokines that bind to CCR5, but CCL3 and CCL7 can also bind to other receptors. These various receptors are expressed on neutrophils, monocytes, macrophages, NK cells, and some T cell populations, and further investigations are required to determine if they drive fluctuations in leukocyte trafficking over the course of the estrous cycle.

Estrus was associated with a reduction in the expression of *Ccl2* and *Ccl4* in the ovary. Macrophages, which can be derived from blood monocytes, are the most abundant leukocyte in the ovary, and their presence peaks during proestrus and metestrus (Petrovska, 1996). CCL2, by binding CCR2, is central to monocyte trafficking in mice, and CCL4 can also contribute so they could contribute to cyclical changes in ovarian macrophages by driving monocyte recruitment. Ovarian macrophages serve a variety of functions (Wu, 2004), and their depletion in mice leads to disruption of ovarian vasculature (Turner, 2011).

In conclusion, our study has revealed the complex chemokine expression profiles of mouse FRT tissues under homeostatic conditions. This provides a framework for future investigations aimed at defining critical molecular mechanisms responsible for directing the trafficking and localisation of specific leukocyte populations in these tissues. It is also

important to note that while the principal function of chemokines is to drive the migration of leukocytes into, within, and out of tissues, chemokines can also control other aspects of leukocyte behaviour and function, as well as regulating the biology of tissue-resident cells, such as endothelial, epithelial and mesenchymal cells, particularly when homeostasis is perturbed (Hughes, 2018). It will therefore be important to determine if chemokines in the FRT do more than direct leukocyte trafficking. The availability of experimental tools means the function of chemokines in the FRT will be easiest to dissect in mice, but key mechanisms are likely to be conserved in other species so such studies will no doubt improve understanding of physiological and pathophysiological processes in the human FRT.

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Figure Legends

Figure 1: Comparative expression of leukocyte markers in the tissues of the FRT. RNA was isolated from the ovary (n=20), uterus (n=20), cervix (n=19) and vagina (n=20) of virgin C57BL/6 mice. The RNA was converted into cDNA, and QPCR was used to compare expression of **(A)** *Emr1*, **(B)** *Itgax*, **(C)** *Prg2*, **(D)** *CD3g*, **(E)** *Itga2* and **(F)** *Ngp*. The proteins encoded by these genes are indicated in parentheses. Expression of the *Gapdh* control gene was used to normalise the data. Data show mean relative expression ± S.E.M. with expression by ovary set to 1. The statistical significance of differences between FRT tissues was determined using the Kruskal-Wallis test followed by a *post hoc* Dunn's test. *p<0.05, **p<0.01, ****p<0.0001.

Figure 2: Uterus and cervix contain diverse populations of lymphocytes and myeloid cells. Single cell suspensions of uterus, cervix, bone marrow and PALN from virgin C57BL/6 mice were immunostained with fluorescently labelled antibodies and examined by flow cytometry. Cell debris, dead cells and cell doublets/aggregates have been excluded from all data shown. **(A)** Myeloid cells. Left panels: Histogram plots showing expression of CD45 on live single cells. Middle panels: Histogram plots showing expression of CD11b by $CD45^+$ cells. The dotplots show expression of F4/80 and Ly6C by $CD45^+CD11b^+$ cells ($F4/80^+$, $Ly6C^{hi}$ and $Ly6C^{lo}$ populations are labelled). **(B)** $CD3^+$ cells. Left panels: Histogram plots showing expression of CD45 on live single cells. Adjacent panels: Histogram plots showing expression of CD3 by $CD45^+$ cells. The dotplots show expression of CD8 and CD4 by $CD45^+CD3^+$ cells. Right panels: Overlaid histogram plots showing CD25 expression by $CD45^+CD3^+CD4^+$ and $CD45^+CD3^+$CD4-CD8- cells. In both A and B, the numbers on the histograms and dotplots show the percentage of cells that fall within the region indicated by the horizontal line

(histograms) or within each box (dotplots). Data are representative of experiments repeated on at least four occasions with 3 or more mice per group.

Figure 3: Four chemokines show higher expression in the uterus than all other FRT tissues. Comparative expression of *Ccl1*, *Ccl5*, *Ccl28* and *Xcl1* in ovary (n=20), uterus (n=20), cervix (n=19), vagina (n=20), skin (n=20), colon (n=20) and small intestine (n=20) of virgin C57BL/6 mice. Expression of the *Gapdh* control gene was used to normalise the data. Data show mean relative expression ± S.E.M. with expression by ovary set to 1. The statistical significance of differences between tissues was determined using the Kruskal-Wallis test followed by a *post hoc* Dunn's test. Only statistically significant differences between FRT tissues are shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 4: Nine other chemokines show preferential expression in the uterus. Comparative expression of **(A)** *Ccl2*, *Ccl6*, *Ccl9*, *Ccl12*, *Cxcl14* and *Cxcl16*, and **(B)** *Ccl8*, *Cxcl12* and *Cx3cl1* in ovary (n=20), uterus (n=20), cervix (n=19), vagina (n=20), skin (n=20), colon (n=20) and small intestine (n=20) of virgin C57BL/6 mice. Expression of the *Gapdh* control gene was used to normalise the data. Data show mean relative expression ± S.E.M. with expression by ovary set to 1. The statistical significance of differences between tissues was determined using the Kruskal-Wallis test followed by a *post hoc* Dunn's test. Only statistically significant differences between FRT tissues are shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 5: Thirteen other chemokines are preferentially expressed in ovary, cervix and/or vagina. Comparative expression of **(A)** *Ccl3*, *Ccl4*, *Ccl7* and *Ccl11*; **(B)** *Ccl17* and *Ccl22*; **(C)** *Cxcl1*, *Cxcl2*, *Cxcl5* and *Cxcl7*; **(D)** *Ccl20*; and **(E)** *Ccl21* and *Ccl27* in ovary (n=20), uterus (n=20), cervix (n=19), vagina (n=20), skin (n=20), colon (n=20) and small intestine (n=20) of

virgin C57BL/6 mice. Expression of the *Gapdh* control gene was used to normalise the data. Data show mean relative expression ± S.E.M. with expression by ovary set to 1. The statistical significance of differences between tissues was determined using the Kruskal-Wallis test followed by a *post hoc* Dunn's test. Only statistically significant differences between FRT tissues are shown. **(F)** ELISA-based quantitation of CCL27 protein in lysates of ovary and uterus (n=12), with statistical differences determined using the Mann-Whitney test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 6: Chemokine expression can vary with estrous cycle stage, particularly in the vagina. Comparative expression of **(A)** *Ccl7* in the cervix; **(B)** *Ccl2* and *Ccl4* in the ovary; and **(C)** *Ccl3*, *Ccl4*, *Ccl7*, *Ccl22*, *Cxcl1*, *Cxcl2*, *Cxcl5* and *Cxcl7* in the vagina of virgin C57BL/6 mice determined to be at P=Protestrus (n=5), E= Estrus (n=7), M=Metestrus (n=5) or D=Diestrus (n=3). Expression of the *Gapdh* control gene was used to normalise the data. Data show mean relative expression ± S.E.M. with expression at proestrus set to 1. The statistical significance of differences between estrous cycle stages were examined using the Kruskal-Wallis test followed by a *post hoc* Dunn's test. *p<0.05, **p<0.01.

Figure 1

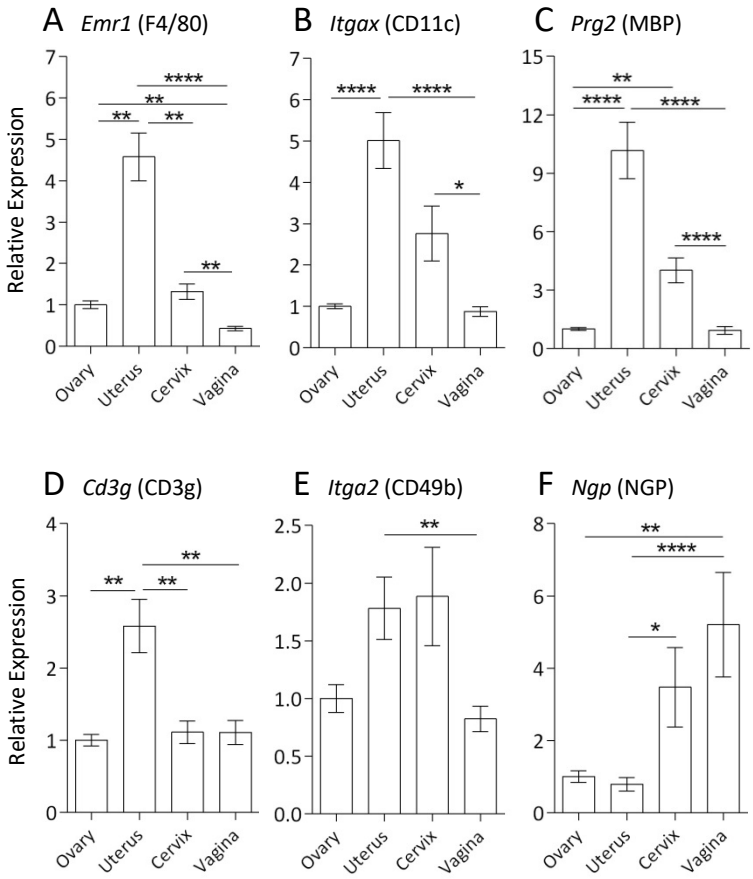

A Emr1 (F4/80)
B Itgax (CD11c)
C Prg2 (MBP)
D Cd3g (CD3g)
E Itga2 (CD49b)
F Ngp (NGP)
Relative Expression
Ovary
Uterus
Cervix
Vagina

Figure 2

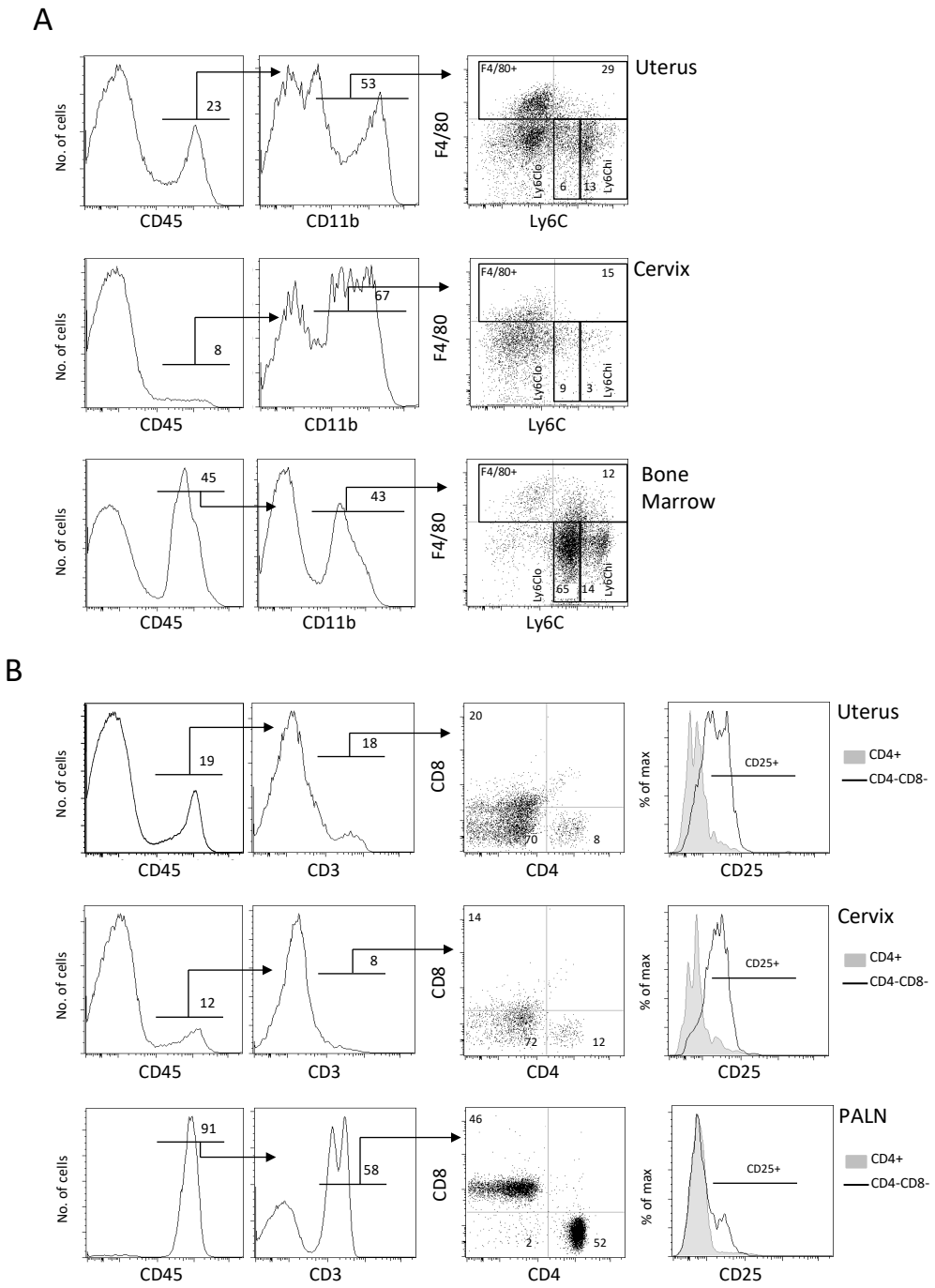

A
No. of cells
CD45
CD11b
F4/80
Ly6C
F4/80+
Ly6Clo
Ly6Chi
Uterus
Cervix
Bone Marrow
B
CD3
CD8
CD4
% of max
CD25
CD25+
CD4+
CD4-CD8-
PALN

Figure 3

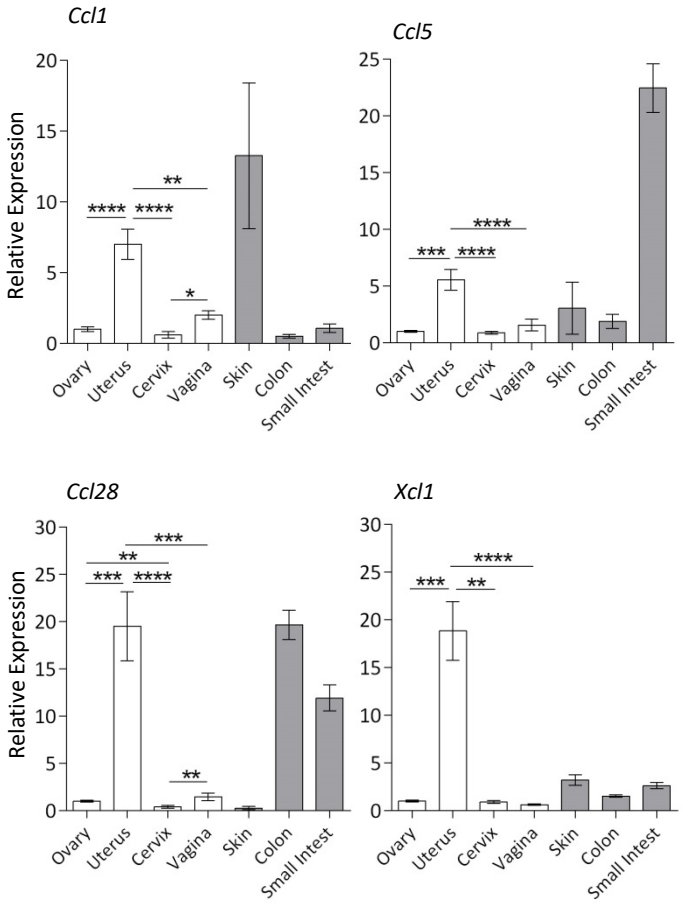

Ccl1
Ccl5
Ccl28
Xcl1
Relative Expression
Ovary
Uterus
Cervix
Vagina
Skin
Colon
Small Intest

Figure 4

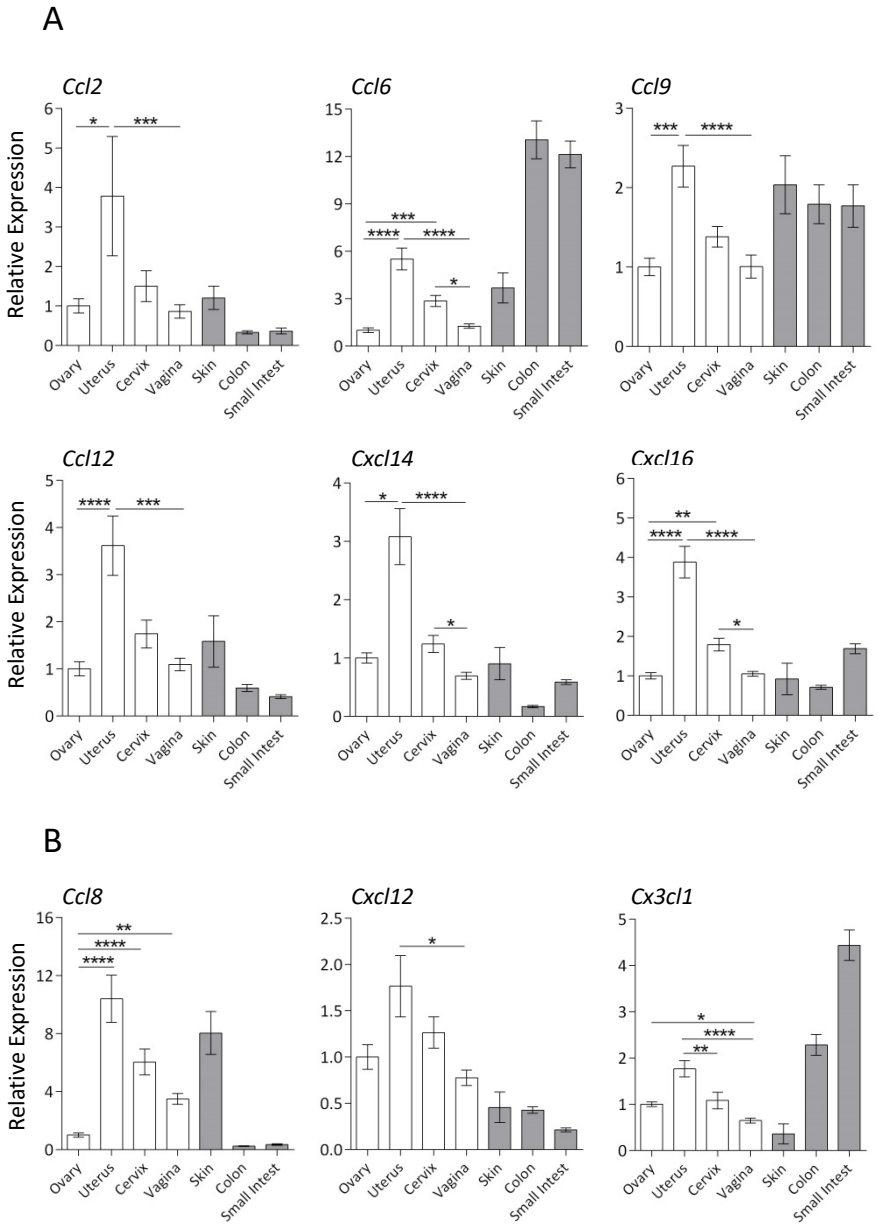

A
Ccl2
Ccl6
Ccl9
Ccl12
Cxcl14
Cxcl16
Relative Expression
Ovary
Uterus
Cervix
Vagina
Skin
Colon
Small Intest
B
Ccl8
Cxcl12
Cx3cl1

Figure 5

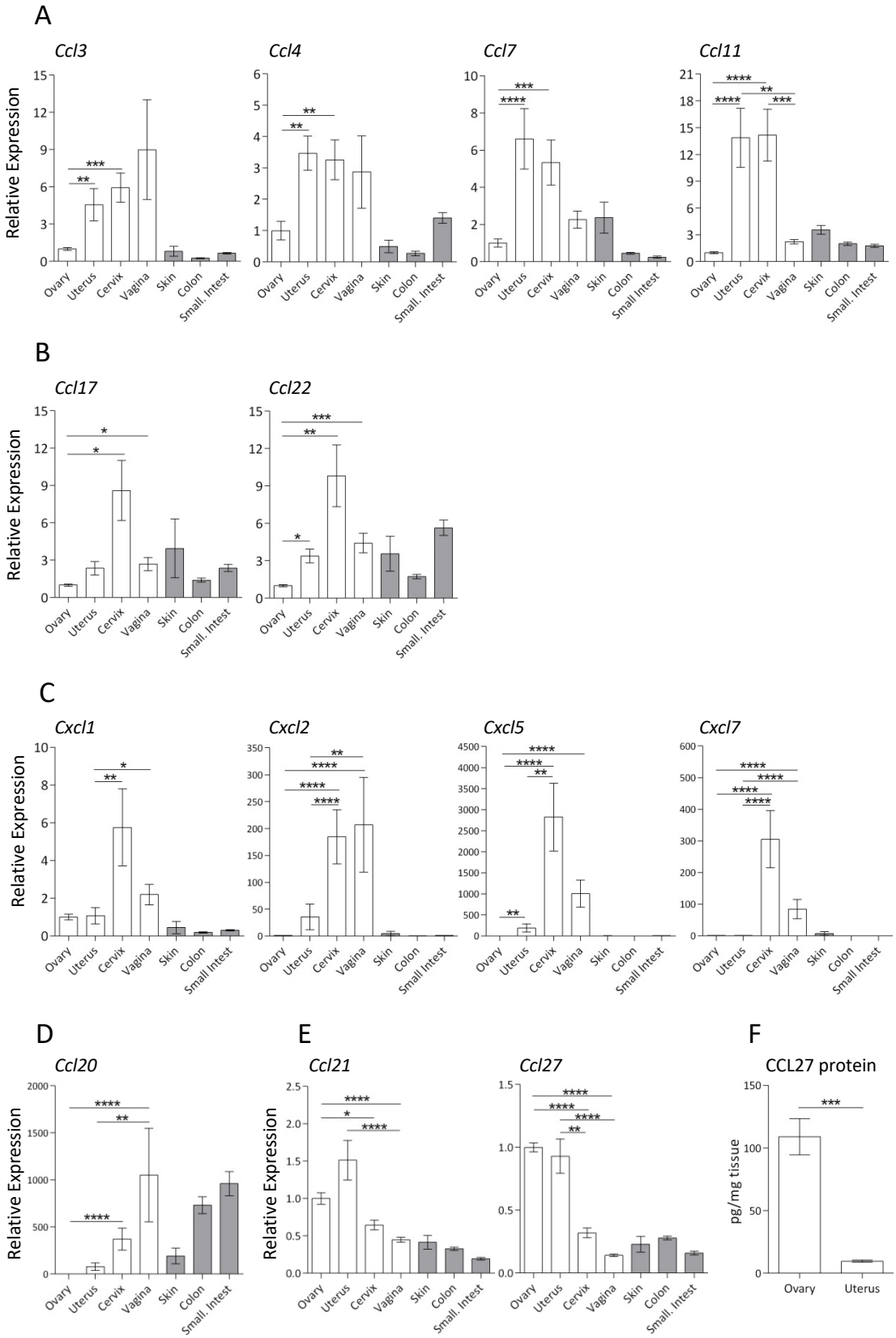
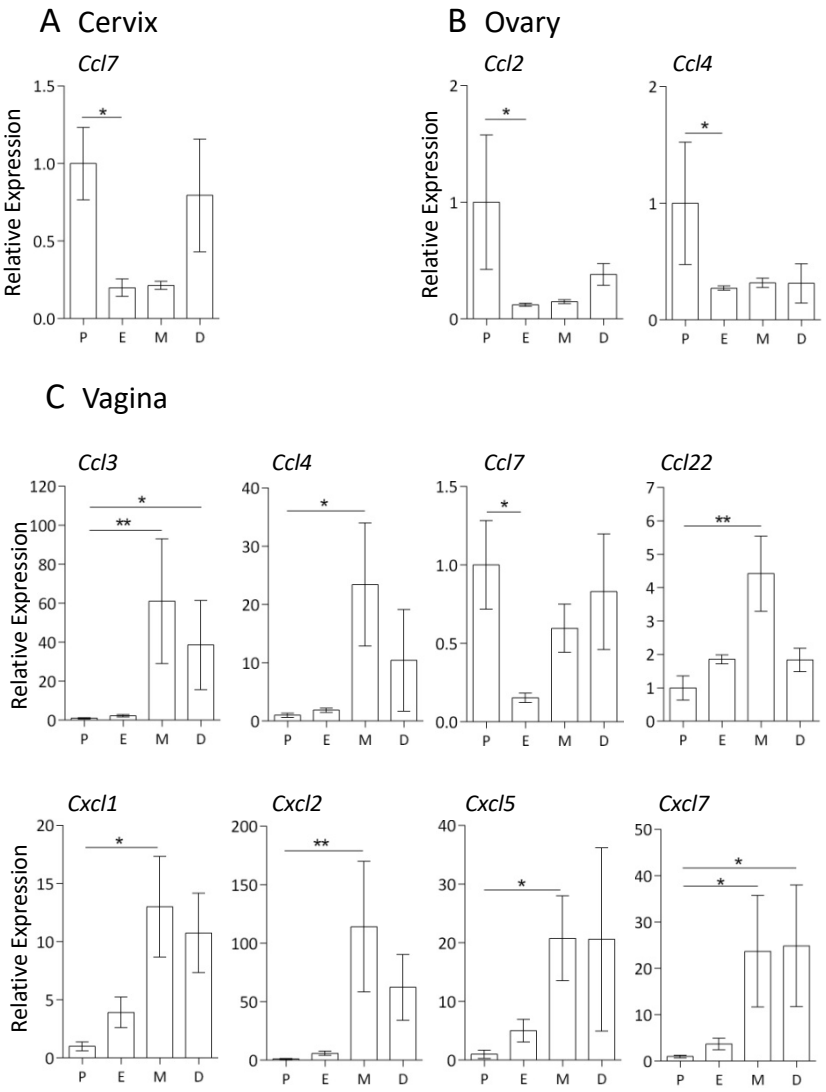

A
Ccl3
Ccl4
Ccl7
Ccl11
Relative Expression
Ovary
Uterus
Cervix
Vagina
Skin
Colon
Small. Intest
B
Ccl17
Ccl22
C
Cxcl1
Cxcl2
Cxcl5
Cxcl7
Small Intest
D
Ccl20
E
Ccl21
Ccl27
F
CCL27 protein
pg/mg tissue

Figure 6


A Cervix
Ccl7
Relative Expression
B Ovary
Ccl2
Ccl4
C Vagina
Ccl3
Ccl4
Ccl7
Ccl22
Cxcl1
Cxcl2
Cxcl5
Cxcl7
P E M D