Characterisation of antigen-presenting cells in the murine female reproductive tract and its draining lymph nodes

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Abstract

Despite the global burden of sexually transmitted diseases, the immunology of the female reproductive tract is poorly understood. An understanding of how the distribution of antigen presenting cells in the tissue and cross talk between cell types both at the site of antigen uptake and in the draining lymph nodes will be important for the design of new tools to manipulate local immune responses. The murine estrous cycle is characterised by large changes in the architecture of the vaginal and cervical epithelia in response to hormonal changes. Here, I show that the distribution of APCs in the epithelia of the vagina and cervix is not uniform. Neutrophils infiltrate the tissue in large numbers during the progesterone high stages of the murine estrous cycle, but this does not affect DC localisation. The iliac lymph nodes that drain the reproductive tract are structurally similar to other mucosal lymph nodes, but DCs are reduced in frequency compared to LNs draining other sites. RALDH expression, a marker for retinoic acid metabolism was also reduced in iliac compared to other peripheral lymph nodes. The murine lower female reproductive tract and its draining lymph nodes are, therefore, distinct from other mucosal tissues and lymph nodes and warrant further investigation.

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Acknowledgements

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Author's Declaration

I declare that this is my own, original work with the exception for Figure 3.2A, Figure 3.3A and B, Figure 3.6A in which staining and imaging were done by Katrein Schäfer as well as all work for Figure 4.5.

1. Introduction

Sexually transmitted infections (STIs) are a worldwide health problem [1, 2]. In 1999 there were 340 million new cases of STIs worldwide [3]. This equates to a huge burden of disease. They cause high mortality; AIDS related deaths account for 1.8 million deaths globally per year [4]. STIs can also cause complications such as infertility and spontaneous abortion and increase risks of developing other diseases; for example 95% of cervical cancers are associated with previous HPV infection [5]. STIs are a growing problem with rates of new infections going up in the UK [6].

Protective immune responses against STIs may rely on long-lived, effective local responses in the female reproductive tract (FRT) to prevent infection. However, these have proven difficult to manipulate, as both systemic and intravaginal immunization strategies are unreliable at initiating protective immune responses in the FRT [7, 8]. Despite the need for comprehensive research, immunity in the female reproductive tract (FRT) remains under-studied.

1.1. Defence of mucosal tissues

The mucosal surfaces of the gastro-intestinal, respiratory and genito-urinary tracts are the main sites of pathogen entry. They must act as barriers to potential pathogens [9], but are also populated by harmless, and in some cases beneficial, commensal organisms [7, 9-11]. Immunity in mucosal tissues must balance prevention of disease with tempered responses to the vast majority of microorganisms without disrupting the normal functioning of the mucosal tissues [12]. In mucosal tissues the majority of antigen elicit tolerogenic responses, which are characterised by regulatory T cells (Tregs) and anti-inflammatory cytokines such as IL-10 and TGF-β [13].

Mucosal tissues facilitate the acquisition of essential nutrients, water and oxygen from the environment and, therefore, must balance the need to allow passage of some molecules while preventing the invasion of potential pathogens. To limit pathogen invasion, mucosal secretions contain a variety of antimicrobial agents. For example there are antimicrobial peptides in saliva, urine, intestinal fluid [14],

seminal plasma [15] and cervicovaginal fluid [2, 9, 12, 14]. The stomach produces acid and the cervicovaginal fluid is also slightly acidic [2, 16]. Mucosal tissues produce a great deal of mucus [2, 12], which captures organisms. Mechanical activity, such as the mucociliary escalator in the lung [17], helps to eliminate many pathogens.

There are many commensal organisms living on mucosal tissues. Some are beneficial, as in the microbiota of the gastro-intestinal tract [18, 19] and *Lactobacilli* [20, 21] in the genitourinary tracts [2]. Commensals may compete with pathogens for attachment to mucosal surfaces, restricting their colonisation, however the normal flora is not always beneficial. *Candida* species, which are normally non-invasive commensals can cause invasive infections in response to changes in the vaginal environment brought on by hormonal changes [22].

1.2. Initiation of the immune response

Cells of the immune system can be divided into innate and adaptive immune cells. Innate phagocytic cells such as macrophages and neutrophils, which engulf and destroy pathogens, recognise broad categories of pathogen and respond rapidly to infection. Adaptive cells, such as T and B cells recognise, species or strain specific protein antigens and tailor the immune response to that particular organism. They mediate more efficient responses both qualitatively and quantitatively to such an extent that they can confer life-long systemic protection from re-infection. There are large numbers of T and B cells, each with different antigen specificities.

At mucosal sites antigen-presenting cells (APCs) survey the local environment for signs of infection and tissue damage. Many cell types can be APCs including macrophages, B cells and neutrophils [23-25]. Dendritic cells (DCs) are phagocytic cells that bridge the gap between the innate and adaptive immune systems. They are ‘professional’ antigen presenting cells that reside within tissues and lymph nodes. Their primary function is to acquire ‘foreign’ peptide antigen and initiate the adaptive immune response. They express an array of pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs) [26, 27], that have a broad specificity for highly conserved pathogen associated molecular patterns (PAMPs) [2]. Acquisition

of antigen and ligation of PRRs results in DC maturation and migration to draining lymph nodes (dLN) where they present antigen to naïve T cells.

T cells recognise a specific peptide antigens bound to major histocompatibility complex (MHC) on APCs. APCs express co-stimulatory molecules, such as CD40 which lower the threshold for activation. They also direct the homing of T cells to different tissues. For example APCs induce T cell homing to mucosal tissues by production of retinoic acid, which induces the upregulation of mucosal addressins (discussed further in chapter 5). Finally, APCs produce cytokines that direct T cell differentiation into functionally different subtypes of effector T cells, which mediate different types of immune response. After clearance of infection some antigen-specific memory T cells patrol the mucosal sites and LNs ready to respond to re-infection [28].

1.3. Effects of the local tissue environment on APCs

There is increasing appreciation within the field of immunology of the role played by non-haematopoietic cells in all stages of immunity [29-39]. DC maturation is affected by the local environment. Cytokines, stress molecules and cellular interactions can all affect DCs [27]. Epithelial cells of the oral mucosa [33], gut [11, 19], FRT [40, 41] and lungs [32, 42], as well as epidermal and endothelial cells [39] also express PRRs and on ligation of those PRRs they can produce chemokines, which recruit APCs and other immune cells, and cytokines, that can influence the maturation of DCs [11, 32, 42]. APCs are also sensitive to molecular markers produced by damaged epithelial cells, which affect maturation and cell recruitment. These can be cytokines, chemoattractants, alarmins such as HMGB1 or heat shock protein which are produced by infected or damaged cells, or intracellular molecules, which are only found outside cells after necrotic cell death [31, 43]. There are different stress indicator molecules produced after different severities of epithelial damage, which bias the immune response in different ways; promoting a regulatory response after mild damage and an inflammatory response after severe damage [31, 32]. Epithelial cells in the intestine condition DCs and macrophages towards tolerogenic responses to commensal bacteria in order to maintain gut homeostasis [11].

It is important not to view immune responses in terms of interactions among innate and adaptive immune cells and pathogens on a background of tissue stromal cells, but to think of the functioning of the organ and its resident stromal cells and how it recognises pathogens, recruits and directs immune cells and resolves infection while retaining its normal functioning. If the small intestine is infected the main event is not the immune response to the infection, but the continuing absorption of food. It is therefore important to understand the immune response in the context of the tissue it occurs within.

1.4. Characteristics of mucosal immune responses

The majority of antigen that enters the body through mucosal surfaces induces a tolerance response. This has been best characterised by the immunosuppressive and anergic responses to orally administered antigen - so called oral tolerance [44]. The antibody response at mucosal surfaces is usually dominated by IgA [13] with lower levels of IgG and IgE [7]. The predominant antibody in serum is IgG [8]. In the small intestine B cells switch to IgA production in the Peyer's patches (PPs) under the influence of TGF-β and IL-10 [13].

T cells primed in the small intestine express distinct mucosal addressins and chemokine receptors, which allow them to traffic to the intestinal mucosa. T cells from the MLNs upregulate $\alpha_4\beta_7$ integrin, which binds MAdCAM-1 expressed on mucosal vasculature. T and B cells express CCR9, which binds CCL25 expressed by intestinal epithelial cells [7, 13].

In PPs and MLNs there are distinct phenotypes of DCs. There are unusually large numbers of $CD8\alpha^-CD11b^-$ DCs and lower percentages of $CD8\alpha^-CD11b^+$ DCs compared to the spleen where the majority of DCs are $CD8\alpha^-CD11b^+$ [13]. $CD8\alpha^-CD11b^+$ DCs in the PPs preferentially produce IL-10 compared to splenic $CD8\alpha^-CD11b^+$ DCs, which produce IL-12 under the same conditions. DCs in the MLNs produce IL-10 and TGF-β in response to oral antigen and promote upregulation of $\alpha_4\beta_7$ on T cells [13].

1.5. Challenges of studying immunology in the FRT

The tissue of the lower FRT consists of layers of squamous epithelial cells, similar to skin, with connective tissue underneath (Fig 1.1) [28, 45]. The epithelia are covered in mucus. In humans the epithelia is non-keratinized, but in mice the outer layers become keratinized [45, 46].

The FRT is a unique mucosal environment. The FRT undergoes homeostatic remodelling in response to sex hormones in mammalian reproductive cycles, which results in dramatic cyclic changes in the epithelia and lamina propria of the uterus, cervix and vagina. The FRT must prevent infection while at the same time promoting conception and gestation of the genotypically different foetus. This presents a challenge for the immune system. The issue of balancing the reproductive function against protection from disease is particularly problematic after ovulation. The FRT must become permissive to sperm to increase the chance of fertilization, but sexual intercourse is also when the female is at highest risk of infection by sexually transmitted pathogens. The environment of the vagina changes to allow insemination. It becomes less acidic, the mucus becomes less thick and more permissive [47].

Unlike other mucosal sites, the FRT tissue and the tissue resident immune cells are similar to those of the skin. There are Langerhans' cells (LCs) in the epithelia with a separate DC population in the lamina propria. As has been shown in the skin [48, 49], different DC subtypes have different roles in immunity [50]. Submucosal DCs rather than the LCs, migrate to the draining lymph nodes and present antigen to T cells [50]. The humoural immune response in the FRT is different to other mucosal surfaces. Like the gut, the FRT has IgA in secretions; however unlike the gut there are much higher levels of IgG in cervicovaginal secretions [8, 40, 51-54]. IgG in cervicovaginal secretions is produced locally by antigen secreting cells and comes from the blood [8, 40, 51-54].

There is some evidence that hormones and their effects on stromal and epithelial cells in the FRT can affect immune responses [40, 55-60] through changes in PRR expression [61], cell recruitment, antibody levels, antigen presentation[40, 55, 58,

59, 62], T cell responses and susceptibility to STDs [58-60] over the estrous cycle or after hormone treatment (discussed further in chapter 4).

Like in the intestinal mucosa, the FRT can induce tolerance [63], but it appears to be estrous cycle stage specific. There was no difference in antigen-trafficking to the draining lymph nodes or in the serum antibody response at different cycle stages, but spleen and lymph node cells also showed significantly less proliferation in response to restimulation after priming in estrus [63].

1.6. Aims

Relative to other mucosal sites little is known about the immunology of the genital tract. The changeable conditions in the FRT pose a challenge to experimental design. Rats & mice have different reproductive cycles to humans and therefore questions remain about their suitability for translational research.

The aim of this study was to characterise the murine FRT in steady state to identify some of the factors which affect the initiation of the immune response to antigen. Specifically;

i. To use immunohistochemistry to characterise the FRT at different stages of the cycle.
ii. To examine the distribution of antigen presenting cells in the vagina and cervix
iii. To compare the FRT dLNs (iliac LNs) to other mucosal/non-mucosal LNs to try to identify factors, which may affect antigen presentation and the downstream adaptive immune response.

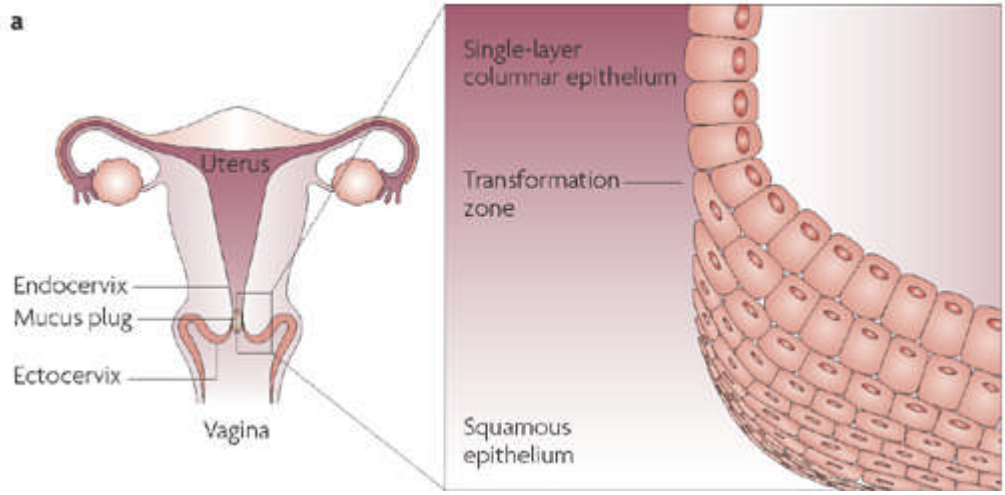

Figure 1.1: The human female reproductive tract.

The FRT can be split into two: The lower FRT, consisting of the vagina and ectocervix and the upper FRT consisting of the endocervix, uterus and ovaries. The lower FRT is covered in layers of squamous epithelial cells, while the upper FRT is covered in a simple columnar epithelial layer. Figure from [45].

2. Materials and Methods

2.1. Mice

6-10 week old female C57BL/6 (B6) or B6.CD45.1 mice (Charles River UK) were housed under specific-pathogen free conditions Mice were kept in a continuous 12 hour light/dark cycle and were caged only with females from weaning. 8 week old female CD19CreR26REYFPVaDsRedxC57xC57 mice were bred under specific-pathogen free conditions. All procedures were done in compliance with the Animal (Scientific Procedures) Act 1986.

2.2. Depo-Provera treatment and vaginal smears

Mice were injected subcutaneously in the base of the tail with 100 µl of 30 mg/ml Depo-Provera^{TM} (Pharmacia) in sterile saline (Baxters, UK) 5 days prior to use. Vaginal smears were taken using a pipette and approximately 30-50 µl sterile saline then placed on polylysine slides (Fisher) with or without further dilution, depending on viscosity of the mucus. Slides were air dried then haematoxylin and eosin (H&E) stained.

2.3. Serial sectioning of the FRT

Reproductive tracts from mice were divided into lower vagina, upper vagina, and cervix and snap frozen in OCT embedding medium (TissueTek) in plastic Cryomolds (TissueTek) and stored at -80°C. Transverse 7 µm serial sections from the lower vagina, upper vagina and cervix were cut on a cryostat and placed on polylysine slides (Fisher). Approximately 6 slides (approx. 48 tissue sections) were prepared sequentially. 60-100 µm of tissue was removed before another 6 slides were prepared to allow representative sampling of the whole lower FRT. Slides were air-dried then stored at -20°C ready for H&E staining or immunohistochemistry.

2.4. Haematoxylin and eosin staining

Air-dried slides were stained in Harris haematoxylin (Sigma) for 5 minutes and then washed in cold running water for 5 minutes. Slides were dipped 12 times in 0.5%

eosin then dipped in distilled water. Slides were then dipped 10 times in 50% ethanol, 10 times in 75% ethanol, left in 95% ethanol for 30 seconds followed by 1 minute in 100% ethanol. Slides were air dried then mounted in DePex (VWR BHD Prolabo) with a cover slip. Images were acquired on Zeiss Axioplan microscope using an Optronics camera and brightness and contrast were adjusted using Photoshop, as required.

2.5. Immunohistochemistry

Sections were fixed in ice cold acetone for 5 minutes, washed in PBS 0.05% BSA (Fisher) and then blocked in PBS 0.05% BSA containing 5% Rat (Sigma), or Hamster (MP Biomedicals) serum or blocking IgG for at least 1 hour. An avidin-biotin blocking kit (Invitrogen) was used according to the instructions, when biotinylated antibodies were used. Briefly, sections were blocked with β-biotin (10 min), washed (PBS 0.05%BSA) and then incubated with avidin (10 minutes) followed by 3 washes. Slides were then stained with CD11c, CD68 or Gr-1 antibodies (see table) or appropriate isotype controls in blocking buffer for 45 minutes. Slides were washed as above. For experiments using biotinylated antibodies Streptavidin-AlexaFluor546 or Streptavidin-AlexaFluor488 (both eBioscience) diluted 1:300 in blocking buffer was then added and incubated at room temperature for 30 minutes. The slides were then washed 3 times as above and then once in PBS. Slides were counterstained with 1 mg/ml DAPI, for 5 minutes, if required, washed in PBS twice and then mounted in Prolong Gold anti-fade reagent (Fisher) with a cover slip. Images were captured using a Zeiss inverted 510 Meta on Axiovert 200M confocal microscope and analysed using Zeiss LSM Image Browser software and cells were counted using ImageJ software. Stitched images of LNs were made using Fiji software.

Antibody	Fluorochrome	Isotype control	Dilution	Supplier
CD11c	Alexafluor 647	Hamster IgG	1:200	eBioscience
CD68	Alexafluor 647	Rat IgG2a	1:100	eBioscience
MHCII	biotinylated	Rat IgG2b	1:200	eBioscience
MHCII	Alexafluor 450	Rat IgG2b	1:200	eBioscience
GR-1	biotinylated	Rat IgG2b	1:200	eBioscience
gp38	Alexafluor 488	Hamster IgG	1:200	eBioscience

CD31	biotinylated	Rat IgG2a	1:200	eBioscience

Table 2.1 Antibodies used for immunohistochemistry

2.6. Quantification of immunohistochemically stained tissue sections

2.6.1. Pixel counts

For each field of view lines were drawn around the epithelia using Photoshop. The epithelia were selected and the total pixel counts performed. The green, red and yellow channels were selected in turn and pixel counts performed for each. The pixel count for one channel was divided by the total pixels (x100) to give a percentage. For the total CD11c or MHCII cells the red and green channels respectively were added to the yellow channel and expressed as a percentage of total pixels.

2.6.2. Cell counts

The lengths of the basal layers of the epithelia were measured using LSM software. The number of cells in the epithelia were counted using ImageJ software and the results were expressed as cells/mm.

2.6.3. Determining neutrophil density

Blind analysis was done using LSM software. Images showing only GR-1^+ and DAPI staining were designated as high or low neutrophil areas. Greater than 50% of the outer epithelial length positive for Gr-1 was designated as neutrophil high. Images showing only $CD11c^+$ and DAPI staining channels were used for DC counts.

2.7. Stereo imaging of CD19CreR26REYFPxC57BL/6xC57BL/6 mice

Single images of mice were taken under white, red fluorescent and green fluorescent light in turn on a stereo microscope (Zeiss) and captured using a CCD camera. Individual images were overlaid in ImageJ.

2.8. Isolation of lymph node cells and surface staining for flow cytometry

Lymph nodes from multiple mice were pooled into groups of cervical lymph nodes, iliac lymph nodes, mesenteric lymph nodes and a group of 'other' lymph nodes, which included axillary and inguinal lymph nodes. Pooled LNs were diced into small pieces with a scalpel and forceps then washed in PBS. These were incubated for 25 minutes at 37°C in 1 ml of enzyme mix containing 1.8 Wünsch units/ml Liberase TL (Roche) and 0.5 mg/ml DNaseI (Roche) in PBS. The resulting digests were then passed through a 70 µm cell strainer (BD Biosciences). Cells were washed (1300 rpm for 5 minutes) twice in PBS 1% FCS. Viable cell counts were determined by Trypan blue exclusion using a haemocytometer. Pooled cells from lymph nodes were blocked in Fc block (eBioscience 0.5 µg/ml) in PBS 1% FCS for 15 minutes on ice. Cells were washed in PBS 1% FCS as above then split into $2x10^6$ per sample. Cells were stained for CD11c, MHCII, CD11b, CD103 and CD45.2 or with appropriate isotype controls (See table 2.2) and incubated on ice, covered in foil to protect from light for 25 minutes. Cells were washed twice in PBS 1% FCS as above. Cells were fixed in 4% PFA for 20 mins on ice and were stored at 4°C until analysis. Samples were analysed on a CyAn flow cytometer using Summit software (Beckman Coulter).

Antibody	Fluorochrome	Isotype control	Dilution	Supplier
CD11c	PE-Cy7	Hamster IgG	1:400	eBioscience
MHCII	e450	Rat IgG2b	1:400	eBioscience
MHCII	APC-Cy7	Rat IgG2b	1:400	eBioscience
CD45.2	APC-780	Rat IgG2a	1:200	eBioscience
CD11b	e450	Rat I gG2b	1:200	eBioscience
CD103	PE	Rat IgG2a	1:200	eBioscience

Table 2.2 Antibodies used for flow cytometry staining

2.9. ALDEFLOUR staining

To identify cells containing active RALDH enzyme activity, ALDEFLUOR reagents (Stemcell Technologies) were used. Cells were stained for surface markers as above but without fixation. After surface staining, cells were washed in PBS 1% FCS as

above then resuspended in 50 µl ALDEFLUOR assay buffer. 50 µl ALDEFLUOR substrate or DEAB inhibitor diluted 1:15 in ALDEFLUOR substrate were added to appropriate wells and incubated in foil at 37°C 5% CO_2 for 30 minutes. Samples were washed in ALDEFLUOR buffer then resuspended in PBS. 1 µl of LIVE/DEAD Fixable aqua dead cell stain (Invitrogen) was added to each sample then incubated on ice for 30 minutes in foil. Samples were washed in PBS then resuspended in PBS 1% FCS before analysis on a CyAn flow cytometer using Summit software (Beckman Coulter).

2.10. Statistical Analysis

Statistical analysis was performed using GraphPad InStat 3 software. Nonparametric Mann-Witney-tests were used for all analysis.

3. Histological characterisation of the lower FRT over the murine estrous cycle

3.1. Introduction

Mice are the common model for immunology and infection research because they are a relatively cheap and easy to use mammalian species that are genetically similar to humans and other mammals. They also have the advantage of the huge range of genetically defined or altered mouse strains available. These allow researchers to design experiments that assess the role of specific genes, which could not be done in humans. Research in mouse models is often used to draw conclusions about human immunology, so it is important to have a thorough understanding of the similarities and differences between them and what this could mean for the application of mouse research to human research. Importantly, humans and mice have different reproductive biology. Human females of reproductive age, not receiving hormonal contraception have a menstrual cycle which lasts around 28 days. It is characterised by a period of endometrial growth, known as the follicular phase, followed by ovulation. This is followed by the luteal or secretory phase, where the ovaries produce large amounts of hormones that facilitate implantation and early growth of the fertilised ovum. If fertilisation does not occur the ovum degenerates and menstruation occurs, in which the endometrium is sloughed off and is expelled from the body [16]. Conversely, mice and rats have estrous cycles. The main difference between mammalian menstrual and estrous cycles is that in the menstrual cycle the epithelial cells shed into the lumen are discharged out of the vagina, whereas in the estrous cycle the epithelial cells are broken down and reabsorbed [16].

Mice have non-seasonal polyestrous cycles and spontaneously ovulate [16, 64], which means that, similar to humans, they ovulate multiple times throughout the year at regular intervals and do not require sexual stimulation to ovulate. Unlike many other estrous animals such as sheep and dogs, the murine estrous cycle is not driven by seasonal cues and mice will not naturally enter a anestrus stage in which the animal is unable to reproduce [16].

The murine estrous cycle lasts 4 to 5 days [64] and ovulation occurs spontaneously whether mating has occurred or not [64]. It comprises a period of preparing the tissue for nourishing a fertilised egg, before a period of 'heat' where the animal is receptive to mating and ovulation occurs. If fertilisation does not occur the reproductive tract undergoes a period of shedding before restarting the cycle. The estrous cycle can be split into 4 stages; *proestrus* in which the ovarian follicles grow and the uterine epithelium thickens, *estrus* in which ovulation occurs, *metestrus* begins when the uterine epithelium sheds and *diestrus* when the epithelia is at its thinnest before the cycle restarts [64, 65]. Metestrus and diestrus are sometimes referred to as metestrus-1 and -2 or diestrus-1 and -2 [64], however, in this study the metestrus will be used to refer to the stage immediately after estrous followed by diestrus. Proestrus and estrus are equivalent to the follicular phase of the human menstrual cycle and metestrus and diestrus are similar to the luteal phase.

Events in the ovaries are almost identical in the estrous and menstrual cycles and the kinetics of luteinising hormone (LH) and follicle-stimulating hormone (FSH) levels in the blood stream follow almost identical patterns [16, 64, 66]. FSH produced in the pituitary promotes maturation of the ovarian follicle. LH causes the maturing follicle to produce estrogen, which results in increased LH production through a positive feedback loop. Rising LH causes ovulation [64]. The ovarian follicle then becomes a corpus luteum and secretes hormones that promote survival and growth of the fertilised ovum. The corpus luteum degenerates and the decreasing hormone concentrations causes epithelial shedding in the uterus [16, 64].

The vaginal epithelium consists of stratified squamous epithelium [2, 16, 41], similar to skin. The stromal and epithelial cells are under hormonal control. The epithelial architecture of the uterus, cervix and vagina changes in response to estrogen and progesterone (Fig3.1). Increased estrogen leads to thickening of the epithelia and ovulation, while high progesterone is associated with thinning of the epithelia. Much of the hormone-induced changes in the vaginal tissue are mediated by the stromal cells in the lamina propria, which control epithelial cell behaviour and immune cells.

3.2. Results

The histology of the FRT over the estrous cycle has been well characterised in rats [67], but less so in mice. In order to assess the physiological changes in the FRT 6-8 week old female mice were vaginally smeared and these smears were H&E stained and used to classify each mouse into an estrous cycle stage. C57BL/6 CD45.2 or C57BL/6 CD45.1 mice were used because they are common laboratory mouse strains. They differ in the allelic form of CD45, which results in no functional differences. The two strains are, otherwise, assumed to be identical. The FRTs were removed and snap frozen in order to preserve the tissue architecture. To characterise the tissue throughout the tract serial transverse tissue sections of the vagina and cervix were H&E stained (Fig 3.2).

3.2.1. Vaginal Smears

The histology of vaginal smears can be used as a predictor of estrous cycle stage, as has been shown in rats [68]. To establish a protocol based on the murine estrous cycle individual mice were smeared everyday for 4 days+ at approximately the same time every day. H&E stained vaginal smears were assigned into one of the 4 estrous cycle stages based on guidelines for the reading of rat vaginal smears [68]. Smears were also taken from high-dose progesterone (Depo-Provera) treated mice at 5 days post treatment and on some occasions at 6 – 30 days post treatment. The vaginal smear classification scheme devised is summarised in table 3.1.

In proestrus (Fig 3.3A) the smears contained few cells and were made up almost exclusively of epithelial cells. The smears contained differing ratios of small, rounded, nucleated epithelial cells with pale cytoplasm and larger, cornified cells, which appear pinker, probably due to keratinisation and increased glycogen.

At estrus there were huge numbers of large non-nucleated cornified epithelial cells in the smear (Fig3.3B). The cornified cells were large, flattened, highly eosinophilic and clumped together. Other cells were almost completely absent in the smear.

At metestrus the predominant cells in the wash were leukocytes (Fig3.3C). The majority of leukocytes were polymorphonuclear neutrophils, which can be identified by their multi-lobed nuclei (open arrows). The smears were generally thick with

mucus, causing leukocytes and non-nucleated cornified epithelial cells to clump together. In some smears the epithelial cells are nearly all large anucleated cornified cells, but in others more nucleated cells were present in the smear.

At diestrus the majority of cells in the smear were leukocytes with some cornified and/or nucleated epithelial cells (Fig3.3D). Leukocytes were less clumped in diestrus compared to metestrus smears, though there was considerable variation.

After Depo-Provera treatment the smears were similar to the smears from metestrus/diestrus. The majority of cells were leukocytes with smaller numbers of nucleated epithelial cells (Fig3.3E). There was a large amount of thick mucus present in the smears which caused clumping of cells; however this did vary from mouse to mouse.

	Proestrus	**Estrus**	**Metestrus**	**Diestrus**	**Depo-Provera**
Nucleated epithelial cells	+	-	-	+	+
Non-nucleated epithelial cells	-	+++	++	-	-
Leukocytes	-	-	+++	++	++
Mucus	+	++	+++	++	+++

Table 3.1: Determining estrous cycle stage using vaginal washes.

3.2.2. Histology at proestrus

The vaginal lumen was devoid of cells in the tissue sections, consistent with the smears which contained few cells (Fig 3.4A). The epithelium was thick at proestrus and was densely packed with very few visible gaps between epithelial cells. The epithelium varied in thickness from approximately 5-25 cells thick (Fig 3.4B&C). In most sections the outermost layer of the epithelium was anucleated, but not keratinised (Fig 3.4B), but in some there was a highly eosinophilic band in the outer epithelium showing keratinisation of the outer epithelium into the stratum corneum (SC) (Fig 3.4C).

3.2.3. Histology at estrus

Strings of cornified epithelial cell sheets were seen filling the lumen in tissue sections (Fig 3.5A). Some areas of the epithelium were relatively thick while others were comparatively thin. In most sections the stratum corneum was partly detached, but the degree of detachment varied. In some smears leukocytes were seen in the epithelia and in the submucosa, but almost never in the lumen (Fig 3.5C). There were very few gaps between epithelial cells. The cervix had relatively less desquamed epithelial cells in the lumen than the vagina (Fig 3.5D).

3.2.4. Histology at metestrus

The stratum corneum was almost completely detached, but can be seen in the lumen along with leukocytes in the tissues sections (Fig 3.6A). The outer epithelium appeared to lose integrity and there was infiltration of leukocytes, which have darker staining nuclei than the surrounding epithelial cells (Fig 3.6B). Individual leukocytes were seen in the submucosa and basal layers of the epithelium and aggregates of cells were observed in the superficial layers of the epithelium, sometimes forming small foci. Some cells were identified as neutrophils based on their distinctive multi-lobed nuclei (Fig 3.6C). The outer epithelia layers were obviously disrupted in the majority of tissue sections and there were gaps between epithelial cells. Leukocyte infiltration is not uniform throughout the lower reproductive tract with some areas experiencing rapid infiltration and loss of epithelial cell layers while other areas have slower infiltration and an extended period of slow loss of the epithelial layers (Fig 3.6A-C). This makes early and late metestrus difficult to differentiate. In this study metestrus is characterised as beginning with the widespread loss of the stratum corneum and infiltration of leukocytes and ends with extensive loss of deeper epithelial layers, decreasing leukocytes in the epithelium and formation of the thick, dark outer epithelial layer characteristic of diestrus (described below and in Fig 3.7A and C).

3.2.5. Histology at diestrus

The lumen was mostly cell free in tissue sections (Fig 3.7B) and the epithelia was generally thin with only 2-3 cell layer present in some areas, though this varied

within the FRT and even within a single field of view. The outer epithelium contained leukocytes. In some places the leukocytes were in clumps in the epithelium, but in others they were largely absent or only visible in the lumen. While the epithelia appeared to be less leaky than in metestrus, they were less tightly packed than in proestrus or estrus as there were visible gaps between the epithelial cells (Fig 3.7B). Where the epithelium was thinnest the outer epithelium contained a thicker solid band of cells (Fig 3.7A and C). The dark nuclear staining in the outer epithelia implies that it was made up of leukocytes. The cervical epithelia were generally slightly thinner than the vaginal epithelia. The cells contained large vacuoles and there were large nucleated cells in the lumen (Fig 3.7D and E).

3.2.6. Histology of progesterone treated mice

The histology of the FRT after progesterone treatment was also characterised because it will be used later in the study. B6.CD45.1 mice were injected subcutaneously with 100 µl (30 mg/ml) Depo-Provera. 5 days later the FRTs were removed and H& E stained. In all sections the epithelia were thin and all layers contained nucleated cells (Fig 3.8A). Large mucus-filled vacuoles were seen in the superficial layers of the epithelia (Fig 3.8C) and there were gaps in the basal layer of the epithelium. Leukocytes, characterised by darker staining nuclei, were still present under the mucified cell layer (Fig 3.8B). The cervical epithelia were slightly thinner than the vaginal epithelia and there was more mucification of cells (Fig 3.8C).

3.3. Discussion

During proestrus the epithelia thickens leading up to estrus, where ovulation occurs. After ovulation the stratum corneum sloughs off in sheets. While the stratum corneum desquames, the underlying epithelia integrity appear to remain intact as there are no visible gaps between epithelial cells. At the very end of estrus leukocytes begin to infiltrate into the tissue and appear in the epithelia. Widespread leukocyte infiltration marks the end of estrus and the beginning of metestrus [67]. At metestrus the squamous layers were less organised, probably due to the influx of leukocytes. At diestrus the epithelia is thin and has a sticky thicker outer layer that shows the beginning of mucification.

High dose progesterone treatment is a common way of synchronising mice into diestrus [50, 69]. The smears and tissue sections of diestrus and DP mice were similar. After Depo-Provera treatment the superficial epithelial cells were more heavily mucified than at diestrus, consistent with pseudopregnancy, which is characterised by high progesterone levels causing mucification of the epithelium [67, 70].

The changes in architecture do not happen simultaneously throughout the tract. Some areas can have characteristics of different estrous cycle stages to other areas. I found no evidence of consistent variation up or across the tracts in the serial sections of numerous mice; instead it is likely these differences are due to individual differences in the geography of the vaginal epithelia and luminal fluid flow. This leads to some, more exposed, areas sloughing faster than others and may lead to variations hormone concentrations and cell recruitment in some areas.

The variation within the local environments of the tissue cannot be reflected in the smears, which provide a rough sample of the outer epithelia and lumen of the lower FRT. Vaginal smears provide an approximate overview of the tissue, but cannot, for example, readily determine between an early estrus mouse with the SC only just starting to desquame or a late estrus mouse with almost complete detachment of the SC and infiltration of leukocytes into the basal epithelia, both of these smears would consist exclusively of large cornified epithelial cells. Vaginal smears are indicative of the state of the tissue, but will never provide the more comprehensive information that serial tissue sectioning can.

Vaginal smears offer a quick and convenient method of determining estrous cycle stage, however they are not always clear cut; it is possible to get transitional smears that are difficult to assign to one stage or another. The rapid and substantial changes in the vaginal epithelia mean that the outer epithelium is in constant flux. The cellular composition of vaginal smears is very dynamic and while a 4 stage estrous cycle seems superficially simpler to classify, it is a little crude and conceals a lot of the subtle differences between the smears.

There is a great deal of variation between individual mice in the overall cellularity and viscosity of the smears, which is difficult to factor in to a classification scheme.

Vaginal smears are therefore a good indicator of estrous cycle stage and conditions in the tissue, but it cannot be assumed that they are 100% accurate or that they reflect every corner of the FRT.

Neutrophils are recruited into the vagina by the chemokine MIP-2, an IL-8 homologue [71] after the drop in estradiol after ovulation [72]. Epithelia cells can produce inflammatory cytokines/chemokines, which recruit granulocytes from the blood [40, 71, 73]. It is unknown whether neutrophils are responsible for the disruption of the outer epithelia and the sloughing of the cell layers under the stratum corneum or if neutrophil infiltration is a side effect of the loss of cells. It is possible that the loss of the superficial layers of epithelium and invasion by bacteria/fungi caused by the increased permeability of the epithelium may trigger stress responses in epithelial cells. The barrier function of uterine epithelial cells has been shown to be effected by hormonal changes [41] and it possible that changes in the vaginal epithelial cell barrier may lead to the penetration of lumen contents into the epithelial layer causing production of chemokines by epithelial cells.

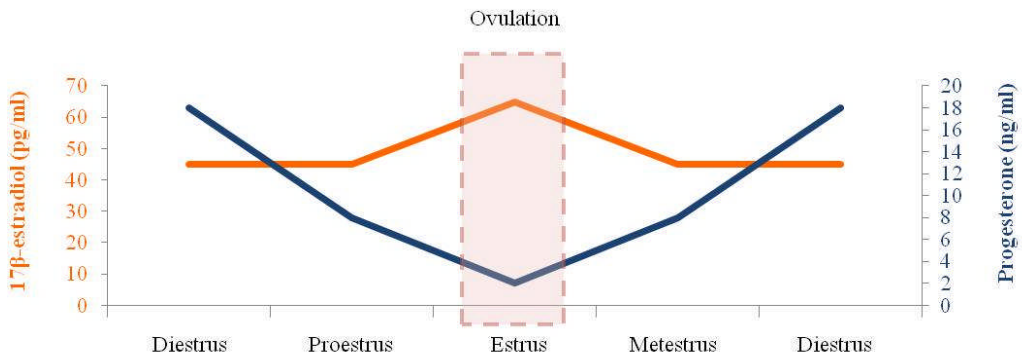

Figure 3.1: Serum hormone concentrations in mice.
The hormones progesterone (blue) and estrogen (orange) fluctuate over the estrous cycle. Estrogen peaks at estrus, when ovulation occurs, then drops off. Progesterone increases after ovulation. Based on data from [74].

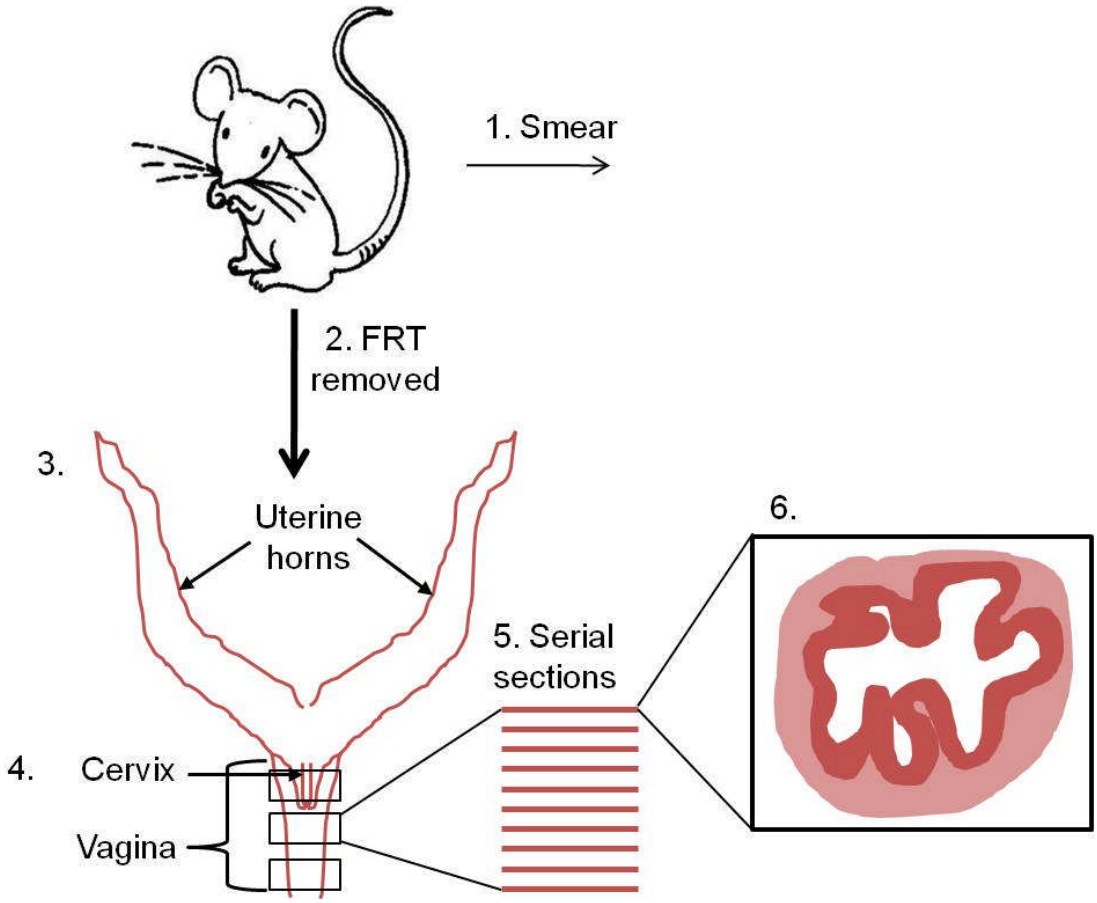

Figure 3.2: Experimental Procedure.

1. Vaginal smears were taken from C57BL/6 or CD45.1 female mice and H&E stained to determine cycle stage. 2. The FRTs were removed. 3. Sagittal view of murine reproductive tract. 4. FRTs were split into cervix, upper vagina and lower vagina (boxes) and snap frozen. 5. 7 µm serial transverse sections of tissue were cut on a cryostat. 6. Diagram of transverse tissue section showing vaginal lumen in centre, with epithelium and surrounding connective tissue.

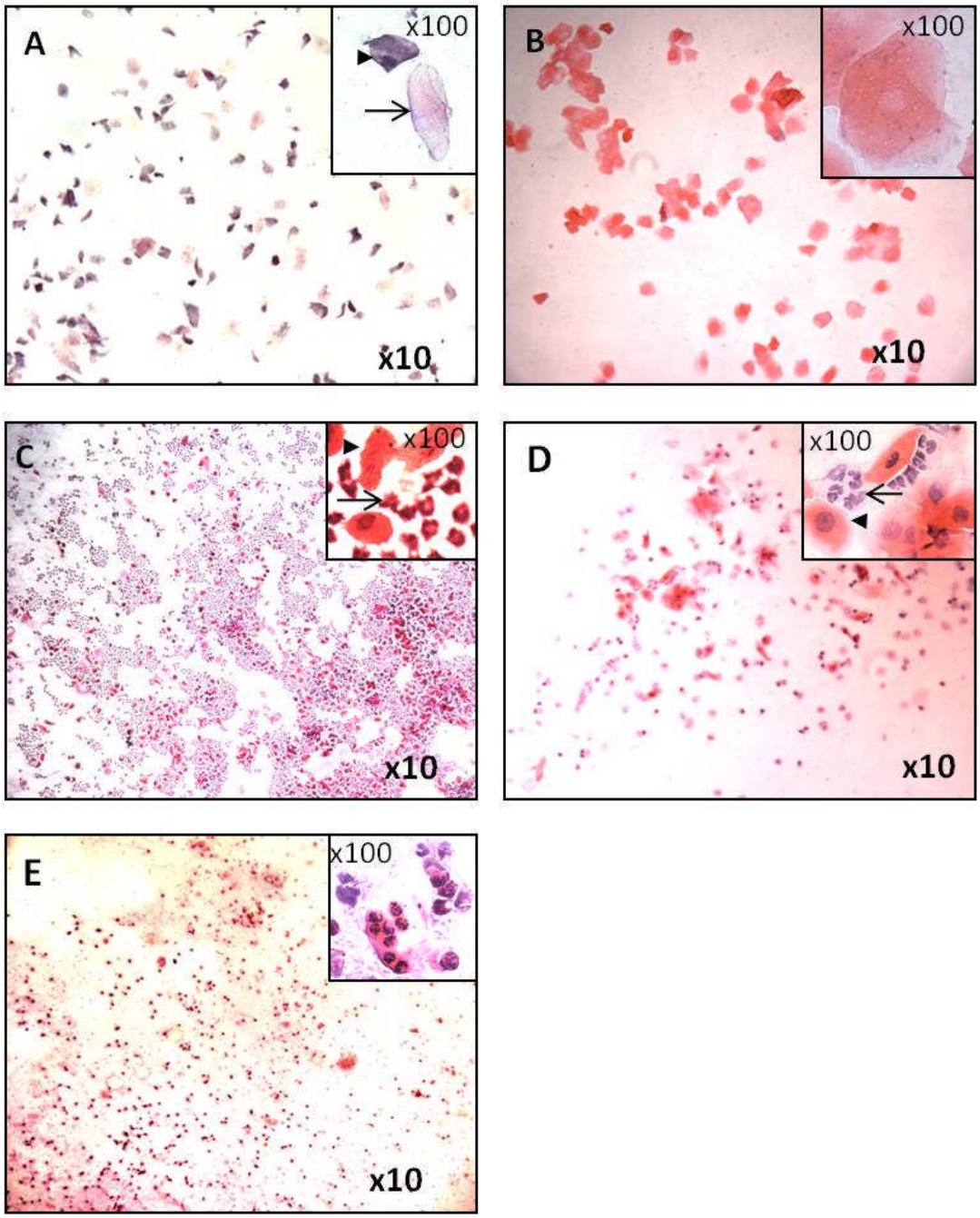

Figure 3.3: Vaginal Smears

A) Proestrus vaginal smear; insert shows a nucleated epithelial cell (arrow head) and an anucleated epithelial cell (arrow) showing early cornification. B) Estrus vaginal smear; insert shows cornified epithelial cells. C) Metestrus vaginal smear; insert shows leukocytes (arrow) and cornified epithelial cell (arrow head). D) Diestrus vaginal smear; insert shows leukocytes (arrow) and nucleated epithelial cells (arrowhead). E) Vaginal smear from Depo-Provera treated mouse; insert shows clumped leukocytes.

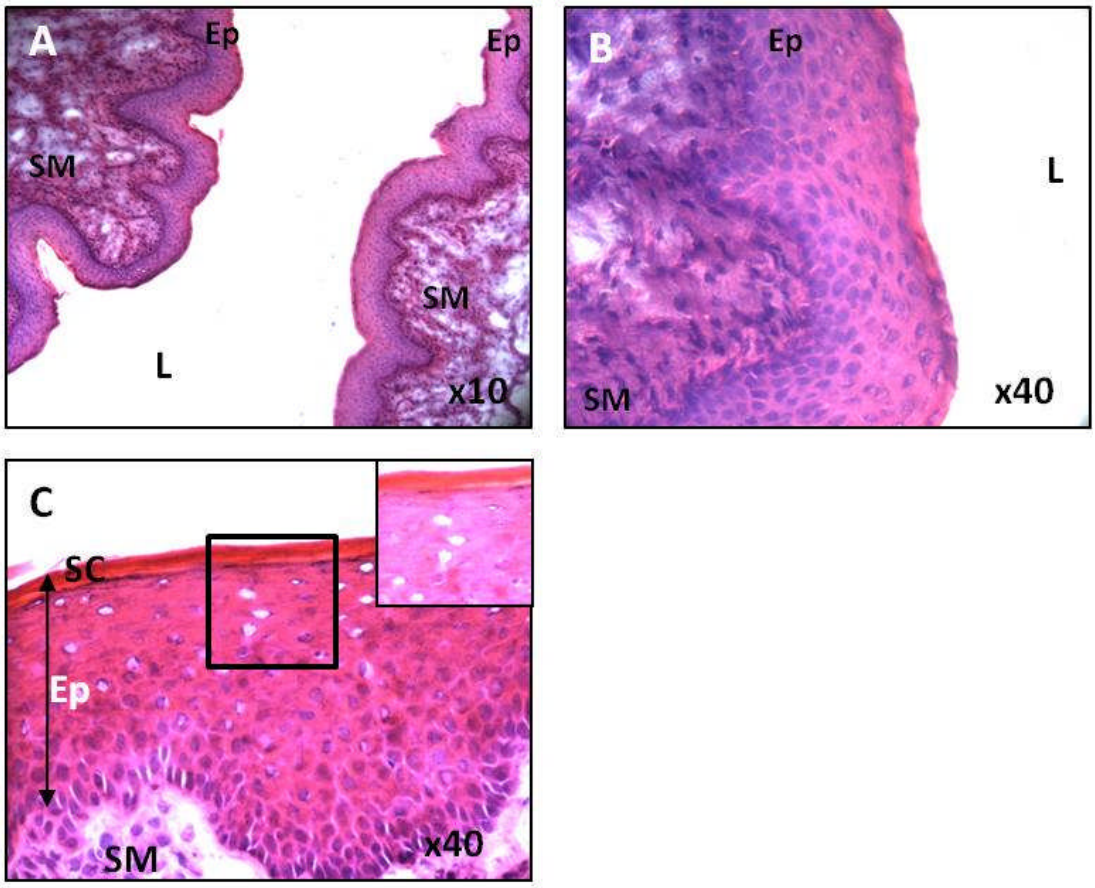

Figure 3.4: Histology of lower FRT at proestrus.

H&E stained vaginal smears and 7 µm transverse sections of lower FRT tissue from 6-8 week old C57BL/6 or B6.CD45.1 female mice. A) x10 image of vagina. A-C) Vagina at early proestrus. B) Early proestrus C) Late proestrus; insert shows the outer epithelium. Ep epithelium, L lumen, SC stratum corneum, SM submucosa

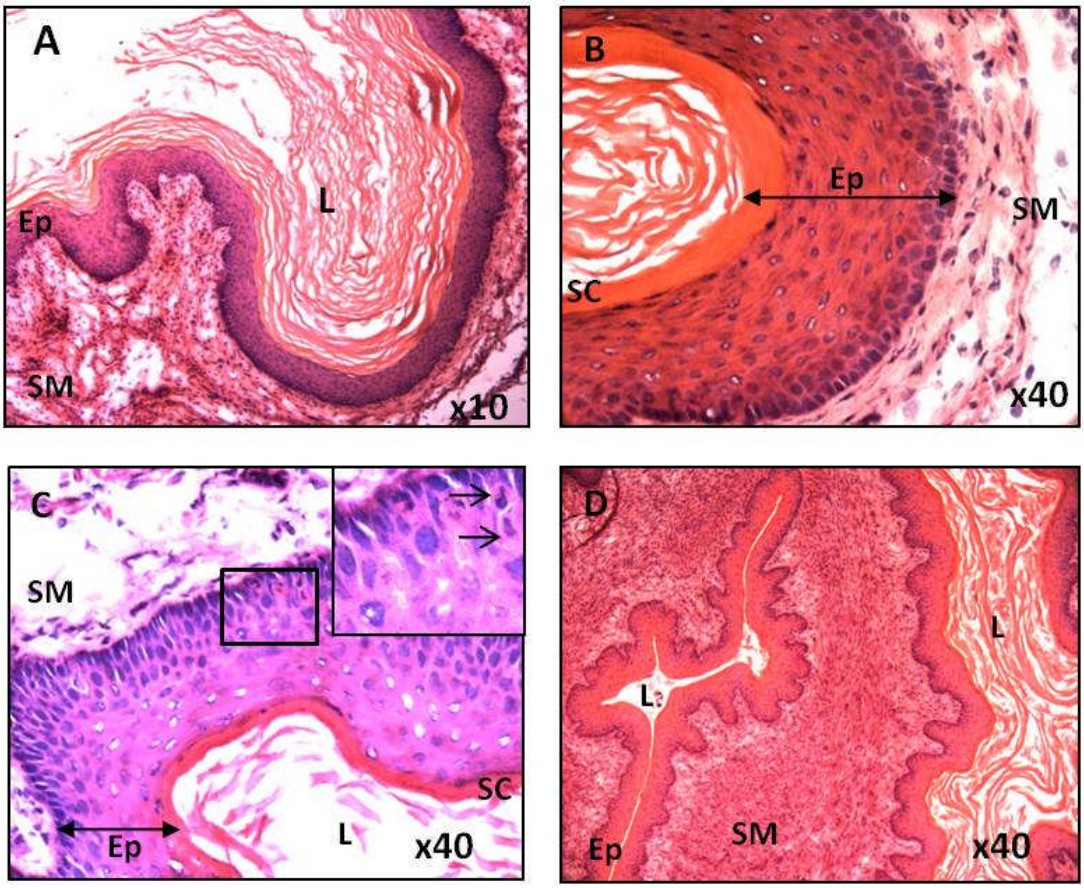

Figure 3.5: Histology of lower FRT at Estrus

H&E images of vaginal smear and 7 µm transverse sections of vagina and cervix at estrus. A) Lower vagina with lumen containing epithelial cell sheets. B) Vagina at early estrus. C) Vagina at late estrus; insert shows leukocytes in epithelium (arrows). D) x10 image of upper vagina epithelium (right) and cervical epithelium (left) showing the difference in the level of epithelial sloughing. Red arrows indicate sloughed cornified epithelial cells. Ep epithelium, L lumen, SC stratum corneum, SM submucosa.

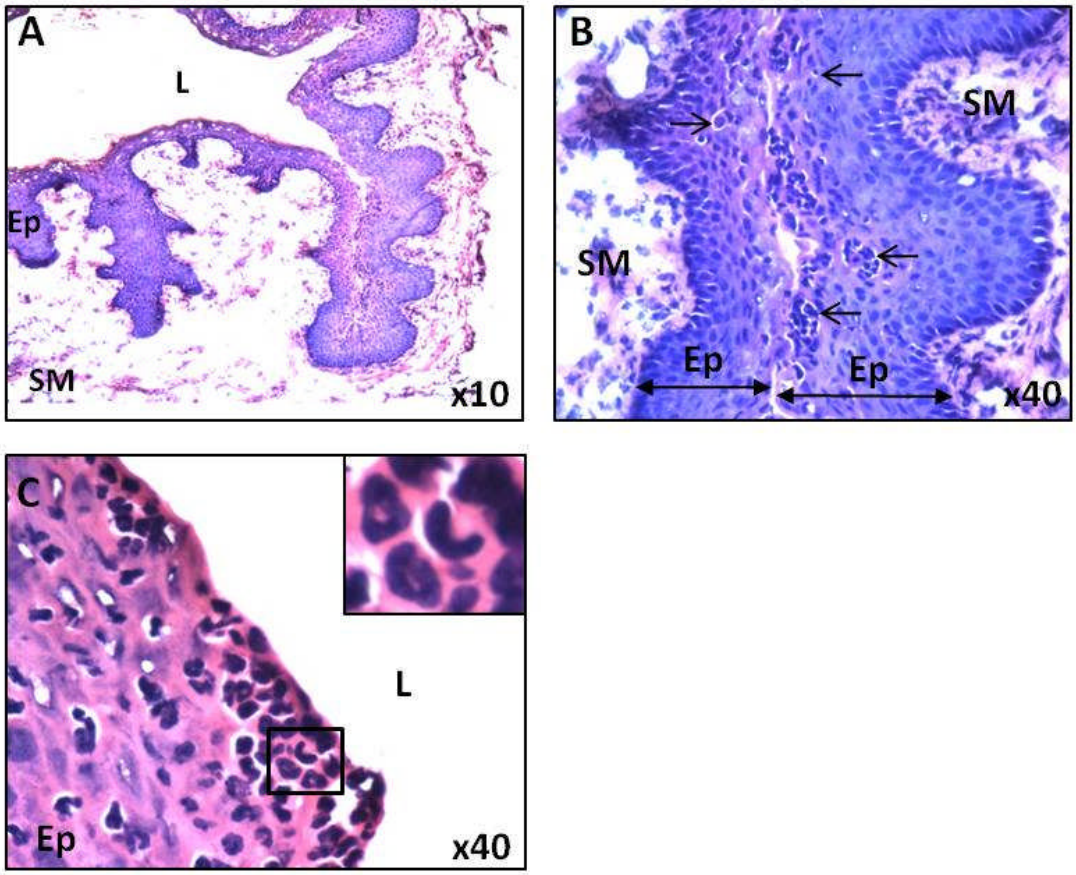

Figure 3.6: Histology of lower FRT at metestrus.

H&E images of 7 µm transverse sections of vagina at metestrus. A) Lower vagina B) Vagina with leukocyte infiltration (arrows). C) Vaginal epithelium; insert shows a close up of neutrophils. Arrows show leukocytes. Ep epithelia, L lumen, SM submucosa.

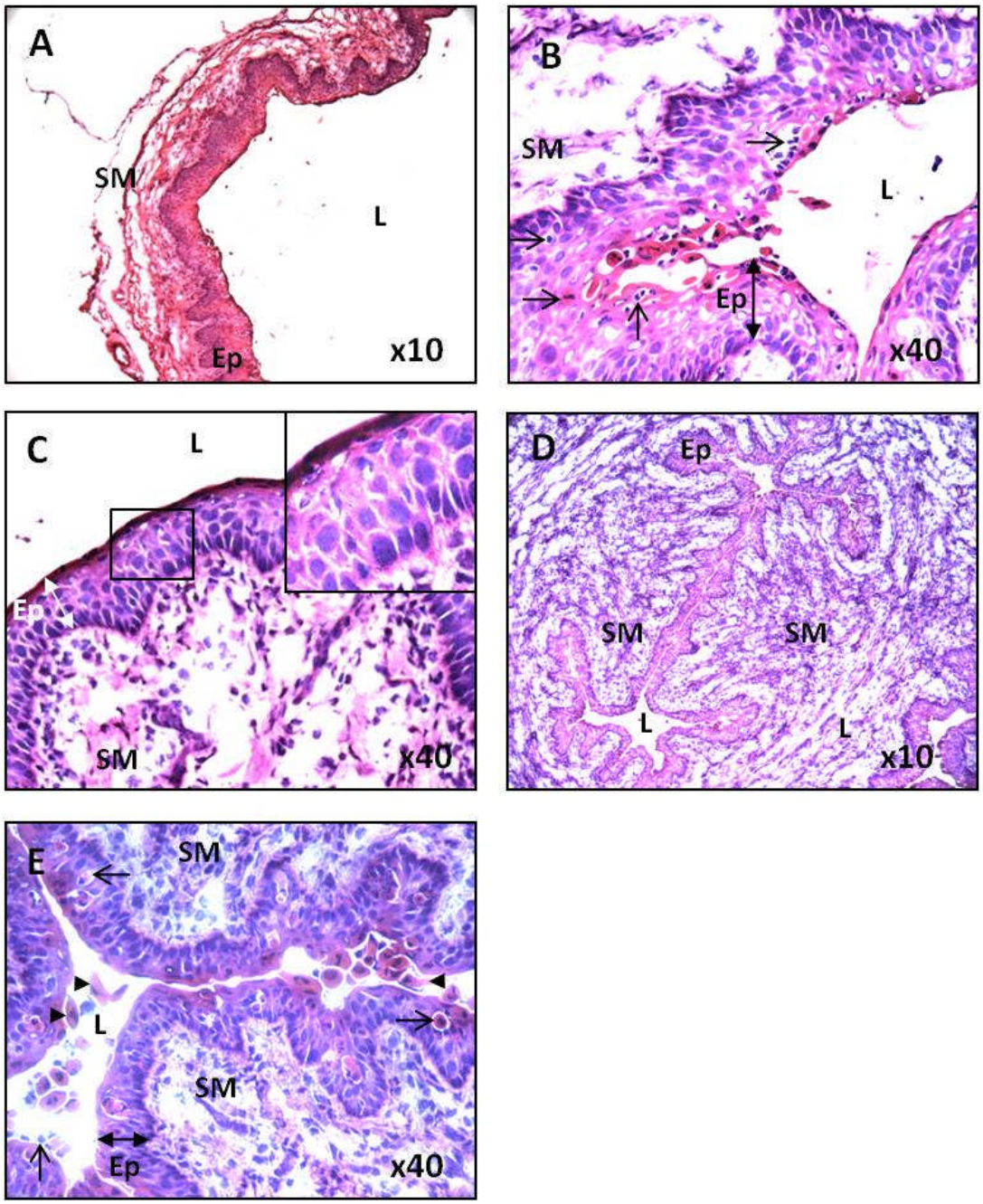

Figure 3.7: Histology of lower FRT at diestrus.

H&E images of 7 µm transverse sections of vagina and cervix at diestrus. A) Vagina. B) Vagina at early diestrus with leukocyte infiltration (arrows). C) Vagina at late diestrus; insert shows the epithelium. D) Cervix. E) Cervix showing leukocytes in the epithelium and lumen (arrows) and nucleated epithelial cells in the lumen (arrow heads) .Arrows show leukocytes. Ep epithelium, L lumen, SM submucosa.

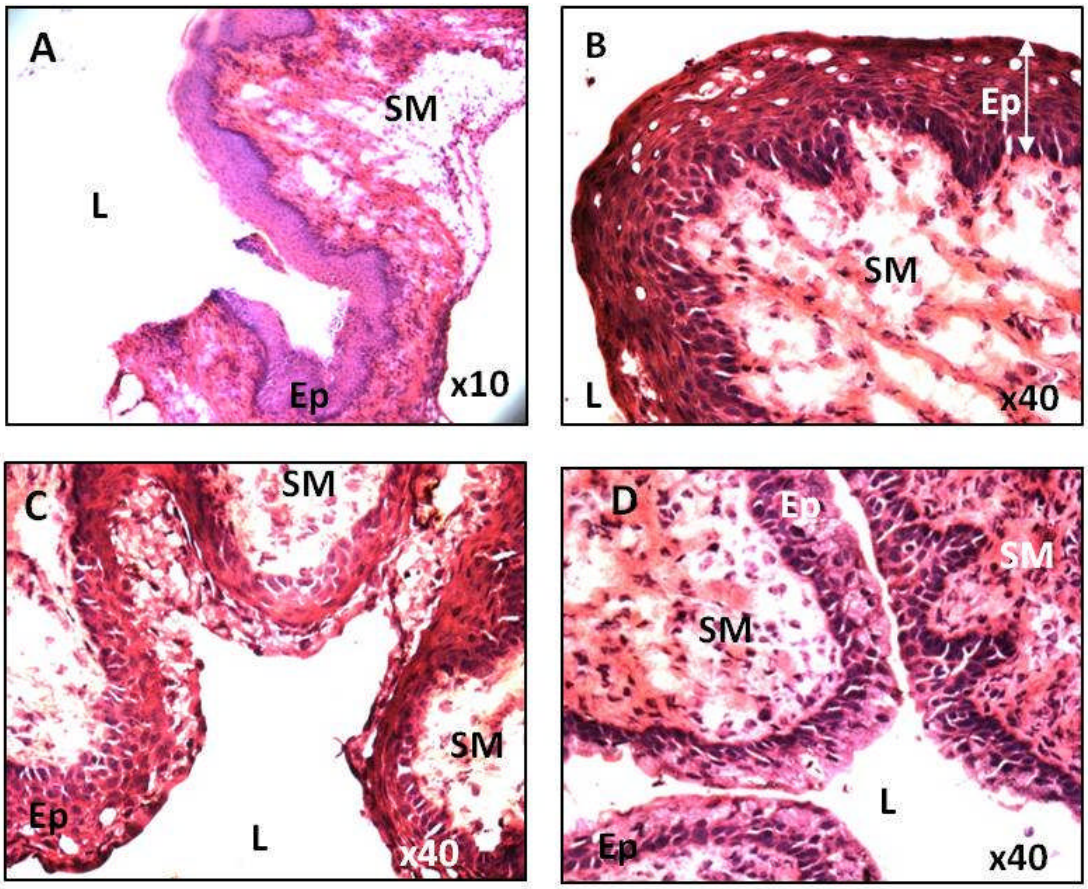

Figure 3.8: Histology of lower FRT after high dose progesterone treatment.
CD45.1 female mice were treated with 100 µl Depo-Provera. 5 days later vaginal smears were taken and H&E stained. FRTs were then frozen and 7 µm sections were cut and stained with H&E. A) Vagina. B) Vagina showing thinned epithelium. C) Vagina showing mucification of the outer epithelia. D) Cervix showing mucification. Ep epithelium, L lumen, SM submucosa.

4. Localisation of innate immune cells in the FRT

4.1. Introduction

Estrogen receptor expression is found both in lymphoid organs and bone marrow and on DCs and macrophages [40]. Differential estrogen levels are thought to affect early DC development by affecting the differentiation of DC precursors in the bone marrow [58]. Estrogen has different effects on different DC development pathways, promoting GM-CSF induced DC development pathways and inhibiting Flt-3L mediated development pathways [58]. These studies show how systemic estrogen may affect the immune system as a whole; however it does not highlight the immediate effects of estrogen on cells within tissues. Macrophages, B cells and T cells can all express progesterone receptors, although it is unclear whether this is constitutive or induced as the data comes from pregnant women [21], which raises the possibility that progesterone can have direct effects on immune cells.

The efficiency of intravaginal vaccination is affected by phase of the estrous cycle [75, 76], a finding that might have significant implications on FRT vaccination strategies. There is some evidence that susceptibility to infections in the FRT may change over the estrous/menstrual cycle [59]. For example mice are more susceptible to HSV-2 infection in metestrus and diestrus [50, 76], but are more susceptible to *Neisseria gonorrhoeae* infection at proestrus [59]. Rhesus macaques are more susceptible to SIV infection during the progesterone high stage of the menstrual cycle, whereas estrogen is protective [21, 77]. Women show increased susceptibility to HIV and other STIs while taking progesterone contraceptives [21, 77] and hormones have effects on susceptibility to candidiasis and Chlamydia [78] .

These differences could be due to a variety of factors. It may simply be the result of epithelia thinning during progesterone high stages which allows easier access for pathogens [21, 77], or changes in epithelium permeability [79]. Antibody and antimicrobial peptide concentrations vary of the course of the estrous cycle, as do chemokine levels [21, 59]. Estrogen inhibits expression of the chemokine MCP-1 by stromal cells in the uterus [21]. Cyclic changes in the commensal bacteria, particularly H_2O_2-producing *Lactobacilli* [21], that line the FRT [80] may affect

susceptibility to viral infections [77]. T cell and B cell populations in the FRT can vary over the reproductive cycle, as does IgG and IgA production [21, 77].

Activation of T cells in the presence of progesterone inhibits Th1 responses and promotes Th2 responses by inducing IL-4 production and inhibiting IL-12 production by APCs [21, 77] [59]. Estrogen has varied affects on immunity; it can be both pro-inflammatory and have inhibitory affects. In rats, estradiol increases antibody levels in the uterus, but reduces levels in the vagina [77]. Low doses of estrogen are pro-inflammatory while high doses are anti-inflammatory [21].

In this chapter the distribution of DC and macrophage in the mouse FRT during cycle will be compared to previously published studies.

4.2. Results

4.2.1. Dendritic cell and macrophage localisation in the FRT in different hormonal conditions

The location of APCs within the tissue is important because it will affect their ability to sample antigen from the lumen and to interact with other cells. To investigate whether APC localization within the tissue is affected by the hormonal changes the vaginas from estrus (the estrogen high stage), metestrus (the progesterone high stage characterized by large infiltration of leukocytes) and progesterone treated mice were stained for CD11c and CD68 (Fig 4.1).

There were greater numbers of $CD11c^+$ cells in the submucosa compared to the epithelia both at estrus and metestrus (Fig 4.1). At estrus only the basal layers of epithelial cells were nucleated and stained $DAPI^+$ (Fig 4.1A). The cornified sheets of cells auto-fluoresced so can be seen adjacent to the lumen. $CD11c^+$ cells were almost completely absent. Despite the fact that the epithelium had increased in thickness, $CD11c^+$ cells were only present in the basal layer or immediately underneath with processes reaching in between the epithelial cells. No $CD11c^+$ cells were observed reaching to the luminal edge of the epithelia. There were fewer cells in the submucosa compared to metestrus. At metestrus $CD11c^+$ interdigitating cells (Fig 4.1C arrows) were present in both the basal and superficial layers of the epithelium (Fig 4.1C).

$CD68^+$ cells were largely absent from the epithelia in both estrus and metestrus (Fig 4.1B and D). They were present throughout the submucosa at similar levels in both estrus and metestrus. There were no obvious differences in $CD68^+$ localisation during cycle.

4.2.2. Localisation of CD11c and MHCII expressing cells throughout the tract

To see if there are any differences in the distribution of APCs in the lower FRT serial tissue sections from Depo-Provera treated mice were stained for the DC markers CD11c and MHCII. Depo-Provera treated mice were used because they have identical hormone conditions which mean that any variation between mice is not due to them being in different estrous cycle stages. In order to effectively quantify cell localisation pixel counting was compared with cell counting (Fig 4.2). There were no significant differences between the two quantification methods.

There was more variation in cellularity between different mice than within one mouse; some showed low cellularity (Fig 4.3A and B), while others showed high cellularity (Fig 4.3C and D). There were no significant differences in the numbers of $CD11c^+$, $MHCII^+$ or $CD11c^+MHCII^+$ cells in the epithelia of lower vagina compared to the upper vagina/cervix (Fig 4.3E-G). There were no significant difference in the numbers of $CD11c^+MHCII^-$ and $CD11c^+MHCII^+$ cells in the epithelia (Fig 4.3E&G), but very few $MHCII^+CD11c^-$ cells in the epithelia (Fig 4.3F).

4.2.3. Localisation of CD68 and MHCII expressing cells throughout the FRT

To compare macrophage cell numbers in the epithelia at different locations in the lower FRT sections of FRT tissue from Depo-Provera treated mice were stained for the tissue macrophage marker CD68 and a marker of antigen presenting capability MHCII.

The majority of stained cells were in the submucosa with smaller numbers in the epithelium (Fig 4.4A-D). There was no significant difference between the numbers of $CD68^+$, $MHCII^+$ or $CD68^+MHCII^+$ in the epithelia of the lower vagina or upper vagina and cervix (Fig 4.4E-G).

4.2.4. Localisation of GR-1 and CD11c expressing cells in the FRT

In progesterone treated mice there is infiltration of neutrophils (Fig 3.8). To investigate if this infiltration has any affect on DCs FRTs from progesterone treated mice were stained for CD11c and a neutrophil/monocyte marker Gr-1.

GR-1^+ staining was absent in tissue sections stained with the isotype control (data not shown) and was also absent from tissue sections from the estrus stage (data not shown). There was a great deal of variation within the tissue. There were areas of very high GR-1 staining (Fig 4.5A and B) and areas of very low Gr-1 staining (Fig 4.5C and D) present within 1 tissue section. There was no significant difference in the number of $CD11c^+$ cells in the upper vs. lower tract or in the level of neutrophil infiltration in the outer epithelia. There was no significant difference in the number of $CD11c^+$ cells in the areas where there were a lot of GR-1^+ cells compared to areas where there were fewer GR-1^+ cells.

4.2.5. Immunohistochemistry versus flow cytometry for determining cell localisation

In Figure 4.6A cells from the pooled, digested vaginal epithelium of mice in diestrus was stained for CD11c vs. MHCII. 2 populations of $CD11c^+MHCII^+$ cells were seen. Split populations like this cannot be detected using immunohistochemistry; either because of the higher background fluorescence present in tissue sections (due to mucus), which would mean low expression is not above the isotype control, or because immunohistochemistry lacks the sensitivity to differentiate between low, intermediate and high expressing cells.

Flow cytometry allows staining for more markers (Fig 4.6B and C), which gives a more comprehensive charactersation of the cell populations present. Immunohistochemistry, however, is superior for showing localisation of cells within tissue. Cryopreservation and fixation of tissue is relatively unintrusive and the tissue and the cells within it remain intact and in place. With flow cytometry the tissue is processed into a single cell solution, so the validity of assumptions about localisation depends on the ability to separate the epithelia from the underlying lamina propria. This is done by manually scraping the epithelia off and using an enzyme mix to

break the intercellular bonds. While this can be done to a certain level of accuracy there is still a much higher chance of cells from the lamina propria becoming washed onto the epithelium or vice versa. It is also lacks the subtlety to measure cells that line the border between the basal layer of the epithelia and the submucosa, or cells that reside in one but extend processes into the other as many of the $CD11c^+$ cells in the FRT do.

4.3. Discussion

The localisation populations of CD11c+ DCs, CD68+ macrophages and Gr-1+ neutrophils within the FRT of progesterone treated mice were examined. Mice from the same estrous cycle stage could be used, however (as shown in chapter 3), cyclic changes are not necessarily consistent throughout the tissue and vaginal smears are not always clear cut. It is therefore better to use Depo-Provera treatment as a control for cycle stage variation.

4.3.1. Hormonal effects on cell localisation

$CD11c^+$ cells (predominantly DCs) are the main antigen presenting cell that are responsible for initiating protective immune responses. There were increased numbers of $CD11c^+$ cells in the epithelia in metestrus and in progesterone treated mice. This is consistent with published literature [81-83], which shows hormone mediated changes in cell recruitment in the vagina. There were differences in $CD11c^+$ cell localisation in different hormonal conditions in the reproductive tract (Fig 4.1), with estrus stage showing extremely low levels of $CD11c^+$ cells in the vaginal epithelia. There is evidence that the number of immune cells fluctuate in the FRT over the estrous cycle [28, 82-84] and that this may affect immunity and susceptibility to disease. The localisation and function of DCs and macrophages in the FRT in rats and humans are affected by sex hormones [21, 56, 59, 81-83]. Langerhans' cell (LC) numbers vary over the reproductive cycle [21, 83]. In mice, the repertoire of DC subtypes also changes at different stages of the estrous cycle [28].

In the gut DCs can reach out into the lumen from beneath the epithelial layer and sample antigen [85-87] and during infection it has been observed that DCs in the outer epithelia of the vagina also extend processes into the lumen [88]. At estrus no

$CD11c^+$ cells were observed reaching to the luminal edge of the epithelia, whereas at metestrus $CD11c^+$ cells were seen throughout the epithelial layers, similar to in the skin, where LCs sit within the stratified cell layers of the epidermis [48, 49, 89]. It appears that luminal antigen-sampling by $CD11c^+$ cells does not occur at estrus, which may have implications for antigen presentation and initiation of the immune response.

$CD68^+$ tissue macrophages are professional phagocytes that perform homeostatic roles within the tissue , including clearance of apoptotic cells and would repair [90, 91]. Macrophages have a homeostatic role in uterus and ovary in humans [40]. Vaginal macrophages are phenotypically different to macrophages in other mucosal sites [92] and peripheral blood [93]. $CD68^+$ tissue macrophages can express MHCII and present antigen to T cells [94] and can also influence the initiation of immune responses by cross-talk with other APCs and T cells [90]. Macrophages express the estrogen receptor and are responsive to progesterone, with macrophage numbers in the endometrium fluctuating over the menstrual cycle [40]. No difference in CD68+ cell localisation was observed in the murine vagina under different hormonal conditions, implying that there would be no difference in vaginal macrophage antigen presentation during the estrous cycle.

4.3.2. Distribution of cells along the lower FRT

In humans immune cells are not distributed throughout the lower FRT uniformly. DCs are most abundant in the cervix and other immune cells are clustered around the cervical transformation zone, where the ecto- and endocervix meet, with very few cells in the vagina [95]. Comparisons of the proximal and distal vagina have shown some differences in T cell distribution [95]. Differences in cell localization and numbers along the tract may affect immunity. Different pathogens invade the FRT at different locations. For example, the vagina is prone to *Candida albicans* and *Trichomonas vaginalis* infection, the cervix is prone to *Chlamydia trachomatis* and *Neisseria gonorrhoea* infection and HPV preferentially infects the transformation zone [28, 47, 95].

There was variation in the localisation of macrophages, DCs and, neutrophils in the FRTs of progesterone treated mice (Fig 4.3 and 4.4), leading to lots of distinct

microenvironments along the length of the lower reproductive tract. However, no statistically significant pattern was observed, that would account for the consistent differences in susceptibility to infection described above.

4.3.3. Affects of neutrophils on APCs

Neutrophils enter the FRT from the blood in response to IL-8 and other chemokines produced by epithelial cells [40]. Infection and insemination results in increased neutrophil recruitment to the FRT in humans [40]. In humans rapid neutrophil recruitment in response to stimuli is particularly associated with the cervix [40]. In the human uterus falling progesterone levels triggers an increase in IL-8 production by uterine epithelial cells [40].

The high progesterone stages of estrous cycle are characterized by physiological infiltration of leukocytes, the majority of which are neutrophils (Fig 3.3, 6 and 7) [40, 82]. GR-1 is expressed in high levels on neutrophils, although, it is also expressed by plasmacytoid DCs and some monocytes [96]. There was variation in the density of GR-1^+ staining in the FRTs of progesterone treated mice (Fig 4.5), however no pattern in distribution of GR-1^+ cells in different areas of the lower FRT were observed. The differences are most likely due to variations in the microenvironments over the epithelium caused by the normal undulations in the tissue.

Neutrophils have been shown to effect APC recruitment and function and may interact directly with APCs [25, 97-101]. To investigate the affect of neutrophil infiltration to the epithelia on $CD11c^+$ cell localization in the epithelia tissue sections from the vaginas and cervixes of Depo-Provera treated mice were stained for Gr-1 and CD11c. There was no significant difference in the number of $CD11c^+$ cells in the areas where there were a lot of GR-1^+ cells compared to areas where there were fewer GR-1^+ cells. This does not exclude the possibility that GR-1^+ cells were responsible for recruitment of $CD11c^+$ cells (Gr-1^+ cells were present in all sections with $CD11c^+$ cells), but shows that increasing numbers of GR-1^+ cells did not cause a proportional increase in $CD11c^+$ cells. There was no correlation between different density of GR-1^+ staining and $CD11c^+$ staining. This shows that while hormonal changes mediate changes in neutrophil infiltration (Fig 3.6-8), the frequency of

neutrophils does not affect DC localisation in the epithelia. This does not rule out the possibility that the presence of neutrophils may affect DC localisation; just that it is not 'dose dependant.'

4.3.4. Quantification of confocal images

While no significant differences in the 2 quantification methods were seen, there are still issues with cell counts and pixel counts. Due to the changes in the epithelia thickness measurement of cells per unit area and pixel counts expressed as a percentage of total pixels (essentially an expression of area) are flawed. For example if the cell numbers in the epithelia remain the same but the epithelia thickens this analysis would show a reduction in cells/mm^2 despite unchanging cell numbers. Measuring the length of the epithelium along the basal edge, which remains relatively unchanged over the estrous cycle (as opposed to measuring the length of the luminal edge of the epithelium, which becomes disrupted), allows for a more accurate prediction of the relative number of cells at different locations and at different cycle stages. Pixel counts cannot discriminate between the same number of cells with greater surface expression and therefore more staining and increased numbers of cells expressing similar levels of surface protein. Both of these scenarios would result in an increased pixel count.

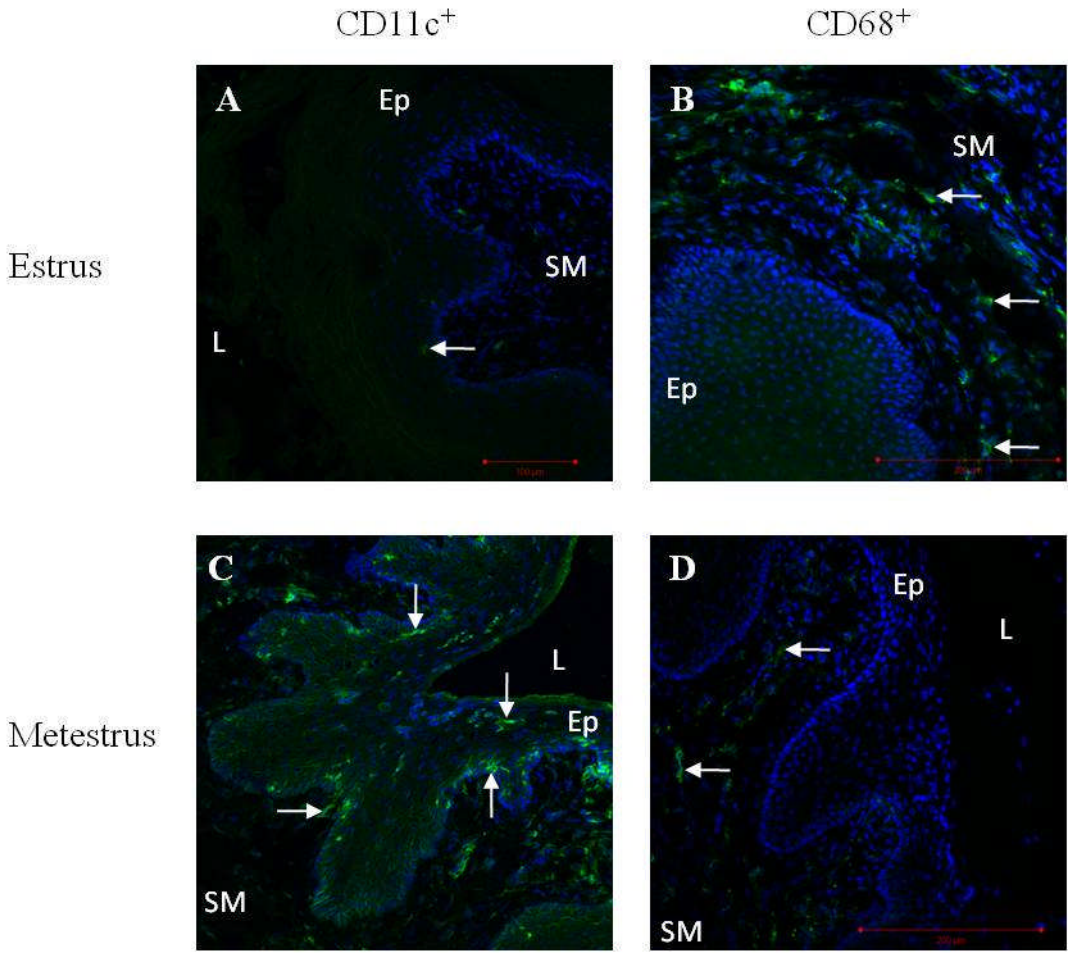

Figure 4.1: Comparison of DC and macrophage localisation within the epithelia of the FRT at different stages of the estrous cycle.

7 µm sections from CD45.1 or C57BL6 mice were stained for CD11c or CD68 (green) and DAPI (blue). Images are representative of 3-6 images per mouse. Estrous cycle stage was determined by H&E staining of tissue sections. A) CD11c stained vagina at estrous. B) CD68 stained vagina at estrus. C) CD11c stained vagina at metestrus D) CD68 stained vagina at metestrus. White arrows show positively stained cells. Ep. Epithelia L. Lumen SM. Submucosa.

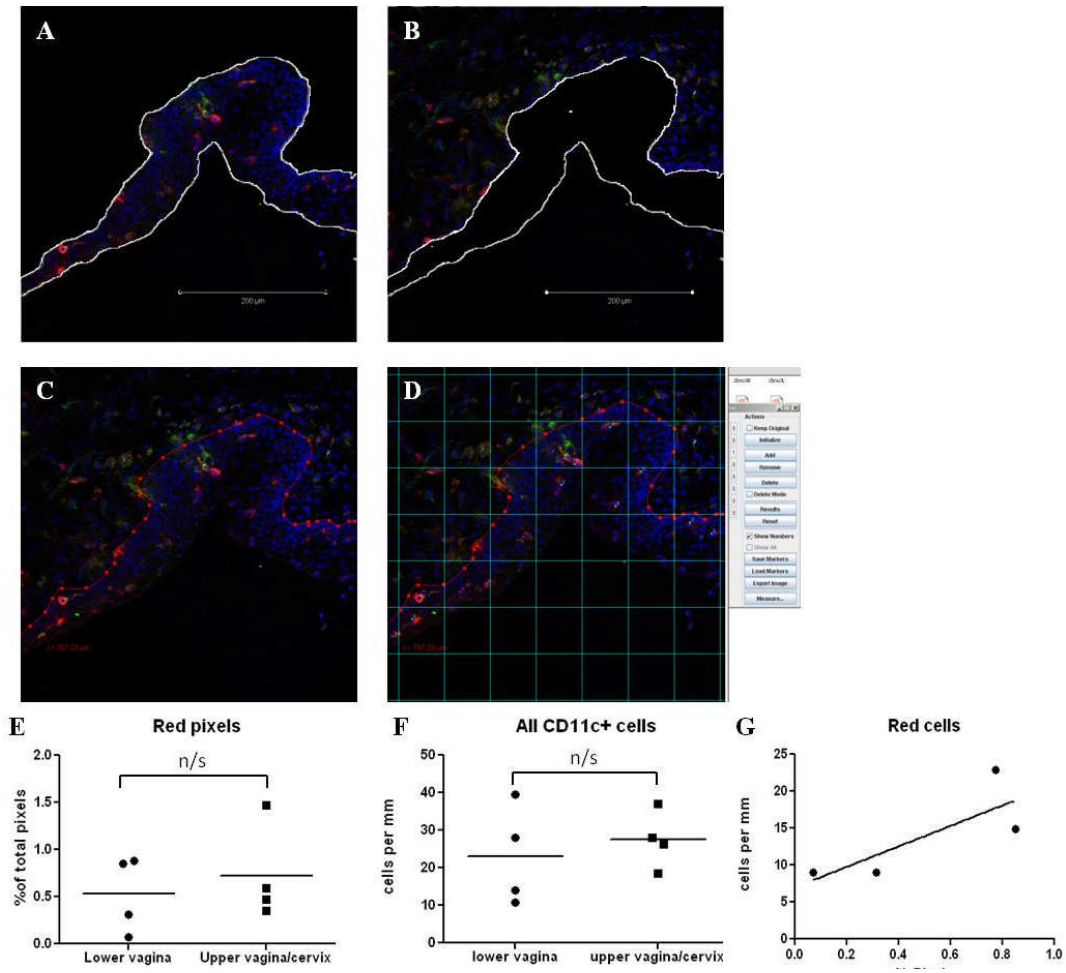

Figure 4.2: Pixel count versus cell count analysis.

A) For pixel counts the analysis was done in Photoshop. A line was drawn around the epithelium and then the epithelium was selected. A total pixel count for the epithelium was performed followed by selected counts on the green, red and yellow channels in turn. B) The epithelium was then excluded and the submucosa selected. Total pixel counts for the submucosa and for each individual channels was performed. C) For cell counts the length of the basal layer of the epithelia was drawn using the LSM image browser software. D) Cell counts were performed using ImageJ software. E) For the total CD11c counts the red and yellow channels were added together and expressed as a percentage of total pixels for each field of view. Percent pixel values from 3-6 fields of view were averaged to give a single point for the lower and upper vagina for each mouse. F) Cell counts were expressed as cells/mm. Each point represents the average cells/mm from 3-5 fields of view. G) To compare the 2 methods of quantification cells/mm was plotted by % total pixels.

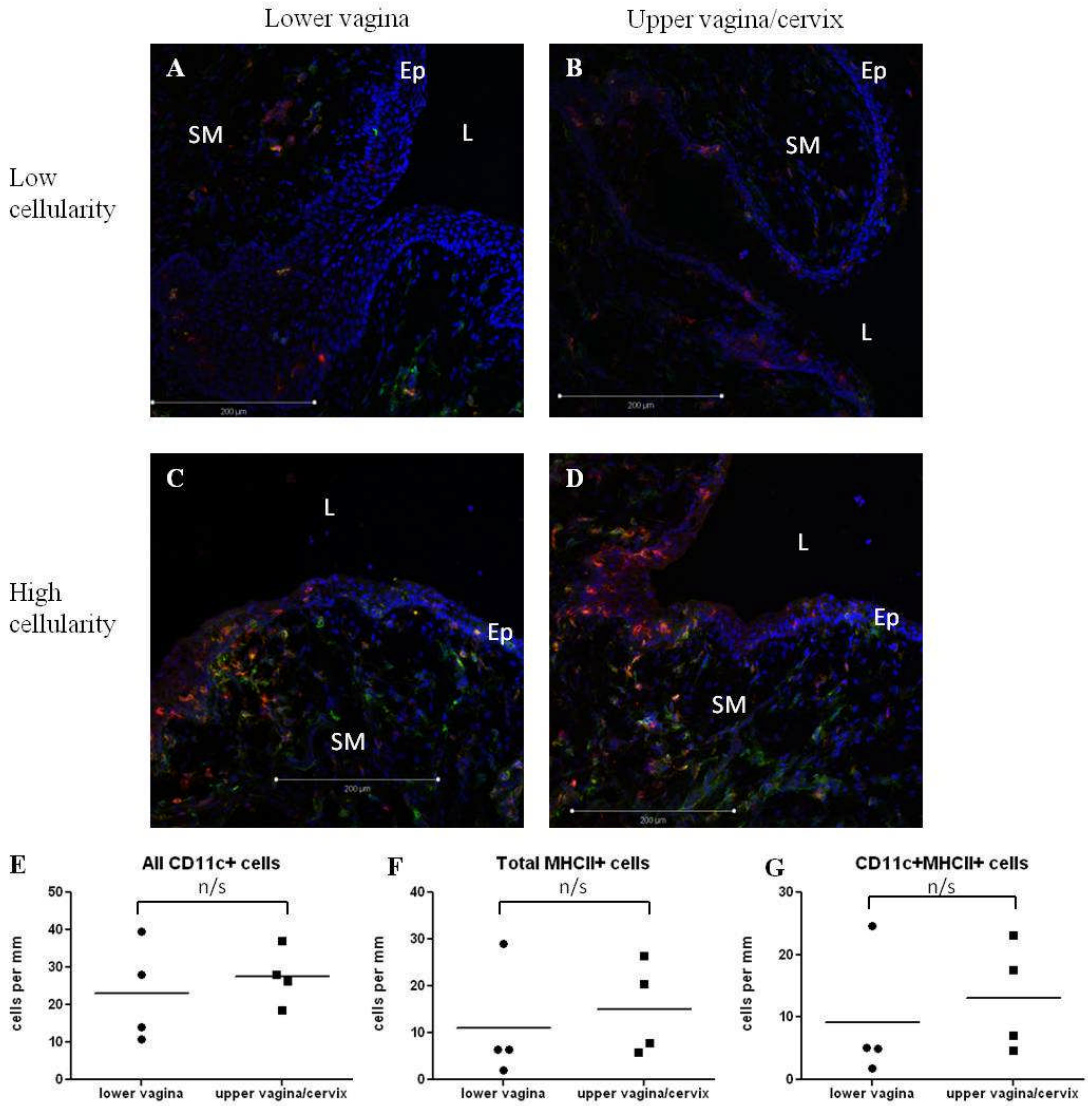

Figure 4.3: Comparison of CD11c$^+$ cells in the epithelia and sub mucosa of the vagina and upper vagina/cervix of Depo-Provera treated mice.

4 female CD45.1 mice were injected subcutaneously with 100 µl of 30 mg/ml Depo-Provera. 5 days later reproductive tracts were removed and 7 µm sections were cut, then stained for CD11c (red), MHCII (green) and DAPI (blue). Representative images of the lower vagina (A and C) and upper vagina/cervix (B and D) of 2 individual mice. A) and B) Mouse with comparatively low numbers of cells in the FRT. C) and D) Mouse with comparatively high numbers of cells in the FRT. (E-G) Average cells/mm CD11c single positive (E), MHCII single positive (F) and CD11c and MHCII double positive (G) cells per mouse. Images are representative of 3-6

images lower vagina and 3-6 images per upper vagina/cervix from 4 different mice. Ep epithelia, L lumen, SM submucosa

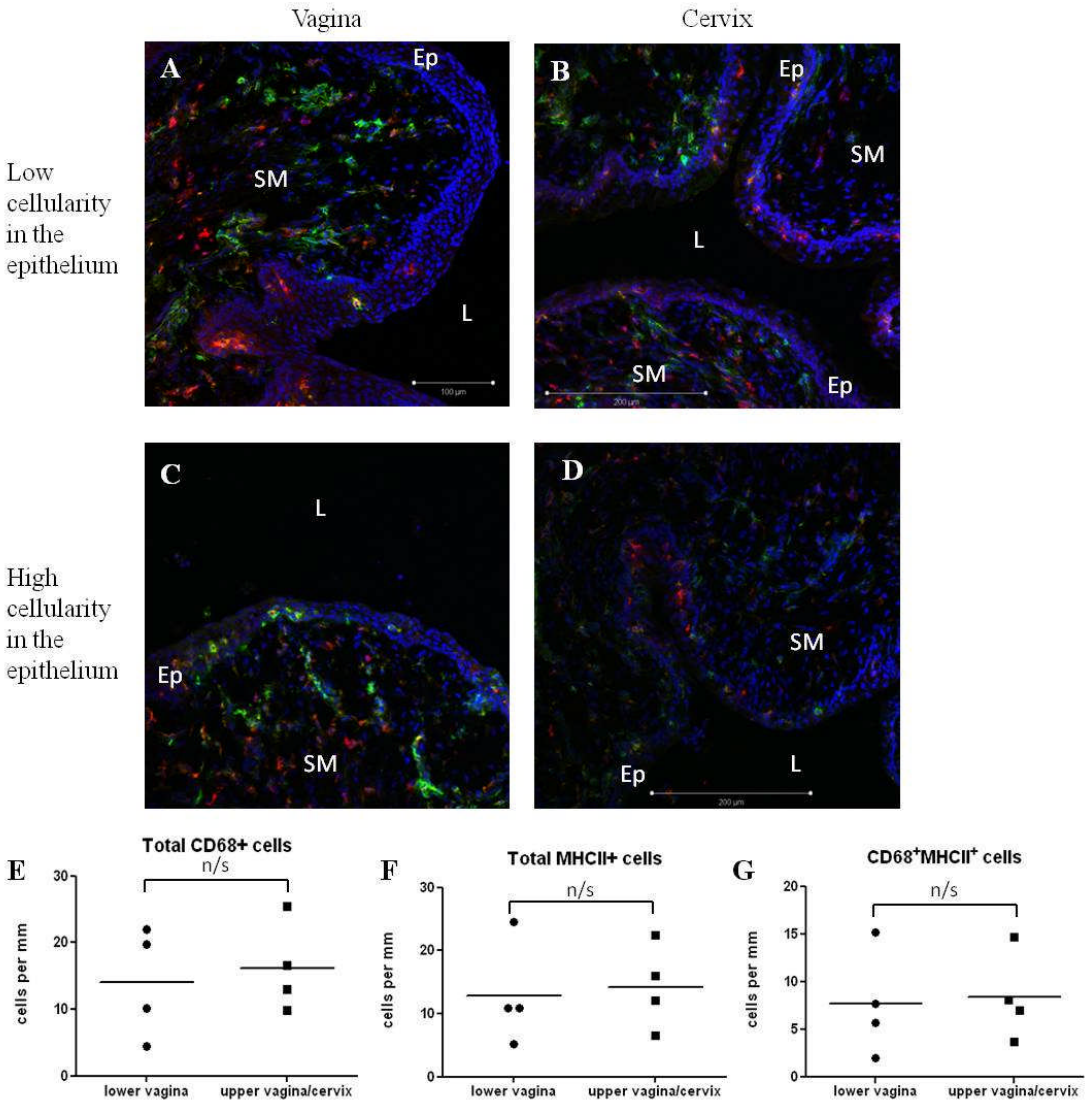

Figure 4.4: CD68 and MHCII positive cells in the epithelia of Depo-Provera treated mice.

Vaginas from 4 B6.CD45.1 mice treated with 100 µl 30 mg/ml Depo-Provera were cut into 7 µm tissue sections. A-D) Representative images of CD68 (red), MHCII (green) and DAPI (blue) stained sections of upper and lower vagina. E-G) Comparison of the average number of CD68+, MHCII+ and CD68+MHCII+ double positive cells per mm in the epithelia of the upper and lower vagina. Images are representative of ≥5 images per group from 4 different mice. Ep epithelia, L lumen, SM submucosa

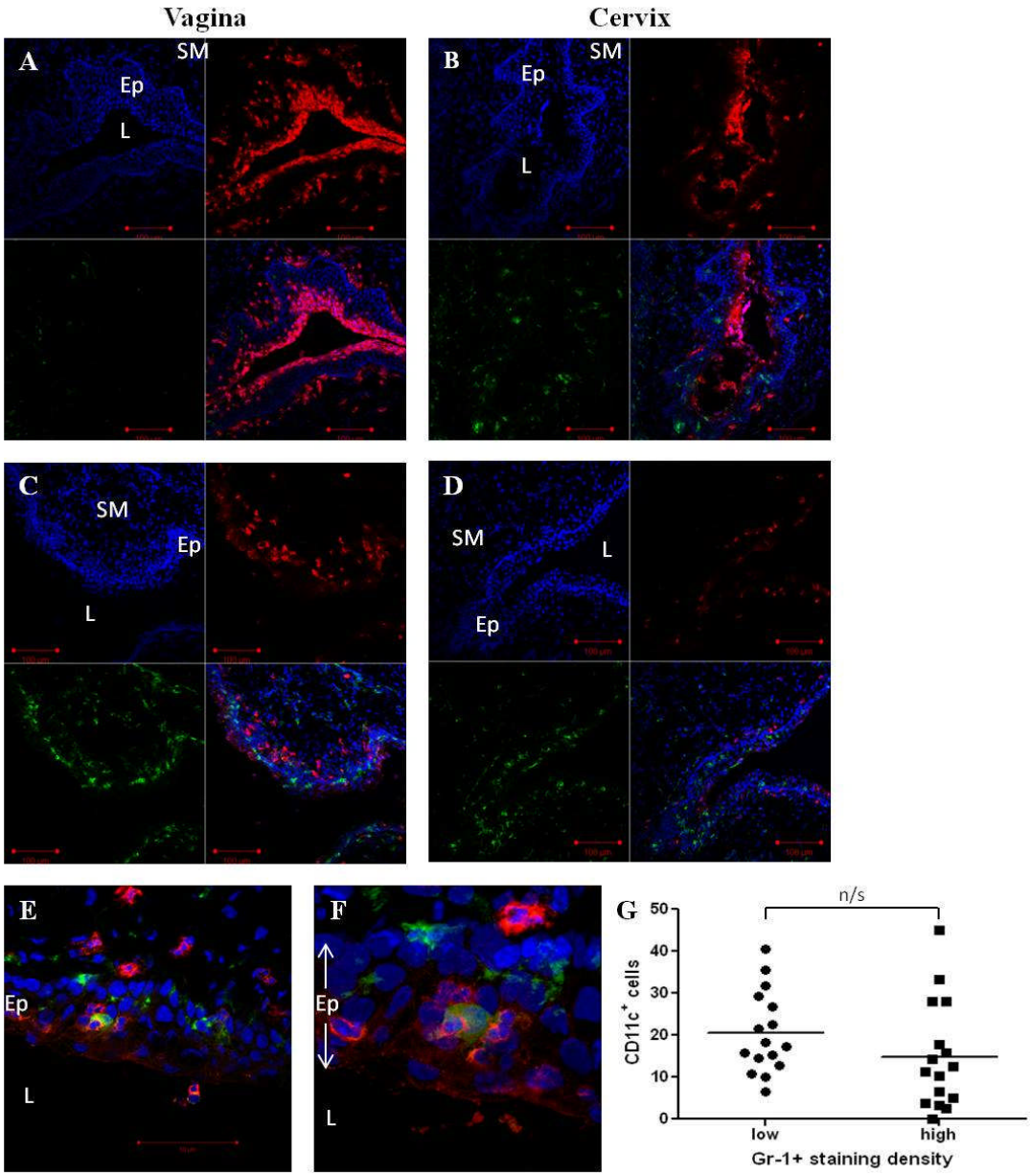

Figure 4.5: DCs and neutrophils in the vagina and cervix of Depo-Provera treated mice.

The vagina and cervix of 4 Depo-Provera treated B6.CD45.1 mice were isolated and 7 µm tissue sections were counterstained with DAPI (blue) and stained with Gr-1 (red) and CD11c (green). A-D) Images are split into from top left to bottom right - blue only, red only, green only and merged image. A) and B) Vagina and cervix of 1 mouse. C) and D) Vagina and cervix of a 2nd mouse. E) $CD11c^+$ cell and $Gr\text{-}1^+$ cell interacting within the epithelia. F) Higher magnification z stack image of E). G) $CD11c^+$ cells numbers in the epithelia of upper and lower vagina. The graph compares DC numbers in areas of high neutrophil density (A and B) and low

neutrophil density (C and D). Each point represents one field of view. Images are representative of ≥3 images per group from 4 different mice. Ep epithelia, L lumen, SM submucosa

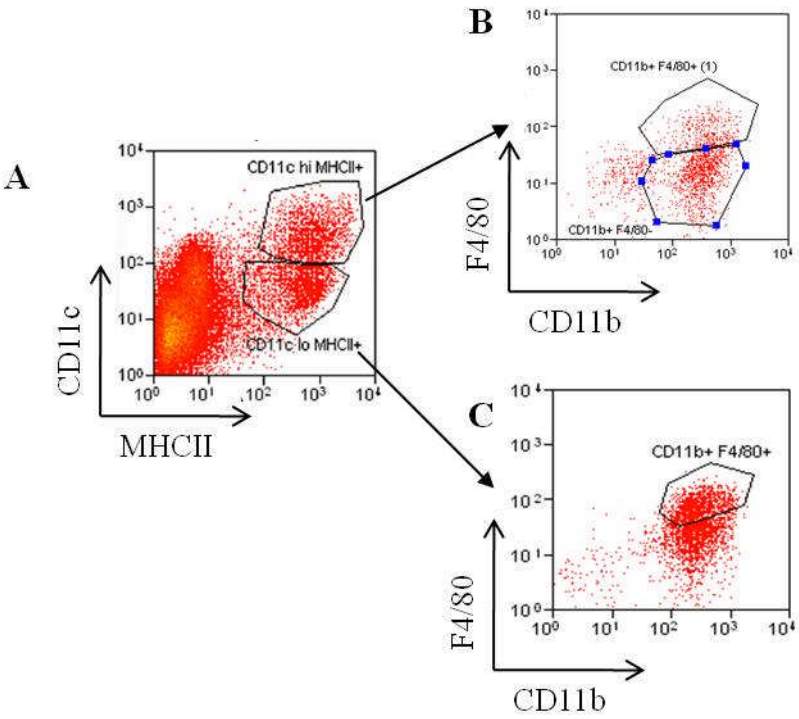

Figure 4.6 Diverse phenotypes of CD11c^{+}MHCII^{+} cells from the vaginal epithelia.

C57BL/6 female mice. Dead cells were excluded using a live/dead discriminator. Live cells were selected based on forward scatter vs. side scatter. Haematopoietic cells were selected based on CD45.2 expression. A) CD11c vs. MHCII expression in CD45.2^{+} cells. B) F4/80 vs. CD11b expression in CD45.2^{+}CD11c^{hi}MHCII^{+} cells. C) F4/80 vs. CD11b expression of CD45.2^{+}CD11c^{lo}MHCII^{+} cells.

5. Characterisation of the lymph nodes draining the reproductive tract

5.1. Introduction

The immune system at mucosal sites is considered to be linked, because of the preferential trafficking of T cells primed in mucosal dLNs to other mucosal lymphoid tissues rather than to the spleen or peripheral LNs [102, 103]. This means that immune responses primed at one mucosal site can elicit protective immunity at distal mucosal sites.

Mucosal lymph nodes have a different ontogeny to other peripheral LNs and are the first secondary LNs to develop in the embryo [104]. In most cases, mesenchymal cells initiate LN development by production of CXCL13, which binds CXCR5 on LTi precursor cells. LTβ signalling between mesenchymal cells and LTi cells causes differentiation of the mesenchymal cells into stromal organiser cells, which give rise to the LN stromal cell populations (MRCs, FRCs etc) [104, 105]. Hence, mice deficient in CXCR5 and CXCL13 lack iliac LNs and other peripheral LNs [106] and LTβ deficient mice lack most peripheral LNs and Peyer's Patches [69, 104, 107, 108]. Surprisingly, MLNs and CLNs are found in mice lacking CXCR5 and CXCL13 [106]. Similarly, MLNs, CLNs and ILNs are present in LTβ deficient mice [69, 104, 107, 108]. These findings imply that mucosal LNs associated with the airways and gut are fundamentally different to other secondary lymphoid tissues.

Lymph nodes function as local hubs for interactions between immune cells. Antigen presentation to naïve T cells as well as T-B cell interactions, which lead to initiation of the cellular and humoural adaptive response, occur here [28, 36, 44, 109-116]. The environment in draining lymph nodes can affect T cell responses [44, 112]. Upon activation naïve T cells change their expression of surface adhesion molecules, which allows them to exit the LN via the lymphatic vessels. The environment produced by APCs and stromal cells in LNs induces expression of different adhesion molecules on T cells [36, 44, 117]. The endothelial cells in different mucosal tissues and lymph nodes express different adhesion molecules meaning that only certain subsets of T cells can enter [69, 71, 84, 118]. For example, MLN stromal cells

produce retinoic acid (RA), which promotes gut-homing by inducing expression of the mucosal addressin MAdCAM-1 [36-38, 44, 114]. Lymph node stromal cells have also been shown to present antigen [119]. There are variations in LN stromal cells in different LNs [36-38], which means that there may be LN specific differences in antigen presentation intrinsic to the stromal cells. In this chapter, the architecture and cellular composition of LNs draining the FRT were compared with other mucosal (mesenteric and cervical) and skin draining LNs.

5.2. Results

5.2.1. Iliac lymph nodes are architecturally similar to other mucosal lymph nodes

While lymph node architecture has been well characterised [34, 114, 115, 120] there has been little comparison of different lymph nodes. Differences in architecture of the LNs especially the locations of APCs and T cells may point to differences in antigen-presentation and subsequent immune responses. Therefore, the macrostructure of the cervical, mesenteric and iliac lymph nodes were compared.

Mice expressing a variety of fluorochromes under the control of cell-specific promoters/locus control regions allow visualisation of lymph nodes in steady state without the need to treat with injectable markers. CD19CreR26REYFPVaDsRedxC57xC57 mice, which express EYFP in CD19 positive cells and DsRed in CD2 positive cells, were dissected on a fluorescent stereo microscope to show the locations of cervical, mesenteric and iliac lymph nodes (Fig 5.1B). Lymph nodes have discreet T cell (depicted blue after image analysis) and B cell (green) zones. There were differences in size of lymph nodes with MLNs being largest. The superficial cervical lymph nodes are also relatively large with smaller deep cervical LNs. Iliac lymph nodes are small and are slightly inconsistent in location (data not shown). They are sometimes both on one side of the aorta and sometimes on both sides, but are always just above the base of the spine. It is unknown if this variability could affect immune responses in different animals.

Sections of frozen iliac, mesenteric and cervical lymph nodes from naïve C57BL/6 mice were stained with markers for DCs ($CD11c^+$, $MHCII^+$), B cells ($MHCII^+$),

marginal reticular cells ($gp38^+$) and lymphatic ($CD31^+$, $gp38^+$) and vascular endothelial cells ($CD31^+$). Fig 5.2 shows representative images of ILNs, CLNs and MLNs. MHCII $^+CD11c^-$ B cells are situated in the cortex in defined follicles. $CD11c^+$ DCs are located in the medulla and surround the B cell follicles but are rarely seen within the B cell follicles. $CD31^+gp38^-$ HEVs are located in the medulla. The $gp38^+$ MRCs form structural support for the T cell zones and subcapsular sinus (SCS) [34, 117]. The localisation of $CD11c^+$ cells around $CD31^+$ vessels, in the T zone and around B cell follicles were comparable, as was the number of B cell follicles (Fig 5.2).

5.2.2. Iliac lymph nodes have comparable antigen-presenting cell subpopulations to cervical lymph nodes, but not mesenteric lymph nodes

Differences in DC numbers and phenotypes in the LN can affect downstream immune responses. The DC populations in iliac LNs were characterised by their expression of surface markers and compared with other mucosal and peripheral LNs. LNs from Depo-Provera treated mice were pooled into groups of cervical lymph nodes, iliac lymph nodes, mesenteric lymph nodes and a group of 'other' lymph nodes, which included axillary and inguinal lymph nodes and then stained for CD11c, MHCII, CD103 and CD11b.

CD11c and MHCII double positive cells were split into 2 subpopulations: $CD11c^{hi}MHCII^+$ and $CD11c^+MHCII^{hi}$ cells (Fig 5.3C) [121, 122]. The percentage of DCs was consistently low, with total $CD11c^{hi}MHCII^+$ and $CD11c^+MHCII^{hi}$ cells accounting for less than 1.5% of total live LN cells in all lymph nodes. Comparison of different lymph nodes showed different percentages of $CD11c^{hi}MHCII^+$ and $CD11c^+MHCII^{hi}$ cells (Fig 3C and D). Iliac lymph nodes had a much lower percentage of both APC populations compared to other lymph nodes.

Both Iliac LNs and mesenteric LNs had lower percentages of $CD11c^{hi}MHCII^+$ antigen presenting cells compared to cervical LNs and the pool of other lymph nodes. Based on their CD103 and CD11b expression DCs could be split into 3 subpopulations (Fig 5.3C): $CD103^{lo}CD11b^+$ cells, $CD103^+CD11b^+$ cells and $CD103^+CD11b^-$ cells. In all lymph nodes $CD103^{lo}CD11b^+$ cells were the largest

population. Percentages of $CD103^{lo}CD11b^{+}$, $CD103^{hi}CD11b^{hi}$ and $CD103^{hi}CD11b^{lo}$ subtypes of $CD11c^{hi}MHCII^{+}$ and $CD11c^{+}MHCII^{hi}$ cells were similar in CLNs, ILNs and other LNs, but MLNs show higher $CD103^{hi}CD11b^{hi}$ and $CD103^{hi}CD11b^{lo}$ cells. In ILNs, CLNs and OLNs $CD103^{lo}CD11b^{+}$ cells dominated. In the MLNs there were similar percentages of all 3 subtypes with a roughly 3-fold increase in the $CD103^{+}$ subsets.

5.2.3. Comparatively low RALDH expression in iliac lymph nodes

To compare RALDH expression of both haematopoietic and non-haematopoietic cells in cervical and iliac lymph nodes pooled LN cells from 5 Depo-Provera treated C57BL6 mice each were stained for RALDH expression and surface molecules.

There were almost no detectable $RALDH^{+}CD45.1^{-}$ non-haematopoietic cells in any LNs (Fig 5.4B). The highest found was 0.7% of the CD45.1- population (or 21 cells), which is below the reliable detection of this assay. Iliac lymph nodes had the lowest percentage of $RALDH^{+}CD45.1^{+}$ cells with roughly equal numbers in the CLNs and 'other' LNs. A lower percentage of iliac lymph node $CD45.2^{+}$ cells showed RALDH activity compared to cervical lymph nodes. Fewer iliac lymph node $CD11c^{+}MHCII^{+}$ cells show RALDH activity compared to cervical lymph node cells.

5.3. Discussion

The FRT is a mucosal tissue, however in the lower FRT the epithelia and the resident immune cells bear similarities to the skin. To determine if the FRT dLNs were more similar to mucosal LNs or peripheral LNs that drain the skin, the iliac LNs were compared to other LNs.

Iliac LNs are architecturally similar to MLNs and CLNs. ILNs have a lower percentage of $CD11c^{+}MHCII^{+}$ APCs than MLNs or CLNs, even after accounting for the lower total cell numbers (Fig 5.3). Different ratios or numbers of DC subtypes may imply that there are functional differences between lymph nodes.$CD11c^{+}$ DCs in both the tissues and lymph nodes can be divided into functionally different subtypes based on the expression of surface molecules. DC subtypes have been well characterised in many other tissues [26, 50, 110, 112, 113, 123-128]. In the lungs

$CD11c^{+}MHCII^{hi}$ cells, but not $CD11c^{hi}MHCII^{+}$ have been shown to be responsible for antigen transport to the dLN [122]. $CD11c^{+}MHCII^{hi}$ cells are thought to be migratory DCs while $CD11c^{hi}MHCII^{+}$ cells are LN resident DCs [128]. $CD103^{+}$ DCs have been best characterised in the gut [85, 110, 129], but are also found in other lymph nodes [113, 130]. CD103 expression is involved in attachment to mucosal epithelial cells and is found in migratory DCs from mucosal lymph nodes [85, 110, 131]. They have been shown to promote tolerogenic responses and effector T cell homing to mucosal tissues [110, 129, 131]. ILNs have similar APC populations to CLNs, but not MLNs.

The vitamin A metabolite retinoic acid (RA) is linked to oral tolerance because it can affect T cell maturation by promoting the differentiation of Tregs and gut homing [37, 113]. Retinal is converted to RA by RALDH enzyme activity [132]. In the MLNs tissue-derived DCs and macrophages [133], as well as lymph-node resident non-haematopoietic stromal cells [36-38], express RALDH and are thought to be important for oral tolerance induction [37, 38, 114]. RALDH expression is associated with the induction of gut-homing T and B cells and tolerance [37, 38, 113, 133, 134]. RALDH is also expressed by $CD11c^{+}MHCII^{+}$ cells in other mucosal lymph nodes such as the mediastinal LNs that drain the lung, but is expressed at much lower levels in skin-draining LNs and in the spleen [113]. In this study RALDH expression in iliac LNs was measured and compared to other LNs, which has not been investigated before. While RALDH expression was lower in iliac LNs compared to other peripheral LNs, there is some evidence that RA may be important in immunity in the FRT because there is increased HIV shedding in women with vitamin A deficiencies [60].

The lower RALDH expression and lower DC ratios implies that ILNs are dissimilar to other mucosal LNs. These differences in APC populations and the environment within the ILNs potentially means that antigen presentation and the subsequent immune response and long term immunity initiated will be different to the immune responses initiated with the same antigen at a different mucosal site. This has implications for treatment and prevention of STIs and for vaccination strategies administered intravaginally or for vaccines that need to induce immunity in the FRT, but are administered at different sites.

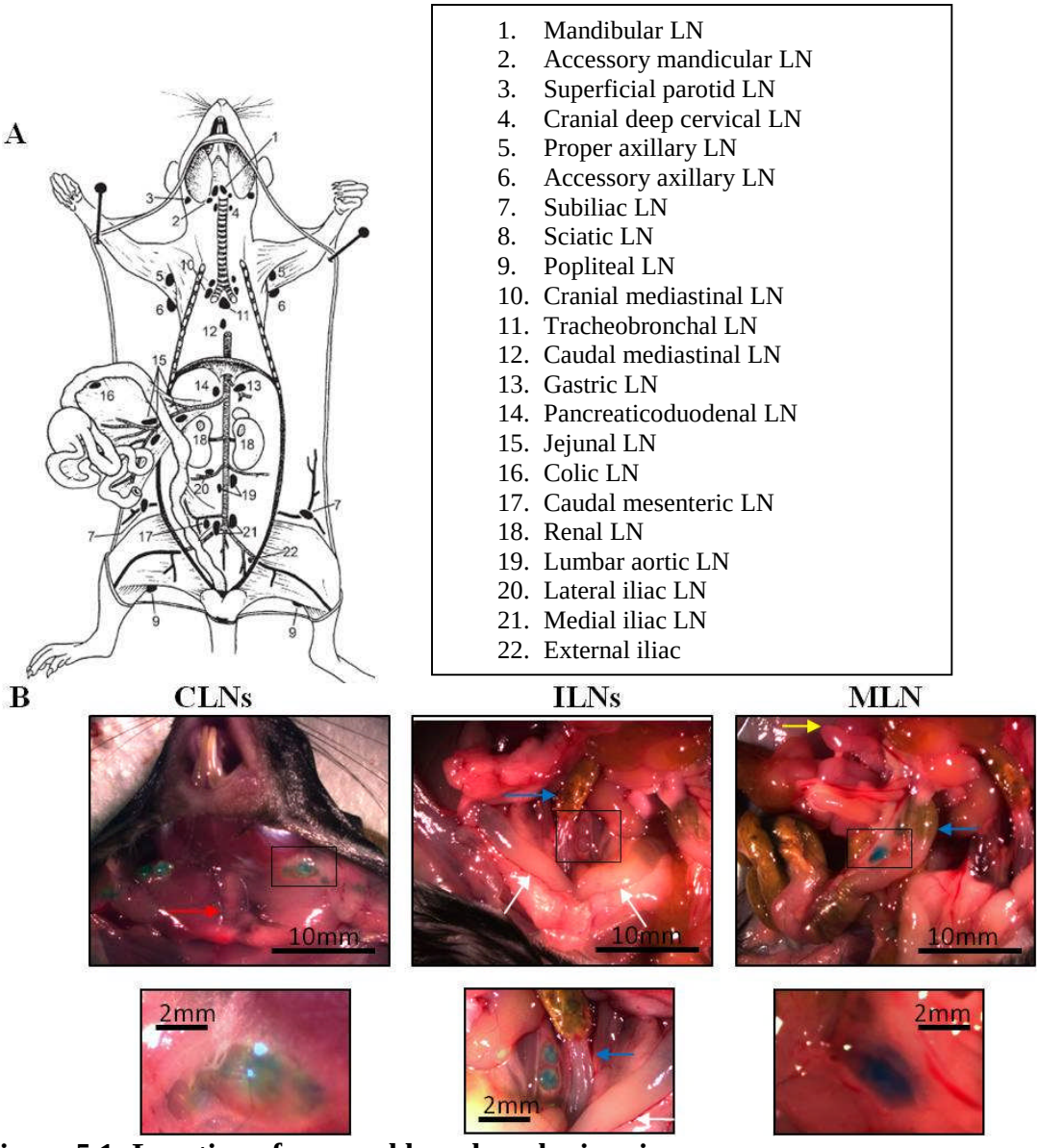

Figure 5.1: Location of mucosal lymph nodes in mice.

A) Nomenclature of lymph nodes in mice, from [120]. B) CD19CreR26REYFPVaDsRedxC57xC57 mouse was imaged. Blue colouration shows T cells and green shows B cells. Arrows show trachea (red), small intestine (yellow), colon (blue) and uterine horns (white). The zoomed images are separate higher magnification images. Data is representative of 2 mice.

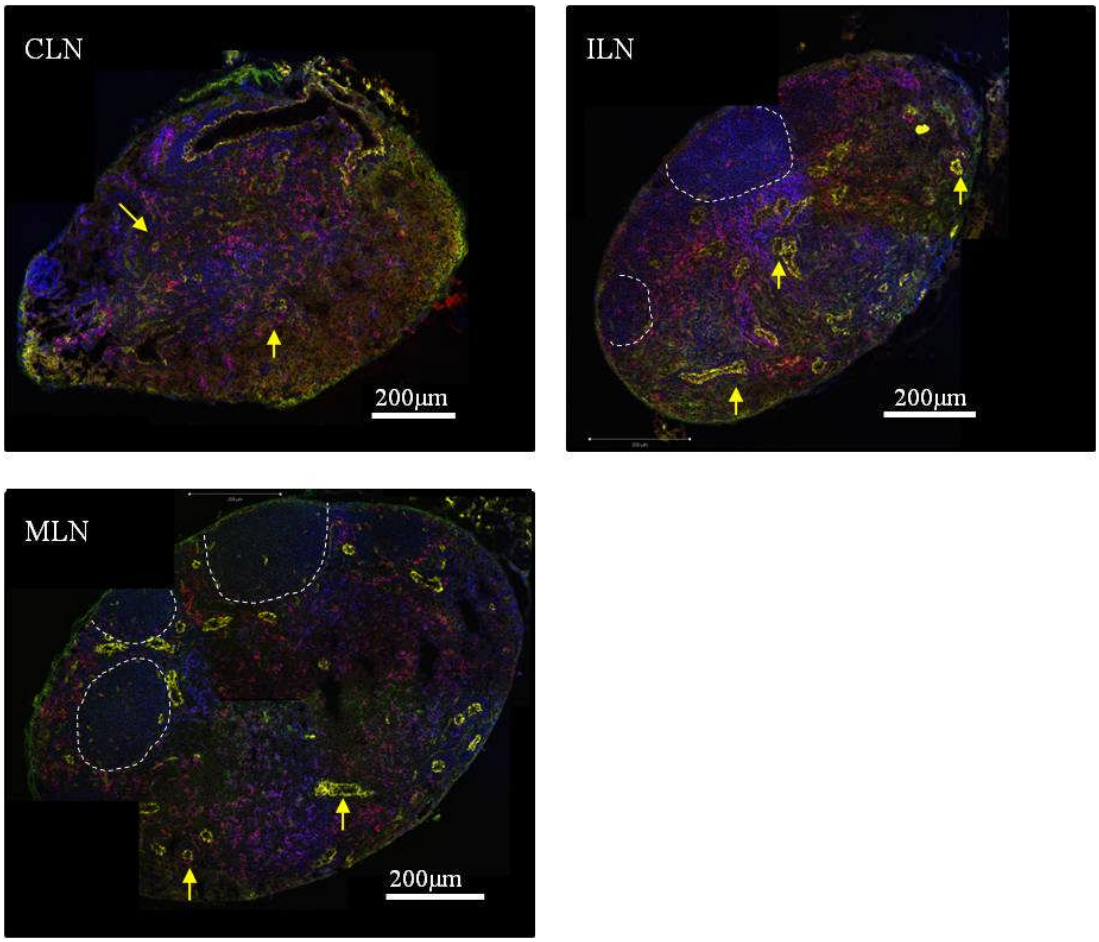

Figure 5.2: Architecture of mucosal lymph nodes.

3 C57BL/6 mice were injected subcutaneously with 100 µl 30 mg/ml Depo-Provera. 5 days later estrous cycle arrest was confirmed by vaginal washes. Lymph nodes were removed and frozen in OCT. 8 µm sections were cut on a cryostat and then stained for MHCII (blue), gp38 (green), CD31 (yellow) and CD11c (red). Dashed lines show B cell follicles. Yellow arrows show HEVs. Overlapping images were taken on a confocal microscope and stitched together using Fiji software.

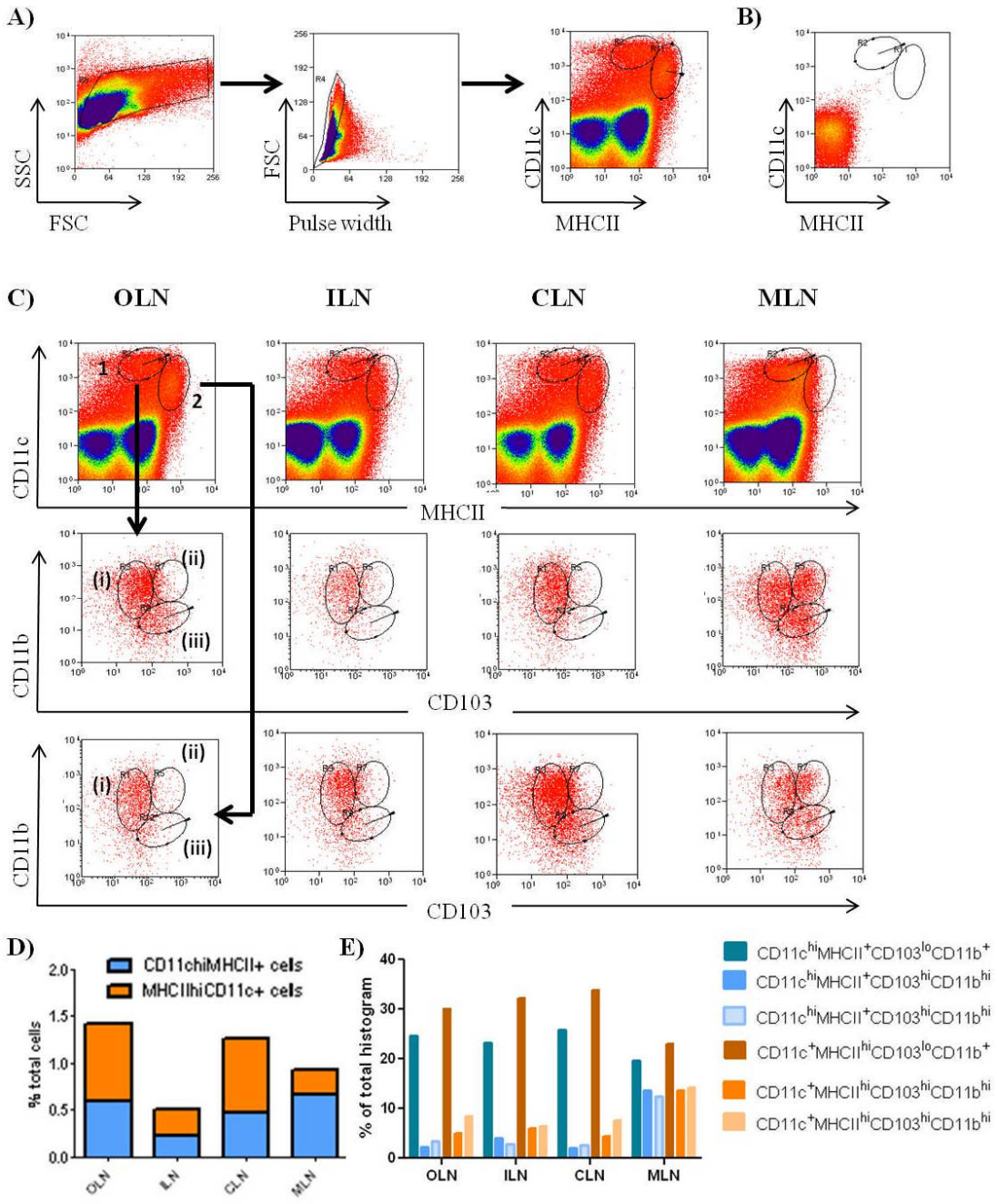

Figure 5.3: Comparison of DC subtypes in iliac, mucosal and peripheral lymph nodes.

Lymph nodes from 5 female Depo-Provera treated C57BL/6 mice were pooled and stained for surface markers. A) Gating strategy. Live cells are selected based on forward scatter vs. side scatter. Doublet discrimination based on pulse width vs. forward scatter. Both $CD11c^{hi}MHCII^{+}$ and $CD11c^{+}MHCII^{hi}$ antigen presenting cells are analysed. B) Isotype control. C) CD103 and CD11b expression of $CD11c^{hi}MHCII^{+}$ and $CD11c^{+}MHCII^{hi}$ antigen presenting cells. D) % of total cells

that are CD11c^{hi}MHCII^{+} and CD11c^{+}MHCII^{hi} in different lymph nodes. E) % of total gate of 3 subpopulations of CD11bCD103 expressing cells. 1) CD11c^{hi}MHCII^{+} 2) CD11c^{+}MHCII^{hi} i) CD103^{lo}CD11b^{+} ii) CD103^{hi}CD11b^{hi} iii) CD103^{hi}CD11b^{lo}

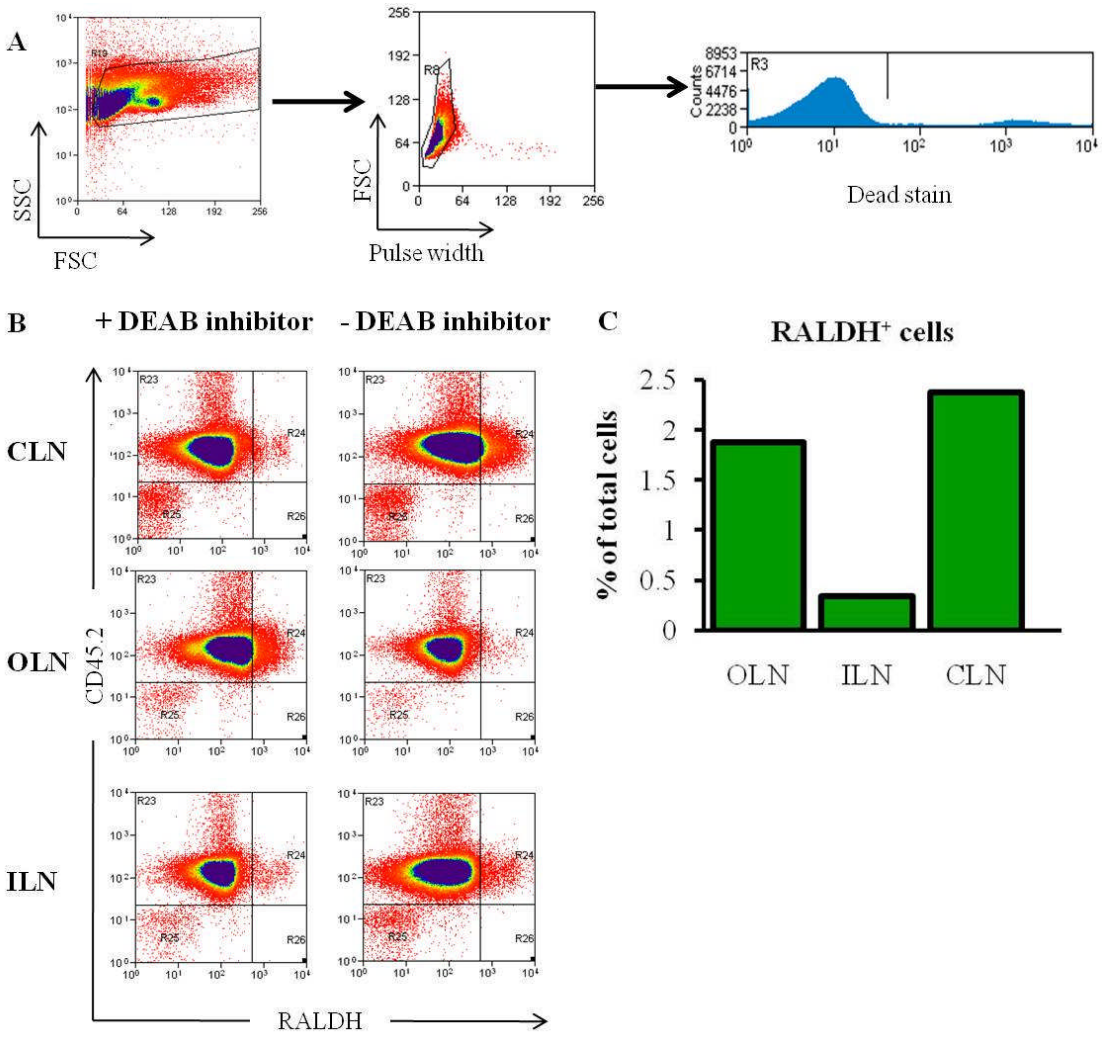

Figure 5.4: RALDH expression in iliac and peripheral lymph nodes.

LNs from 5 female Depo-Provera treated mice were pooled into groups of CLNs, ILNs and OLNs and stained for CD45.2 and with ALDEFLUOR for RALDH activity. A) Gating strategy. Cells were gated on based on forward scatter vs. side scatter. Doublets were excluded based on pulse width vs. forward scatter and dead cells were excluded based on positive staining for the live/dead cell marker. B) Cells were assessed for RALDH activity with or without the DEAB inhibitors. DEAB inhibitors prevent RALDH activity. C) RALDH^{+} cells as a percentage of total cells.

6. Discussion

6.1. General conclusions

The FRT undergoes drastic changes over the reproductive cycle. In progesterone high stages there is a large influx of leukocytes. The distribution of APCs is constant along the length of the vagina and cervix. The numbers of Gr-1^+ cells varies greatly over the course of the reproductive cycle. The distribution of Gr-1^+ cells within the tissue varies, with some areas showing relatively high numbers of neutrophils in the outer epithelia and some areas showing much lower infiltration. This study found no significant pattern to the areas of low or high neutrophil influx. It is possible that this represents individual differences in the architecture of the epithelia caused by differences in epithelial folding, thickness and mucus flow. This shows that the frequency of APCs is constant throughout the tract, but that the local environments within the tract may have differences in antigen capture and presentation. There is a lot of variation along the length of the lower reproductive tract and between mice, which may affect the design of future experiments. For example more mice may be needed to get statistically significant results.

There is some evidence that neutrophils in the human fallopian tube [40, 135] are phenotypically different to normal blood neutrophils. In mice anti-Gr-1^+ cell depletion results in estrous cycle arrest [136], implying a physiological role for neutrophils. However, monocytes also express Gr-1 and depletion with the neutrophil specific anti-Ly6G antibodies does not result in estrous cycle arrest (Schäfer unpublished observations). It is not known if neutrophils recruited to the vagina in the murine estrous cycle are different to blood neutrophils. There is a rapidly growing literature on newly characterised neutrophil phenotypes and functions [25, 97-101, 135]. This has led to a growing appreciation that neutrophils are not merely rapidly recruited killer cells, but may have varied roles in the immune response. [99, 137]

Iliac lymph nodes are architecturally similar to mesenteric and cervical nodes in normal mice, as judged by distribution of B cell follicles, vascular and lymph endothelia and DC localisation. Hence, there is unlikely to be any major differences

in the cellular organisation or trafficking between iliac and other mucosal lymph nodes. This does not exclude the possibility of differences in architecture occurring during an immune response or as a result of cyclic changes.

The lymph nodes draining the FRT have a lower percentage of APCs compared to other mucosal lymph nodes. APC's can be divided into subpopulations based on their expression of the surface molecules CD103 and CD11b. The ratios of different subpopulations in FRT dLNs are comparable to cervical lymph nodes, but are different to MLN ratios. In MLNs there are higher percentages of the $CD103^{hi}$ subtypes. CD103 is involved in binding to epithelial cells so CD103 expression is associated with cells that have migrated from the epithelia of mucosal tissues [138]. This implies that there are either fewer migratory DCs coming from the FRT and nasal tissue or that $CD103^{+}$ represent only a minor subpopulation of the migratory DCs from these sites.

RALDH expression is lower in iliac LNs compared to cervical and other peripheral LNs. Low RALDH expression implies a bias away from tolerogenic responses and trafficking to mucosal tissues. However, tolerogenic responses in the skin are associated with increased vitamin D metabolites and not RA [134], so it is possible that vitamin D could induce tolerogenic responses in the FRT. Vitamin A deficiency is associated with susceptibility to HIV infection [60], but the implication of this is unclear. One possibility is that T cells primed in mucosal lymph nodes do not traffic to mucosal tissues because they have not upregulated MAdCAM-1 and would traffic to other LNs.

DCs represent a lower percentage of iliac LNs than in other LNs, this could mean that the FRT has a lower potential for stimulating immune responses. This may be due to either or both lower LN resident DCs and lower migratory DCs. Since the ratios of DC subpopulations are equivalent to other lymph nodes, the data in this study would imply an overall drop in DC numbers rather than changes in specific subpopulations. If antigen presentation is equivalent in ILNs this could mean that another cell type is providing antigen presentation, for example macrophages.

6.2. Disadvantages of Mouse Models

The observation of a large infiltration of neutrophils during metestrus/diestrus and in progesterone treated animals raises questions about the appropriateness of this model to study human FRT biology. Neutrophils are not seen in large numbers in humans either during the normal menstrual cycle or after hormone contraceptive treatment [40]. There is no increase in neutrophils during the progesterone high stage of the menstrual cycle despite increases in neutrophils chemoattractants [40, 139].

Mouse models have advantages over doing work on humans or macaques. Firstly, there are lower ethical restraints. Secondly, there is a greater potential for genetic manipulation due to the diverse genetic models in mice. Whilst not exactly the same as humans, mice provide a good model for proof of concept studies.

An alternative would be to use *in vitro* experiments based on human FRT cell lines or explants. Due to the high turnover of immune cells coming into the tissue from the blood and that many of the important immunological events take place in the lymph node, these methods come with a lot of caveats and cannot effectively mimic complex inter-system interactions involved in immunity.

6.3. Future Work

Phenotyping of the neutrophils of the lower FRT and comparison with both blood and uterine neutrophils would shed some light onto the homeostatic function of neutrophils. It would also be interesting to compare the early events in primary infection with or without neutrophils present to see if they have any effect on APCs or the initiation of the adaptive immune response.

There is some evidence that macrophages in the human FRT are phenotypically different to other macrophages [92] and it would be interesting to see what differences this would have on immunity and if similar differences were also observed in mouse models. FRT DC phenotypes have been more thoroughly studied in mice [81].

'Mucosalness' of LNs is difficult to define. Immune responses to administered antigen may prove a better way of comparing mucosal lymph nodes. It would be important to study the trafficking of T cells primed in the FRT and their expression

of MAdCAM-1 and other tissue addressins. This would have important implications for systemic immunity and vaccine design. The data produced here provides a foundation for such studies.

Abbreviations

AIDS	acquired immunodeficiency syndrome
APC	antigen-presenting cell
APC	allophycocyanin (Materials and Methods only)
BSA	bovine serum albumin
CLN	cervical lymph node
Cy	cyanin
DAPI	4',6'-diamidino-2-phenylindole
DC	dendritic cell
DEAB	diethylaminobenzaldehyde
dLN	draining lymph node
FACS	fluorescence-activated cell sorting
FCS	Foetal calf serum
Flt-3L	fms-like tyrosine kinase-3 ligand
FRC	fibroblastic reticular cell
FRT	female reproductive tract
FSH	follicle-stimulating hormone
gp38	podoplanin
GM-CSF	granulocyte-macrophage colony stimulating factor
Gr-1	granulocyte differentiation antigen 1
H&E	haematoxylin and eosin
HEV	high endothelial venule

HIV	human immunodeficiency virus
HPV	human papilloma virus
HSV	herpes simplex virus
IL	interleukin
ILN	iliac lymph node
LC	Langerhans' cells
LH	luteinizing hormone
LN	lymph node
LPS	lipopolysaccharide
LT	lymphotoxin
LTi	lymphoid tissue inducer
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MCP-1	monocyte chemoattractant protein -1
MHCII	major histocompatibility complex type two
MIP-2	macrophage inflammatory protein 2
MLN	mesenteric lymph node
MRC	marginal reticular cell
OCT	optimal cutting temperature
OLN	'other' (mixture of axillary and inguinal) lymph nodes
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PE	phycoerythrin

PerCP	peridinin chlorophyll A protein
PFA	paraformaldehyde
PP	Peyer's patch
PRR	pathogen-recognition receptor
RA	retinoic acid
RALDH	retinaldehyde dehydrogenase
SC	stratum corneum
SCS	subcapsular sinus
STI	sexually transmitted infection
TGF	transforming growth factor
Th1	type 1 helper T cell
TLR	toll-like receptor
T_{reg}	regulatory helper T cell

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