



The role of eutopic endometrial CD8+ T cells in adverse reproductive outcomes

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Michaelmas Term 2023



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Michaelmas Term 2023, St Catherine's College

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Oxford

Abstract

Endometrial immune dysregulation is indicated in adverse reproductive outcomes, such as endometriosis-associated subfertility (EAS) and recurrent pregnancy loss (RPL). Endometriosis, defined as the growth of endometrium-like tissue outside of the uterus, affects 1 in 10 women, of which 35–50% suffer from EAS. RPL is defined as the loss of two or more subsequent clinically recognised pregnancies before week 24 and affects 1 in 50 pregnant women. This thesis investigates the role of CD8+ T (CD8 T) cells in endometriosis and RPL, the major T cell population constituting 25–35% of all endometrial leukocytes.

I performed a systematic review of CD8 T cells in endometriosis, revealing conflicting data in endometriosis-associated tissues with no detailed phenotypic analysis of CD8 T cells in the endometrium and peripheral blood. Bulk mRNA sequencing of endometrial CD8 T cells from RPL patients (n = 13) and fertile controls (n = 11) demonstrated significantly altered CD8 T cell transcriptome between RPL patients with miscarriage versus live birth in the next pregnancy and between RPL groups and fertile controls. A 35-parameter panel was developed for full spectrum flow cytometry (Cytek®Aurora), and matched endometrial and peripheral blood samples of 10 RPL, 20 endometriosis and 8 control patients were analysed. I confirmed significant variations

between the endometrium and peripheral blood. Endometrial CD8+ MAIT-like cells were increased in endometriosis versus non-endometriosis controls ($p.adj < 0.05$), and naïve CD8 T cells were increased in RPL patients with miscarriage versus live birth in their next pregnancy ($p.adj = 0.021$). Finally, samples were histologically examined across the menstrual cycle with singleplex and multiplex (ZellScannerONE®) immune imaging to study the spatial organisation of single immune cells, including intraepithelial lymphocytes and lymphoid aggregates (LAs) across the cycle in patients and controls.

My findings on endometrial CD8 T cell transcriptomes offer valuable insights into modified immune responses during the window of implantation in RPL. Flow cytometry revealed systemic immune changes in endometriosis and localised CD8 T cell dysregulation in EAS and RPL. Qualitative differences in endometrial and myometrial immune populations across menstrual cycle phases in endometriosis indicate opportunities for future research to understand mechanisms at the foeto-maternal interface impacting embryo implantation and immune tolerance.

Acknowledgements

As a good book, hopefully, this thesis keeps you turning the pages about my scientific journey, driven by curiosity and uncertainty. It all began with the idea to undertake a wet lab project as a freshly minted clinician who had barely ever entered the lab, with my supervisors willing to accept the uncertainty of my ability to deliver what was needed to complete it. And finally, as I conclude this writing in circumstances that have forever changed my view of life, I am reminded not to take anything for granted – a curious mind, health, people, help ...

I will do my best to thank everyone who was irreplaceable during my research and personal DPhil endeavours. First and foremost, Jen, Ingrid and Christian, who have been an absolute blessing in every sense possible. With their unwavering support, encouragement, and guidance, I cannot imagine better supervisors. Jen, thank you for the exceptional mentorship throughout my journey, for always making yourself available and for balancing seriousness with humour when needed. Your trust and belief in me have been invaluable in overcoming doubts and anxiety.

My thank you extends to some research and administration team members from the Nuffield Department of Women's and Reproductive Health, many of whom became my Oxford family. Starting with Krina, who opened the doors to our wonderful group. Kurtis and Becca, for all the administrative backing. My lab gang, Bec, Yifan, Zhixing, Monica, Magda, Adriana and Tiff for helping out whenever I did not know my way around the benches and pipettes. Tatjana, Lydia, Danielle, and Fitnat, thank you for encouraging a great climate in the group and motivating me to engage in public outreach activities. Ginny, Kelly, Lisa, Shanice, Sandra, Carol, Fenella, Tabitha and others for recruiting patients and collecting tissue samples. And last but absolutely not least.

Thank you, Ruth, Omar, Toni and Nilay, for adopting me into your team and Magdalena, Maryam and Edie for being so much more than the best office mates.

There are also several people and organisations whom I did not think about every day but were indispensable for this wonderful project. The funders who made my dreams possible: Rotary Klub Ljubljana, The City Municipality of Ljubljana, Die Lucas Knaffel'sche Privatstiftung, St. Catherine's College, British Society for Immunology, Human Immune Discovery Initiative (HIDI) and Oxford/Bayer-Alliance for Women's Health. Collaborators: Adam Davison, Helen Ferry, Kate Powell, Paul Klenerman and John Cole. And, of course, the patients who were willing to combat our lengthy questionnaire and donate their precious tissue samples.

My other Oxford family members, including housemates Grace, Fabian, Giannis, Lottie, Chris, Douglas and Tobias, thank you for listening to me complain and thrive, sometimes all in a matter of seconds. I could not agree more with Grace on this: PhD life means thriving while sucking. Klara went with me, among many other things, through the thick and thin of the full spectrum flow cytometry panel optimisation, and Martin reinvented the statistical analysis in a manner I would never be able to undertake myself. Many others supported me in various ways and kept me sane throughout. I cannot list all, but thank you, Jernej, Flurin, Micon, Annalisa, the Oxford University Swiss Society crew and Luna, my ironwoman, my forever moon, *en retour*, my inspiration.

Friends back home and in Switzerland, my trustees, and ego-boosters. Matjaž, who ensured my thesis looks pretty and well-organised. Fika, my biggest fan and a puppy overachiever, who challenged my organisational and patience skills but rewarded me with endless love, destressing cuddles and motivational park runs. And finally.

Ati, mami, Urban, Veronika, Milena, Zvone, dedi, bica in mama. Hvala, ker ste me oblikovali v to, kar sem. Ker ste moji vzorniki za trdo delo, vzdržljivost in iznajdljivost. Ker podpirate moje nekonvencionalne sanje in mi dajete pogum ter podporo, da jih uresničim. Ta dosežek je toliko vaš kot moj.

A Wayfarer

(Tone Pavček)

When you walk, follow your way to the end.

In spring, to the rose in flower,

in summer to the ripened wheat,

in autumn, to the well-stocked shelf,

in winter, to the snow-white queen,

in a book, to the last line recorded,

in life, to the very truth,

and in yourself – to a colour shed

over your cheeks blushing red.

If you've failed the first time, the second, then,

in attaining sorts and summits, you pursue,

try

again,

once more,

and anew.

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List of abbreviations

A	APC	Antigen presenting cell
	APS	Antiphospholipid syndrome
	ART	Assisted reproductive technologies
	AUC	Area under the receiver operating characteristic curve
	ASRM	American Society for Reproductive Medicine
C	cDNA	Complementary DNA
D	DC	Dendritic cell
	DN	Double negative
	DP	Double positive
E	EAS	Endometriosis-associated subfertility
	ESHRE	European Society of Human Reproduction and Embryology
	EVT	Extravillous trophoblast
F	FCS	Foetal calf serum
	FRT	Female reproductive tract
	FSC	Forward scatter
	FSH	Follicular stimulating hormone
	FWER p	Family-wise error rate p-value
G	GC	Germinal centre
	GO	Gene Ontology
	GSEA	Gene Set Enrichment Analysis
	γδ T	γδ+ T cell
	GWAS	Genome-wide association study
H	HLA	Human leukocyte antigen

	HEC	Human endometrial single cell
I	IEL	Intraepithelial lymphocyte
	IFN	Interferon
	IL	Interleukin
	IHC	Immunohistochemistry
	ILC	Innate lymphoid cells
	IVF	*In vitro* fertilisation
K	KEGG	Kyoto Encyclopedia of Genes and Genomes
L	LA	Lymphoid aggregate
	LH	Luteinizing hormone
M	MAIT	Mucosal-associated invariant T cell
	MFI	Median fluorescence intensity
	MHC	Major histocompatibility complex
	mRNA	Messenger RNA
N	N/A	Not available, not applicable
	NGS	Next generation sequencing
	NK	Natural killer
	NK T	Natural killer T
	NES	Normalised enrichment score
O	OR	Odds ratio
P	PBMC	Peripheral blood mononuclear cells
	PBS	Phosphate-buffered saline
	PCA	Principal component analysis
	PD-1	Programmed cell death protein 1
	PD-L1	Programmed death-ligand 1

	PICO	Population, intervention, comparator, and outcomes
	PID	Pelvic inflammatory disease
	PRPL	Primary recurrent pregnancy loss
R	rAFS/ASRM	Revised criteria for endometriosis developed by the American Fertility Society and American Society of Reproductive Medicine
	RCOG	Royal College of Obstetricians and Gynaecologists
	RIF	Recurrent implantation failure
	RNAseq	RNA sequencing
	ROC	Receiver operating characteristic curve
	RPL	Recurrent pregnancy loss
	RR	Relative risk
S	SCC	Spearman Correlation Coefficient
	scRNAseq	Single cell RNA sequencing
	SOP	Standard operating protocol
	SRPL	Secondary recurrent pregnancy loss
	SSC	Side scatter
T	TCR	T cell receptor
	TGR-β	Tumour growth factor β
	TNF-α	Tumour necrosis factor α
	TLS	Tertiary lymphoid structure
	Treg	T regulatory cell
	tRNA	Transfer RNA
U	UMAP	Uniform manifold approximation and projection
W	WOI	Window of implantation

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1 Introduction

1.1 ENDOMETRIAL IMMUNE ENVIRONMENT

1.1.1 Healthy human endometrium

Human endometrium, the inner lining of the uterus, is a dynamic mucosal tissue that consists of a columnar epithelium and underlying multicellular stroma. It has two main layers: the functional and the basal layer. The functional layer (*stratum functionalis*) undergoes structural changes in response to hormonal regulation during the menstrual cycle to prepare for potential embryo implantation. The basal layer (*stratum basalis*), located underneath, serves as a source of progenitor stem cells for the renewal of the endometrium in each cycle and its thickness remains relatively constant despite hormonal fluctuations [1]. The cyclic nature of the menstrual cycle, which on average lasts 28 days, results in three phases, each with unique histological and functional properties (Figure 1). It begins with menstruation, characterised by the shedding of the functional endometrial layer, after a gradual drop in pregn-4-ene-3,20-dione (progesterone; P4) due to the absence of implantation and the subsequent degradation of the corpus luteum, which produces progesterone. Subsequently, in the proliferative phase, rising 17-estradiol (oestrogen; E2) levels drive endometrial growth, including angiogenesis and an increase in glandular and stromal cell proliferation. Ovulation, triggered by the luteinising hormone (LH) surge, marks the end of the proliferative and the beginning of the secretory phase. During this phase, rising progesterone levels cause additional changes in the endometrium to create a receptive environment for embryo implantation. The blood flow to the endometrium increases, stromal cells become more compact, the endometrial glands grow convoluted and secrete factors that facilitate embryo implantation and early pregnancy [2, 3].

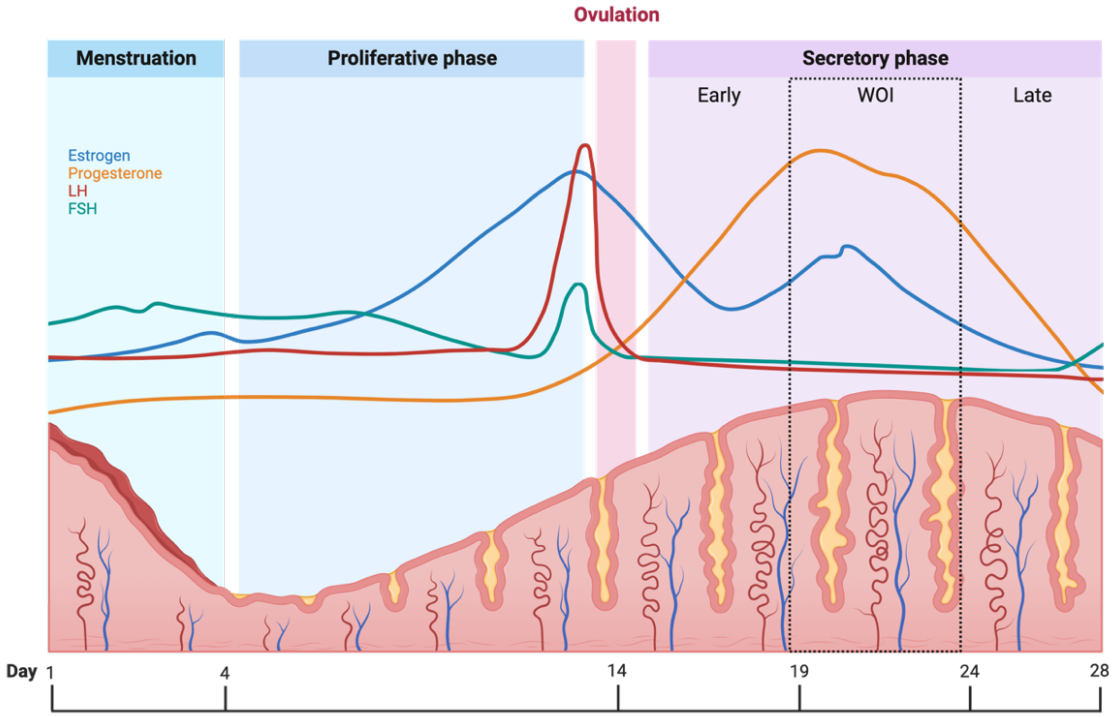


Figure 1: Diagram detailing the human menstrual cycle.

The menstrual cycle starts with menstrual phase (days 1–4), continues with proliferative phase (days 4–14), which stops with ovulation (day 14) and is followed by secretory phase (days 14–28). Implantation happens during the window of implantation (WOI), when the endometrial lining is receptive to the embryo and is usually between days 19 and 24 of the average 28-day cycle. Created with BioRender.com.

1.1.2 Endometrial immune cell variation across the menstrual cycle

Endometrial mucosa is populated by various immune cells, which account for 6–20% of total endometrial cells across the cycle [4, 5]. They can be found scattered throughout the endometrium either as single cells surrounded by stromal cells, as intraepithelial cells adjacent to the luminal or glandular epithelium or as a part of large “lymphoid aggregates” (LAs) (Figure 2) [6]. LAs consist of a B cell core surrounded by T cells and macrophages. They are located in the basal layer of the endometrium and vary in size throughout the menstrual cycle. Even though their function is unclear, they have not been found in postmenopausal females, indicating that their development is hormonally influenced [7].

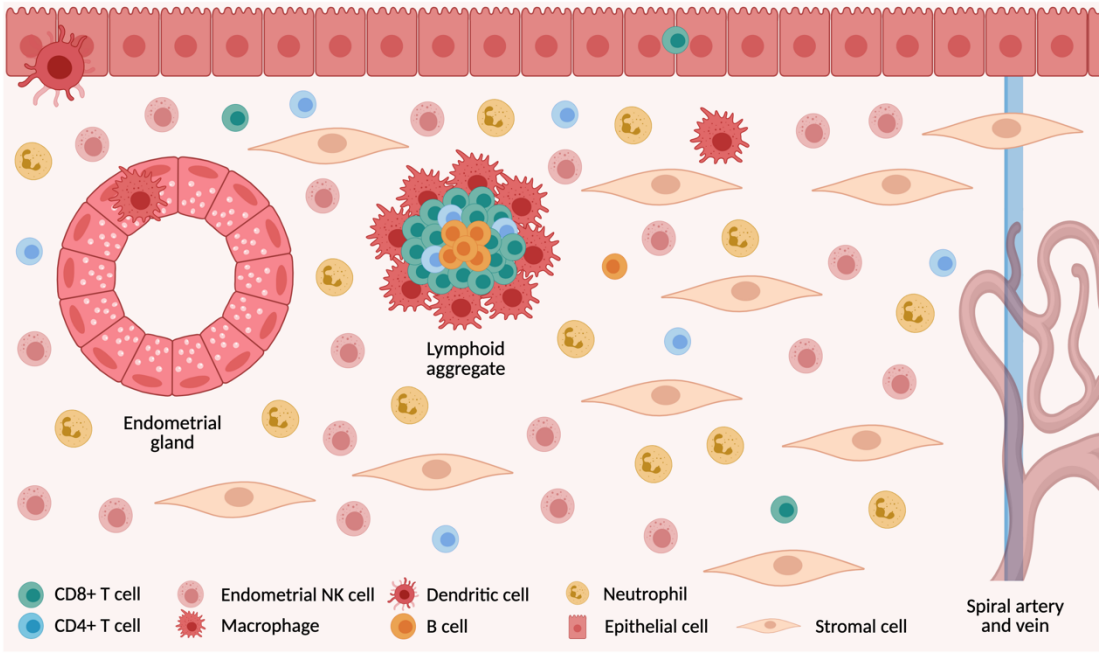


Figure 2: Immune cells in peri-implantation endometrial mucosa.

The endometrium is comprised of a variety of cell types during the window of implantation (WOI). Luminal and glandular epithelium are surrounded by stromal cells, which are interspersed with a variety of immune cells. These are either found as single cells or in lymphoid aggregates. NK, natural killer. Created with BioRender.com.

To date, the complexities of cyclical immune cell dynamics and their impact on reproductive health have not been fully elucidated, but it is clear that not only do endometrial leukocytes provide immunity against pathogens but following hormonal regulation, also help facilitate embryo implantation and pregnancy [6]. During the menstrual phase, shedding of the endometrial lining triggers the release of proinflammatory cytokines and the influx of immune cells, such as macrophages and neutrophils, to remove tissue debris and pathogens. In the proliferative phase, T cells are the most abundant leukocytes, comprising 40–60% of all endometrial leukocytes. While their absolute numbers remain relatively constant, their proportion decreases to < 10% in the late secretory phase due to the accumulation of endometrial natural killer (NK) cells post ovulation [6, 8].

1.1.3 Embryo implantation and pregnancy

During the hormonally regulated menstrual cycle, the immune system is critical in safeguarding the female reproductive tract (FRT) from potential infections and cancer [9]. However, during the window of implantation (WOI), the immune system undergoes temporary modifications to facilitate embryo attachment and invasion of trophoblast cells from the developing conceptus, which expresses alloantigens derived from the father and can trigger an inflammatory response that poses a potential threat to reproductive success [10]. To ensure the development of a healthy placenta and the maintenance of pregnancy, it is crucial to establish an active state of maternal immune tolerance while maintaining protection from pathogens. During the WOI, oestrogen and progesterone enhance luminal innate cells specialised in tissue remodelling and suppress CD8+ T (CD8 T) and NK cell cytotoxicity, providing a tolerogenic environment that effectively prevents T cell-mediated allograft rejection [11]. Following successful fertilisation, the blastocyst develops and adheres to the decidua. This thicker, nutrient-rich lining forms from the endometrium in the secretory phase via a process known as decidualisation. Decidualisation involves changes in the form and function of endometrial stromal cells, causing them to become more prominent, rounder, and packed with glycogen and lipids, which is essential to support the embryo [12]. In early pregnancy, decidual leukocytes represent around 40% of all decidual cells and comprise approximately 70% NK cells, 20–25% macrophages, 3–10% T cells and 1.7% dendritic cells (DCs). However, very few decidual B cells are found and cells that bridge innate and adaptive immune systems, such as invariant NK T cells and innate lymphoid cells (ILCs), are poorly researched [13-15]. Later, during pregnancy, NK cells make up less than 50% of the total leukocytes in the decidua, while the proportion of T cells increases to 40–80%. This dynamic shift in the composition of leukocytes in the decidua highlights the vital role of NK cells and T cells in supporting different stages of gestation [16].

1.2 IMMUNOLOGY OF ADVERSE REPRODUCTIVE OUTCOMES

1.2.1 Definitions and epidemiology

Disruptions in cyclical structural and immune changes may have a detrimental impact on embryo implantation and subsequent pregnancy. The term adverse reproductive outcomes refers to the inability to achieve or maintain a successful pregnancy, encompassing a range of conditions and complications that can occur throughout the reproductive process, including infertility, implantation failure, pregnancy loss, and preterm birth [12, 17]. In the general population, difficulties in conceiving are surprisingly common, with an initial chance of success of around 30% per cycle that may decrease over time [18, 19]. However, the current clinical definition of subfertility refers to the inability to conceive naturally after one year of trying to achieve pregnancy through unprotected intercourse during the fertile phase of the menstrual cycle. Infertility affects approximately 10% of couples trying to conceive [20]. After a successful conception and embryo implantation, about 40–50% of pregnancies result in pregnancy loss [18, 19], very often before a woman has even missed a period or had a positive pregnancy test. Pregnancy loss refers to the natural termination of a pregnancy before the foetus reaches the stage of viability and encompasses all losses from conception up to 20–24 weeks of gestation [14]. Underlying factors leading to subfertility and miscarriage may occur in both men and women. They may require medical intervention and fertility treatments such as assisted reproductive technologies (ART) like *in vitro* fertilisation (IVF) to achieve a successful pregnancy.

1.2.2 Immunological aetiology

Adverse reproductive outcomes are associated with a wide range of factors, including lifestyle, structural abnormalities in the reproductive organs, hormonal imbalance, genetic disorders,

infections and other immune conditions [21]. Several immunological disorders are linked to reproductive failure, such as anti-phospholipid syndrome and autoimmune thyroid diseases [22]. Nevertheless, it is unclear how systemic immune alterations influence the endometrium, which plays a crucial role in orchestrating a highly sophisticated immune response that supports establishing and maintaining pregnancy [23]. Interactions between the maternal immune system and the developing embryo encompass a vast array of immune cells and molecules with immunoregulatory properties, which need to be first cautiously investigated across healthy cycling endometrium before drawing conclusions about potential immunological factors during the WOI and their involvement in adverse reproductive outcomes. Only then will we be able to identify individuals at risk, develop diagnostic tests, and explore potential therapeutic interventions to improve fertility and pregnancy outcomes [12].

1.2.3 Endometriosis

Endometriosis is a common disease affecting women, defined as the presence of endometrium-like tissue outside the uterus [24]. It affects one in ten women, mostly of reproductive age, translating to approximately 190 million women worldwide [25]. Using revised criteria developed by the American Fertility Society and American Society of Reproductive Medicine (rAFS/ASRM), endometriosis can be divided into four stages considering lesion size, location, and extent of adhesions [24]. However, this does not necessarily correlate with the severity of associated conditions, such as chronic pelvic pain and subfertility [26, 27]. The most common causes for endometriosis-associated subfertility (EAS) are adhesions, which can affect the uterus, uterine tubes and ovaries, thereby disrupting the normal function of these reproductive organs, including reduced oocyte quality and ovarian reserve [28]. However, considering that the operative treatment does not improve pregnancy rates in endometriosis patients undergoing ART, altered endometrial immune receptivity has been suggested as one of the

additional factors in EAS [29-31]. A recent meta-analysis of 39 studies has demonstrated increased miscarriage rates after natural conception in endometriosis patients when compared with healthy women (odds ratio (OR): 1.81, 95% CI: 1.44–2.28, I2: 96%), and a higher risk of adverse events, such ante- and post-partum haemorrhage, preterm birth, stillbirth and placenta praevia, after natural conception as well as after ART [32]. Moreover, a meta-analysis of 27 studies demonstrated significantly lower implantation (relative risk (RR): 0.79, 95% CI: 0.67–0.93, p = 0.006) and clinical pregnancy rates (RR: 0.79, 95% CI: 0.69–0.91, p = 0.0008) in patients with severe endometriosis undergoing ART when compared with mild disease and healthy women [33]. Studies investigating pregnancy outcomes with donated oocytes from healthy women implanted in endometriosis patients remain controversial [34, 35]. Several other authors have confirmed a higher risk of pregnancy complications in patients with endometriosis, such as ectopic pregnancy, placenta praevia, miscarriage, preterm birth, and preeclampsia [36-39]. Although the exact pathophysiology of EAS and other adverse pregnancy outcomes remains to be determined, it has gained significant interest in recent years from the immunological perspective. Nevertheless, apart from conflicting studies on endometrial NK cells, comprehensive data on other immune subtypes and their role in EAS are scarce [40, 41].

1.2.4 Recurrent pregnancy loss

According to the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM), recurrent pregnancy loss (RPL) refers to the failure of two or more clinically recognised pregnancies. In the guidelines from the Royal College of Obstetricians and Gynaecologists (RCOG), RPL is defined as three or more first trimester miscarriages. RPL can be further categorised into primary RPL (PRPL), referring to women who have experienced RPL but have never had a live birth, in contrast to secondary RPL (SRPL), which includes women who have had a previous live birth before experiencing recurrent

pregnancy loss [42-44]. In contrast to sporadic pregnancy loss, recurrent pregnancy loss is classified as a disorder and warrants referral to specialised clinics to undertake relevant investigations, as well as increased support and monitoring during subsequent pregnancies [14]. Empirical treatments with immunologic modulators, such as aspirin and corticosteroids, have a long history of use in ART, and immunologic testing is increasingly offered despite limited evidence-based justification [45, 46]. As the predominant immune cell population in the peri-implantation endometrium and decidua, NK cells have been most widely studied in RPL. The presence of elevated levels of NK cells in the endometrium of patients with RPL suggests potential immune alterations in this population with limited clinical applicability of peripheral NK cell measurements as indicators of endometrial immune occurrences. Nevertheless, a better understanding of the underlying pathophysiology is needed to support the development of standardised diagnostic tests and effective targeted therapy [47]. Being the second most abundant immune cell population, T cells have been increasingly studied in the context of RPL, with T regulatory (Treg) cells getting significant attention due to their vital role in fetal-maternal tolerance, which has been confirmed in studies on mice [23, 48]. In human studies, altered peri-implantation Treg phenotypes and decreased cell counts were observed in patients with RPL [49-51]. Consistent with a presumed immune imbalance in favour of cytotoxic rather than regulatory features, an altered endometrial-resident CD8 T cell population has been described in RPL patients [52, 53]. Importantly, the persistence of this CD8 T phenotype beyond three months after the last miscarriage suggests its involvement in the pathophysiology of the embryo implantation and RPL rather than playing a role in the post-miscarriage immune response, which could be the case in numerous studies focusing on decidual tissues obtained from surgical treatment of miscarriage [52].

1.3 PERIPHERAL AND ENDOMETRIAL CD8+ T CELLS

1.3.1 CD8+ T cell phenotypes

Based on their distinct thymic developmental pathways, CD8 T cells can be divided into two subsets [54, 55]. The “unconventional” types, such as T cell receptor (TCR) γδ+ T (γδ T) cells, natural killer T (NK T) cells and mucosal-associated invariant T (MAIT) cells resemble innate immune cells and express TCRαβ/TCRγδ and typically CD8αα homodimers. In contrast, the “conventional” CD8 T cells express TCRαβ along with CD8αβ. The latter are further differentiated by multiple cell surface markers based on their function and the phases of the typical adaptive immune response, as shown in Figure 3 [56].

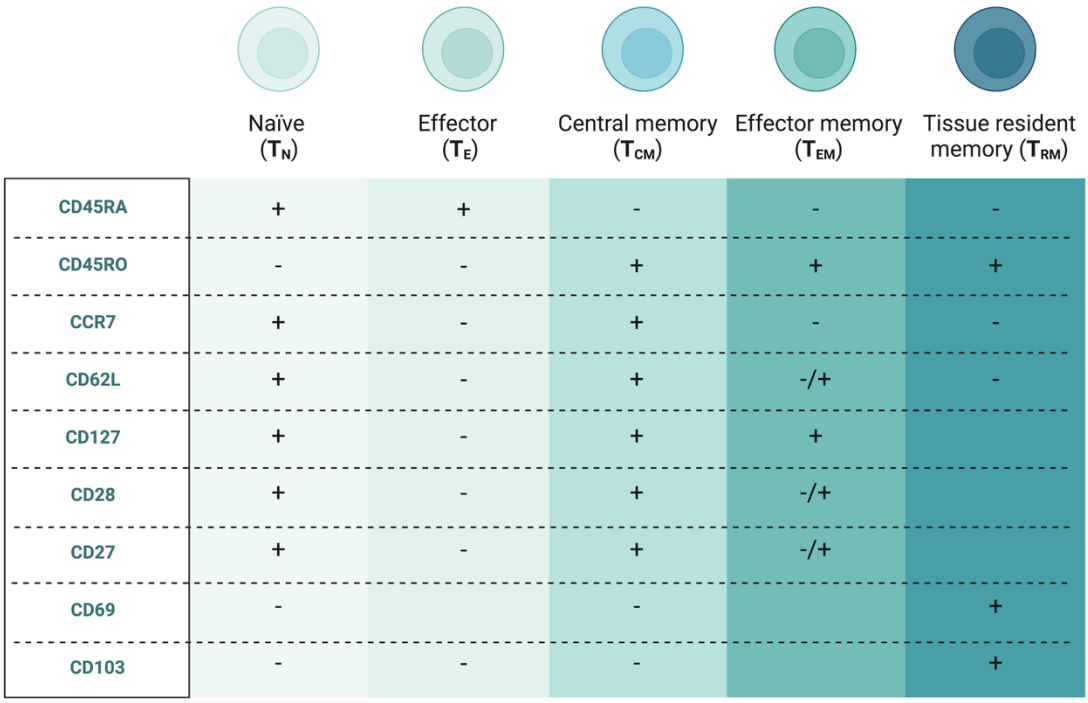


Figure 3: Conventional CD8+ T cell subsets in peripheral blood and endometriosis-associated tissues.

Adapted from Kieffer et al. 2019 [56]. Created with BioRender.com.

During the initial activation and expansion, naïve CD8 T cells (T_N) expressing CD45RA circulate between the peripheral blood and secondary lymphoid organs, where they recognise antigens presented via major histocompatibility complex (MHC) class I on antigen-presenting cells (APC) and start expanding to develop into effector CD8 T cells (T_E) [57]. T_E cells do not express lymph-node homing markers such as CCR7 and CD62L and consequently migrate to the tissue site where they secrete pro-inflammatory cytokines, such as interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α) and kill infected cells with cytolytic molecules, such as perforins and granzymes. Most of these CD8 T cells die by apoptosis during the contraction phase. In the last phase, surviving cells develop into several forms of memory CD8 T subtypes, such as central (T_{CM}), effector (T_{EM}) and tissue-resident (T_{RM}) memory subsets [58], which do not express CD45RA, but CD45RO.

1.3.2 Endometrial CD8+ T phenotypes

Of all T cells in the endometrial mucosa, approximately 60% are CD8 T cells, in contrast to peripheral blood, where CD4+ T (CD4 T) cells predominate [7, 59]. Similar to the decline in the proportion of T cells across the cycle due to the influx of NK cells, the proportion of CD8 T cells within the T cell population decreases from 63% in the proliferative to 54.2% in the secretory phase [8]. As with many other immune cells, the CD8 T population can be found across the endometrium either as single cells or in CD8+ LAs, encompassing the B cell core and surrounded by macrophages. Even though the role of LAs remains poorly understood, they are believed to be hormonally regulated, reaching their maximum size (> 100 µm diameter) due to cell trafficking during the secretory phase [7, 60]. Simultaneously with the growth of LAs, CD8 T cell cytotoxicity ceases following the rise of oestrogen and progesterone levels [5, 60-62]. While oestrogen directly suppresses cytotoxic activity by regulating the expression of perforin and

granzymes, progesterone has an indirect effect through the induction of tumour growth factor β (TGF-β) expression by epithelial and stromal cells [63].

1.3.2.1 Conventional CD8+ T cells

Conventional mucosal CD8 T cells usually acquire a tissue resident memory phenotype, widely but not exclusively determined by CD69 and CD103 markers [64, 65]. By remaining in tissue without constant antigen stimulus, the CD8 T_{RM} subtype rapidly responds to previously exposed pathogens without requiring co-stimulatory signals [66]. Over 90% of peri-implantation endometrial CD8 T cells are memory cells, CD45RA-/CD45RO+ as described in Figure 3, strongly suggestive of being tissue-resident phenotypes with more than 90% and 50% expressing CD69 and CD103, respectively [52, 59, 67]. CD69 is commonly regarded as a prototypical marker for tissue residency. It acts as a signal for retaining T cells within tissues by immobilising the sphingosine-1-phosphate receptor (S1PR1), essential for T cell migration out of tissues. Despite being initially recognised as an early marker of T cell activation due to its expression on recently activated effector T cells, the expression of CD69 on T_{RM} subtypes is continuous. It is not linked to activation markers such as CD25, CD38, and human leukocyte antigen class DR (HLA-DR). Moreover, gene expression analysis of CD69+ memory T cells from various human organs has revealed a consistent transcriptional profile that differs from memory T cells in the bloodstream [68]. CD103 is the αE subunit of the αEβ7 integrin and binds to E-cadherin found on epithelial cells. Anchoring CD8 T_{RM} in specific regions within the mucosal epithelium is more limited in lineage and location within the tissue than CD69 and is also less commonly expressed by CD4+ T cells. Interestingly, CD103+ CD8 T cells were shown to have reduced cytotoxicity and reduced content of perforins as well as decreased proliferative capacity when compared to CD103- counterparts in pre- as well as post-menopausal women [67]. Similar to tissue resident

phenotypes in other human tissues, endometrial CD8 T cells were also found to express programmed cell death protein 1 (PD-1), CCR5, and CD49a [69].

In addition to tissue residency markers CD69 and CD103, most endometrial CD8 T cells also express several chemokine receptors, which ensure precise migration and localisation to enable efficient immunosurveillance in peripheral tissues [70]. CCR5 is a receptor for CCL3, CCL4 and CCL5 (RANTES) and is expressed by activated and memory CD8 T cells [71]. Its expression is well established on CD8 T cells in the endometrium, where it is considered an important factor for cell trafficking [60]. CCR6, attributed to a Th17 (RORγt) phenotype, modulates porcine endometrial receptivity during early pregnancy through CCL20 signalling and has been implicated in the pathogenesis of endometriosis [72, 73]. CXCL9, CXCL10 and CXCL11 are IFN-γ inducible ligands that act through CXCR3 expressed by T cells, essential for their localisation around APCs and subsequent activation [74]. CXCR5 binds to CXCL13 and promotes T cell trafficking to B cell follicles and germinal centres in secondary lymphoid organs and marks a distinct CD8 T cell subset capable of indirectly suppressing B cell differentiation [75]. Additional surface markers, including CD127, PD-1, CD57, CD38, and HLA-DR, provide further insights into the characterisation of CD8 T cell subsets. The IL-7 receptor (CD127) plays a role in the homeostatic proliferation and survival of memory T cell precursors, while CD38 and HLA-DR serve as markers of T cell activation [76, 77]. CD57 indicates cell senescence, an inability to proliferate, and susceptibility to activation-induced cell death, while PD-1, an inhibitory marker, is upregulated in activated T cells to prevent excessive immune activation and is highly expressed on decidual CD8 T cells [15, 78, 79]. Beyond conventional CD8 T subtypes, these phenotypic signature molecules can also be expressed by unconventional γδ T, NK T and MAIT cells [80].

1.3.2.2 Unconventional CD8+ T cells

In addition to conventional tissue resident memory cells that migrate into tissue after antigen encounter in lymphoid organs, mucosal tissues also contain "unconventional" populations that naturally home to specific organs during development without needing prior antigen priming such as γδ T cells [81]. This immune cell type expresses TCRs composed of γ and δ chains in contrast to conventional T cells with α and β chains. Unlike conventional subtypes, γδ T cells recognise a diverse range of antigens, including non-peptides, independently of MHC molecules. These cells are an important bridge between innate and adaptive immunity, exhibiting protective and pathogenic roles in pathogen clearance and autoimmune diseases [80]. In recent murine studies, γδ T cells were largely found in the stroma and produced interleukin 17A (IL-17A) upon stimulation, with a minor subset producing IFN-γ. Like gut subsets, they were established early in life microbe-dependently [82].

Other innate-like T cell populations recognising non-peptide antigens, such as vitamins and glycolipids, are NK T and MAIT cells. In addition to sharing surface markers with conventional T cells (CD3, CD8 and CD4) and NK cells (CD56), NK T cells have a similar thymic developmental pathway to conventional T cells and mostly express TCRα chains combined with a limited subset of TCRβ chains. Upon activation through the CD1d molecule, they release cytokines, such as IFN-γ, IL-4 and various chemokines [83]. Although they have been poorly described in healthy non-pregnant endometrium, they are highly prevalent in the human decidua as compared to the peripheral blood (0.48% of all T cells) and are thought to have an immunoregulatory role at the maternal–foetal interface by producing Th1-related cytokines and could contribute to pregnancy complications such as miscarriage and preeclampsia [84].

MAIT cells exhibit semi-invariant Vα7.2-Jα33/12/20 TCRα chain and can be activated via TCR-dependent recognition of microbial riboflavin metabolites presented by MR1, independent of the TCR by inflammatory cytokines such as IL-12 and IL-18 or through a combination of both. Depending on tissue localisation, stimulation environment, and clonotype, MAIT cells differ in phenotypic and functional characteristics [85]. Circulating MAIT cells are mostly CD8+ (70%) and double negative (DN) (15%) with low proportions of CD4+ (5%) and are commonly identified with anti-Vα7.2 TCR antibodies in combination with markers for CD161, CD26, or IL-18Rα [86]. Endometrial MAITs are enriched for CD4+ subsets and exhibit minimal dynamic changes across the menstrual cycle, after menopause or during pregnancy [87, 88]. When activated, they display a bias towards IL-17 and IL-22 expression, indicating a role in the immunological homeostasis and control of microbes at this site, whereas blood MAIT cells produced primarily IFN-γ, TNF-α, and granzyme B [87]. Interestingly, data on transplanted uteri showed that endometrial MAIT cells most likely represent a transiently tissue-resident immune cell population and suggests that the endometrial niche can be replenished by circulating MAIT cells entering the tissue and starting to express tissue-residency markers [88].

1.3.3 Potential roles of CD8+ T cells in adverse pregnancy outcomes

Given that altered endometrial CD8 T characteristics were found in endometrial biopsies obtained during WOI from patients with RPL, pre-eclampsia and preterm labour, it is likely that different phenotypes contribute to adverse pregnancy outcomes [52, 89-91]. Two recent genome-wide association studies (GWAS), one on endometriosis and one on RPL, revealed genetic modifications potentially implicated in the function of CD8 T cells [92, 93]. There are two ways by which CD8 T cells could influence the embryo and foetus [94, 95]. This could either happen indirectly by releasing inflammatory cytokines or directly through antigen recognition of foetal extravillous trophoblast (EVT) cells (Figure 4) [94].

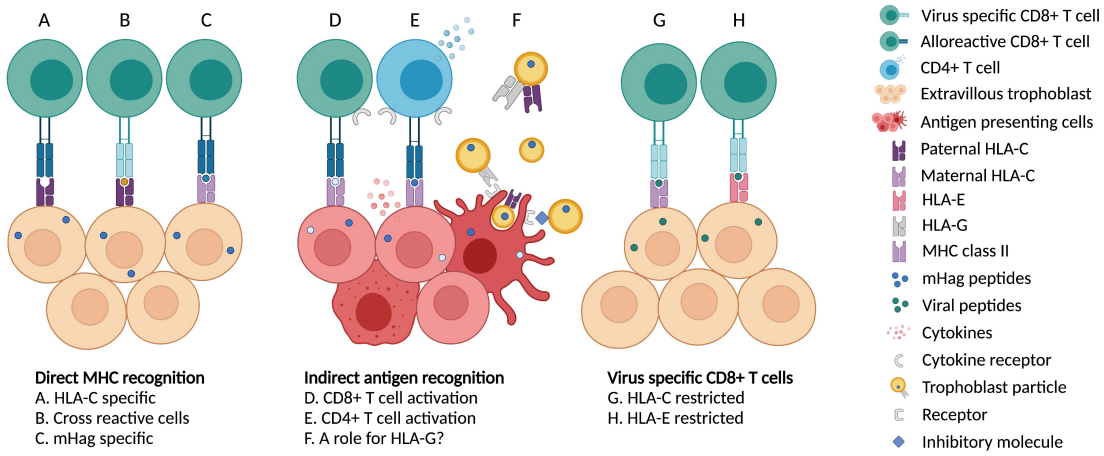


Figure 4: Possible mechanisms of maternal CD8+ T cell antigen recognition of foetal extravillous trophoblast (EVT) cells leading to subfertility and pregnancy complications.

(**A**) The human leukocyte antigen class C (HLA-C) is the only polymorphic major histocompatibility complex (MHC) molecule present on foetal EVT cells. Some pregnant women have naïve or memory CD8+ T (CD8 T) with a T cell receptor (TCR) that can directly bind and respond to paternal HLA-C expressed by EVT cells. (**B**) Pre-existing virus-specific cross-reactive CD8 T cells could react with external MHC molecules, such as paternal HLA-C expressed by EVT cells. These T cells' alloreactivity and viral specificity are mediated by the same TCR. (**C**) Foetal minor histocompatibility antigens (e.g., HY antigens) may be introduced by maternal HLA-C molecules on EVT cells. (**D**) Major and minor histocompatibility antigens produced by EVT cells can activate maternal CD8 T cells by indirect antigen recognition. Maternal antigen-presenting cells (APCs) cross-present peptides on MHC class I to maternal CD8 T cells. (**E**) Maternal APCs in decidual tissue and draining lymph nodes can collect trophoblast cell debris, digest allogeneic antigens, and deliver peptides to maternal CD4+ T lymphocytes via MHC class II. (**F**) HLA-G can suppress the alloimmune response by binding to HLA-G receptors expressed on APCs and NK cells. By polarising APCs and causing them to emit more anti-inflammatory cytokines they encourage CD4+ T and CD8 T cell differentiation with more suppressive T cell phenotypes. (**G**) Viruses, bacteria, and parasites can all be found in placental and EVT cells. It has been demonstrated that HLA-C molecules on EVT cells may introduce viral peptides to virus-specific CD8 T. (**H**) It has also been shown that HLA-E molecules on EVT cells could introduce viral and bacterial peptides to antigen-specific CD8 T cells. Adapted from Tilburgs et al. 2013 [94].

1.4 THESIS OBJECTIVES

The introduction of this thesis outlined how immune dysregulations could lead to adverse reproductive outcomes, such as EAS and RPL. Endometrial immune cells provide immunity against pathogens, but must also facilitate embryo implantation and pregnancy [6]. Disruptions in cyclical immune changes could have a detrimental impact on embryo implantation and subsequent pregnancy by either compromising endometrial receptivity, leading to subfertility, or by triggering immune rejection of the foetus, resulting in a miscarriage [13, 16, 29-31]. Our understanding of the physiologic and pathophysiologic role of CD8 T cells at the foeto-maternal interface is limited, with several mechanisms proposed on how aberrant activation of this immune population could impair embryo implantation and pregnancy [94, 95]. Studies in both mice and humans suggest that a significant number of women have naïve and effector memory CD8 T subtypes capable of directly binding to paternal HLA-C molecules on EVT or reacting to foetal minor histocompatibility antigens (mHags). Despite mismatches in foeto-maternal HLA-C and mHags leading to increased lymphocyte activation, this typically does not result in pregnancy failure, indicating that various mechanisms are in place to prevent harmful maternal CD8 T cell response to foetal alloantigens [94]. Therefore, a better characterisation of the CD8 T cell phenotypes and their dynamics across the menstrual cycle, especially during WOI, is crucial in deciphering immune responses during pregnancy and adverse outcomes and holds clinical potential to identify diagnostic and therapeutic targets for improving pregnancy outcomes and managing related complications. This thesis aims to comprehensively explore the role of endometrial CD8 T cells to optimise future clinical research to discover non-invasive diagnostic tools and targeted immunomodulating therapy for adverse reproductive outcomes.

My thesis is divided into four research chapters, each addressing some of the gaps in our current knowledge on endometrial CD8 T cells in endometriosis and RPL:

- **Chapter 2:** A systematic review of the literature to ascertain the known roles of CD8 T cells in endometriosis and EAS.
- **Chapter 3:** CD8 T cell transcriptome analysis of RPL patients and fertile controls during WOI to identify differentially expressed genes associated with RPL and miscarriage.
- **Chapter 4:** Deep immune phenotyping of endometrial and circulating immune cells to delineate altered CD8 T phenotypes and other immune populations in patients with endometriosis, EAS and RPL.
- **Chapter 5:** Single- and multiplex immune imaging to spatially observe immune changes across the menstrual cycle and relevant CD8 T cell subtypes

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2 Systematic review of the literature on CD8+ T cells in endometriosis and associated subfertility

2.1 INTRODUCTION

2.1.1 Pathophysiology of endometriosis

Endometriotic lesions located in the abdominal cavity exhibit diverse features and appearances, typically classified into three main categories: peritoneal (or superficial) lesions, ovarian lesions (referred to as endometriomas), and deep endometriosis lesions. A shared characteristic among all these lesions is the presence of endometrial-like epithelial and/or endometrial-like stromal cells [24]. Although the exact pathophysiology of endometriosis is still unclear, the oldest and most widely accepted explanation is the retrograde menstruation theory proposing that endometrial cells travel through the uterine tubes into the abdominal cavity, adhere to the peritoneum and invade the mesothelium and deeper layers [96]. Retrograde menstruation is a physiological process occurring in most women, but only in some do these cells persist and contribute to inflammation together with cytokines in the effluent, potentially resulting in pelvic pain and infertility. Therefore, additional factors have been suggested to play a role, including altered systemic and local immunity, leading to impaired clearance of endometrial cells from the abdominal cavity [24].

2.1.2 Endometriosis-associated tissues

Findings indicate that immune cells, including macrophages, NK cells, DCs, neutrophils, T cells, and B cells, along with cytokines and inflammatory mediators, play a role in the establishment and maintenance of endometriotic lesions by supporting vascularisation and fibrogenesis [97]. Comparing ectopic endometrium to both matched eutopic and healthy control endometrium, significant variations were observed in the relative proportions of immune cells [98]. Endometriotic lesions exhibit a substantial increase in the ratio of CD8 T cells and macrophages (23.5% in lesions, 8.7% in eutopic endometrium, 5.8% in control endometrium), coupled with a lower percentage of NK cells (10.9% in lesions vs 40.9%, eutopic endometrium, 42.2% in normal endometrium). Moreover, a distinct immune environment was also observed among endometriotic lesions, indicating a highly dynamic ectopic endometrial immunity closely related to the peritoneal environment [99].

In contrast to the mucosal tissue in the eutopic and ectopic endometrium, the peritoneal fluid mainly derives as an ovarian exudate induced by enhanced vascular permeability. However, small amounts have been found in healthy men [100, 101]. Although the peritoneal fluid immune milieu strongly depends on the hormonal milieu, macrophages remain the most prevalent population of peritoneal immune cells throughout the uterine cycle in women with endometriosis (55%), followed by T cells (20%) [102]. Similar to the eutopic endometrium, the peritoneal fluid CD4/CD8 ratio favours CD8 T cells with increased relative frequencies of this population when compared to the peripheral blood [103].

2.2 CHAPTER OBJECTIVES

Despite being such an abundant immune cell population in the human endometrium and peritoneal fluid, data regarding CD8 T cells in endometriosis, a disease commonly associated with immune disorders, remain conflicting. To my knowledge, there has been no systematic assessment of the data on CD8 T cells in this highly prevalent disease. I conducted systematic searches in four databases (MEDLINE, EMBASE, Web of Science, and CINAHL) to summarise and critically assess the available literature. Ultimately, I aimed to provide the rationale for the potential role of the CD8 T population in the pathophysiology of endometriosis and associated conditions (e.g., pelvic pain, subfertility, miscarriage) as well as for its predisposition to disease recurrence. This systematic review was registered in the international prospective database of systematic reviews - PROSPERO in January 2021 (PROSPERO ID Number: CRD42021233304) and published in the Frontiers in Immunology in July 2023 [104, 105].

Objectives:

1. To identify papers related to CD8 T cells in endometriosis following a systematic and rigorous methodology.
2. To critically appraise the quality of existing studies on CD8 T cells in endometriosis.
3. To offer an evidence-based summary of the relevant, sufficient- and high-quality research findings.
4. To enhance the overall understanding of the role of CD8 T cells in endometriosis and associated conditions.
5. To inform about future research and clinical implications based on the available information and limitations of the existing data.

2.3 METHODS

2.3.1 Database search

The methodology followed the standardised PRISMA statement guidelines for systematic reviews [106]. Multiple searches were conducted from January 2021 through December 2022. Medline database (Ovid MEDLINE® Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Ovid MEDLINE® Daily and Ovid MEDLINE®; 1946 to present), Embase (Ovid; 1974 to present), The Web of Science (Clarivate; 1900 to present) and CINAHL (HDAS; 1937 to present) were searched for the relevant terms from two categories: CD8+ T cells and endometriosis. The following keywords were used to search text words and Medical Subject Headings (MeSH-terms): CD8, CD8+, cytotoxic T or CTL; natural killer T or NKT; mucosal-associated invariant T or MAIT; intraepithelial lymphocyte* or intra-epithelial lymphocyte*; endometriosis; endometriotic; endometrioma. Results were limited to the English language with no publication period restrictions. Additionally, relevant papers that reviewers had been aware of were also considered.

2.3.2 Study selection

Duplicates were removed, and all studies were screened for eligibility using the adapted population, intervention, comparator, and outcomes (PICO) framework (Table 1) [107]. Non-peer-reviewed studies such as editorial letters, expert opinions and conference abstracts were excluded (wrong publication type). For the first round of screening, myself and JHS independently screened the titles, abstracts, and keywords, applying eligibility criteria. If the abstract did not clearly indicate whether a study met the initial inclusion criteria, the entire article was assessed. In the second round, full records of the selected articles were retrieved and

screened, and articles not complying with eligibility criteria were excluded. We assessed papers independently through stages 1 and 2 then compared decisions and discussed discrepancies. In this instance, IG and CMB were consulted, and final decisions were reached. The screening was undertaken using Rayyan QCRI [108].

2.3.3 Data extraction

I extracted the following data from each study: first author's last name, publication year, study design, study period, sample size (patients with endometriosis and controls), participant demographics and baseline characteristics, outcomes related to CD8 T cells (e.g., concentration, activation and proliferation status, cytokine production, an association between CD8 T cells and disease stage, treatment response, recurrence rate after surgery, pregnancy rate, live birth rate, and miscarriage rate). The data were extracted into an Excel spreadsheet and were checked by JHS.

2.3.4 Quality assessment

Quality assessment of included studies was conducted independently by myself and JHS using the Newcastle-Ottawa Scale (Selection, Comparability, Exposure/Outcome) [109]. Each paper was graded and defined accordingly as "good", "fair", or "poor" quality. Any disagreements or uncertainties were resolved with IG and CMB.

Table 1: PICO framework to select eligible research papers.

	INCLUDED	EXCLUDED
Population	Women between menarche and menopause with all stages of surgically confirmed endometriosis and animal models with menstrual cycle.	Women with adenomyosis or other gynaecological diseases, idiopathic infertility, immune diseases, undergoing immune or hormonal therapy. Women with no menstrual cycles, and animal models with oestrous cycle.
Intervention	*In vitro* and *in vivo* studies of CD8+ T cells in endometriosis related tissues, such as but not limited to peripheral blood, peritoneal fluid, eutopic endometrium and ectopic lesions of patients and animal models with endometriosis.	Meta-analyses of papers (but not datasets), systematic reviews and other reviews, case reports or case series, and organizational guidelines.
Control	Healthy women and animal models without endometriosis.	Studies with no controls, poorly defined controls, or poor controls e.g., women with adenomyosis, acute pelvic inflammatory disease, idiopathic infertility, hydro/pyo/hematosalpinx, immune diseases, undergoing immune or hormonal therapy. Women with no menstrual cycle, and animal models with oestrous cycle.
Outcome	Differences in measurable parameters (e.g., concentration, activation status, cytokine production) related to CD8+ T cells in patients and animal models with endometriosis when compared to healthy controls. Association between CD8+ T cells and disease stage, treatment response, recurrence rate after surgery, pregnancy rate, live birth rate, miscarriage rate.	Studies with no CD8 + T cell outcomes.

2.4 RESULTS

The following sections detail the properties and outcomes of all papers that I included in the systematic review following rigorous screening process and data extraction as previously described. Each subchapter focuses on one of the endometrios-associated tissues and peripheral blood that were investigated by the respective studies.

2.4.1 Study selection and characteristics

Initially, we identified 517 papers, of which 28 met all the inclusion criteria and were included in the narrative synthesis. A detailed overview of their key characteristics is presented in Table 2. In summary, most studies were conducted in Asia (11), followed by Europe (8), North America (4) and South America (3), and were conducted in a university hospital setting (19).

2.4.2 Quality of evidence

Most of the papers were scored as “good” or “fair” (Table 2). Overall, all papers scored in the definition of cases and controls and the ascertainment of exposure. Only 2 papers reported on the non-response rate showing a potential risk of bias in the sample selection in most of the studies. One paper was graded as “low” quality due to the low score in the comparability category. One paper could not be graded as it was an animal study performed on baboons.

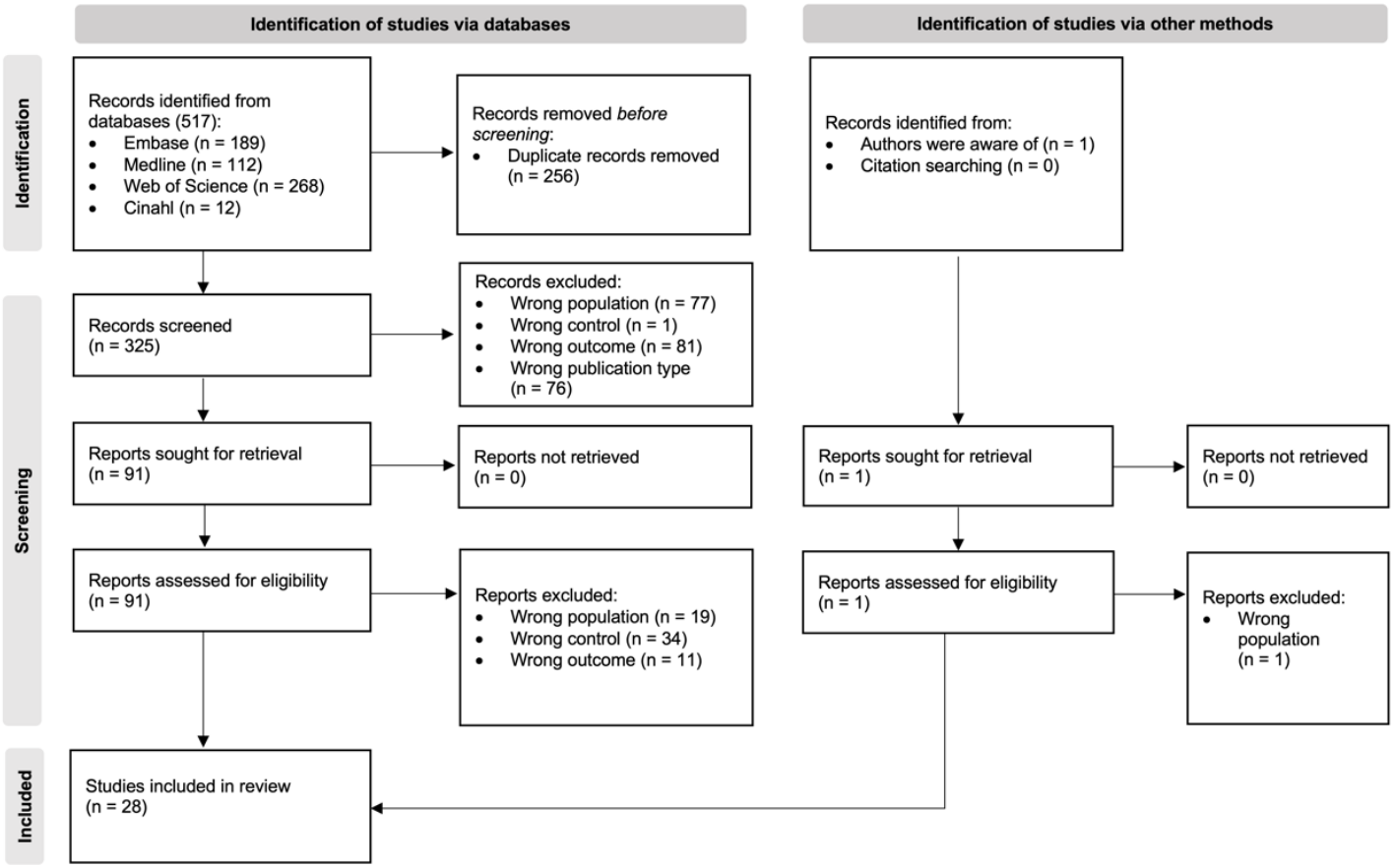


Figure 5: PRISMA flow diagram of the systematic review study selection.

Adapted from Page et al. 2021 [106].

Table 2: Study characteristics.

FIRST AUTHOR, YEAR	STUDY PERIOD	STUDY DESIGN				TISSUE					OBTAINING METHOD	ANALYSIS METHOD	QUALITY ASSESSMENT
		Case-control	Cohort	Transcriptome meta-analysis	Animal	Peripheral blood	Eutopic endometrium	Ectopic endometrium	Peritoneal fluid	Menstrual effluent			
Ahn, 2016	N/A	✓					✓	✓			Endometrial biopsy and laparoscopy	scRNAseq	Good
Bulmer, 1998	N/A	✓					✓	✓			Unknown for endometriosis, hysterectomy for controls	IHC	Fair
Bunis, 2022	N/A			✓			✓				Microarray data	GSEA	Good
Chen, 2017	N/A	✓				✓			✓		Venepuncture and laparoscopy	FC	Fair
Correa, 2022	2013-2015	✓				✓					Venepuncture and laparoscopy	FC	Good
D'Hooghe, 1996	N/A				✓	✓			✓		Venepuncture and laparoscopy	FC	Good
Deng, 2022	2018-2019	✓							✓		Laparoscopy	FC	Good
Gagne, 2003	1997–2001	✓				✓					Venepuncture	FC	Good
Gallinelli, 2004	1999–2001	✓							✓		Laparoscopy	FC	Poor
Gmyrek, 2008	N/A	✓				✓					Venepuncture	FC	Good
Ho, 1997	1993–1996	✓				✓			✓		Venepuncture and laparoscopy	FC	Good
Hsu, 1997	1993–1994	✓				✓					Venepuncture	FC	Good
Klentzeris, 1995	N/A	✓					✓				Endometrial biopsy	IHC	Good
Li, 2019	2018–2019	✓				✓			✓		Venepuncture and laparoscopy	FC	Good
Liu, 2020	2018–2019		✓			✓					Venepuncture	FC	Fair
Ma, 2021	N/A	✓					✓	✓			Endometrial biopsy and laparoscopy	scRNAseq	Fair
Mettler, 1997	N/A	✓					✓				Endometrial biopsy	IHC	Good
Mier-Cabrera, 2011	N/A	✓				✓			✓		Venepuncture and laparoscopy	FC	Fair
Muharam, 2022	2011	✓				✓					Venepuncture	FC	Good
Opsahl, 1994	N/A	✓				✓			✓		Venepuncture and laparoscopy	FC	Fair
Poli-Neto, 2021	N/A			✓			✓				Microarray data	GSEA	Fair
Schmitz, 2021	2018-2019	✓				✓				✓	Venepuncture and menstrual cup	FC	Fair
Shih, 2022	N/A	✓								✓	Menstrual cup	scRNAseq	Fair
Sobstyl, 2020	N/A	✓				✓					Venepuncture	FC	Fair

Wu, 2019	N/A	✓					✓	✓			Venepuncture, laparoscopy and hysterectomy	IHC	Fair
Wu, 2021	2006-2013, 2016, 2018-2019	✓		✓			✓				Microarray data, N/A	GSEA, IHC	Good
Zhong, 2021	2019			✓			✓	✓			Microarray data	GSEA	Fair
Zou, 2021	N/A	✓							✓		Laparoscopy	scRNAseq	

N/A - Not available; scRNAseq - Single cell RNA sequencing; IHC - Immunohistochemistry; GSEA - Gene set enrichment analysis; FC - Flow cytometry.

2.4.3 CD8+ T cells in endometriosis-associated tissues

2.4.3.1 Eutopic endometrium

Recent bioinformatic analyses looking at several CD8 T cell subtypes found changes in naïve, central memory and effector memory CD8 T cell scores between the eutopic endometrium of patients and control (Table 3). However, 5 out of 9 papers reported no difference in the concentration of the total CD8 T cell population between patients with endometriosis and healthy controls.

Using gene set enrichment analysis (GSEA) on data from eutopic endometrial biopsies taken during the proliferative phase with CIBERSORT, Zhong et al. found no difference in CD8 T cell fraction between patients with severe endometriosis and controls [110]. Nevertheless, in a similar analysis, investigators found increased CD8 T_{EM} cell gene enrichment scores in women with rAFS/ASRM stage I/II as well as stage III/IV disease ($p = 0.000$ and $p = 0.027$, respectively) and decreased CD8 T_N and T_{CM} scores ($p = 0.001$ and $p = 0.000$; $p = 0.035$ and $p = 0.782$) when compared to controls [111]. Another GSEA by a different group shows an increased CD8 T cell score in endometriosis versus controls when analysed using CIBERSORT (0.1659 ± 0.0968 vs 0.1086 ± 0.0939, respectively; $p = 0.0033$), MCP-counter ($p = 0.00018$) and ImmuneCellAI ($p = 7.2e\text{-}8$) [112]. Comparing different subsets with ImmuneCellAI, decreased CD8 T_N cells ($p = 7.6e\text{-}5$), T_{CM} ($p = 0.00076$), and T_{EM} cells ($p = 1.1e\text{-}6$) were present in the endometrium of women with endometriosis when compared with controls. In a second analysis (GSE120103), performed with MCP-counter, increased CD8 T cell scores were revealed in the endometrium of infertile versus fertile women with endometriosis ($p = 0.0019$, AUC (area under the receiver operating characteristic curve (ROC)) = 0.722). Immunohistochemical validation of their transcriptomic

data confirmed increased proportions of CD8+ cells among all leukocytes in the eutopic endometrium of endometriosis patients versus controls in both the proliferative and early secretory phases (0.2292 ± 0.0591 vs 0.1790 ± 0.0562, p = 0.0132, AUC = 0.727) [112].

Similarly, increased quantity of CD8+ cells per mm^2 was detected in severe endometriosis versus controls, of note samples were collected in the proliferative phase (0.019 ± 0.004 vs 0.007 ± 0.002 cell/mm^2, respectively; $p < 0.01$) [113]. Focusing on the eutopic endometrial tissue in the secretory phase, RNA sequencing analysis did not reveal any difference in the expression of the CD8A gene between endometriosis and controls [114]. Again, no difference was found in the microscopic analysis of the secretory phase tissue from infertile women with endometriosis and fertile controls [115]. In samples equally distributed across the uterine cycle, no difference was found in either glandular or intraepithelial CD8+ cell count in either of the uterine cycle phases between endometriosis and controls [116], or between endometriosis and controls with laparoscopically proven tubal factor infertility resulting from prior pelvic inflammatory disease (PID) [117].

Finally, in the findings from Bunis et al., the endometrial CD8 T_{EM} signature had higher gene enrichment scores across all menstrual phases in endometriosis patients when compared to the control [118]. Additionally, they were significantly increased in the mid-secretory phase when compared to the preceding phases in both disease and control (Normalised enrichment score (NES) = 1 for unstratified samples). The activation potential of eutopic endometrial CD8 T cells was decreased in the endometriosis patients when compared to controls and genes with functions known to be relevant to phagocytosis and complement activation were stimulated in the eutopic endometrium of patients. No papers were found assessing the proliferative and cytotoxic capacities of eutopic endometrial CD8 T cells in endometriosis.

Table 3: CD8+ T cell outcomes in the eutopic endometrium.

FIRST AUTHOR, YEAR	TOTAL (N)	ENDOMETRIOSIS (N)				CONTROLS (N)			UTERINE CYCLE PHASE (N)		ANALYSED SAMPLES (N)		CD8+ T CELL FINDINGS IN ENDOMETRIOSIS VERSUS CONTROLS
		Stage I/II	Stage III/IV	Subfertility	Age (yrs)*	Diagnosis	Subfertility	Age (yrs)*	Proliferative	Secretory	Endometriosis	Controls	
Klentzeris, 1995	34	11	5	16	33.2 (±1.6, 22–41)	Tubal ligation/ reanastomosis	0	35.4 (±2.8, 24–40)	0	34	16	18	**NO DIFFERENCE** in precipitate area for CD8+ cells.
Mettler, 1997	143	*	*	143	29.8 (24–36)	Tubal factor infertility	110	29.6 (24–35)	**	**	253	110	**NO DIFFERENCE** in CD8+ quantity per mm2 and no differences in lymphocyte aggregations.
Bulmer, 1998	60	N/A	N/A	N/A	N/A	Non-endometrial pathology	N/A	N/A	20	40	30	30	**NO DIFFERENCE** in neither glandular nor surface intraepithelial CD8+ cells.
Ahn, 2016	16	0	8	8	33.1 (±7.3)	Tubal ligation	0	26 (±5.8)	0	16	8	8	**NO DIFFERENCE** in the expression of CD8A gene.
Wu, 2019	15	0	15	N/A	44.60 (±2.57)	Non-endometrial pathology	N/A	47.00 (±4.04)	30	0	15	15	**INCREASED** CD8+ quantity per mm^2 ($p < 0.01$).
Poli-Neto, 2021	59	16	43	N/A	N/A	Healthy	N/A	N/A	113	0	59	54	**INCREASED** effector memory CD8+ T cell score in mild and severe disease ($p = 0.000$; $p = 0.027$). **DECREASED** naive and central memory CD8+ T cell scores in in mild and severe disease ($p = 0.001$; $p = 0.000$ and $p = 0.035$; $p = 0.782$).
Wu, 2021	67	N/A	N/A	N/A	N/A	Healthy	N/A	N/A	107*	*	67	40	**INCREASED** CD8+ T cell score (CIBERSORT: $p = 0.0033$; MCP-counter: $p = 0.00018$; ImmuneCellAI: $p = 7.2e-8$). **DECREASED** naïve ($p = 7.6e-5$), central memory ($p = 0.00076$), and effector memory CD8+ T cells ($p = 1.1e-6$).
	18	N/A	N/A	9	N/A	Healthy	9	N/A	36*	*	18	18	**INCREASED** CD8+ T cell score in infertile women with endometriosis versus fertile women with endometriosis ($p = 0.0019$, AUC = 0.722).
	40	N/A	N/A	N/A	N/A	Healthy	N/A	N/A	40*	*	20	20	**INCREASED** CD8+ cell ratio ($p = 0.0132$, AUC = 0.727).
Zhong, 2021	180	20	62	N/A	N/A	N/A	N/A	N/A	204	0	112	68	**NO DIFFERENCE** in CD8+ T cell ratio. **NO DIFFERENCE** in CD8+ T cell ratios between mild and severe disease.
Bunis, 2022	105	24	47	N/A	20–50	Tubal ligation/ reanastomosis	0	20–50	47	58	71	34	**INCREASED** effector memory CD8+ T cell score across all cycle phases (FDR < 0.05, log2 fold change > 1).

*Mixed with early secretory; **Up to 7 patients for every day of the menstrual cycle (28–29 days); N/A - Not available, Age (yrs)*: Mean ± SD or Median (Range).

2.4.3.2 Ectopic endometrium

All 5 studies researching ectopic endometrium reported an increased proportion of CD8 T cells in endometriotic lesions when compared to matched eutopic endometrium and/or healthy eutopic endometrium (Table 4). Additionally, 2 studies also investigated their activation and cytotoxic properties.

Zhong et al. performed transcriptome meta-analysis with CIBERSORT on 24 ectopic, 82 eutopic and 68 healthy endometrial samples in the proliferative phase (GSE120103, GSE51981, GSE25628, GSE37837, GSE7846, GSE6364, GSE7305) [110]. They described an increased CD8 T cell fraction among all infiltrating immune cells in the endometriotic lesions when compared to the eutopic endometrium of endometriosis patients ($p = 0.085$) and endometrium of controls ($p = 0.033$). Similarly, analysing single-cell RNA sequencing (scRNAseq) data of proliferative phase timed samples, a significantly higher percentage of CD8 T_N cells was reported in endometriotic lesions when compared to matched eutopic endometrium and normal endometrium (43.48% vs 0.91% and 0.33%, respectively), and a lower proportion of T_E subtypes [99]. In addition, the authors describe decreased cytotoxic T cell populations in lesions, however, data was not shown. Interestingly, a smaller immunohistochemistry study on proliferative phase samples confirmed increased CD8+ cell counts in ectopic endometrium when compared to the control endometrium (0.019 ± 0.002 vs 0.007 ± 0.002 per mm^2; $p < 0.01$), but not in ectopic versus matched eutopic endometrium [113].

Investigating samples in the secretory phase of the menstrual cycle, genes associated with several immune cell surface markers, including CD8A, were upregulated in ectopic tissues compared with eutopic endometrium of patients as well as control endometrium ($p < 0.05$)

[114]. In the same study, they also found increased expression of immune genes for T cell co-stimulation (e.g., CD27 and CD28) as well as less specific HLA genes associated with T cell activation, such as HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DRA, and HLA-DMA ($p < 0.05$). On the other hand, a different immunohistochemistry study found no difference in the CD8+ cell count between the glandular epithelium of ectopic lesions and matched eutopic endometrium in the proliferative and late secretory phase but confirmed an increased count in the early secretory phase ($p = 0.0002$) [116].

Table 4: CD8+ T cell outcomes in the ectopic endometrium.

FIRST AUTHOR, YEAR	TOTAL (N)	ENDOMETRIOSIS (N)				CONTROLS (N)			UTERINE CYCLE PHASE (N)		ANALYZED SAMPLES (N)			CD8+ T CELLS FINDINGS IN ECTOPIC ENDOMETRIUM COMPARED TO EUTOPIC AND HEALTHY ENDOMETRIUM
		Stage I/II	Stage III/IV	Subfertility	Age (yrs)*	Diagnosis	Subfertility	Age (yrs)*	Proliferative	Secretory	Eutopic	Ectopic	Controls	
Bulmer, 1998	60	N/A	N/A	N/A	N/A	Non-endometrial pathology	N/A	N/A	20	40	30	30	30	**INCREASED** number in the glandular epithelium in the early secretory compared to the matched eutopic endometrium ($p = 0.0002$).
Ahn, 2016	16	0	8	8	33.1 (±7.3)	Tubal ligation	0	26 (±5.8)	0	16	8	8	8	**INCREASED** expression of CD8A gene ($p < 0.05$).
Wu, 2019	30	0	15	N/A	44.60 (±2.57)	Non-endometrial pathology	N/A	47.00 (±4.04)	30	0	15	15	15	**INCREASED** CD8+ quantity per mm^2 when compared to control endometrium ($p < 0.01$). **NO DIFFERENCE** when compared to matched eutopic endometrium ($p > 0.01$).
Ma, 2021	6	0	3	1	31.7	Non-endometrial pathology	N/A	30.4	6	0	3	3	3	**INCREASED** ratio of naive CD8+ T cells ($p < 0.05$). **DECREASED** ratio of effector CD8+ T cells ($p < 0.05$).
Zhong, 2021	204	20	62	N/A	N/A	N/A	N/A	N/A	204	0	112	24	68	**INCREASED** CD8+ T ratio when compared to control endometrium ($p = 0.033$). **NO DIFFERENCE** in CD8+ T cell ratio when compared to matched eutopic endometrium.

N/A - Not available; Age (yrs)*: Mean ± SD or Median (Range).

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2.4.3.3 Peritoneal fluid

Of the 9 studies researching CD8 T cell ratios in the peritoneal fluid, 3 found no difference between endometriosis patients and controls, 1 paper noted no difference in CD8 T cell fraction among lymphocytes but increased proportions among all peritoneal cells in patients with endometriosis and 2 found increased concentrations in the disease (Table 5). Similarly, as shown, no consensus was reached on the activation status of the peritoneal CD8 T cells: 3 studies found no difference, 2 reported decreased and 1 increased activation in endometriosis patients.

The proportion of CD8 T cells among total mononuclear cells, between patients with rASRM stage I/II endometriosis and fertile controls are unchanged in the proliferative and secretory phases as well as when uncontrolled for the cycle phase [119]. Similarly, some authors found comparable rates of CD8 T cells among mononuclear cells in women with and without the disease in the proliferative phase [120] and others reported no difference in the percentage of CD8+ cells among all lymphocytes in the periovulatory phase between endometriosis patients and healthy controls [121]. While Deng et al. identified no difference in the proportion of CD8+ cells among lymphocytes in the proliferative phase, they found increased numbers of CD8+ cells among all peritoneal fluid cells in samples from patients with endometriosis ($p = 0.0032$) [122].

Increased proportions of specific CD8 T cell subsets were reported in studies studying baboons and MAIT cells. In baboons, a significant increase in the proportion of peritoneal CD8 T cells was detected in the group with long-term spontaneous endometriosis, when compared to other groups with no disease, induced disease, and recent spontaneous endometriosis (74.3 ± 6% vs 67.1 ± 8.3% vs 65.8 ± 8% vs 54.8 ± 21.9%, respectively; $p = 0.01$) [123]. In humans among MAIT cells defined as CD3+CD161+Vα7.2+ enhanced CD8+ MAIT cell subsets were found in the disease compared to control (6.52 ± 1.05 vs 2.77 ± 0.42, respectively; $p = 0.0454$) [124]. In the same

study, peritoneal fluid CD8+ MAIT cells from endometriosis patients displayed higher levels of the activation marker CD38, when compared to non-endometriosis participants (2.98 ± 0.53 vs 0.36 ± 0.09, respectively; $p = 0.0071$). The difference was more pronounced in the group with stages I/II than with stages III/IV when compared to controls.

On the other hand, Gallinelli et al. showed decreased proportions of CD3+CD8+HLA-DR+ cells in the endometriosis versus the control group (42.8 ± 6.8 vs 57.1 ± 7.6, respectively; $p < 0.02$) [125]. Additionally, they found a significantly higher CD8/CD4 ratio in the disease (2.8 ± 0.5 vs 2.1 ± 0.4, respectively; $p < 0.05$). Nevertheless, 2 publications report no difference in activation potential between endometriosis and non-endometriosis groups [121, 126]. In a study from Ho et al., they found no differences between stage I/II and III/IV endometriosis and controls in any of the identified activated phenotypes, including HLA-DR+, CD25+, CD28+, and CD69+ CD8 T cells [126].

Table 5: CD8+ T cell outcomes in peritoneal fluid.

FIRST AUTHOR, YEAR	TOTAL (N)	ENDOMETRIOSIS (N)				CONTROLS (N)			UTERINE CYCLE PHASE (N)		ANALYSED SAMPLES (N)		CD8+ T CELL FINDINGS IN ENDOMETRIOSIS VERSUS CONTROLS		
		Stage I/II	Stage III/IV	Sub-fertility	Age (yrs)*	Diagnosis	Sub-fertility	Age (yrs)*	Proliferative	Secretory	Endometriosis	Controls	Cell proportion	Activation and cytotoxicity	CD4/CD8 ratio
Chen, 2017	63	14	18	N/A	29 (18–38)	Non-endometrial pathology	N/A	32 (19–44)	63	0	4	4	**NO DIFFERENCE** among CD3+ cells.		
D'Hooghe, 1996	57	20	11	N/A	N/A	Healthy	N/A	N/A	21	18	36	21	**INCREASED** in long-term spontaneous endometriosis when compared with other groups (p = 0.01).		
Deng, 2022	49	0	24	N/A	31 (± 6.8)	Non-endometrial pathology	N/A	40 (±5.6)	49	0	15	18	**NO DIFFERENCE** among lymphocytes. **INCREASED** among all peritoneal fluid cells (p = 0.0032).		**NO DIFFERENCE.**
Gallinelli, 2004	63	21	22	0	33.1 (±4.4)	Tubal ligation/ reanastomosis	3 ± 0.6 yrs	31.7 (±3.7)	80	0	0	20		**DECREASED** concentration of HLA-DR+ subsets (p < 0.02).	**DECREASED** (p < 0.05).
Ho, 1995	29	8	11	N/A	N/A	Tubal ligation	0	N/A	29	0	19	10		**NO DIFFERENCE** in HLA-DR+, CD25+, CD28+ and CD69+ subsets between mild and severe endometriosis and controls.	
Li, 2019	50	17	15	N/A	32.6 (±1.10)	Non-endometrial pathology	N/A	33.3 (±1.23)	50	0	29	10	**INCREASED** CD8+ MAIT cell subsets (p = 0.0454).	**INCREASED** expression of CD38 among CD8+ MAIT cells (p = 0.0071).	
Mier-Cabrera, 2011	62	32	0	32	32.7 (±2.5)	Tubal ligation	0	33.8 (±5.4)	0	62	32	30	**NO DIFFERENCE** among lymphocytes.	**NO DIFFERENCE** in the ratio of IFN-γ and IL-2 expressing cells.	
Opsahl, 1994	20	8	0	8	N/A	Tubal ligation	0	N/A	11	9	8	12	**NO DIFFERENCE** among all phases.	**NO DIFFERENCE** in the ratio of HLA-DR+ subsets**.**	
Zou, 2021	2	0	1	N/A	36	Septate uterus	N/A	28	2	0	1	1		**DECREASED** mean gene expression of GNLY, GZMB, GZMH and GZMK (p < 0.0001).	

N/A - Not available; Age (yrs)*: Mean ± SD or Median (Range).

2.4.3.4 Menstrual effluent

Schmitz et al. investigated the variance in immune cells between menstrual effluent of patients with endometriosis and healthy controls [127]. They found no statistically significant differences in either the proportion of CD8 T cells or the CD4/CD8 ratio. However, a decreased proportion of perforin-high CD8 T cells was present in the menstrual effluent of patients versus controls (4.0 ± 4.8 vs 11.2 ± 10.4, respectively; p = 0.029). In the study of Shih et al. four distinct CD8 T cell clusters were differentially enriched between patients with surgically confirmed endometriosis and community controls, although the differential markers for those four clusters have not been explored [128].

2.4.4 CD8+ T cells in peripheral blood

As presented in Table 6, data from 10 papers looking at the general CD8 T cell population in peripheral blood indicate no difference in ratios amongst the immune compartment between patients with endometriosis and controls, but one study on different subtypes revealed potential changes in the levels of T_{CM} and T_{EM} in the disease [129]. Additionally, there seem to be no differences in their activation status between patients and controls and their proliferative capacity has not been assessed.

A study comparing leukocyte subsets in peripheral blood samples that were equally distributed across the menstrual cycle reported no differences in either CD8+ or CD8 T cell proportions between patients with endometriosis and healthy controls neither within the unadjusted model nor the model adjusted for age, smoking, oral contraception, parity, and history of an acute infection (CD8+: 16.4 ± 6.6 vs 16.7 ± 5.7, respectively; CD3+CD8+: 14.3 ± 5.3 vs 13.8 ± 5.7) [130]. Focusing on the proliferative phase of the cycle, the median percentages of CD8 T cells among

CD3+ cells and CD4/CD8 ratio in the peripheral blood between patients and healthy controls were unchanged [120] [127]. Additionally, no difference was revealed in perforin-high CD8 T cells (9.0 ± 6.6 vs 12.9 ± 17.3, respectively) [127] or in the proportions of CD8 T cells expressing activation markers, such as HLA-DR, CD25, CD28 and CD69 in different disease stages when compared to controls [131]. The only study investigating peripheral blood CD8 T cells solely in the secretory phase found no difference in either concentration or activation status [121].

A significant number of papers did not consider the menstrual cycle phase in their analysis. [132] identified comparable findings with no difference in CD8 T cell proportions among leukocytes between endometriosis and control group (29.3 ± 5.5 vs 30.2 ± 4.9, respectively) and no difference in CD4/CD8 ratio (1.4 (0.73–2.7) vs 1.3 (0.93–4.5), respectively). Interestingly, no difference was observed even when they compared CD8 T rates and CD4/CD8 ratio between different disease stages. Similarly, no significant changes were observed in the percentages of CD8 T cells among total mononuclear cells [119] and no variations revealed in CD8 T cell rates and CD4/CD8 ratio among CD3+ cells in endometriosis regardless of the stage [133]. However, the latter group found lower expression of TNF-α and IFN-γ in CD8 T cells in the endometriosis group when compared with the control group (17.4 ± 1.6 vs. 25.5 ± 2.0, respectively; $p = 0.0045$). Focusing on the cytotoxic activity of peripheral blood mononuclear cells, authors found no difference in CD8+CD28+ T cell ratios between endometriosis patients and controls (15.16 ± 3.26 vs 11.89 ± 2.65, respectively; $p = 0.45$) [134]. Finally, the study on baboons discovered no differences in the CD8 T cell ratio between baboons with spontaneous endometriosis and those with the induced disease as well as between animals with the disease and healthy subjects [123].

In addition to these studies which largely compare bulk CD8 T cell ratios, some papers report on CD8 expression within other subpopulations of T cells, such as NK T [135] and MAIT [124], or

subsets of the global CD8 T repertoire such as T_{CM} and T_{EM} subtypes [129]. CD8 expression on NK T cells in combination with the regulatory cytokines IL-6, IL-17 and IL-10 were unaltered between health and endometriosis controls [135]. Although Li et al. did not identify differences in the overall CD8+ MAIT population between endometriosis patients and controls, they found an increased frequency of CD8+ cells among MAIT subtypes in the peripheral blood of patients as well as controls when compared to global CD8 T cells ($p = 0.003$ and $p = 0.0002$, respectively) [124]. Importantly, the only study looking into distinct CD8 T subtypes found increased levels of T_{CM} (CD3+CD8+CD45RA-CCR7+; $p < 0.05$) and decreased levels of terminally differentiated T_{EM} subtypes (CD3+CD8+CD28-; $p = 0.006$) [129].

Table 6: CD8+ T cell outcomes in peripheral blood.

FIRST AUTHOR YEAR	TOTAL (N)	ENDOMETRIOSIS (N)				CONTROLS (N)			UTERINE CYCLE PHASE (N)		ANALYSED SAMPLES (N)		CD8+ T CELL FINDINGS IN ENDOMETRIOSIS VERSUS CONTROLS	
		Stage I/II	Stage III/IV	Subfertility	Age (yrs)*	Diagnosis	Subfertility	Age (yrs)*	Proliferative	Secretory	Endometriosis	Controls	Cell proportion	Activation and cytotoxicity
Opsahl, 1994	20	8	0	8	N/A	Tubal ligation	0	N/A	11	9	8	12	**NO DIFFERENCE** among PBMCs.	
Ho, 1995	29	8	11	N/A	N/A	Tubal ligation	0	N/A	29	0	19	10		**NO DIFFERENCE** in HLA-DR+, CD25+, CD28+ and CD69+ subsets between mild and severe endometriosis and controls.
D'Hooghe, 1996	60	20	13	N/A	N/A	Healthy	N/A	N/A	21	20	37	23	**NO DIFFERENCE** among leukocytes. **NO DIFFERENCE** among leukocytes between spontaneous and induced endometriosis.	
Hsu, 1997	52	0	26	26	26–41	Healthy	0	22–38	N/A	N/A	26	26	**NO DIFFERENCE** in CD8+CD11b- or CD8+CD11b+ level and ratio among lymphocytes.	
Gagne, 2003	306	N/A	N/A	29	31–44 (69%)	Tubal ligation/ reanastomosis	0	31–44 (72%)	160	137	175	131	**NO DIFFERENCE** among leukocytes. **NO DIFFERENCE** in CD8+ cells among leukocytes.	
Gmyrek, 2008	48	13	23	N/A	23–53	Non-endometrial pathology	N/A	27–51	20	28	25	11	**NO DIFFERENCE** among CD3+ cells. **NO DIFFERENCE** among CD3+ cells between different stages.	**NO DIFFERENCE** in the ratio of IFN- γ, TNF-α or IL-8 expressing cells.
Mier-Cabrera, 2011	62	32	0	32	32.7 (±2.5)	Tubal ligation	0	33.8 (±5.4)	0	62	32	30	**NO DIFFERENCE** among lymphocytes.	**NO DIFFERENCE** in the ratio of IFN-γ and IL-2 expressing subsets
Chen, 2017	63	14	18	N/A	29 (18–38)	Non-endometrial pathology	N/A	32 (19–44)	63	0	4	4	**NO DIFFERENCE** among CD3+ cells.	
Li, 2019	50	17	15	N/A	32.6 (±1.10)	Non-endometrial pathology	N/A	33.3 (±1.23)	50	0	32	18	**NO DIFFERENCE** among MAIT population.	
Liu, 2020	101	0	33	33	30 (27–33)	Healthy	0	30 (27–32)	N/A	N/A	33	68	**INCREASED** levels of central memory subtypes (CD3+CD8+CD45RA-CCR7+; $p < 0.05$). **DECREASED** levels of terminally differentiated effector memory subtypes (CD3+CD8+CD28–; $p = 0.006$).	

Sobstyl, 2020	80	22	21	14	35.5 (±8.0)	Healthy	N/A	36.1 (±10.7)	N/A	N/A	40	40	**NO DIFFERENCE** among leukocytes. **INCREASED** CD8+TLR-2+ among CD8 T cells (p = 0.0054). **NO DIFFERENCE** in CD3+CD8+ and CD8+ TLR-2+ between different stages of endometriosis.	
Schmitz, 2021	23	6	6	N/A	33.7 (± 6.3)	Healthy	N/A	32.5 (±7.2)	23	0	12	11	**NO DIFFERENCE** among CD3+ cells.	**NO DIFFERENCE** in the ratio of perforin high subsets.
Correa, 2022	73	0	47	N/A	34.3 (±6.2)	Tubal ligation	N/A	34.5 (±4.6)	29	44	47	26	**NO DIFFERENCE** in CD8+ iNKT cell subset.	**NO DIFFERENCE** in levels of IL-17, IL-10 or IL-6 expressing CD8+ iNKT cells.
Muharam, 2022	14	N/A	N/A	N/A	30.57 ± 2.33	Non-endometrial pathology	N/A	33.71 ± 2.14	N/A	N/A	7	7		**NO DIFFERENCE** in CD8+CD28+ cells.

N/A - Not available; Age (yrs)*: Mean ± SD or Median (Range).

2.5 DISCUSSION

2.5.1 Summary

Endometriosis-associated immune alterations are complex [98]. This systematic review aimed to provide a rationale for further research into the CD8 T cells in endometriosis and associated conditions by summarizing and critically appraising the currently available data. Due to the observational study design of the included papers, it does not provide evidence for the direct involvement of the CD8 T cells in the development of endometriosis and associated disorders. Nevertheless, the findings highlight significant gaps in the literature that must be addressed in future research to determine the pathophysiological significance, non-invasive diagnostic potential and targeted treatment possibilities for this immune population. Out of 28 papers included in this systematic review, 14 could provide high-quality findings, which show that CD8 T cell levels are higher in endometriotic lesions than in the eutopic endometrium of patients as well as healthy controls, with no changes in peripheral blood levels between patients and healthy controls. However, the evidence for changes to CD8 T in peritoneal fluid and eutopic endometrium is debatable. Although older studies mostly found no difference in eutopic endometrial CD8 T cell concentrations, recent genomic analyses of distinct subtypes revealed a strong tendency of enriched CD8 T effector memory cells in the eutopic endometrium of patients with the disease. In one study investigating CD8 T cells in menstrual effluent, cytotoxicity was increased in patients while no difference was found in concentration and CD4/CD8 ratio.

2.5.2 Strengths and limitations

Numerous review articles have been published describing CD8 T cells in endometriosis [98, 136-148]. However, none of them has comprehensively investigated the literature on this immune cell population in the relevant tissues, including peripheral blood and endometriosis-associated tissues, such as eutopic endometrium, ectopic lesions, peritoneal fluid, and menstrual effluent. This review is the first to systematically summarise adequate quality evidence of CD8 T cells in endometriosis and was based on a comprehensive search strategy and strict inclusion criteria to ensure better comparability and reliability. For example, I have excluded studies investigating participants on hormonal contraception and menopausal women since it is known that oestrogen and progesterone significantly impact the CD8 T cell milieu and might undermine the accuracy of the findings [63, 149]. Additionally, I have excluded papers recruiting individuals with certain gynaecological pathologies, which could also have a significant impact on the relevant outcomes, such as adenomyosis, acute pelvic inflammatory disease, hydro/pyo/hematosalpinx, idiopathic infertility, and immune diseases [22, 150-152].

Despite the strict inclusion criteria, one of the weaknesses of this systematic review is that it is based on observational studies with small sample sizes which did not consistently account for confounders, so bias cannot be ruled out. While it is widely accepted that the menstrual cycle phase influences CD8 T cell counts and activation status in endometriosis-associated tissue, one of the included studies also found a clear correlation between the levels of several blood leukocytes and age, parity, previous use of oral contraceptives, smoking, and recent history of acute infection, but no other studies accounted for this [6, 130]. The method of choice in the older studies on eutopic and ectopic endometrium was immunohistochemical staining, which has several limitations regarding quantification, including the small and inadequately representative tissue size, interobserver differences and variations in technology. With surgery

being the gold diagnostic standard, it was expected that this review would suffer from the heterogeneity of the control population, with 8 studies having recruited control participants with various gynaecological conditions, such as uterine fibroids, ovarian cysts, and tubal factor infertility, and 4 of them including “healthy” controls without having undergone surgery to rule out potential pathological conditions. Additionally, some participants might have undergone several diagnostic and therapeutic surgeries before being enrolled in the study, which might have impacted the outcomes as significant immune changes are associated with the postoperative period [153].

2.5.3 Potential roles of CD8+ T cells in endometriosis

Imaging techniques have recently been recognised as a reliable diagnostic alternative to invasive procedures but they still have significant limitations for the detection of early disease [154]. Therefore, the development of novel non-invasive biomarkers is urgently needed to provide alternatives to surgery for diagnosis, and to reduce the prolonged time to diagnosis that is widely recognised for this condition [155]. Furthermore, biomarkers are a dynamic and effective tool to understand the range of the disease and provide a means for a consistent disease and risk factor assessment, which could help us learn more about the underlying aetiology of the disease [156]. Regarding the pathophysiology, none of the studies included in this review were designed to elucidate whether an altered CD8 T population could be the cause or the consequence of endometriosis. The closest is the study by D’Hooghe et al. in which they investigated peripheral blood and peritoneal fluid of baboons with spontaneous and induced disease compared to healthy controls [123]. While no difference was found in blood, an increased CD8 T cell ratio was found in peritoneal fluid of long-term spontaneous endometriosis when compared to animals with the recent spontaneous disease, induced disease and healthy controls, which could point out to the altered immune environment being a consequence rather than a cause of the disease.

No conclusions can be drawn from human studies, which did not find any differences in the CD8 T cells between different disease stages and healthy controls.

2.5.3.1 Local findings

The eutopic endometrium is an important mucosal barrier and the location of embryo implantation. Not only is immune dysregulation in eutopic endometrium associated with subfertility it may also play a role in lesion establishment [29, 157]. Therefore, along with other immune cell populations, CD8 T cells are an important consideration in endometriosis. Although the data regarding eutopic endometrial CD8 T cells in endometriosis altogether are controversial, our systematic review gives an overview not only of the whole CD8 T cell population, but of alterations in other CD8 T cell subtypes in the disease. Naïve and central memory CD8 T cell gene enrichment scores were found to be decreased while CD8 T_{EM} cells seem to be increased [111, 112, 118]. There is increasing evidence demonstrating an association between endometriosis and autoimmunity, and CD8 T_{EM} cells are likely an important player in the pathogenesis of autoimmune diseases due to their effectiveness and durability [152]. Nevertheless, evidence is not uniform in other autoimmune disease where, for example, CD8 T_{EM} cells are increased in the cerebrospinal fluid of patients with multiple sclerosis. While the opposite was found in the gut of patients with inflammatory bowel disease [158, 159]. More research is certainly needed to ascertain their role in the pathogenesis of endometriosis [160].

In adenomyosis, CD8 T cell subsets are increased not only in the eutopic, but also in the ectopic endometrium of patients compared to healthy controls [161]. Similar seems true for endometriosis, although there are discrepancies in the results between the lesions and matched eutopic endometrium. However, I emphasise that studies reporting no difference in CD8 T cells between eutopic and ectopic endometrium did not investigate specific phenotypes [110, 113].

Although the potential roles of CD8 T cells in lesions are not fully defined, some parallels may be drawn from cancer research, where CD8 T cells are regarded as the most potent effectors in the anti-cancer immune response and form the basis of some current cancer immunotherapies [162]. For example, increased intraepithelial CD8 T cell count in the tumour microenvironment is associated with prolonged survival in colorectal, ovarian and endometrial cancer [163-165] and the intraepithelial subpopulation of CD8 T cells was found to be increased in ectopic lesions in the early secretory phase when compared to matched eutopic and healthy endometrium [116]. However, no studies were conducted comparing intraepithelial CD8 T cells between lesions of patients with different endometriosis stages and between those who benefited from the surgery or relapsed, which would give us valuable insights into the prognostic role of this population.

It has been suggested that endometriotic lesions ought to be investigated along with peritoneal fluid since this dynamic environment strongly influences their pathophysiology [166]. Nevertheless, to date, studies have examined lesions and peritoneal fluid in isolation. Similar to eutopic endometrium, data on peritoneal CD8 T cells are contradictory and no definitive conclusions can be made about the role of the CD8 T cell population in this regard. On the other hand, macrophages are a well-researched population in this setting as they are the most abundant peritoneal immune cell population, representing around 50% of peritoneal cavity leukocytes [167]. They are required for lesion growth, development, vascularisation, and innervation and are even associated with pain symptoms. In endometriosis, an increased ratio of altered peritoneal macrophages has been indicated, and since they are involved in antigen presentation to CD8 T cells, this points to potentially related dysfunctions within the CD8 T cell compartment. Similarly, changes were found in Tregs in endometriosis, with findings showing an increased number of activated Tregs in the peritoneal fluid of patients with endometriosis

[168]. Like macrophages, Tregs are involved in CD8 T cell activation and play an important role in the generation of high-avidity initial responses and effective memory [169].

One aim of this review was to gain a better understanding of the activation characteristics of the CD8 T cells in endometriosis. Unfortunately, no functional analyses have been performed to date on either eutopic or ectopic endometrial CD8 T cells, despite clear evidence of endometriosis-related dysfunctions in key regulators of CD8 T function, such as PD-1, programmed death-ligand 1 (PD-L1) and Tregs [98, 113, 170]. Functional analyses of CD8 T cells in peritoneal fluid demonstrate inconsistent findings, where data mostly report no difference or decreased activation potential of peritoneal CD8 T cells, with only one study reporting an increase in activated CD8+ MAIT cells. There is some evidence of upregulated activation marker CD69 expression on peritoneal fluid T cells in endometriosis patients, although it is important to emphasise that the investigated cohort had been taking hormonal treatment hence this study was not included in our systematic review [103].

Studies on immune cells in the female genital tract are challenging given the invasive nature of collecting mucosal and peritoneal samples [171]. As previously mentioned, most research depends on biopsies and hysterectomies from women with underlying pathologies and samples from healthy women are limited. Menstrual effluent contains cells of endometrial origin and could be a valuable non-invasive source of endometrial immune cells but has so far been overlooked in the endometriosis research [127]. Authors found decreased expression of perforin in CD8 T cells of patients when compared with controls and argued that perforin-mediated cytotoxicity may play a crucial role in the establishment of the lesions, drawing from research on CD8 T cells in susceptibility and elimination of cancer cells. Importantly, this could provide some answers to why only a few women develop endometriosis while retrograde menstruation

occurs in most females of reproductive age [172]. However, further investigation of menstrual effluent is required to understand the processes responsible for endometrial cell attachment and development outside of the endometrium, especially given the key differences in immune milieu between eutopic and ectopic endometrium and peritoneal fluid, which I have confirmed in this review.

2.5.3.2 Systemic findings

The ease of sampling makes peripheral blood one of the most studied tissues in endometriosis leading to valuable insights into the systemic nature of endometriosis and its association with autoimmune diseases, including abnormalities in the T cell population [152, 168, 173]. Peripheral blood immune cells are influenced by sex hormones, with higher CD8 T cell numbers in males, but greater activation, proliferation and cytotoxic capacity in females [174]. Although longitudinal studies investigating CD8 T in the peripheral blood at different menstrual phases are limited, modifications in Treg cells [175] and Th1/Th2 ratios [176] indicate that it is important to consider the menstrual cycle when investigating CD8 T population in the peripheral blood. However, this was not the case in some of the included studies. While most of our data does not suggest discrepancies in the peripheral CD8 T cells, it is important to highlight that most papers did not describe CD8 T cell subpopulations. They were shown to be significantly changed in patients in one of the recent analyses with increased central memory and decreased effector memory subtypes in the disease [129]. Interestingly, several authors confirmed a decreased frequency of peripheral effector memory CD8 T cells in patients with multiple sclerosis at the onset of the disease and throughout its clinical course, which underlines its importance in the initiation of the disease rather than being its consequence [177]. Additionally, there is evidence of changes in innate immune cells in the peripheral blood of patients with endometriosis, such as monocytes, which are important for antigen presentation and CD8 T cell activation [178].

Reduced numbers of classical and intermediate monocytes were found in the peripheral blood of endometriosis patients, while the levels of plasmacytoid dendritic cells and non-classical monocytes were increased. Nevertheless, to better understand the systemic role of CD8 T cells in endometriosis, detailed phenotypic studies are needed, which will address specific subtypes and their activation characteristics found to be changed in the peripheral blood of other immune diseases [179].

2.5.3.3 Endometriosis-associated subfertility

Although the pathophysiology of EAS remains uncertain, several findings support altered endometrial immune receptivity as one of the possible causes [29, 30]. The exact nature of CD8 T in eutopic endometrium is poorly understood, yet the suggested hormonal influence on LA formation and CD8 T cell cytotoxicity underlines the importance of optimal regulation during implantation and pregnancy [6, 63]. As noted in our review, authors found significantly increased CD8 T cell scores in infertile women with endometriosis when compared to fertile women as well as in infertile versus fertile women with or without endometriosis [112]. Despite the limited sample size of this GSEA analysis, it reveals possible changes in CD8 T cells in patients with endometriosis-associated infertility and urges for more investigation into the phenotypic characterisation of the population in this related disorder. It is likely that different CD8 T phenotypes could contribute to subfertility and pregnancy complications as their findings correspond to the results of several authors, who confirmed altered endometrial CD8 T characteristics in endometrial biopsies obtained during WOI from patients with recurrent pregnancy loss, pre-eclampsia, and preterm labour [52, 89-91].

2.5.4 Future research

By combining cutting-edge technologies, such as single-cell RNA sequencing, mass cytometry and spectral flow cytometry, with established high-resolution, high-plex and multi-omic spatial biology solutions, such as spatial transcriptomics and multiplex immunostaining, I could spatially verify immune phenotypes, their functional capacities, and cell-cell interactions to ascertain the physiological and pathophysiological function of endometrial CD8 T cells. Importantly, structures similar to LA and intraepithelial lymphocytes (IELs) are known to play a role in intestinal inflammatory disorders; therefore, a deeper understanding of endometrial LA and intraepithelial CD8 T subsets may finally unravel their involvement in endometrial pathologies and infertility. For example, Tan et al. undertook image mass cytometry in addition to single-cell transcriptomic characterisation of ectopic and eutopic endometrium, providing a cell atlas of the endometriosis microenvironment [180]. This is a promising direction for future research, and the analysis of a larger cohort, including those not taking hormonal contraceptives, will enable us to draw conclusions on the disease pathophysiology and contribute critical information for future diagnostics and therapies. Importantly, further research should also provide more detailed insights into menstrual effluent, as well as functional and cytotoxic properties of the population in relevant tissues with consistent considerations for the phase of the uterine cycle and parity.

2.6 CONCLUSIONS

Understanding of CD8 T cells in endometriosis is limited, with significant discrepancies in current data in endometriosis-associated tissues. I have identified relevant gaps in the literature, such as deficient phenotypic and functional analyses in all relevant tissues. In the future, a detailed pathophysiological characterisation could lead to the discovery of non-invasive diagnostic biomarkers as well as successful drug repurposing and the development of targeted treatments to alter the immunological niche for better lesion clearing as well as endometrial homeostasis, vital for pregnancy success.

3 Transcriptome analysis of endometrial CD8+ T cells during the window of implantation

3.1 INTRODUCTION

3.1.1 RNA sequencing

Cutting-edge technologies, such as next-generation sequencing (NGS), have been propelled by scientific advancements in molecular biology and have revolutionised the analysis of messenger RNA (mRNA), which is transcribed from DNA and then subsequently translated into proteins. Bulk mRNA sequencing (RNAseq) enables simultaneous sequencing of thousands to millions of mRNA molecules from various biological samples, thereby yielding complete and intricate insights into gene expression profiles of different phenotypes of interest [181]. RNA extraction, purification, and quantification are critical steps in profiling a cell's or tissue's transcriptome, all available from multiple platforms, of which the filter-based spin basket format was used for RNA extraction and purification in this study, offering convenience, high yield, and mRNA enrichment by using RLT buffer and silica-membrane-based RNeasy Micro Kits [182]. Next, many sequencing and quantification systems are commercially available, including simple, scalable and reliable NovaSeq6000 and Smart-seq2 known for its high sensitivity using the SMART reaction (Switching Mechanism At the end of the 5'-end of the RNA Transcript) and employing the properties of reverse transcription and template switching facilitated by the Moloney Murine Leukemia Virus reverse transcriptase, specifically at the 5'-end of the RNA transcript [183]. Finally, sophisticated bioinformatics tools and algorithms are essential to facilitate tasks not only for read alignment and transcript quantification but also quality control, data normalisation, differential gene

expression analysis, functional annotation and data visualisation, enabling us to extract meaningful biological information from the sequencing results [183, 184]. Several tools have been created for each stage of the analysis. For example, DESeq2 is a bioinformatics tool designed to analyse RNAseq data, specifically addressing differential gene expression by implementing statistical methods that account for variability [185]. Gene Ontology (GO) enrichment is a bioinformatics analysis method for evaluating the overrepresentation of specific gene sets in a list of differentially expressed genes, revealing functional characteristics of those genes regarding cellular components, molecular functions, and biological processes [186]. In contrast to GO enrichment, GSEA utilises the list of all sequenced genes to determine whether predefined gene sets exhibit statistically significant, concordant differences between two biological states, thereby assisting in identifying biological pathways or functional gene sets associated with specific conditions or phenotypes [187]. Thanks to recent advancements, transcriptomic characterisation enables us to understand the molecular elements and their processes in various cells and tissues, including human endometrium.

3.1.2 Peri-implantation endometrial transcriptome

The endometrium exhibits extraordinarily dynamic transcriptome alternations compared to other tissues in the body. Daily and often hourly fluctuations in gene expression are caused by increased circulating oestrogen during the proliferative phase of the cycle, followed by progesterone after ovulation, with changes in gene clusters associated with processes such as adhesion, invasion, survival, growth, differentiation, wound healing and immunological responses [188, 189]. Focusing on the WOI, studies describe fundamental dynamic changes in gene expression in epithelial cells (upregulation of *PAEP, GPX3,* and *CXCL14*) that signal the opening of the WOI. In contrast, changes in stromal cells (upregulation of *FOXO1* and *IL15*) seem to be more continuous [190]. There is a significant scientific and commercial interest in markers

associated with endometrial receptivity to diagnose and treat impaired endometrial receptivity that extends beyond considerations of tissue remodelling and encompasses a comprehensive exploration of the role played by endometrial immunity [191]. A systematic review and meta-analysis of endometrial receptivity transcriptome studies revealed the significance of the inflammatory response, immunoglobulin-mediated immune responses, and the complement in aiding implantation success [192]. CD3+ cells have greater cytotoxic capability during WOI, notably in *CD8* expression, whereas cytotoxic granule genes, such as *GZMA, GZMB, PRF1, NKG7* and *GNLZ*, have modest elevation. During decidualisation, CD3+ and CD3- cell subsets exhibit increased expression of genes associated with IL-2 receptors and IL-2-elicited cell activation. CD3- cells have a high amount of chemokine expression, whereas CD3+ cells have a more diversified cytokine repertoire with lower chemokine expression. Upregulated ligand-receptor combinations in stromal fibroblasts and lymphocytes, such as IL-15 and IL-2RB, imply that these cell types interact directly in the decidualising endometrium, which was confirmed by spatial proximity with immunofluorescence imaging [190]. Compared to their circulating counterparts, peri-implantation endometrial Tregs display increased expression of CXCR6 and ICOS, consistent with their functions in tissue adaptability and IL-10 production, as well as increased expression of immunological checkpoint molecules such as CTLA-4, TIGIT, PD-1, Tim-3, and LAG-3. Notably, the PD-1/PD-L1 pathway, which is important in murine pregnancy, emphasises the possible influence of the Treg phenotype on Th17/Treg balance and reproductive outcomes, although it has not been explored how this reflects on CD8 T cell population [49].

3.1.3 CD8 T cell transcriptome in recurrent pregnancy loss

CD8 T cells are the predominant subset of decidual T cells, which comprise approximately 5–20% of total decidual leukocytes during the first trimester of healthy human pregnancy [16]. scRNAseq analysis showed expansion of CD8 T cells in the early decidua, with decreased

expression levels of perforin and granzyme B compared to peripheral blood CD8 T counterparts and increased expression of immune checkpoint molecules, such as TIM3, CTLA4, LAG3 and PD-1 with the corresponding ligands (PD-L1 and PD-L2) highly expressed by EVT [15, 193]. Interestingly, the total proportion of decidual CD8 T_{EM} cells without PD-1 expression was increased in miscarriage versus normal pregnancy along with clonal expansion in certain TCRβ repertoires, potentially recognising foetal or placental antigens, when compared to peripheral blood, suggesting that decidual CD8 T cells at the foeto-maternal interface could lead to foetal rejection [194]. However, human studies have predominantly examined early and term decidua, neglecting to investigate CD8 T cells during the WOI, which is crucial for establishing an optimal immune environment for successful implantation and pregnancy outcomes. Furthermore, differences have been explored only after the disorder has clinically manifested, making it challenging to discern whether these differences are the cause or consequence of the primary pathogenesis. This is important to better understand the pathophysiology of RPL and to provide justified evidence-based immune testing in the future. Hence, it is imperative to investigate the population during the WOI, a research gap that has not been addressed previously.

3.2 CHAPTER OBJECTIVES

This chapter discusses transcriptome analysis by bulk RNA sequencing of sorted endometrial CD8+ T cells from 26 subjects during WOI, including patients with RPL and fertile controls. The chapter describes human subject recruitment, cell isolation, library preparation, RNA sequencing and pipeline developed for quality control (QC) assessment conducted by Dr Mengni Shen as well as the bioinformatic analysis of the data that I undertook myself. Due to potential diagnostic implications, experimental groups for RPL patients were designed based on the next pregnancy outcome, which was either another miscarriage or live birth. Additionally, possible modulators of endometrial immunity have been investigated, such as age and parity. Differentially expressed genes were identified in different patient groups' GO enrichment analysis and GSEA were undertaken to identify altered pathways between them. Finally, I have discussed the findings in the context of current knowledge in this setting and highlighted potential implications for future research on CD8 T cell transcriptome in RPL.

Objectives:

1. To implement a comprehensive pipeline for bioinformatic analysis of endometrial CD8 T RNAseq data.
2. To investigate human endometrial CD8 T cell transcriptome in fertile women.
3. To assess how patients' factors such as age and parity modulate endometrial immunity.
4. To identify differentially expressed genes and enriched pathways between RPL patients with miscarriage and live birth as well as between RPL patients and fertile controls.

3.3 METHODS

3.3.1 Study design

Females under 40 years of age with regular menstrual cycles at least three months post miscarriage or hormonal treatment were recruited through TFP Oxford Fertility or Recurrent Miscarriage Clinic of Oxford University Hospitals Foundation Trust between 2016 to 2018. All samples were collected during WOI, determined as 6–11 days after LH pre-ovulatory surge. The RPL group consisted of participants who experienced at least 3 consecutive miscarriages and was further divided into RPL miscarriage and RPL live birth groups based on the next pregnancy outcome. Fertile control group participants were undergoing *in vitro* fertilisation (IVF) for male factor infertility and had proven fertility by either a previous live birth or had a pregnancy with live birth outcome in the subsequent IVF cycle. All RPL patients had normal thyroid function, negative anti-phospholipid screen (cardiolipin IgG Ab, anti-beta-2 glycoprotein 1 IgG and DRVVT ratio), negative thrombophilia screen (including Factor V Leiden, Prothrombin 20210 mutation, Antithrombin III, protein C, protein S) and no evidence of uterine structural abnormalities (identified by ultrasound scan or hysteroscopy). According to the Royal College of Obstetricians and Gynaecologists guidelines, maternal and paternal karyotypes were only carried out if an unbalanced translocation was identified in karyotyped miscarriage tissue. The study was approved by the Oxford Research Ethics Committee C (ref:08/H0606/94). All participants gave written informed consent in accordance with the Helsinki Declaration of 1975. Patient recruitment is illustrated in Figure 6.

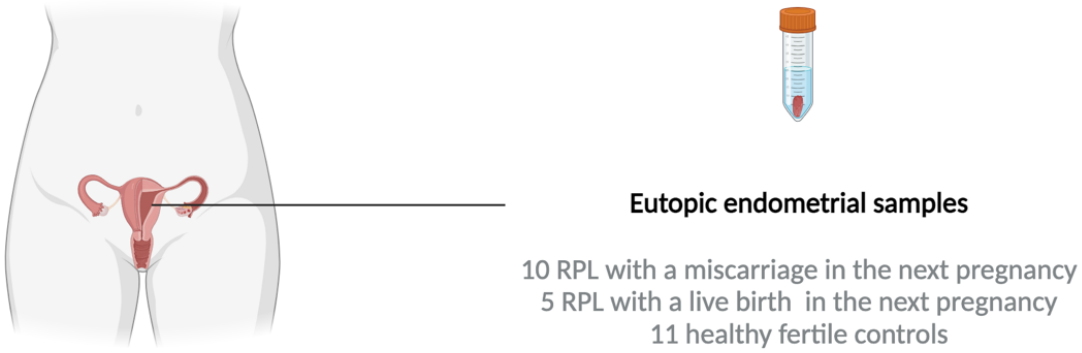


Figure 6: Patient recruited for the endometrial CD8+ T cell transcriptome analysis.

Pipelle biopsies were taken from 26 participants, who were grouped based on the underlying pathology either as recurrent pregnancy loss (RPL) (n = 15) or fertile controls (n = 11). RPL group was further divided to RPL miscarriage (n = 10) and RPL live birth (n = 5) based on the next pregnancy outcome. Created with BioRender.com.

3.3.2 Sample preparation

Sample preparation, including tissue processing, cell sorting, RNA extraction and quantification, was undertaken by Dr Mengni Shen as part of her DPhil studies in 2019 [195] and is shown in Figure 7.

3.3.2.1 Tissue processing

Endometrial biopsies were collected using an Endocell® disposable cell sampler (Cooper Surgical, USA). After sample collection, endometrial tissue was digested using 1X Liberase TM. Single cell suspensions were frozen in freezing media (10% DMSO/foetal calf serum (FCS)) and stored in liquid nitrogen.

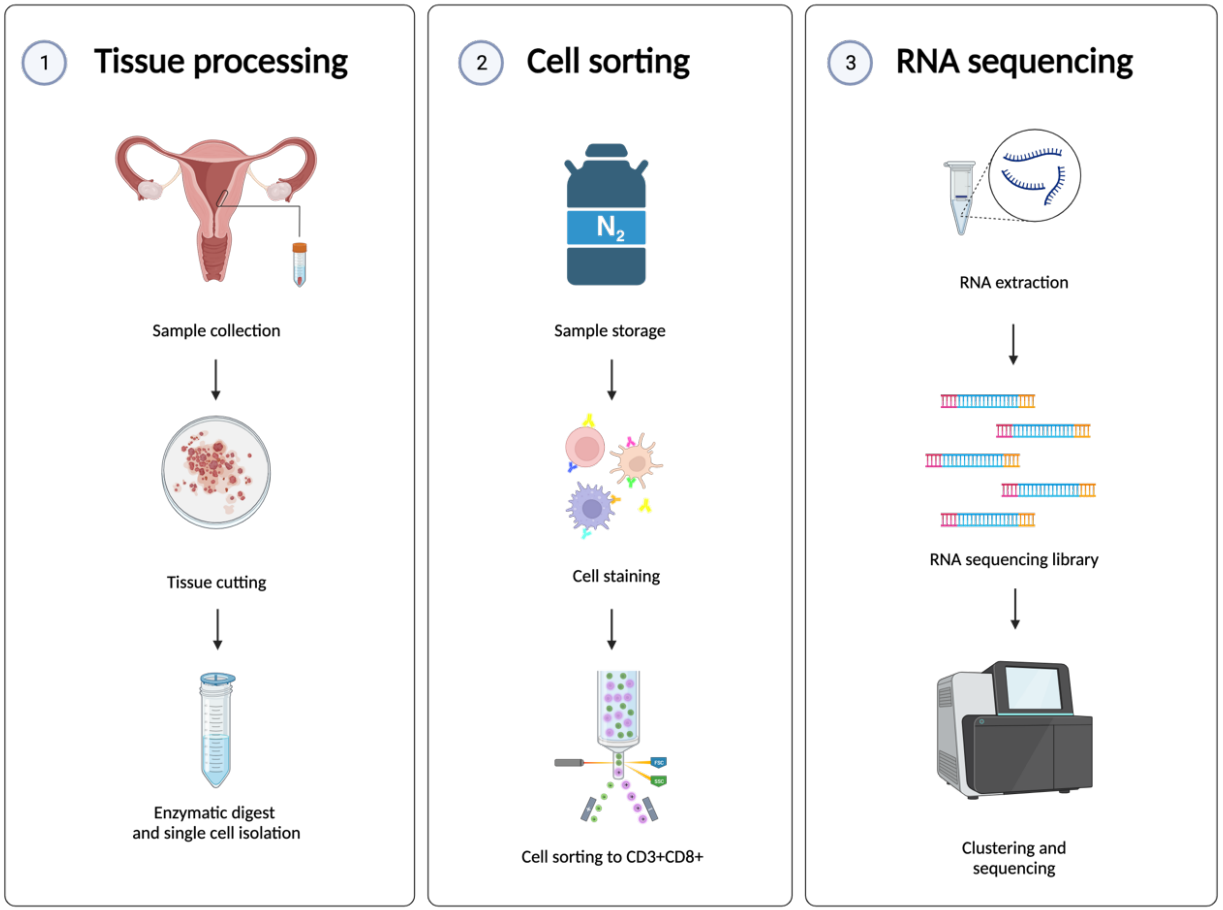


Figure 7: Sample workup for the endometrial CD8+ T cell transcriptome analysis.

Created with BioRender.com.

3.3.2.2 Cell sorting

For cell sorting, frozen cell suspensions were thawed in complete RPMI media (RPMI 1640 supplemented with 10% FCS, 1% Human Serum, 2mM glutamine, 100 IU/mL penicillin and 100 g/mL streptomycin), centrifuged, and re-suspended. Non-adherent cells were removed to deplete stromal and epithelial cells. CD8 T cells were sorted out from the remaining cells based on viability dye Zombie Aqua and surface markers CD45, CD3 and CD8 using FACSAria™ III Sorter (BD Biosciences, USA).

3.3.2.3 RNA extraction and quantification

RNA extraction was performed immediately after sorting using RNeasy Micro Kit (QIAGEN, The Netherlands), RNA quantity and quality were assessed using the 4200 TapeStation System (Agilent, USA), all performed according to the manufacturer's protocol.

3.3.3 RNA sequencing

3.3.3.1 Data acquisition

The RNA sequencing library was prepared in collaboration with the Weatherall Institute of Molecular Medicine Single Cell Facility, clustering and sequencing were conducted by Novogene Experimental Department, Novogene, UK.

RNA sequencing library

Complementary DNA (cDNA) conversion for single cells was performed with Smart-Seq2 protocol [183]. The library was created using the Standard Nextera Illumina Library Prep kit, employing a unique dual-index strategy for cDNA fragment barcoding. DNA amplification was carried out for 16 cycles unless cDNA concentrations suggested more cycles. During library

quality control, cDNA quantities were assessed using the 2100 Bioanalyzer and Quant-iT PicoGreen, with cDNA masses ranging from 0.9 to 15 ng/µL.

Clustering and sequencing

Index-coded samples were clustered on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina, USA) per the manufacturer's instructions. Following cluster generation, paired-end RNA sequencing was performed on the Novoseq 6000 platform, with read lengths of 125 bp/150 bp and an approximate yield of 25 million reads per sample.

3.3.3.2 Data processing

Data processing, including quality control, read alignment and quantification, was performed by Novogene Experimental Department, Novogene, UK.

Quality control

First, reads containing adapter, reads containing ploy-N and low-quality reads were removed from raw fastq files by processing through Novogene in-house perl scripts. Additionally, trimming of reads was carried out to eliminate bases located at the ends of the reads that exhibited a high error rate and had skewed GC content.

Read alignment and quantification

Obtained clean raw reads were mapped to Ensembl Homo sapiens cDNA database release 94 using TopHat v2.0.12 [196]. To assess the mapping status of RNAseq data against the reference genome, only reads filtered by chosen parameters (e.g., the longest intron length) were used. HTSeq v0.6.1 was utilised to count the number of reads that mapped to individual genes and the resulting non-normalised count table was subsequently used in downstream analyses [197].

3.3.3.3 Data analysis

All steps of the data analysis were performed in R Studio (R version 4.3.1) unless described otherwise. This part of the analysis was performed by myself.

Sample variance visualisation

DESeq2 package (1.40.2) was used with no group comparison to normalise the data prior to exploratory sample variance visualisation [197]. To ensure an unbiased comparison among experimental groups, blind dispersion estimation was conducted. This involved applying two statistical methods to the transformed data: (1) Sample to sample Spearman Correlation Coeficient (SCC) was computed for 26 endometrial CD8 T cell samples after rlog transformation. (2) Principal Component Analysis (PCA) plots were created using rlog-transformed count data.

Differential gene expression analysis

I identified and mitigated confounding variables using sva package (3.48.0) before determining differentially expressed genes between groups of interest using a model based on the negative binomial distribution with DESeq2 package (1.40.2) [185, 198]. Genes with an adjusted p-value (p.adj) < 0.05 and absolute $\log_2$ fold change ($|\log_2 FC|$) > 1 were assigned as differentially expressed. Open-source software tool Searchlight2 (V2.03) was employed for the automated analysis visualisation of differentially expressed genes [199].

Pathway enrichment analysis

GO analysis was performed with goseq package on count matrix generated by DESeq2 [186]. The dataset was subjected to a probability weighting function, assigning weights to individual genes based on their respective lengths. Finally, I utilised Wallenius approximation to determine the enriched GO terms among the differentially expressed genes, with the GO annotation based on the hg19 build of the human genome. Pathway enrichment analysis was conducted with the

clusterProfiler package [200]. Ensembl IDs in differentially expressed gene list were linked to the Entrez database for GO, which was performed with a false discovery rate control using a Homo sapiens (human) reference source. The rich factor in a specific pathway was computed by dividing the number of differentially expressed genes by the number of background genes. GSEA was undertaken using GSEA software (4.3.2) [187]. Normalised read count table generated by the DESeq2 package was used as input data with genes ranked based on the signal-to-noise metric. Hallmark gene sets (ftp.broadinstitute.org://pub/gsea/genesets/h.all.v2023.2.Hs.symbols.gmt), GO gene sets (ftp.broadinstitute.org://pub/gsea/genesets/c5.all.v2023.2.Hs.symbols.gmt) and immunologic gene sets (ftp.broadinstitute.org://pub/gsea/genesets/c7.immunesigdb.v2023.2.Hs.symbols.gmt) were selected as mapping gene sets, and 1000 permutations were performed on the "gene set" category for all analyses. Phenotype labels were defined to represent different groups of interest in the analysis. Collapse of gene symbols was applied to Human Ensembl Gene ID MSigDB.v2023.1.hS chip platform. Family-Wise Error Rate p-value (FWER p) < 0.05 and False Discovery Rate q-value (FDR) < 0.05 were set as a threshold for significantly enriched gene sets.

3.4 RESULTS

3.4.1 Patient characteristics

Table 7: Patient characteristics for RNAseq samples.

	RECURRENT PREGNANCY LOSS		FERTILE CONTROLS
	Miscarriage (n = 10)	Live birth (n = 5)	n = 11
Number of previous live births	6	1	4
Number of previous miscarriages	5 ± 3	4 ± 1	0
Age*	37 ± 4	35 ± 4	34 ± 4

Mean ± SD values for each group are shown. * Differences in age were not statistically significant.

3.4.2 Cell sorting and RNA quantification

Endometrial CD8 T cells were separated from all endometrial cells via flow cytometric cell sorting as CD3+CD4-CD8+ single live events. In total, 784,580 CD8 T cells were collected with 29,058 ± 23,021 cells (mean ± SD) per sample. Sorting purity was over 95% for all samples, and RNA concentration for all CD8 T cells was 495.6 ± 731.7 pg/L (mean ± SD). To reduce the noise of the dataset, only genes with count > 1 and variance > 1 were included in further analysis, which leaves 23,092 genes out of 60,448.

3.4.3 Sample variance

SCC was used as a statistical measure to calculate the strength of the relationship between the 26 endometrial CD8 T cell samples. As shown in the sample to sample correlation heatmap in Figure 8, hierarchical clustering based on SCC identified no major clusters. Therefore, similarities in correlation values cannot serve as an indicator in separating pathological groups.

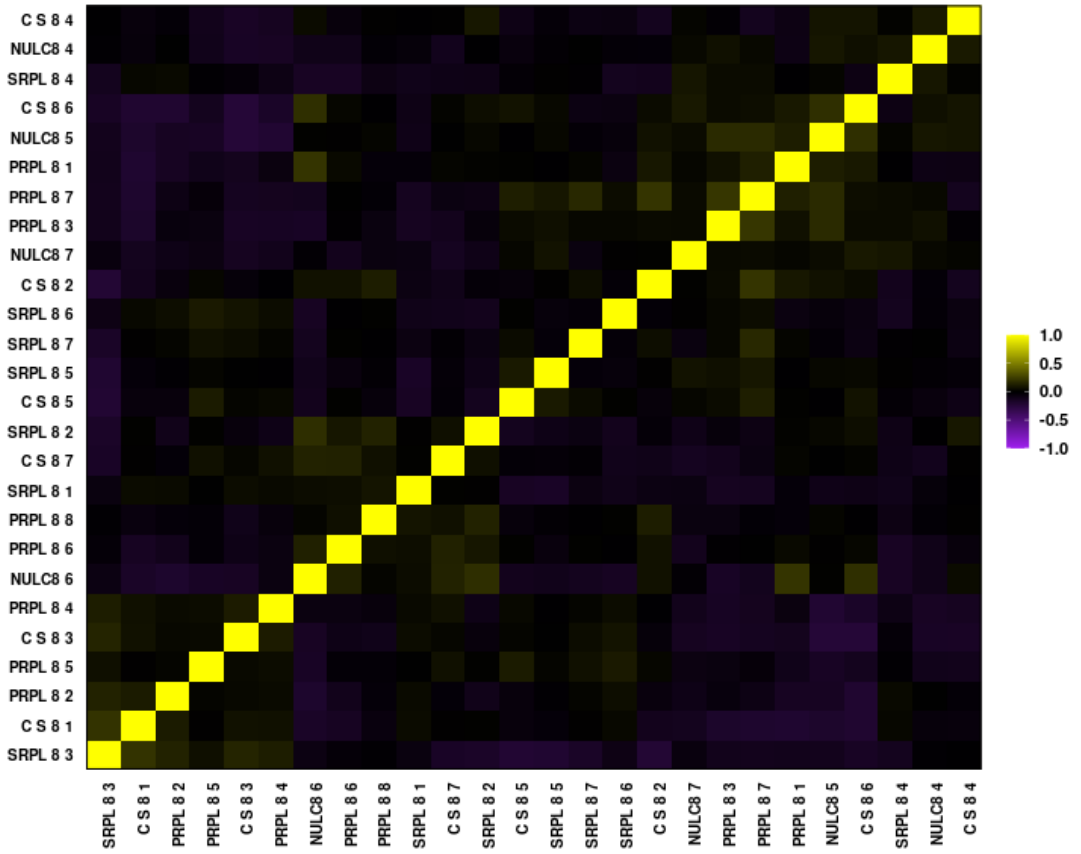


Figure 8: Sample to sample correlation heatmap.

Correlations were determined using all genes and a Spearman Correlation Coefcient (SCC) and clustered accordingly. Colour indicates SCC with - 1 as purple and 1 as yellow. Data was generated and visualised with Searchlight 2 [199].

Similarly, as shown in Figure 9 PCA of 26 endometrial CD8 T cell samples displayed a limited segregation between three experimental groups. Auto clustering was unable to identify any distinct groups, demonstrated that this transcriptomic-wide analysis was unable to provide evidence of major transcriptomic CD8 T cell variations between two RPL groups and fertile controls.

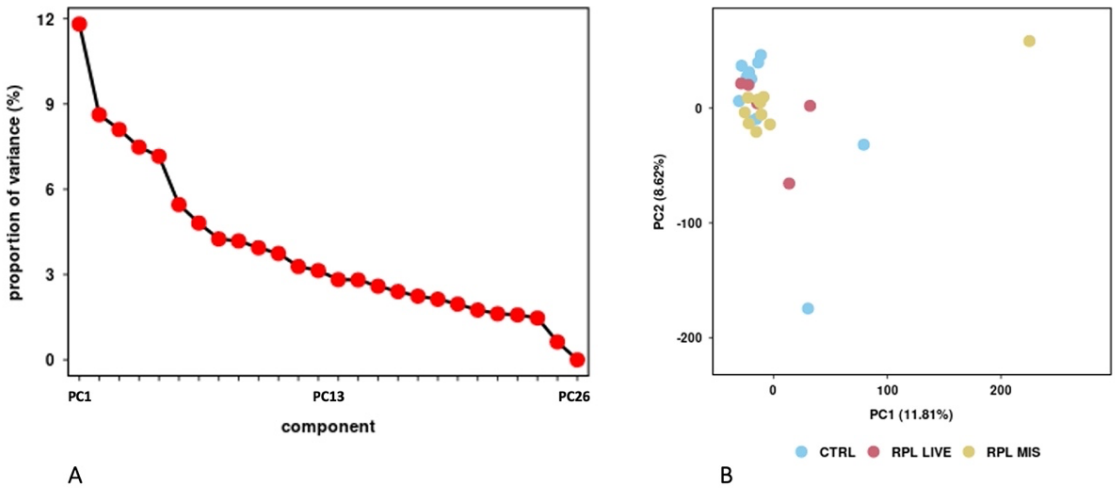


Figure 9: Visualisation of principal component analysis (PCA) and auto clustering of 3 experimental groups.

(A) PCA proportion of variance plot. The % of variation explained by each component is given on the Y-axis. (B) PC1 versus PC2 scatter plot. The % of variation explained by each component is given on the axis label. RPL LIVE, recurrent pregnancy loss with live birth; RPL MIS, recurrent pregnancy loss with miscarriage. Data was generated and visualised with Searchlight 2 [199].

3.4.4 Healthy endometrial CD8+ T cell transcriptome

3.4.4.1 Most expressed genes

Normalised gene expression values derived from DESeq2 analysis revealed the top 10 expressed genes in samples from fertile controls, which are comparable across the other two experimental groups. As shown in Table 8, most of the highly expressed genes are housekeeping genes, such as *TMSB4X, DNAJB1, MT-RNR2, UBC, MT-CYB* and *FOS* with some genes that highly associated with CD8 T cells, such as *B2M, ACTB* and *TNFAIP3*. These findings confirm the efficacy of cell sorting and RNA sequencing.

Table 8: Top 10 expressed genes in endometrial CD8+ T cells of fertile control patients.

Gene	Encoding protein function	Fertile control	RPL live birth	RPL miscarriage
B2M	Serum protein found in association with the major histocompatibility complex (MHC) class I heavy chain.	231299.04	247893.00	243357.19
ACTB	One of six different actin proteins that are involved in cell motility, structure, integrity, and intercellular signalling.	208638.92	239097.67	253970.93
TMSB4X	Actin sequestering protein which plays a role in regulation of actin polymerization.	171222.81	199685.27	203091.42
DNAJB1	Molecular chaperone that stimulates the ATPase activity of Hsp70 heat-shock proteins to promote protein folding and prevent misfolded protein aggregation.	153547.09	136614.45	133208.42
TNFAIP3	Zinc finger protein and ubiqitin-editing enzyme, shown to inhibit NF-kappa B activation and TNF-mediated apoptosis.	142801.38	130053.08	131046.58
**MT-RNR2*	Involved in negative regulation of execution phase of apoptosis.	132001.05	127408.61	165508.08
UBC	Polyubiquitin precursor, associated with protein degradation, DNA repair, cell cycle regulation, kinase modification, endocytosis, and regulation of other cell signaling pathways.	120771.90	109538.18	107809.52
MT-CYB	Enables metal ion binding activity and is involved in several processes, including electron transport, coupled proton transport and response to cobalamin and glucagon.	86422.17	82826.52	82146.17
FOS	Implicated as one of the regulators of cell proliferation, differentiation, and transformation.	83700.15	87024.15	100905.40
HSPA8	Constitutively expressed heat-shock protein that functions as a chaperone and binds to nascent polypeptides to facilitate correct folding.	79825.12	77132.85	67067.82

The data is presented as the mean gene count of normalised counts generated with DESeq2 [185]. * Differences in age were not statistically significant.

3.4.4.2 Modulating factors

To inform my final analysis design in the next chapter, I first investigated how different factors, such as patient's age and parity could influence endometrial CD8 T cell transcriptome. Therefore, I compared gene expression between patients younger versus older than 35 years and those who have previously given live birth versus those who have not. PCA of 26 endometrial CD8 T cell samples revealed minimal segregation between samples grouped by age and parity (data not shown). DESeq2 analysis controlled for history of miscarriage returned 23,002 encoding non-zero read counts genes in the endometrial CD8 T cell sample set. No changes were found in patients older than 35 years (n = 16) when compared with those younger than 35 years (n = 10) (data not shown). To observe changes in CD8 T cell transcriptome after term pregnancy, differential gene expression analysis was performed between parous (n = 11) and nulliparous (n = 15) women. I found 38 genes to be significantly downregulated in women with a history of live birth when compared to those without, including the most differentially expressed *MEX3A* (Mex-3 RNA Binding Family Member A), involved in nucleic acid binding and RNA binding, *CHRM4* (Cholinergic Receptor Muscarinic 4), the muscarinic cholinergic receptor, and *FSCN2* (Fascin Actin-Bundling Protein 2, Retinal), which crosslinks actin into filamentous bundles within dynamic cell extensions

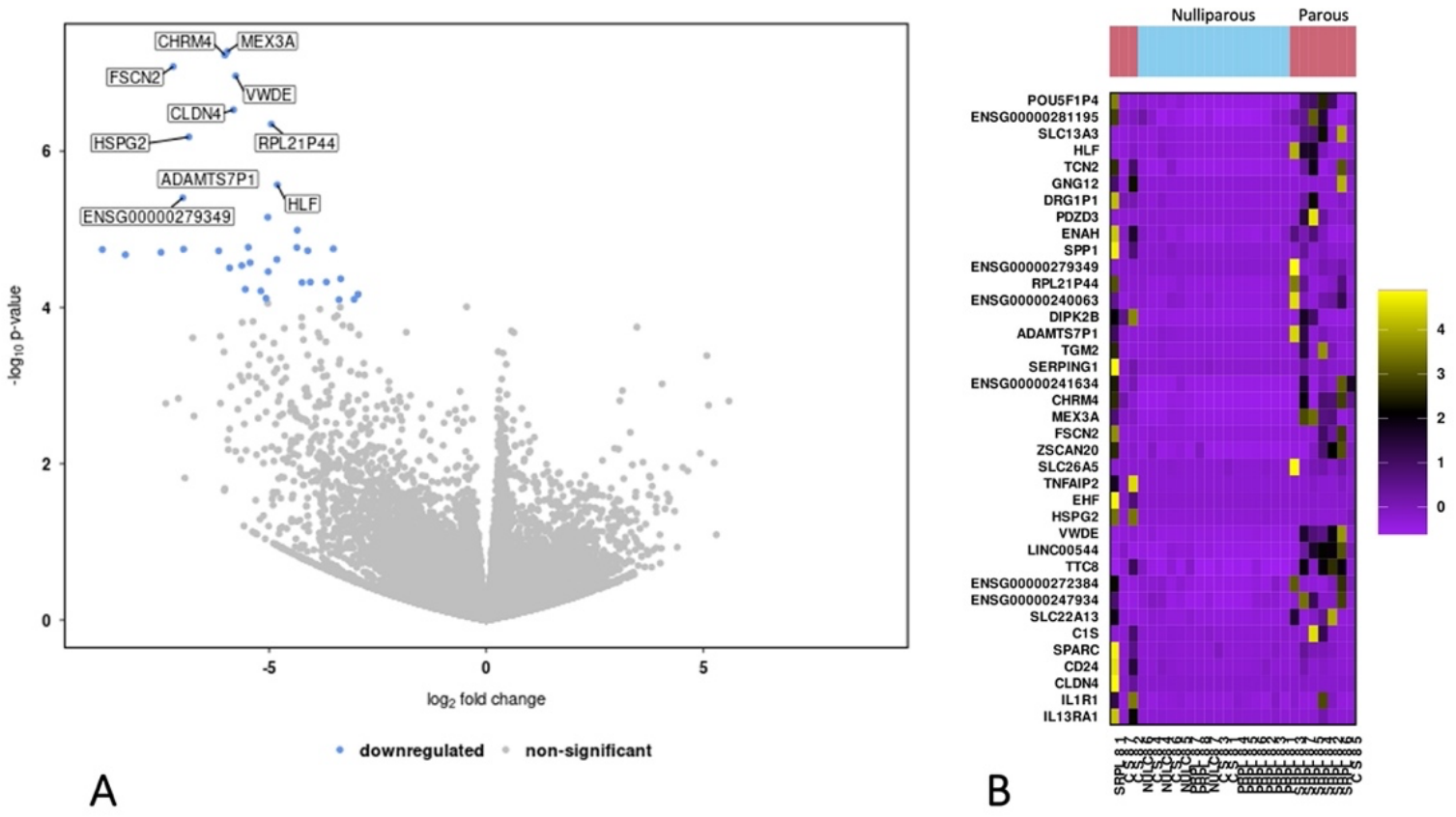


Figure 10: Altered endometrial CD8+ T transcriptome between parous and nulliparous women.

(**A**) Volcano plot of differentially expressed genes between parous versus nulliparous women. Each point represents one gene. Genes were coloured according to figure legend. Top 10 differentially expressed genes (ranked by p.adj and $|\log_2 FC|$) in both groups were labeled. (**B**) Heatmap of differentially expressed genes between between parous versus nulliparous women. (**A-B**) p.adj < 0.05, $|\log_2 FC| > 1$ were applied. Data was generated with DESeq2 [185] and Searchlight 2 [199].

3.4.5 Endometrial CD8+ T cell transcriptome in recurrent pregnancy loss

3.4.5.1 Next pregnancy outcome

DESeq2 analysis controlled for parity returned 23,002 encoding non-zero read counts genes (out of 23,092) in the endometrial CD8 T cell sample set. First, I compared RPL patients with miscarriage versus live birth in the next pregnancy and found 11 significantly differentially expressed genes between the groups, with upregulated *MTCO1P12, C5, SEMA5A,* and *ZNF709* genes and downregulated *HSPG2, PLLP, SFN, REPS2, TRIM2, AP1G2-AS1 (ENSG00000258727)*, and *LINC02363* genes in the miscarriage group (Figure 11).

Among the top significantly changed genes, *HSPG2* was the most expressed in the RPL group with a subsequent live birth and *MTCO1P12* in the RPL group with a miscarriage. *HSPG2* (Heparan sulfate proteoglycan 2) encodes the perlecan protein, a large multidomain proteoglycan that binds to and cross-links many extracellular matrix components and cell-surface molecules. *MTCO1P12* (MT-CO1 Pseudogene 12) is a pseudogene, which is a segment of DNA that structurally resembles a gene but is not capable of coding for a protein.

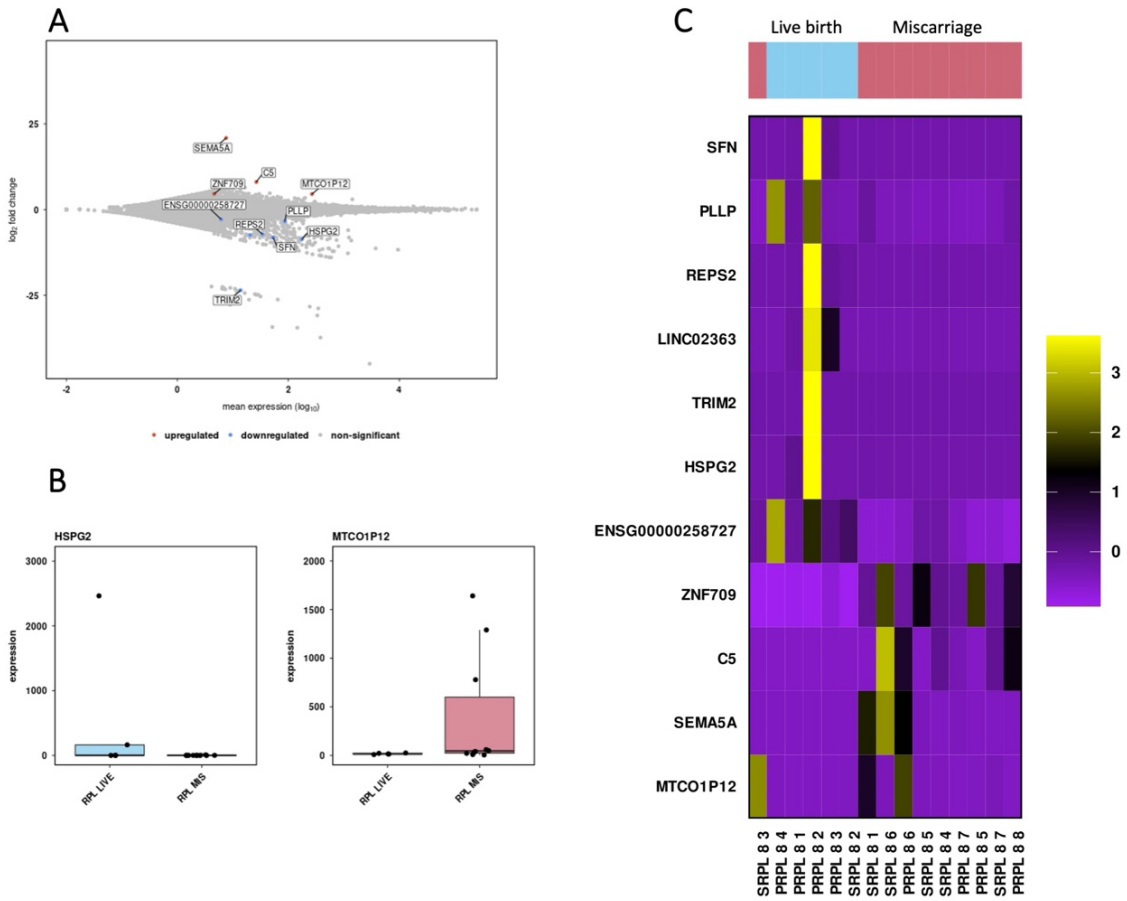


Figure 11: Altered endometrial CD8+ T transcriptome in patients with recurrent pregnancy loss and miscarriage versus live birth in the next pregnancy.

(**A**) MA plot of differentially expressed genes between patients with recurrent pregnancy loss (RPL) and miscarriage versus live birth in the next pregnancy. Each point represents one gene. Genes were coloured according to figure legend. Top 10 differentially expressed genes (ranked by p.adj and $|\log_2 FC|$) in both groups were labeled. (**B**) Boxplots representing two highest expressed genes and significantly changed between patients with recurrent pregnancy loss (RPL) and miscarriage versus live birth in the next pregnancy. *HSPG2* upregulated in the RPL LIVE and *MTCO1P12* upregulated in the RPL MIS group. Each point represents one sample. Samples were grouped according to the x axis. RPL LIVE, recurrent pregnancy loss with live birth; RPL MIS, recurrent pregnancy loss with miscarriage. (**C**) Heatmap of differentially expressed genes between RPL patients with miscarriage versus live birth in the next pregnancy.(**A-C**) p.adj < 0.05, $|\log_2 FC| > 1$ were applied. Data was generated with DESeq2 [185] and Searchlight 2 [199].

Differentially expressed genes (p.adj < 0.1, $|\log_2 FC| > 1$) that have been implicated in the function of CD8 T cells but were not necessarily significant are shown in Figure 12. *PLLP, SFN, HMOX1* and *ATPC2C2-AS1* were upregulated in the group with a subsequent live birth while *C5, SEMA5A, LLGL1* and *CCL20* were upregulated in the group with miscarriage in the next pregnancy. *PLLP* (Plasmolipin) is important in the process of myelination but also participates in ion transport by inducing the formation of ion channels. *SFN* (Stratifin) encodes a cell cycle checkpoint protein. *HMOX1* (Heme oxygenase 1) gene encodes an essential enzyme in heme catabolism and *ATPC2C2-AS1* (ATP2C2 Antisense RNA 1) belongs to the class of long non-coding RNAs. *SEMA5A* (Semaphorin 5A) encodes membrane proteins containing a semaphorin domain and several thrombospondin type-1 repeats, which are involved in axonal guidance during neural development and angiogenesis. *C5* (Complement C5) encodes a component of the complement system and is proteolytically processed to generate multiple protein products, including the C5α chain, C5β chain, C5a anaphylatoxin and C5b. *LLGL1* (LLGL Scribble Cell Polarity Complex Component *1*) transcribes into a protein that is part of a cytoskeletal network and the protein encoded by *CCL20* (C-C Motif Chemokine Ligand 20) displays chemotactic activity for lymphocytes and can repress proliferation of myeloid progenitors. Pathway enrichment analysis was performed to explore which biological processes involve significantly upregulated genes and could be influenced by alterations in the CD8 T cell transcriptome.

Figure 13 shows enriched gene sets and significantly increased genes in patients with RPL and miscarriage in the next pregnancy when compared to patients with live birth. Importantly, 3 significant genes (*SEMA5A* and *C5* in the miscarriage and *HSPG2* in the live birth group) are involved in the regulation of vascular development and angiogenesis, while *SEMA5A* and *C5* also play a role in chemotaxis.

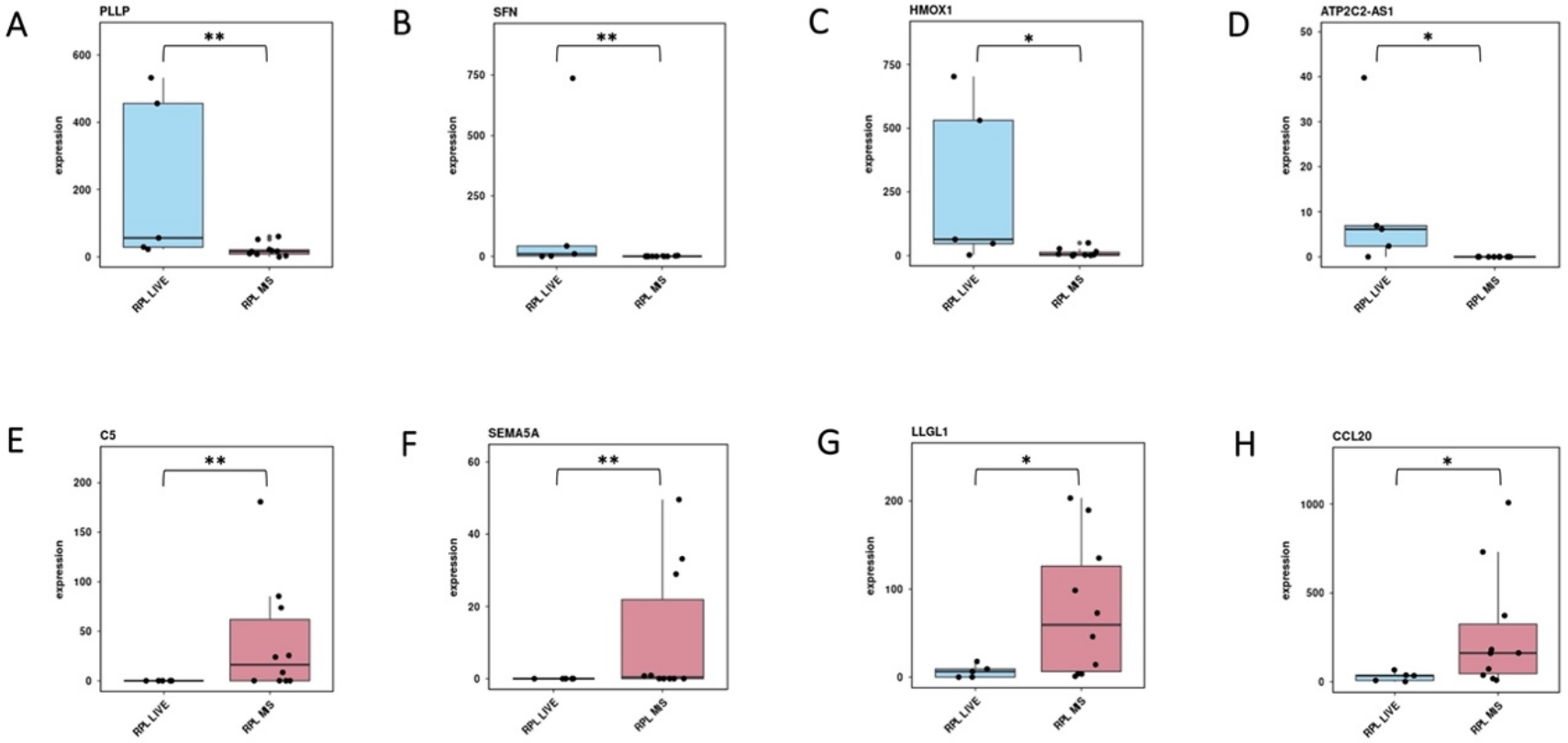


Figure 12: Boxplots representing genes relevant to CD8+ T cell function that are differentially expressed between patients with recurrent pregnancy loss and miscarriage versus live birth in the next pregnancy.

Each point represents one sample. Samples were grouped according to the x axis. RPL LIVE, recurrent pregnancy loss with live birth; RPL MIS, recurrent pregnancy loss with miscarriage. ** p.adj < 0.05, $|\log_2 FC| > 1$; * p < 0.005, $|\log_2 FC| > 1$. Data was generated with DESeq2 [185] and Searchlight 2 [199].

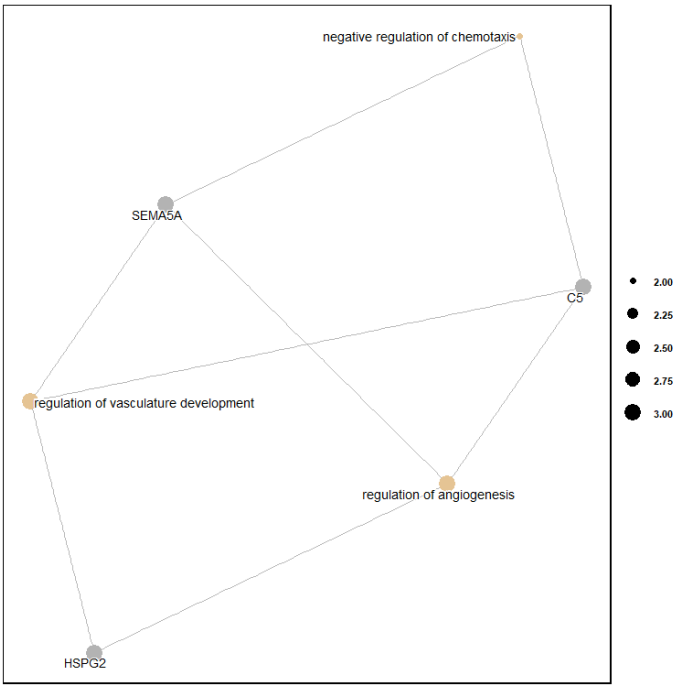


Figure 13: Changes in CD8+ T cell transcriptome pathways of patients with recurrent pregnancy loss and miscarriage in the next pregnancy are related to angiogenesis, vasculature development and chemotaxis.

Gene-concept network illustrates enriched gene sets ($p < 0.05$, $q < 0.05$) with significantly upregulatedupregulated genes ($p.adj < 0.05$) CD8+ T cells of patients with recurrent pregnancy loss and subsequent miscarriage compared to live birth. Nodes denote enriched gene sets and significant genes. Edges connect gene sets and their constituent genes. Node size shown on the right represents the number of signifIcant genes in the gene-set (1.0–3.0).

GSEA is an additional method for interpreting patient group specific gene enrichment. In order to rank genes, a signal to noise measure was created using the normalised count table created by DESeq2. This metric was then utilised to compare endometrial CD8 T cells between RPL patients who had a miscarriage and those who had a live delivery in the subsequent pregnancy in order to ascertain the differential expression of CD8 T cell genes in hallmark gene sets, GO gene sets, and immune gene sets. Hallmark gene sets database contains 50 gene sets of which 29 were enriched in the RPL with miscarriage and 21 in the RPL with live birth group. No gene sets passed the threshold (FWER $p < 0.05$, FDR $q < 0.25$) in either of the groups. In all GO sets, 4930 out of 7925 gene sets were enriched in the RPL patients with miscarriage and 2995 in the

live birth group, but again no gene set passed the significance threshold in either group. However, in the hallmark gene set, *inflammatory response* was enriched ($p < 0.05$, FWER $p > 0.05$, FDR $q < 0.8$) in the RPL miscarriage group, while in the GO gene set, two enriched but not significant gene sets were interesting in the context of CD8 T cells and pregnancy in both groups: *antiviral innate immune response* and *negative regulation of T cell mediated immunity* in the miscarriage group and *cell differentiation involved in embryonic placenta developme*nt and *peptide assembly with MHC protein complex* in the live birth group. Using immunologic gene sets, 3210 out of 4872 gene sets were upregulated in the miscarriage group and 3 were significantly enriched, with GSE15750 cluster related to CD8 T cells including genes upregulated in comparison of wild type CD8 effector T cells at day 6 versus those at day 10. None of the gene sets was significantly enriched in the live birth group.

3.4.5.2 Compared to fertile controls

Next, I compared RPL patients with miscarriage in the next pregnancy with fertile controls and also discovered 11 significantly differentially expressed genes, including *MTCO1P12,* ENSG0000*0280800, CPNE2-DT (*ENSG00000260148*)* and *RPSAP46* which were upregulated and *PRPF39, ALPL, EHD2, CA4, TRIM2*, ENSG00000279137 and *DAB2,* which were downregulated in the RPL group with miscarriage (Figure 14). Investigated genes with the highest significant difference exhibiting a recurring pattern of modifications observed in most patients within one of two groups were *TRIM2,* which was upregulated in the fertile control group and *MTCO1P12,* which was upregulated in the RPL patients with miscarriage in the next pregnancy. *TRIM2* (Tripartite Motif Containing *2*) gene is a member of the tripartite motif (TRIM) family, which includes three zinc-binding domains, a RING, a B-box type 1 and a B-box type 2, and a coiled-coil region. It plays a neuroprotective role and functions as an E3-ubiquitin ligase in proteasome-mediated degradation of target proteins.

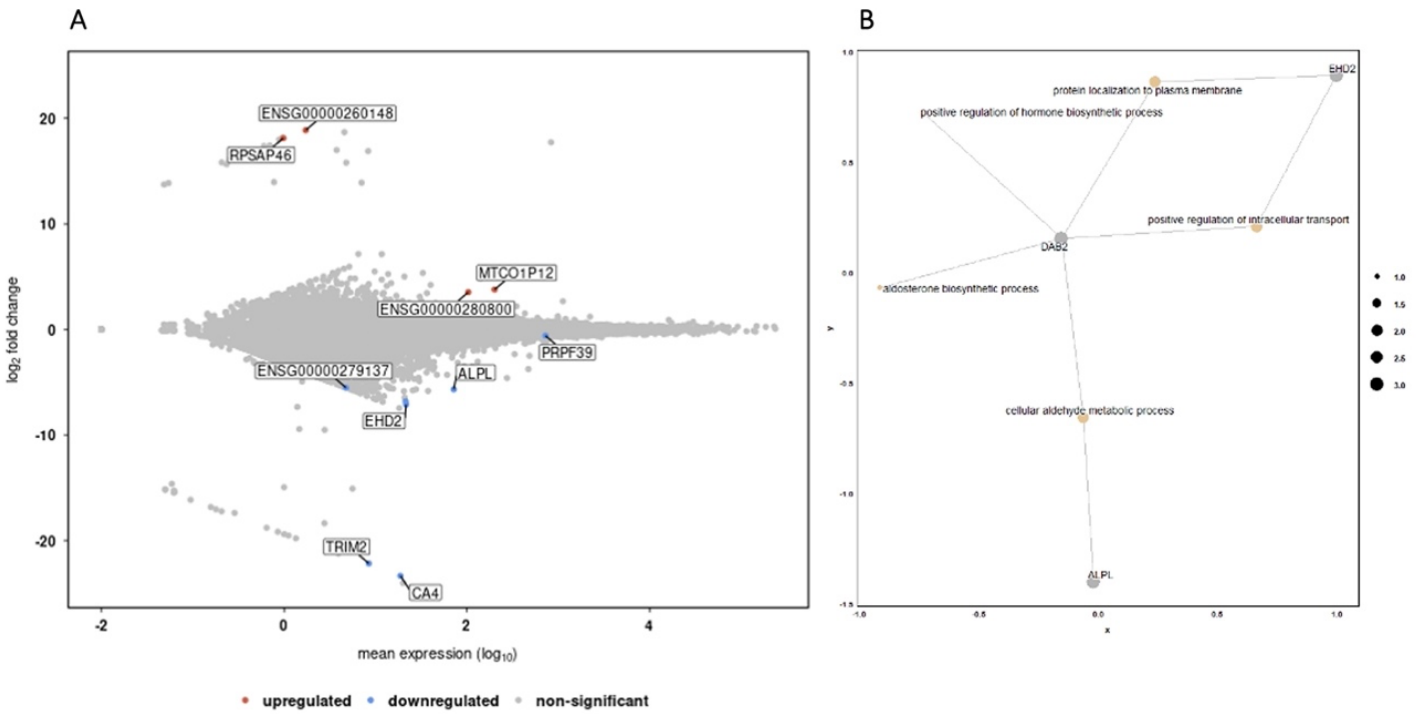


Figure 14: Altered endometrial CD8+ T transcriptome in patients with recurrent pregnancy loss and miscarriage in the next pregnancy versus fertile controls.

(**A**) MA plot of differentially expressed genes between patients with recurrent pregnancy loss (RPL) and miscarriage in the next pregnancy versus fertile controls. Each point represents one gene. Genes were coloured according to figure legend. Top 10 differentially expressed genes (ranked by p.adj and $|\log_2 FC|$) in both groups were labeled. (**B**) Gene-concept network illustrates enriched gene sets ($p < 0.05$, $q < 0.05$) with significantly upregulated genes (p.adj < 0.05, $|\log_2 FC| > 1$) in CD8+ T cells of patients with recurrent pregnancy loss and subsequent miscarriage compared to fertile controls. Nodes denote enriched gene sets and significant genes. Edges connect gene sets and their constituent genes. Node size shown on the right represents the number of significant genes in the gene-set (1.0–3.0). Data was generated with DESeq2 [185] and Searchlight 2 [199].

Utilising GO enrichment pathway analysis, I found changes in CD8 T cell transcriptome pathways of patients with RPL and miscarriage in the next pregnancy related to cellular transport (significantly increased genes *EHD2* and *DAB2)* and mineralocorticoid processes (genes *DAB2* and *ALPL)* as shown on the right-hand side of Figure 14.

Again, I used GSEA to identify patient group specific gene set enrichments with GO gene sets, curated gene sets, hallmark gene sets, and immune gene sets. Among 50 gene sets in the hallmark gene set database, 22 were enriched in the RPL miscarriage and 28 in the fertile control group. Among 22 enriched, two gene sets passed the threshold (FWER $p < 0.05$, FDR $q < 0.05$) and were considered significantly enriched. These were *interferon alpha response* and *oxidative phosphorylation* gene clusters. Interestingly, gene cluster for *allograft rejection* and *interferon gamma response* were enriched but not statistically significant ($p < 0.05$, FWER $p > 0.05$, FDR $q < 0.05$). In the fertile control group, significantly enriched *epithelial mesenchymal transition* cluster includes significantly upregulated gene *DAB2*. In all GO sets, 2608 out of 7925 gene sets are enriched in the RPL patients with miscarriage, but only one passed the significance threshold: *ATP synthesis coupled electron transport*, which also includes significantly upregulated gene *MTCO2P12* described above. In the fertile control group, gene set for *extracellular matrix structural constituent* is significantly enriched. Using immunologic gene sets, 3210 out of 4872 gene sets were upregulated in the miscarriage group and 19 were significantly enriched. Of these, there were some related to CD8 T cells:

- KAECH_NAIVE_VS_DAY8_EFF_CD8_TCELL_UP: genes upregulated in naïve CD8 T cells compared to effector CD8 T cells at the peak expansion phase (day 8 after LCMV-Armstrong infection),
- GSE15930_NAIVE_VS_48H_IN_VITRO_STIM_CD8_TCELL_DN: genes downregulated in comparison of CD8 T cells at 0 h versus those at 48 h,

- GSE21360_PRIMARY_VS_TERTIARY_MEMORY_CD8_TCELL_DN: genes downregulated in memory CD8 T cells: 1' versus 3',
- GSE24026_PD1_LIGATION_VS_CTRL_IN_ACT_TCELL_LINE_DN: genes down-regulated in comparison of Jurkat T cells stimulated in the presence of PD-1 versus controls.

Finally, I compared RPL patients with live births in the next pregnancy after sample collection and fertile controls. Among 10 significantly differentially expressed genes, *HSPG2, CPNE2-DT (ENSG00000260148), SFN, RPSAP46,* and *LINC02363* were upregulated in the RPL group while *SFRP4, SLC2A14, CPXM1, CA4* and *SEMA5A* were downregulated (Figure 15). There is a lack of prominent differences in upregulated gene expression with most of them being highly expressed in only one or two patients, which accordingly makes the clustering of patients into two groups based on their gene expression profiles less distinct (data not shown). Conversely, upregulated genes in the control group are more evenly distributed, with *CPXM1* and *SLC2A14* being the leading two. *CPXM1* (Carboxypeptidase X, M14 Family Member 1) is associated with metallocarboxypeptidase activity, and SLC2A14 (Solute Carrier Family 2 Member 14) is a member of the glucose transporter family, involved in the transport of hexoses such as glucose and fructose into all mammalian cells. GO enrichment analysis (Figure 15) identified changes in gene sets related to epithelial and epidermal differentiation including significantly changed genes *SFRP4* and *SFN*.

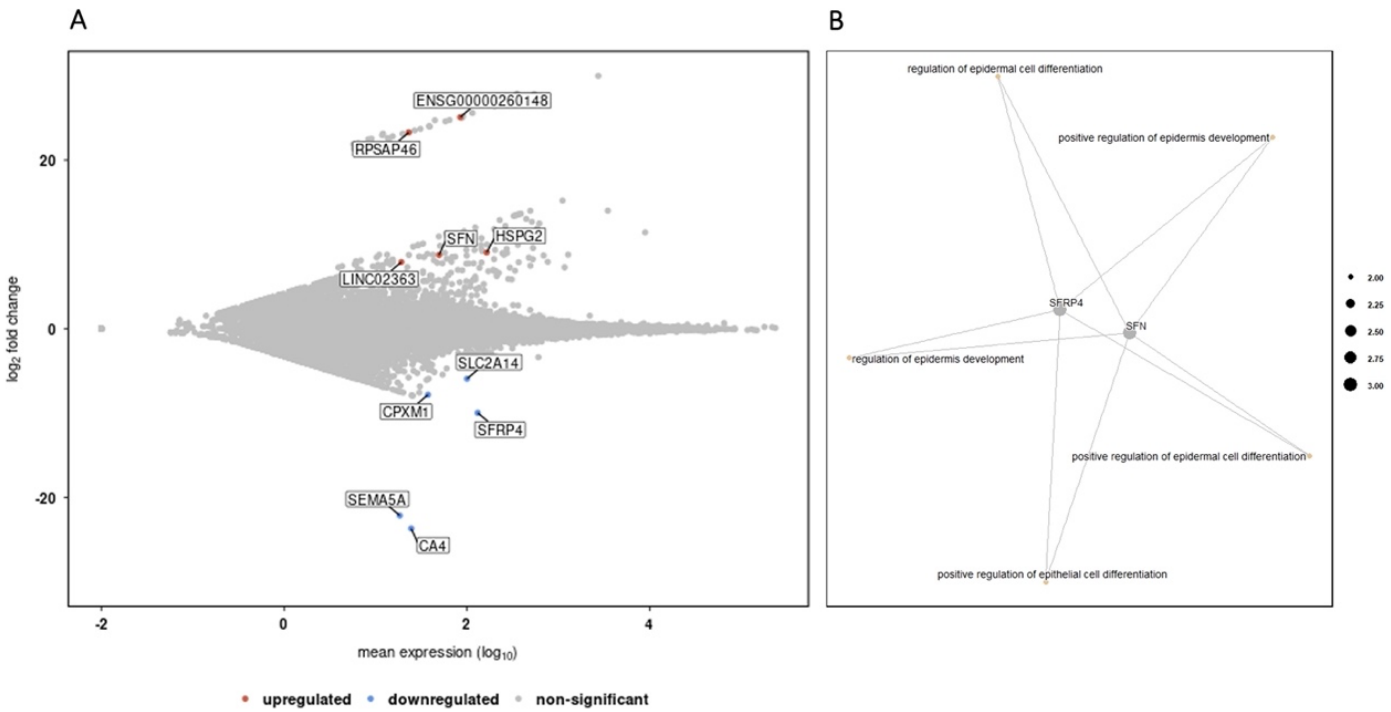


Figure 15: Altered endometrial CD8+ T transcriptome in patients with recurrent pregnancy loss and live birth in the next pregnancy versus fertile controls.

(**A**) MA plot of differentially expressed genes between patients with recurrent pregnancy loss (RPL) and live birth in the next pregnancy versus fertile controls. Each point represents one gene. Genes were coloured according to figure legend. Top 10 differentially expressed genes (ranked by p.adj and $|\log_2 FC|$) in both groups were labeled. (**B**) Gene-concept network illustrates enriched gene sets ($p < 0.05$, $q < 0.05$) with significantly upregulated genes ($p.adj < 0.05$, $|\log_2 FC| > 1$) in CD8+ T cells of patients with recurrent pregnancy loss and subsequent live birth compared to fertile controls. Nodes denote enriched gene sets and significant genes. Edges connect gene sets and their constituent genes. Node size shown on the right represents the number of significant genes in the gene-set (1.0-3.0). Data was generated with DESeq2 [185] and Searchlight 2 [199].

GSEA was performed to elucidate potential changes in gene sets between RPL patients with live birth and fertile controls. In the hallmark gene set database, 21 gene sets were enriched in the RPL miscarriage group and 29 in the fertile control group with *oxidative phosphorylation* gene cluster being significant (FWER $p < 0.05$, FDR $q < 0.05$) in the first group and *myogenesis* and *epithelial mesenchymal transition* gene clusters in the latter, which corresponds to the findings between the RPL miscarriage group and fertile controls. None of the GO gene sets passed significance threshold. Among the immune gene set clusters, 16 were significantly enriched in the RPL live birth group, including:

- GSE3720_UNSTIM_VS_LPS_STIM_VD2_GAMMADELTA_TCELL_UP: genes upregulated in Vd2 gamma delta T cells: untreated versus LPS,
- GSE41867_DAY8_EFFECTOR_VS_DAY30_MEMORY_CD8_TCELL_LCMV_ARMSTRONG_UP: genes upregulated in CD8 T cells, acute infection with LCMV-Armstrong: effectors at day 8 versus memory at day 30.

In the fertile control group, three gene clusters were increased, however, none of them was specifically related to CD8 T cells.

3.4.5.3 Overlapping genes

Table 9: Nine significantly changed genes that overlap in different group comparisons.

Gene symbol	Fertile control	RPL live birth	RPL miscarriage
TRIM2	16.12 ± 22.56	41.42 ± 82.19	0
MTCO1P12	28.21 ± 21.44	16.85 ± 5.77	392.32 ± 586.08
CA4	35.77 ± 86.71	0	0
CPNE2-DT	0	270.69 ± 458.38	3.60 ± 7.50
RPSAP46	0	74.02 ± 139.90	2.03 ± 6.10
HSPG2	1.02 ± 2.51	525.59 ± 971.10	1.28 ± 2.38
SFN	0.35 ± 0.66	158.11 ± 289.68	0.50 ± 1.04
LINC02363	0.18 ± 0.38	60.93 ± 82.20	0.31 ± 0.69
SEMA5A	26.81 ± 61.32	0	11.33 ± 17.63

The data is presented as the mean gene count ± SD of normalised counts generated with DESeq2 [185].

Among all significantly differentially expressed genes, there were 9 overlapping genes, which were significant in at least two comparisons. *TRIM2* and *MTCO1P12* were considered as markers of adverse reproductive outcomes, with decreased expression of *TRIM2* in RPL miscarriage versus RPL live birth (p.adj = 2.83E-31, log_2FC = 23.51) and versus fertile control group (p.adj = 1.35E-41, log_2FC = -22.15) and increased expression of *MTCO1P12* in RPL miscarriage group when compared to RPL live birth (p.adj = 0.009, log_2FC = -4.54) and fertile control group (p.adj = 0.001, log_2FC = 3.79). On the other hand, *CA4, CPNE2-DT* and *RPSAP48* were identified as RPL genes. Expression of *CA4* was decreased in the RPL groups when compared to fertile controls (p.adj = 5.04E-12 and p.adj = 3.68E-07) while *CPNE2-DT* and *RPSAP48* were increased in the RPL groups (p.adj = 6.77E-05, p.adj = 3.02E-06 and p.adj < 0.05, p.adj = 2.53E-05, respectively). Interestingly, *HSPG2*, SFN and *LINCC02362* gene expression was increased in RPL live birth versus miscarriage andt increased in RPL live birth versus fertile controls, indicating potential gene involvement in the pathophysiology of the RPL as well as their role in the subsequent pregnancy outcomes. Similar is true for *SEMA5A*, however its role seems to be more complex as it was

significantly increased in RPL miscarriage group when compared to RPL live birth (p.adj = 7.45E-11, log_2FC = 20.9), but decreased in RPL live birth group versus fertile controls (p.adj = 1.24E-12, log_2FC = -22.14).

3.5 DISCUSSION

3.5.1 Summary

In summary, in this chapter I aimed to develop a comprehensive pipeline for the bioinformatic analysis of endometrial CD8 T RNAseq data, which was first used to explore the transcriptome of human endometrial CD8 T cells in fertile control women. Next, I investigated endometrial immune modulators to inform the study design for determining differentially expressed genes and enriched pathways between RPL patients with miscarriage and live birth as well as between RPL patients and fertile controls. To the best of my knowledge, this is the largest transcriptome analysis of endometrial CD8 T cells in terms of patient and cell numbers and the first transcriptome study of CD8 T cells in RPL. In total, 784,580 CD8 T cells were investigated from 26 patients, including RPL patients with miscarriage in the next pregnancy (n = 10), live birth in the next pregnancy (n = 5) and fertile controls (n = 11). Comparative analysis revealed alterations in CD8 T cell transcriptome of RPL patients with a subsequent miscarriage when compared to those with live birth as well as changes between both RPL groups and fertile controls. As expected, differences were most pronounced between RPL patients with miscarriage versus fertile controls.

3.5.2 Strengths and limitations

Previous reports have suggested alterations in CD8 T cells in RPL, but no transcriptome analysis has been performed so far on samples strictly timed to WOI, which is transcriptionally significantly different from other cycle phases [52, 53, 189]. Moreover, there has been no analysis to date comparing RPL patients with different pregnancy outcomes, which could guide the design of clinically relevant studies to optimise patient management and identify individuals

at risk for another miscarriage. However, there are some limitations to our study. Bulk RNA sequencing of sorted immune cells is a valuable method for determining the overall gene expression patterns of cell groups of interest at a good cost-benefit ratio. It is not, however, appropriate for examining cellular heterogeneity, uncommon cell types, or intercellular and spatial information, and it lacks the granularity afforded by the scRNAseq [191]. Additionally, mRNA expression is a poor predictor of total protein production, regardless of cell differentiation or activation state [201]. I plan to address this limitation by employing full spectrum flow cytometry discussed in the next chapter.

3.5.3 Transcriptome analysis of endometrial CD8+ T cells

3.5.3.1 Healthy endometrium during the window of implantation

This is the biggest transcriptomic study of CD8 T cells in human endometrium and the first one to investigate CD8 T cell gene expression during the WOI. The most detailed study by Chen et al. has focused on genes that differentiate CD8 T cells from CD4 T cells and Tregs in the first trimester decidua, therefore, it is impossible to compare our top expressed genes to their data set [53]. In the meta-analysis of 164 endometrial samples, *CLDN4,* epithelial cell tight junction protein, was identified as one of the markers for endometrial receptivity and the only gene among our top 10 expressed ones described in their study [192]. While gut resident CD8 T subtypes were shown to express various cytotoxic profiles, given the current literature on decreased cell cytotoxicity and activation during the secretory phase, I was not surprised to find that genes related to cytotoxicity such *as GZMK, GZMA, GZMM, XCL1, XCL2*, and *CCL4* were not among the top 10 [6, 53, 63]. Furthermore, one of the top expressed genes was *TNFAIP3*, which is a key suppressor of CD8 T cell activity, impacting their activation, differentiation, and immune

response. Indirect inhibition of its expression in mice led to increased sensitivity to antigen stimulation with the production of large amounts of IL-2 and IFN-γ by CD8 T cells [202].

3.5.3.2 Modulating factors

Next, I assessed the influence of age and previous life birth on endometrial CD8 T cell transcriptome and found changes in the parous women only indirectly linked to CD8 T cell function. For example, top three significantly upregulated genes in nulliparous women were found to be important in cell migration and invasion, negatively related to infiltrating levels of CD8 T cells in ovarian cancer and enriched among memory CD4 T population when compared to naïve subtypes [203-205]. Although I could not draw any significant conclusions on CD8 T cell phenotypes in parous versus nulliparous women, these findings helped me design the subsequent analysis in which I regarded parity as a potential confounder and have controlled for this accordingly. Although some authors did report significant changes in the transcriptome of women of different age, my findings did not confirm that. However, it is important to highlight that in the reported study the age span of the participants was 25–45, while in my limited-number cohort it was 28–41 years of age [189].

3.5.3.3 Recurrent pregnancy loss and next pregnancy outcome

Findings suggest a greater proinflammatory and cytotoxic environment in RPL patients with a subsequent miscarriage when compared to the live birth group and fertile control group including not only transcriptomic changes involved in angiogenesis and chemotaxis but also more specific changes in the regulation of T cell mediated immunity. The gene *MTCO1P12* was the most expressed and significantly increased in the RPL miscarriage group when compared to the live birth group as well as fertile control group. *MTCO1P12* is a pseudogene closely related to the mitochondrial energy function [206]. It exhibits altered methylation patterns in cytotoxic

T cells when exposed to *Staphylococcus aureus* infection leading to distinct accessibility patterns in different regions of the gene, potentially impacting CD8 T cell function [207]. Additionally, *MTCO1P12* was found to be significantly upregulated in the blood of patients with severe COVID-19 compared to those with mild disease, again indicating its involvement in immune responses [208]. On the other hand, *TRIM2* the top significantly upregulated gene in the RPL live birth and fertile control group when compared to the RPL miscarriage group, binds to phosphorylated SIRPA, blocking virus entry and regulates phagocytosis. By this mechanism it negatively impacts the engulfment of apoptotic cells by macrophages, which suggests that TRIM2 protein may have overlapping roles in virus entry and macrophage phagocytosis [209]. Looking at differentially expressed genes between the two RPL groups with a more direct impact on CD8 T cell function that could be detrimental to a successful pregnancy, complement protein C5 promotes CD8 T cell activation and function by influencing their cytokine, granzyme, and perforin production [210, 211]. While *LLGL-1* does not directly impede CD8 T cell activation, it can alter CD8 T cell responses by affecting effector and memory type differentiation, leading to more powerful antitumor and antiviral CD8 T cell responses [212, 213]. In the latter, chemokines, including CCL20, are crucial for T cell recruitment [214]. However, CCL20 also plays a role in CD8 T cell responses by activating the chemokine receptor CCR6 often expressed by tissue resident CD8 T cells [215, 216]. CCR6+ CD8 T cells exhibit characteristics of early effector memory cells, which express granzyme A with low perforin but not granzyme B and produce IFN-γ and TNF-α but not IL-2 [216]. Importantly, decidual T cells exhibit a distinctive gene profile with heightened expression of proteins linked to the response to IFN signalling in normal pregnancy, holding significance for understanding normal placentation and exploring the potential relevance of foetal-specific alloreactive immune responses in pregnancy disorders [217]. On the other hand, the *HMOX1* gene upregulated in the RPL live birth group is expressed by the trophoblast cells, placenta-infiltrating leukocytes, and decidua and myometrium. Interestingly, female mice

exposed to stress had substantially reduced placental *HMOX1* mRNA expression, associated with a reduction of tolerogenic CD122+ CD8 T cells and an increase of cytotoxic CD8 T cells, leading to foetal growth restriction. According to the aforementioned findings in mice, *HMOX1* expression regulated by progesterone enhances CD8 T cell immunological tolerance [218]. PLLP, a SNARE-associated proteolipid, plays a crucial role in the intracellular machinery involved in ICAM-1-mediated adhesion of leukocytes to epithelial cells. PLLP depletion increased T cell adherence to hepatic epithelial cells, highlighting the PLLP as an important regulator of the epithelial immune and inflammatory response [219]. This has been confirmed in studies on mice, in which the authors demonstrated intensive communication between endometrial stromal cells and CD8 T cells during the initial stages of embryo implantation, primarily facilitated via ICAM-1 [220]. In breast cancer, *ATP2C2* expression was found to be increased in the tumour tissue when compared to normal breast tissues or paired breast non-tumour tissue. Its expression was negatively associated with the level of cytotoxic T lymphocytes and γδ T cells, which correlates to unfavourable prognosis in various cancer types and underlines the importance of ATP2C2 in immune response/tolerance regulation [221]. Further research into the precise genes and pathways involved is needed to deliver important insights into the underlying processes of miscarriage in RPL patients to inform possible treatment targets that would potentially improve immunotolerance.

3.5.3.4 Recurrent pregnancy loss compared to fertile controls

Studying the overlapping genes and genes involved in the enriched pathways, I confirmed literature findings on altered CD8 T cell populations in RPL when compared to fertile controls [52, 53]. Similar to changes between RPL patients with different reproductive outcomes, differentially expressed genes between RPL groups and fertile controls implied functional changes in CD8 T cell activation and cytotoxicity, but also more fundamental changes impacting

cell metabolism, which was supported by pathway enrichment analysis showing changes in gene sets associated with *epithelial mesenchymal transition* and *epithelial differentiation* as well as *intracellular transport, mineralocorticoid processes, oxidative phosphorylation* and *ATP synthesis coupled electron transport.* The literature on the genes identified as RPL markers (*CA4, CPNE2-DT* and *RPSAP48*) and especially their role in endometrial immunity is scarce, but they have been experimentally detected in human extracellular vesicles [222]. Extracellular vesicles are membrane-bound structures released by cells, serving as mediators for intercellular communication and signalling. It has been suggested that extracellular vesicles, containing distinct RNAs such as microRNAs and proteins, are discharged into the uterine cavity. These vesicles may be transferred to trophoblast cells or endometrial epithelial cells, playing a role in promoting implantation [223]. I will briefly discuss relevant differentially expressed genes shown to be most involved in CD8 T cell function. HSPGs, including HSPG2 increased in the RPL groups, are expressed by activated T cells, and are critical for the attachment and entry of certain viruses, including herpesvirus and papillomavirus, into host cells [224]. It has been observed that an elevated infiltration of immune response cells (e.g., CD8 T cells and M1 macrophages) and a decreased infiltration of immune suppressive cells (e.g., Tregs and mast cells) were enriched in *HSPG2*-mutated patients with melanoma and non-small cell lung cancer, which suggests that *HSPG2* mutations mediate a favourable immune infiltration and tumour microenvironment [225]. EHD2, increased among fertile controls, is a protein involved in the internalisation and recycling of T cell surface receptors and is constitutively expressed by naïve and resting T cells. It regulates the recycling of TCR-CD3 complexes, which helps maintain T cell activation levels. Its shortage can result in embryonic mortality, male infertility, and abnormalities in ocular and brain development. EHD2 influences antigen-driven cell proliferation and cytokine production in T cells, contributing to the immunological response [226]. Another gene overexpressed in the fertile control group, DAB2 is emerging as an immunoregulatory molecule in T lymphocytes,

where it plays roles in immunosuppression and the regulation of Toll-like receptor signalling in APC [227]. The most statistically significant but contradictory at the same time is *SEMA5A* gene expression, which was increased in the RPL miscarriage group when compared to controls but also in the control group when compared to the RPL live birth group. Identified as an immunological semaphorin, SEMA5A demonstrates possible significance in influencing CD8 T cell responses and cytokine production, by inducing the expression of *TNF-α* and *IL-8* genes both of which are important components of CD8 T cell activity. Moreover, recombinant SEMA5A stimulated *in vitro* proliferation of T cells and increased expression of serum SEMA5A has been found in patients with rheumatoid arthritis [228]. Overall, the developing pregnancy paradigm is that embryo implantation does not occur in an immunologically suppressed environment. Rather, during the WOI and throughout pregnancy the endometrium and decidua experience a regulated inflammatory response emphasising the importance of immune tolerance, which was shown to be disrupted in the T cell compartment of RPL patients by other authors and indicated in my findings [49, 52, 53, 229, 230].

3.5.4 Future research

As a pilot study, this chapter demonstrates the viability of transcriptome analysis on endometrial CD8 T cells in a bigger cohort. Ideally, the next stage would entail scRNAseq to unravel cellular heterogeneity and identify distinct immune cell subsets necessary for endometrial immune homeostasis along with proteomics validation to ensure functional relevance of the findings. Only a comprehensive approach considering relevant clinical aspects such as parity and immunological comorbidities will provide a full perspective of immune events in the endometrium during WOI and potentially justify the benefit of immunomodulatory therapies for RPL.

3.6 CONCLUSIONS

My preliminary findings on endometrial CD8 T cell transcriptome provide valuable insights into the complex interplay of genes and their potential roles in immune responses, immunotolerance and immune cell function in RPL during the WOI but do not offer sufficient conclusions to infer potential roles in diagnosis or therapy in RPL. The molecular mechanisms of the identified genes require further investigation into the endometrial immunity and pathophysiology. However, I have shown that history of live birth, commonly overlooked, is an important factor to consider when designing clinical studies in reproductive immunology to deliver reliable results and thereby improving the overall quality of clinical research in the field.

4 Deep immune phenotyping of endometrial and circulating CD8+ T cells

4.1 INTRODUCTION

4.1.1 Full spectrum flow cytometry

Technological advancements in fluorescence flow cytometry, combined with a growing comprehension of the intricate immune system, have paved the way for extensive panels, some incorporating up to 40 fluorophores [70]. By utilising instruments designed to efficiently detect emitted light, highly effective avalanche photodiodes and mathematical algorithms for spectral unmixing, it becomes possible to differentiate fluorophores with very similar emission maxima but unique overall spectral fingerprints [231]. Unlike conventional flow cytometry, which mainly measures the peak emission of each fluorochrome, full spectrum flow cytometry captures the entire spectral signature for each fluorochrome, spanning all laser lines. Ultimately, these sophisticated panels allow researchers to simultaneously analyse numerous immune cell markers and functions, offering a deeper insight into the complexity and diversity of cellular immunity while necessitating careful sample management and limiting the risk of bias [232].

4.1.2 The CD8+ T cell immune network

As in other complex systems, no immune cell population, either systemically or locally, exists and operates alone Figure 16.

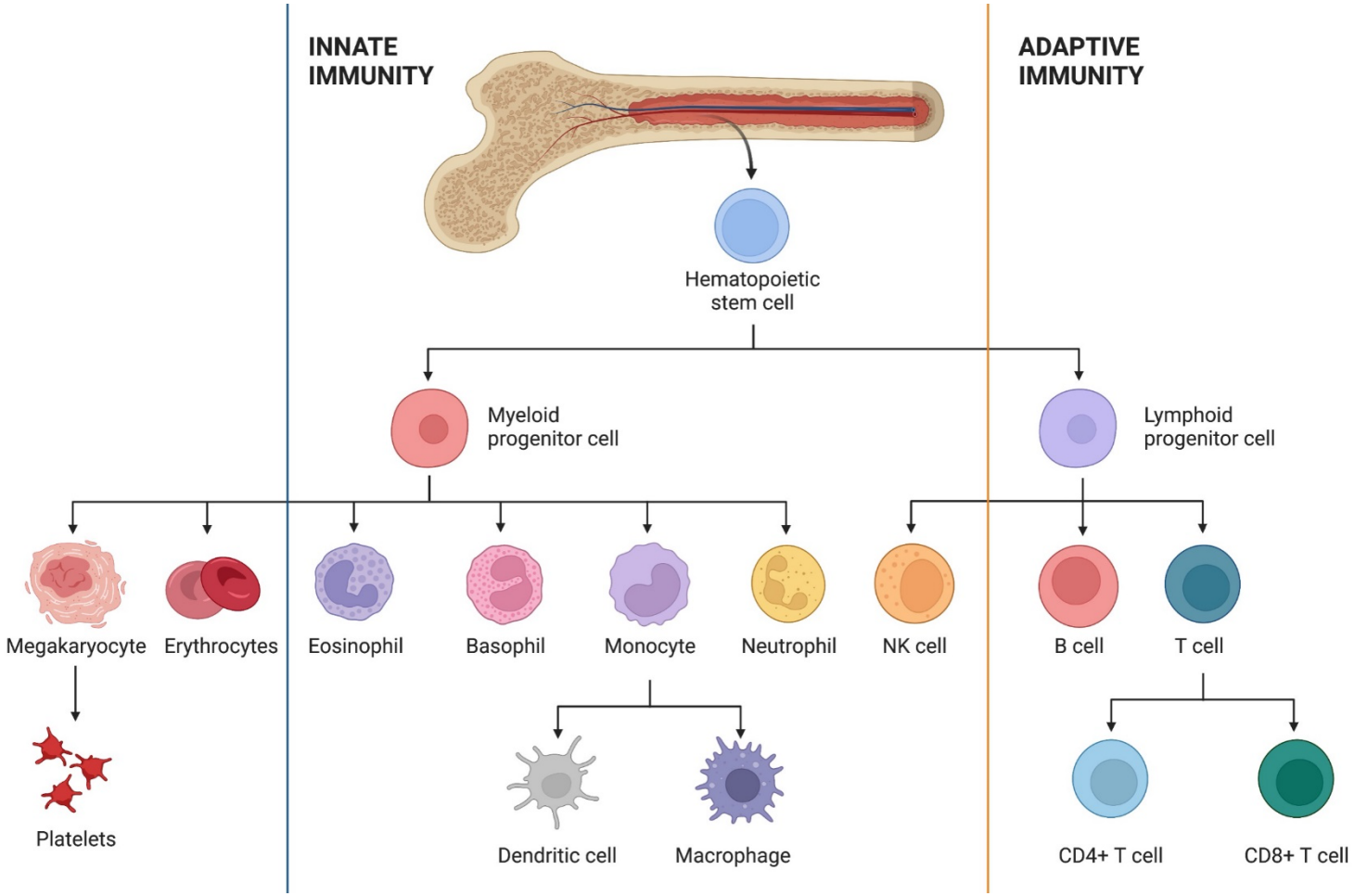


Figure 16: A schematic representation of the human immune cell network based on developmental pathways.

Cellular networks cooperate for functions. CD8 T cells, for example, depend on APCs for activation, differentiation, and effector functions, while their activities are also regulated by NK and CD4 T cells [57]. APCs, such as dendritic cells (DCs), macrophages, and B cells, capture and process pathogenic and tumorigenic antigens to prime CD8 T cells and elicit cytotoxic immune responses. This happens through the engagement of TCRs on CD8 T cells with MHC class I, including HLA-A, -B and -C, on APCs [233]. The dynamics of the DC-CD8 T circuitry can be altered by NK and CD4 T cells through promoting DC maturation and increasing antigen content thereby enhancing CD8 T cell activation [234, 235]. On the other hand, both cell types can also limit CD8 T immune response: NK cells can act either by direct lysis of CD8 T cells or through cytokine production, such as IL-10, while CD4 T cells, specifically Tregs, secrete inhibitory IL-10, tumour growth factor β (TGF-β) and IL-35 but can also inhibit CD8 T cells through the CTLA-4 pathway [234, 236].

Based on their phenotypic and functional properties, DCs are classified into conventional and plasmacytoid DCs [237]. Common markers to identify main endometrial subsets include HLA-DR, CD11c, CD1c and CD123 in the absence of other immune lineage-specific markers such as CD3, CD14, CD19, CD20 and CD56 [70]. In the endometrium, CD11c+ conventional DCs were found in or near the epithelium, while CD123+ plasmacytoid DCs were also found deep in the endometrial stroma [238]. In the decidua, higher proportions of conventional DCs type I (CD1c-) were found compared to type II (CD1c+), potentially leading to the enrichment of decidual CD8 T cells [15].

Blood monocytes differentiate into tissue resident macrophages and are distinguished into classical, non-classical and intermediate monocytes based on the expression of surface markers CD14 and CD16 [239]. Human macrophages are identified by the expression of CD11b and HLA-

DR, the differential expression of the Fc receptors CD64 and CD16, and the downregulation of CD14. However, marker expression can vary significantly in different tissues and is affected by inflammation, which makes macrophages a highly heterogeneous group not necessarily organised in clearly defined subpopulations [240]. Found throughout the stroma and epithelium and most abundant in the menstrual phase, macrophages represent around 10–20% of endometrial leukocytes and are indicated to play a role in several gynaecological disorders [6, 241].

B lymphocytes have clonally different cell surface immunoglobulin (Ig) receptors that recognise unique antigenic epitopes. Upon antigen encounter, naïve B cells (IgD+CD27-) can differentiate into short-lived plasmablasts (CD20-IgD-CD27+) or migrate to germinal centres, where they acquire a memory B cell phenotype (CD20+IgD−CD27+). Due to class switch recombination, B cells can exhibit several B cell receptor isotypes, including IgG and IgM, which are most frequent in the circulation [70, 242]. Endometrial B cells typically account for 5% of total endometrial CD45+ cells, with high variation in the abundance and phenotypes in the gynaecological disorders [243]. Tissue resident B cells are recognised by expression of CD69 and CXCR3 markers, with CD69 expression being previously confirmed on endometrial subsets by our group [244, 245].

NK cells are the most common kind of innate lymphocytes that lack antigen receptors and are divided into two subgroups based on the expression of CD56 and CD16. Circulating NK cells are predominantly $CD56^{dim}CD16+$ cells, whereas barrier tissue residents, including endometrial subsets, are $CD56^{bright}CD16+$ [240]. Tissue resident NK cells have a less cytotoxic profile and commonly express CD69, CD103, and CXCR6, with CD69 expression previously confirmed in endometrial populations. Endometrial NK cell levels increase after ovulation, representing

around 30% of all CD45+ cells during WOI and peak in frequency in the late secretory phase and early pregnancy, in which they account for over 70% of decidual leukocytes [246].

Similar to CD8 T cells, CD4 T cells are characterised based on memory and differentiation markers as naïve (CD45RA+CCR7+), central memory (CD45RA−CCR7+), effector (CD45RA+CCR7-), effector memory (CD45RA-CCR7-) and tissue resident (CD69+/CD103+) subtypes [247]. Tregs are commonly identified by the combination of α subunits of the IL-2 and IL-7 receptors (CD25 and CD127, respectively), expressed on the cell surface, while another relevant marker, FoxP3, requires intracellular staining [248]. Although CD4 T cells are the predominant T cell subset in the peripheral blood and lower FRT, the CD4/CD8 T cell ratio is inversed in the endometrial mucosa [6]. Like other T cells, Tregs are hormonally dependent and decrease in the peripheral blood and endometrium during the secretory phase. They are critical immune mediators to ensure implantation success and foetal tolerance [10].

4.2 CHAPTER OBJECTIVES

Considering the low risk of bias and small tissue sizes and availability, I employed full spectrum flow cytometry as a comprehensive approach to elucidate the distinct subtypes of CD8 T cells and other relevant immune populations in the endometrium and peripheral blood of patients and control cohorts. A 36-marker panel allowed for a precise and in-depth characterisation of the immune cell populations from 32 participants with endometriosis, RPL or both. The panel and protocol optimisation process is briefly outlined in Appendix 7.1. Sample processing followed a well-established protocol developed by our group, while the staining protocol was provided and adapted from Cytek. The analysis of the endometriosis cohort compared patients with endometriosis and controls, but also subfertile and fertile endometriosis patients, while RPL patients were again grouped based on their next pregnancy outcomes. Firstly, potential confounders were explored similarly to the previous chapter and differences between endometrial and systemic immunity were assessed. The final comparisons revealed several modulated immune populations in study groups, which were discussed from the broader immunological perspective and within the current knowledge of reproductive immunology.

Objectives:

1. To establish a robust protocol for full spectrum flow cytometry of matched immune cells from the endometrium and peripheral blood.
2. To characterise immune cell populations in the endometrium and peripheral blood of the study cohorts.
3. To assess how patients' characteristics and menstrual cycle phase influence endometrial immunity.

4. To determine how peripheral blood reflects endometrial immunity and to evaluate its diagnostic value.
5. To identify modulations in endometrial and systemic immunity in patients with adverse reproductive outcomes.

4.3 METHODS

4.3.1 Study design

Following PICO criteria, females of reproductive age with regular uterine cycles at least three months post miscarriage or hormonal treatment were eligible for this deep immune phenotyping study (Table 10) [107]. Participants were screened for eligibility before undergoing surgical intervention for endometriosis diagnosis and/or treatment at the Endometriosis CaRe Centre or during clinical evaluation at the Recurrent Miscarriage Clinic, both of which are situated at the Women's Centre in John Radcliffe Hospital, Oxford. They were recruited into two prospective cross-sectional studies: FENOX (REC Ref: 17/SC/0664) and PIP study (REC ref: 18/SC/0216). After inclusion, we divided them into two main analysis groups based on their underlying pathologies: Endometriosis and RPL, with some patients fulfilling inclusion criteria for both groups (Figure 17).

Table 10: PICO framework to select eligible samples for deep immune phenotyping study.

<table>
<tr><td rowspan="4">Population</td><td colspan="2">INCLUDED</td></tr>
<tr><td>Endometriosis group</td><td>RPL group</td></tr>
<tr><td>• 18–39 years old female
• All stages of surgically confirmed endometriosis
• Any phase of a regular uterine cycle</td><td>• 18–39 years old female
• ≥ 2 consecutive miscarriages
• Samples collected between 7–11 days post LH pre-ovulatory surge
• Subsequent pregnancy resulted in another miscarriage</td></tr>
<tr><td colspan="2">EXCLUDED</td></tr>
<tr><td colspan="2">Participants experiencing a miscarriage or undergoing hormonal treatment within the last three months. Those having polycystic ovarian syndrome (PCOS), hydrosalpinx, pelvic inflammatory disease or cancer.</td></tr>
<tr><td>Intervention</td><td colspan="2">Full spectrum flow cytometry analysis.</td></tr>
<tr><td rowspan="4">Control</td><td colspan="2">INCLUDED</td></tr>
<tr><td>Endometriosis group</td><td>RPL group</td></tr>
<tr><td>• 18–39 years old female
• Endometriosis surgically/or sonographically ruled out with no other gynaecological pathologies
• Medical history < 3 miscarriages
• Any phase of a regular uterine cycle</td><td>• 18–39 years old female
• ≥ 2 consecutive miscarriages
• Samples collected between 7–11 days post LH pre-ovulatory surge
• Subsequent pregnancy resulted in live birth</td></tr>
<tr><td colspan="2">EXCLUDED</td></tr>
<tr><td colspan="2">Participants experiencing a miscarriage or undergoing hormonal treatment within the last three months. Those having polycystic ovarian syndrome (PCOS), hydrosalpinx, pelvic inflammatory disease or cancer.</td></tr>
<tr><td>Outcome</td><td colspan="2">Deep immune phenotyping of eutopic endometrium and matched peripheral blood to identify altered immune populations in study groups.</td></tr>
</table>

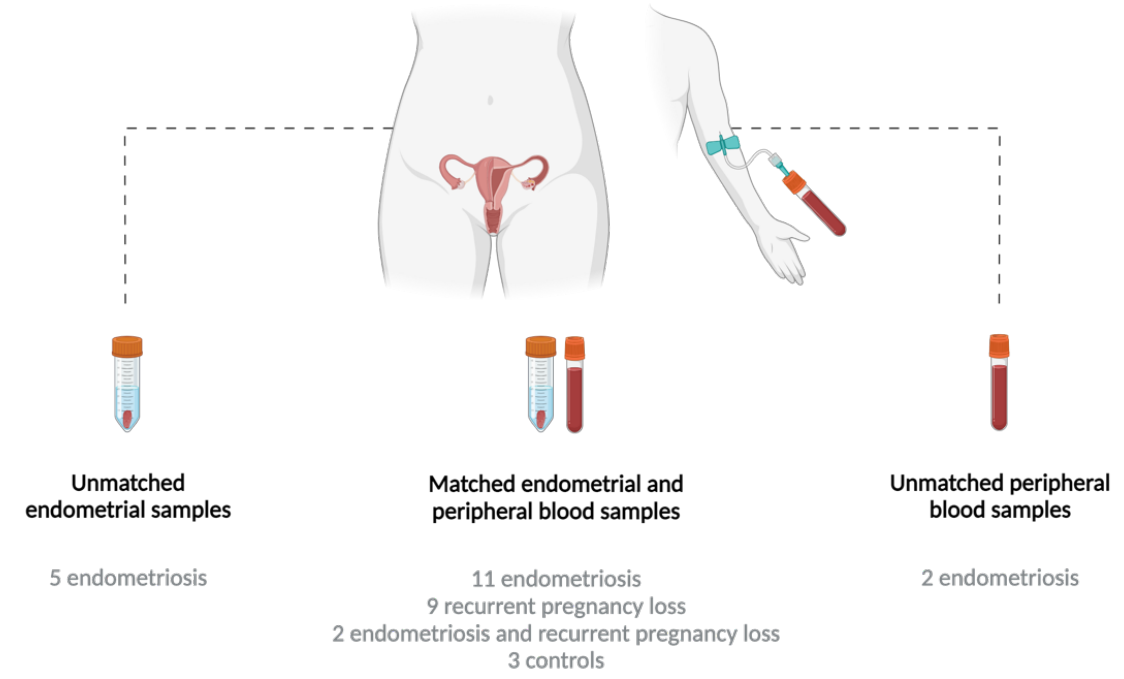


Figure 17: Participants recruited for the deep immune phenotyping study.

Matched pipelle biopsy and peripheral blood samples were taken from 25 participants, who were grouped based on the underlying pathology either as endometriosis (11), recurrent pregnancy loss (9), endometriosis and recurrent pregnancy loss (2), and controls (3). 5 endometrial biopsies from endometriosis patients were taken without matched peripheral blood samples and 2 peripheral blood samples from endometriosis patients without matched endometrial biopsy.

4.3.2 Sample preparation

4.3.2.1 Tissue collection and processing

All steps in sample collection, tissue processing, cell suspension storage and staining followed established protocols and are graphically shown below (Figure 17). Peripheral blood samples were acquired before surgery for endometriosis or during clinical examination for RPL into 4.5 mL/9 mL Lithium/Sodium Heparin vacutainers. Endometrial biopsies were collected using an Endocell® disposable cell sampler (Cooper Surgical, USA) during surgery for endometriosis or at the recruitment visit for the PIP study.

All samples were obtained according to the manufacturer's instructions at the Women's Centre, JR Hospital and processed within 6 hours from collection as described in Appendix 7.2.1. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation using Lymphoprep™ (Gen Group, USA). Endometrial tissue was mechanically digested in digest media (Iscove's Modified Dulbecco's Medium supplemented with 10% FCS, 1% HS and 1% p/s/g) and passed through sequential 70 μm and 40 μm cell strainers to obtain a human endometrial single cell (HEC) suspension. All single-cell suspensions were stored in freezing media (10% DMSO/FCS) in liquid nitrogen containers until cell staining

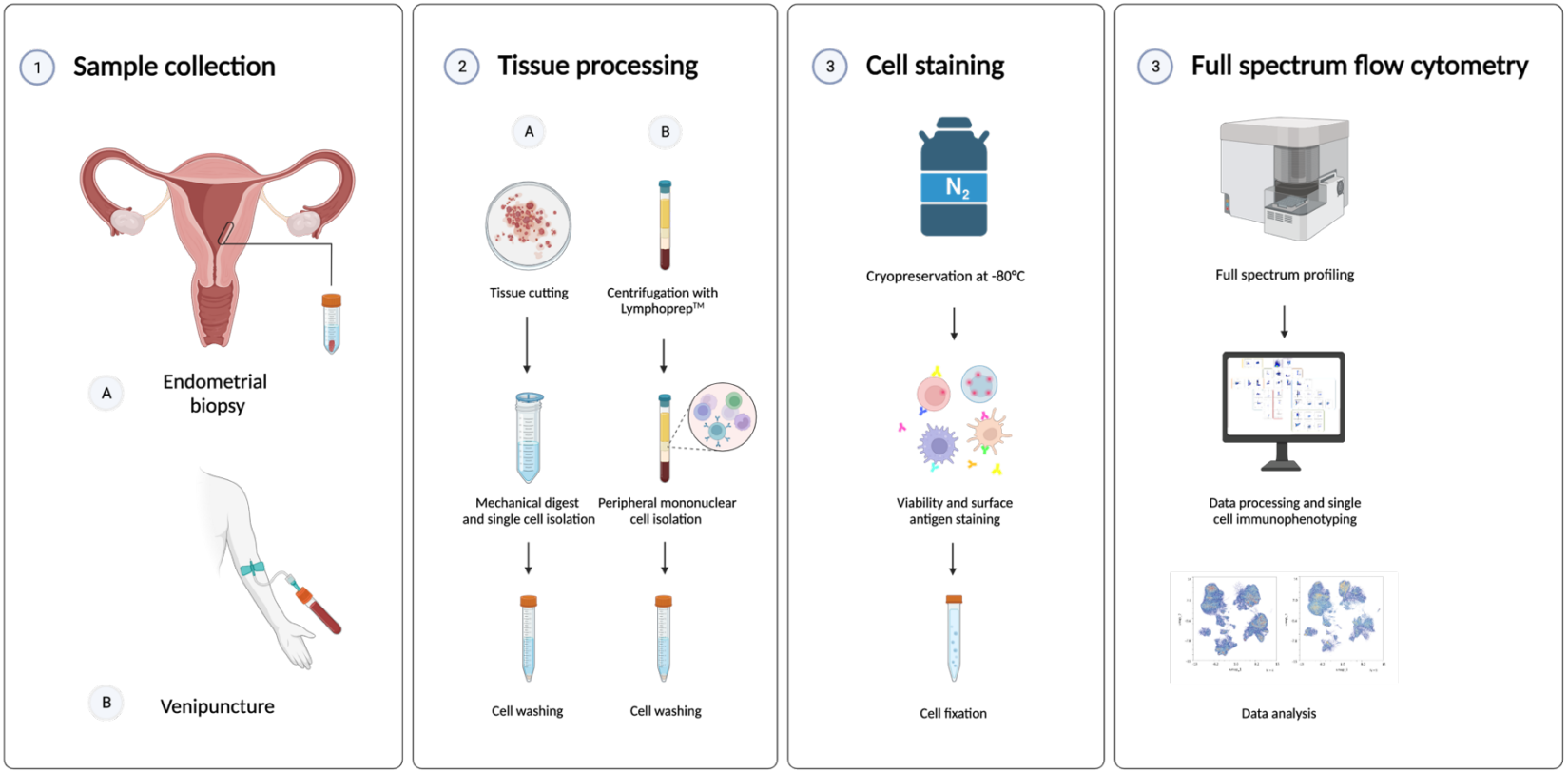


Figure 18: Sample workup for full spectrum flow cytometry analysis.

Endometrial and peripheral blood samples were processed following standard protocols at the University of Oxford's Nuffield Department of Women's and Reproductive Health, within 6 hours of collection. Endometrial tissue was mechanically digested into single-cell suspensions and blood mononuclear cells were isolated via Lymphoprep™ density gradient centrifugation. Samples were then stored in liquid nitrogen until cell staining and data acquisition.

4.3.2.2 Cell staining

After thawing in complete RPMI media (RPMI 1640 supplemented with 10% FCS, 2mM glutamine, 100 IU/mL penicillin and 100 g/mL streptomycin), cells were washed by centrifugation (300 x g) and then incubated with viability dye Zombie NIR (Biolegend) (1:1000 dilution in phosphate-buffered saline (PBS)) for 20 minutes at room temperature in the dark and then washed with PBS. Following protocol and antibody titration optimisation (Appendix 7.2.2), all antibodies were diluted at validated concentrations (Table 11) in the Cell Staining Buffer (Biolegend, USA). Some antibodies were stained before applying the multi-antibody mix to minimise steric hindrance. Each group of selected antibodies was stained for 10 minutes at room temperature before final staining with the remaining antibodies for 30 minutes on ice. Finally, all samples were fixed with FluoroFix™ Buffer (Biolegend, USA) before data acquisition.

4.3.3 Full spectrum flow cytometry

4.3.3.1 Data acquisition

Data was acquired with Cytek® Aurora cytometer (5-laser 16UV-16V-14B-10YG-8R) and using SpectroFlo® software (Cytek Biosciences, USA). To minimise batch effects, all data was collected within one week. Before data acquisition, instrument performance was ensured with quality control beads. We maintained consistency by employing the same event rate and threshold settings throughout the data acquisition process. Additionally, identical single stain controls were used for live unmixing of all the samples.

Table 11: Reagents used for deep immune phenotyping with full spectrum flow cytometry.

Marker	Target cell types	Clone	Per test (µL)	Lot Nr	Fluorochrome
Viability	Viability				Zombie NIR
CD45	Leukocytes	HI130	2	B331250	PerCP
CD3	T cells, NKT-Like cells	SK7	5	B330335, B370758	BV510
CD4	CD4 T and NKT-Like cells	SK3	2	F-050721-01	cFluor® B532
CD8	CD8 T, NK, and NKT-Like cells	SK1	5		cFluor® V610
CD25	Regulatory T cells	BC96	2	B354215	PE-Cy5
TCRγδ	γδ T cells	B1.1	1.2	2311939, 2527383	PerCP-eFluor 710
CD14	Monocyte differentiation	63D3	2.5	B313132, B329322	Spark Blue 550
CD16	Monocyte, NK cell, and dendritic cell differentiation	3G8	0.6	1099493	BUV496
CD11c	Dendritic Cell differentiation	B-ly6	2.5	1148605, 2111332	BUV661
CD11b	Monocyte/macrophages, granulocytes, and NK cells	ICRF44	5	B319299	PerCP-Cy5.5
CD19	B cells	HIB19	1.2	B324542	Spark NIR 685
CD20	B cells	HI47	5	2375623, 2532779	Pacific Orange
CD24	B cell differentiation	SN3	5	2363760, 2412844	PE-eFluor 610
IgD	B cell differentiation	IA6-2	0.6	1195093	BV480
IgG	B cell differentiation	G18.145	5	300956, 1292726, 2052950	BV605
IgM	B cell differentiation	MHM-88	2.5	B316253, B334624	BV570
CD1c	Dendritic cells, NKT-Like cells	L161	5	B293062	Alexa Fluor 647
CD123 (IL3R)	Plasmacytoid dendritic cells	6H6	2.5	2305276	Super Bright 436
CD56	NK cells, γδ T cell activation	NCAM16.2	1.2	1048151	BUV737
CD197 (CCR7)	T cell differentiation	G043H7	5	B332066, B361376	BV421
CD27	T and B cell differentiation	O323	2.5	2288534, 2443439	APC
CD28	T cell and NK cell differentiation	CD28.2	2.5	B324232	BV650
CD45RA	T cell and dendritic cell differentiation	5H9	1.2	1210597	BUV395
CD127	Cytokine receptor; T cell differentiation	HIL-7R-M21	6	1208740, 1341730, 2292425	APC-R700
CD196 (CCR6)	Chemokine receptor; T cell and B cell differentiation	g034e3	1.25	B309645 , B369286	BV711
CD195 (CCR5)	Chemokine receptor; monocyte, dendritic cell, T cell, and B cell differentiation	2D7/CCR5	2.5	1210625, 3051963	BUV563
CD185 (CXCR5)	Chemokine receptor; T cell differentiation	RF8B2	1.2	1210649	BV750
CD183 (CXCR3)	Chemokine receptor; dendritic cell, T cell, and B cell differentiation	G025H7	5	B354664	PE
HLA DR	T cell and monocyte activation, NK cell lineage discrimination, dendritic cell lineage marker	L243	2.5	2406121	APC-eFluor 780
CD38	Monocyte, dendritic cell, T cell, and B cell activation/differentiation	HIT2	3	B325373	APC/Fire 810
CD57	NK and CD8+ T cell immune senescence	NK-1	0.2	1273657	BB515
CD161 (KLRB1)	NK cell cytotoxicity and T cells	HP-3G10	5	2480316	eFluor 450
CD279 (PD1)	T cell inhibitory receptor	EH12.2H7	5	B318729, B373725	BV785
CD69	Tissue residency	FN50	5	B350377	PE-Cy7
CD103	Tissue residency	Ber-ACT8	0.5	2236472	BUV805

4.3.3.2 Data processing

Raw high-dimensional flow cytometry data from all endometrial and peripheral blood samples was processed with the Omiq^{M} (Dotmatics, USA) platform.

Data cleaning

To enhance data quality, we first applied the flowAI tool, which corrects baseline fluorescence shifts, ensures uniform data acquisition by addressing flow rate variations, and minimises fluorescence spillover between channels to enhance data quality [249]. This was followed by excluding debris and non-single cell events by utilising forward scatter (FSC) and side scatter (SSC) parameters and then gating on live (Zombie NIR-) immune cells (CD45+).

Data normalisation

To achieve data comparability across samples, we performed normalisation with fdaNorm for 31 parameters targeted to one of the peripheral blood samples. Then, the normalisation process was reapplied for the three remaining parameters highly expressed in the endometrium (CD69, CD183, CD56) and the baseline set to one of the endometrial samples [250] Unmixing accuracy for CD45+ events was assessed before and after normalisation by examining Nx1 and NxN plots with minimal compensation adjustments before normalisation.

4.3.3.3 Data analysis

Pre-processed multiparameter flow cytometry data was first analysed with Omiq^{M} (Dotmatics, USA) platform and is described in detail in Appendix 7.2.4. Analysis of CD45+ cells was first performed on matched endometrial and peripheral blood samples. Next, we investigated all endometrial and peripheral blood CD45+ cells separately, first assessing immune modulating

factors and then comparing different patient groups. Finally, the same steps were repeated for subsampled endometrial and peripheral blood CD8+ cells.

Dimensionality reduction and clustering

Apart from standard gating strategies (Appendix 7.2.3), a non-linear dimensionality reduction technique - uniform manifold approximation and projection (UMAP) - was applied before clustering to visualise the diverse cell populations within our dataset [251]. We then performed unsupervised clustering with FlowSOM based on lineage markers and visualised meta-clusters using UMAP [252]. After cluster annotation with a conventional clustered heatmap analysis, the data were exported and further analysed in R Studio (R version 4.3.1).

Differential analysis of cell counts and percentages of total

We identified and mitigated confounding variables using the sva package (3.48.0) before the DESeq2 package (1.40.2) was used to calculate differences in meta-cluster cell counts and percentages of the total population between different groups of interest using a model based on the negative binomial distribution [185, 198]. Outcomes with p.adj < 0.05 and $|\log_2 FC| > 1$ confirmed with both approaches using count and percentage of total data were assigned as statistically significant.

Differential analysis of marker expression

One-way ANOVA on ranks test from stats package (4.4.0) was used to assess if patient groups and controls share the same distribution, with 1088 tests conducted for 34 markers and 31 cell clusters. Adjusted p-values were calculated with the Benjamini/Hochberg procedure for all tested comparisons.

4.4 RESULTS

4.4.1 Patient characteristics

Based on their underlying pathology and reproductive outcomes, 32 study participants were included in two analysis groups, as shown in Table 12, alongside their detailed characteristics. Altogether, 883,181 endometrial CD45+ and 1,950,292 circulating CD45+ cells were analysed.

Table 12: Patient characteristics from both analysis groups.

	ENDOMETRIOSIS-ASSOCIATED SUBFERTILITY		RECURRENT PREGNANCY LOSS	
	Endometriosis (n = 20)	**Control (n = 8)**	**Miscarriage (n = 5)**	**Live birth (n = 5)**
Age	33 ± 5	32 ± 6	35 ± 1	34 ± 3
BMI	25.7 ± 5.0	29.3 ± 6	29.5 ± 7.4	26.2 ± 4.4
Parous	8	2	0	2
Cycle phase				
Proliferative	6	2	N/A	N/A
Early secretory	7	0	N/A	N/A
WOI	2	5	5	5
Late secretory	5	1		0
rASRM stage				
I/II	12	N/A	N/A	N/A
III/IV	8	N/A	N/A	N/A
Subfertility	8	0	0	0
Previous miscarriage	2	5	5	5
Endometrial sample	17	8	5	5
Peripheral blood sample	15	8	5	5
Endometrial CD45+ cells	615,475	246,321	44,575	51,096
Circulating CD45+ cells	1,111,110	592,592	370,370	320,664

WOI, Window of implantation; Mean ± SD values for age and BMI of each group are shown.

4.4.2 Local and systemic immunity

4.4.2.1 Endometrial immune populations

Altogether, 883,181 endometrial CD45+ cells from 30 participants were analysed. I identified 31 immune cell clusters, presented in Figure 19, which were annotated based on the existing literature and manual gating strategy, described in detail in Appendix 7.2.3 [70]. Based on different phenotypic traits and established markers of tissue residency, clusters expressing CD69 and CD103 were identified and characterised as “endometrial”. Overall, T cells were the main population (38.18%), followed by NK cells (28.96%) and macrophages (7.87%). Not accounting for unconventional subtypes, the CD4/CD8 ratio was slightly in favour of CD4 T cells, with 18.20% versus 19.97% of all endometrial leukocytes. The majority of CD8+ subtypes were tissue resident clusters including endometrial CXCR5+ and CCR5+ CD8 T cells.

4.4.2.2 Circulating immune populations

As illustrated in **Error! Reference source not found.**, unsupervised clustering delineated 30 immune cell clusters from 1,950,292 cells from 27 participants. I annotated them using a combination of existing literature and a manual gating strategy, elucidated in detail in Appendix 7.2.3 [70]. Unsurprisingly, no tissue resident immune cells were identified as they only represent minor populations. In contrast to the endometrium, where CD8 T cells predominate, CD4 T cells represented the major immune cell population (43.92%) followed by monocytes (17.45%) and CD8 T cells (15.93%).

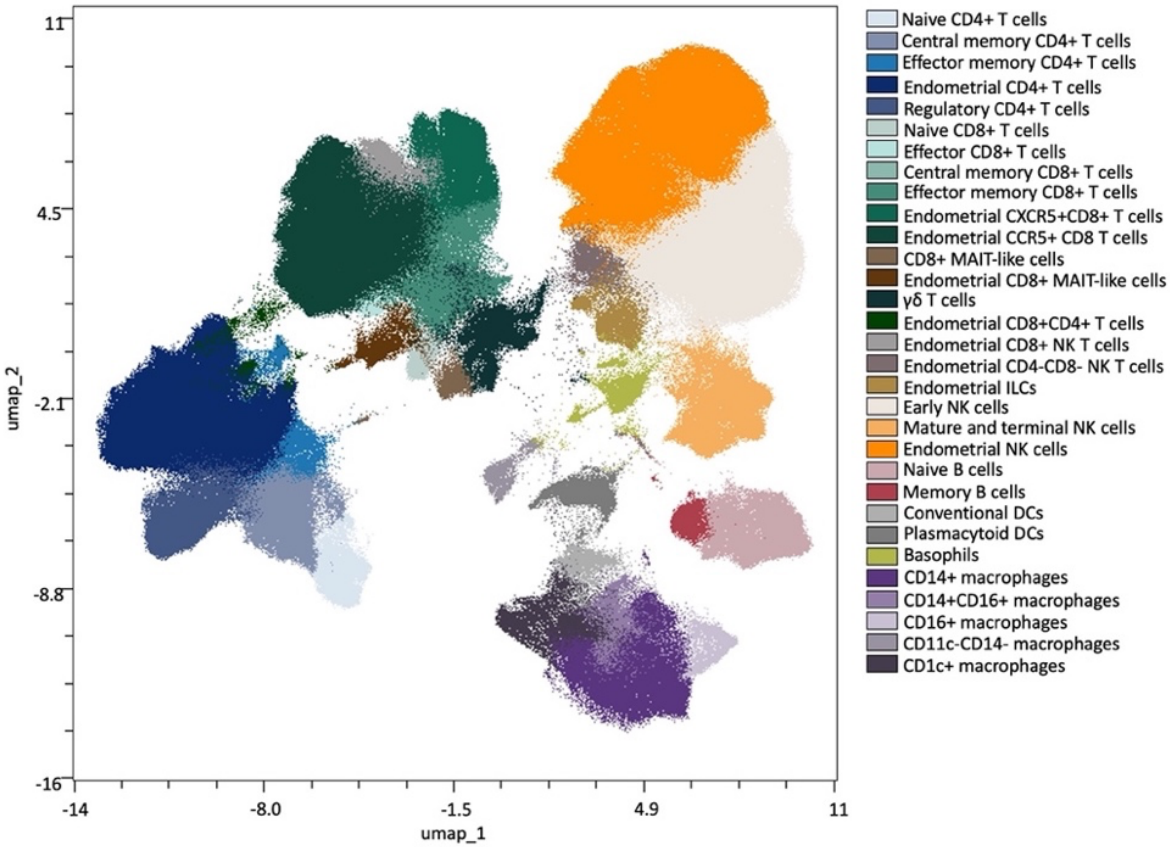


Figure 19: Visualisation of high-dimensional data analysis on endometrial immune cells from all participants displaying 31 distinct immune populations.

FlowSOM clustering was performed on single endometrial CD45+ lymphocytes and resulted in 31 immune cell clusters, which were mapped to the two UMAP dimensions. Altogether, 883,181 cells from 30 participants were analysed. Data was analysed with FlowSOM and visualised with UMAP via Omiq^{M} (Dotmatics, USA).

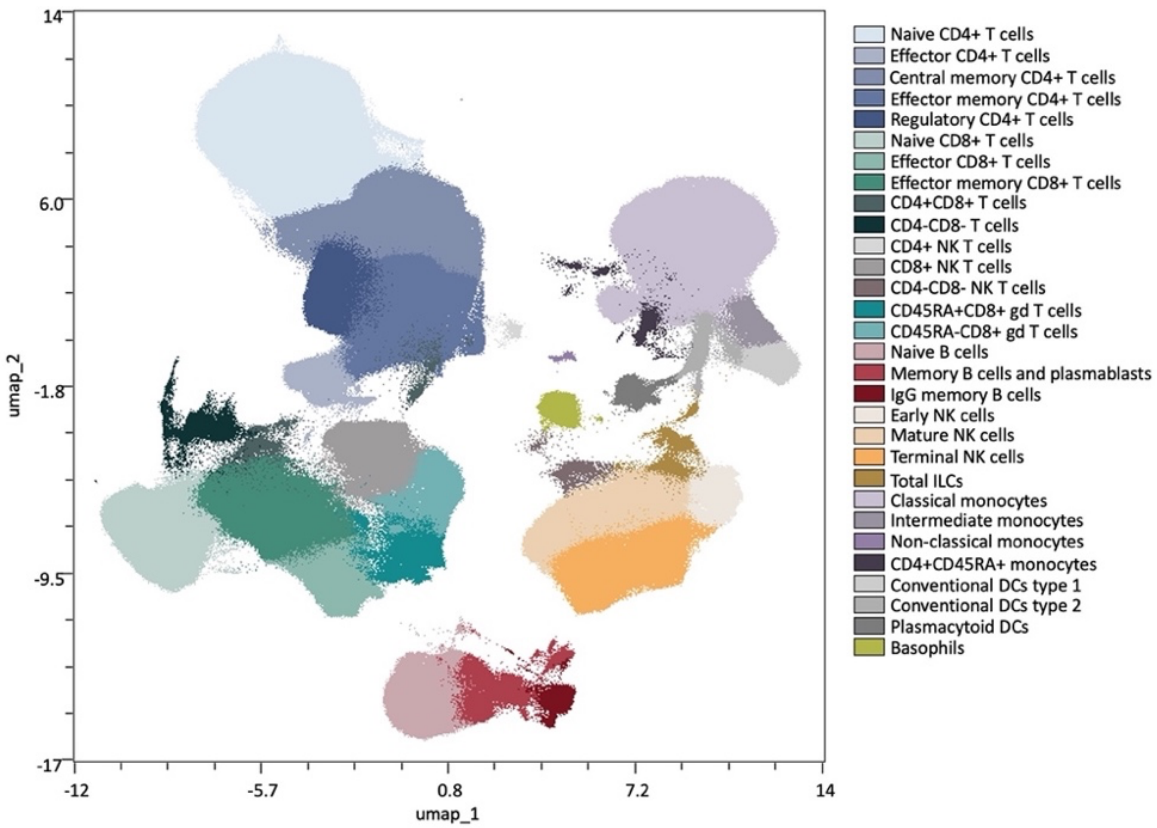


Figure 20: Visualisation of high-dimensional data analysis on circulating immune cells from all participants displaying 30 distinct immune populations.

FlowSOM clustering was performed on single circulating CD45+ leukocytes and resulted in 30 immune cell clusters, which were mapped to the two UMAP dimensions. Altogether, 1,950,292 cells from 27 participants were analysed. Data was analysed with FlowSOM and visualised with UMAP via OmiqM (Dotmatics, USA).

4.4.2.3 Physiological changes

Prior to comparing patient groups, my investigation focused on evaluating the impact of patient characteristics on endometrial and systemic immunity. All analyses were performed on 883,181 endometrial CD45+ cells from 30 participants and 1,950,292 circulating CD45+ cells from 27 participants unless stated otherwise.

Age and BMI

The results from differential analysis of endometrial and circulating CD45+ cells indicate that factors such as age under/over 35 and BMI under/over 30 do not significantly influence any of the 31 endometrial and 30 circulating immune phenotypes (p.adj < 0.05, $|\log_2 FC| > 1$, data not shown).

History of miscarriage and live birth

A history of miscarriage and live birth revealed a significant difference to immune cell populations. As shown in Figure 21, decreased levels of CD8+ MAIT cells in the participants with a history of miscarriage were found in the endometrium (p.adj = 0.023, $\log_2 FC = -1.900$), while effector CD8 T cells were increased in the same group in peripheral blood (p.adj = < 0.001, $\log_2 FC = 2.017$). Parous participants had increased levels of CD8+ MAIT cells (p.adj = 0.004, $\log_2 FC = 2.190$) and decreased levels of effector CD8 T cells (p.adj = 0.002, $\log_2 FC = -1.870$) in the endometrium but differences in peripheral blood were not found. Importantly, these factors have been controlled for in the following analyses.

Menstrual cycle phase

I observed fewer changes in immune populations across the menstrual cycle than anticipated. As described in the Table 13, increased levels of effector CD8 T cells in the endometrium were

observed in the proliferative when compared to late secretory phase (p.adj = 0.048, log_2FC = 1.653). There were no significant differences in endometrial immune populations between early secretory phase and WOI but decreased naïve CD8 T cells (p.adj = 0.021, log_2FC = -2.501) in the WOI compared to late secretory phase. There was a rise of NK cells in the late secretory phase, especially early NK cells (p.adj = 0.026, log_2FC = 1.241) and CD4-CD8- NK T cells (p.adj = 0.026, log_2FC = 1.890). Even fewer differences were demonstrated when investigating the influence of menstrual cycle on the circulating immune populations, with effector CD4 T cells rising through the early (p.adj < 0.001, l log_2FC = 5.636) and late secretory phase (p.adj < 0.001, log_2FC = -4898). Visualisation of the results through the cycle is presented in Figure 23.

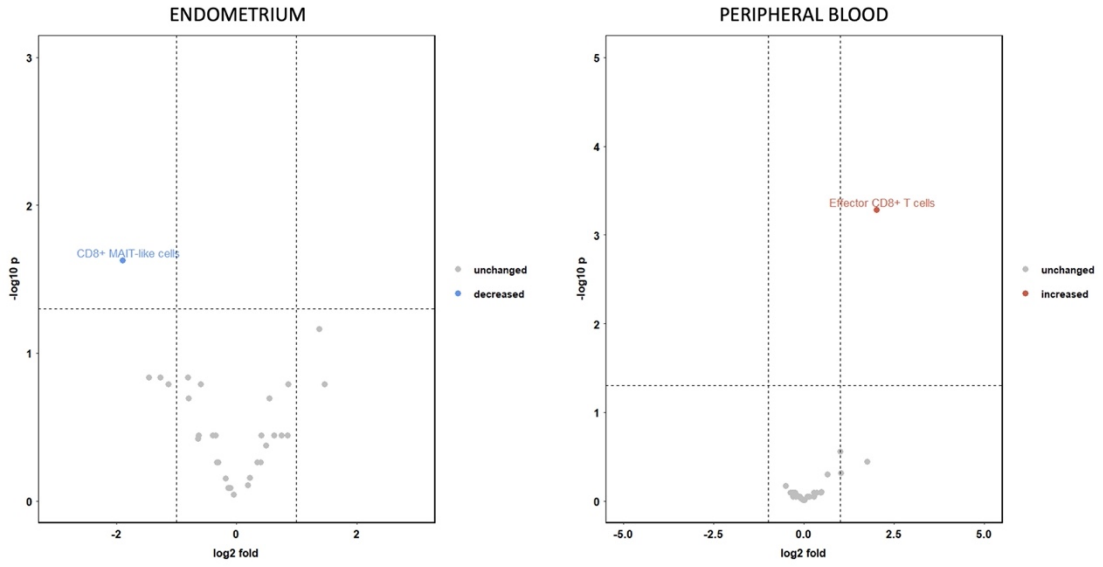


Figure 21: History of miscarriage indicates endometrial and systemic immune changes.

Volcano plots showing differences in cell cluster counts between participants with a history of miscarriage versus no history in the endometrium (left) and peripheral blood (right). Each point represents one cell cluster. Only significant clusters were labelled and coloured according to figure legend. p.adj < 0.05 and $|log_2FC| > 1$ were applied. Analysis was performed on cluster cell counts from 883,181 endometrial and 1,950,292 circulating CD45+ cells from 30 and 27 participants, respectively, with DESeq2 [185].

Table 13: The summary of immune cell fluctuations across the menstrual cycle.

Cell cluster	Proliferative phase	Early secretory phase	Window of implantation	Late secretory phase
Endometrium	**↑ Effector CD8+ T cells**		**↓ Naïve CD8+ T cells**	**↑ Early NK cells** **↑ CD4-CD8- NK T cells**
Peripheral blood		**↑ Effector CD4+ T cells**		**↑ Effector CD4+ T cells**

Differences in each phase are calculated based on the previous phase. E.g. effector CD8+ T cell population is increased in the proliferative phase when compared to the late secretory phase.

Examining CD8 T cell populations throughout the WOI is critical for understanding the immunological dynamics required for successful embryo implantation. Therefore, I have outlined the percentages of 11 CD8 T clusters in the total leukocyte population in the RPL group with a live birth, which comprised 51,096 CD45+ endometrial cells in total (Figure 22).

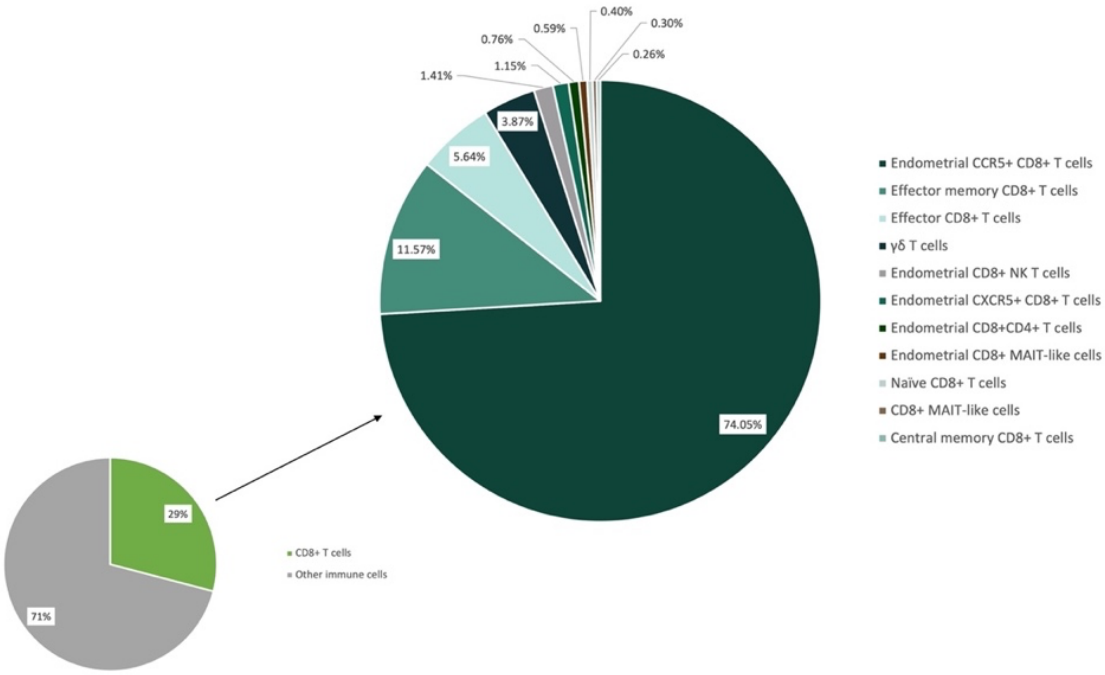


Figure 22: Percentages of CD8 T cell subtypes among all endometrial immune cells during window of implantation (WOI).

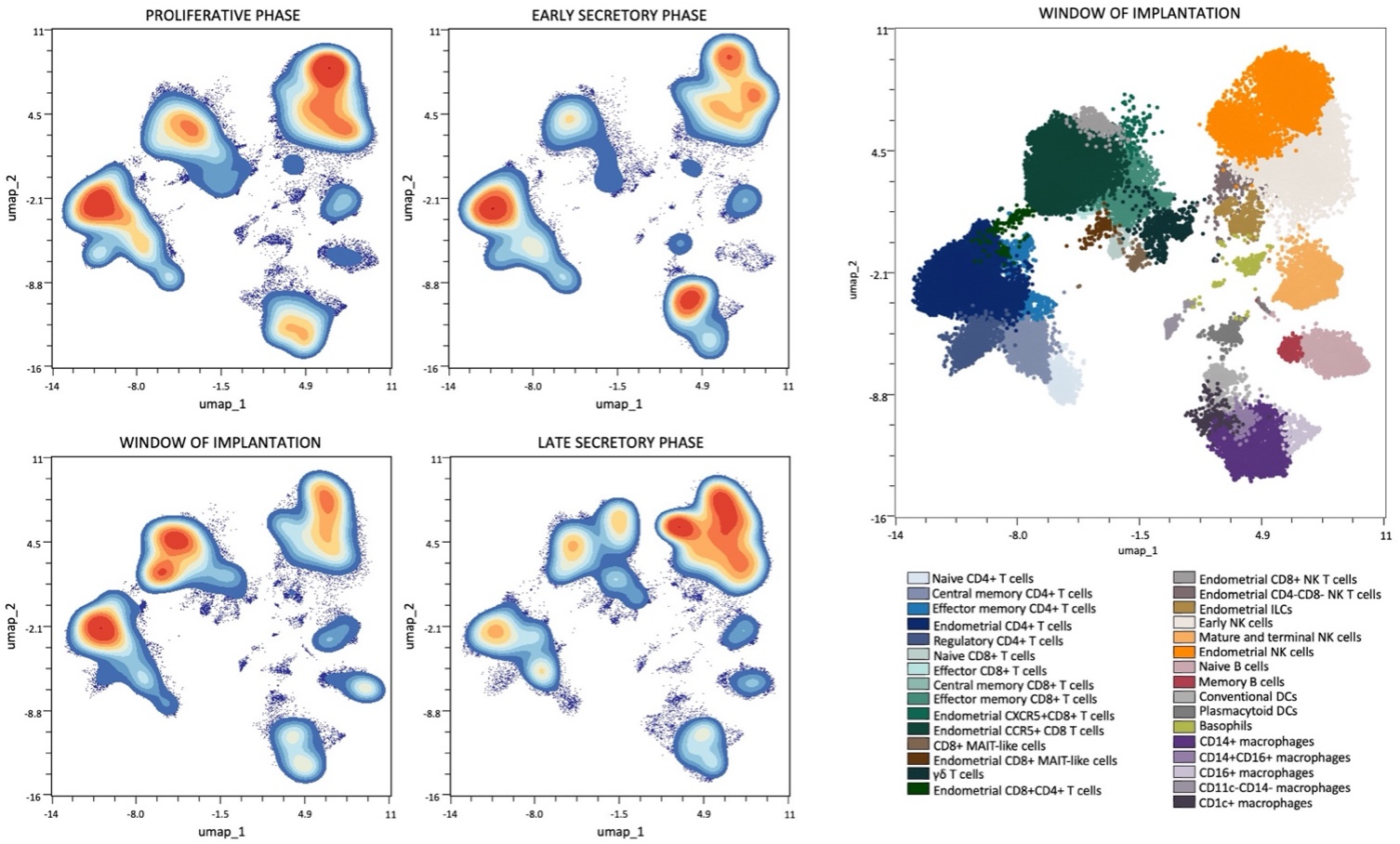


Figure 23: Changes in endometrial immune cell populations across the menstrual cycle.

Clustering and differential analysis were performed on 883,181 single endometrial CD45+ cells from 30 participants and resulted in 31 immune cell clusters. 61,479 cells from each cycle phase were mapped to the two UMAP dimensions, each dot represents one cell. Data was analysed with FlowSOM and visualised with UMAP via Omiq[M] (Dotmatics, USA).

4.4.2.4 Endometrial versus systemic immunity

To investigate differences in immune populations between endometrium and peripheral blood, I analysed 4,371,755 single CD45+ cells from 25 patients with matched endometrial and peripheral blood samples, which were clustered into 16 distinct groups. Comparative analysis revealed significant differences in 12 populations, of which the most significant were increased tissue resident populations of CD4 (p.adj < 0.001, $log_2FC = 6.0177$) and CD8 T cells (p.adj < 0.001, $log_2FC = 4.430$), along with NK cells (p.adj < 0.001, $log_2FC = 5.035$) in the endometrium and are presented in the Figure 24 below. While tissue resident CD4 T, CD8 T and NK cells constitute approximately 60% of all endometrial immune cells, they represent approximately 2% of circulating leukocytes and can be of various origins, such as from the gut, lungs, liver, endometrium etc. The 3 most significantly increased in the peripheral blood compared to the endometrium were naïve CD4 (p.adj < 0.001, $log_2FC = -3.480$) and CD8 T cells (p.adj < 0.001, $log_2FC = -2.733$) followed by central memory CD4 T cells (p.adj < 0.001, $log_2FC = -1.690$). Effector CD8 T cells, γδ T cells, B cells and plasmacytoid dendritic cells did not exhibit statistically different levels when comparing endometrial and circulating populations. Nevertheless, observing marker expression patterns (Figure 25) across CD8 T cell clusters between the two tissues, even these cell groups exhibited different phenotypes especially in tissue-homing chemokine receptors such as CCR5, CXCR5 and CCR6, but also differences in markers such as PD-1 and CD57. Interestingly, tissue resident CD8 T cells from the endometrium and from peripheral blood did not cluster together but instead tissue resident CD8 T cells from the endometrium phenotypically resembled effector CD8 T cells while the tissue resident CD8 T cell cluster from the peripheral blood was more similar to circulating effector memory CD8 T cells.

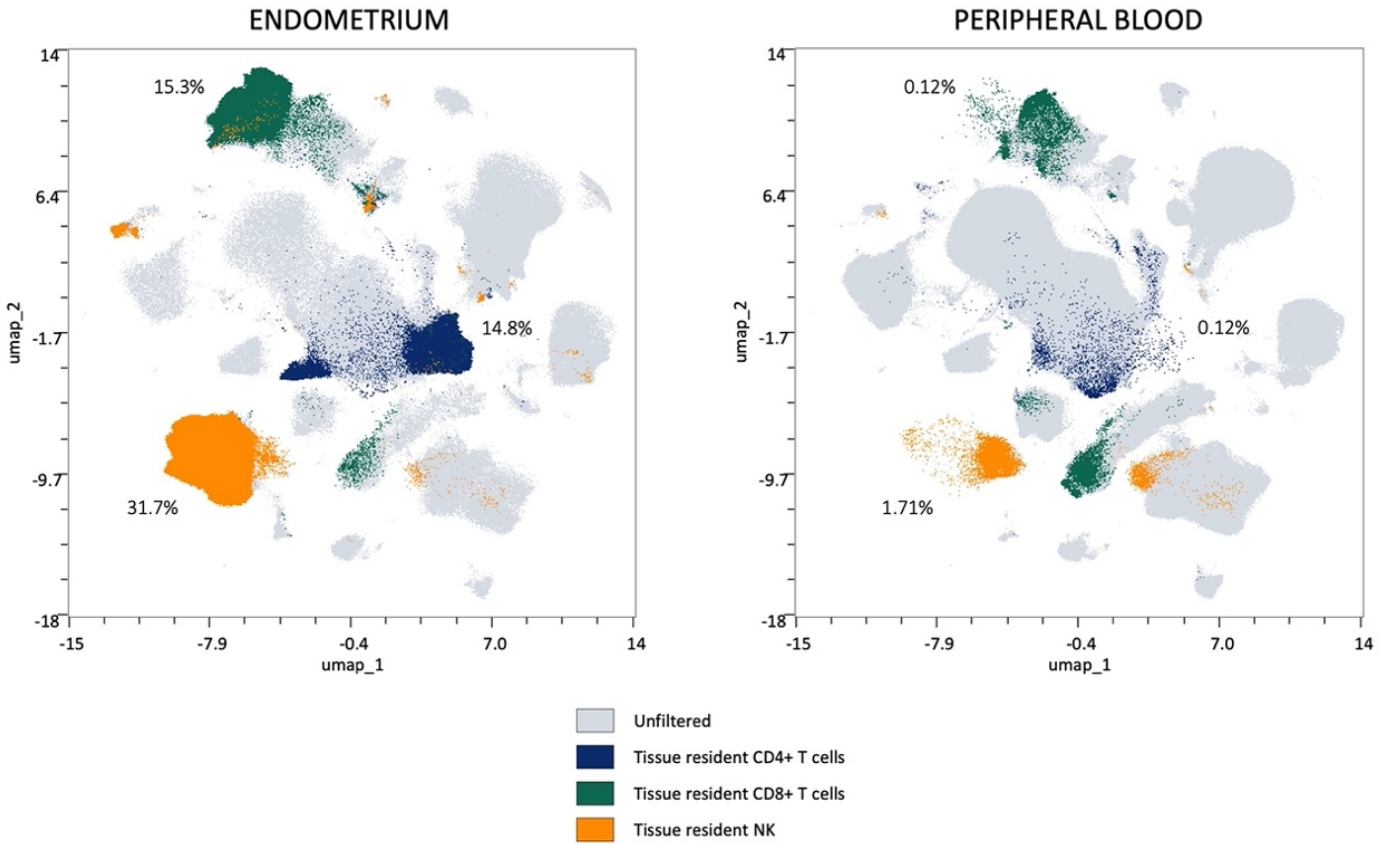


Figure 24: Changes between endometrial and circulating immune cell populations.

Clustering and differential analysis were performed on 4,371,755 single CD45+ cells from 50 matched endometrial and peripheral blood samples. 559,960 cells from each tissue were mapped to the two UMAP dimensions, each dot represents one cell. Data was analysed with FlowSOM and visualised with UMAP via OmiqM (Dotmatics, USA).

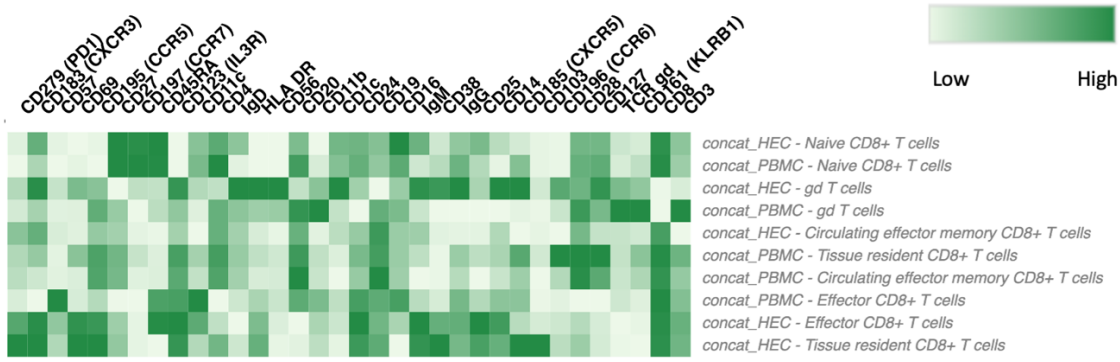


Figure 25: Heatmap of marker expression for all CD8+ T (CD8 T) cell clusters from the comparison analysis between endometrial and circulating immune cells.

The figure depicts a heatmap, with each column representing a specific marker and each column representing one of the CD8 T cell clusters from either endometrium (HEC) or peripheral blood (PBMC). FlowSOM clustering was performed on single CD45+ lymphocytes. Markers were only qualitatively studied between the two analysis groups. Expression levels are displayed using a colour scale, with darker colours indicating higher expression. Analysis was performed and visualised with OmiqM (Dotmatics, USA).

4.4.3 Endometriosis

4.4.3.1 Endometriosis

Differential analysis was performed on 861,796 cells from 25 patients, 17 in the endometriosis and 8 in the non-endometriosis control group. An example of the analysis with DESeq2 on cluster cell counts is shown in Figure 26. The endometrial CD8+ MAIT-like cell cluster was the only CD8 T population consistently identified as altered between the two groups (p.adj = 0.011 and $\log_2$FC = 1.714) in addition to decreased early endometrial NK cells (p.adj = 0.004 and $\log_2$FC = -1.593) and increased CD11c-CD14- macrophages (p.adj = 0.011 and $\log_2$FC = 2.478) in patients with endometriosis group when compared to controls. No differences were observed between endometriosis patients with stage I/II versus III/IV disease neither in cluster cell counts nor percentages of total leukocyte population.

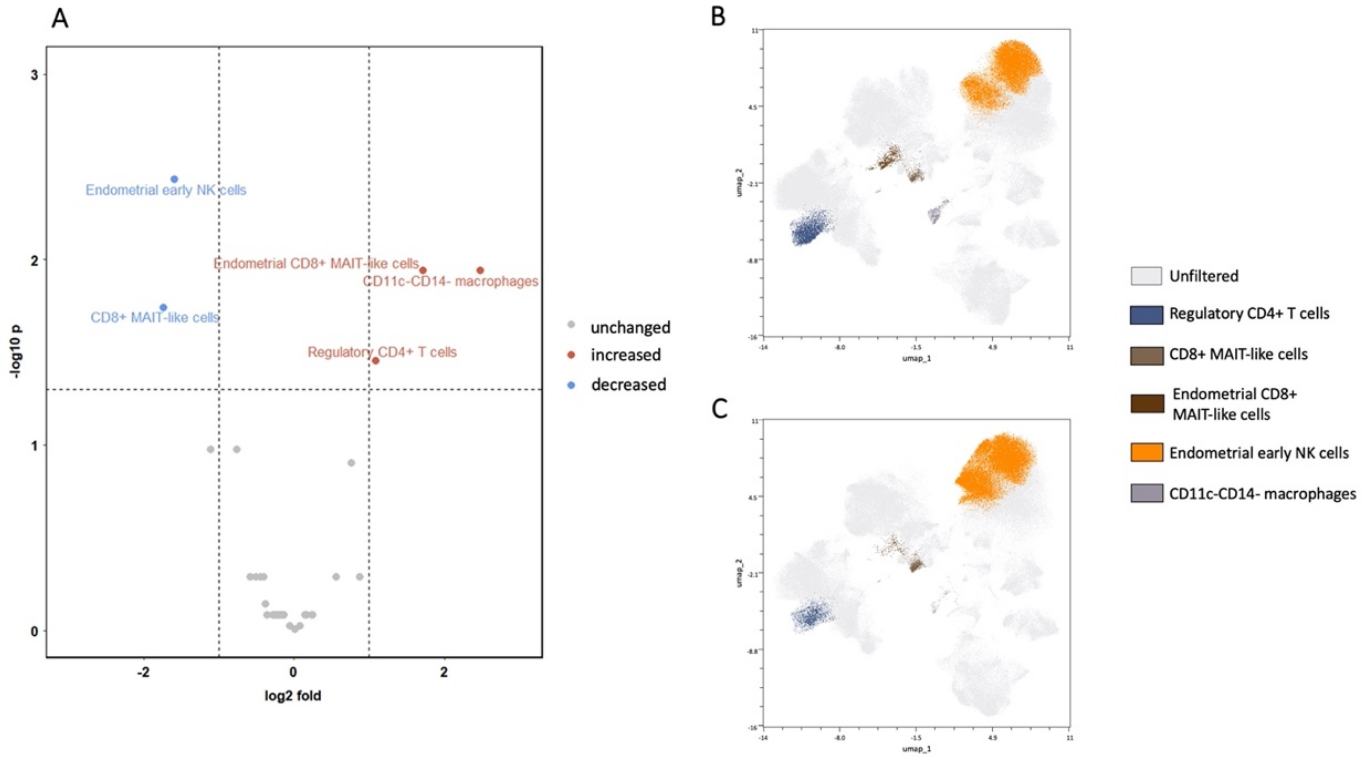


Figure 26: Altered endometrial immune cell populations between patients with endometriosis and non-endometriosis controls.

(A) Volcano plot showing changed endometrial cell clusters in endometriosis patients when compared to healthy controls. Each point represents one cell cluster, which were coloured according to figure legend. Significantly changed clusters were labelled. p.adj < 0.05 and|log2FC| > 1 were applied. Analysis was performed on 861,796 cells from 25 patients with DESeq2 [174] and visualised with OmiqM (Dotmatics, USA). (B) High-dimensional data visualisation of endometrial immune populations from endometriosis group displaying altered cell clusters projected onto two UMAP dimensions. (C) High-dimensional data visualisation of endometrial immune populations from control group displaying altered cell clusters projected onto two UMAP dimensions. (B–C) 327,051 cells from each patient group were mapped to the two UMAP dimensions, each dot represents one cell.

I further explored endometrial CD8+ MAIT-like cells, which represented $0.38 \pm 0.52\%$ of the total CD45+ population. The annotation was confirmed by comparing its characteristics to the manual gating strategy and another MAIT-like cluster, characterised as a non-resident population (Figure 27). Endometrial CD8+ MAIT-like cells aligned with manually set gates for CD3+TCRγδ- lymphocytes. The majority of cells were CD56- and CD8+ with some double positives and double negatives, which corresponds well to the existing literature on endometrial MAIT cells [88]. Importantly, in contrast to the circulating CD8+ MAIT-like population, these cells were positive for tissue residency markers CD69 and CD103. No other differences were observed.

Next, I investigated the expression patterns of immune markers in different CD8 T cell clusters between the groups and revealed distinct differences in various populations, which are illustrated in Figure 28. In general, the expression of PD-1 and CD103 markers was significantly increased across most clusters in the endometriosis group. PD-1 is a cell surface receptor protein that plays a crucial role in immune regulation by inhibiting T cell activation and promoting immune tolerance while CD103 is an integrin subunit predominantly expressed on tissue resident memory T cells, facilitating their adhesion to epithelial cells within mucosal tissues. In endometriosis patients, endometrial CD8+ MAIT-like cells displayed significantly higher levels of tissue-homing markers CD103 and CCR6 as well as regulatory PD-1 while having a lower expression of markers indicating cell differentiation and activation C127, CD27, CD28, CD69 and CXCR3.

Lastly, I observed systemic changes in immune populations in patients with endometriosis when compared to non-endometriosis controls (Figure 29). Differential analysis on 1,703,702 circulating CD45+ cells from 23 patients revealed increased CD45RA+CD8+ γδ T cells ($p.aj < 0.001$ and $\log_2FC = 1.379$) and decreased effector CD8 T cells ($p.adj = 0.0$ and $\log_2FC = 1.194$).

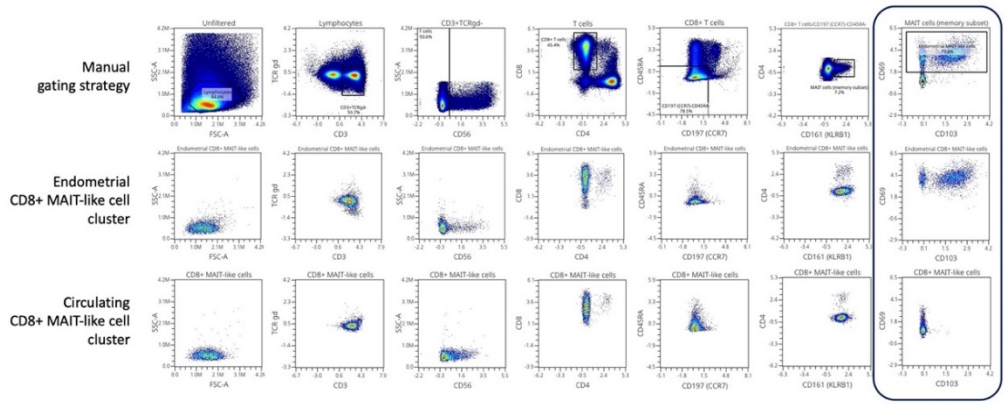


Figure 27: Endometrial CD8+ MAIT-like cell cluster annotation.

FlowSOM clustering was performed on single endometrial CD45+ lymphocytes. CD8+ MAIT-like cell clusters were annotated based on similarity with manually set gates for the population of interest, delineated down to single live lymphocytes CD45+TCRγδ-CD56-CD3+CD8+CD161+. Based on the expression of CD69+ and CD103+, they were then identified as endometrial and circulating CD8+ MAIT-like cell populations. In the representative figures, each dot represents one cell and all cells from the endometriosis analysis were concatenated, analysed and visualised with Omiq[M] (Dotmatics, USA).

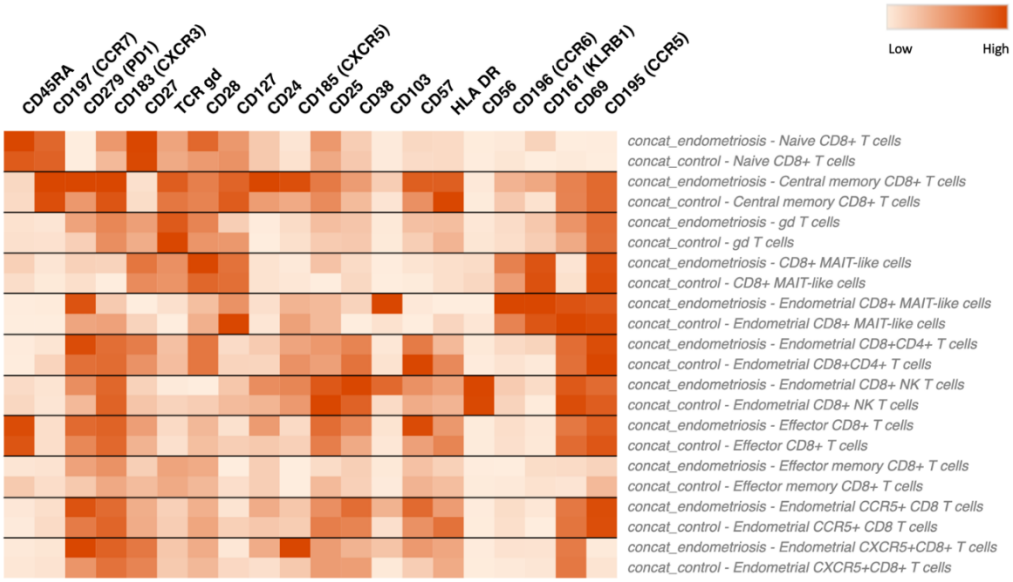


Figure 28: Heatmap of relevant marker expression for all endometrial CD8+ T cell clusters from the endometriosis and non-endometriosis control groups.

The figure depicts a heatmap, with each column representing a specific marker and each row representing one of 11 CD8+ T cell clusters from either the endometriosis or non-endometriosis control group. Only markers relevant to T cell function were selected and compared between the two analysis groups. Expression levels are displayed using a colour scale, with darker colours indicating higher expression. Unsupervised FlowSOM clustering and differential analysis with one-way ANOVA were performed on 861,796 single endometrial CD45+ cells. p.adj < 0.05 was applied. Data was visualised with OmiqM (Dotmatics, USA).

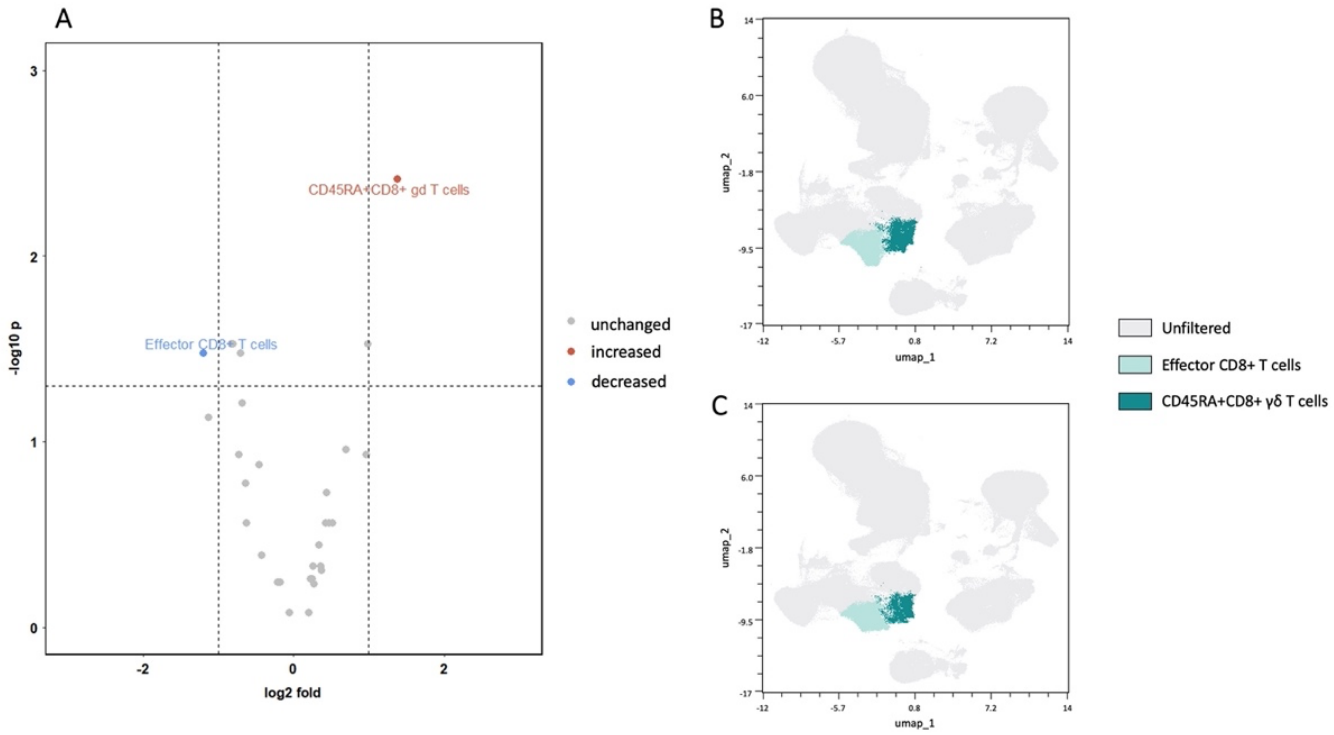


Figure 29: Altered circulating immune cell populations between patients with endometriosis and non-endometriosis controls.

(**A**) Volcano plot showing changed circulating cell clusters in endometriosis patients when compared to healthy controls. Each point represents one cell cluster, which was coloured according to the figure legend. Significantly changed clusters were labelled. p.adj < 0.05 and $|\log_2 FC| > 1$ were applied. Analysis was performed on 1,703,702 cells from 23 patients with DESeq2 [185] and visualised with Omiq^M (Dotmatics, USA). (**B**) High-dimensional data visualisation of circulating immune populations from the endometriosis group displaying altered cell clusters projected onto two UMAP dimensions. (**C**) High-dimensional data visualisation of circulating immune populations from the control group displaying altered cell clusters projected onto two UMAP dimensions. (**B–C**) 592,591 from each patient group were mapped to the two UMAP dimensions, each dot represents one cell.

4.4.3.2 Endometriosis-associated subfertility

Next, alterations in endometrial immune populations in EAS were investigated on 214,237 cells from 8 samples (4 with EAS and 4 fertile endometriosis patients) from the secretory phase. Figure 30 depicts an example of DESeq2 analysis on cluster cell counts and in addition to Tregs (p.adj = 0.001 and log_2FC = -2.215) and naïve B cells (p.adj = 0.008 and log2FC = 2.331) the endometrial CCR5+ and CXCR5+ CD8 T cells were the two CD8 T clusters consistently identified as significantly altered in the EAS group when compared to fertile patients with endometriosis (p.adj < 0.001 and log_2FC = -4.664, p.adj = 0.008 and log_2FC = 4.522, respectively). No systemic differences were observed between the two groups.

Endometrial CCR5+ represent 14.88 $\pm$ 12.01% while CXCR5+ CD8 T cells constitute 2.67 $\pm$ 7.00% of all endometrial immune cells. As validated with the manual gating shown in the Figure 31 both clusters display tissue resident effector memory phenotype with distinct expression of CCR5 and CXCR5 markers.

Figure 32 showcases alterations in expression levels of T cell relevant immune markers across different CD8+ clusters within the studied groups, highlighting the unique patterns and differences between them. CCR5 stands out as a marker consistently increasingly expressed in the EAS group as opposed to CXCR3, which was decreased across most CD8 T cell clusters in this group. Endometrial CCR5+ CD8 T cells from EAS patients had a significantly lower expression of CD103 marker but a higher expression of CD27, CD28 and PD-1 than fertile endometriosis patients. On the other hand, endometrial CXCR5+ CD8 T cluster exhibited higher expression of CD103, but a lower expression of PD-1 than the same cluster in the fertile group.

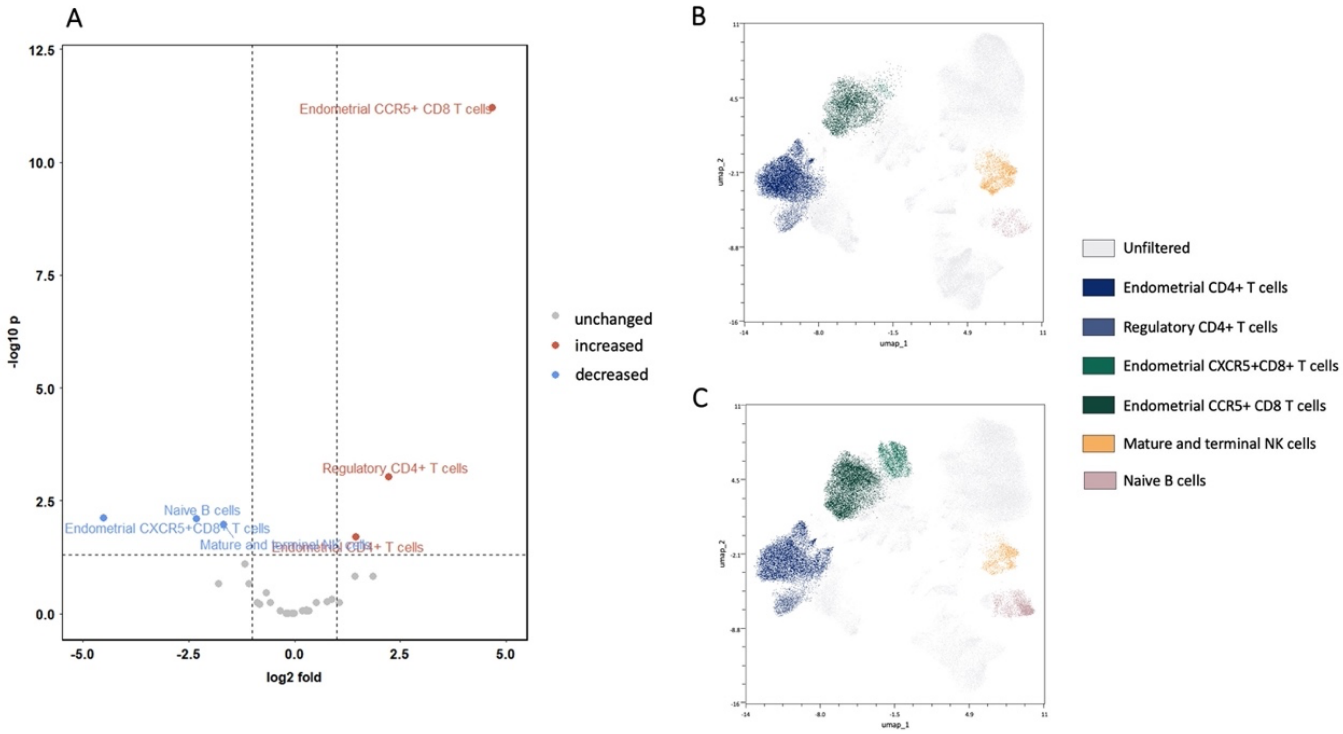


Figure 30: Altered endometrial immune cell populations between patients with endometriosis-associated subfertility (EAS) and fertile-endometriosis controls.

(**A**) Volcano plot showing changed endometrial cell clusters in EAS when compared to fertile endometriosis controls. Each point represents one cell cluster, which was coloured according to the figure legend. Significantly changed clusters were labelled. p.adj < 0.05 and|log2FC| > 1 were applied. Analysis was performed on 214,237 cells from 8 patients with DESeq2 [185] and visualised with Omiq^{M} (Dotmatics, USA). (**B**) High-dimensional data visualisation of endometrial immune populations from the EAS group displaying altered cell clusters projected onto two UMAP dimensions. (**C**) High-dimensional data visualisation of endometrial immune populations from fertile endometriosis group displaying altered cell clusters projected onto two UMAP dimensions. (**B–C**) 43,274 cells from each patient group were mapped to the two UMAP dimensions, each dot represents one cell.

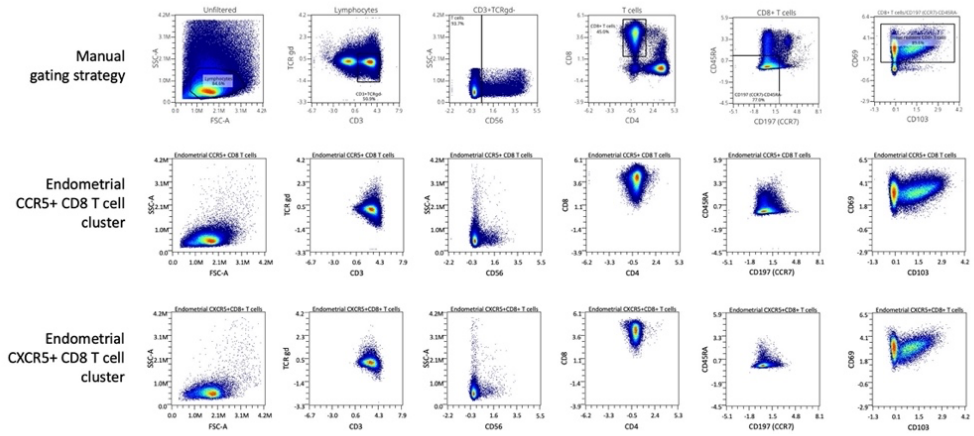


Figure 31: Endometrial CCR5+ CD8+ and CXCR5+ T cell cluster annotation.

FlowSOM clustering was performed on single endometrial CD45+ lymphocytes. Endometrial CCR5+ and CXCR5+ CD8+ T cell clusters were annotated based on similarity with manually set gates for the population of interest, delineated down to single live lymphocytes CD45+TCRγδ-CD56-CD3+CD8+CD103+CD69+ CCR5+/CXCR5+. In the representative figures, each dot represents one cell and all cells from the endometriosis analysis were concatenated, analysed and visualised with Omiq^{M} (Dotmatics, USA).

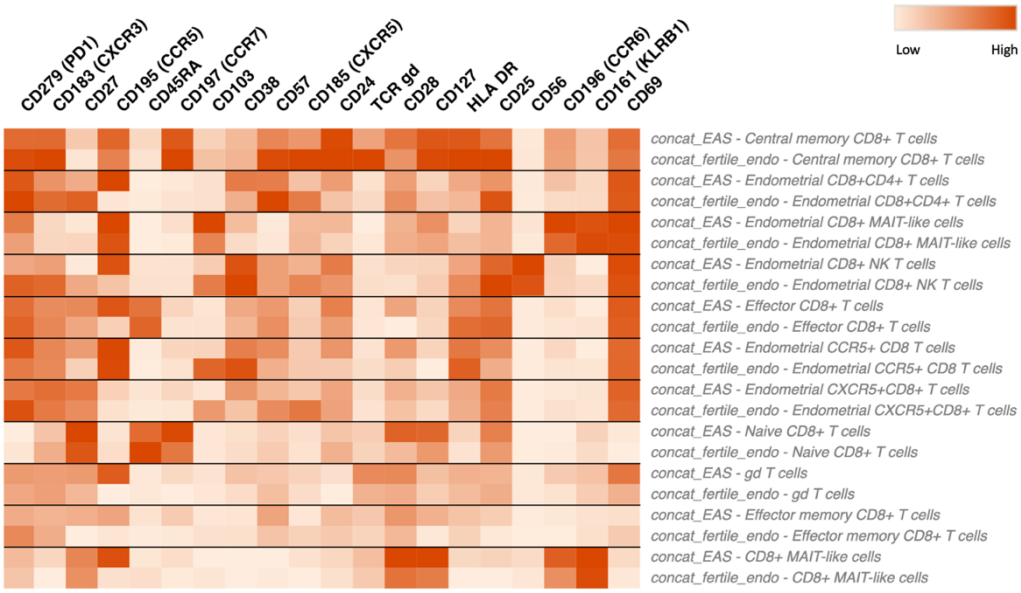


Figure 32: Heatmap of relevant marker expression for all endometrial CD8+ T cell clusters from the endometriosis-associated subfertility (EAS) and fertile endometriosis groups.

The figure depicts a heatmap, with each column representing a specific marker and each row representing one of 11 CD8+ T cell clusters from either the EAS or fertile endometriosis group. Only markers relevant to T cell function were selected and compared between the two analysis groups. Expression levels are displayed using a colour scale, with darker colours indicating higher expression. Unsupervised FlowSOM clustering and differential analysis with one-way ANOVA were performed on 214,237 single endometrial CD45+ cells. p.adj < 0.05 was applied. Data was visualised with OmiqM (Dotmatics, USA).

4.4.4 Recurrent pregnancy loss

4.4.4.1 Next pregnancy outcome

Differential analysis of protein expression on endometrial immune clusters was performed between RPL patients with miscarriage in the subsequent pregnancy (n = 5) compared to RPL patients with a subsequent live birth (n = 5). In total, 96,671 CD45+ cells from the peri-implantation endometrium were clustered and analysed. Figure 33 illustrates a case of DESeq2 analysis on cluster cell counts, revealing that naïve CD8 T cells stood out as the only immune cluster consistently significantly altered between the RPL patients with a subsequent miscarriage versus live birth (p.adj = 0.021 and $\log_2$FC = 2.076). No significant differences were observed in the circulating immune populations regarding cell counts. Still, naïve and effector memory CD8 T cells in the RPL miscarriage had significantly increased expression of CXCR3 marker essential for trafficking of activated CD8 T cells into peripheral tissues during infection.

The validation of the population annotation that constitutes 0.48 $\pm$ 0.66 % of the total CD45+ population was performed by comparing its characteristics with the naïve CD8 T cells obtained through the manual gating approach (Figure 8).

Across all CD8 T cell clusters, I found significantly increased expression of CD57 and decreased expression of PD-1 in the miscarriage group, indicating reduced proliferative capacity but increased cytokine production and cytotoxicity, while CD127, a marker for memory and effector CD8 T cells, was increased in the live birth group (Figure 35). In addition to the two markers with generally altered expression patterns, naïve CD8 T cells had a significantly higher expression of CD24, a marker widely used to determine immune cell differentiation and essential for stimulation in T cell activation, especially in non-lymphoid target organs.

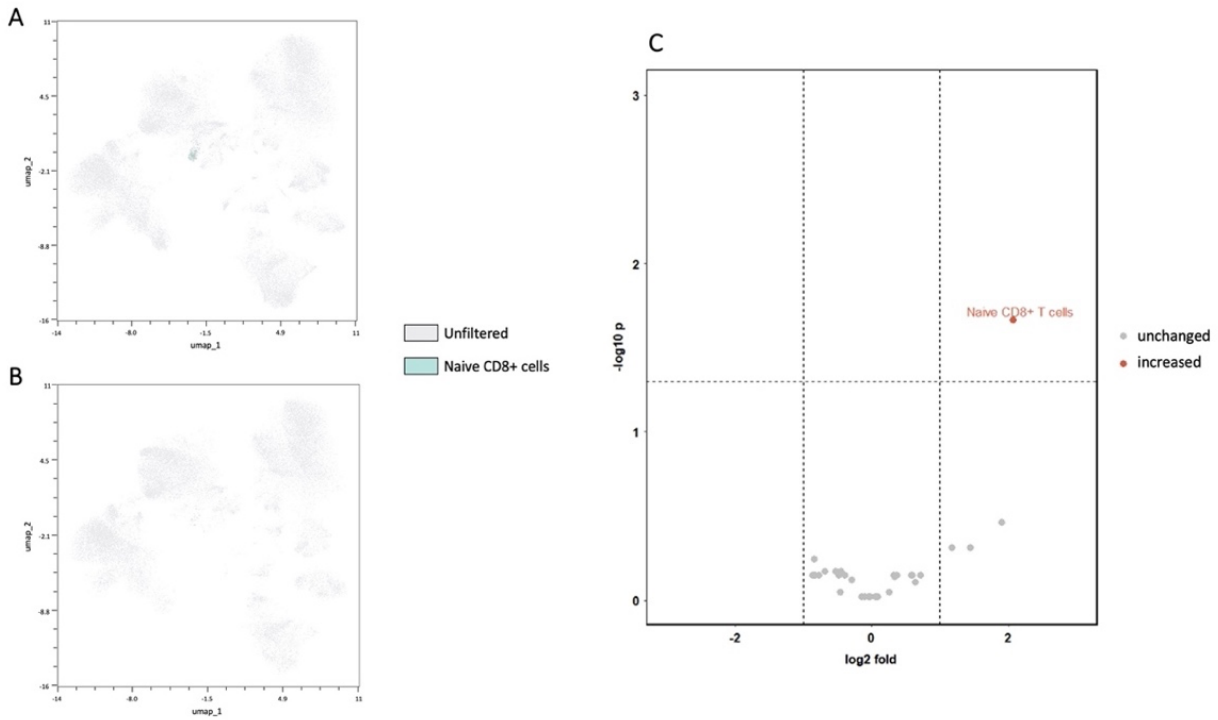


Figure 33: Naïve CD8+ T cells are enriched in recurrent pregnancy loss (RPL) with another miscarriage in the next pregnancy when compared to those with a subsequent live birth.

(**A**) High-dimensional data visualisation of endometrial immune populations from RPL group with miscarriage in the next pregnancy displaying altered cell cluster projected onto two UMAP dimensions. (**B**) High-dimensional data visualisation of endometrial immune populations from RPL group with live birth in the next pregnancy displaying altered cell cluster projected onto two UMAP dimensions. (**A–B**) 30,891 cells from each patient group were mapped to the two UMAP dimensions, each dot represents one cell. (**C**) Volcano plot showing changed cell clusters in RPL patients with different outcomes in the next pregnancy. Each point represents one cell cluster, which were coloured according to figure legend. Only significantly changed cluster was labelled. p.adj < 0.05 and|log2FC| > 1 were applied. Analysis was performed on 96,671 cells from 10 patients with DESeq2 [185] and visualised with OmiqM (Dotmatics, USA).

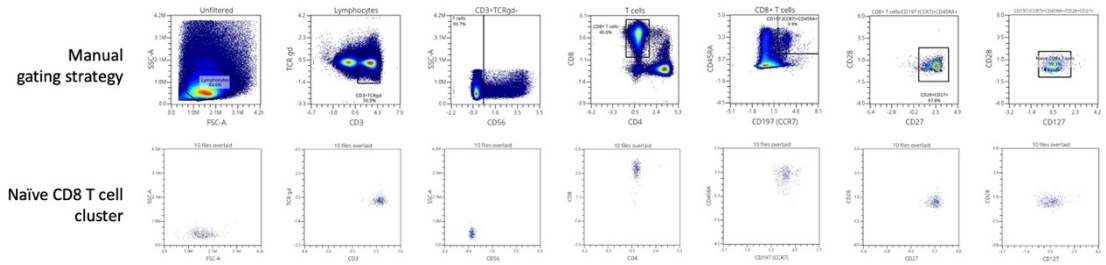


Figure 34: Naïve CD8+ T cell cluster annotation.

FlowSOM clustering was performed on single endometrial CD45+ lymphocytes. Naïve CD8+ T cell cluster were annotated based on similarity with manually set gates for single live lymphocytes CD45+TCRγδ-CD56-CD3+CD8+CCR7+CD45RA+CD27+CD28+CD127+. In the representative figures, each dot represents one cell and all cells from the endometriosis analysis were concatenated, analysed and visualised with Omiq[M] (Dotmatics, USA).

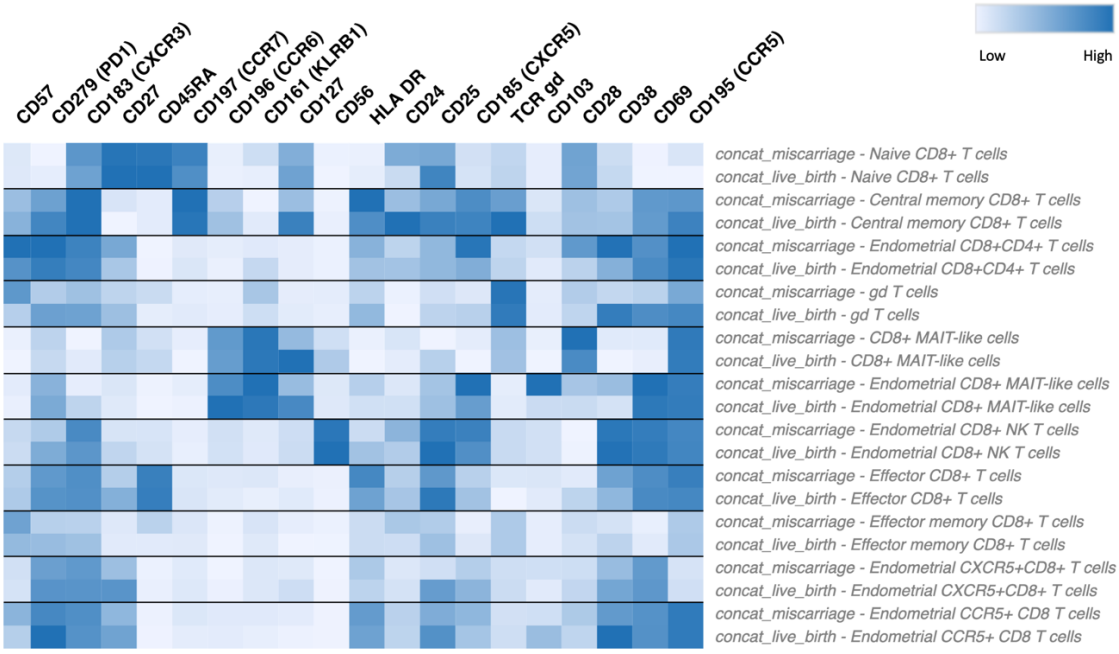


Figure 35: Heatmap of relevant marker expression for all endometrial CD8+ T cell clusters from the recurrent pregnancy loss (RPL) patients with miscarriage and live birth in the next pregnancy.

The figure depicts a heatmap, with each column representing a specific marker and each row representing one of 11 CD8+ T cell clusters from either the RPL patients with miscarriage or live birth in the next pregnancy. Only markers relevant to T cell function were selected and compared between the two analysis groups. Expression levels are displayed using a colour scale, with darker colours indicating higher expression. Unsupervised FlowSOM clustering and differential analysis with one-way ANOVA were performed on 96,671 single endometrial CD45+ cells. p.adj < 0.05 was applied. Data was visualised with OmiqM (Dotmatics, USA).

4.5 DISCUSSION

4.5.1 Summary

This chapter describes the first phenotypic study on endometrial and matched circulating immune cells utilising full spectrum flow cytometry with 36 markers, and the first study to investigate endometrial CD8 T subtypes in endometriosis. Firstly, I examined differences in immune populations between the endometrium and peripheral blood and demonstrated that peripheral blood is a poor indicator of endometrial immune activity and should, therefore, be mainly considered for diagnostic purposes. Having gained information on potential immune modulating factors such as a history of miscarriage and live birth, I went on to explore local and systemic immunity in patients with RPL and endometriosis. A total of 883,181 endometrial and 1,950,292 circulating leukocytes from 32 participants were analysed. I observed increased levels of endometrial MAIT-like CD8 T cells in patients with endometriosis (n = 17) when compared to non-endometriosis controls (n = 8) and decreased numbers of endometrial CCR5+ CD8 T cells in patients with EAS (n = 4) versus fertile endometriosis patients (n = 4). In RPL, naïve CD8 T cells were significantly increased in patients with a miscarriage in the next pregnancy (n = 5) versus live birth (n = 5). Systemic changes were observed in patients with endometriosis but not between subfertile and fertile endometriosis patients and RPL patients with different pregnancy outcomes.

4.5.2 Strengths and limitations

Analysing 883,181 endometrial and 1,950,292 circulating CD45+ cells across the cycle, this is the most extensive study to date investigating and comparing endometrial and systemic immunity. Furthermore, it is the first full spectrum flow cytometry study of human endometrium and

matched peripheral blood, the first deep immunophenotyping study detailing adaptive tissue resident immune populations in endometriosis and EAS, and the first prospective study to investigate peri-implantation endometrial immunity in RPL patients based on the outcome of the subsequent pregnancy. Only patients without hormonal therapy and regular menstrual cycles were included to mitigate the lack of histological confirmation of the menstrual cycle stage. No gynaecological or immunological comorbidities were accepted in either of the analysis groups. Due to the strict eligibility criteria, this pilot study has a limited number of patients, especially when investigating EAS and RPL. Additionally, it is essential to highlight that EAS patients could suffer from subfertility due to surgically resolving causes, but we lack prospective data to assess this. In both endometriosis analyses, findings should be interpreted in the context of association rather than causation. Conversely, in the RPL group, comparing two different pregnancy outcomes during the WOI strengthens the predictive value of the analysis. Still, it lacks comparison with fertile controls to inform us about underlying pathophysiology.

4.5.3 Studying endometrial immunity

Not only is the answer to how endometrial immunity is mirrored in the peripheral blood of diagnostic value, but it is also the key to understanding the pathophysiology of the disease through studying more accessible systemic immunity. Nevertheless, having identified significant changes in 12 out of 16 immune cell clusters, my findings underscore the inadequacy of peripheral blood as a reliable indicator of endometrial immune pathophysiology while acknowledging its potential role for biomarker discovery. While differences in BMI and age did not seem to have relevant effects on either endometrial or systemic immunity in premenopausal women, a history of miscarriage and live birth should be considered when designing clinical studies, as my findings have indicated modifications in the CD8 T cell compartment in women with previous miscarriage when compared to no miscarriage (parous and nulliparous) as well as

in parous women when compared to nulliparous. Interestingly, the menstrual cycle phase played a slightly less pronounced role than I had anticipated, given the existing literature. However, it is essential to note that differences might only seem less evident on a bigger scale despite significant day-to-day changes recently described in the most detailed study on the endometrial gene expression across the menstrual cycle [6, 8, 189]. Interestingly, despite the commonly accepted concept of a decrease in CD8 T cell levels across the secretory phase, our findings only demonstrated a significant decrease in the naïve subtypes during the WOI. Moreover, high-dimensionality data visualisation figures show enhanced CD8 T cells during the mid-secretory phase, a time crucial for favourable endometrial receptivity, when the population accounted for 29% of all endometrial leukocytes with NK cells (33%) representing the majority in RPL patients with a live birth in the next pregnancy. Several confounding factors could explain differences in these findings, including immunohistochemistry approaches utilised in the majority of established studies, which only look at broader populations rather than specific phenotypes and poorly reflect the whole endometrium, especially the basal layer with LAs [6, 7, 60]. In addition, controlling for a history of miscarriage in my analysis could result in masking prominent endometrial CD8 T cell population as a consequence of the menstrual cycle changes rather than underlying pathology since all the RPL patients were timed to WOI while endometriosis patients samples were from across the menstrual cycle. However, transcriptomic differences in the CD8 T population between RPL patients with a subsequent live birth and fertile controls during WOI described in the previous chapter were minimal and naïve CD8 T cells were found to be increased in the RPL patients with another miscarriage rather than decreased what was demonstrated in the cycle phase analysis.

4.5.4 Endometriosis and associated subfertility

T_{RM} are thought to be the most prevalent CD8 T subpopulation in mucosal tissues but our understanding of this subtype in the human endometrium is poor and in my second chapter, I have not identified any existing studies on tissue resident subpopulations in endometriosis [58, 105]. However, T_{RM} in the gut are widely researched, and there is strong evidence they are implicated in the pathogenesis of inflammatory bowel disease and other intestinal immunopathologies such as coeliac disease [66, 253]. For example, authors investigating T_{RM} in inflammatory bowel disase show considerably lower levels of CD103+ T_{RM} in patients with flares versus patients with endoscopic remission and healthy controls, which suggests their contribution to tissue homeostasis and immune regulation [254]. Similarly, the previous work in our group revealed a modified eutopic endometrial CD8 T_{RM} phenotype with considerably lower expression of tissue residency marker CD69 in RPL when compared to fertile controls [52]. On the other hand, I found an increased tissue resident population of CD8+ MAIT-like cells and a higher expression of CD103 in the endometriosis group, which was also discovered in vitiligo, with higher levels of CD69+CD103+ T_{RM} associated with active disease, and importantly, these cells attracted cytotoxic effector CD8 T cells from the blood, which is critical for disease persistence [179]. On the other hand, in endometriosis, three tissue resident CD8 T cell subtypes are of particular interest, including endometrial CD8+ MAIT-like cells in the comparison of endometriosis versus non-endometriosis, and endometrial CCR5+ and CXCR5+ CD8 T cells which were altered between subfertile and fertile endometriosis patients.

MAIT cells have been implicated in the outcome of various diseases, from microbial infection and autoimmune inflammatory diseases to malignancy [85]. MAIT cell frequency increases in infected and inflamed tissues through migration from peripheral blood or local proliferation at infected sites [86]. Most studies investigating MAIT cells in inflammatory bowel disease report

increased frequencies of the tissue resident population while functional analysis on circulating MAIT cells repeatedly demonstrated increased IL-17 production in inflammatory bowel disease patients propagating chronic inflammation [255-257]. In addition to an enriched MAIT-like cell population, the universally heightened expression of CD161 marker reveals a shift toward increased IL-17 production in endometriosis; this has already been implicated in various other immune-mediated diseases associated with endometriosis, including systemic lupus erythematosus and rheumatoid arthritis [85, 152]. In rheumatoid arthritis, MAIT cells in the synovial fluid displayed elevated levels of activation, including the production of IL-17, compared to the peripheral blood, and the degree of activation has been positively correlated with the severity of the disease in the lungs. As in the gut, locally increased expression of IL-17 by MAIT cells can be harmful for epithelial barrier integrity, thus aggravating infection and potentially facilitating dissemination of cancer metastases by inhibiting NK cell antimetastatic response and increasing blood vessel permeability [258, 259]. This is particularly interesting in the context of the lymphatic and vascular metastasis hypothesis of the endometriosis histogenesis [24, 260, 261]. Findings that could support this include increased levels of IL-18, an important mediator of TCR-independent MAIT cell activation, in the peritoneal fluid of patients with endometriosis, and increased levels of IL-17 found in stage I/II disease and EAS when compared to stage III/IV and control participants [262-264]. Furthermore, enriched CD4+ and CD8+ MAIT subtypes were confirmed in peripheral blood and peritoneal fluid of patients with endometriosis along with heightened MAIT-cell activation and IL-8, IL-12 and IL-17 production, while control participants had increased DN subtypes and higher expression of PD-1 [124]. Despite limited markers for more precise cell identification, my findings suggest MAIT cells as a promising target for further investigations, with the first step being a detailed characterisation of eutopic and ectopic endometrial MAIT cells in the disease as no studies have been published to date [105].

Elucidating the immune mechanisms underlying endometriosis pathology could lead to the development of targeted endometriosis therapy in the future.

The contribution of the immune system to the endometrial receptivity and decidualisation remains poorly understood, with data indicating alterations associated with EAS in several mediators of CD8 T cell immunity, including endometrial CD4 T cells, NK cells and macrophages [28, 98]. In contrast to existing findings, we found increased levels of Tregs in the endometrium of women with EAS when compared to fertile endometriosis patients [265]. Focusing on CD8 T cell populations for the purpose of this thesis, I have found increased levels of CCR5+ and decreased levels of CXCR5+ endometrial CD8 T cells in patients with EAS in addition to significantly increased CCR5 marker expression across most CD8+ clusters. However, a previous study showed a significant increase in endometrial expression of CCR5 during the secretory phase and in the co-culture with a human blastocyst suggesting a potential role in embryo implantation [266]. This underscores potential pitfalls in data interpretation without accounting for physiologic changes across the menstrual cycle, which I could control for in the differential analysis of cell counts but not for differential marker expression. Therefore, I can confidently conclude that increased levels of CCR+ CD8 T cells are likely pathologic, but increased CCR5 expression needs further validation. It has been previously reported that endometrial T cells exhibited elevated expression of CCR5 with a higher susceptibility to activation as effector memory cells in comparison to circulating T cells, which corresponds to my findings, with endometrial CCR5+ CD8 T cells being the most pronounced CD8 T population, comprising 22% of the total leukocyte population [59, 180]. In addition to their crucial role in the successful management of viral replication in the early phases of a secondary immune response, CCR5+ CD8 T cells have been described as important mediators of antibody-mediated allograft rejection in mice together with CXCR5+ CD8 T cells [267-269]. More specifically, CXCR5+ CD8 T

cells derived from CCR5 knock-out (KO) recipients exhibited a 30% reduced capability to eliminate antibody-producing IgG+ B cells *in vitro* when compared to their wild-type (WT) counterparts and the adoptive transfer of CXCR5+IFN-γ+ CD8 T cells into CCR5 KO kidney transplant recipients led to a significant decrease in alloantibody production compared to WT recipients and improved transplant outcomes [269]. In peripheral organs, CXCR5+ CD8 T cells interact with B cells and promote B cell proliferation, class-switching, and antibody production, which could explain the associated decrease of these two populations in our findings [270]. Although altered proportions of endometrial B cells and even B cells expressing CXCR5 have been demonstrated in the endometrium of endometriosis patients versus controls, we have not identified any studies investigating endometrial B cells in EAS to compare our outcomes [243, 271].

4.5.5 Recurrent pregnancy loss

Our group has previously demonstrated altered peri-implantation endometrial CD8 T cell phenotypes in RPL patients versus fertile controls in addition to determining the roles of regulatory and conventional CD4 T and B cell populations [49, 52, 245]. Nevertheless, there has been no analysis to date considering prospective pregnancy outcomes despite diagnostic immunological testing continually being offered to this patient group. In my analysis, I discovered increased naïve CD8 T cells in RPL patients with a miscarriage versus live birth in the next pregnancy and consistent changes in CD57, PD-1 and CD127 marker expression among several endometrial CD8 T cell clusters. While naïve CD8 T cells from the endometrium displayed minimally significant changes in marker expression and there were no quantitative differences observed in the peripheral blood, circulating naïve CD8 T cells from the patients with miscarriage expressed CXCR3 at significantly higher levels than naïve CD8 T cells from the live birth group. This could indicate a higher tendency to migrate to peripheral tissues and explain an increased

population in the endometrium, which seems to be especially tightly regulated during the WOI, when significantly lower numbers were observed compared to early secretory phase [272]. Moving to the marker expression, CD57 antigen is used to detect mature cells with low proliferative ability but increased cytotoxicity often identified as senescent cells. The presence of CD57+ cells in peripheral blood and tissues has clinical significance in various conditions, including chronic infection and cancer [273, 274]. Although alloantigen-activated CD8+CD57+PD-1- cells were shown to be more cytotoxic than CD57- cells, their cytotoxic ability was not related to the development of kidney transplant rejection [275]. In my analysis, PD-1 expression was decreased in the miscarriage group in most CD8 T cell clusters, and similar findings were shown in the decidual CD8 T cells of patients with miscarriage and preeclampsia, setting the ground for hypotheses about mechanisms of foetal rejection [194]. To support this, studies on mice have shown that in response to a paternally inherited foetal antigen, antigen-specific maternal T cells multiply and upregulate PD-1 *in vivo* in mid-gestation. Furthermore, the cells with PD-1 deletion accumulate in the uterus-draining nodes due to reduced apoptosis demonstrated *in vitro* and could pose a significant risk to the foetal allograft [276]. In lower FRT, PD-L1, rather than PD-1, is thought to control T-cell function, indicated by an increase in PD-L1 expression on pre-menopausal CD8+ T cells during suppressed cytotoxic activity through oestrogen [61]. PD-1/PD-L1 pathway has been successfully targeted in several cancer types by immune checkpoint inhibitors, which block PD-1 on T cells and prevent them from interacting with PD-L1/L2 expressed by cancer cells, making malignant cells susceptible to immune clearance [277]. However, the data on the effect of immunotherapy with checkpoint inhibitors on organ transplantation are scarce and the potential to enhance transplant survival with a reverse approach has so far not been explored [278]. Therefore, further research is needed to understand the exact mechanism of how the PD-1/PD-L1 pathway is associated with allograft

rejection to justify CD8 T cell inhibition by PD-1 immunomodulation to improve foetal immune tolerance and reproductive outcomes.

4.5.6 Future research

Despite its limitations, this chapter points to endometrial immune populations of interest for further research in endometriosis and RPL while highlighting factors to be considered when designing clinical studies to investigate human endometrium in health and disease, such as cycle phase and parity. In the next step, it will be important to characterise the functional properties of CD8 T cells, especially during WOI, focusing on their activation and exhaustion status. Having gained insights into their cytotoxic activity and potentially detrimental roles in reproductive outcomes, unfavourable effects of dysregulated immunity on endometrial receptivity could be mitigated with timely diagnosis and appropriate targeted immune therapy in the future.

4.6 CONCLUSIONS

Local and systemic immune changes in patients with endometriosis confirm the systemic character of the disease, while subgroup analysis on patients with EAS indicated predominantly localised immune modifications. Similarly, localised dysregulation in the CD8 T cell compartment was found in RPL patients with a subsequent miscarriage, suggesting an important role of this immune population in adverse reproductive outcomes and potential to be explored in bigger, well-controlled cohorts. Again, I highlighted important factors that should be considered in clinical study design, not only parity and miscarriage, as previously discussed, but also the menstrual cycle phase. Although peripheral blood could be of high diagnostic and therapeutic value in reproductive disorders due to its accessibility, its findings should be taken cautiously considering big disparities between systemic and endometrial immunity.

5 Immunohistochemical evaluation of endometrial CD8+ T cells and lymphoid aggregates

5.1 INTRODUCTION

5.1.1 Immunohistochemistry

Immunohistochemistry is a well-established method that leverages the selective interaction between antibodies and antigens to identify specific molecules within cells and tissues, visualised with microscopy [279]. Traditional singleplex staining is restricted in the number of markers that can be simultaneously investigated, thereby limiting the understanding of complex biological interactions [280]. To address this, significant progress has been achieved by integrating a deepened understanding of cellular and tissue biology and advanced methodologies, such as immunofluorescence. In fluorescence microscopy, antibodies against antigens of interest are conjugated with fluorophores/or a secondary antibody tagged with a fluorophore that targets the primary antibody. These molecules emit light upon excitation, enabling specific and targeted fluorescence labelling of more than one biomolecule within tissue samples [281]. Advancements in multiplexing techniques have led to the sequential use of different fluorophores and antibodies with intermittent bleaching steps to remove residual fluorescence from the previous labelling round, allowing for the simultaneous staining of the sample with up to 50 markers [282]. Furthermore, the ChipCytometry™ platform operates by loading single cells or whole tissue samples onto a specialised chip or coverslip, forming an

enclosed chamber that acts as a live biorepository, where a dropwise flowthrough process facilitates antibody staining, washing, and multiplexing through reiterative cycles, ensuring extended sample preservation of markers for up to two years, allowing for non-destructive, repeated analysis [283]. This proves especially advantageous for research in reproductive immunology studying highly complex and dynamic endometrial immunity with anticipated constraints on tissue sample availability.

5.1.2 Endometrial immunohistology

T cells are the predominant endometrial immune population during the proliferative phase, in which they are mostly found dispersed across the endometrium as single stromal and intraepithelial cells but also in small LAs, which grow throughout the cycle due to cell trafficking [6]. IELs are distinctive immune cells located within the mucosal epithelium and act as the primary defence mechanism against pathogens and inflammatory disease mediators in the gut, where they are predominantly TCRγδ+CD4-CD8αα+CD8αβ- and TCRαβ+CD4-CD8αα+CD8αβ- [284]. Endometrial IELs display cycle phase-dependent phenotypes, yet their functional characteristics, along with their role in embryo implantation, placentation, and pregnancy, remain elusive despite being the first to come into contact with the allogeneic blastocyst [116]. The most utilised marker for IELs identification is CD103, a cell surface glycoprotein and a member of the integrin family, also known as integrin αE (ITGAE) that binds to E-cadherin, a cell adhesion molecule on epithelial cells, which assures its specificity for intraepithelial localisation [285]. In the proliferative phase, most IELs are CD8 T cells with higher levels in the luminal than in the glandular epithelium. In contrast, in the late secretory phase and early pregnancy, NK cells become predominant, and the ratio favours glandular IELs [286].

Endometrial CD8 T cell levels remain stable in the secretory phase. Still, the population seems less numerous in the functional layer of the endometrium as they migrate to the basal layer, where CD8 T cells are primarily concentrated in LAs [6]. As previously described, these aggregates consist of various leukocytes, including adaptive CD8 T, CD4 T and B cells, and innate immune types, such as macrophages and NK cells. They can increase up to 100-fold in cellular size with the cycle progression [7, 60]. The role of LAs remains speculated, nevertheless, they are only found in the lower FRT during infection, while their physiological presence in the endometrium points towards their importance in preserving endometrial memory reservoir during endometrial shedding [6, 287, 288]. The expression of proliferation marker Ki-67 in LAs shows higher levels of cell division during the secretory phase compared to the proliferative phase. However, relatively low proliferation rates in LAs propose that CD8 T cells do not arise from single precursors but from cell trafficking into the endometrium [60, 117]. High endothelial venules (HEV), initially described as active recruiters of circulating lymphocytes into lymphoid organs, have recently been identified in human endometrium and decidua [289, 290]. They were surrounded by LAs, and their density strongly correlated with the abundance of T cells in the tissue. This again supports the hypothesis that LAs form through lymphocyte trafficking rather than *in situ* cell proliferation [290]. Regarding their function, HLA-DR expression in LAs suggests an immunoprotective role for LAs, potentially through antigen presentation function, as they resemble a germinal centre-like structure [117]. However, despite the increase in the expression of commonly used markers for immune cell activation, namely HLA-DR and CD38, in the late versus early secretory phase, CD8 T cell cytotoxicity in the secretory phase decreases through hormonal regulation [5, 117, 291]. While perforin and granzyme A depend on oestrogen and progesterone, intracellular granzyme B expression in CD8 T cells seems to be affected by progesterone only via indirect signalling facilitated by TGF-β [63].

5.1.3 Myometrial immunohistology

Myometrium, the middle layer of the uterine wall, consists mainly of smooth muscle tissue and is the largest volume component of the uterus [292]. It plays a pivotal role in uterine contractions during menstruation and parturition, which are considered inflammatory processes [3, 293]. Even though myometrium is involved in various reproductive pathologies with implicated immune modulations, such as adenomyosis and preterm birth, its immune environment remains relatively unexplored [150, 294, 295]. In the non-pregnant uterus, neutrophils, monocytes, DCs, macrophages, NK cells, T cells (CD8+ and CD4+) and B cells have been identified. T cells were the predominant population, and no significant variations were found between peri- and post-menopausal women [296].

5.2 CHAPTER OBJECTIVES

In this final experimental chapter, I aimed to spatially evaluate previous findings and gain information about potential cell-to-cell interaction by visualising CD8 T cells and other immune cell populations across the endometrium and myometrium in different cycle phases as well as in health and disease. Altogether, I investigated 33 endometrial samples, of which three were full-thickness biopsies. After quality screening, protocol optimisation and preliminary brightfield microscopy, specimens were stained with fluorophore-conjugated antibodies and images were taken with the confocal microscope. In addition, three full-thickness biopsies underwent chip cytometry with an extended panel to gain in-depth insights into endometrial immunohistology. Qualitative analysis was performed to assess significant immunophenotypical differences in the endometrium across the cycle but also in the myometrium. Spatial and functional properties of CD8 T cells were evaluated along with a detailed analysis of LAs in the basal layer of the endometrium and IELs. The immunohistochemistry pilot study importantly complemented our current knowledge of the spatial distribution of endometrial CD8 T cells and improved the understanding of the utilisation of CD103 for IEL identification.

Objectives:

1. To describe the main differences in immune cell distribution between the proliferative and secretory phases of the menstrual cycle.
2. To investigate the distribution of tissue residency markers across the endometrium and myometrium.
3. To determine the histological organisation of CD8 T cells and LAs.
4. To identify functional characteristics of endometrial CD8 T cells, such as proliferative capacity, activation status and cytotoxic potential.

5. To observe differences in endometrial and myometrial immunohistological properties between endometriosis patients and controls.
6. To assess the location of endometrial CD8+ MAIT-like cells in endometriosis patients and controls.

5.3 METHODS

5.3.1 Study design

As part of standard operating procedure (SOP) optimisation and quality screening, 283 snap-frozen eutopic endometrial samples from women undergoing surgery between 1993 and 2010 for various gynaecological pathologies and tubal ligation/anastomosis were investigated. Following PICO criteria (Error! Reference source not found.), 1,334 samples were screened, and of those, 283 were stained to assess sample quality [107]. Finally, 30 eligible samples of high quality from the secretory phase were included and divided into four groups according to their gynaecological history: subfertile patients with endometriosis (n=8), fertile patients with endometriosis (n=6), healthy fertile controls (n=8) and controls with unexplained subfertility (n=8) (Figure 36). In addition, three full-thickness endometrial biopsies from endometriosis patients in the proliferative phase (n=2) and non-endometriosis control from the secretory phase were selected. Patients were recruited into two studies (REC reference: 19/LO/1802): Investigation of gene expression in normal endometrium and endometriosis COREC 00.156 and Investigating the causes of infertility OxREC No.C02.358.

Table 14: PICO framework to select eligible samples for immunohistochemistry staining.

Population	**INCLUDED** • 18–39 years old female • **All stages of surgically confirmed endometriosis** with no other gynaecological pathologies • **Fertile and subfertile** • Any phase of a regular uterine cycle • High-quality endometrial biopsy sample
	EXCLUDED Participants experiencing a miscarriage or undergoing hormonal treatment within the last three months. Those having polycystic ovarian syndrome (PCOS), hydrosalpinx, pelvic inflammatory disease or cancer.
Intervention	Immunohistochemistry.
Control	**INCLUDED** • 18–39 years old female • **Endometriosis surgically ruled out** with no other gynaecological pathologies • **Fertile and subfertile** • No previous miscarriages or ART-treatments • Any phase of a regular uterine cycle • High-quality endometrial biopsy sample
	EXCLUDED Participants experiencing a miscarriage or undergoing hormonal treatment within the last three months. Those having polycystic ovarian syndrome (PCOS), hydrosalpinx, pelvic inflammatory disease or cancer.
Outcome	Qualitative differences in histological organization of CD8+ T cells and lymphoid aggregates in eutopic endometrium of fertile and subfertile women with endometriosis compared with fertile and subfertile women without endometriosis.

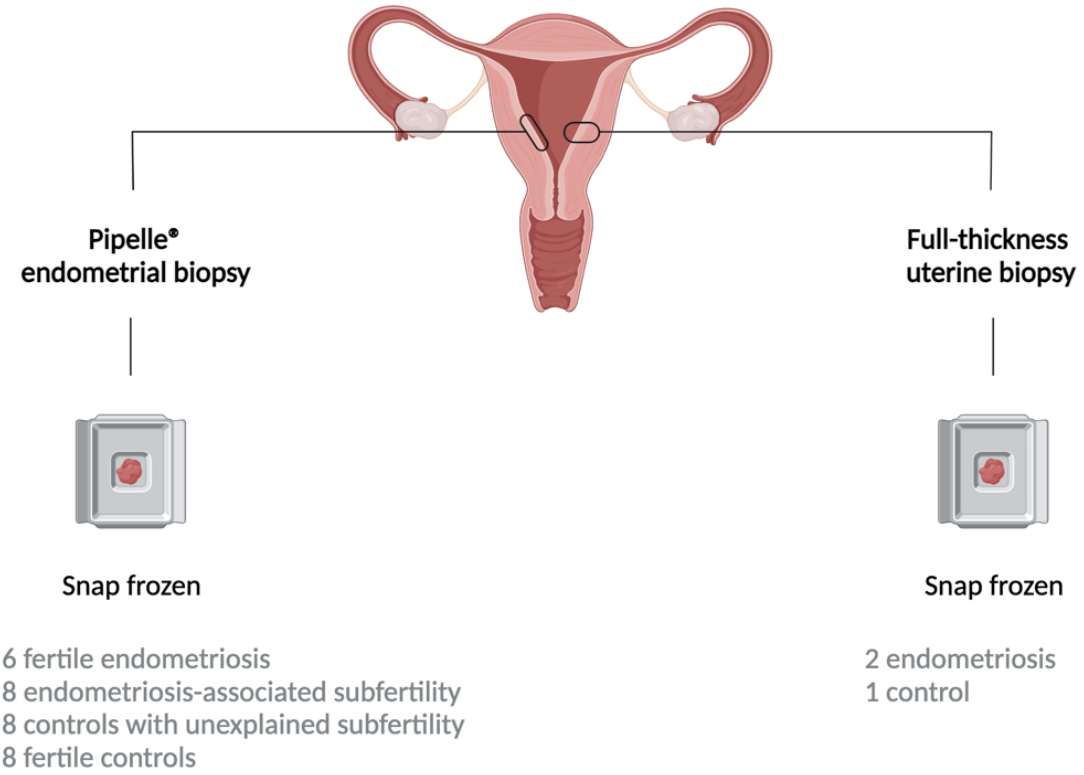


Figure 36: Participants recruited for immunohistochemistry staining.

30 pipelle endometrial biopsies and 3 full-thickness biopsies were included in the study. Of the 30 pipelle endometrial biopsies, 6 were from fertile endometriosis patients, 8 from patients with endometriosis-associated subfertility, 8 from controls with unexplained subfertility and 8 from fertile controls. 2 of full-thickness uterine biopsies were from endometriosis patients in the proliferative phase and 1 was from a fertile control patient in the secretory phase.

5.3.2 Conventional immunohistochemistry

5.3.2.1 Sample preparation

Tissue collection

Endometrial biopsy samples were collected using an Endocell® disposable cell sampler (Cooper Surgical, USA), except for the three full-thickness biopsy samples obtained after the hysterectomy. Histological dating was performed by a pathologist. They were stored at -80°C at the Nuffield Department of Women's and Reproductive Health, University of Oxford.

Cryosectioning and immunohistolochemical staining

Cryosectioning and immunohistochemical staining followed established protocols detailed in Appendix 7.3. Serial cryostat sections (5–7 µm thickness) were mounted on SuperFrost® Plus (VWR, USA) slides, dried overnight and stored at -80°C until immunolabelling. All tissue sections were fixed in acetone for five minutes at -20°C, washed twice with PBS and blocked with 10% FCS/PBS at room temperature for one hour. Initially, sections were incubated at room temperature for one hour with a primary antibody and 30 minutes with a secondary antibody. I performed 3,3'-Diaminobenzidine (DAB) staining with horseradish peroxidase (Vector Laboratories, USA) and haematoxylin (Sigma-Aldrich, USA) counterstain to gain better insights into LAs in human endometrium and to assess tissue quality of 283 samples. Finally, 30 high-quality samples in the mid-secretory phase s have been stained. After blocking, they were incubated with directly conjugated primary antibodies at 4°C overnight. All antibodies were diluted in 0.5% FCS and washes in PBS were carried out twice after each incubation.

Table 15: Antibodies and concentrations used in DAB-staining.

Marker	Purpose	Clone	Concentration	Isotype	Supplier
CD45	Leukocytes	2B11+PD7/26	10 µg/mL	Mouse IgG1, κ	Dako
CD8	T cells, NKT-Like cells	C8/144B	22 µg/mL	Mouse IgG1, κ	Santa Cruz Biotechnology
CD68	Monocytes, macrophages	Y1/82A	2.4 µg/mL	Mouse IgG2b, κ	Biolegend
Negative control	NA	Goat anti-mouse	22 µg/mL	Mouse IgG1	Dako
Secondary antibody	NA	Immunoglobulins/HRP	10 µg/mL	Various	Dako

Table 16: Antibodies and concentrations used in direct immunofluorescence staining.

Marker	Purpose	Clone	Concentration	Conjugate	Isotype
CD45	Leukocytes	HI30	10 µg/mL	Alexa Fluor®488	Mouse IgG1, κ
CD8	T cells, NKT-Like cells	C8/144B	7.5 µg/mL	Alexa Fluor®594	Mouse IgG1, κ
		RPA-T8	5 µg/mL	Alexa Fluor®594	Mouse IgG1, κ
CD4	T cells	OKT4	10 µg/mL	Alexa Fluor®647	Mouse IgG2b, κ
Ki67	Cell proliferation	Ki67	1 µg/mL	Alexa Fluor®488	Mouse IgG1, κ
HLA-DR	Cell activation	L243	0.1 µg/mL	Alexa Fluor®647	Mouse IgG2a, κ
Pan-cytokeratin	Epithelium	AE1/AE3	0.5 µg/mL	Alexa Fluor®488	Mouse IgG1, κ
Isotype controls		MOPC-21	10 µg/mL	Alexa Fluor®488	Mouse IgG1, κ
		MOPC-21	7.5 µg/mL	Alexa Fluor®594	Mouse IgG1, κ
		MOPC-173	0.1 µg/mL	Alexa Fluor®647	Mouse IgG2a, κ
		MPC-11	10 µg/mL	Alexa Fluor®647	Mouse IgG2b, κ
		MOPC-21	10 µg/mL	Alexa Fluor®647	Mouse IgG1, κ
		MPC-11	2.4 µg/mL	Alexa Fluor®488	Mouse IgG2b, κ

All antibodies were purchased from Biolegend except pan-cytokeratin (AE1/AE3) which was purchased from Invitrogen.

5.3.2.2 Imaging

DAB-stained samples were observed with the optical microscope Leica DM2500 (Leica Microsystems, Germany). Immunofluorescent sections from 30 samples were observed and analysed using the spinning disc confocal microscope Axiovert 200M (Carl Zeiss AG, Germany). The exposure time and threshold fluorescence for positive stains were set according to the

isotype control antibodies. Five random non-overlapping fields were captured under x20 and x40 magnification. The location and relation of CD8 T and LAs to the epithelium and glands were qualitatively described. Proliferative and activation markers were examined. No statistical analysis was performed.

5.3.3 Multiplex immunohistochemistry

Multiplex immunohistochemistry with ZellScannerONE™ ChipCytometry™, including sample preparation, was undertaken with Dr Kate Powell as part of a collaboration with the Klenerman Group and will continue beyond the submission of this thesis [283]. OCT-embedded endometrial samples were cryosectioned onto coverslips and inserted in cytometer chips (Zellsafe Tissue chips, Zellkraftwerk, Germany). Sections were fixed for 10 minutes at room temperature with 4% paraformaldehyde before being rinsed with 10 mL PBS. Non-specific binding was inhibited by incubation in 5% goat serum in PBS at room temperature for one hour. For staining, fluorophore-conjugated antibodies (Table 17) were diluted in PBS. Immunostaining was performed with up to three colours used simultaneously, and the sample was repeatedly photobleached after the image had been taken. The steps were repeated as many times as needed to complete the panel [297]. Images were captured using a ZellScannerONE™ ChipCytometry™ instrument (Canopy Biosciences, USA) and ZellExplorer software (Canopy Biosciences, USA).

Table 17: Antibodies used for multiplex immunohistochemistry.

Marker	Purpose	Fluorophore	Clone	Company	Catalogue number	Lot number	µg antibody in 800 µg total
CD3	T cells, NKT-Like cells	PerCP	UCHT1	Biolegend	300428	B225944	5:800
CD4	CD4 T and NKT-Like cells	PE	REA623	Miltenyi	130.113.787	52203903498	1.5:800
CD8	CD8 T, NK, and NKT-Like cells	PerCP/Cy5.5	SK1	Biolegend	344710	B311545	5.5:800
CD14	Monocyte differentiation	PE	599	Miltenyi	130-110-577	5210404237	8:800
CD19	B cells	PE	REA675	Miltenyi	130-114-172	5220505258	4:800
CD20	B cells	PE	LT20	Miltenyi	130-113-936	5210508017	6:800
CD26	Endothelium	PE	FR10-11G9	Miltenyi	130-126-411	5220601927	8:800
CD31	Endothelium	PerCP/Cy5.5	WM59	Biolegend	303132	B224559	1:1000
CD38	Monocyte, dendritic cell, T cell, and B cell activation/differentiation	PerCP/Cy5.5	HIT2	Biolegend	303522	B229737	5:800
CD45	Leukocytes	FITC	HI10	Biolegend	304038	B212277	1:800
CD56	NK cells, γδ T cell activation	FITC	REA196	Miltenyi	130-114-740	5220910174	8:800
CD68	Macrophages	AF488	KP1	Santa Cruz	sc-20060	B0521	1:1000
		PE	REA886	Miltenyi	130-114-460	5210806056	8:800
CD69	Tissue residency	PE	REA824	Miltenyi	130-112-802	5190124376	4:800
CD103	Tissue residency	PE	Ber-ACT8	Biolegend	350205	B258753	10:800
CD161	NK cell cytotoxicity and T cells	PE	REA631	Miltenyi	130-113-596	5210606249	6:800
E-cadherin	Epithelium	PE	67A4	Miltenyi	130-128-886	5220801931	10:800
Pan-cytokeratin	Epithelium	FITC	C-11	GeneTex	GTX11212	821900751	1:4000
Granzyme B	Cytotoxicity	PE	REA226	Miltenyi	130-116-654	5210910374	8:800
HLA-DR	T cell and monocyte activation	PerCP/Cy5.5	L243	BD	339216	0224809	25:800
		FITC	REA805	Miltenyi	130-111-788	5230306077	1:800
Ki67	Cell proliferation	PE	REA183	Miltenyi	130-120-557	5220910165	10:800
SMA	Myoepithelium	FITC	REAL650	Miltenyi	130-123-363	5220201791	1:800

5.4 RESULTS

5.4.1 Endometrial immune cells

5.4.1.1 Across the menstrual cycle

A representative endometrial sample from the proliferative phase of the menstrual cycle (Figure 37) demonstrates the presence of LAs in the basal layer of the endometrium, consisting predominantly of CD8 T cells, whereas various single cells, including CD4 T, B, NK cells and macrophages are found across the epithelium, endometrium and myometrium. Importantly, no LAs were found in the functional layer or myometrium; cell aggregation seen in the picture is the content of a blood vessel rather than LA. Qualitatively, I did not observe decreased levels of CD8 T cell population during the secretory phase (Figure 38). However, CD8 T cells were less spatially dispersed across the stroma and primarily found in the epithelium and LAs. CD4 T cells, NK cells and macrophages were found throughout all endometrial and myometrial layers, with NK cells present in LAs in the secretory but not proliferative phase.

5.4.1.2 Tissue residency markers

To study tissue resident endometrial immune populations, I investigated the expression of CD69 and CD103 markers (Figure 39). These were expressed on most CD8 T cells and other immune cells that occupy epithelial and mucosal tissues. They were highly expressed in LAs and the epithelium, less so in the stroma, especially CD103. While CD103 is crucial in enabling immune cell adhesion to the epithelium, CD69 expression is not specific to tissue residency and can be a sign of cell activation. Therefore, the interpretation of the findings regarding CD69 is limited without markers delineating effector memory subsets.

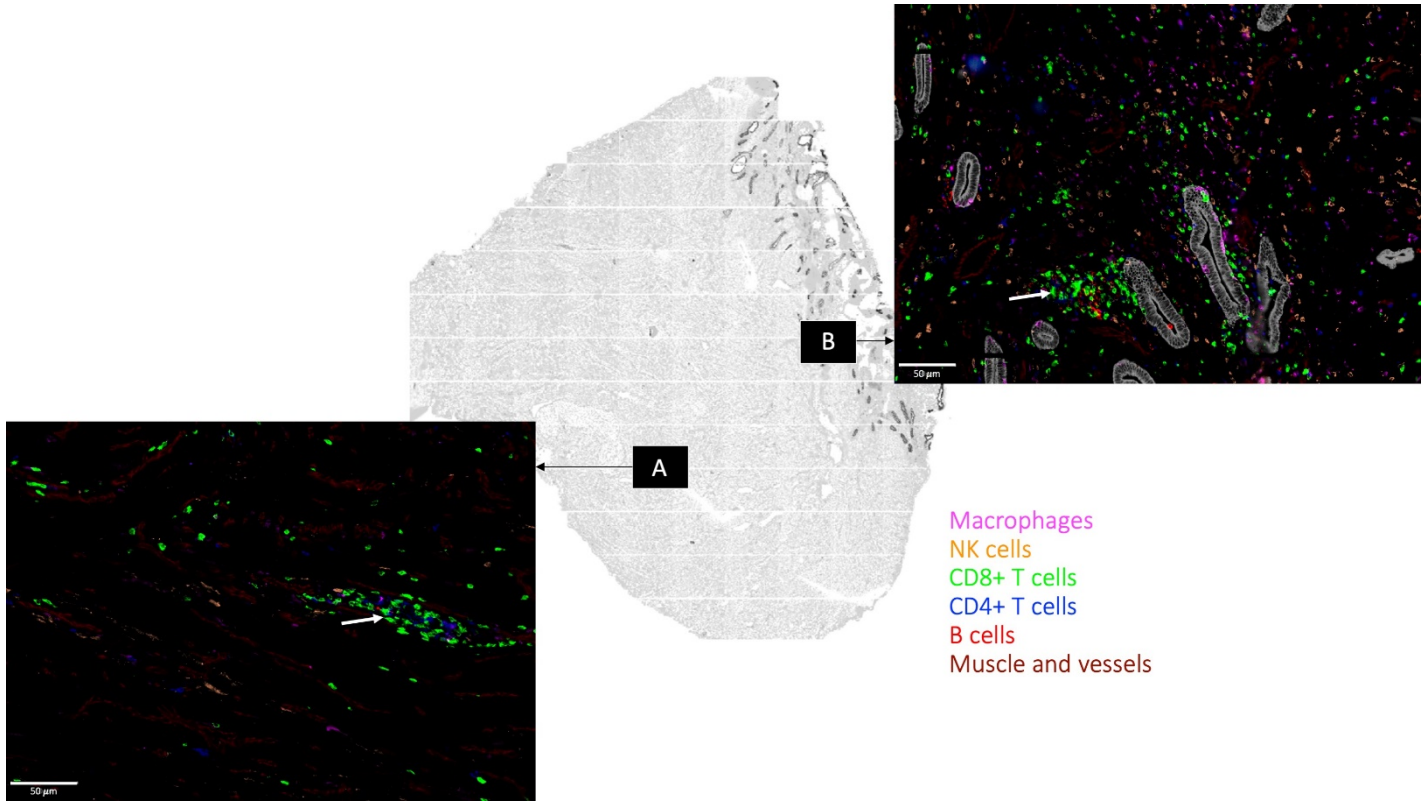


Figure 37: Endometrial immune cells in the proliferative phase of the menstrual cycle.

Image shows a section of full-thickness endometrial biopsy with myometrium taken from a patient with endometriosis-associated subfertility. (**A**) Macrophages, NK cells, CD8+ and CD4+ T cells dispersed throughout the myometrium and concentrated in the blood vessel (arrow). (**B**) Immune cells as single cells in the stroma and epithelium and lymphoid aggregates (arrow) in the basal layer of the endometrium. (**A and B**) False-color fluorescence imaging macrophages in pink (CD14, CD68), NK cells in orange (CD16, CD56), CD8+ T cells in green, CD4+ T cells in dark blue, B cells in red (CD19, CD20), epithelium in light grey (pan-cytokeratin), cell nuclei in dark grey (hoechst), muscle cells (SMA) and blood vessels (CD26, CD31) in dark red. Multiplex imaging was performed with ZellScannerONE™ ChipCytometry™ instrument.

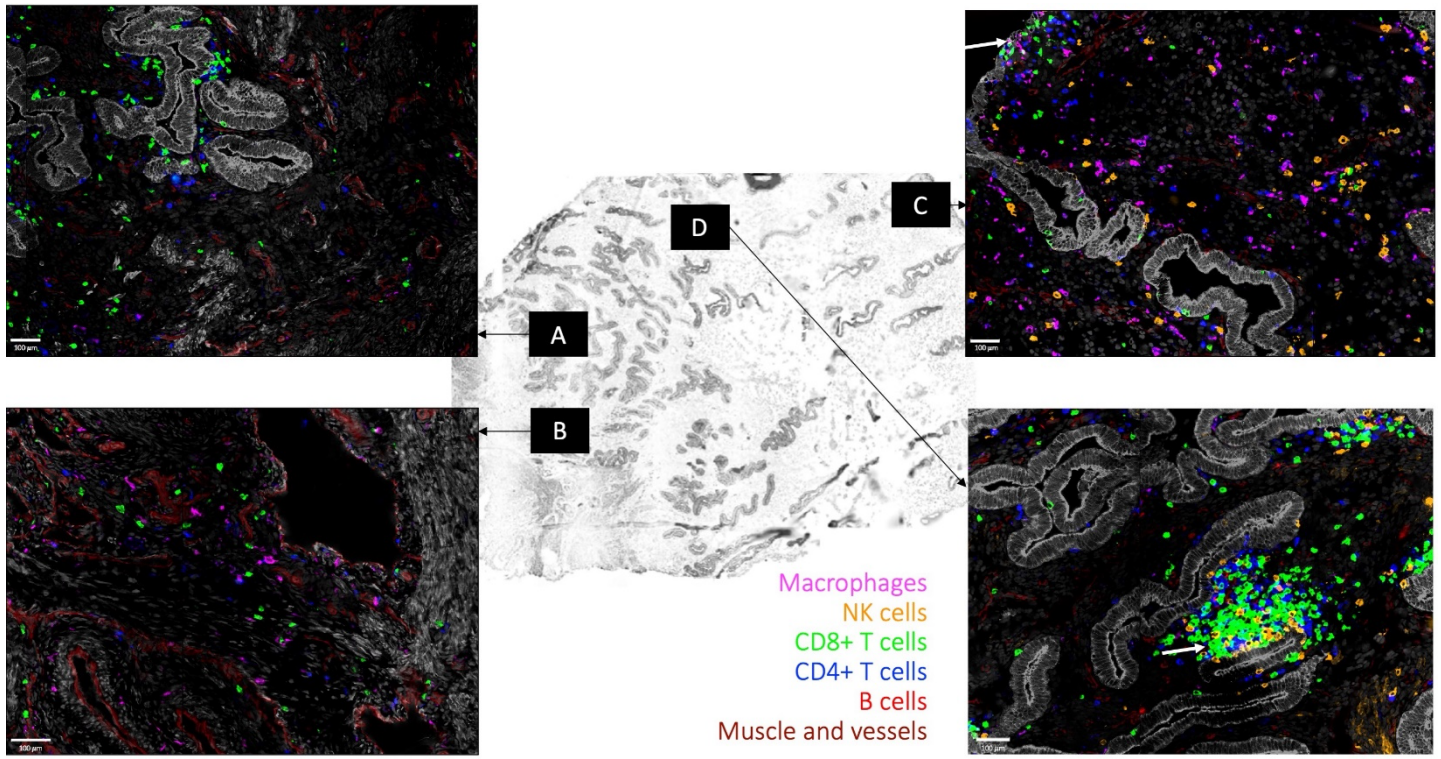


Figure 38: Endometrial immune cells in the late secretory phase of the menstrual cycle.

Image shows a section of full-thickness endometrial biopsy with myometrium taken from a fertile patient without endometriosis. (**A**) Predominantly single CD8+ and CD4+ T cells in the transitional layer between the myometrium and the endometrium. (**B**) Macrophages, CD8+ and CD4+ T cells dispersed throughout the myometrium. (**C**) Luminal epithelium (arrow) is rich with CD8+, CD4+ T cells and macrophages. NK cells are dispersed across stroma along with more CD4+ T cells and macrophages. (**D**) Lymphoid aggregates (arrow) in the basal layer predominantely consist of CD8+ T cells, with some CD4+ T cells, B cells and NK cells. (**A–D**) False-color fluorescence imaging macrophages in pink (CD14, CD68), NK cells in orange (CD16, CD56), CD8+ T cells in green, CD4+ T cells in dark blue, B cells in red (CD19, CD20), epithelium in light grey (pan-cytokeratin), cell nuclei in dark grey (hoechst), muscle cells (SMA) and blood vessels (CD26, CD31) in dark red. Multiplex imaging was performed with the ZellScannerONE™ ChipCytometry™ instrument.

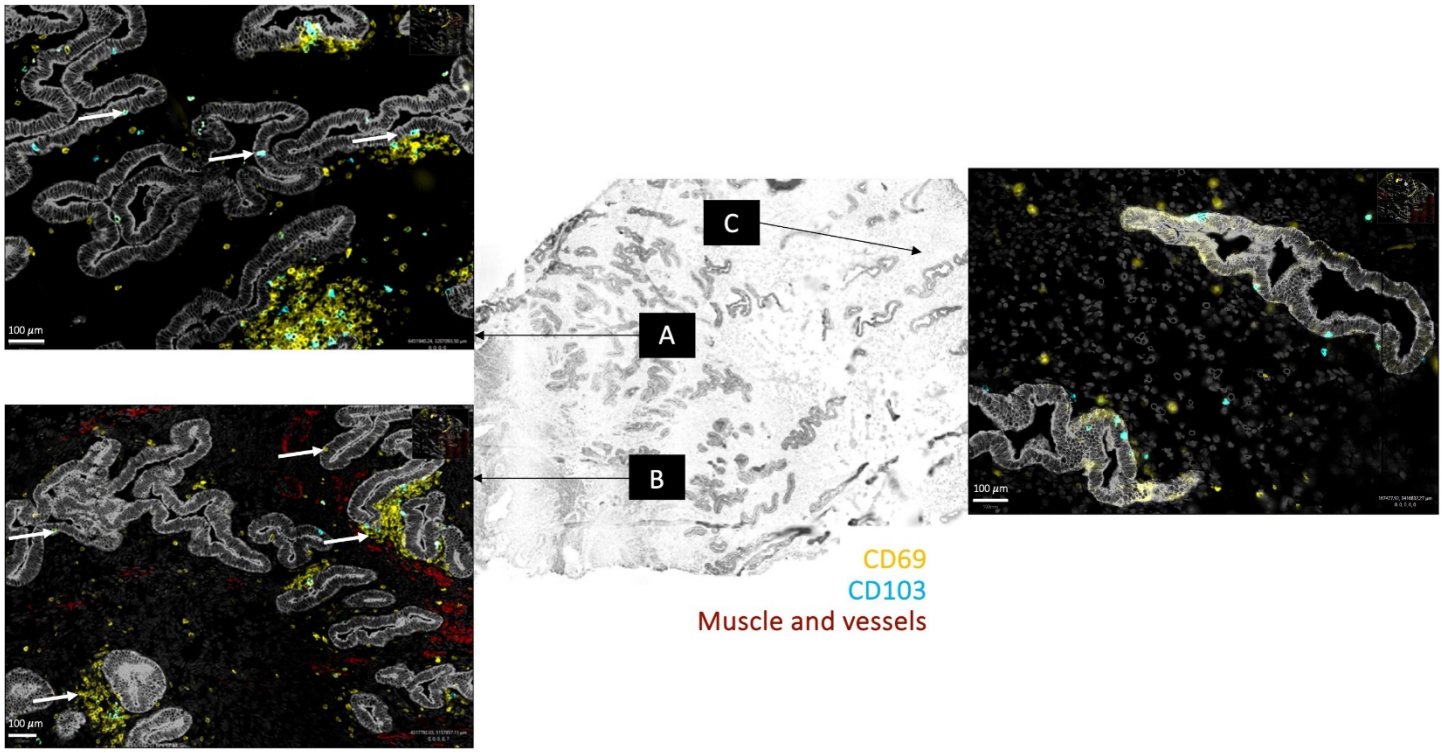


Figure 39: Tissue residency markers in the late secretory phase endometrium.

Image shows a section of full-thickness endometrial biopsy with myometrium taken from a fertile patient without endometriosis. (**A**) Aggregates in the basal layer express high levels of CD69 and also CD103, which is predominantly found in the glandular epithelium (arrows). (**B**) In the transition phase between the basal layer and myometrium, CD69 and CD103 decreased along with less abundant lymphoid aggregates and intraepithelial cells (arrows). (**C**) Tissue residency markers CD69 and CD103 were less abundant in the functional layer of the epithelium. (**A–C**) False-color fluorescence imaging for tissue residency and activation marker CD69 in yellow and tissue residency marker CD103 in light blue. Epithelium in grey (pan-cytokeratin), cell nuclei in dark grey (hoechst), muscle (SMA) and blood vessels (CD26, CD31) are shown in dark red. Multiplex imaging was performed with ZellScannerONE™ ChipCytometry™ instrument.

5.4.2 Endometrial CD8+ T cells

Having gained evidence regarding the location of CD8+ cells in the endometrium, I next progressed to perform confocal microscopy to find detail structures and to investigate multiparameter protein identification.

5.4.2.1 Histological organization

CD8 T cells were found throughout the endometrium either as part of LAs (Figure 40 and Figure 41) or scattered around as single stromal cells (Figure 42) and IELs (Figure 43). Epithelial cells were detected using the pan-cytokeratin marker, found in the intracytoplasmic cytoskeleton of epithelial tissue. Staining with CD103 shows high but not complete specificity for IELs, with marker expression found on stromal CD8 T cells and CD8 T cells in LAs (Figure 44). Qualitatively, most IELs observed in the luminal and glandular epithelium were CD8+, with others being CD4+, CD19+, CD56+ and CD68+.

5.4.2.2 Functional characteristics

Human endometrium contains proliferating (Figure 45) and activated (Figure 46) CD8 T cells. Proliferation capacity in pipelle biopsy samples was assessed with the coexpression of Ki-67 marker and the coexpression of HLA-DR in addition to CD8 was considered for cell activation. In the full-thickness endometrial biopsy from the secretory phase (Figure 47), very few CD8 T cells expressed activation markers HLA-DR and CD38 despite HLA-DR being highly expressed in the luminal and glandular epithelium in the functional layer and CD38 predominantly indicated in the epithelium, where the majority of IELs are CD8 T cells as previously observed. Similarly, no granzyme B positive CD8 T cells were found despite the cytotoxicity marker being highly expressed near the luminal epithelium with numerous CD8 T cells.

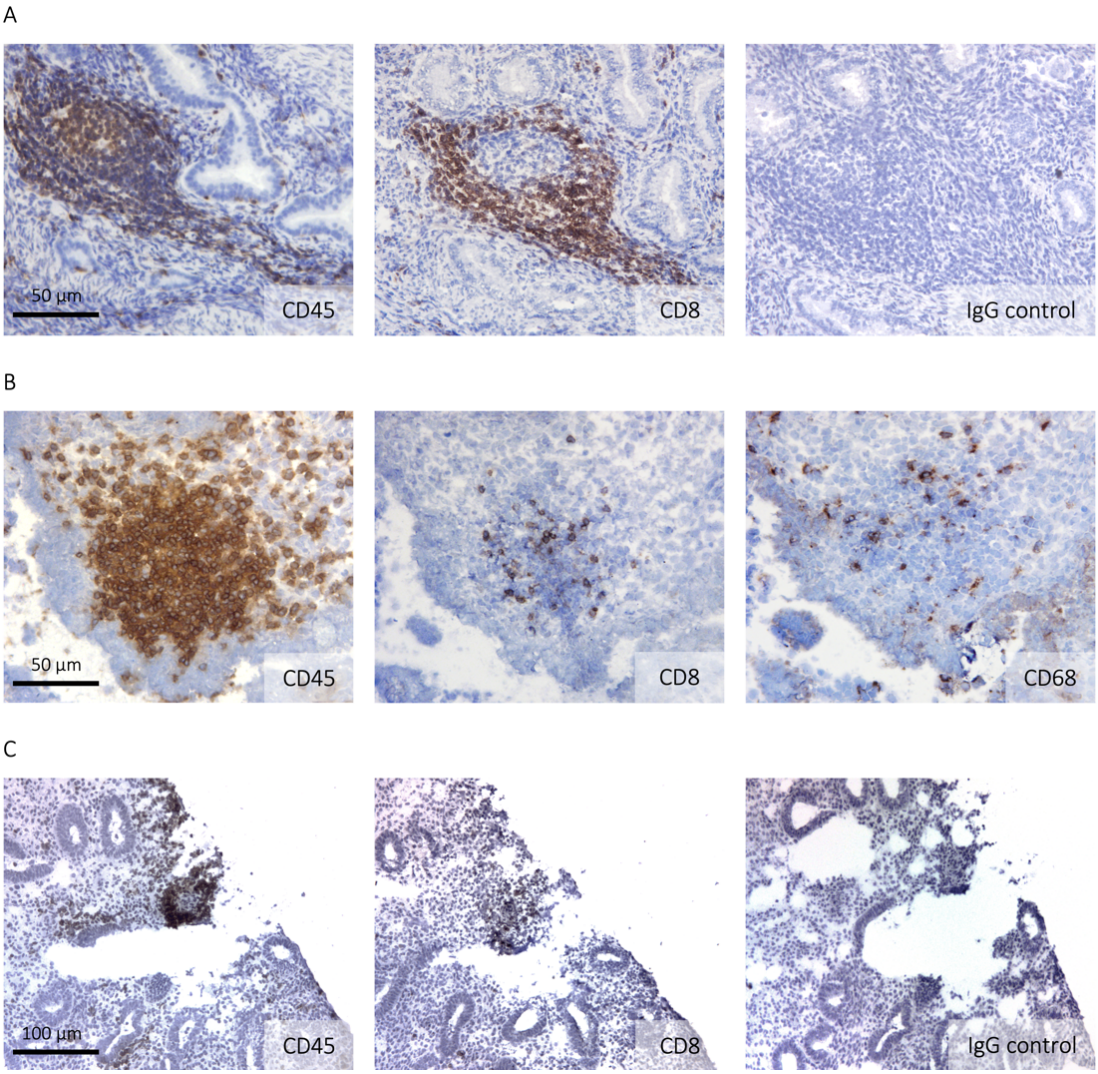


Figure 40: Representative examples of lymphoid aggregates in the secretory endometrium.

DAB staining with horseradish peroxidase for pan-leukocyte marker CD45 shows (**A**) a lymphoid aggregates (LA) of a healthy patient in the late secretory phase, (**B**) of a subfertile patient with an ovarian cyst in the mid-secretory phase and (**C**) of a subfertile patient with endometriosis in the early secretory phase. CD8 marker identifies cells forming the medium layer of LAs and CD68 marker reveals macrophages in the outer layer. Images were taken with the optical microscope Leica DM2500 (Leica Microsystems). Magnification: x20 (**A and B**) and x10 (**C**).

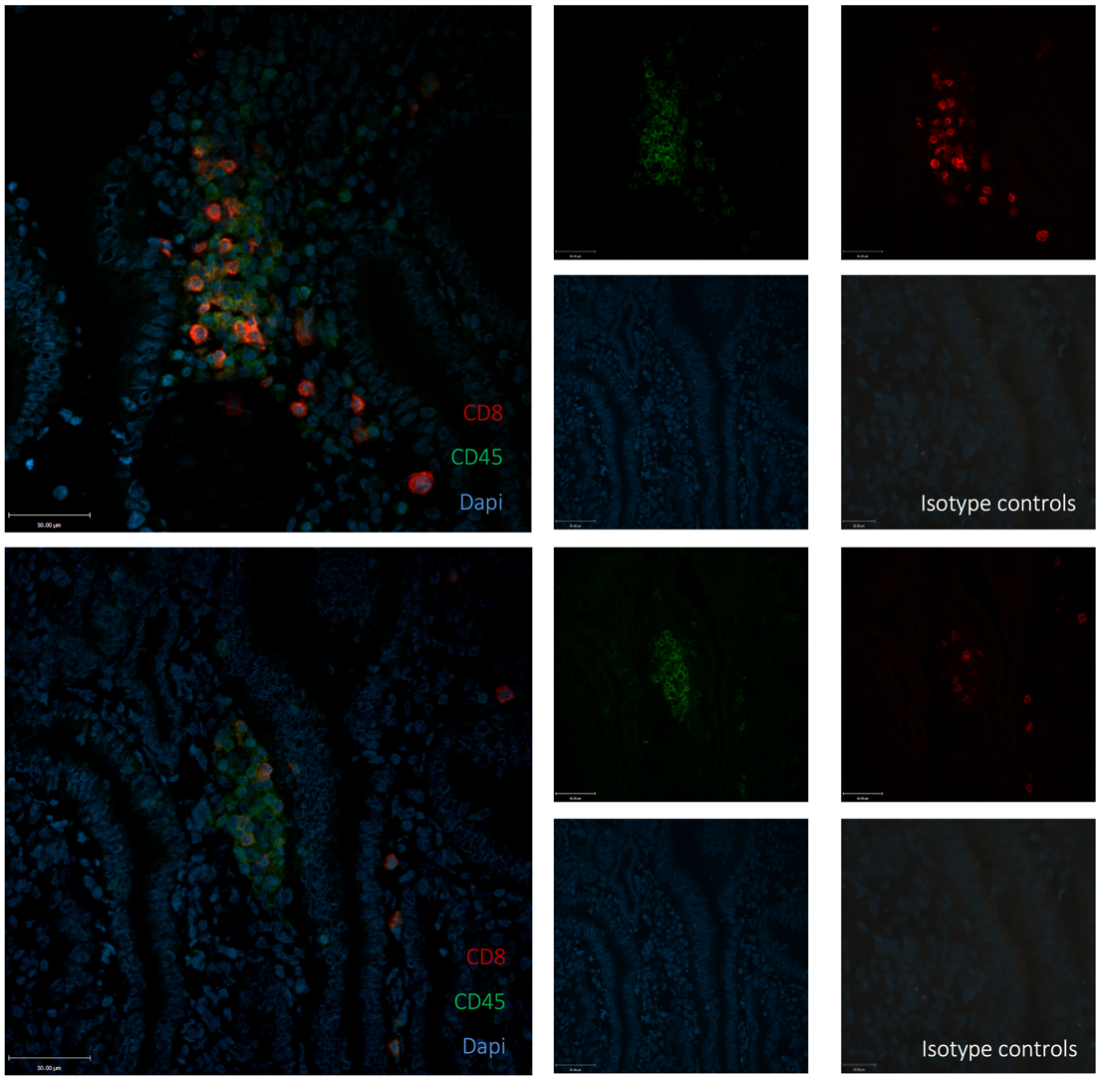


Figure 41: CD8+ T cells in lymphoid aggregates (LAs).

A human endometrial biopsy sample from the mid-secretory phase shows LAs consisting of various immune cells, which express a pan-leukocyte marker CD45 in green. CD8+ cells are coloured red/orange, depending on the coexpression of CD45. Separate channels are shown on the right. IgG controls served to adjust the appropriate threshold and exposure. Sections were stained with direct immunofluorescence and the images were taken with the spinning disc confocal microscope Axiovert 200M (Carl Zeiss AG). Magnification: x20.

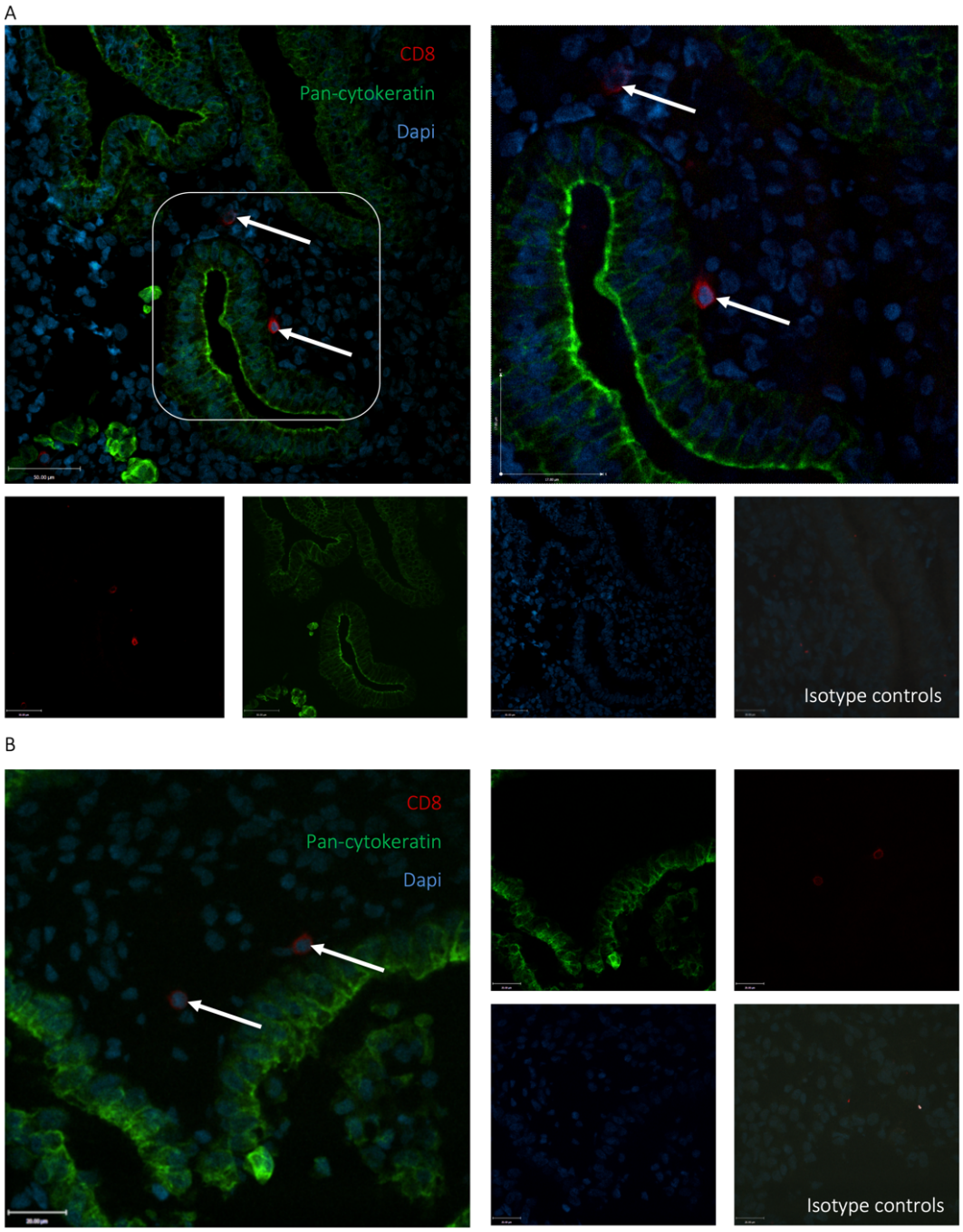


Figure 42: Single stromal CD8+ T cells.

Arrows show CD8 expressing cells in red, found in human endometrial biopsy samples from the mid-secretory phase. Glands are coloured green with pan-cytokeratin marker, found in the intracytoplasmic cytoskeleton of epithelial tissue. Separate channels are shown below (**A**) and on the right (**B**). IgG controls served to adjust the appropriate threshold and exposure. Sections were stained with direct immunofluorescence and the images were taken with the spinning disc confocal microscope Axiovert 200M (Carl Zeiss AG). Magnification: x20 (**A**) and x40 (**A and B**).

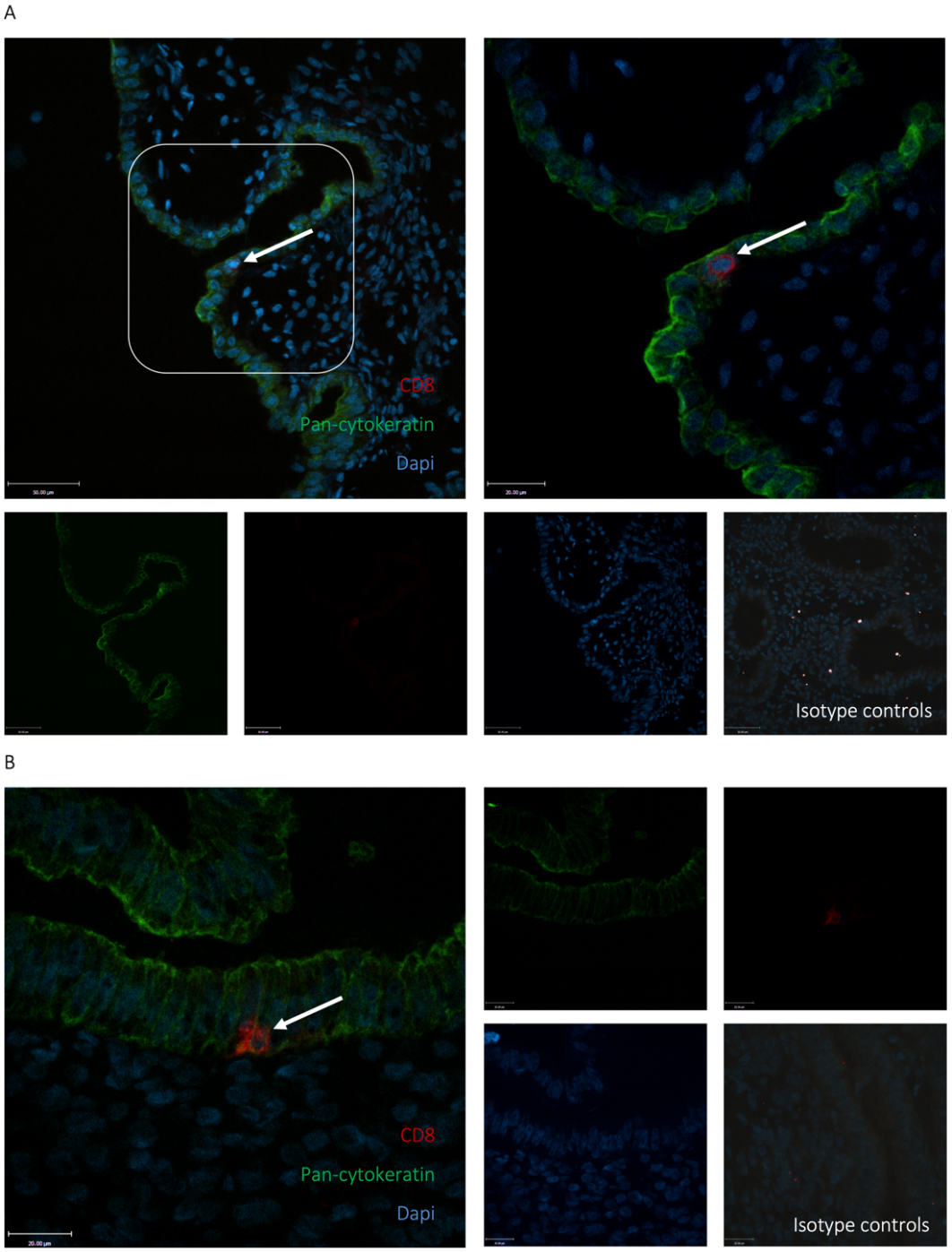


Figure 43: Intraepithelial CD8+ T cells.

Arrows show CD8+ cell insterted in the epithelium in human endometrial biopsy samples from the mid-secretory phase. CD8+ cell can be (**A**) either inserted into the luminal epithelium or (**B**) into the endometrial gland epithelium. Epithelial cells were detected using the pan-cytokeratin marker (green). Separate channels are shown below (**A**) and on the right (**B**). IgG controls served to adjust the appropriate threshold and exposure. Sections were stained with direct immunofluorescence and the images were taken with the spinning disc confocal microscope Axiovert 200M (Carl Zeiss AG). Magnification: x20 (**A**) and x40 (**A and B**).

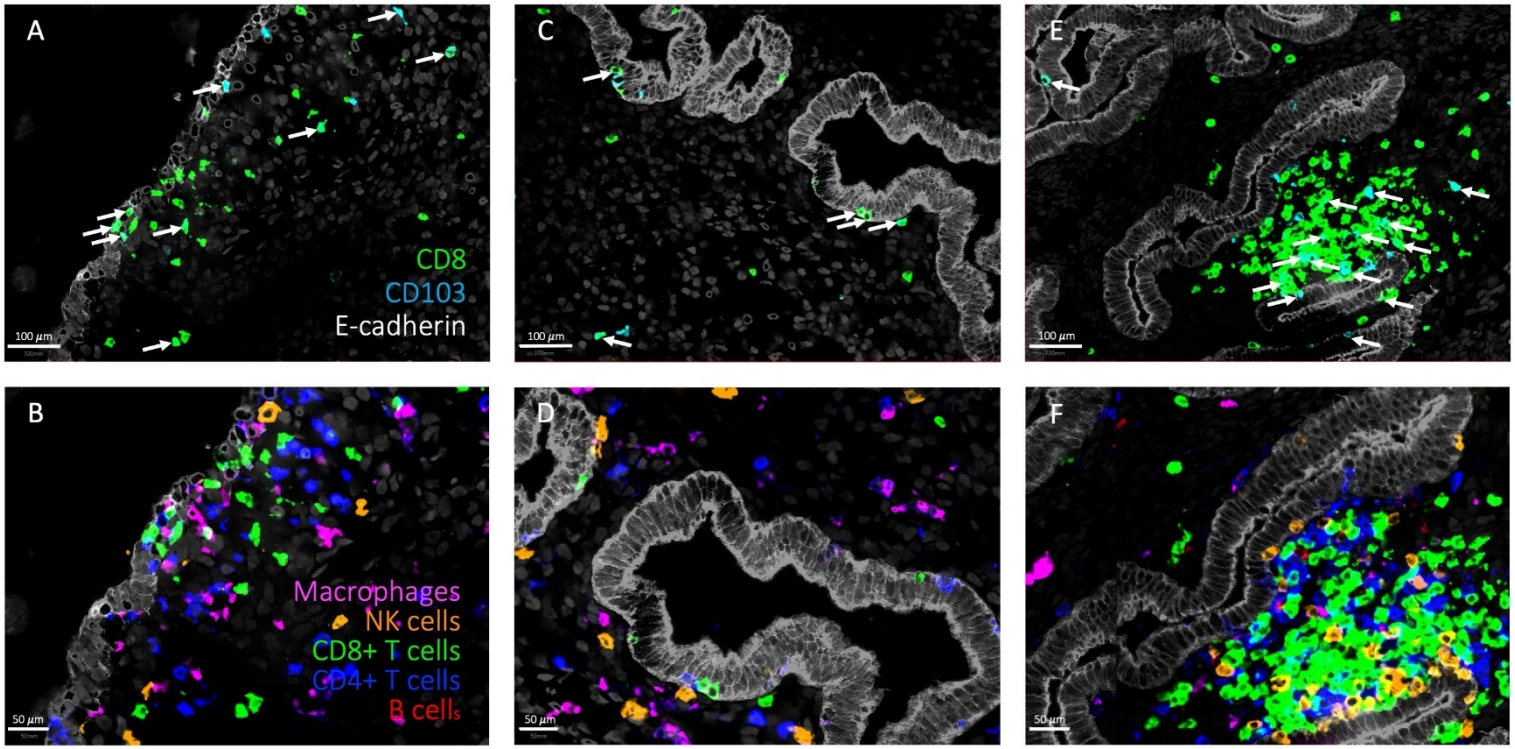


Figure 44: CD103 expression as a marker for intraepithelial lymphocytes.

Image shows a section of full-thickness endometrial biopsy taken from a fertile patient without endometriosis. (**A-B**) Several intraepithelial lymphocytes (IELs) were found in the luminal epithelium, the majority of which were CD8+ and CD4+. Only intraepithelial CD8+ were positive for CD103. (**C–D**) In the stromal part of the functional layer, CD8+CD103+ were found inserted into the glandular epithelium along with some CD4+ T cells and macrophages. (**E–F**) Lymphoid aggregates in the basal layer expressed high levels of CD103, which was also expressed on intraepithelial cells. (**A–F**) False-color fluorescence imaging for tissue residency marker CD103 in light blue, macrophages in pink (CD14, CD68), NK cells in orange (CD16, CD56), CD8+ T cells in green, CD4+ T cells in dark blue, B cells in red (CD19, CD20), epithelium in grey (pan-cytokeratin) and cell nuclei in dark grey (hoechst). Multiplex imaging was performed with ZellScannerONE™ ChipCytometry™ instrument.

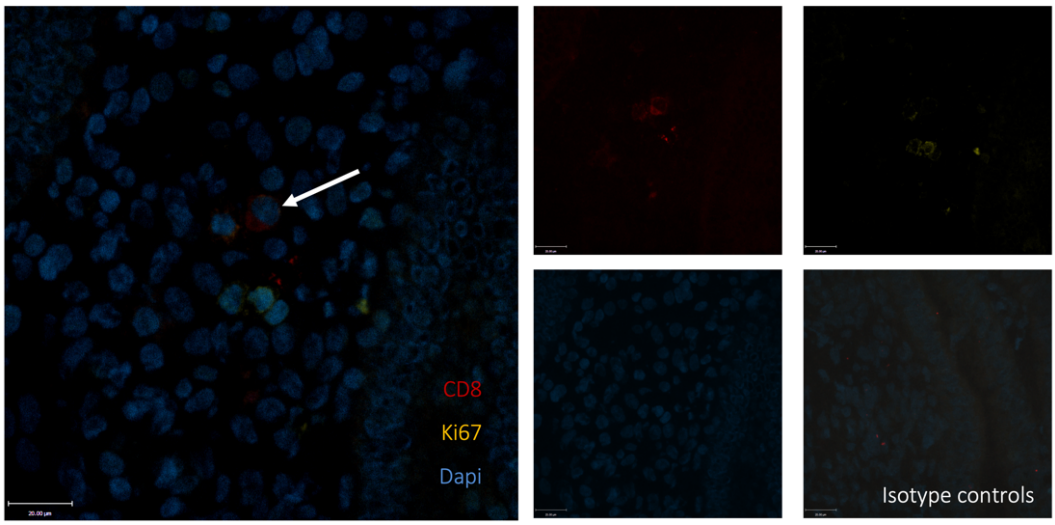


Figure 45: A representative example of a proliferating CD8+ T cell in human endometrium.

An arrow shows a CD8 and Ki67 coexpressing cell in human endometrial biopsy sample. Ki67 in yellow is a marker of cell proliferation. Separate channels are shown on the right. IgG controls served to adjust the appropriate threshold and exposure. Section was stained with direct immunofluorescence and the image was taken with the spinning disc confocal microscope Axiovert 200M (Carl Zeiss AG). Magnification: x40.

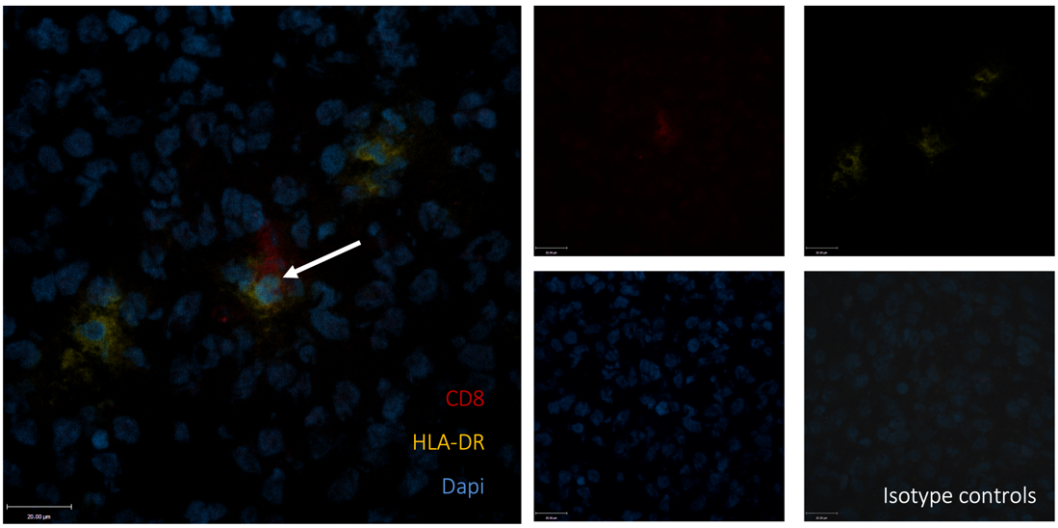


Figure 46: A representative example of an activated CD8+ T cell in human endometrium.

An arrow shows a CD8 and HLA-DR coexpressing cell in human endometrial biopsy sample. HLA-DR in yellow is a marker of T cell activation. Separate channels are shown on the right. IgG controls served to adjust the appropriate threshold and exposure. Section was stained with direct immunofluorescence and the image was aquired with the spinning disc confocal microscope Axiovert 200M (Carl Zeiss AG). Magnification: x40.

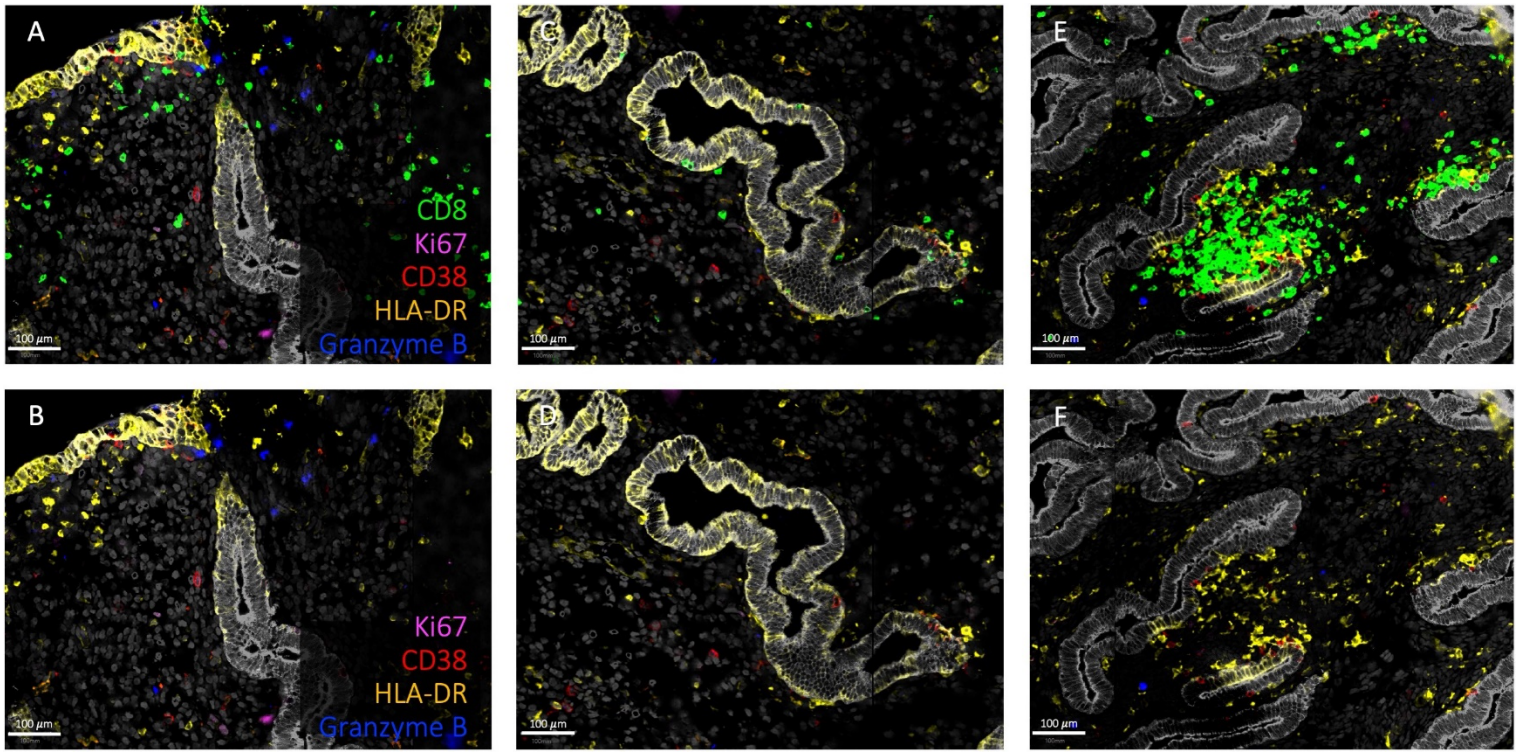


Figure 47: Functional properties of CD8+ T cells across the endometrium.

Image shows a section of full-thickness endometrial biopsy with myometrium taken from a fertile patient without endometriosis. (**A–B**) In the luminal part of the functional layer, high expression of HLA-DR is seen in the epithelium and several granzyme B and CD38 positive cells are scattered around it with no CD8+ cells being positive for either. (**C–D**) In the stromal part of the functional layer, high HLA-DR expression was observed in the glandular epithelium. (**E–F**) In the basal layer, HLA-DR was expressed on immune cells in lymphoid aggregates but not on the glandular epithelium. Some Ki-67, CD38 and granzyme B expressing cells were also observed none of which were CD8+. False-color fluorescence imaging for CD8+ T cells in green, proliferation marker Ki67 in pink, activation markers CD38 and HLA-DR in red and orange, respectively, cytotoxicity marker granzyme B in blue, epithelium in grey (pan-cytokeratin) and cell nuclei in dark grey (hoechst). Multiplex imaging was performed with ZellScannerONE™ ChipCytometry™ instrument.

5.4.2.3 CD8+ MAIT-like cells

To complement the full spectrum flow cytometry analysis findings (Chapter 4), I visualised endometrial CD8+ MAIT-like cells in three full-thickness biopsy samples. First, CD8+ MAIT-like cells were determined by coexpression of CD8 and CD161 and were found across the epithelium, stroma, and LAs of a fertile patient in the secretory phase (Figure 48). Next, tissue-resident CD8+CD161+ were identified with CD103, although CD69 marker expression was also explored (Figure 49). Finally, I observed endometrial CD8+ MAIT-like cells in the myometrium of a patient with endometriosis, while no cells of a similar phenotype were found in the other non-endometriosis sample (

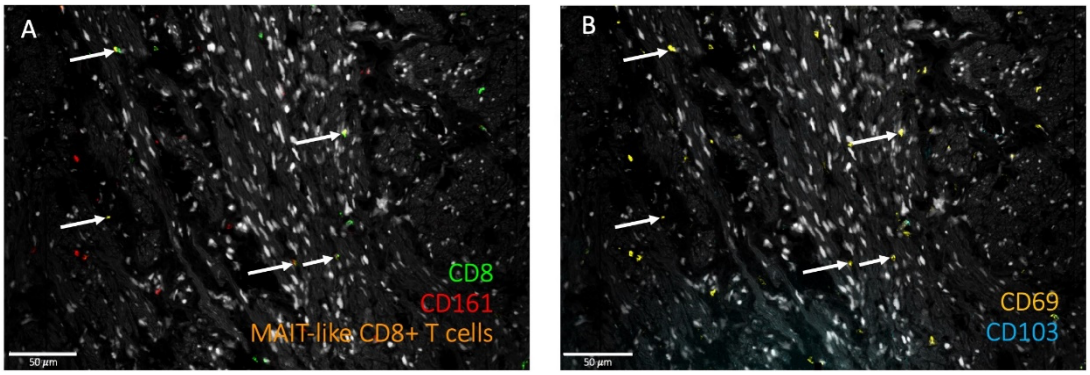


Figure 50: Myometrial CD8+ MAIT-like cells.

Image shows a section of full-thickness endometrial biopsy with myometrium taken from a patient with endometriosis. Similar to the endometrium, all CD8+ MAIT-like cells in the myometrium were tissue resident cells expressing CD69 and CD103. False-color fluorescence imaging for tissue residency markers CD69 (yellow) and CD103 (light blue) on CD8+ MAIT-like cells (CD8 and CD161) in orange, CD8+ T cells in green, CD161+ cells in red and cell nuclei in dark grey (hoechst). Multiplex imaging was performed with ZellScannerONE™ ChipCytometry™ instrument.

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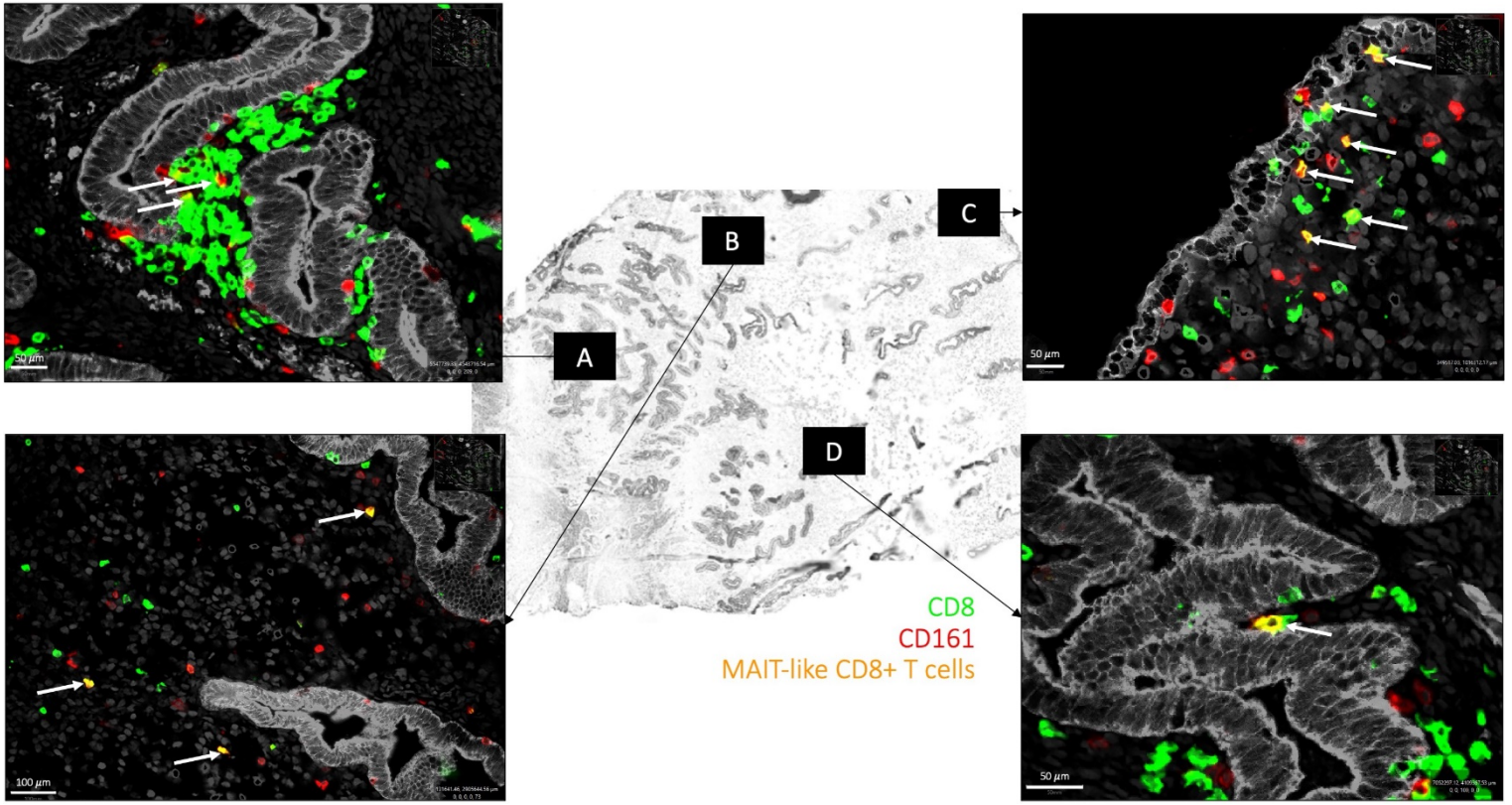


Figure 48: CD8+ MAIT-like cells in the endometrium.

Image shows a section of full-thickness endometrial biopsy taken from a fertile patient without endometriosis. (**A**) CD8+ MAIT-like cells were present in the lymphoid aggregate, (**B**) as single cells across the endometrial stroma, (**C**) in the luminal and (**D**) glandular epithelium. False-color fluorescence imaging for CD8+ MAIT-like cells (CD8 and CD161) in orange, CD8+ T cells in green, CD161+ cells in red, epithelium in grey (pan-cytokeratin) and cell nuclei in dark grey (hoechst). Multiplex imaging was performed with ZellScannerONE™ ChipCytometry™ instrument.

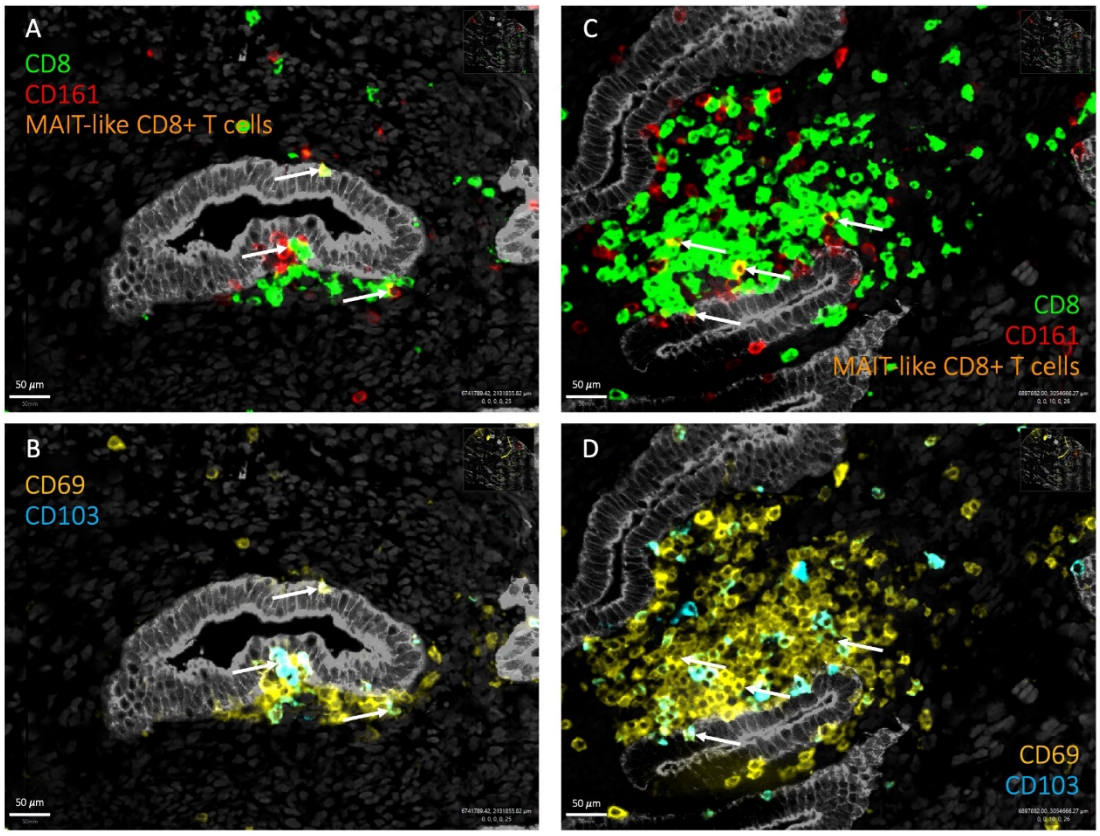


Figure 49: Endometrial CD8+ MAIT-like cells.

Image shows a section of a full-thickness endometrial biopsy taken from a fertile patient without endometriosis. In the functional layer (**A–B**) as well as in the basal layer (**C–D**), all observed CD8+ MAIT-like cells were tissue resident expressing CD69 and CD103 markers.False-color fluorescence imaging for tissue residency markers CD69 (yellow) and CD103 (light blue) on CD8+ MAIT-like cells (CD8 and CD161) in orange, CD8+ T cells in green, CD161+ cells in red, epithelium in grey (pan-cytokeratin) and cell nuclei in dark grey (hoechst). Multiplex imaging was performed with ZellScannerONE™ ChipCytometry™ instrument.

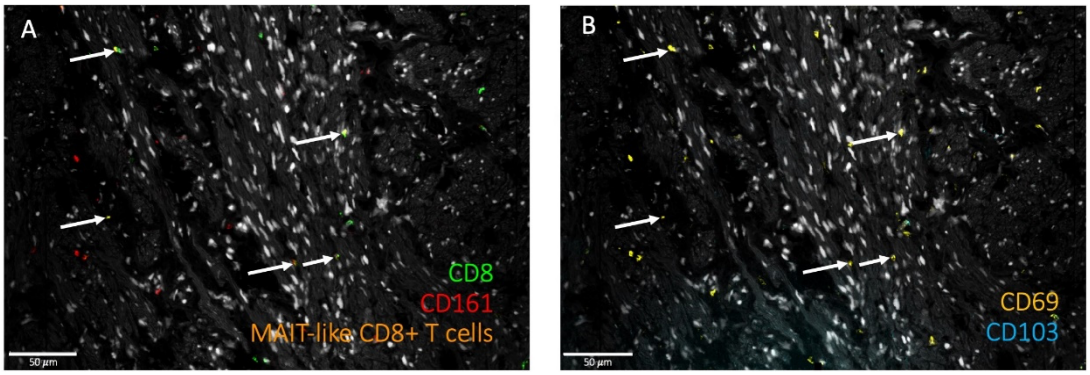


Figure 50: Myometrial CD8+ MAIT-like cells.

Image shows a section of full-thickness endometrial biopsy with myometrium taken from a patient with endometriosis. Similar to the endometrium, all CD8+ MAIT-like cells in the myometrium were tissue resident cells expressing CD69 and CD103. False-color fluorescence imaging for tissue residency markers CD69 (yellow) and CD103 (light blue) on CD8+ MAIT-like cells (CD8 and CD161) in orange, CD8+ T cells in green, CD161+ cells in red and cell nuclei in dark grey (hoechst). Multiplex imaging was performed with ZellScannerONE™ ChipCytometry™ instrument.

5.5 DISCUSSION

5.5.1 Summary

Using immunofluorescence microscopy, I conducted a preliminary investigation into the composition and location of LAs, the location and distribution of CD8 T cells and their tissue residency and functional characteristics in samples from endometriosis and non-endometriosis as well as fertile and subfertile patients. The proliferative phase revealed small LAs in the basal layer primarily comprised of CD8 T cells. During the secretory phase, CD8 T cell levels persisted, but their distribution became less dispersed in the stroma, concentrating in and around the epithelium and LAs. CD4 T cells, B cells, NK cells, and macrophages were found scattered across the epithelium, endometrium, and myometrium in both cycle phases, with no LAs present in the functional layer and myometrium. Examining tissue-resident immune populations, I observed heightened expression of CD69 and CD103 markers on CD8 T cells and other immune cells, particularly in LAs and the epithelium. Investigation into proliferative and activated states indicated limited expression of activation markers (HLA-DR and CD38) and the absence of granzyme B in CD8 T cells, suggesting intricate regulation in the endometrial microenvironment during the secretory phase. My findings highlight the complex dynamics of CD8 T cells in the endometrium across menstrual phases, offering insights into their roles in immune surveillance and tolerance to facilitate embryo implantation and pregnancy.

5.5.2 Strengths and limitations

Once again, only samples from patients without hormonal therapy and with regular menstrual cycles were included, without gynaecological comorbidities that could potentially influence the endometrial immune milieu, such as PCOS, pelvic inflammatory disease, etc. Myometrial

immune populations were identified in the non-pregnant uterus for the first time, marking a milestone in understanding the immune dynamics within this context. This is especially important for myometrial diseases closely associated with adverse reproductive outcomes such as adenomyosis [39, 298]. Due to technical issues with imaging, my analysis lacked the robustness for quantification of the findings. However, this limitation has been addressed with the more comprehensive full spectrum flow cytometry data, utilising a larger tissue sample in a cell suspension that is more representative of the entire endometrium compared to a thin section of a few millimetres in the case of immunohistochemistry. Unfortunately, specific markers, including CD45RA, CCR7, CCR5 and CXCR5, were not incorporated into the panel, and the visualisation was not possible for all CD8 T populations, which were found to be altered in the flow cytometry findings. Future studies are required to address this pitfall.

5.5.3 Endometrial CD8+ T cells

Histologically, endometrial CD8 T cells are dispersed throughout the endometrial mucosa either in large LAs or as single stromal or intraepithelial cells [6]. We lack an understanding of endometrial IELs, which precipitate intestinal inflammatory disorders and could, given their proximity to the embryo and foetus, impair fertility [299]. They have been under-researched in healthy human endometrium and even more so in reproductive pathologies, such as RPL and endometriosis, in which no changes in cell numbers were found between endometriosis and controls [105, 116]. Interestingly, the menstrual cycle in the lower reproductive tract did not seem to impact their numbers, but increased levels of CD8+, CD4+ and APC IELs were found in cervicitis and vaginitis [300]. Focusing on the secretory phase at the time of embryo implantation, I confirmed intraepithelial CD8 T, CD4 T, NK, B cells and macrophages, while dendritic cells could only be observed by the absence of other lineage markers on CD45+ cells, which were also found in small numbers across the luminal and glandular epithelium in all

endometrial layers. Intraepithelial CD8 T cells were further investigated with CD103, which is more straightforward in determining tissue residency than CD69 and highly associated with IELs. Nevertheless, as demonstrated in the images, it is not 100% specific for IELs since CD103 expression was also confirmed on CD8 T cells in the stroma and LAs unrelated to E-cadherin expression. Indeed, this leukocyte integrin is now recognised not only by its function in cell adhesion but also as a dynamically modulated receptor crucial for the functional differentiation of specific cytotoxic T cells and Tregs, potentially playing a role in the precise modulation of immune responses within epithelial compartments [285]. Numerous tissue resident CD8 T cells were found in the secretory endometrium, while only a few were proliferating (Ki67+) and activated (HLA-DR+) with no cells expressing granzyme B. These findings are consistent with the current literature on decreased CD8 T cell activity and cytotoxicity during the secretory phase, which proposes CD8 T cell regulation as an essential mechanism to avoid T cell-mediated rejection of the semi-allogeneic foetus [5, 61, 63, 94]. For example, granzyme B expression in CD8 T cells is indirectly inhibited by progesterone, which peaks during WOI, the time when the investigated full-thickness biopsy was taken and PD-L1 expression is upregulated in pre-menopausal when compared to post-menopausal women [61, 63]. Finally, like other authors, I identified CD8+ MAIT-like cells as single cells scattered near and in the glandular epithelium but also in LAs, where they were found dispersed among other CD8 T cells [87]. All of the identified CD8+ MAIT-like (CD8+CD161+) cells also expressed CD69 and CD103 markers, confirming my flow cytometry data that the majority of CD8+ MAIT-like cells are a tissue resident population.

5.5.4 Lymphoid aggregates

LAs bear similarity to ectopic or tertiary lymphoid structures (TLS) observed in different tissues under conditions of prolonged immune activation due to infection, autoimmune conditions and cancer with either protective or destructive roles [301]. These antigen-driven leukocyte

aggregations in extra-lymphoid sites develop upon stimulation with pro-inflammatory signals from immune-stromal interactions and support (re)activation of precursor and memory subtypes, leading to clonal expansion and terminal effector response [302]. Importantly, they diminish upon antigen clearance in conditions like *Helicobacter pylori* gastritis and their presence in tumours is associated with a favourable prognosis likely attributed to a superior adaptive immune response and response to immunotherapy [301, 303]. Similar to TLS, LAs consist of a central B cell core, surrounded by T cells and macrophages in close proximity to HEVs to allow cell trafficking from the peripheral blood [7, 304]. Despite higher proliferation rates previously observed in LAs during the secretory phase when compared with the proliferative phase, Ki-67 expression was still very low, which is in line with the existing knowledge disputing LAs as the source of immune cells due to clonal expansion and instead points towards their importance for antigen presentation and tissue retention [60, 117]. Indeed, high expression of HLA-DR (activation, antigen presentation) and CD69 (activation, tissue residency) was confirmed, along with a relative abundance of CD38 (activation) and CD103 (tissue residency), which were predominantly found in and around the epithelium. Only a few granzyme B positive cells were found near LAs, indicating that despite being activated, cells in the LAs probably traffic elsewhere to exhibit their cytotoxic function. Given that luminal epithelium is the point of contact with the uterine microbiota, a much higher expression of granzyme B in that area is unsurprising. The existing literature debates whether LAs could represent a novel TLS, and another argument in favour is the presence of HEV in the endometrium, commonly defined with the MECA-79 marker [305]. As mentioned, HEVs play a vital role in initiating and sustaining immune responses by capturing both naïve and memory lymphocytes from the peripheral blood, irrespective of their antigen receptor specificity, and transporting them to APCs within lymph nodes and TLS [306]. In RPL patients, a decreased density of HEVs with correspondently reduced levels of T cells was found in the decidua when compared to age-matched controls

[290]. On the other hand, no difference was observed in LA features such as the size, location, or cell types between endometriosis patients and controls [117]. The potential involvement of LAs in the pathophysiology of adverse reproductive outcomes remains unclear, but they seem to have a largely unexplored research potential. However, before any definitive parallels can be drawn with TLS, it is imperative to gain an additional understanding of their cellular phenotypes, structural characteristics, and functional properties.

5.5.5 Myometrial CD8+ T cells

Immunohistological findings in the myometrium of a non-pregnant uterus have revealed all the main immune populations from the innate and adaptive immune compartments, which were found as single cells dispersed throughout the tissue with no LAs. Identifying endometrial CD8+ MAIT-like cells in the myometrium of a patient with endometriosis but not in a fertile control signifies the potential role of specific immune cell populations in this condition, which is strongly related to adenomyosis. However, it is important to first explore the influence the cycle phase could play in this since endometriosis and non-endometriosis samples were from different menstrual cycle phases [307, 308]. In pregnancy, the myometrial immune milieu changes significantly just before the onset of labour, indicating marked inflammation, which is associated with cervical dilatation and foetal membrane rupture [309-311]. ScRNAseq revealed an increased presence of M1 macrophages (CD86) and heightened CXCL8 expression mainly observed in neutrophils during labour compared to term pregnancy [311]. Leukocytes are hypothesised to infiltrate the myometrium around the time of parturition. This suggests their potential role in labour activation (both term and preterm) and a significant role in the post-partum uterine involution [310]. Additionally, myometrial immunity undergoes substantial changes during ageing, especially among monocytes, leading to important implications in its functionality and immune regulation [296].

5.5.6 Further research

This study serves as a small pilot observational investigation, which will prospectively include the missing markers from the flow cytometry panel (CD45RA, CCR5) to illustrate and investigate the location and cell-to-cell networks of naïve and CCR5+ CD8 T cells. However, scaling up to include a larger patient cohort throughout the menstrual cycle using a comprehensive multiparameter technique such as chip cytometry is beyond the scope of my DPhil project and would necessitate significant funding. Additionally, the current methods for analysing cellular interactions detected by multiplex immunofluorescence are not fully developed. Future approaches may involve assessing cell-cell distances, quantifying cellular expression protein levels, and similar parameters, ideally through spatial transcriptomics.

5.6 CONCLUSIONS

Several endometrial and myometrial immune populations were identified with qualitatively observed differences in their distribution between the proliferative and secretory phases as well as between endometriosis and non-endometriosis participants. Cells were characterised across all layers, with single cells found in the epithelium and stroma and LAs discovered in the basal layer only. Most cells expressed tissue resident markers CD69 and CD103, of which CD69 can also be an activation marker, and CD103 is not 100% specific for IELs, as previously indicated. Further research is needed to quantify differences between patients with adverse reproductive outcomes and fertile controls to determine potential mechanisms by which IELs could compromise embryo implantation and immune tolerance through high activation and cytotoxicity observed in and close to the luminal epithelium.

6 Final discussion and concluding remarks

6.1 SUMMARY

My thesis explores the role of CD8 T cells in endometrial immunity and their potential implications in adverse reproductive outcomes, such as EAS and RPL. By systematically reviewing the literature, analysing the transcriptome of 784,580 CD8 T cells from 26 patients, deep immune profiling of 883,181 endometrial and 1,950,292 circulating immune cells from 32 patients and immunohistochemical imaging of 33 endometrial samples, I have explored the potential pathophysiologic significance of CD8 T cells in mediating endometrial function and receptivity. As I transition into my upcoming postdoctoral role as a bioinformatician, working on scRNAseq of T and B cells in RPL, and later a trainee in obstetrics and gynaecology, my DPhil studies conclude with robust preliminary data, instrumental in shaping further validation and investigation into functional and spatial properties of the identified immune phenotypes. This concluding chapter presents a concise summary of the CD8 T cell findings in adverse reproductive outcomes. It discusses their clinical significance in the context of current evidence to guide patient counselling and optimise ongoing clinical research.

6.2 FACILITATING THE TRANSITION BETWEEN BENCH AND BEDSIDE

6.2.1 Endometriosis and endometriosis-associated subfertility

A systematic review of the existing literature, encompassing 28 case-control studies, revealed conflicting data on CD8 T cells in endometriosis and EAS [105]. Previous findings indicate no difference in circulating CD8 T levels between patients and controls, with inconclusive evidence regarding CD8 T cell numbers in the eutopic endometrium and limited outcomes regarding EAS

[110-121, 123, 124, 127, 129-135, 312]. While earlier studies generally reported no disparities in CD8 T cell concentrations in the eutopic endometrium, recent genomic analyses delineating several CD8 T subtypes suggest notable modifications in CD8 T_{EM} in the eutopic endometrium of patients with endometriosis [111, 112, 118]. However, no existing studies investigated the predominant phenotype of CD8 T_{EM}, tissue resident cells [105]. I have addressed this literature gap by implementing tissue residency markers CD69 and CD103 into the 36-parameter panel for full spectrum flow cytometry (Table 11) and the 21-parameter panel for multiplex immunohistochemistry (Table 17). Going beyond the scope of previous studies, I identified 7 subtypes of CD8 T_{EM} population in the endometrium (Figure 19), of which 5 were tissue resident. I found increased levels of endometrial CD8+ MAIT-like cells in patients with endometriosis when compared to non-endometriosis controls (Figure 26). CD161, one of the common markers for MAIT cells, was also incorporated into the immunohistochemistry panel, hence, I was able to characterise the spatial properties of the endometrial and circulating CD8+ MAIT-like subpopulations in fertile control and endometriosis samples [86]. They were found across the endometrium (glandular and stromal compartments) and myometrium of the two endometriosis patients but not in the myometrium of the fertile control, again indicating modifications in endometrial CD8+ MAIT-like population (Figure 49 and Figure 50). Despite the lack of quantitative analysis, these qualitative observations support full spectrum flow cytometry findings and justify further investigation into this population to unravel more immunological context, especially regarding adenomyosis, an understudied condition which is closely associated with endometriosis and adverse reproductive outcomes [298]. Despite the discussed limitations in inferring endometrial immune pathophysiology from the peripheral blood, systemic changes hold great diagnostic potential. In contrast to the findings from the systematic review, I have identified altered systemic immunity in patients with endometriosis while carefully controlling for the menstrual cycle phase, parity and miscarriage, which has

previously been poorly considered in the clinical study design [119-121, 123, 124, 127, 129-135, 312]. In addition to already established associations with some autoimmune diseases, systemic immune changes in endometriosis were also suggested in the recent GWAS study on common genetic variants in 60,674 endometriosis cases and 701,926 controls [92, 152]. Significant changes in gene expression or methylation were revealed, among others, for genes *ESR1* and *SYNE1,* implicated in CD8 T cell function modulation via oestrogen and TCR signalling, respectively [313, 314]. Given the invasiveness of endometrial sampling, this could significantly aid the development of non-invasive diagnostics as no reliable biomarkers for the diagnosis of endometriosis have been identified to this day [315]. Nevertheless, the pathophysiology of reproductive failure in patients with endometriosis seems to be more localised. There were several modifications in endometrial immunity, including increased numbers of CCR5+ and decreased numbers of CXCR5+ CD8 T cells in patients with EAS compared to fertile endometriosis patients (Figure 30). At the same time, no differences were observed in the circulating populations between the two groups. While acknowledging that these preliminary findings come from a very limited number of participants, it is essential to recognise their significant contribution to enhancing our understanding of the immune landscape in EAS, suggesting that additional distinct mechanisms are involved with adverse reproductive outcomes in endometriosis. This could explain why not all patients with endometriosis experience subfertility, why only some benefit from ART and why this subgroup of patients should be studied separately from the main population [28].

6.2.2 Recurrent pregnancy loss

My findings on endometrial CD8 T cell transcriptome provide valuable insights into the complex interplay of genes and their potential roles in immune responses and immunotolerance in RPL during the WOI. The pathophysiology of such a heterogeneous condition is challenging even

with strict inclusion criteria, including only samples from the same menstrual cycle phase and excluding patients with underlying immune conditions, immune or hormonal therapy and coexisting gynaecological disorders. While GWAS involving 750 cases of multiple consecutive miscarriages and 359,469 female controls pointed towards significant genetic modifications associated not only with placental and embryo development but also with immune cell functions in RPL, the predictive role of the immune environment for the subsequent pregnancy remains unexplored [93]. Identifying patients at risk for a subsequent miscarriage carries significant diagnostic possibilities for early recognition of high-risk pregnancies to guide patient counselling and medical management [316]. When comparing RPL patients who experience a miscarriage in the subsequent pregnancy to those with live births, transcriptome and flow cytometry findings indicate a heightened proinflammatory and cytotoxic environment in the first group. This was evident not only in transcriptomic alterations related to angiogenesis and chemotaxis, as highlighted in the gene set enrichment analysis (Figure 13), but also in genetic modifications related to the regulation of T cell-mediated immunity (Figure 12). Moreover, differentially expressed genes such as *PLLP* and *C5* are closely associated with cell trafficking and activation of naïve CD8 T cells, which were found to be significantly increased in the miscarriage group with full spectrum flow cytometry analysis (Figure 33) [210, 219]. Additionally, decreased expression of the negative regulator of T cell activity PD-1 in the miscarriage group might indicate increased activation and functionality of CD8 T cells [78]. These differences were even more pronounced in the CD8 T cell transcriptome comparison between RPL with miscarriage versus fertile controls, where several pathways were found enriched in the RPL with miscarriage group, including genes upregulated in naïve CD8 T cells compared to effector CD8 T cells and genes down-regulated in the presence of PD-1. Even though I have included RPL patients in the sample selection for immune imaging, my panel lacked the relevant markers to visualise the altered population, which will be addressed in future experiments. Similar to EAS, only localised dysregulation was

found in the CD8 T cell compartment in RPL patients with a subsequent miscarriage. This highlights an essential pathophysiologic role of the CD8 T population in subsequent adverse reproductive outcomes with limited systemic implications for all circulating immune cells, which could be exploited to ease the diagnostics for identifying high-risk pregnancies.

6.3 CLINICAL IMPLICATIONS

6.3.1 Diagnostics

While gene-expression-based diagnostic tools remain expensive and laborious with several limitations in studying heterogeneous immune populations altogether, flow cytometry has paved its way not only in hematolymphoid neoplasias but also in immune disorders, such as multiple sclerosis, systemic lupus erythematosus and primary immunodeficiency diseases [317-321]. Peripheral blood is an ideal diagnostic material due to its accessibility and the ability to distinguish and screen multiple cell types, with additional translational research potential in tailoring and following up personalised treatment approaches [320]. Nevertheless, as I have demonstrated with the findings in my thesis, systemic immunity does not seem to play a major role in the manifestation of adverse reproductive outcomes, such as EAS and RPL, and endometrial immunity is poorly mirrored in the peripheral blood. Although I was not able to identify any studies assessing the feasibility of immunological testing for endometriosis, existing data in EAS and RPL are scarce and, given the complexity of endometrial immunity, likely oversimplified. Circulating NK, Th17, T regulatory cells and Th1/Th2 ratio have been suggested as promising diagnostic targets in addition to proposed endometrial tests.

- Endometrial Immune Profile Test measures the expression of genes that regulate IL-15, IL-18, TWEAK, Fn14, and CD56, factors essential for endometrial NK cell regulation, uterine receptivity and trophoblast invasion [322].
- Endometrial Decidualization Score assesses the expression of *FOXO1, SGK1, SCNN1A*, *SLC2A1, IL-15* and *GZMB* genes related to progesterone signalling, decidualisation, tissue and cellular homeostasis, immunoregulatory and tissue remodelling factors [323].
- Human Herpesvirus 6A Test detects the presence of HHV-6A infection in the endometrium, associated with unexplained subfertility via modifications in the immune profile of endometrial NK and epithelial cells [324].
- Endometrial B Cell CLL/Lymphoma 6 (BCL6) Test to assess endometrial BCL6 expression is based on the implication of aberrant BCL6 expression reported in patients with unexplained subfertility and endometriosis [325].

All so far with a low-level of evidence [46]. Therefore, systematic reviews and more comprehensive investigations are needed to draw parallels that would help us infer endometrial immune pathology from the circulating populations and make immunological testing of peripheral blood robust and justifiable.

6.3.2 Therapy

Unfortunately, despite the prevalent utilisation in clinical settings and an increase in the clinical trials investigating non-specific immunologic treatments, there is a substantial lack of high-quality clinical evidence supporting their efficacy [45, 326-328]. A detailed pathophysiological characterisation of CD8 T and other immune populations is key to successful drug repurposing and the development of targeted treatments to alter the immunological niche for improving

pregnancy outcomes [45, 329]. While CD8 T cells are a common target in cancer therapy, e.g., through immune checkpoint inhibitors and engineering into CAR T cells, studies specifically examining their therapeutic potential in adverse reproductive outcomes are understandably lacking due to a limited understanding of their immunomodulatory role [330, 331]. Closely linked to the CD8 T cell function is the pro-inflammatory cytokine TNF-α, a popular target in reproductive disorders research due to its association with inflammatory mechanisms in implantation, placentation, and pregnancy outcomes [332]. TNF-α can stimulate and increase the activity of CD8 T cells, thereby boosting their ability to destroy target cells. However, an overabundance or improper regulation of TNF-α production may result in immune system dysfunction, playing a role in developing chronic inflammatory conditions [333]. No randomised controlled trials have been conducted for EAS so far, but a retrospective study with 68 patients with endometrioma undergoing ART reported doubled clinical pregnancy rates with etanercept (anti-TNF-α monoclonal antibody) in an antagonist protocol versus no etanercept ($\chi 2 = 5.547$; $p = 0.019$), while the live birth rates were not statistically different ($\chi 2 = 3.179$; $p = 0.075$) [334]. Analysing the efficacy of another TNF-α inhibitor, infliximab, in 21 patients with endometriosis and pelvic pain noted no significant difference in either pain or the lesion characteristics between the infliximab and placebo groups [335]. A recent meta-analysis of the data from randomised control trials by our group assessed the literature on various immunomodulating agents in the context of subfertility, ART, and implantation failure [45]. Findings suggest that aspirin does not significantly impact the live birth in ongoing pregnancies, corticosteroids and subcutaneous low-molecular-weight heparin showed no clear increase in live birth rates, and the effect of intralipids and intrauterine PBMC treatment on live birth remains uncertain due to limited available evidence. Only one randomised control trial has been found for other immunomodulatory agents, such as granulocyte colony-stimulating factor, intravenous immunoglobulins or recombinant human leukaemia inhibitory factor and none evaluating anti-

TNF-α therapies [45]. Additional challenges arise as most studies do not consider embryo aneuploidy despite representing the leading cause of implantation failure, abnormal implantation, and miscarriage, especially with advancing female age [45, 336]. While the potential benefits of immunotherapy for specific patient subgroups, such as intravenous immunoglobulin therapy in RPL patients with abnormal NK cell levels, are recognised, caution is warranted due to limited high-quality evidence [326, 328]. In light of the current situation, clinicians are responsible for counselling patients about the lack of conclusive evidence regarding the efficacy of immune therapies, especially to mitigate expectations and the considerable financial strain they can impose on patients and their partners [45].

6.3.3 Study design

The future of immunologic testing and treatments to improve reproductive outcomes remains a promising possibility, essentially built on high-quality evidence from well-designed randomised control trials with clearly defined patient phenotypes for the optimal personalised approach. Although my findings do not offer sufficient conclusions to infer the potential roles of CD8 T cells in diagnosis or therapy in endometriosis, EAS and RPL, I have shown that history of miscarriage and live birth, as well as the menstrual cycle phase, are important but commonly overlooked factors to be considered when designing clinical studies [105, 243]. A detailed patient history is crucial when investigating immunity in endometriosis and RPL, not only due to their strong correlation with other immune disorders but also due to the recently implicated clinical overlap of both conditions, which was also observed in my cohort [337]. Endometriosis has been associated with systemic lupus erythematosus, Sjogren's syndrome, rheumatoid arthritis, autoimmune thyroid disorder, coeliac disease, multiple sclerosis, inflammatory bowel disease, and Addison's disease, while RPL patients often present with antiphospholipid syndrome, systemic lupus erythematosus, autoimmune connective tissue disease, autoimmune thyroid

disorders and recently a genetic overlap with asthma has been revealed [93, 152, 338-341]. Beyond uncovering shared pathophysiologic pathways with these immune comorbidities, it is essential to consider the impact of immune-modulating therapies these patients may be undergoing before the recruitment to clinical studies for endometriosis and RPL. To summarise, like several other authors, I confirmed that acquiring a detailed patient history, considering endometrial dynamics across the menstrual cycle and following strict inclusion criteria leading to big and representative cohorts are crucial steps to delivering robust results and thereby improving the overall quality of clinical research in reproductive immunology [41, 45, 46, 327, 329, 342].

6.4 CONCLUSIONS

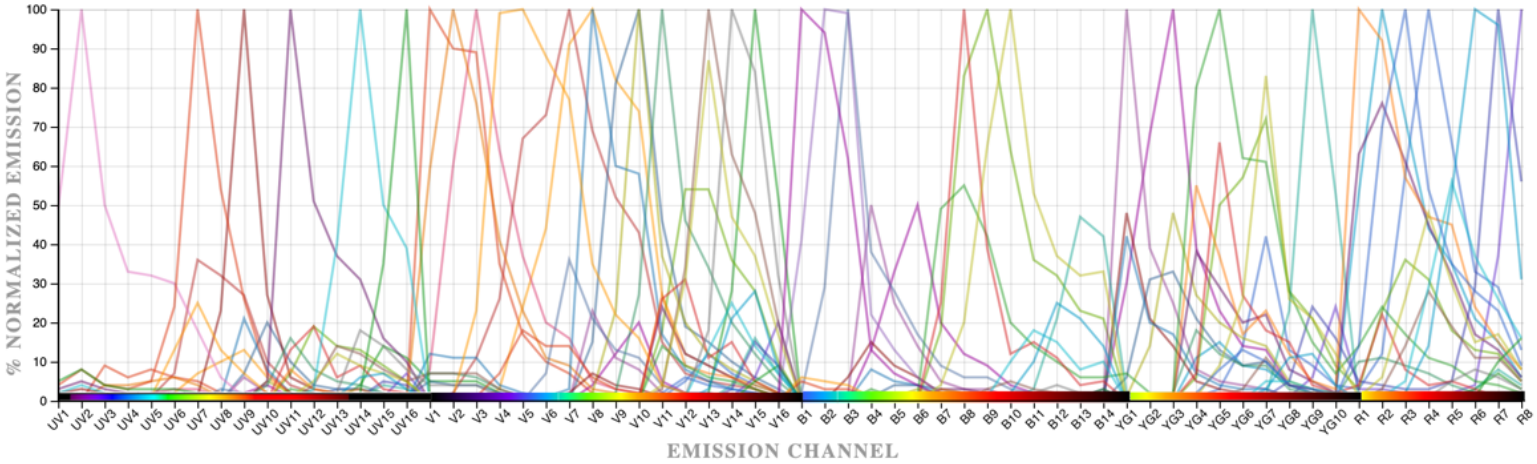
The interplay between the maternal immune system and the developing embryo involves various immune cells and molecules possessing immunoregulatory properties. Since CD8 T cells seem to be the major population during WOI, it is crucial to thoroughly examine the population and its interactions across the menstrual cycle and during healthy pregnancy before making any definitive conclusions regarding the potential impact on adverse reproductive outcomes. This careful examination is necessary for developing diagnostic tests to identify individuals at risk for adverse pregnancy outcomes and exploring possible therapeutic interventions aimed at enhancing fertility and pregnancy outcomes in the future.

7 Appendices

7.1 ESTABLISHMENT OF A FULL SPECTRUM FLOW CYTOMETRY PANEL FOR DEEP IMMUNE PROFILING

Panel design

In collaboration with Cytek and the Division of Experimental Medicine in Oxford, I developed a 36-parameter panel focused on adaptive immune subtypes. The development of the panel involved selecting an optimal combination of 36 fluorochromes (Figure 51) based on specific criteria, including unique spectral signature (similarity index) and overall fluorochrome compatibility (complexity index), as shown in Figure 52. Following a pre-determined spectral flow cytometry protocol with modifications, I ascertained optimal titres for 13 out of 36 reagents from our spectral flow panel for both HEC and PBMC based on the median fluorescence intensity (MFI) and followed principles for panel testing advised by Cytek [70, 195]. I tested and determined optimal staining concentrations for the following antibodies: CD103, CD11b, CD11c, CD161, CD24, CD25, CD4, CD45RA, CD57, CD69, CD8, CXCR3, HLA DR and Zombir-NIR. After antibody titration, spectral unmixing accuracy, marker resolution, steric hindrance and spread impact on the resolution were assessed before fine-tuning of the staining protocol and data acquisition.



APC-R700	BV421	Pacific Orange	BV785	PE-eFluor® 610	Alexa Fluor® 647	cFluor® V450
PerCP-Cy™5.5	BV510	BV605	PE	PE-Cy™7	cFluor® V610	APC-Fire 810
Super Bright 436	BUV563	BUV661	BV650	Spark Blue™ 550	APC-eFluor® 780	BV570
APC	PE-Cy™5	PerCP	cFluor® B532	BV711	BUV395	BV750
PerCP-eFluor® 710	BUV737	Spark NIR™ 685	BUV496	BV480	BUV805	BB515
Zombie NIR™						

Figure 51: Spectral signatures of 36 fluorochromes included in the panel.

The Cytek Spectrum Viewer was used to visualise the spectral fingerprints of 35 fluorochromes. For direct comparison, all signatures were normalised to peak channels.

Similarity™ Indices

Configuration:5L 16UV-16V-14B-10YG-8R

	APC-R700	BV421	Pacific Orange	BV785	PE-eFluor 610	Alexa Fluor 647	cFluor V450	PerCP-Cy5.5	BV510	BV605	PE	PE-Cy7	cFluor V610	APC-Fire 810	Super Bright 436	BUV563	BUV661	BV650	Spark Blue 550	APC-eFluor 780	BV570	APC	PE-Cy5	PerCP	cFluor B532	BV711	BUV395	BV750	PerCP-eFluor 710	BUV737	Spark NIR 685	BUV496	BV480	BUV805	BB515	Zombie NIR
APC-R700	1	0	0.01	0.07	0.04	0.63	0	0.41	0.01	0.05	0.01	0.1	0.07	0.2	0	0.01	0.5	0.18	0	0.32	0.02	0.57	0.28	0.17	0	0.39	0	0.11	0.49	0.45	0.78	0	0	0.04	0	0.51
BV421	0	1	0.07	0.07	0	0	0.78	0	0.17	0.06	0	0	0.01	0	0.97	0.01	0	0.09	0.02	0	0.15	0	0	0.01	0	0.09	0.05	0.05	0	0	0	0.09	0.28	0.01	0	0
Pacific Orange	0.01	0.07	1	0.03	0.11	0	0.17	0.04	0.88	0.62	0.16	0	0.46	0	0.09	0.14	0.04	0.25	0.33	0	0.77	0.03	0.02	0.03	0	0.08	0.01	0.05	0.04	0.02	0.01	0.22	0.58	0	0	0.01
BV785	0.07	0.07	0.03	1	0	0.01	0.06	0.19	0.03	0.07	0	0.17	0.06	0.18	0.07	0	0.05	0.15	0.01	0.22	0.03	0.04	0.03	0.07	0	0.49	0	0.82	0.26	0.25	0.04	0	0.02	0.21	0	0.27
PE-eFluor 610	0.04	0	0.11	0	1	0.05	0	0.18	0.07	0.47	0.59	0.04	0.54	0.01	0	0.32	0.11	0.14	0.16	0.01	0.35	0.14	0.36	0.21	0.05	0.03	0.01	0.01	0.11	0.01	0.03	0.01	0.03	0	0.03	0.01
Alexa Fluor 647	0.63	0	0	0.01	0.05	1	0	0.29	0	0.03	0.01	0.03	0.06	0.12	0	0.01	0.71	0.18	0	0.2	0.01	0.9	0.32	0.23	0	0.18	0	0.02	0.23	0.22	0.92	0	0	0.02	0	0.27
cFluor V450	0	0.78	0.17	0.06	0	0	1	0.01	0.36	0.07	0.01	0	0.02	0	0.86	0.01	0	0.08	0.05	0	0.15	0	0	0.01	0	0.07	0.01	0.04	0	0	0	0.13	0.58	0	0	0
PerCP-Cy5.5	0.41	0	0.04	0.19	0.18	0.29	0.01	1	0.02	0.17	0.05	0.23	0.22	0.09	0.01	0.03	0.39	0.38	0.06	0.15	0.06	0.37	0.72	0.81	0	0.5	0	0.28	0.86	0.31	0.35	0.01	0.01	0.06	0.01	0.25
BV510	0.01	0.17	0.88	0.03	0.07	0	0.36	0.02	1	0.4	0.12	0	0.27	0	0.22	0.22	0.04	0.16	0.3	0	0.54	0.02	0.01	0.01	0.02	0.06	0.02	0.04	0.02	0.02	0	0.48	0.84	0	0.01	0.01
BV605	0.05	0.06	0.62	0.07	0.47	0.03	0.07	0.17	0.4	1	0.28	0.03	0.89	0.01	0.06	0.2	0.17	0.53	0.16	0.02	0.7	0.14	0.21	0.18	0.01	0.17	0.01	0.1	0.14	0.05	0.03	0.05	0.16	0.01	0	0.02
PE	0.01	0	0.16	0	0.59	0.01	0.01	0.05	0.12	0.28	1	0.02	0.22	0	0.01	0.51	0.03	0.05	0.24	0	0.52	0.04	0.14	0.06	0.09	0.01	0.01	0	0.03	0	0.01	0.03	0.05	0	0.05	0
PE-Cy7	0.1	0	0	0.17	0.04	0.03	0	0.23	0	0.03	0.02	1	0.04	0.2	0	0.01	0.05	0.04	0.01	0.3	0.01	0.05	0.14	0.09	0	0.11	0	0.15	0.29	0.14	0.04	0	0	0.08	0	0.26
cFluor V610	0.07	0.01	0.46	0.06	0.54	0.06	0.02	0.22	0.27	0.89	0.22	0.04	1	0.02	0.01	0.12	0.17	0.56	0.11	0.02	0.53	0.19	0.31	0.23	0	0.17	0.01	0.09	0.16	0.04	0.06	0.02	0.1	0	0	0.02
APC-Fire 810	0.2	0	0	0.18	0.01	0.12	0	0.09	0	0.01	0	0.2	0.02	1	0	0	0.1	0.05	0	0.75	0	0.13	0.06	0.03	0	0.12	0	0.12	0.13	0.21	0.16	0	0	0.24	0	0.43
Super Bright 436	0	0.97	0.09	0.07	0	0	0.86	0.01	0.22	0.06	0.01	0	0.01	0	1	0.01	0	0.09	0.03	0	0.16	0	0	0.01	0	0.09	0.03	0.05	0	0	0	0.1	0.36	0	0	0
BUV563	0.01	0.01	0.14	0	0.32	0.01	0.01	0.03	0.22	0.2	0.51	0.01	0.12	0	0.01	1	0.06	0.04	0.15	0	0.37	0.03	0.07	0.04	0.07	0.01	0.05	0	0.02	0.01	0.01	0.29	0.07	0.01	0.04	0
BUV661	0.5	0	0.04	0.05	0.11	0.71	0	0.39	0.04	0.17	0.03	0.05	0.17	0.1	0	0.06	1	0.4	0.01	0.16	0.06	0.78	0.42	0.38	0	0.25	0.04	0.09	0.29	0.37	0.65	0.02	0.01	0.1	0	0.21
BV650	0.18	0.09	0.25	0.15	0.14	0.18	0.08	0.38	0.16	0.53	0.05	0.04	0.56	0.05	0.09	0.04	0.4	1	0.06	0.07	0.25	0.37	0.29	0.38	0	0.45	0	0.23	0.31	0.13	0.19	0.02	0.06	0.02	0	0.09
Spark Blue 550	0	0.02	0.33	0.01	0.16	0	0.05	0.06	0.3	0.16	0.24	0.01	0.11	0	0.03	0.15	0.01	0.06	1	0	0.24	0.01	0.06	0.06	0.77	0.02	0.02	0.01	0.05	0.01	0	0.09	0.22	0	0.53	0.01
APC-eFluor 780	0.32	0	0	0.22	0.01	0.2	0	0.15	0	0.02	0	0.3	0.02	0.75	0	0	0.16	0.07	0	1	0.01	0.2	0.09	0.05	0	0.18	0	0.18	0.18	0.31	0.27	0	0	0.2	0	0.78
BV570	0.02	0.15	0.77	0.03	0.35	0.01	0.15	0.06	0.54	0.7	0.52	0.01	0.53	0	0.16	0.37	0.06	0.25	0.24	0.01	1	0.05	0.09	0.06	0.02	0.07	0.01	0.04	0.05	0.02	0.01	0.09	0.26	0	0.01	0.01
APC	0.57	0	0.03	0.04	0.14	0.9	0	0.37	0.02	0.14	0.04	0.05	0.19	0.13	0	0.03	0.78	0.37	0.01	0.2	0.05	1	0.52	0.34	0	0.23	0	0.07	0.29	0.24	0.75	0	0	0.02	0	0.27
PE-Cy5	0.28	0	0.02	0.03	0.36	0.32	0	0.72	0.01	0.21	0.14	0.14	0.31	0.06	0	0.07	0.42	0.29	0.06	0.09	0.09	0.52	1	0.77	0.01	0.14	0	0.05	0.47	0.1	0.28	0	0	0.01	0.01	0.11
PerCP	0.17	0.01	0.03	0.07	0.21	0.23	0.01	0.81	0.01	0.18	0.06	0.09	0.23	0.03	0.01	0.04	0.38	0.38	0.06	0.05	0.06	0.34	0.77	1	0	0.23	0	0.13	0.46	0.15	0.21	0.01	0.01	0.03	0.01	0.09
cFluor B532	0	0	0	0	0.05	0	0	0	0.02	0.01	0.09	0	0	0	0	0.07	0	0	0.77	0	0.02	0	0.01	0	1	0	0.01	0	0	0	0.06	0.05	0	0.89	0
BV711	0.39	0.09	0.08	0.49	0.03	0.18	0.07	0.5	0.06	0.17	0.01	0.11	0.17	0.12	0.09	0.01	0.25	0.45	0.02	0.18	0.07	0.23	0.14	0.23	0	1	0	0.69	0.65	0.41	0.27	0	0.03	0.09	0	0.32
BUV395	0	0.05	0.01	0	0.01	0	0.01	0	0.02	0.01	0.01	0	0.01	0	0.03	0.05	0.04	0	0.02	0	0.01	0	0	0	0.01	0	1	0	0	0.03	0	0.25	0.02	0.11	0.01	0
BV750	0.11	0.05	0.05	0.82	0.01	0.02	0.04	0.28	0.04	0.1	0	0.15	0.09	0.12	0.05	0	0.09	0.23	0.01	0.18	0.04	0.07	0.05	0.13	0	0.69	0	1	0.38	0.37	0.06	0	0.02	0.14	0	0.31
PerCP-eFluor 710	0.49	0	0.04	0.26	0.11	0.23	0	0.86	0.02	0.14	0.03	0.29	0.16	0.13	0	0.02	0.29	0.31	0.05	0.18	0.05	0.29	0.47	0.46	0	0.65	0	0.38	1	0.39	0.31	0	0	0.07	0	0.34
BUV737	0.45	0	0.02	0.25	0.01	0.22	0	0.31	0.02	0.05	0	0.14	0.04	0.21	0	0.01	0.37	0.13	0.01	0.31	0.02	0.24	0.1	0.15	0	0.41	0.03	0.37	0.39	1	0.28	0.01	0	0.39	0	0.53
Spark NIR 685	0.78	0	0.01	0.04	0.03	0.92	0	0.35	0	0.03	0.01	0.04	0.06	0.16	0	0.01	0.65	0.19	0	0.27	0.01	0.75	0.28	0.21	0	0.27	0	0.06	0.31	0.28	1	0	0	0.03	0	0.36
BUV496	0	0.09	0.22	0	0.01	0	0.13	0.01	0.48	0.05	0.03	0	0.02	0	0.1	0.29	0.02	0.02	0.09	0	0.09	0	0	0.01	0.06	0	0.25	0	0	0.01	0	1	0.38	0.03	0.07	0.01
BV480	0	0.28	0.58	0.02	0.03	0	0.58	0.01	0.84	0.16	0.05	0	0.1	0	0.36	0.07	0.01	0.06	0.22	0	0.26	0	0	0.01	0.05	0.03	0.02	0.02	0	0	0	0.38	1	0	0.06	0
BUV805	0.04	0.01	0	0.21	0	0.02	0	0.06	0	0.01	0	0.08	0	0.24	0	0.01	0.1	0.02	0	0.2	0	0.02	0.01	0.03	0	0.09	0.11	0.14	0.07	0.39	0.03	0.03	0	1	0	0.14
BB515	0	0	0	0	0.03	0	0	0.01	0.01	0	0.05	0	0	0	0	0.04	0	0	0.53	0	0.01	0	0.01	0.01	0.89	0	0.01	0	0	0	0.07	0.06	0	1	0
Zombie NIR	0.51	0	0.01	0.27	0.01	0.27	0	0.25	0.01	0.02	0	0.26	0.02	0.43	0	0	0.21	0.09	0.01	0.78	0.01	0.27	0.11	0.09	0	0.32	0	0.31	0.34	0.53	0.36	0.01	0	0.14	0	1

Complexity™ Index: 37.73

Figure 52: Panel similarity and complexity indeces.

The Similarity™ Index calculates how similar two spectra are to one other. A value of 1 implies almost no difference between two fluorochromes, whereas a value of 0 indicates that two fluorochromes are entirely different. Each pair of fluorochromes identified for use in the panel is shown numerically in the chart. Based on several fluorochrome combinations tested in the OMIP-069 study, it was established that any fluorochrome pair with a similarity index of 0.98 or below could be accurately unmixed with adequate single stained controls [70, 195]. The Complexity™ Index is provided at the bottom of the matrix as a metric for evaluating the difficulty of the overall fluorochrome combination.

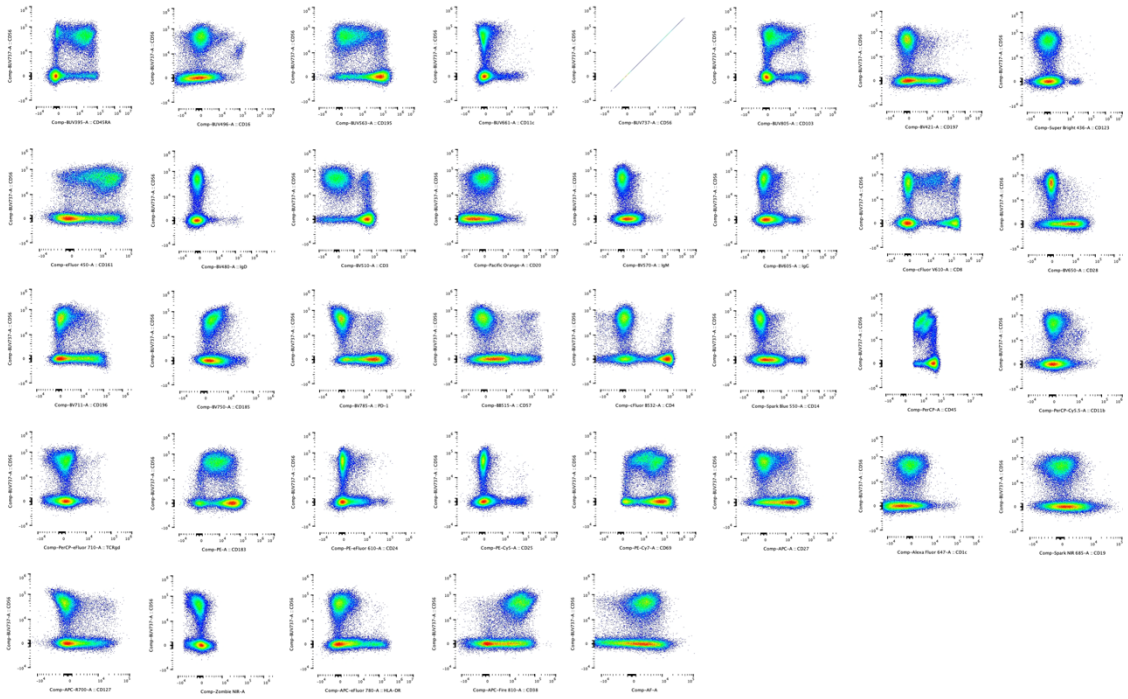


Figure 53: Unmixing accuracy of multi-stained endometrial sample.

NxN plots were generated to visually assess the unmixing accuracy. Data plotted for evaluation was gated on singlets, scatter, non-aggregates, live and CD45+ events. In each of the presented NxN plots, the CD56 marker on BUV737 is plotted on the x-axis and the y-axes represent every other fluorochrome in the panel. Minimal corrections were required to align the negative and positive populations.

7.2 FULL SPECTRUM FLOW CYTOMETRY STUDY PROTOCOL

7.2.1 Sample processing protocol

Materials and reagents

- Cryovials
- Fetal Calf Serum (FCS) (Sera Laboratories International Ltd, EU-000-F)
- PBS (SIGMA, D8537)
- Lymphoprep™ (Axis Shield)
- Iscove's Modified Dulbecco's Medium (Sigma, 13390)
- DNase (Sigma, 10104159001)
- DMSO (Sigma, 5.89569)

Reagent's preparation

- **Digestion Buffer:** Add 50 mL FCS, 5 mL Human Serum and 5 mL p/s/g (100 IU/mL penicillin, 100 g/mL streptomycin and 2mM glutamine) into 500 mL Iscove's Modified Dulbecco's Medium.
- **DNase I aliquot:** DNase I powder in 0.15 M NaCl (5 mg/ml), 10 mg/ml 100 µl stock aliquots
- **Freezing Media** (10% DMSO)**:** 10 mL DMSO + 90 mL FCS

Isolation of peripheral blood mononuclear cells (PBMCs)

- Prepare PBS/2% FCS, at room temperature (15–25°C).
- Transfer 4 mL blood from Lithium/Sodium Heparin vacutainer to a 15 mL tube, dilute with 4 mL of PBS (equal volume).
- Add 3 mL Lymphoprep™ in a separate 15 mL tube.
- Gently lay 8 mL of diluted blood on top of Lymphoprep™ in each 15 mL tube.
- Centrifuge at 800 x g, room temperature for 20 minutes without centrifugal brake.
- After centrifugation, carefully remove the PBMCs layer in the Lymphoprep™/plasma interface with a transfer pipette into a 15 mL tube.
- Top up the 15 mL tube to 15 mL with PBS/2% FCS. Centrifuge for 10 minutes at 300 x g, room temperature. Aspirate the supernatant. Repeat.
- Re-suspend cell pellet in 1 mL PBS.
- Transfer 10 µL of collected cell suspension to 1.5 mL Eppendorf. Keep the 15 mL tube with cell suspension on ice.
- Dilute cell sample in Trypan Blue by preparing a 1:1 dilution of the cell suspension (10 µL + 10 µL) using a 0.4% Trypan Blue solution.
- Perform cell counting using a Neubauer counting chamber.

Tissue digestion and isolation of HECs

- Ensure sterility. Work in a Class II microbiological safety cabinet and ensure full aseptic technique.
- Prepare Digestion Buffer and warm to 37°C. Turn on the shaking incubator and set the temperature to 37°C.
- Transfer endometrial tissue with PBS to a Petri-dish. Record shape, size and patient number of the biopsy. Take a photo of the sample.
- Take 10 mm^3 of the tissue, cut into 1 mm^3 fragments using scalpel and pipette and remove mucus. Ensure tissue fragments are small enough to fit through the tip of the transfer pipette (excess ES with good structure will be snap-frozen for immunohistochemistry).
- Transfer all the tissue fragments into 15 mL tubes, maximum 20 mm^3 per tube (I usually do around 500ul amount of tissue in each tube – that is the tissue size reach 500 µL in 15 mL tube). If blood contamination exchange solution to PBS. Remove supernatant to 1 mL solution of PBS with endometrial tissue.
- Add 10 mL Digestion Buffer to each 15 mL tubes. Remove the excess buffer with only 1 mL left in each tube.
- Add 100 uL DNase I aliquot to each 15 mL tube, vortex for 20 seconds.

- Add 10 mL Digestion Buffer to each 15 mL tubes and pass through sequential 70 µm and 40 µm cell strainers, wash the cell strainers twice with 3 mL Digestion Buffer, collect all the run-through in a new 50 mL Falcon tube.
- Centrifuge the collected run-through for 10 minutes at 300 x g, room temperature, aspirate the supernatant then re-suspend cell pellet in 1 mL PBS.
- Transfer 10 µL of collected cell suspension to 1.5 mL Eppendorf. Keep the 15 mL tube with cell suspension on ice.
- Dilute cell sample in Trypan Blue by preparing a 1:1 dilution of the cell suspension using a 0.4% Trypan Blue solution.
- Carefully and continuously fill the haemocytometer chamber.
- Incubate the haemocytometer and cells for 1–2 minutes at room temperature.
- Perform cell counting.
- Cell storage
- Transfer the rest of cells to one Eppendorf, centrifuge for 5 minutes at 300 x g. Aspirate all supernatant then re-suspend cell pellet with 1 mL Freezing Media.
- Transfer 1 mL cell suspension to one labelled Cryo tube vial (sample number, cell type, cell number, date, name, number of samples), place the vial in Cryo Freezing container with isopropyl alcohol, stored in -80°C overnight.

7.2.2 Staining protocol

Materials and reagents

- RPMI 1640-Medium (Sigma, R8758)
- Fetal Calf Serum (FCS) (Sera Laboratories International Ltd, EU-000-F)
- PBS (Wash buffer) (SIGMA, D8537)
- Zombie NIR™ dye (Biolegend, 423106)
- DMSO (Sigma, 5.89569)
- Cell Staining Buffer (Stain buffer) (Biolegend, 420201)
- Brilliant Stain Buffer Plus (BD Biosciences, 563794)
- True-Stain Monocyte Blocker™ (Biolegend, 426103)
- Specific antibodies (Table 11)
- FluoroFix™ Buffer (Biolegend, 422101)

Reagent's preparation

- **Complete RPMI:** Add 50 mL of FBS, 5 mL of Penicillin-Streptomycin into 500 mL of RPMI 1640 (with L-glutamine and sodium bicarbonate).
- **Zombie:** Pre-warm the kit to room temperature; spin down the vial of lyophilised reagent in a microcentrifuge to ensure the reagent is at the bottom of the vial and add 100 µL of DMSO to one vial of Zombie NIR™ dye and mix until fully dissolved. Aliquot to 1 µL in an Eppendorf tube and store at -4°C.
- Spin antibodies at 13,000 x g for 1 second.

Thawing

- Pre-warm 30 mL of complete RPMI at 37°C for at least 30 minutes.
- Prepare MULTI-ANTIBODY MIX excluding the antibodies listed below.
- Thaw cryo-vial in 37°C water bath, until only small piece of ice remains.
- Transfer 10 mL of warm complete RPMI to 15 mL conical tube.
- Add contents of cryo-vial with a transfer pipette.
- Add additional volume of complete RPMI to complete to 15 mL.
- Spin at 400 x g for 10 minutes (1500 rpm for 10 minutes in the TC room).
- Decant supernatant and resuspend pellet in 1 mL of warm complete RPMI.
- If not ready to proceed:
- Add complete RPMI to 15 mL.
- Repeat steps 6 and 7.
- Resuspend in 7.5 mL of complete RPMI or to an approximate concentration of $3.5x10^6$/mL.

- Leave in incubator until ready to proceed (rest is not necessary, but once staining has begun, samples will need to be processed without delay).

Single antibody control staining

- Label Eppendort Tubes® for single antibody controls (SAC): *unstained*, *SAC-marker*
- Add approximately $3x10^5$ cells or beads to the unstained and each of the SAC tubes.
- Add 1.5 mL PBS to SAC-Zombie NIR (ZNIR) tube.
- Add 1.5 mL wash buffer to all other SAC controls.
- Spin at 13,000 x g for 1 second.
- Decant supernatant and leave on ice.
- Prepare antibody dilutions to the total volume of 140 uL and add to the SAC tubes.
- Incubate on ice in the dark for 30 minutes.
- After incubation, add 1 mL wash buffer to the SAC-ZNIR and MAC tubes.
- Spin at 13,000 x g for 1 second.
- Decant supernatant and proceed with fixation.
- If not ready to proceed:
- Resuspend cell pellet in 1.5 mL BioLegend's Cell Staining Buffer.
- Keep on ice in dark until ready to use.

Viability dye staining

- Thaw 1 µL aliquot of Zombie NIR™ viability dye and dilute in 1.5 mL PBS.
- Decant supernatant and resuspend cells in the SAC-ZNIR and MAC tube in diluted 100 µL Zombie NIR™ solution.
- Vortex and incubate at room temperature in the dark for 15 minutes.
- After incubation, add 1 mL wash buffer to the SAC-ZNIR and MAC tubes.
- Spin at 13,000 x g for 1 second.
- Decant supernatant.
- If not ready to proceed:
- Resuspend cell pellet in 1.5 mL BioLegend's Cell Staining Buffer.
- Keep on ice in dark until ready to use.

Multi antibody cocktail staining

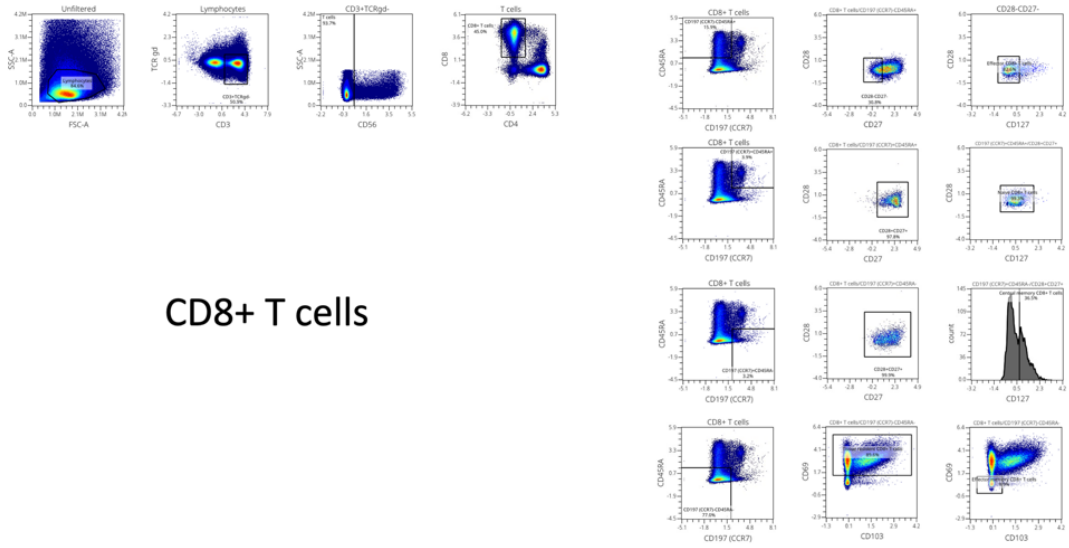
- Label Eppendorf Tubes® for multiple antibodies cocktail (MAC) for samples:
- *Unstained*
- *MAC-PBMC-Sample ID*
- *MAC-HEC- Sample ID tubes*
- Add approximately $2x10^6$ cells to each MAC tube with a transfer pipette.
- Add 1 mL PBS to all tubes.
- Spin at 13,000 x g for 1 second.
- Add 10 µL of Brilliant Stain Buffer Plus to all MAC tubes, vortex well.
- Add 5 µL of True-Stain Monocyte Blocker, vortex.
- CHEMOKINE RECEPTORS FIRST
- Add anti-CXCR5 (1.2 µL), vortex.
- Add anti-CCR6 (1.25 µL), vortex.
- Add anti-CCR7 (5 µL), vortex.
- Add anti-CCR5 (2.5 µL), vortex.
- Incubate for 10 minutes at room temperature in the dark.
- Add anti-TCRγδ (1.2 µL), vortex.
- Add anti-IgD, (0.6 µL), vortex.
- Add anti-PD-1 (5 µL), vortex.
- Incubate for 10 minutes on ice in the dark.
- Add anti-CD20 (5 µL), vortex.
- Add anti-CD161 (5 µL), vortex.
- Add anti-CD28 (2.5 µL), vortex.
- Incubate for 10 minutes on ice in the dark.
- Add the remainder of antibodies from the MAC, vortex.

- Incubate for 30 minutes on ice in the dark.
- After incubation, add 1 mL wash buffer to the SAC-ZNIR and MAC tubes.
- Spin at 13,000 x g for 1 second.
- Decant supernatant and proceed with fixation.
- If not ready to proceed:
- Resuspend cell pellet in 1.5 mL BioLegend's Cell Staining Buffer.
- Keep on ice in dark until ready to use.

Fixation

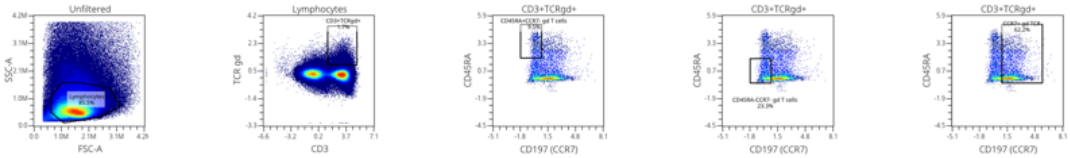
- Resuspend pellet in 250 µL of FluoroFix™ Buffer including SAC tubes, vortex.
- Incubate for 15 minutes at room temperature.
- Add 1 mL of wash buffer.
- Spin at 13,000 x g for 1 second.
- Resuspend pellet in 250 µL of wash buffer.
- Store at 4°C protected from light until ready to acquire on the instrument.

7.2.3 Gating strategy

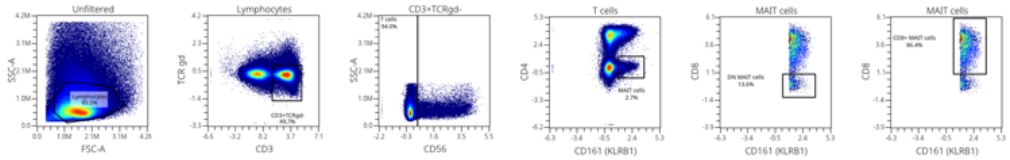


CD8+ T cells

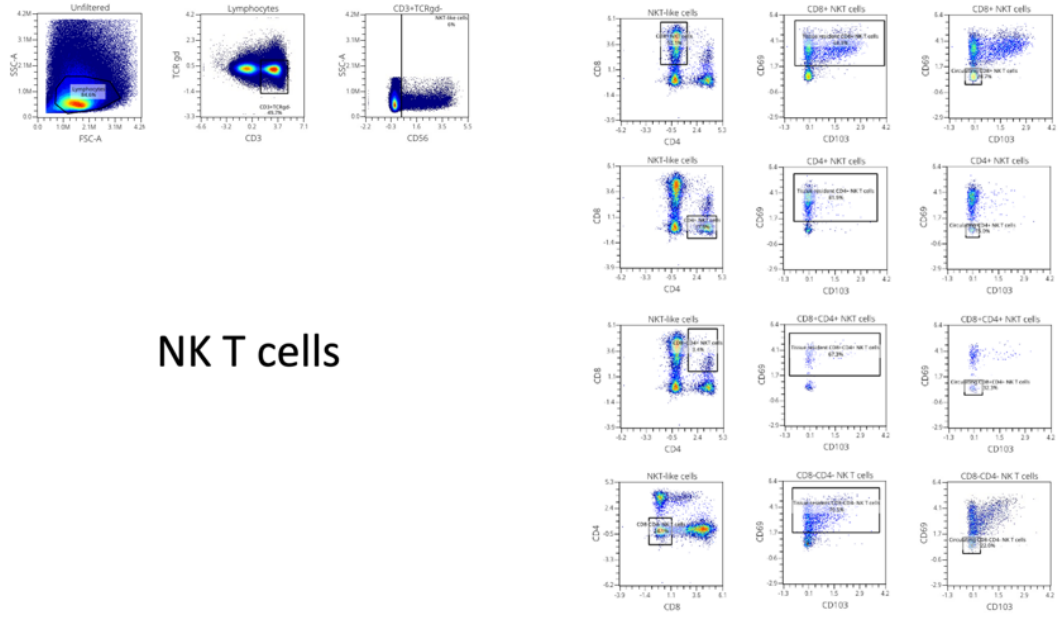
gd T cells



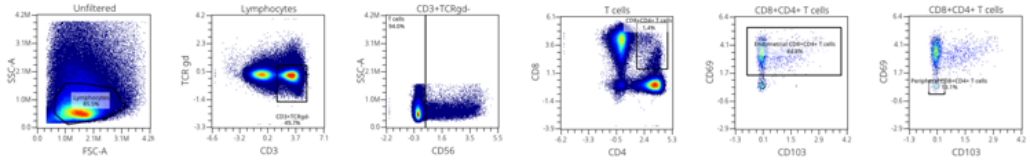
MAIT-like cells



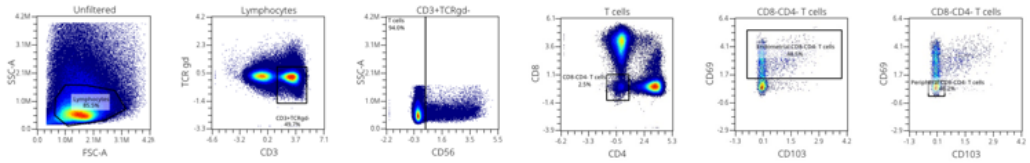
NK T cells



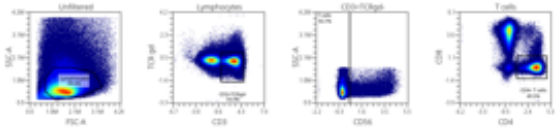
CD8+CD4+ T cells

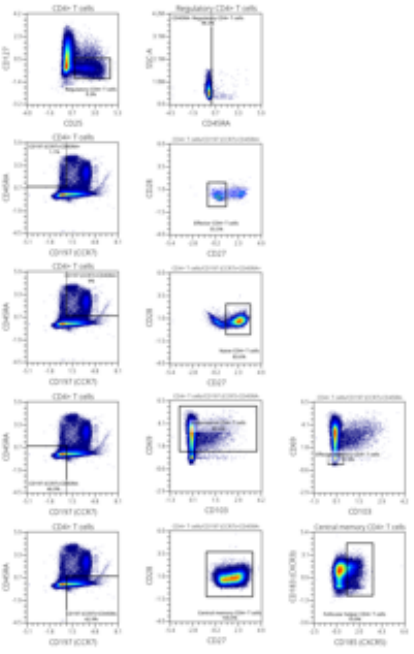


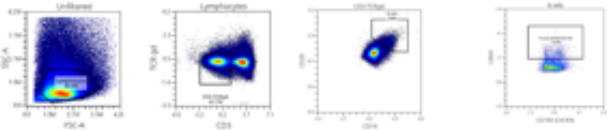
CD8-CD4- T cells



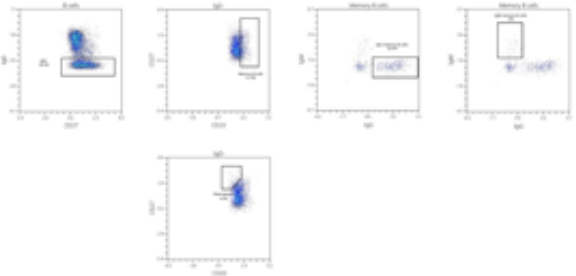
CD4+ T cells

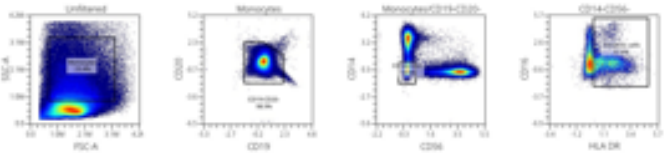




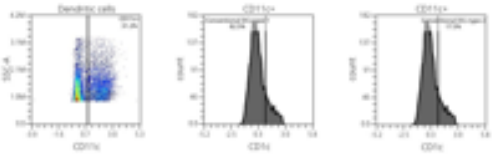


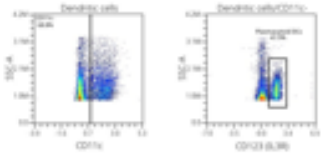
B cells

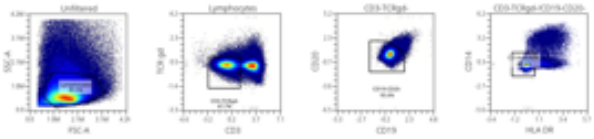




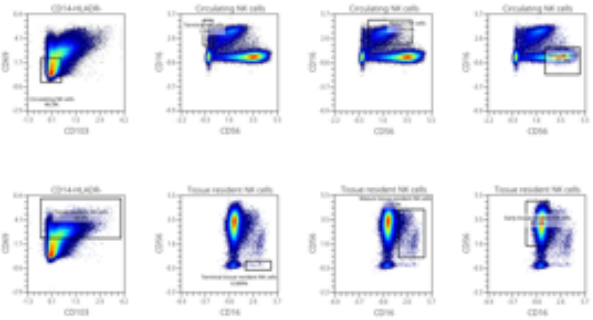
Dendritic cells

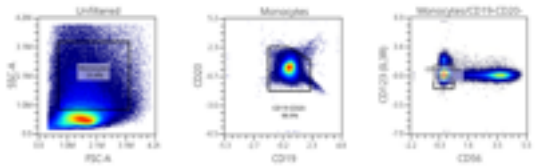




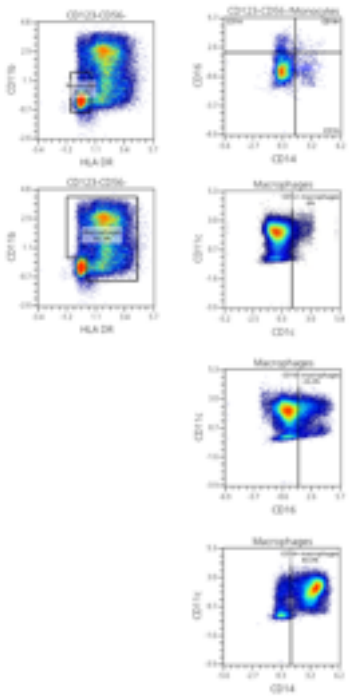


NK cells





Monocytes/macrophages



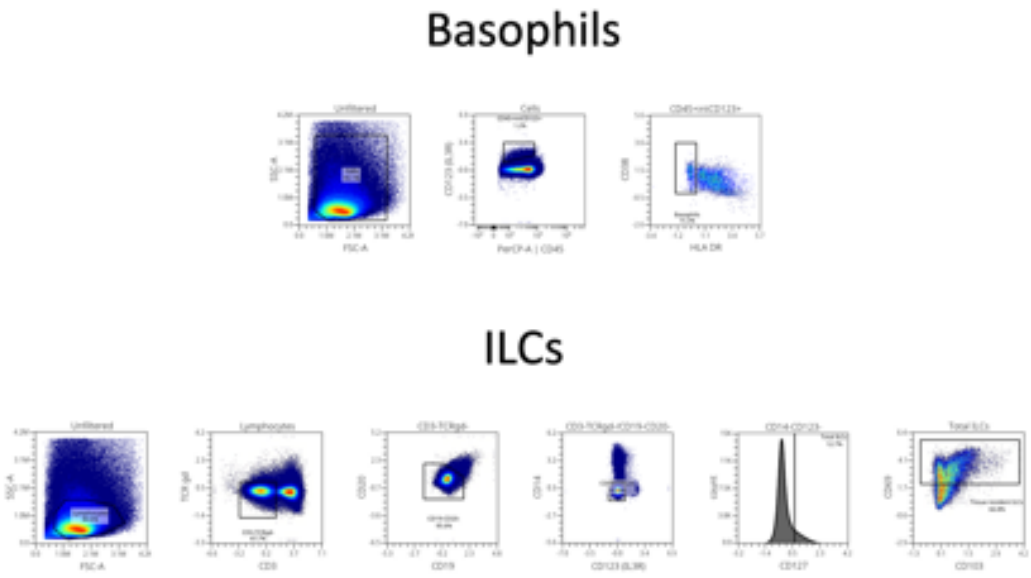


Figure 54: Standard gating strategy for peripheral and endometrial immune cells.

After doublets and dead cells were excluded, the different cellular subsets were characterized by manual gating. For each main population, the main lineage markers that showed different levels of expression across the various subsets of that specific population are presented. Data presented corresponds to concatenated cells from all endometrial samples.

7.3 IMMUNOHISTOCHEMISTRY STUDY PROTOCOL

7.3.1 Sample processing protocol

Reagents

- Isopropanol (Sigma, 59300-2.5L)
- OCT mounting media (Sakura, 4583)

Sample freezing

- Prepare a small container with dry ice, put a plastic mount on it, add mounting media to the mount.
- Simultaneously, place tissue in mounting media, add isopropanol to the container.
- After around 30 seconds, ensure the tissue is snap frozen.
- Transfer frozen tissue to a pre-cooled 5 mL tube for -80℃ storage.

Tissue cutting

- Set trimming on the cryostat to 20 µm and thickness to 7 µm.
- Retrieve a sample from -80℃, ensuring it is buried with dry ice.
- Allow the tissue to rise in temperature in the CryoStat for 10–20 minutes.
- Carefully take the tissue out using tweezers.
- Load mounting buffer, place tissue section onto it, and apply pressure from the side.
- Clean the glass and blade with a brush.
- Position the tissue opposite the blade, secure it, and adjust the sample distance.
- Trim and cut the tissue.
- Collect the tissue on the marked side of the glass.
- Clean the cryostat, keeping the safety handle on and spraying ethanol on the paper towel.

7.3.2 Staining protocol

Reagents

- Edge pen (Vector, H-400)
- DAB peroxidase substrate kit (Vector, SK-4100)
- Mayer’s haematoxylin (Sigma, MHS32ML)
- Aqua mounting (Thermo Scientific, TA-125-AM)
- Vectashield mounting media with DAPI (Vector, H-1200-10)

Prepare one day in advance

- Cut tissues dried overnight (in clear box) or stored in -80°C.
- Prepare cooled acetone in -20°C before start .
- 1–2 L PBS with tablet and MQ water (1 tablet per 200 mL).
- (Add 100% Tween to prepare 0.05% solution).
- Mark PBS with your name, date and leave it overnight on a mixer, no heating.

Prepare before starting

- Humidified chamber: glass chamber with wet tissue placed under.
- Make sure you have calculated all the dilutions in advance.

Fixation

- Take the cut slides out and let them warm up for 10 minutes.
- Place directly into a box with -20°C acetone → tube lying down.
- Leave in -20°C fridge for fixation for 5 minutes.
- After fixation, let the tissue section dry for 10 minutes (Do not overdry).
- Circle the tissue with Edge pen, make sure its circled properly otherwise liquid bubble will burst during incubation.
- After fixation do not let the slide dry out at any time in the following steps.

Washing

- Rinse the slides in glass box with slide holder with prepared PBS.
- Make sure the PBS covers the slides.
- Put it on a mixer.
- Leave it for 5 minutes, repeat twice.

Blocking

- Circle the tissue with Edge pen, make sure its circle properly otherwise liquid bubble will burst during incubation.
- Incubate the slides in 0.3% H_2O_2 solution at room temperature for 10 min to block endogenous peroxidase activity (look for the bubbles).
- 0.3% H_2O_2 solution: 10 µL of 30% H_2O_2 with 990 µL PBS.
- Repeat WASHING twice.
- Add 100µl blocking buffer (10% fetal bovine serum (FBS) in PBS) onto the sections of the slides and incubate in a humidified chamber at room temperature for 1 hour.
- **Blocking buffer:** 200 µL PBS + 20 µL FBS
- No wash after blocking.

Primary antibody

- Prepare primary antibody with 0.5% FBS.
- Drain off the blocking buffer from the slides.
- Apply 100 µL primary antibody to the sections on the slides and incubate in a humidified chamber overnight (12 hours) at 4°C.
- Repeat WASHING twice.

Secondary antibody*

- Prepare secondary antibody with 0.5% FBS.
- Apply 100 µL of the diluted secondary antibody (using the antibody dilution buffer) to the sections on the slides and incubate in a humidified chamber at room temperature for 30 min.
- Repeat WASHING twice.

DAB staining and hematoxylin*

- Prepare DAB & white A4 paper.
- Add 1 drop of buffer stock to 2.5ml of distilled water, mix well.
  Add 2 drop of DAB, mix well.
  Add 1 drop of H_2O_2, mix well.
- Place the slide on white paper, add 100 µL DAB to the tissue circle, develop for less than 10 mins (incubation time depends on antibodies, markers need to be detected, temperature, *etc.*), stop when brown colour start to emerge, make sure you stop before control sample start to have brown staining.

- Repeat WASHING twice.
- Counterstain slides by immersing sides in Hematoxylin for 3 dips.
- Rinse the slides in running tap water for > 15 min.

Fixation

- Apply the mounting media:
    a) Aqua mounting for DAB-staining
    b) Vectashield mounting media with DAPI for immunofluorescence staining
- Cover with a coverslip and seal.

***** These steps were only performed for DAB staining (brightfield microscopy) as directly conjugated antibodies were used for immunofluorescence staining.

8 REFERENCES

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