Investigating sex differences in the mitochondria of $CD4^+$ and $CD8^+$ T lymphocytes

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work with the following exceptions:

- Cell sorting for proteomic analysis was carried out by Dr. Barry Moran, Trinity College Dublin.
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Abstract

Sex differences in immunity have been observed in many contexts including infectious diseases, cancer, and autoimmunity. Females generally mount stronger innate and adaptive responses, and as a result tend to have lower rates of infectious disease and non-reproductive cancers, but are more prone to inflammatory and autoimmune diseases. These sex differences in the immune system and immune response can be caused by genetic and hormonal factors. In light of increasing evidence that mitochondria are sexually dimorphic in their structure and function, a growing area of interest is the role mitochondria might play in the observed sex differences in immunity. Mitochondrial morphology can dictate the metabolic phenotype of the cell, and in turn this can modulate cellular responses to immunological activation. Determination of how these mitochondrial properties differ across the sexes could therefore provide insight into the mechanisms that drive sex differences in immunity. Using confocal microscopy we found that $CD8^+$ T cells isolated from murine liver showed the greatest sexual dimorphism in mitochondrial morphology, with females exhibiting a greater mitochondrial count than males and a more branched mitochondrial network, while cells isolated from the spleen and lymph nodes did not show significant sex differences. Flow cytometry revealed that there were no sex differences in mitochondrial mass or polarisation ex vivo, but that an in vivo immune challenge might have the potential to decrease these paraments. We further demonstrated that CD69 expression by $CD4^+$ and $CD8^+$ T cells isolated from both spleen and lymph node was upregulated to a greater extent in females than males following in vivo immune challenge. Finally, proteomic analysis of $CD4^+$ and $CD8^+$ T cells revealed sex differences in the expression of a number of mitochondrial proteins including components of the ATP synthase enzyme, and mitochondrial fission and fusion proteins such as Fis1 and Opa1. The results of this project therefore provide preliminary evidence suggesting males and females differ in their mitochondrial content, and this is therefore an area that warrants further investigation.

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Table of Contents

List of Figures

Chapter 1 Introduction

Chapter 2 Materials & Methods

Chapter 3 Results

List of Tables

Chapter 1 Introduction

Chapter 2 Materials & Methods

Chapter 3 Results

Abbreviations

AD	Alzheimer's disease
AIDS	Acquired immunodeficiency syndrome
ARE	Androgen response elements
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cRPMI	Complete Roswell Park Memorial Medium
CS	Citrate synthase
CVD	Cardiovascular disease
DAPI	4′,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
ER	Oestrogen receptor
EREs	Oestrogen response elements
FACS	Fluorescence Activated Cell Sorting
FCCP	Fluoro-carbonyl cyanide phenylhdrazone
FCS	Foetal calf serum
GPER	G protein-coupled oestrogen receptor
HBSS	Hanks Balanced Salt Solution
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HRE	Hormone response elements
ICI	Immune checkpoint inhibitor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
mPTP	Mitochondrial permeability transition pore
MS	Multiple sclerosis
mtDNA	Mitochondrial DNA
MTG	Mitotracker Green
NF-κB	Nuclear factor kappa B
NRF	Nuclear respiratory factor
Nrf-1	Nuclear respiratory factor-1
OXPHOS	Oxidative phosphorylation
P/S	Penicillin-streptomycin
PBS	Phosphate buffered saline
PFA	Paraformaldehyde

Pgc-1α	Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha
Poly(I:C)	Polyinosinic-polycytidylic acid
PPAR	Peroxisome proliferator activated receptor
PRE	Progesterone response element
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SLE	Systemic lupus erythematosus
SOD1	Superoxide dismutase
TCR	T-cell receptor
TFAM	Mitochondrial transcription factor A
TMRM	Tetramethylrhodamine, Methyl Ester, Perchlorate
TNF	Tumour Necrosis Factor

Chapter 1
Introduction

1.1 Biological Sex vs Gender

In the study of immunity, sex and gender have historically been overlooked. However, in light of mounting evidence that biological sex can impact immune responses, propensity for certain diseases, and health outcomes, this is now an area of active investigation[1].

In the context of this research, it is important to differentiate between the terms 'sex' and 'gender'. Biological sex is defined as male, female or intersex based on a combination of sex chromosomes (46XX or 46XY), gonads (ovaries or testes), and gonadal hormones (oestrogen or androgen dominance)[1]. Gender on the other hand refers to how a person self-identifies, based on psychosocial and cultural differences between men and women, and this may or may not align with their biological sex.

Biological sex will contribute to physiological differences in disease, such as hormonal regulation of immune responses, whereas gender is most likely to impact behaviour that influences exposure to pathogens, as well as an individual's access to health care, the likelihood they will seek care and the type of care they recieve[1-3].

The focus of this research is on biological sex differences and how these differences influence immunity. The terms male/female and men/women will be used interchangeably throughout this thesis, but always in reference to biological sex.

1.2 Sex differences in immune responses

Sex differences have been demonstrated in a variety of immunological contexts and components, and in response to both self and foreign antigens. A number of studies have shown that the inflammatory innate immune response is higher in females, with higher numbers and activity of innate immune cells[3]. Furthermore, adaptive immune responses also exhibit sex differences, as females have higher basal levels of immunoglobulin and mount stronger antibody responses to pathogens and vaccines[4]. Such differences mean that females can clear pathogens more quickly, and respond with greater efficacy to vaccination, but may also contribute to the higher burden of inflammatory and autoimmune diseases seen in females[5]. On the other hand, there is a lower prevalence of many viral infections (e.g., dengue virus, hepatitis B, hepatitis C) in females compared to males[1].

Sex differences in the production of cytokines and chemokines by innate immune cells has been widely reported[5]. For example, plasmacytoid dendritic cells (pDCs) from females produce higher levels of IFN-α in response to HIV-1 infection[6]. Another example is that of the Toll-like receptor (TLR) signalling pathways. TLR7 is expressed to a greater extent on female innate immune cells, which results in higher cytokine expression when TLR7 is bound by its ligands[5]. On the other hand, TLR4 is more highly expressed on innate immune cells in males, and therefore LPS stimulation results in greater pro-inflammatory cytokine production by males, an effect which can be reversed by removal of androgens in male rodents[7]. Conversely, activation of TLR9 results in a higher IL-10 response from male peripheral blood mononuclear cells (PBMCs), promoting the anti-inflammatory response, and this effect is positively correlated with androgen concentration in males[8]. Sex differences in pattern recognition receptor (PRR) expression can therefore be key to interpreting differences in innate immune responses. Vaccination in adults has been shown to result in higher expression of the TLR pathway components and pro-inflammatory genes (for example, TLR7, nuclear factor κB (NFKB), interferon-γ (IFNG) and tumour necrosis factor (TNF)) in females compared to males, and the same has been shown in adult rats following viral challenge[4, 9]. Therefore the effective or optimal vaccination dose may differ between the sexes and should be investigated to ensure both efficient use of resources and most optimal protection[4, 5].

The observable sex differences in immunity can in part be attributed to differences in immune cell frequency. Males have higher natural killer (NK) cell frequencies, whereas females, both pre- and post- puberty, have higher $CD3^+$ and $CD4^+$ T cell counts and higher CD4/CD8 T cell ratios than age-matched males[10]. Males on the other hand have higher $CD8^+$ T cell frequencies[5]. Sex differences may also impact innate lymphoid cells (ILCs), as females reportedly have reduced numbers of type 2 ILCs, which is hypothesised to contribute to the development of autoimmune diseases[11].

The activity of the immune cells may also explain immunological sex differences. Phagocytic activity of neutrophils and macrophages is higher in females than males, and antigen presenting cells (APCs) from females are more efficient at peptide presentation, all of which may contribute to the heightened innate immune response in females[12, 13]. Following *in vitro* stimulation, female PBMCs have higher numbers of activated $CD4^+$ and $CD8^+$ T cells and proliferating T cells[10]. In a murine model of H1N1 influenza, females were shown to generate more robust memory $CD8^+$ T cell responses[14]. *In vitro* stimulation using a combination of phorbol myristate acetate (PMA) and ionomycin causes females to upregulate more antiviral and proinflammatory genes than males, and the polarisation of

the T helper subsets also differs between the sexes[1, 15, 16]. In response to *in vitro* stimulation, $CD4^+$ T cells isolated from females are biased towards a Th1 response, producing higher levels of IFNγ as well as proliferating to a greater extent than male $CD4^+$ T cells. The male cells instead produce higher levels of IL-17, indicating a greater Th17 response[16]. Such biases may contribute to the higher rate of autoimmune disease occurrence in women. Studies have shown that this sex difference in Th1 and Th17 polarisation is at least in part dictated by the T cell expression of peroxisome proliferator activated receptor (PPAR)α and PPARγ, a notable observation in the context of this project, as PPARα and PPARγ are important regulators of mitochondrial protein expression[16]. A summary of the sex differences in immune system components and responses can be found in Table 1.1.

1.3 Sex differences in disease pathogenesis

Sex differences in immunity have been observed in a multitude of pathological contexts including cancer incidence and response to cancer immunotherapy, vaccine response, autoimmune diseases and viral infections.

1.3.1 Autoimmunity

Females are much more likely to develop a variety of autoimmune diseases including systemic lupus erythematosus (SLE; 9:1 female-to-male ratio) and multiple sclerosis (MS; 2-3:1 female-to-male ratio)[17]. In total, females account for 80% of autoimmune disease occurrences in the USA[18, 19]. The increased incidence of autoimmune and inflammatory diseases can be attributed to generally stronger innate and adaptive immune responses mounted by adult females[5].

The incidence of autoimmune diseases in animal models, such as development of spontaneous SLE in (NZBxNZW)F1 mice, can be increased by orchidectomy of males, or decreased by ovariectomy of females, demonstrating the impact of the sex hormones on the pathogenesis of disease[19]. Sex hormones may even have therapeutic applications, as it has been shown in women with relapsing-remitting forms of MS that administration of high doses of oestriol can reduce relapse occurrences[20]. Additionally, in men with MS, treatment with testosterone reduced the number of CD4 T cells, increased the number of NK cells and promoted the production of TGFβ by PBMC[21].

Immune component	Characteristic	Sex difference
T cells	CD4/CD8 T cell ratio	Higher in females
	$CD8^+$ T cell counts	Higher in males
	$CD4^+$ T cell counts	Higher in females
	T cell proliferation	Higher in females
	Th1 versus Th2 cell bias	Th1 bias in males Th2 bias in females
NK cells	NK cell numbers	Higher in males
Innate lymphoid cells (ILCs)	Type 2 ILCs	Higher in males
Plasmacytoid dendritic cells	Type 1 interferon activity	Higher in females
Macrophages	Phagocytic activity	Higher in females
	IL-10 production	Higher in females
Neutrophils	Phagocytic activity	Higher in females
Antigen presenting cells	Peptide presentation	Higher efficiency in females
Immunoglobulins	Basal levels	Higher in females
	Antibody responses	Higher in females
Peripheral Blood Mononuclear Cells	IL-10 production following TLR9 activation	Higher in males
	LPS stimulated TNFa production	Higher in males
	LPS stimulated IL-6 production	Higher in females
	IFNa production following TLR7 activation	Higher in females
TLR pathways	TLR pathway gene expression	Higher in females
	TLR 7 expression	Higher in females
	TLR 4 expression	Higher in males

Table 1.1 A summary of the sex differences in immune system components and responses

1.3.2 Infectious diseases

Not only are there sex differences in the prevalence of autoimmune conditions, but there are marked differences in occurrence and severity of diseases caused by viruses, bacteria, parasites, and fungi between the sexes[22]. In general, males are more susceptible to these infections, with both a higher intensity (pathogen load within an individual) and prevalence (number of infected individuals within a population)[3]. This is seen from birth, before the establishment of significant differences in sex hormone profiles, as male newborns are more susceptible to infections than females[23].

Despite lower infection rates amongst females, heightened immunity to pathogens may increase disease symptoms and severity[3]. For example, women infected with human immunodeficiency virus (HIV) are at a 1.6-fold higher risk of developing acquired immunodeficiency syndrome (AIDS) when compared to men matched on their HIV RNA loads. This is despite women having approximately 40% less circulating HIV RNA compared to men in general[24]. Similarly, women have a higher fatality rate following infection with pathogenic influenza A viruses[3].

1.3.3 Cancer

Throughout life, males are at a higher risk of developing non-reproductive cancers, and are twice as likely to die from malignant cancers than their female counterparts. Outcome disparities between the sexes are greatest for oesophagus, bladder and lung cancers[5, 25, 26]. It is thought that this imbalance in mortality rates may be due to sex differences in cancer aetiology, in turn reflecting sex differences in a number of factors including immune function, hormonal regulation, gene expression and sex chromosome complement[25].

Sex differences are also evident in cancer treatment, particularly with immunomodulatory treatments such as immune checkpoint inhibitor (ICI) therapies[27]. These differences are therapy dependent, with anti-CTLA-4 treatment appearing to have more significant sex differences than anti-PD-L1 treatment[28]. Treatment regime also affects outcomes, with males who receive anti-CTLA-4 or anti-PD-1 antibodies as monotherapy gaining a greater survival benefit, but there is evidence to suggest female patients may benefit more than males from a combination of chemotherapy with an anti-PD-1 or anti-PD-L1 antibody treatment[29, 30]. It is therefore clear that cancer research, particularly cancer

immunotherapeutics, is an area that could greatly benefit from improved understanding of immunological sex differences.

1.4 Mediators of sex differences in immunity

There are a number of factors which may contribute to the observed sex differences in disease susceptibility and immune response, such as the sex chromosomes, sex chromosome-linked genes and the sex hormones. It can be difficult to isolate the impacts of each of these contributing factors, and there is likely to be a great deal of interplay. While many of the observed immunological sex differences are detectable throughout life, some of these differences are only apparent after puberty and before reproductive senescence, indicating that both chromosome and hormone related factors have a role to play in contributing to immunological differences[5]. Current studies seek to understand the sex differences in immunity caused by these contributing factors both in combination and in isolation.

1.4.1 Sex chromosomes and sex chromosome-linked genes

Owing to the fact that males and females differ in their innate immune responses, it is hypothesised that some sex differences are germline encoded. The X chromosome is the location of many genes involved in regulating immune function, which code for proteins ranging from PRRs such as TLR7 and TLR8, IRAK1, a regulatory molecule in the TLR-dependent signalling pathway, to cytokine receptors such as IL2RG[5, 31]. Taking TLR7 as an example, due to the potential to escape X chromosome inactivation, females can express higher levels of TLR7 than males[32]. This in turn affects the immune response, as has been shown when PBMCs are exposed to TLR7 ligands *in vitro*, there is a higher IFNα response from females compared to males[33].

To study the impact of sex chromosomes and hormones in isolation, the four core genotype mouse model was developed. The *Sry* gene (sex-determining region Y gene) is found on the Y chromosome, and results in testes formation and testosterone synthesis, and is therefore the gene responsible for male phenotypic development[34]. In this model the *Sry* gene is translocated to an autosome. Therefore, irrespective of whether the mouse has an XX or XY chromosomal complement, it will develop testes when the Sry transgene is present (XX*Sry*+ and XY) and ovaries when it is absent (XX and XY*Sry*−). As the name

indicates, this model produces four genotypes - gonadal females with an XX or XY chromosome complement (*Sry*−) and gonadal males with an XX or XY chromosome complement (*Sry*+)[1]. By comparing XX and XY mice that have the same gonads, the effect of the sex chromosomes can be determined separately from gonads and gonadal hormones. Use of this model has led to some interesting observations. For example, regardless of the gonadal type, XX mice are still more susceptible than XY mice to certain autoimmune diseases[35]. This highlights the importance of the X chromosome in contributing to autoimmune disease susceptibility.

A striking example of the effects of the X chromosome on immunity is Klinefelter syndrome, which occurs when males have an extra X chromosome, resulting in low testosterone and increased oestrogen[5]. Men with Klinefelter syndrome are more immunologically similar to females, with higher CD4 T cell numbers and CD4/CD8 T cell ratios. Strikingly, these effects can be reversed via testosterone therapy, thereby exemplifying how both sex chromosomes and sex steroids regulate the immune system[36]. In addition, men with Klinefelter syndrome have a higher propensity to develop autoimmune disease, providing more evidence for the role of the X chromosome in influencing susceptibility to autoimmunity[5, 37].

1.4.2 Sex hormones

One of the major defining factors of biological sex is the concentrations at which the major sex hormones (also referred to as sex steroids) testosterone, oestrogen, and progesterone are found. During the reproductive years, males typically have higher levels of testosterone and females have higher levels of oestrogen and progesterone.

Sex hormones can influence immune cell function by binding to receptors expressed in most immune cells. Hormone-receptor complexes then bind to DNA segments containing specific hormone response elements (HREs). Indeed, one of the key factors suggesting that sex steroids may directly cause sex differences in innate immune responses is the presence of putative androgen response elements (AREs) and oestrogen response elements (EREs) in the promoters of several innate immunity genes[9]. Such binding events can then influence signalling pathways involved in the production of cytokines and chemokines[3]. Gene expression can also be altered by sex hormones, acting as transcription factors, because some genes encoding immunological proteins have HREs in their promoters. One such example is IFNγ, which has three EREs in its promoter[38].

The sex steroids can directly impact disease pathogenesis, as well as the response to infection. One such example is that of human papillomavirus (HPV), the genome of which contains a progesterone response element (PRE). Once activated, this PRE regulates part of the HPV life cycle and transformation process, which may contribute to the higher incidence of malignant HPV lesions in females[39].

Oestrogen receptors (ERs) are expressed by various lymphocyte populations, and ERα in particular is highly expressed in T cells[5]. The effects of oestrogen on the immune response can sometimes be concentration dependent, as well as being contingent on the frequency and type of ER expressed in the immune cells. Generally, low oestrogen promotes Th1-type responses and cell mediated immunity, whereas high oestrogen can promote Th2-type responses and humoral immunity. For example, production of the pro-inflammatory cytokines IL-1, IL-6 and TNF by human monocytes and macrophages is enhanced in conditions of low oestrogen concentration but reduced with high concentration[40, 41]. Oestrogen can also affect downstream adaptive immune responses, and interestingly, the numbers of antibody secreting cells and antibody levels peak before ovulation in females, when oestrogen levels are highest[42].

Androgens, such as testosterone, are produced in higher concentrations in adult men than women and generally suppress immune cell activity. Men with lower androgen levels tend to have higher concentrations of inflammatory cytokines such as IL-1β, IL-2 and TNF, as well as higher CD4/CD8 T cell ratios. Following orchidectomy, male mice have higher numbers of $CD4^+$ and $CD8^+$ T cells, and in one striking example, following viral infection, the castrated males had higher numbers of macrophages and antigen specific $CD8^+$ T cells when compared to gonadally intact males[43]. Such studies demonstrate the stark effects of androgen deprivation on the immune system. Additionally, exposing murine NK cells to testosterone *in vivo* dramatically reduces their activity[44]. Such inhibitory effects of testosterone can also be demonstrated in females, where testosterone treatment inhibits secretion of IFNγ by natural killer T cells[45].

The importance of hormone receptors, specifically ERα, in determining T cell responses has been shown in a murine model of colitis. Deletion of ERα resulted in a less severe disease phenotype, potentially indicating that ERα helps promote the pathogenic potential of $CD4^+$ T cells. T cell activation and proliferation was also decreased following deletion of ERα, and the proportion of Tregs was increased concurrent to a decrease in Th1 an Th17 subsets[46]. Other evidence points to a role for androgens in promoting Treg populations through the upregulation of a transcription factor important in Treg differentiation, Foxp3.

This protein is important for $CD4^+$ Treg differentiation. This is achieved through binding the androgen receptors located on the *Foxp3* locus. However, this effect was only seen in female Tregs, and due to the low circulating levels of androgens in females, the clinical relevance of this finding is questionable[1, 47].

Taking these findings as examples, it is therefore clear that sex steroids can act as potent regulators of immune responses, and their role in determining T cell responses is not yet fully elucidated and is an area worthy of further investigation.

1.5 Mitochondrial sex differences

Aside from chromosomes and sex hormones, sex differences in mitochondrial content and function could be another factor influencing sex differences in immune response. The mitochondria's primary function, as the site of oxidative phosphorylation (OXPHOS), is to provide most of the required energy to the cell, as well as functioning in processes such as reactive oxygen species (ROS) generation and regulation, apoptosis, pH regulation, steroid hormone synthesis, calcium homeostasis, thermogenesis, lipid and carbohydrate metabolism[48]. There is mounting evidence illustrating sex differences in mitochondrial function, particularly in oxidative capacities, calcium handling, biogenesis, and resistance to oxidative stress[48].

Mitochondrial proteins are encoded by the nuclear genome and so mitochondria are typically very well suited to the tissue specific needs of their host cells. Thus, mitochondria themselves exhibit a wide variety of structure and function, and the number and activity of the mitochondria will be dependent on the energy requirements of the cell[48]. Mitochondria are maternally inherited, and the mitochondrial genome spends relatively more time under selection in females, therefore it is hypothesised that these genes will be better optimised for function in females than males[48]. Oestrogen and androgen receptors are found in mitochondria in multiple cell types and tissues. The ERs are bound to the mitochondrial DNA, indicating the oestrogen can alter mtDNA transcription and replication[48, 49].

1.5.1 Oxidative capacity

Sex differences in oxidative capacity have been observed in many tissues, such as liver, brain, adipose tissue and skeletal muscle, wherein ADP-stimulated respiration is higher in

females than males[48]. Specifically, in mitochondria isolated from rat cerebral arteries, basal and maximal respiration, ATP production, proton leak, and spare respiratory capacity are higher in females compared to males[50]. In addition, genes associated with mitochondrial function are expressed at higher levels in females in adipose tissue[51]. The impact of such mitochondrial sexual dimorphism can be illustrated by the endurance capacities of male and female rats. Females have much higher endurance capacities, which could be explained by their higher mtDNA and protein content, OXPHOS activity, and glutathione peroxidase activity[48].

One human study determined that citrate synthase (CS) activity was higher in females compared to males, which is notable because CS can be interpreted as a mitochondrial mass marker[52, 53]. The same study found that the mitochondrial complexes I, III and IV had a significantly higher activity in female PBMCs and, given that complexes I – IV are the driving force for ATP generation by complex V, females had trended towards higher ATP production, although this was not significant[52].

1.5.2 Oxidative stress

Mitochondria are a major source of ROS, which can lead to damaging effects to the mitochondria themselves, such as mtDNA mutations, with chronic exposure. The generation of mitochondrial ROS is typically lower in females and females produce higher levels of antioxidant enzymes such as glutathione peroxidase resulting in less oxidative damage to female mitochondria[54, 55]. There is evidence to suggest that oestrogen, through biding to ERs and the resulting signalling cascade, is responsible for the increased antioxidant enzyme expression, thereby granting female mitochondria a greater antioxidant defence capacity[55]. One proposed mechanism for this is the activation of mitogen-activated protein kinase (MAPK) by oestrogen, which in turn activates NFκB, resulting in the increased expression of antioxidant enzymes[52].

1.5.3 Calcium handling

Calcium handling by mitochondria is a key determinant of cell fate. It is essential that mitochondria maintain the correct levels of calcium, balancing calcium entry along the electrochemical gradient and calcium extrusion[48]. Calcium overload can lead to the opening of the mitochondrial permeability transition pore (mPTP), which results in an increase in permeability of the inner mitochondrial membrane. The mPTP may be opened

for a short time to relieve build-up of calcium or ROS, but if it remains open for too long, the mitochondria will swell, the outer membrane will rupture, and the membrane potential will collapse. As a result, OXPHOS and ATP synthesis will cease. In addition, release of cytochrome c and other pro-apoptotic factors will initiate the mitochondrial pathway of apoptosis[56].

Cardiac mitochondria isolated from female rats show a greater resistance to mitochondrial swelling at high calcium concentration. This ability to maintain membrane potential and control the uptake of calcium under high calcium conditions is dependent on modulation of the calcium uniporter[57]. Even in normal conditions, oestrogen has been shown to protect cardiac mitochondria from high calcium-induced release of cytochrome c[58].

1.5.4 Biogenesis

The ability of oestrogen to activate mitochondrial biogenesis has been widely demonstrated. Oestrogen receptors can bind to mtDNA and thereby oestrogen can induce transcription of mtDNA and respiratory chain proteins[48]. Pgc-1α is the major regulator of energy metabolism and mitochondrial biogenesis, and oestrogen has been shown to increase the expression of this protein through ERα and the presence of an oestrogen responsive element (ERE) in the promoter of the transcription factor nuclear respiratory factor-1 (Nrf-1)[48, 59]. Nrf-1 controls the expression of nuclear-encoded mitochondrial genes such as mitochondrial transcription factor A (Tfam), which in turn regulates transcription of mtDNA. Treatment of ovariectomised rats with oestrogen can increase Nrf-1 levels, but the oestrogen mediated stimulation of mitochondrial biogenesis is inhibited following knockdown of Nrf-1, providing further evidence of this mechanism[59].

The effects of androgens on mitochondrial structure and function have not yet been as extensively studied. One study, utilising 3T3-L1 adipocytes, found that testosterone decreased mitochondrial biogenesis and proliferation as measured by a decrease in expression of biogenesis markers Gabpa, Cox4 and Pgc1b. Furthermore, a decrease in CS activity and mitochondrial mass measured by Mitotracker Green (MTG) fluorescence indicated a decrease in mitochondrial content, and therefore either decreased biogenesis, disrupted mitochondrial dynamics, or a combination thereof. Interestingly, Pgc1a was unaffected by testosterone treatment, indicating that oestrogen and testosterone affect mitochondrial biogenesis via different pathways[60]. They further reported that testosterone treatment increased Mfn1 mRNA levels, a marker of increased mitochondrial fusion, which

was reversible with oestrogen supplementation. They also noted that testosterone decreased Fis1 and Drp1 expression, markers of mitochondrial fission.

On the other hand, a study using the L6E9 skeletal muscle cell line, reported that testosterone did not appear to affect the expression of markers of mitochondrial biogenesis, Pgc1a and Tfam. In addition, they saw no differences in MTG staining following testosterone treatment[61]. It is clear therefore that much more work must be done to determine the effects, if any, of testosterone on mitochondrial function.

Sex differences in mitochondrial composition and function have been described in a number of tissues and species and these differences affect, among other factors, biogenesis, oxidant and antioxidant capacities and respiratory capacity (Fig. 1.1). There may still be sex differences in other mitochondrial functions or mitochondrial properties that have yet to be elucidated. Understanding the landscape of these sex differences may provide useful insight in the context of pathologies in which mitochondria are known to play a role.

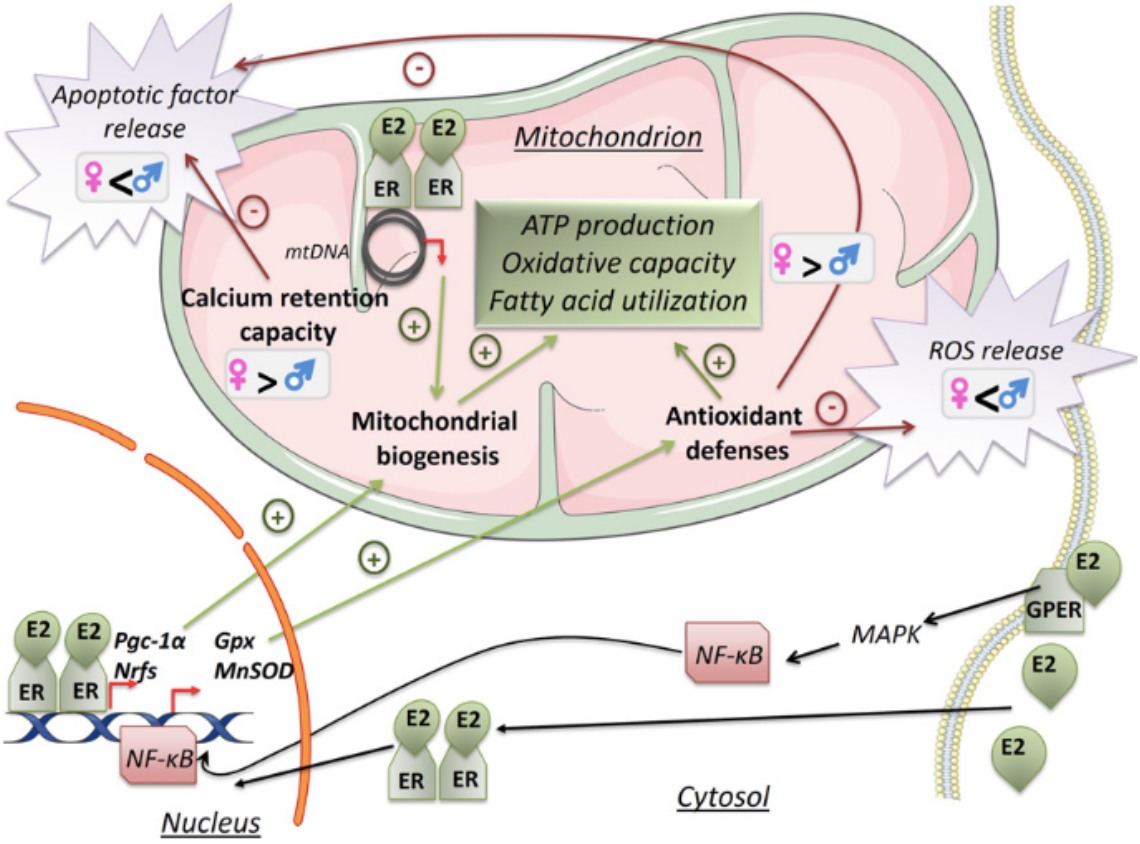

Figure 1.1 The influence of oestrogens on mitochondrial processes

The oestrogen (E2)/oestrogen receptor (ER) complex that is formed when oestrogen binds to its cytosolic receptors is responsible for promoting processes that result in mitochondrial sex differences. Interaction of the oestrogen/oestrogen receptor complex with the nuclear DNA results in transcription of Pgc-1α and Nrfs, while binding of oestrogen to ERs on mtDNA results in replication and transcription of the mtDNA. Oestrogen can also bind to GPER (G protein-coupled oestrogen receptor), resulting in MAPK-dependent NF-κB activation, leading to transcription of genes encoding antioxidant enzymes. The result is higher mitochondrial biogenesis, antioxidant defences, fatty acid utilisation, oxidative capacity, and ATP production in female mitochondria.

Figure adapted from Ventura-Clapier et al., 2016.

1.6 Mitochondria and sex differences in pathologies

Mitochondrial dysfunction is associated with numerous pathologies and disorders such as cardiovascular, metabolic and neurodegenerative diseases and these disorders exhibit marked sex biases (Fig. 1.2). Women are at greater risk of developing these diseases until after menopause at which point their risk matches that of men, strongly indicating the involvement of sex hormones[48]. Pre-menopausal women exhibit better mitochondrial function, lower levels of ROS, better antioxidant capacity and calcium handling (Fig. 1.2), but conversely, reduced antioxidant capacity and increased ROS production after menopause. These decreased mitochondrial differences following reproductive senescence in women further suggests involvement of sex hormones such as oestrogen[48, 52]. Decreased oestrogen levels following menopause may also impact mitochondrial metabolism. Ovariectomised mice demonstrated a decrease in mitochondrial respiratory function and fatty acid oxidation[62]. Such functional deficits may further contribute to the increased incidence of disease in post-menopausal women.

1.6.1 Cardiovascular diseases

Cardiovascular diseases (CVD) are the leading causes of death globally, however there is greater prevalence of CVD in men compared with pre-menopausal women, though this risk profile is reversed following menopause. Dysfunctional mitochondria and atypical mitochondrial biogenesis are markers of CVD. As discussed, oestrogen promotes mitochondrial biogenesis, and therefore oestrogen deficiency, which may occur following menopause, can induce mitochondrial compromise through Pgc-1α down-regulation.

Ischemic heart disease, a common form of CVD, often caused by atherosclerosis is characterised by cardiac ischemia. Ischemia, when there is an inadequate supply of blood to the heart muscles, and post-ischemic reperfusion, results in functional and structural mitochondrial injury due to excess ROS production and calcium overload. Both ROS and calcium can cause mPTP opening, leading to a decrease in ATP supply, and ultimately to cell death[56]. As has been shown in rats, ischemic reperfusion injury results in a smaller infarct size in females[63]. This protection from ischemic reperfusion injury in females may arise as a result of lower calcium uptake and better ability to maintain membrane potential under conditions of high calcium[57]. The lower levels of ROS production in female mitochondria may also play a significant role in the protection against CVD[48].

1.6.2 Metabolic diseases

Sex differences in metabolism have been documented, particularly how the sexes differentially rely on fuels for energy. Males are more reliant on carbohydrates and females more so on lipids[48]. Oestrogen is known to exert a protective effect against insulin resistance and type II diabetes, as well as regulating energy intake and expenditure. Loss of circulating oestrogen rapidly alters whole body metabolism, fat distribution, and insulin action[48]. The mechanisms through which oestrogen and its receptors can regulate metabolism and glucose homeostasis are not well understood, but are suggested to include genomic, non-genomic, and mitochondrial mechanisms[64].

Clinical studies have shown that the decrease in oestrogen levels following menopause increases the risk of developing diabetes in women, but treatment with hormone replacement therapy can reduce this risk by up to 62%[64, 65]. Furthermore, treatment of ovariectomised mice (fed a high fat diet) with physiological levels of oestrogen restores insulin sensitivity and glucose tolerance. The same effect was not seen in ERα-deficient mice[66]. Such studies highlight the importance of oestrogen in regulating insulin action. Testosterone is also known to impact metabolism. Low testosterone levels are associated with a threefold higher risk of developing metabolic syndrome, as well as a lower oxidative profile, suggesting that low testosterone levels and impaired mitochondrial function give rise to insulin resistance in men[67].

There is also a sexual dimorphism in response to extrinsic dietary factors. When fed a high fat diet, male rats exhibit enhanced mitochondrial differentiation (an increase in the functional capability of pre-existing mitochondria) in their brown adipose tissue, whereas in female rats there is a decrease in mitochondrial functionality but a rise in mitochondrial proliferation[68]. Sex differences are also seen in the liver, where a high fat diet increases mitochondrial protein content and oxidative capacity to a greater extent in females[69].

1.6.3 Neurodegenerative diseases

Dysfunctional mitochondria are involved in the pathogenesis of, or are a symptom of, many neurodegenerative diseases[48]. Females are more protected from neurological conditions at younger ages, indicating sex hormones as a factor in this protection. This protection may be because of the greater resistance to oxidative stress and antioxidant defences in female mitochondria.

Alzheimer's disease (AD) is the most common neurodegenerative dementia. Cases are lower in young women than men but are increased in postmenopausal women, once again highlighting the protective effect of oestrogen[70]. Mitochondrial dysfunction, including decreased mitochondrial biogenesis and increased ROS production, is an underlying event in the progression of the disease[48]. In the presence of the amyloid-β plaques, mitochondria will produce free radicals, triggering mitochondrial and extra-mitochondrial pathways of apoptosis. Mitochondria from young females generate less ROS and produce less signals for apoptosis than those from males, but these protective effects are lost with age[48, 71]. Oestrogen can also promote antioxidant protection from the generated ROS and activate anti-apoptotic mechanisms[72, 73].

Parkinson's disease is another neurodegenerative disease with a higher incidence in men than women. There is a mounting body of evidence to suggest that mitochondrial dysfunction may be a driving factor in this disease, specifically the impairment of energy production and subsequent oxidative stress[74, 75]. The mitochondrial biogenesis regulator, Pgc-1α, has been identified as a crucial factor in the gender-dependent susceptibility to Parkinson's disease, and Pgc-1α therefore presents a potential therapeutic target[48, 76].

These ongoing findings relating to the involvement of mitochondrial sex differences in disease pathogenesis may have potential implications for development of novel therapeutic strategies in a number of pathologies ranging from cancer to cardiovascular disease.

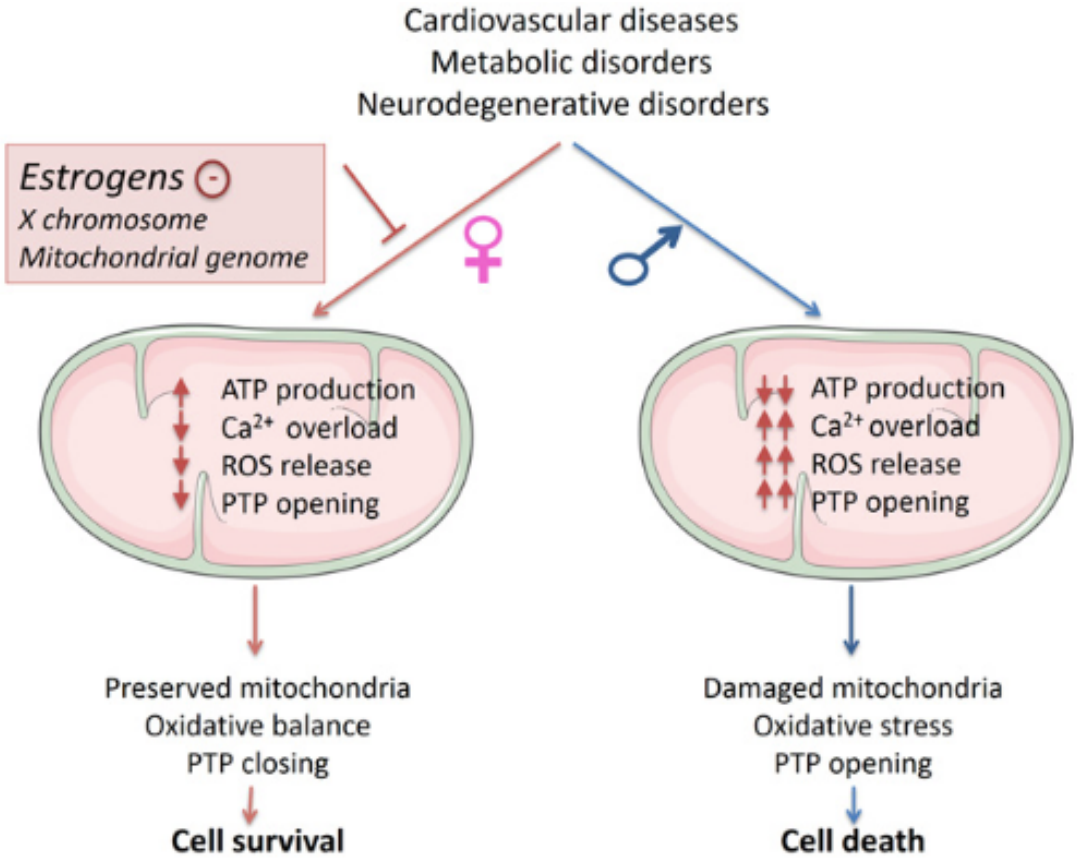

Figure 1.2 The effect of mitochondrial sex differences on disease pathogenesis

The pathogenesis of several diseases exhibits a mitochondrial component, and women are generally better protected from these diseases before menopause. Increased calcium overload, decreased mitochondrial ATP production and increased ROS generation lead to mitochondrial damage in males. Oestrogens therefore appear to exert a protective effect. *Figure adapted from Ventura-Clapier et al., 2016.*

1.7 Aims & Objectives

The field of immunology has now begun to appreciate the importance of considering sex as a biological variable in research. Recent work has made progress in identifying sex differences in the immune response, and in determining the factors driving this, such as sex chromosome complement and sex hormones. Numerous studies have pointed to different cellular responses to immune challenge, and different outcomes in pathologies, however, the mitochondrial and metabolic contribution to these different responses is less clear. Furthermore, specific studies into sex differences in the responses of $CD4^+$ and $CD8^+$ T cells are limited in number and scope, however the existing studies have suggested that these differences could have significant consequences in multiple pathologies.

There is increasing evidence to suggest that mitochondria and their functioning are sexually dimorphic. The aim for this project was to develop a better understanding of how mitochondria can contribute to sex differences in immunity, with the hypothesis that mitochondrial morphological and metabolic differences contribute to sex differences in immune responses in $CD4^+$ and $CD8^+$ T cells.

1.7.1 Objectives

- Outline baseline sex differences in mitochondrial mass and polarisation in $CD4^+$ and $CD8^+$ T cells in spleen, lymph node, and liver tissue.
- Determine sex differences in mitochondrial morphology in $CD8^+$ T cells in spleen, lymph node, and liver tissue.
- Investigate sex differences in mitochondrial parameters in response to immune challenge in $CD4^+$ and $CD8^+$ T cells in spleen and lymph node tissue.

Chapter 2
Materials & Methods

2.1 Materials

2.1.1 Chemicals and reagents

Ammonium chloride (NH_4Cl), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), Dulbecco's phosphate buffered saline (PBS), fluoro-carbonyl cyanide phenylhdrazone (FCCP), hanks buffered saline solution (HBSS), lipopolysaccharide (LPS), L-glutamine solution, oligomycin, paraformaldehyde (PFA), penicillin-streptomycin (P/S), Percoll, Roswell Park memorial institute medium (RPMI-140), and trypan blue were purchased from **Merck**. MagniSort™ Mouse CD8 T cell Enrichment Kit was purchased from **Invitrogen**. Poly (I:C) was purchased from **Invivogen.** 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and OneComp eBeads™ compensation beads were purchased from **Fisher Scientific**. Foetal calf serum (FCS) was purchased from **Gibco**. PBS tablets were purchased from **Medical Supplies Company.**

2.1.2 Equipment

Falcon tubes (15 ml, 50 ml), sterile tissue culture plates (6, 12, 24, 48, 96 well), sterile tissue culture flasks (T25, T75, T175), pipette tips (p10, p200, p1000), petri dishes (15 mm, 60 mm), syringes (2ml, 5 ml, 20 ml), Eppendorf tubes (0.5 ml, 1.5 ml), serological pipettes (5 ml, 10 ml, 25 ml) were purchased from **Cruinn Diagnostics Ltd**. Pipette sets (p2, p10, p20, p200, p100), aerosol barrier pipette tips (10µl, 20µl, 200µl, 1000µl), transfer pipettes, pipette boy, cell strainers (70 µm), FACs tubes (with and without lids), needles, scalpels, dissection tools (scissors, tweezers), and cover slips (#1.5; 0.16 to 0.19 mm) were purchased from **Fisher Scientific**. EasySep™ magnet for column-free separation was purchased from **Stemcell Technologies**. Glass microscope slides were purchased from **VWR International.** Leica SP8 scanning confocal microscope and Leica LAS X acquisition software were purchased from **Leica**. Centrifuges (5424 R, 5425 R, 5804 R) were purchased from **Eppendorf**. Haemocytometer was purchased from **Hausser Scientific**. LSR Fortessa, FACs Canto, and FACs Aria Fusion were purchased from **BD Biosciences**. FlowJo v10 software was purchased from **Treestar.** GraphPad Prism 9 was purchased from **GraphPad Software.**

2.1.3 Mice

In all experiments, adult (8-12 weeks) WT C56BL/6J mice were used. Mice were bred in-house in the comparative medicine unit in Trinity Biomedical Sciences Institute, Trinity College Dublin under specific pathogen-free condition in accordance with Irish and European Union regulations. Mice were housed in accordance with the Health Products Regulatory Authority (HPRA) Ireland guidelines with access to food and water *ad libitum*. All murine experiments were carried out in compliance with the Lydia Lynch laboratory project license (Project Authorisation Number: AE19136/P169; Individual Authorisation Number: AE19136/I670), with ethical approval from the Trinity College Dublin Animal Research Ethics Committee and from the HPRA.

2.1.4 Solutions & Buffers

Buffer	**Composition**
T cell culture media (cRPMI)	RPMI-1640 + 20μM L-glutamine, 10% FCS, 1% P/S
FACs buffer	PBS + 1% FCS
RBC lysis buffer	1 L: 1L dH_20 + 8.7g NH_4Cl
MACs cell separation buffer	PBS + 2% FCS + 1mM EDTA

Table 2.1 List and composition of commonly used buffers

2.1.5 Cell culture stimulants

Reagent	**Vehicle**	**Stock Concentration**	**Working Concentration**	**Supplier**
Anti-CD28	PBS	1mg/ml	3μg/ml	BD Pharmingen
Anti-CD3	PBS	1mg/ml	1μg/ml	BD Pharmingen
Recombinant mouse IL-2	dH2O + 0.1% BSA	100μg/ml	5ng/ml	Immunotools

Table 2.2 List and concentrations of stimulants used in cell culture

2.1.6 Flow cytometry antibodies & flow cytometry/confocal microscopy stains

Target	Fluorophore	Clone	Supplier
CD3	APC	17A2	Biolegend
CD45	FITC	30-F11	BD Biosciences
	BV421	30-F11	Biolegend
CD4	PE	GK1.5	Biolegend
	PerCP-eFluor710	RM4-5	Invitrogen
CD8a	APC-eFluor780	SK1	eBioscience
	APC/Cy7	43-6.7	Biolegend
CD69	PE/Cy7	H1.2F3	Biolegend
CD44	BV605	IM7	Biolegend
CD62-L	PE-CF594	MEL-14	BD Biosciences
CD25	BV785	PC61	Biolegend

Table 2.3 List of flow cytometry antibodies

Stain	Stock Concentration	Working Concentration	Supplier
4',6-diamidino-2-phenylindole, dihydrochloride (DAPI)	3mM	300nM	Thermo Fisher Scientific
MitoSpyTM Red CMXRos	1mM	250nM	Biolegend
MitoTracker™ Green FM (MTG)	1mM	100nM	Invitrogen
Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM)	100μM	100nM	Invitrogen
Live/Dead Aqua Fixable Viability Dye	1000X solution in DMSO	Dilute 1:800	Thermo Fisher Scientific

Table 2.4 List of stains used for confocal microscopy imaging and flow cytometry

2.2 Methods

2.2.1 *In vivo* Drug Administration

Immunomodulatory compounds were administered via intra-peritoneal (i.p.) injection. Mice were injected with Poly (I:C) (200 µg/mouse) and euthanised 72 hours post injection. Mice were injected with lipopolysaccharide (LPS) (500 µg/kg) and euthanised 24 hours post injection.

2.2.2 Tissue Isolation & Processing

Mice were euthanised by CO_2 inhalation, and tissues were immediately harvested and placed in cold cRPMI until further processing steps. Lymph nodes were disrupted through a 70 µm filter into cRPMI and centrifuged for 5 minutes at 300g to pellet. Cells were then resuspended in cRPMI. Spleens were disrupted through a 70 µm filter into cRPMI, then centrifuged for 5 minutes at 300g to pellet. Red blood cell lysis was carried out by resuspending cell pellets in 2 mL of RBC lysis buffer for 5 minutes followed by washing and resuspending cells in cRPMI. Livers were disrupted through a 70 µm filter into cRPMI, before resuspension in 42% Percoll solution. Samples were then centrifuged at 800g at room temperature for 20 minutes with the centrifuge accelerator and break switched off. The resulting cell pellet was resuspended in 5 mL of RBC lysis buffer for 5 minutes followed by washing and resuspending cells in cRPMI.

2.2.3 Cell Isolation

$CD8^+$ T cells used for confocal microscopy were isolated using the MagniSort™ Mouse CD8 T cell Enrichment Kit (Invitrogen) following manufacturers protocols. $CD4^+$ and $CD8^+$ T cells used for proteomic analysis were isolated by Fluorescence-Activated Cell Sorting (FACS) using the BD Aria.

2.2.4 Proteomics

2.2.4.1 Sample Preparation

For proteomic analysis, live $CD4^+$ and $CD8^+$ T cell populations, isolated from lymph node lymphocytes, were sorted into FACS buffer using the BD Aria. Cell sorting was carried out by Dr. Barry Moran, Trinity College Dublin. Cells were divided between groups for *ex vivo* analysis or 24 hour activation before analysis. Cells taken for *ex vivo* analysis were washed twice in PBS. The cells were then pelleted, the supernatant was removed and the cell pellet was snap frozen using liquid nitrogen. Cell pellets were stored at -80°C until further processing. Cells in the activation group were cultured for 24 hours in cRPMI in the presence of anti-CD3 (1 µg/mL), anti-CD28 (3 µg/mL) and IL-2 (5 ng/mL), before harvesting and freezing as described.

2.2.4.2 Proteomic sample processing and analysis

Proteomics sample processing was carried out by Dr. Linda Sinclair, University of Dundee, as previously described[77]. Copy numbers were obtained and normalised using histones as reference standards. The processed data underwent cleaning using Python's pandas and numpy packages. Independent t-tests were performed to assess differences in protein expression between distinct groups within the dataset. The following comparisons were conducted for each individual protein:
CD4 (Male) vs CD4 (Female) *ex vivo*
CD4 (Male) vs CD4 (Female) with stimulation
CD8 (Male) vs CD8 (Female) *ex vivo*
CD8 (Male) vs CD8 (Female) with stimulation

Log2 Fold Changes were computed for each of the group comparisons, quantifying the magnitude of expression changes between groups. Volcano plots were generated using R and the ggvolc package. The most noteworthy genes were identified based on Log2 Fold Change exceeding 1 or falling below -1, coupled with a p-value below 0.05.

2.2.5 Flow Cytometry

For quantification of mitochondrial mass and membrane potential, cells were co-stained with MitoTracker Green (Invitrogen; 100 nM) and TMRM (Invitrogen; 100 nM) dyes. Cells

were co-stained in RPMI for 30 minutes at 37°C. Cells were washed with PBS for further staining. Cell suspensions were incubated with Live/Dead Aqua viability dye (Thermo Fisher Scientific; 1:800 in PBS) for 20 minutes on ice. Cells were then washed with PBS and incubated with extracellular fluorochrome-labelled antibodies (1:200 in FACS buffer) for 25 minutes on ice. Cells were then washed with and resuspended in FACS buffer for acquisition using either the BD Canto, BD LSR Fortessa, or Cytek Aurora. Data was analysed with FlowJo_v10 software.

2.2.6 Confocal microscopy

Isolated CD8 T cells were stained with MitoSpy CMX Ros (250nM, Biolegend) for 30 minutes in serum free RPMI at 37°C, 5% CO_2. Cells were then fixed using 2% paraformaldehyde (Merck) at room temperature for 15 minutes. Nuclear staining was achieved using 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; 300nM). Cells were mounted on glass slides (VWR International) using Mowiol mounting medium containing 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO; Sigma) and #1.5 cover slips (Fisher). Images were acquired using Leica SP8 scanning confocal equipped with ×63/1.4 N.A. oil objective lens. Z-stacks at 0.8µm increments were captured using an HyD detector in conjunction with Leica LAS X acquisition software.

Analysis of the mitochondrial morphology was performed using ImageJ Fiji in addition to the "Mitochondrial Analyser" plugin (Github; https://github.com/AhsenChaudhry/Mitochondria-Analyzer)[78]. Image analysis was carried out on the projected maximum intensity of the z-stack. Mitochondrial images were pre-processed by using the "enhance contrast" and "despeckle" commands to remove background noise. Mitochondria were then delineated as regions of interest (ROIs) using the adaptive thresholding method, where block size was set to an equivalent of 0.75µm and the optimal C value of 3 was determined using the Mitochondrial Analyzer plugin.

These pre-processed images were then subject to the "2D analysis" command which quantifies mitochondrial area, perimeter as well as generating a numerical descriptor of mitochondrial shape. Form Factor (FF) was calculated as the inverse of the "circularity" output value ($P^2/(4\pi A)$, where 1 indicates a round object and increases with elongation. For network connectivity analysis, the "skeletonize 2D/3D" command was applied to the threshold image to produce a skeleton map, and the "analyse skeleton" command was

used to calculate the number of branches, branch lengths, and branch junctions in the skeletonized network[78, 79].

2.2.7 Statistical Analysis

Statistical analyses were performed GraphPad Prism 9 using unpaired two-tailed Student's t tests. The p value <0.05 was considered statistically significant. In all figures, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. The data are presented as mean ± SEM.

2.2.8 Gating strategy for flow cytometry analysis

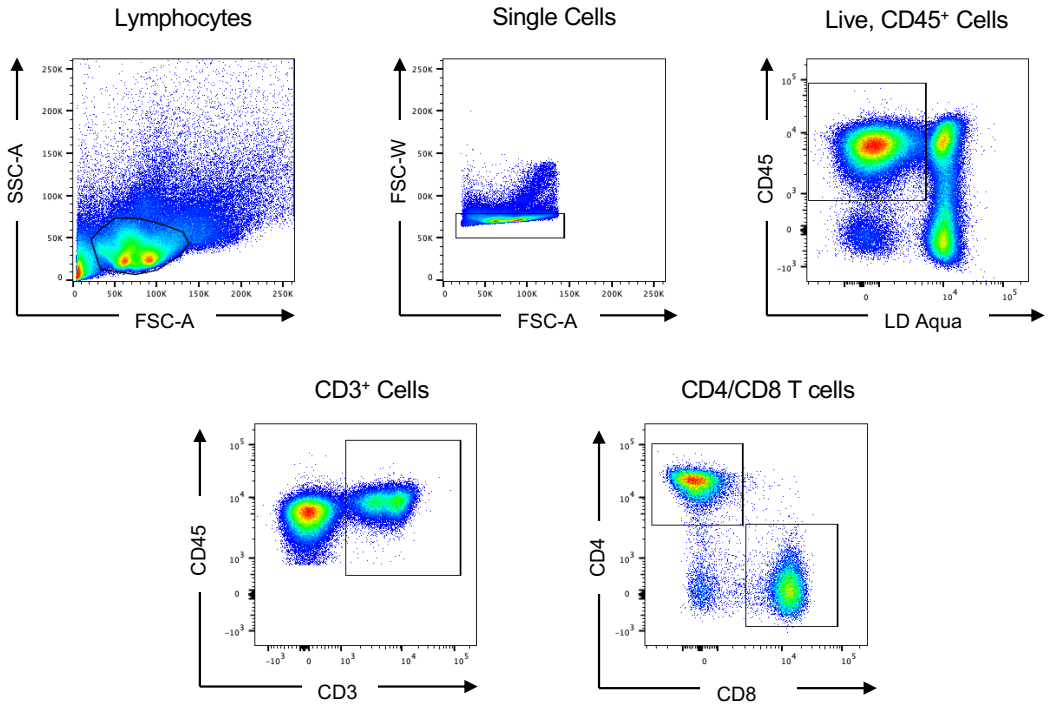

Figure 2.1 Gating strategy to identify $CD4^+$ and $CD8^+$ T cell populations

Cells were isolated and stained for flow cytometry as described in 2.2.2.2 and 2.2.1. Lymphocytes were identified based on forward and side scatter (size and granularity) and doublets were excluded based on size. Dead cells, identified as LD $Aqua^+$, and $CD45^-$ non-immune cells were removed. $CD3^+$ cells were selected. CD8 T cells were selected as $CD3^+CD8^+CD4^-$ cells. CD4 T cells were selected as $CD3^+CD4^+CD8^-$ cells.

Chapter 3

Results

3.1 Male and female mitochondria do not exhibit robust differences in mitochondrial mass and polarisation *ex vivo*

To investigate sex differences in mitochondrial mass and polarisation, male and female $CD4^+$ and $CD8^+$ T cells were isolated from spleens and lymph nodes. The cells were stained for flow cytometry as in 2.2.5. $CD4^+$ T cell and $CD8^+$ T cell populations were identified as per Figure 2.1. Mitochondrial polarisation was determined by utilising an oligomycin and FCCP control, representing highly polarised and depolarised mitochondrial populations, respectively (Fig. 3.1 G). Mean fluorescence intensity (MFI) for MTG and TMRM was calculated for each sample.

No significant differences were observed between male and female mitochondrial mass or polarisation in $CD8^+$ or $CD4^+$ T cells isolated from spleens or lymph nodes (Fig 3.1 and 3.2 A, B, D, E). However, while not significant, as shown in the representative histograms (Fig. 3.1 and 3.2 H), females might tend towards a slightly higher mitochondrial mass and polarisation than males, particularly in T cells isolated from lymph nodes. However, power calculations determined a sample size ranging from 15 – 356 would be required for significance in both parameters, indicating that any observations of trends or significance are spurious and there are no significant differences.

When polarisation was normalised to mass, male lymph node $CD8^+$ and $CD4^+$ T cells had a higher charge to mass ratio (Fig. 3.2 C, F). This finding might suggest that, when total mitochondrial mass is taken into consideration, males may have more polarised mitochondria compared to females. However, the same result was not observed in splenic T cells.

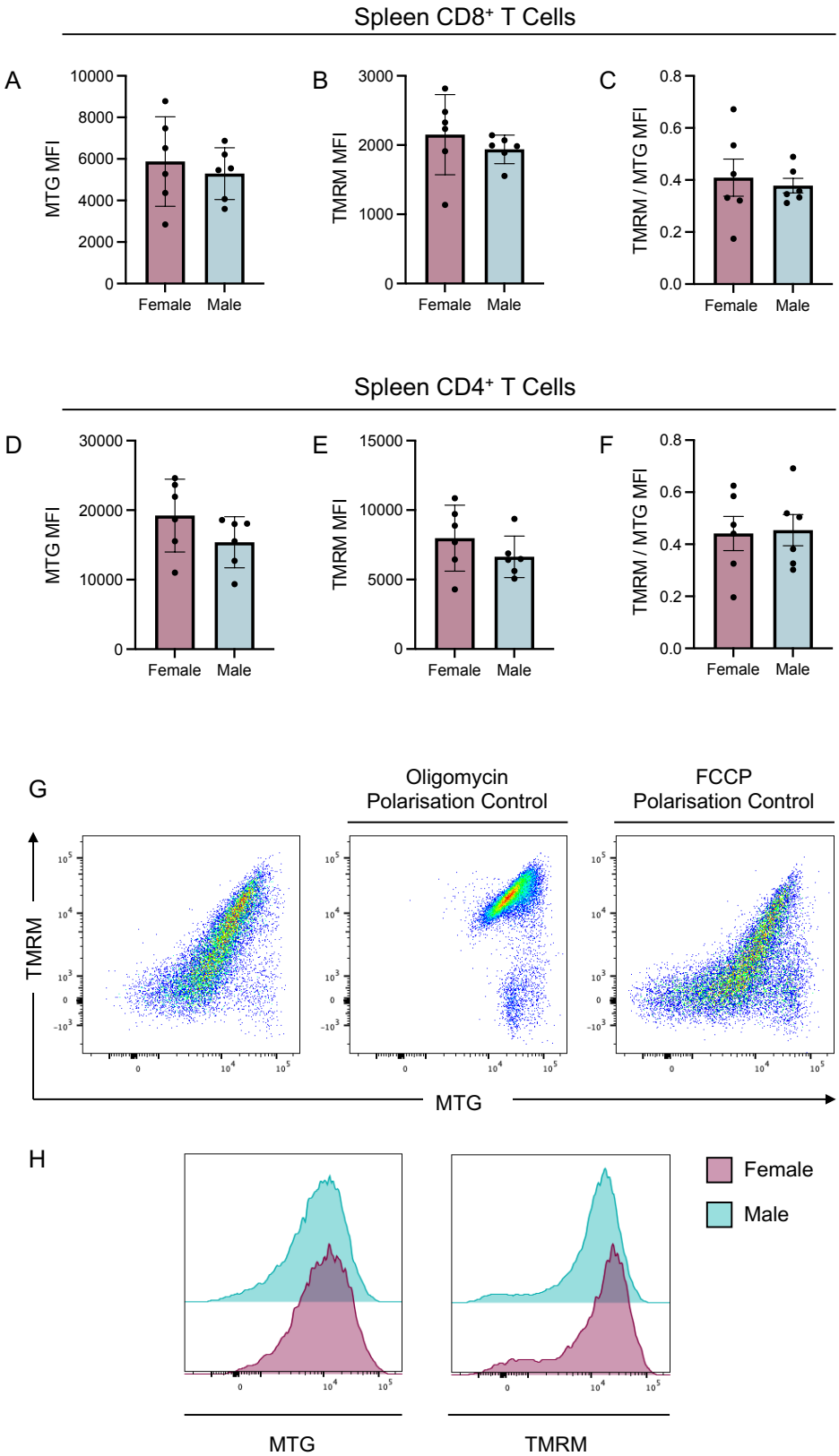
Spleen CD8⁺ T Cells
A
MTG MFI
10000
8000
6000
4000
2000
0
Female
Male
B
TMRM MFI
3000
2000
1000
0
Female
Male
C
TMRM / MTG MFI
0.8
0.6
0.4
0.2
0.0
Female
Male
Spleen CD4⁺ T Cells
D
MTG MFI
30000
20000
10000
0
Female
Male
E
TMRM MFI
15000
10000
5000
0
Female
Male
F
TMRM / MTG MFI
0.8
0.6
0.4
0.2
0.0
Female
Male
G
Oligomycin
Polarisation Control
FCCP
Polarisation Control
TMRM
MTG
H
Female
Male
MTG
TMRM

Figure 3.1 Male and female splenic $CD8^+$ and $CD4^+$ T cells do not have significant differences in mitochondrial mass or polarisation

Splenocytes from naïve mice were isolated and stained according to 2.2.5 to determine baseline mitochondrial content. $CD4^+$ and $CD8^+$ T cells were identified as in Figure 2.1. Graphs show the mean fluorescence intensity (MFI) of MTG and TMRM and the mitochondrial polarisation to mass ratios of splenic $CD8^+$ T cells **(A-C)** and $CD4^+$ T cells **(D-F)**, representative flow cytometry plot of TMRM vs MTG and oligomycin and FCCP polarisation controls **(G)**, representative histograms of male vs female MTG and TMRM MFI **(H)**. Data shown as mean ± SEM from 1 experiment, n = 6 mice/biological replicates per group. Dots represent individual mice. Significance was calculated using two-tailed unpaired students t-test. no * is indicative of ns (not significant).

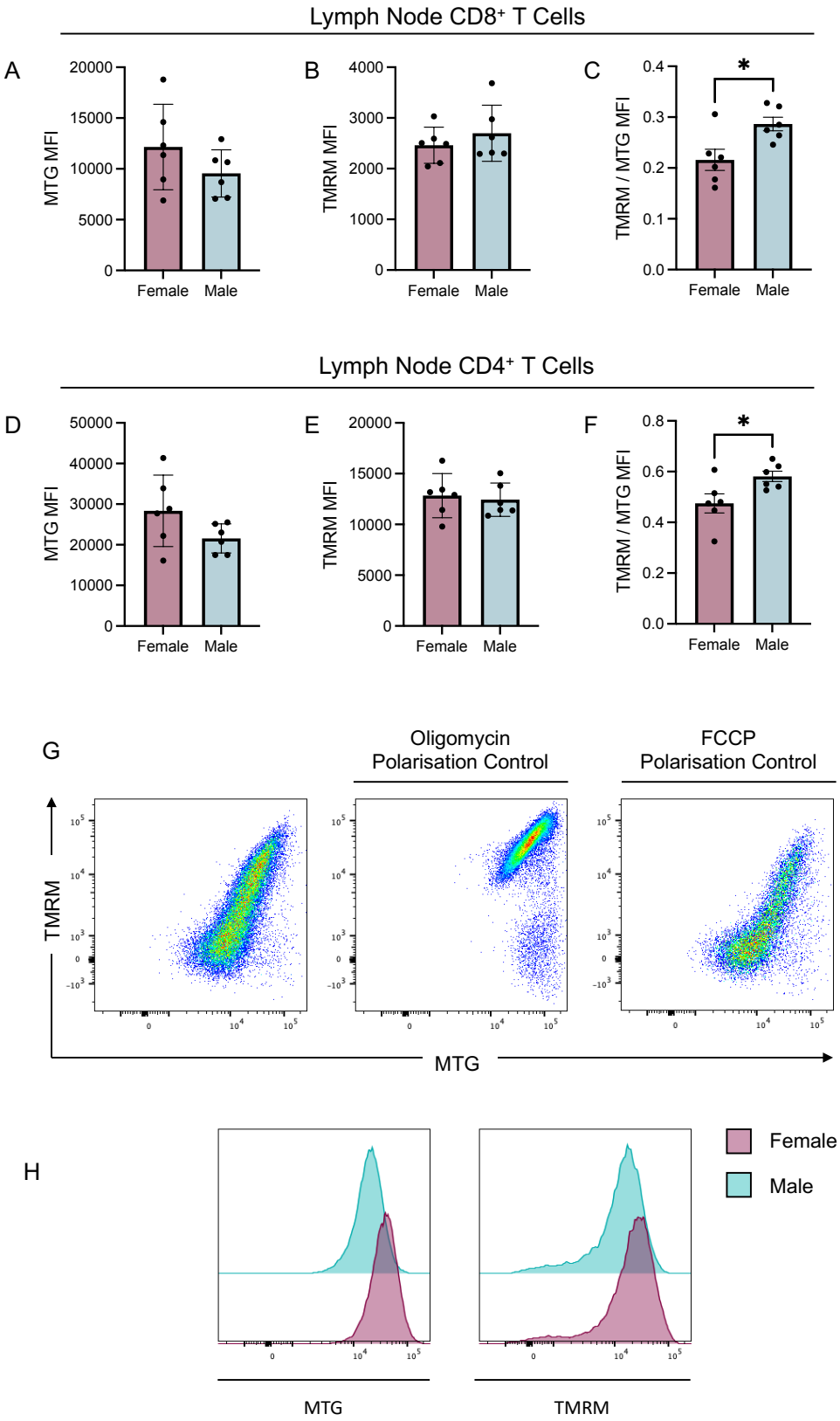
Lymph Node CD8⁺ T Cells
A
MTG MFI
20000
15000
10000
5000
0
Female
Male
B
TMRM MFI
4000
3000
2000
1000
0
Female
Male
C
TMRM / MTG MFI
0.4
0.3
0.2
0.1
0.0
*
Female
Male
Lymph Node CD4⁺ T Cells
D
MTG MFI
50000
40000
30000
20000
10000
0
Female
Male
E
TMRM MFI
20000
15000
10000
5000
0
Female
Male
F
TMRM / MTG MFI
0.8
0.6
0.4
0.2
0.0
*
Female
Male
G
Oligomycin
Polarisation Control
FCCP
Polarisation Control
TMRM
MTG
H
Female
Male
MTG
TMRM

Figure 3.2 $CD8^+$ and $CD4^+$ T cells isolated from male lymph nodes have a higher mitochondrial polarisation to mass ratio than female cells

Lymph node cells from naïve mice were isolated and stained according to 2.2.5 to determine baseline mitochondrial content. $CD4^+$ and $CD8^+$ T cells were identified as in Figure 2.1. Graphs show the mean fluorescence intensity (MFI) of MTG and TMRM and the mitochondrial polarisation to mass ratios of $CD8^+$ T cells **(A-C)** and $CD4^+$ T cells **(D - F)** isolated from lymph nodes, representative flow cytometry plot of TMRM vs MTG and oligomycin and FCCP polarisation controls **(G)**, representative histograms of male vs female MTG and TMRM MFI **(H)**. Data shown as mean ± SEM from 1 experiment, n = 6 mice/biological replicates per group. Dots represent individual mice. Significance was calculated using two-tailed unpaired students t-test. * $p \leq 0.05$.

3.2 Sex differences in mitochondrial morphology are most prominent in the liver

3.2.1 $CD8^+$ T cells from female liver have a higher mitochondrial count compared to $CD8^+$ T cells from male liver

Differences in mitochondrial mass, when measured via fluorescent dye and flow cytometry, could be explained by either differing numbers of mitochondria, or by a higher or lesser degree of cristae folding in a similar number of mitochondria. Since this cannot be determined through flow cytometric methods, it was pertinent to directly image the mitochondria to determine if there were sex differences in their mitochondrial content and structure. This was achieved by staining the isolated, live lymphocytes with a mitochondrial dye, Mitospy. This allowed the mitochondria to be visualised and analysed directly by confocal microscopy (Fig. 3.3 - 3.8).

$CD8^+$ T cells isolated from lymph nodes and spleens showed no significant differences in mitochondrial count per cell between males and females. Similarly, on a per cell or a per mitochondrion basis, no differences were seen in mitochondrial area or perimeter (Fig. 3.9 - 3.10 A, D - G). However, in $CD8^+$ T cells isolated from the liver, females had a greater mitochondrial count per cell. Females also had a higher total mitochondrial area per cell and mitochondrial perimeter per cell. The differences in perimeter and area were not significant when males and females were compared on a per mitochondrion basis (Fig. 3.11 A, D – G). This can be explained by the higher total mitochondria count. On account of the higher number of mitochondria, total perimeter and area increased accordingly, suggesting that mitochondria in female liver may be more abundant but are not generally greater in size.

In addition, two measures of mitochondrial circularity, form factor and aspect ratio, were calculated using the MitoAnalyser software. These results determined no differences between males and females in all three tissue types, indicating a similar level of mitochondrial elongation between the sexes (Fig. 3.9 - 3.11 B, C). This study was limited by the small sample size in the lymph node and liver samples, and so should be repeated with a larger sample size to confirm the phenotype.

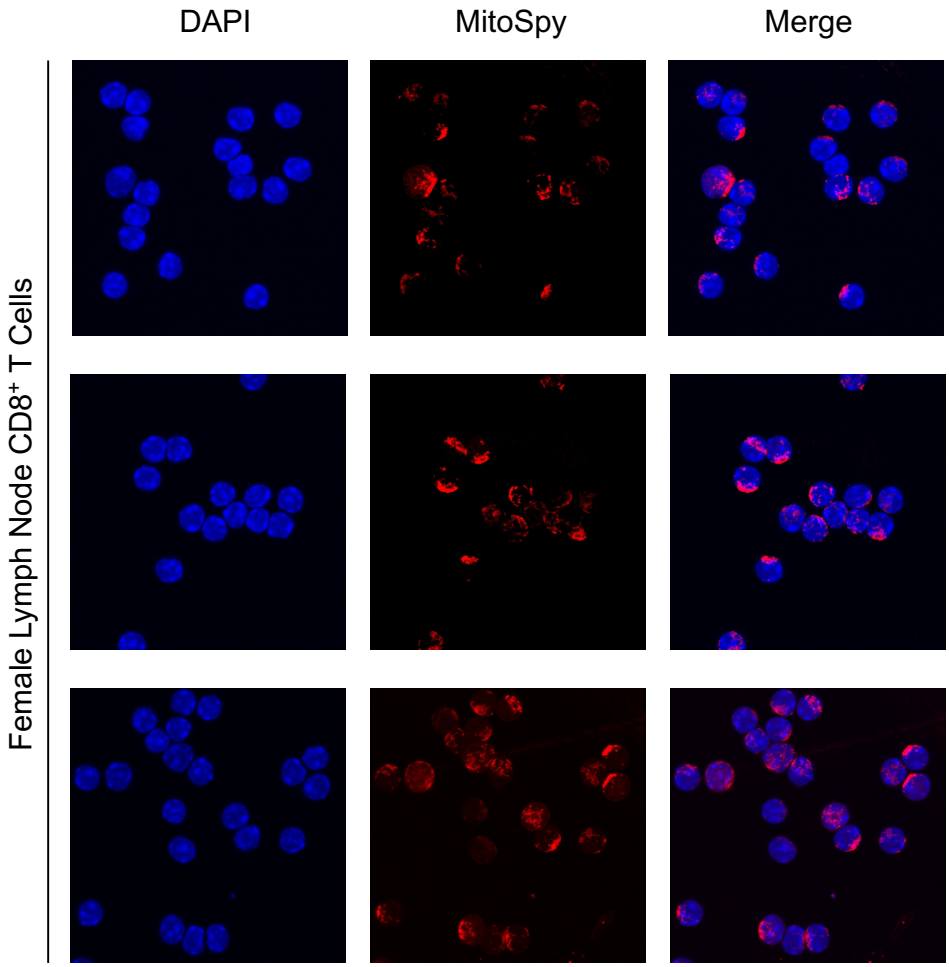

Figure 3.3 Representative confocal images of mitochondrial content of female lymph node $CD8^+$ T cells

Female lymph node $CD8^+$ T cells were isolated and stained for total mitochondrial content using MitoSpy (250nM; shown in red). Nuclear staining was achieved with DAPI (shown in blue). Images were acquired using Leica SP8 scanning confocal equipped with ×63/1.4 N.A. oil objective lens. Images are representative of 3 independent experiments, n = 1 mouse per group.

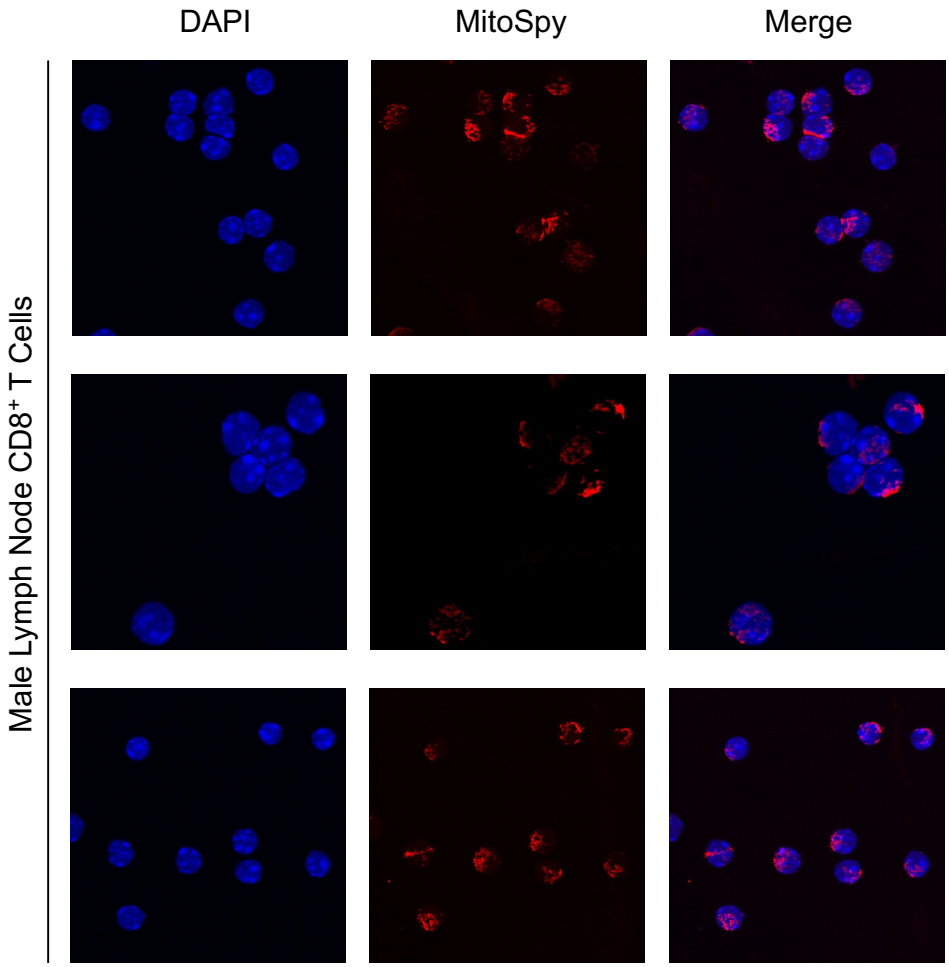

Figure 3.4 Representative confocal images of mitochondrial content of male lymph node $CD8^+$ T cells

Male lymph node $CD8^+$ T cells were isolated and stained for total mitochondrial content using MitoSpy (250nM; shown in red). Nuclear staining was achieved with DAPI (shown in blue). Images were acquired using Leica SP8 scanning confocal equipped with ×63/1.4 N.A. oil objective lens. Images are representative of 3 independent experiments, n = 1 mouse per group.

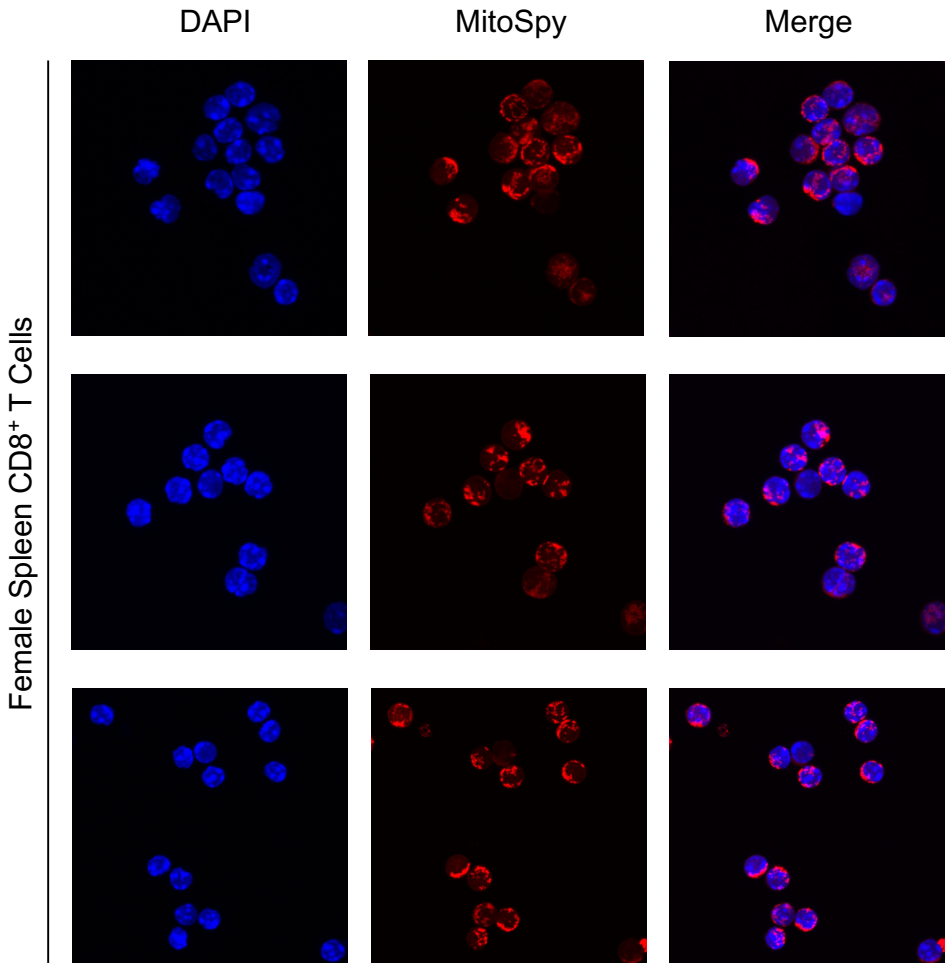

Figure 3.5 Representative confocal images of mitochondrial content of female spleen $CD8^+$ T cells

Female splenic $CD8^+$ T cells were isolated and stained for total mitochondrial content using MitoSpy (250nM; shown in red). Nuclear staining was achieved with DAPI (shown in blue). Images were acquired using Leica SP8 scanning confocal equipped with ×63/1.4 N.A. oil objective lens. Images are representative of 3 independent experiments, n = 2 mice per group.

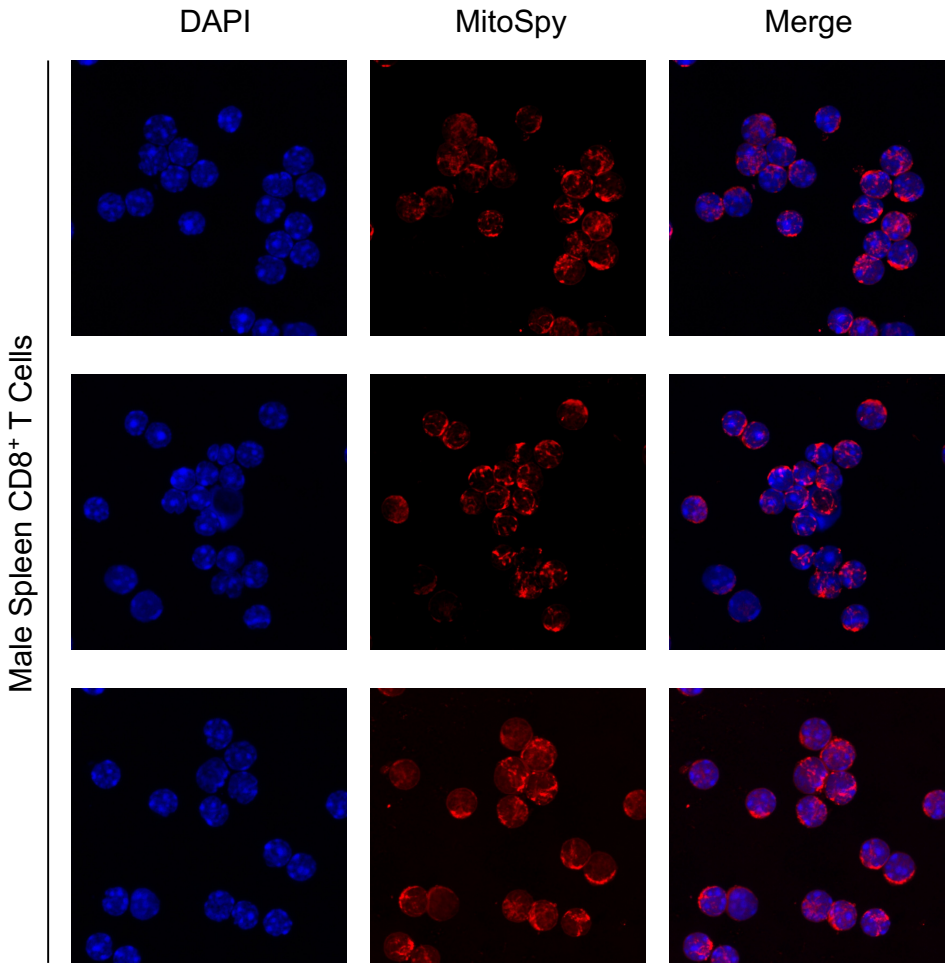

Figure 3.6 Representative confocal images of mitochondrial content of male spleen $CD8^+$ T cells

Male splenic $CD8^+$ T cells were isolated and stained for total mitochondrial content using MitoSpy (250 nM; shown in red). Nuclear staining was achieved with DAPI (shown in blue). Images were acquired using Leica SP8 scanning confocal equipped with ×63/1.4 N.A. oil objective lens. Images are representative of 3 independent experiments, n = 2 mice per group.

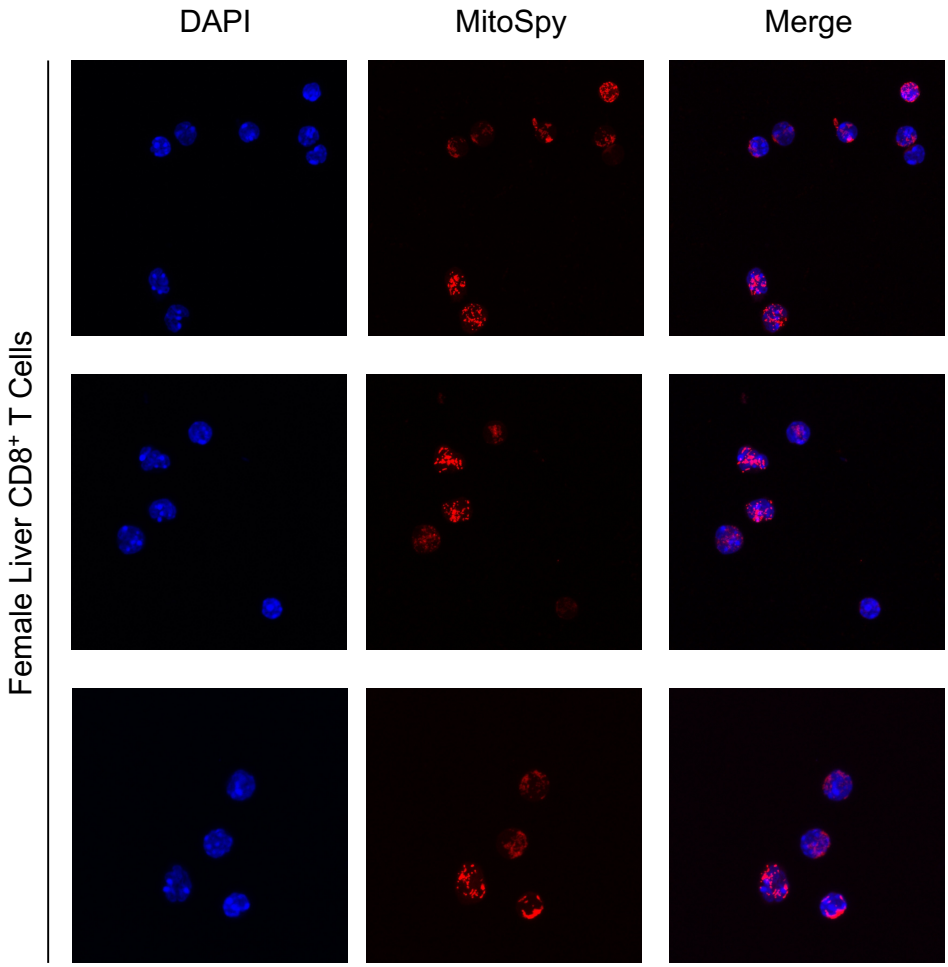

Figure 3.7 Representative confocal images of mitochondrial content of female liver $CD8^+$ T cells

Female liver $CD8^+$ T cells were isolated and stained for total mitochondrial content using MitoSpy (250 nM; shown in red). Nuclear staining was achieved with DAPI (shown in blue). Images were acquired using Leica SP8 scanning confocal equipped with ×63/1.4 N.A. oil objective lens. Images are representative of 3 independent experiments, n = 1 mouse per group.

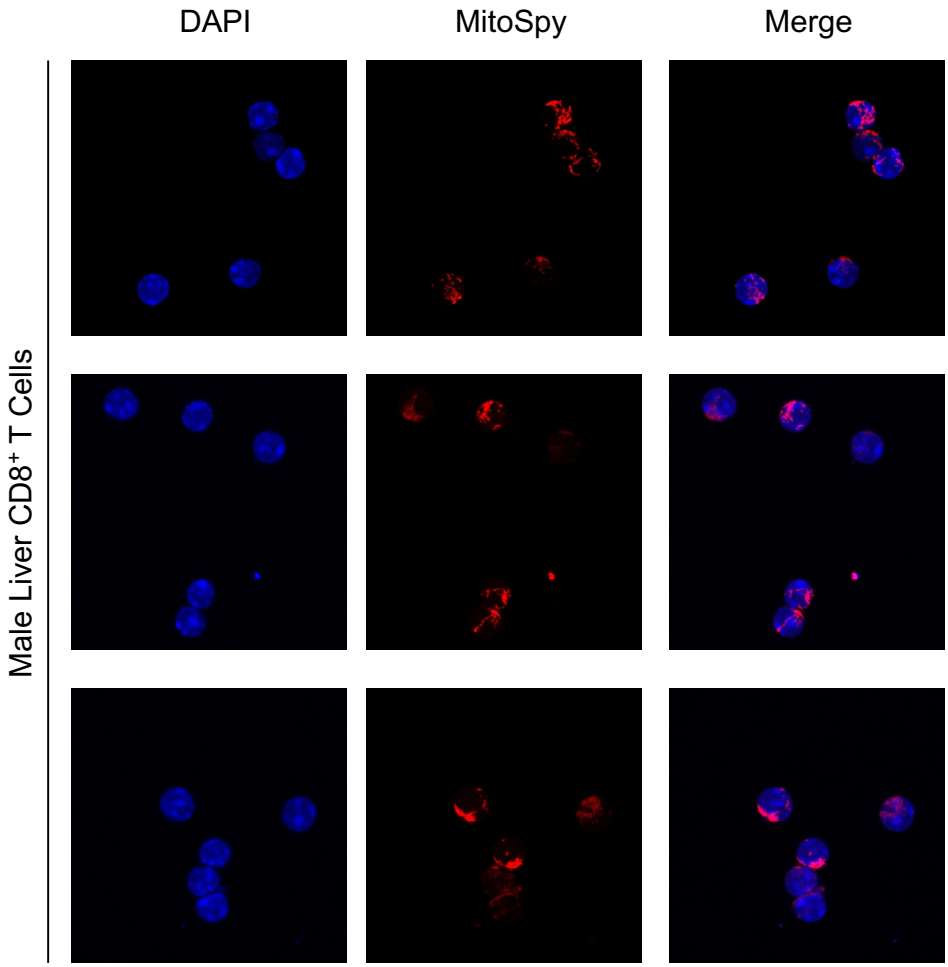

Figure 3.8 Representative confocal images of mitochondrial content of male liver $CD8^+$ T cells

Male liver $CD8^+$ T cells were isolated and stained for total mitochondrial content using MitoSpy (250 nM; shown in red). Nuclear staining was achieved with DAPI (shown in blue). Images were acquired using Leica SP8 scanning confocal equipped with ×63/1.4 N.A. oil objective lens. Images are representative of 3 independent experiments, n = 1 mouse per group.

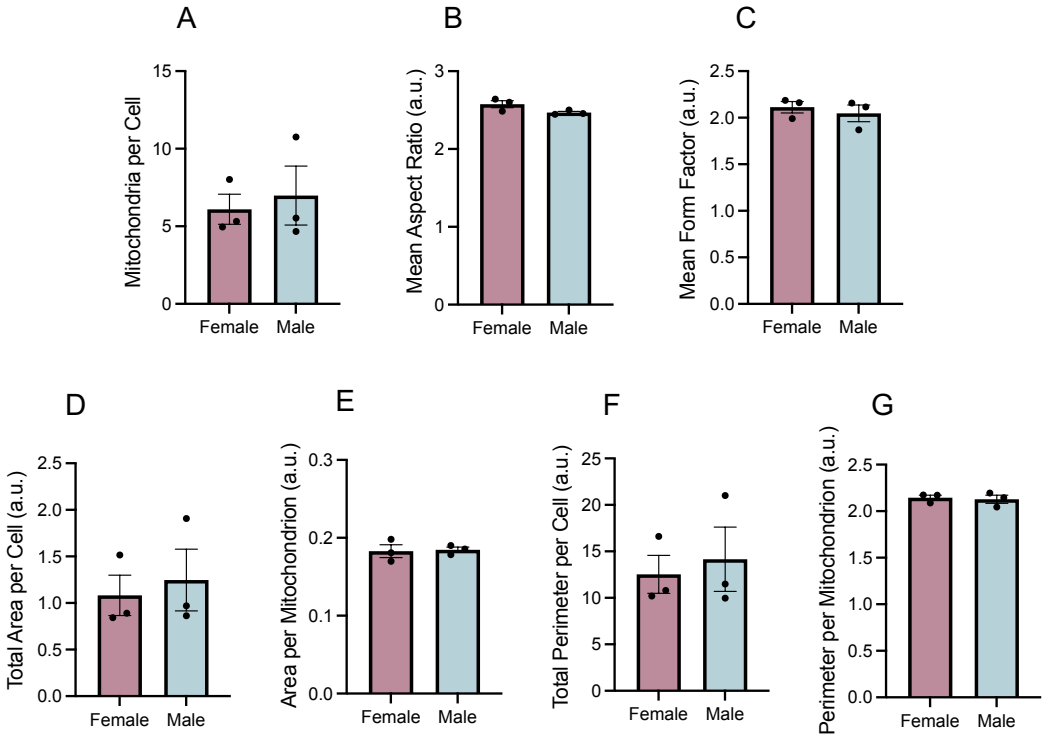

Figure 3.9 Male and Female CD8$^+$ T cells isolated from lymph nodes do not show differences in the mitochondrial parameters of count, area, perimeter or circularity

Lymph node CD8$^+$ T cells were isolated and stained for total mitochondrial content using MitoSpy and images were acquired using Leica SP8 scanning confocal equipped with ×63/1.4 N.A. oil objective lens. Analysis of confocal images was carried out using FIJI equipped with the MitoAnalyser plug-in. Graphs show mitochondrial parameters such as number of mitochondria per cell **(A)**, morphological parameters indicating mitochondrial circularity - aspect ratio and form factor **(B, C)** and mitochondrial area and perimeter on a per cell and per mitochondrion basis **(D - G)**. Dots represent individual mice, 70-100 cells analysed per mouse. Data shown as mean ± SEM from 3 independent experiments, n = 1 mouse per group. Significance was calculated using two-tailed unpaired students t-test.

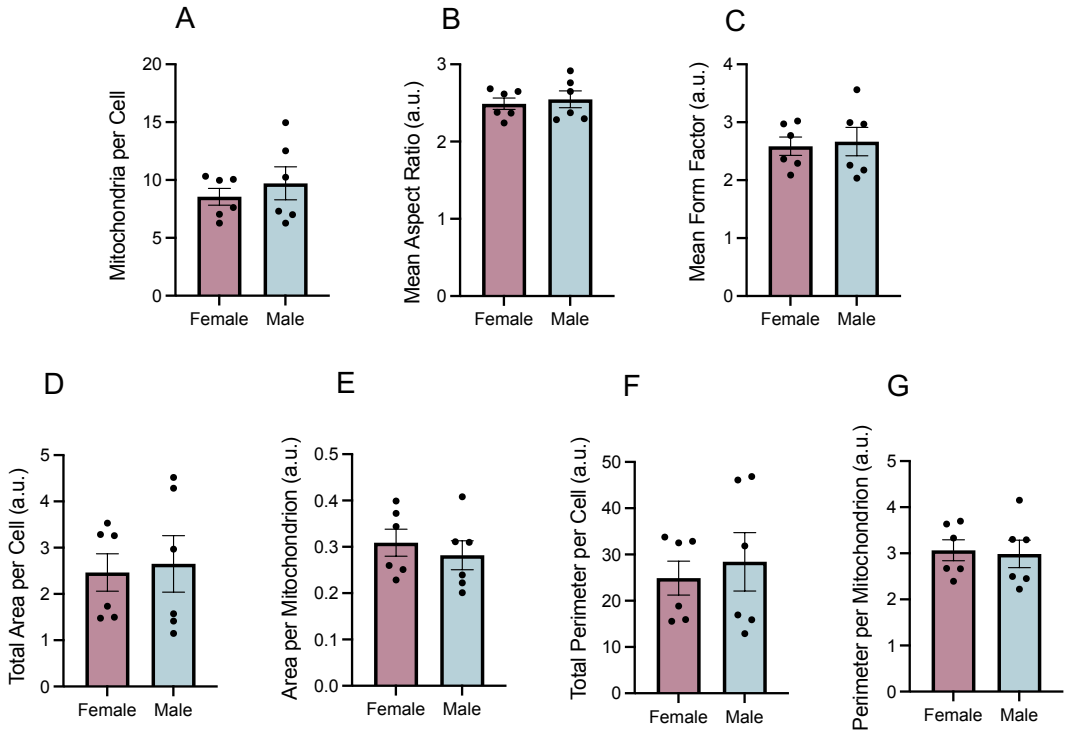

Figure 3.10 Male and female splenic CD8⁺ T cells do not show differences in the mitochondrial parameters of count, area, perimeter or circularity

Splenic CD8⁺ T cells were isolated and stained for total mitochondrial content using MitoSpy and images were acquired using Leica SP8 scanning confocal equipped with ×63/1.4 N.A. oil objective lens. Analysis of confocal images was carried out using FIJI equipped with the MitoAnalyser plug-in. Graphs show mitochondrial parameters such as number of mitochondria per cell **(A)**, morphological parameters indicating mitochondrial circularity - aspect ratio and form factor **(B, C)** and mitochondrial area and perimeter on a per cell and per mitochondrion basis **(D - G)**. Dots represent individual mice, 70-100 cells analysed per mouse. Data shown as mean ± SEM from 3 independent experiments, n = 2 mice per group. Significance was calculated using two-tailed unpaired students t-test.

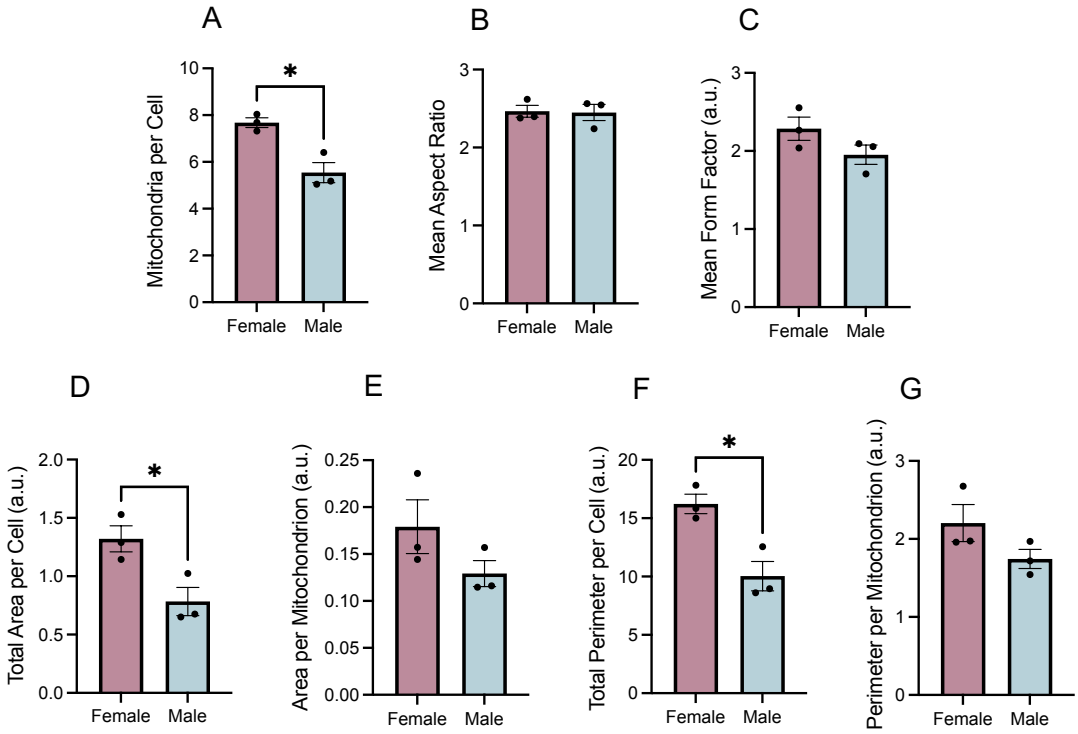

Figure 3.11 Female CD8⁺ T cells isolated from liver have greater mitochondrial count, area and perimeter than male cells, but do not differ in mitochondrial circularity

CD8⁺ T cells were isolated from the liver and stained for total mitochondrial content using MitoSpy and images were acquired using Leica SP8 scanning confocal equipped with ×63/1.4 N.A. oil objective lens. Analysis of confocal images was carried out using FIJI equipped with the MitoAnalyser plug-in. Graphs show mitochondrial parameters such as number of mitochondria per cell **(A)**, morphological parameters indicating mitochondrial circularity - aspect ratio and form factor **(B, C)** and mitochondrial area and perimeter on a per cell and per mitochondrion basis **(D - G)**. Dots represent individual mice, 70-100 cells analysed per mouse. Data shown as mean ± SEM from 3 independent experiments, n = 1 mouse per group. Significance was calculated using two-tailed unpaired students t-test. * $p \leq 0.05$.

3.2.2 $CD8^+$ T cells from female liver have a more branched mitochondrial network compared to $CD8^+$ T cells from male liver

Using the same MitoAnalyser software, the degree of mitochondrial branching was determined based on parameters such as number of branches per cell, branch length and diameter, branch junctions and branch end points. $CD8^+$ T cells isolated from male and female lymph nodes and spleens showed no significant differences in mitochondrial branching (Fig. 3.12 – 3.13). However, in cells isolated from liver, females tended to have a more branched mitochondrial network. Female cells had more branches per cell, a greater total branch length per cell and a greater number of branch junctions and end points per cell. Similar to the observed differences in mitochondrial content, the differences in mitochondrial branching between males and females were not significant on a per mitochondrion basis (Fig. 3.14). Again this points to a greater number of mitochondria in female liver $CD8^+$ T cells, but that are branched to a similar degree.

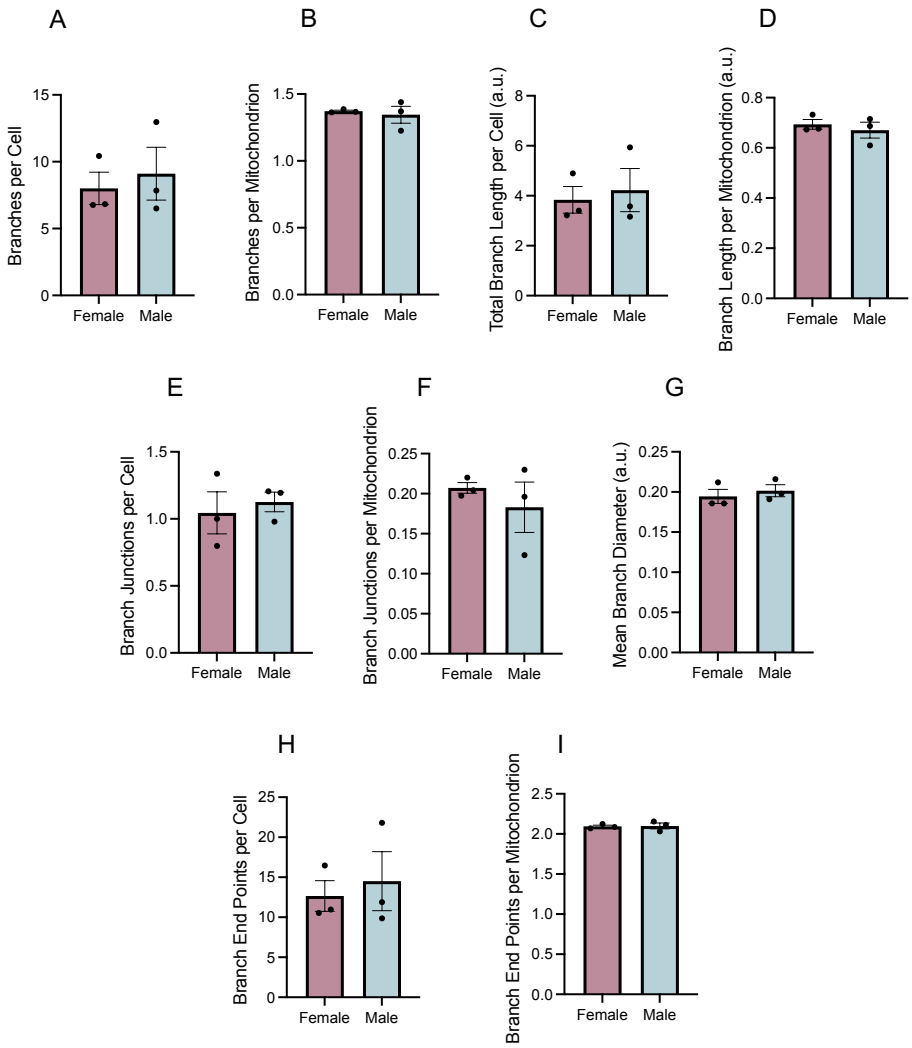

Figure 3.12 Male and Female CD8$^+$ T cells isolated from lymph nodes do not show differences in mitochondrial branch morphology

Lymph node CD8$^+$ T cells were isolated and stained for total mitochondrial content using MitoSpy and images were acquired using Leica SP8 scanning confocal equipped with ×63/1.4 N.A. oil objective lens. Analysis of confocal images was carried out using FIJI equipped with the MitoAnalyser plug-in. Graphs show mitochondrial parameters such as number of mitochondrial branches per cell and per mitochondrion **(A, B)**, length of mitochondrial branches per cell and per mitochondrion **(C, D)**, number of branch junctions per cell and per mitochondrion **(E, F)**, average diameter of each mitochondrial branch **(G)** and number of branch end points per cell and per mitochondrion **(H, I)**. Dots represent individual mice, 70-100 cells analysed per mouse. Data shown as mean ± SEM from 3 independent experiments, n = 1 mouse per group. Significance was calculated using two-tailed unpaired students t-test.

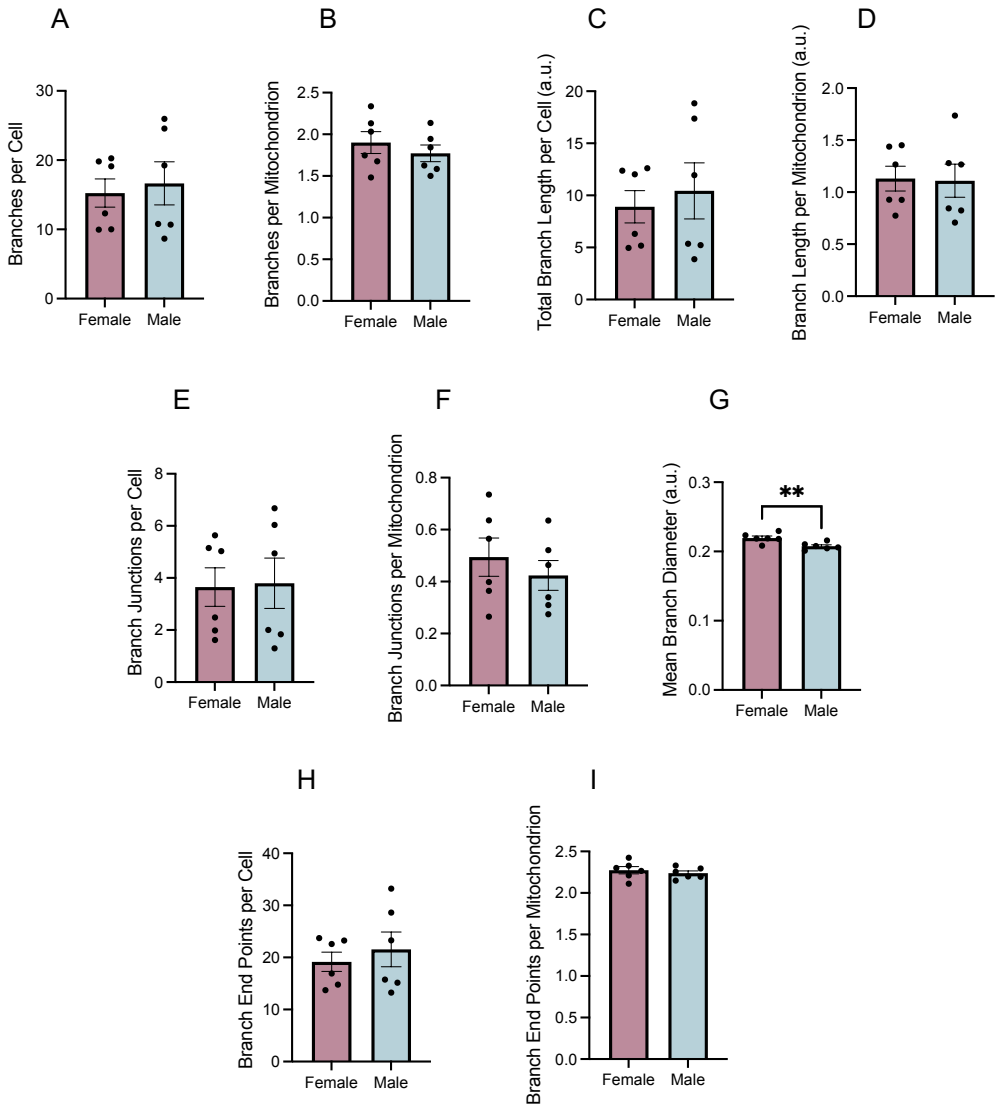

Figure 3.13 Female splenic CD8⁺ T cells have a greater mean branch diameter than male cells, but do not show differences in other parameters of mitochondrial branch morphology

Splenic CD8⁺ T cells were isolated and stained for total mitochondrial content using MitoSpy and images were acquired using Leica SP8 scanning confocal equipped with ×63/1.4 N.A. oil objective lens. Analysis of confocal images was carried out using FIJI equipped with the MitoAnalyser plug-in. Graphs show mitochondrial parameters such as number of mitochondrial branches per cell and per mitochondrion **(A, B)**, length of mitochondrial branches per cell and per mitochondrion **(C, D)**, number of branch junctions per cell and per mitochondrion **(E, F)**, average diameter of each mitochondrial branch **(G)** and number of branch end points per cell and per mitochondrion **(H, I)**. Dots represent individual mice, 70-100 cells analysed per mouse. Data shown as mean ± SEM from 3 independent experiments, n = 2 mice per group. Significance was calculated using two-tailed unpaired students t-test. ** $p \leq 0.01$.

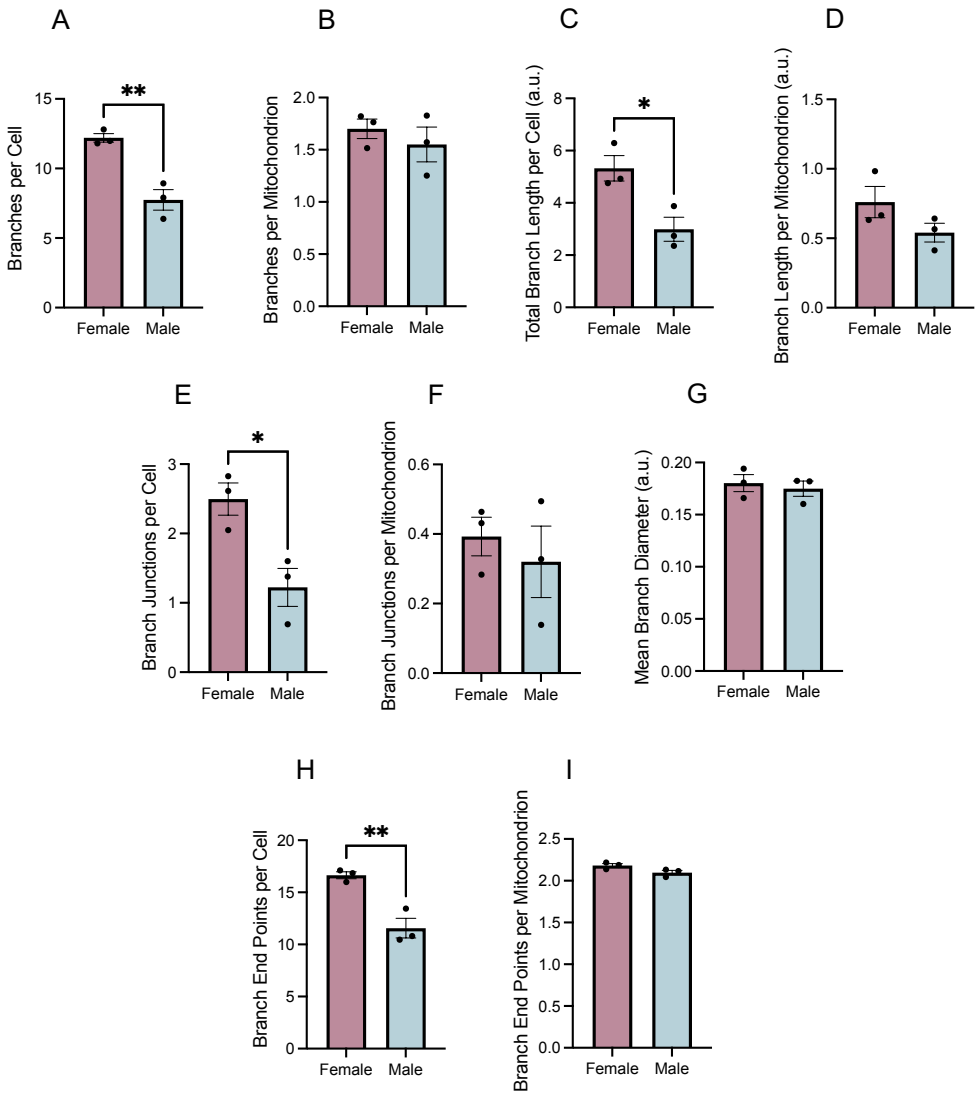

Figure 3.14 Female CD8⁺ T cells isolated from liver have a more branched mitochondrial network than males cells

CD8⁺ T cells were isolated from the liver and stained for total mitochondrial content using MitoSpy and images were acquired using Leica SP8 scanning confocal equipped with ×63/1.4 N.A. oil objective lens. Analysis of confocal images was carried out using FIJI equipped with the MitoAnalyser plug-in. Graphs show mitochondrial parameters such as number of mitochondrial branches per cell and per mitochondrion **(A, B)**, length of mitochondrial branches per cell and per mitochondrion **(C, D)**, number of branch junctions per cell and per mitochondrion **(E, F)**, average diameter of each mitochondrial branch **(G)** and number of branch end points per cell and per mitochondrion **(H, I)**. Dots represent individual mice, 70-100 cells analysed per mouse. Data shown as mean ± SEM from 3 independent experiments, n = 2 mice per group. Significance was calculated using two-tailed unpaired students t-test. * $p \leq 0.05$, ** $p \leq 0.01$.

3.3 Poly (I:C) stimulation does not differentially affect male and female $CD8^+$ and $CD4^+$ T cell activation or mitochondrial content

To determine if mitochondrial differences become exacerbated or lessened through immunological challenge, mice were injected with 200 µg Poly (I:C), and tissues were harvested 72 hours post injection. Poly(I:C) is a synthetic analogue of double-stranded RNA (dsRNA), a molecular pattern associated with viral infection. Poly(I:C) is recognized by endosomal Toll-like receptor 3 (TLR3). This activates the transcription factors NF-κB and AP-1, resulting in the production of type I interferons (IFNs) and other inflammatory cytokines and chemokines[80].

Across both males and females, no activation of $CD4^+$ and $CD8^+$ T cells was detected (Fig. 3.15 – 3.16). Poly (I:C) injected mice did not have a higher expression level of the activation marker CD69 when compared to the PBS injected control mice. Similarly, no increased expression of CD25 or CD44 was observed. Unsurprisingly, given the lack of detectable cellular activation, mitochondrial parameters also remained unchanged between control and Poly (I:C) injected mice, and between males and females (Fig. 3.17 - 3.18).

The half-life of poly (I:C) is short and it is quickly degraded in an RNase-mediated manner, and therefore the immunogenic stimulation will be short. Given that no activation was observed, it is possible the time point at which tissues were harvested was too long after injection, and if any activation had occurred, this infection had been resolved by the time the tissues were harvested.

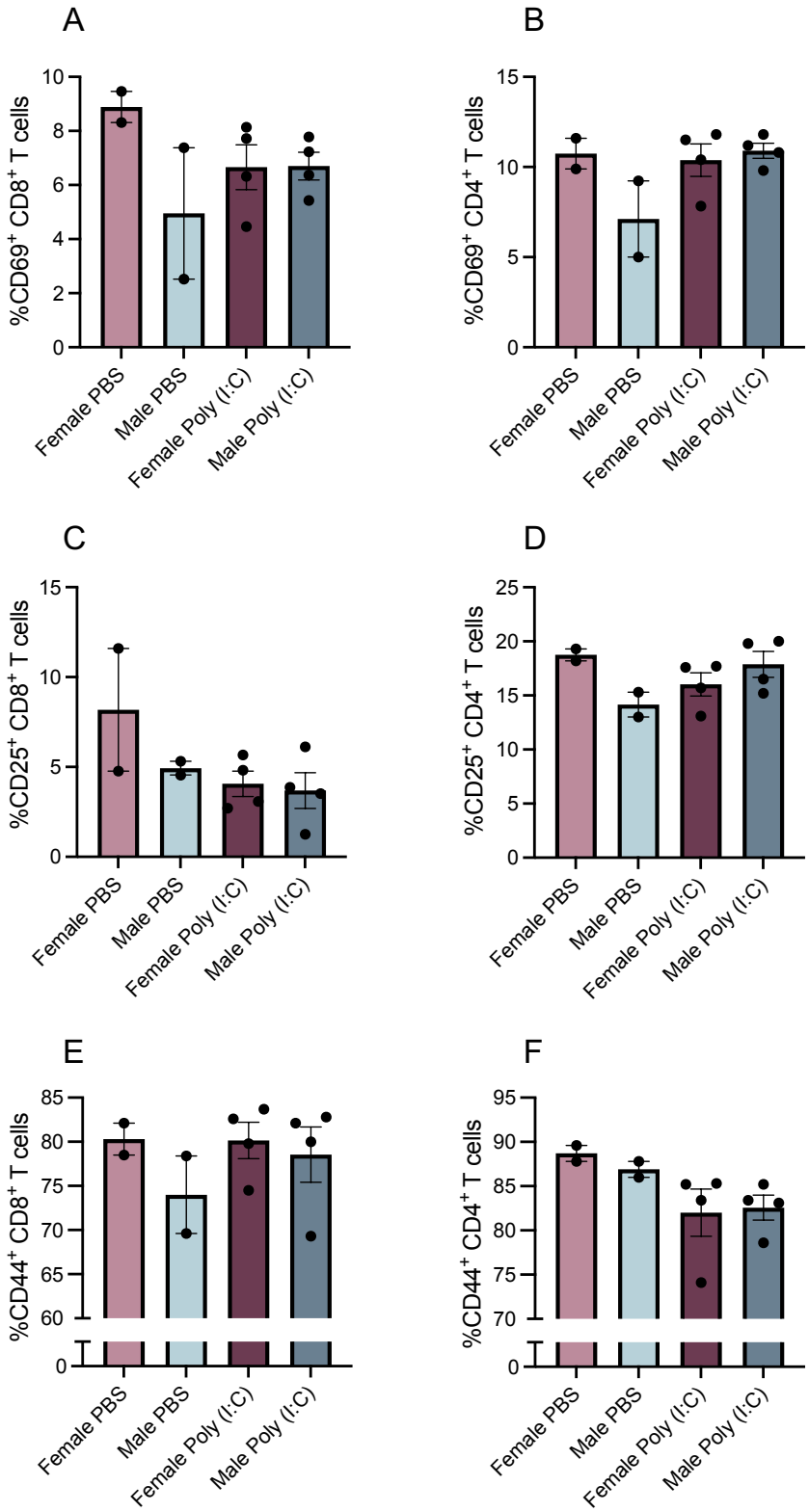
A
%CD69+ CD8+ T cells
10
8
6
4
2
0
Female PBS
Male PBS
Female Poly (I:C)
Male Poly (I:C)
B
%CD69+ CD4+ T cells
15
10
5
0
Female PBS
Male PBS
Female Poly (I:C)
Male Poly (I:C)
C
%CD25+ CD8+ T cells
15
10
5
0
Female PBS
Male PBS
Female Poly (I:C)
Male Poly (I:C)
D
%CD25+ CD4+ T cells
25
20
15
10
5
0
Female PBS
Male PBS
Female Poly (I:C)
Male Poly (I:C)
E
%CD44+ CD8+ T cells
85
80
75
70
65
60
0
Female PBS
Male PBS
Female Poly (I:C)
Male Poly (I:C)
F
%CD44+ CD4+ T cells
95
90
85
80
75
70
0
Female PBS
Male PBS
Female Poly (I:C)
Male Poly (I:C)

Figure 3.15 Poly (I:C) administration does not result in significant changes in the expression levels of common activation markers on splenic $CD8^+$ or $CD4^+$ T cells

Mice were i.p. injected with 200ug Poly (I:C), and tissues harvested 72 hours post-injection. Cells were stained according to 2.2.5 and $CD4^+$ and $CD8^+$ T cells were identified as in Figure 2.1. The expression levels of the activation markers CD69 **(A ,B)**, CD25 **(C, D)**, CD44 **(D, F)** on both $CD4^+$ and $CD8^+$ T cells were determined via flow cytometric analysis using FlowJo software. Data shown as mean ± SEM from 1 experiment, n = 2-4 mice per experimental group. Dots represent individual mice. Significance was calculated using two-tailed unpaired students t-test.

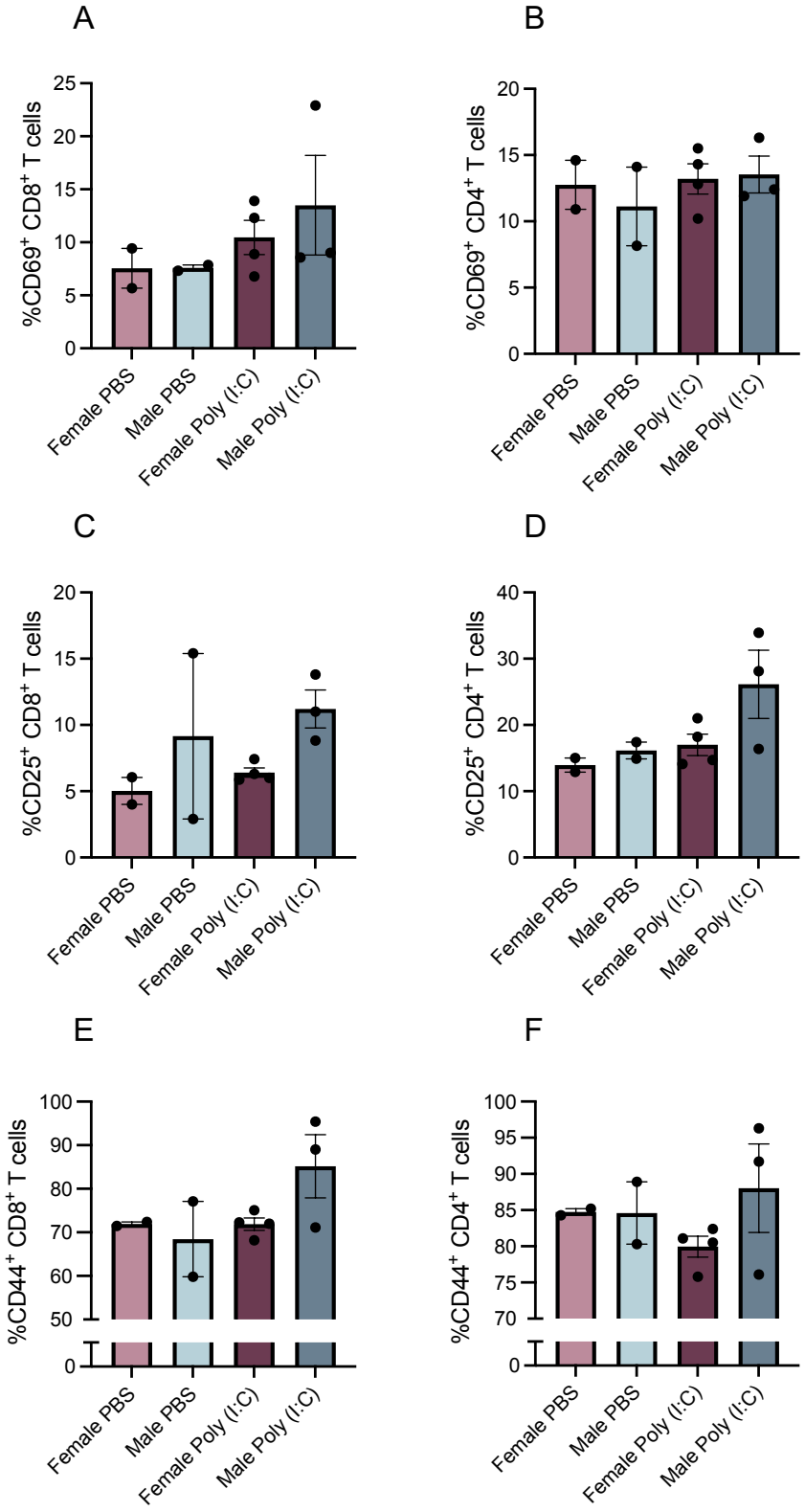
A
%CD69+ CD8+ T cells
25
20
15
10
5
0
Female PBS
Male PBS
Female Poly (I:C)
Male Poly (I:C)
B
%CD69+ CD4+ T cells
20
15
10
5
0
Female PBS
Male PBS
Female Poly (I:C)
Male Poly (I:C)
C
%CD25+ CD8+ T cells
20
15
10
5
0
Female PBS
Male PBS
Female Poly (I:C)
Male Poly (I:C)
D
%CD25+ CD4+ T cells
40
30
20
10
0
Female PBS
Male PBS
Female Poly (I:C)
Male Poly (I:C)
E
%CD44+ CD8+ T cells
100
90
80
70
60
50
0
Female PBS
Male PBS
Female Poly (I:C)
Male Poly (I:C)
F
%CD44+ CD4+ T cells
100
95
90
85
80
75
70
0
Female PBS
Male PBS
Female Poly (I:C)
Male Poly (I:C)

Figure 3.16 Poly (I:C) administration does not result in significant changes in the expression levels of common activation markers on $CD8^+$ or $CD4^+$ T cells isolated from lymph nodes

Mice were i.p. injected with 200ug Poly (I:C), and tissues harvested 72 hours post-injection. Cells were stained according to 2.2.5 and $CD4^+$ and $CD8^+$ T cells were identified as in Figure 2.1. The expression levels of the activation markers CD69 **(A, B)**, CD25 **(C, D)**, CD44 **(D, F)** on both $CD4^+$ and $CD8^+$ T cells were determined via flow cytometric analysis using FlowJo software. Data shown as mean ± SEM from 1 experiment, n = 2-4 mice per experimental group. Dots represent individual mice. Significance was calculated using two-tailed unpaired students t-test.

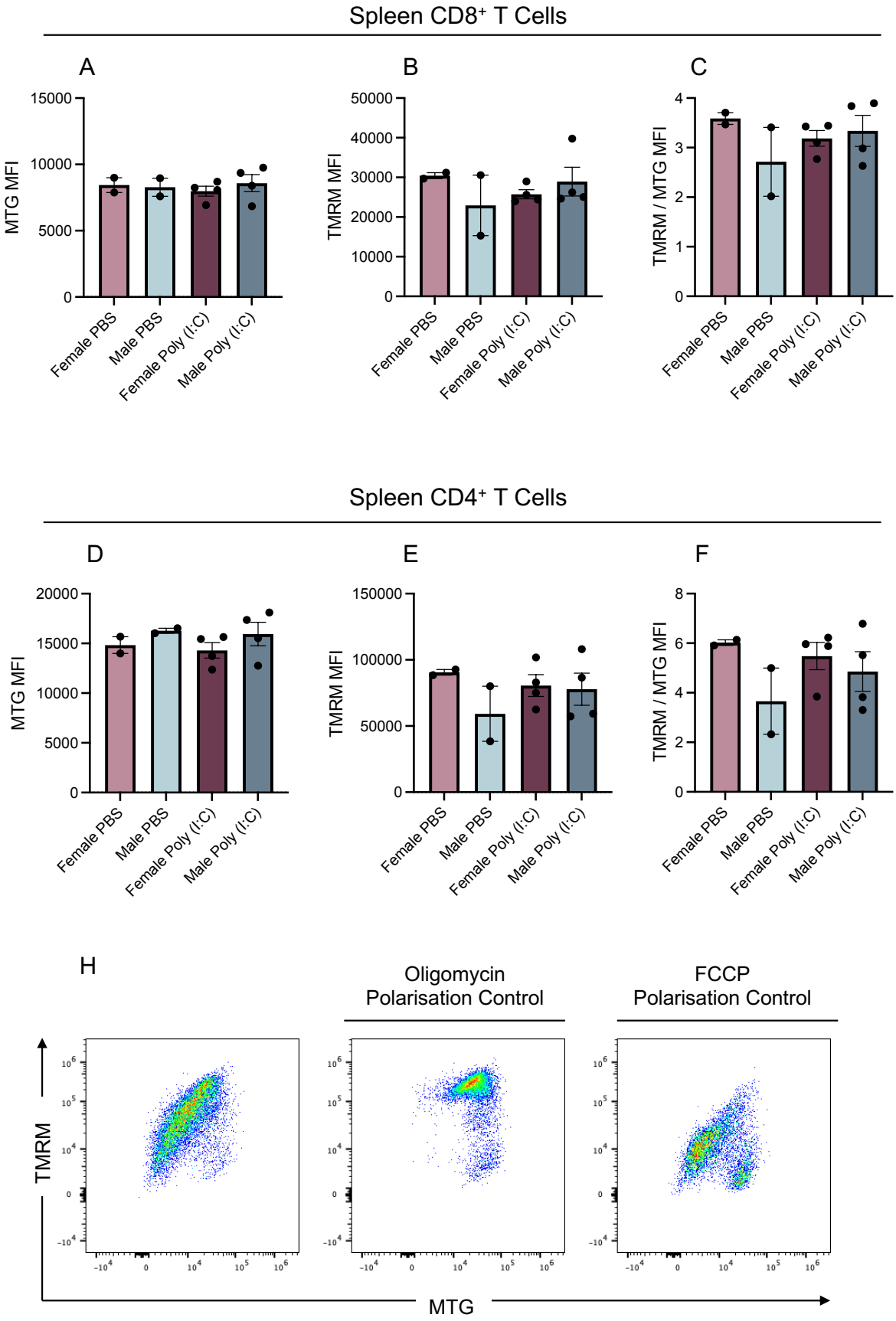
Spleen CD8⁺ T Cells
A
B
C
MTG MFI
TMRM MFI
TMRM / MTG MFI
Female PBS
Male PBS
Female Poly (I:C)
Male Poly (I:C)
Spleen CD4⁺ T Cells
D
E
F
H
Oligomycin
Polarisation Control
FCCP
Polarisation Control
TMRM
MTG

Figure 3.17 *In vivo* administration of Poly (I:C) does not alter mitochondrial mass or polarisation of splenic $CD8^+$ or $CD4^+$ T cells

Mice were i.p. injected with 200ug Poly (I:C), and tissues harvested 72 hours post-injection. Cells were stained according to 2.2.5 to determine mitochondrial content. $CD4^+$ and $CD8^+$ T cells were identified as in Figure 2.1. Graphs show the mean fluorescence intensity (MFI) of MTG and TMRM and the mitochondrial polarisation to mass ratios of splenic $CD8^+$ T cells **(A-C)** and $CD4^+$ T cells **(D - F)**. Representative flow cytometry plot of TMRM vs MTG and oligomycin and FCCP polarisation controls shown in **(H)**. Data shown as mean ± SEM from 1 experiment, n = 2 mice per control group and n = 4 mice per experimental group. Dots represent individual mice. Significance was calculated using two-tailed unpaired students t-test.

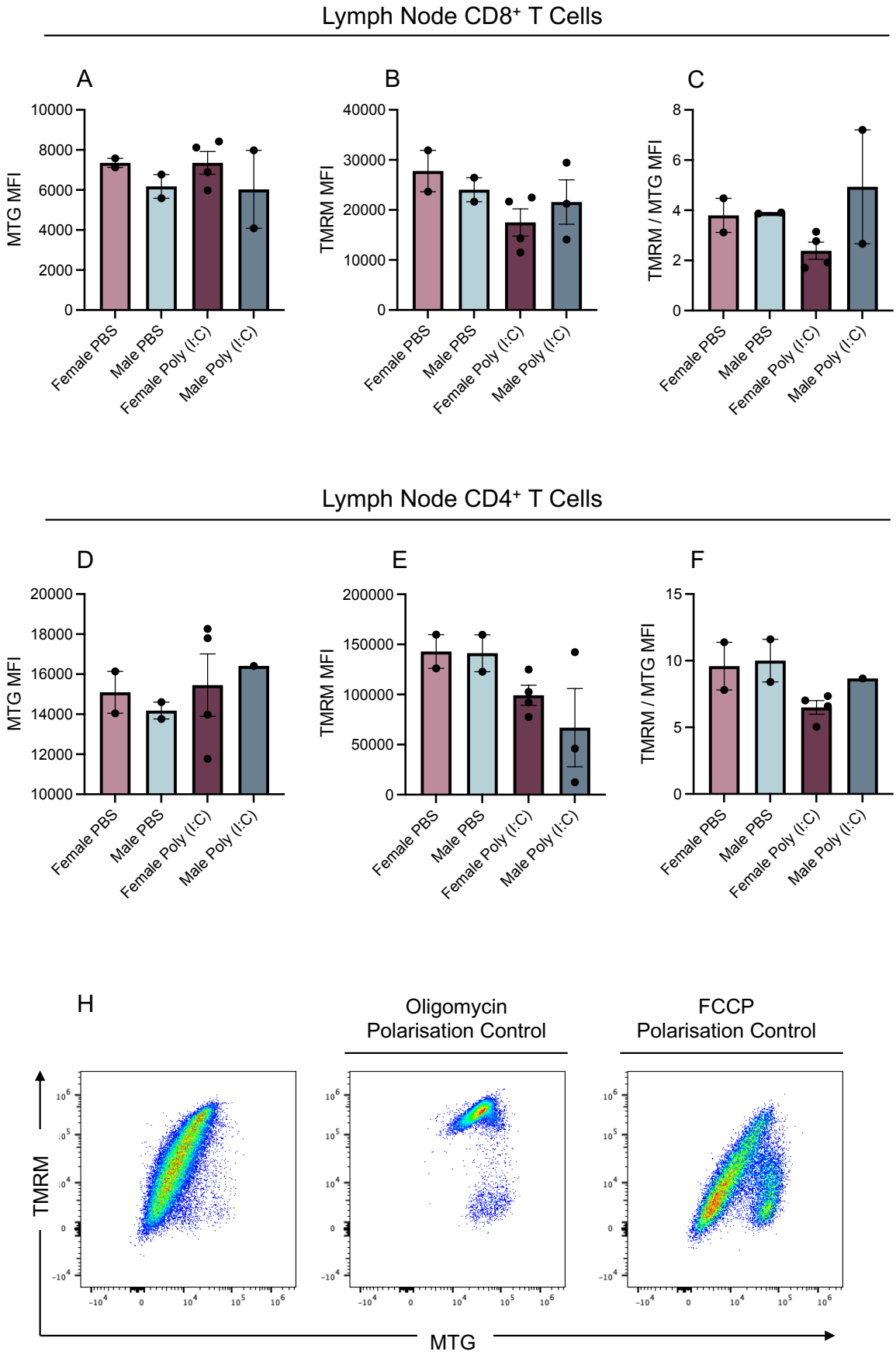
Lymph Node CD8⁺ T Cells
A
MTG MFI
10000
8000
6000
4000
2000
0
B
TMRM MFI
40000
30000
20000
10000
0
C
TMRM / MTG MFI
8
6
4
2
0
Female PBS
Male PBS
Female Poly (I:C)
Male Poly (I:C)
Lymph Node CD4⁺ T Cells
D
MTG MFI
20000
18000
16000
14000
12000
10000
E
TMRM MFI
200000
150000
100000
50000
0
F
TMRM / MTG MFI
15
10
5
0
H
Oligomycin
Polarisation Control
FCCP
Polarisation Control
TMRM
MTG

Figure 3.18 *In vivo* administration of Poly (I:C) does not alter mitochondrial mass or polarisation of $CD8^+$ or $CD4^+$ T cells isolated from lymph nodes

Mice were i.p. injected with 200ug Poly (I:C), and tissues harvested 72 hours post-injection. Cells were stained according to 2.2.5 to determine mitochondrial content. $CD4^+$ and $CD8^+$ T cells were identified as in Figure 2.1. Graphs show the mean fluorescence intensity (MFI) of MTG and TMRM and the mitochondrial polarisation to mass ratios of $CD8^+$ T cells **(A - C)** and $CD4^+$ T cells **(D - F)** isolated from lymph nodes. Representative flow cytometry plot of TMRM vs MTG and oligomycin and FCCP polarisation controls shown in **(H)**. Data shown as mean ± SEM from 1 experiment, n = 2 mice per control group and n = 1-4 mice per experimental group. Dots represent individual mice. Significance was calculated using two-tailed unpaired students t-test.

3.4 Females express higher levels of the activation marker CD69 following LPS administration

To study the effect of immune activation on mitochondrial parameters, we next tried LPS as an *in vivo* immune challenge. Mice were injected with LPS at 500 µg/kg, and tissues were harvested 24 hours post injection. LPS signals through TLR4 expressed on dendritic cells, causing release of inflammatory cytokines and upregulation of costimulatory molecules on antigen presenting cells. The resulting combination of signals from antigen, co-stimulation, and cytokines leads to activation of the lymphoid cells[81, 82]. TLR2, expressed on activated T cells as a costimulatory receptor, also plays a role in response to LPS[83]. TLR2 is particularly important in the activation of Tregs, allowing them to inhibit the inflammatory immune response[84].

In response to this immune challenge, both males and females had a substantial increase in CD69 expression on both $CD4^+$ and $CD8^+$ T cells in both the spleen and lymph node (Fig. 3.19 – 3.20 A, B). One female and two male mice in the LPS groups did not appear to have any immune system activation. Due to these outliers the difference in male versus female CD69 expression is not significant across each of the cell subsets and organs. This study is also limited by the small number of animals used and should be confirmed using a larger sample size. Nonetheless, when considering only the mice that did demonstrate immune activation, the female mice clearly upregulated CD69 expression more than their male counterparts.

There were also changes in CD25 and CD44 expression levels, although there were less obvious sex differences in this case (Fig. 3.19 – 3.20 C – F). CD25 expression was marginally increased on $CD4^+$ T cells; this was most evident in splenic $CD4^+$ T cells. Expression of CD25 on $CD8^+$ T cells was more variable, with apparently higher levels of expression at baseline by females. CD44 expression appeared to decrease following activation in both $CD4^+$ and $CD8^+$ T cells in spleen and lymph node, which could point to an intriguing role as an adhesion receptor.

3.5 LPS stimulation may induce reduction in mitochondrial mass and mitochondrial potential

In both $CD8^+$ and $CD4^+$ T cells isolated from spleen and lymph nodes, the activation with LPS appears to induce a downward shift in mitochondrial mass and polarisation (Fig. 3.21 – 3.22). These results were not significant, and the study is limited by the small number of animals used, and so this area requires further investigation to definitively determine the impact of an immune challenge on the mitochondria of immune cells. The results may however indicate that the mitophagy response was triggered in response to the immune stress.

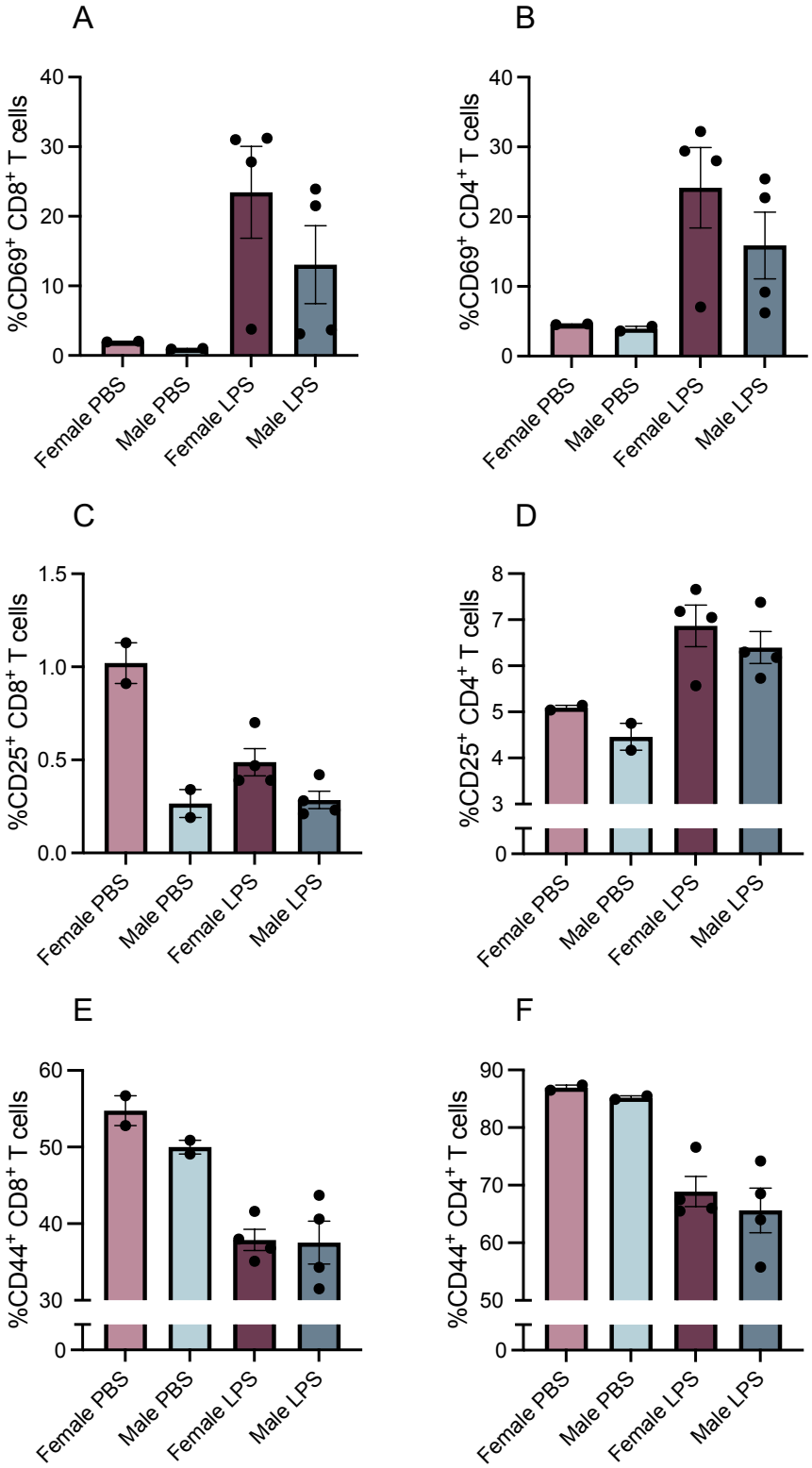
A
%CD69+ CD8+ T cells
40
30
20
10
0
Female PBS
Male PBS
Female LPS
Male LPS
B
%CD69+ CD4+ T cells
40
30
20
10
0
Female PBS
Male PBS
Female LPS
Male LPS
C
%CD25+ CD8+ T cells
1.5
1.0
0.5
0.0
Female PBS
Male PBS
Female LPS
Male LPS
D
%CD25+ CD4+ T cells
8
7
6
5
4
3
0
Female PBS
Male PBS
Female LPS
Male LPS
E
%CD44+ CD8+ T cells
60
50
40
30
0
Female PBS
Male PBS
Female LPS
Male LPS
F
%CD44+ CD4+ T cells
90
80
70
60
50
0
Female PBS
Male PBS
Female LPS
Male LPS

Figure 3.19 LPS administration results in CD69 upregulation in splenic $CD8^+$ and $CD4^+$ T cells

Mice were i.p. injected with 500 ug/kg LPS, and tissues harvested 24 hours post-injection. Cells were stained according to 2.2.5 to determine mitochondrial content. $CD4^+$ and $CD8^+$ T cells were identified as in Figure 2.1. Graphs show the percentage of $CD8^+$ and $CD4^+$ T cells positive for CD69 expression **(A, B)**, CD25 expression **(C, D)** or CD44 expression **(E, F)**. Data shown as mean ± SEM from 1 experiment, n = 2 mice per control group and n = 4 mice per experimental group. Dots represent individual mice. Significance was calculated using two-tailed unpaired students t-test.

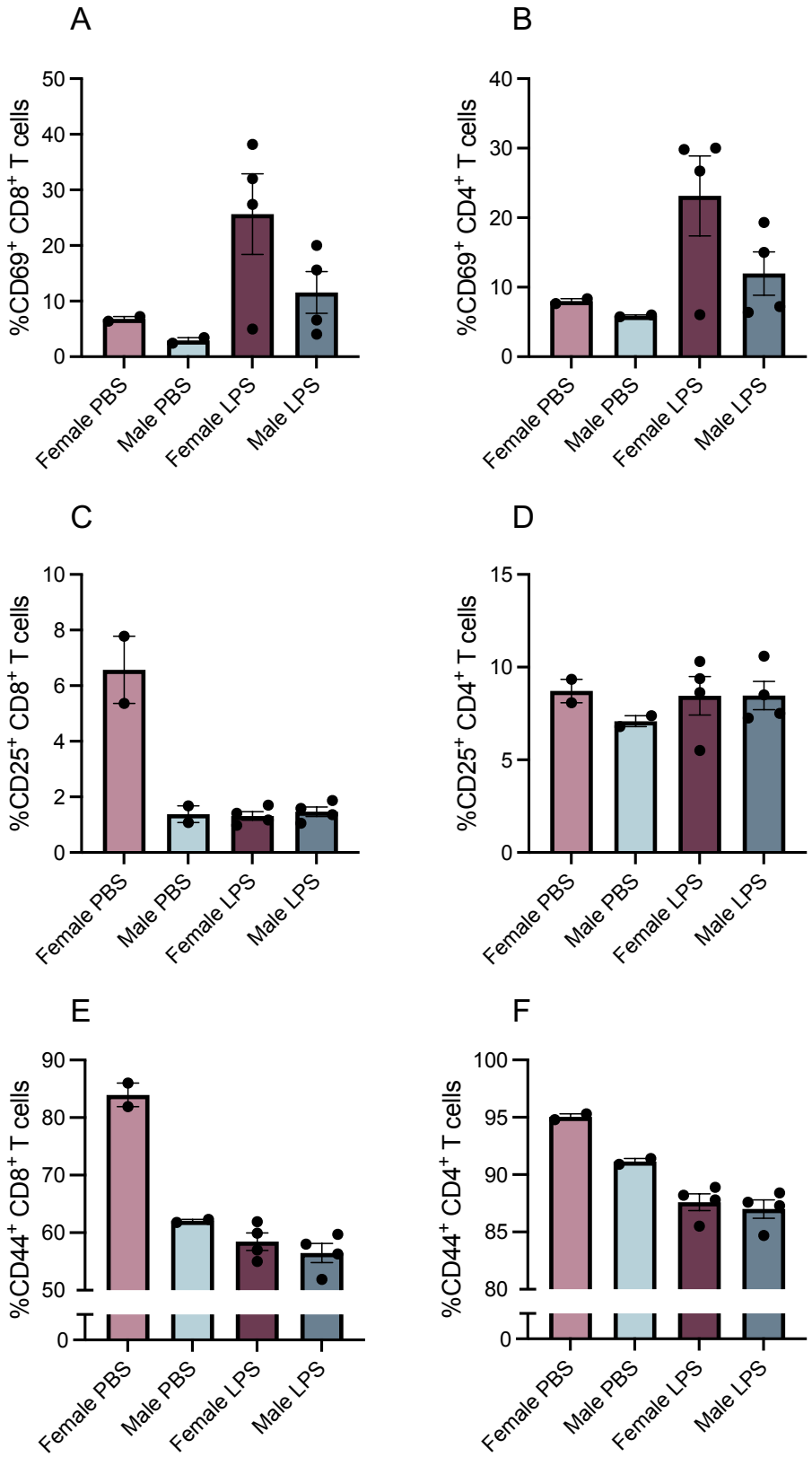
A
%CD69+ CD8+ T cells
50
40
30
20
10
0
Female PBS
Male PBS
Female LPS
Male LPS
B
%CD69+ CD4+ T cells
40
30
20
10
0
Female PBS
Male PBS
Female LPS
Male LPS
C
%CD25+ CD8+ T cells
10
8
6
4
2
0
Female PBS
Male PBS
Female LPS
Male LPS
D
%CD25+ CD4+ T cells
15
10
5
0
Female PBS
Male PBS
Female LPS
Male LPS
E
%CD44+ CD8+ T cells
90
80
70
60
50
0
Female PBS
Male PBS
Female LPS
Male LPS
F
%CD44+ CD4+ T cells
100
95
90
85
80
0
Female PBS
Male PBS
Female LPS
Male LPS

Figure 3.20 LPS administration results in CD69 upregulation in $CD8^+$ and $CD4^+$ T cells isolated from lymph nodes

Mice were i.p. injected with 500 ug/kg LPS, and tissues harvested 24 hours post-injection. Cells were stained according to 2.2.5 to determine mitochondrial content. $CD4^+$ and $CD8^+$ T cells were identified as in Figure 2.1. Graphs show the percentage of $CD8^+$ and $CD4^+$ T cells positive for CD69 expression **(A, B)**, CD25 expression **(C, D)** or CD44 expression **(E, F)**. Data shown as mean ± SEM from 1 experiment, n = 2 mice per control group and n = 4 mice per experimental group. Dots represent individual mice. Significance was calculated using two-tailed unpaired students t-test.

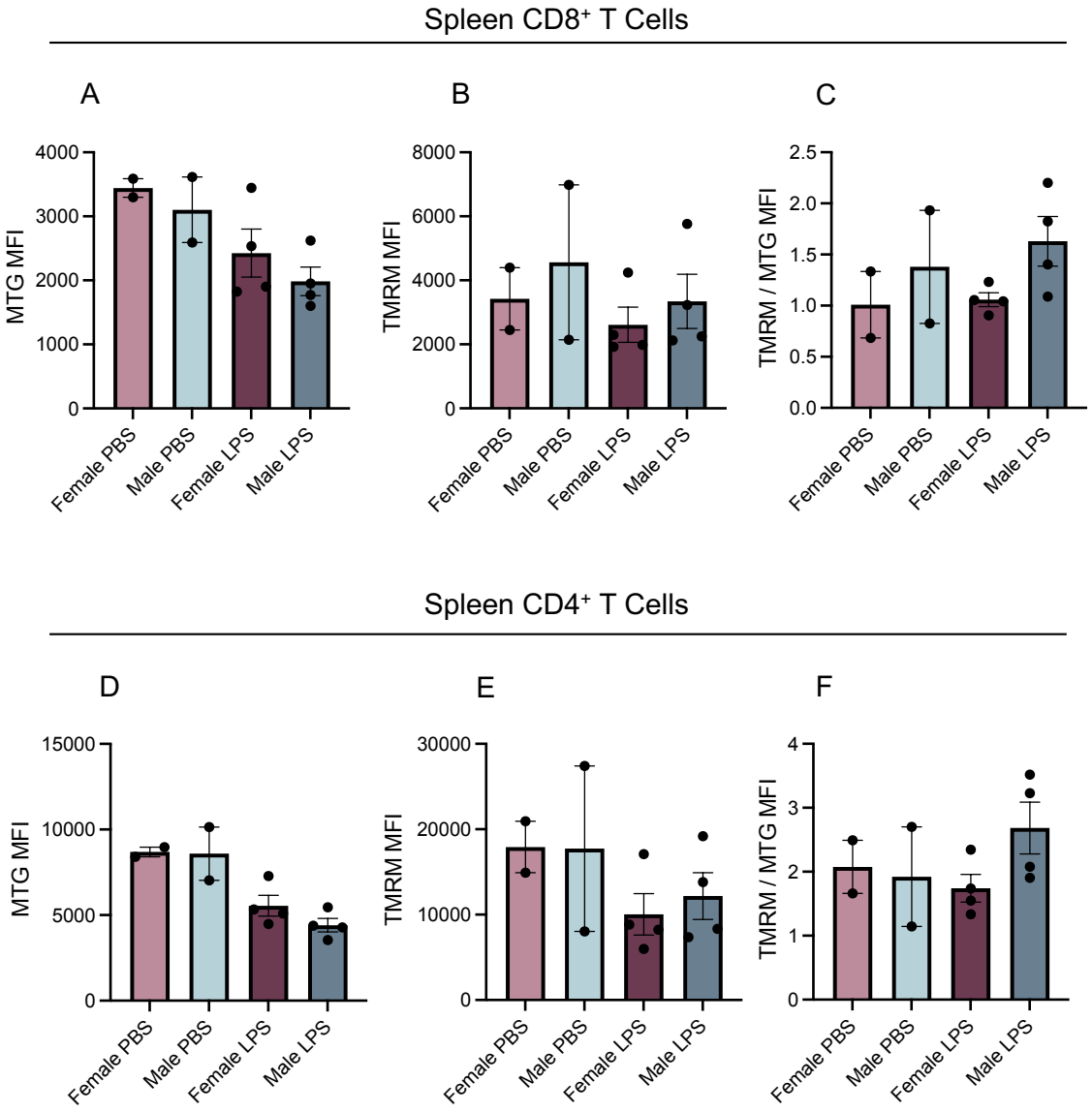

Figure 3.21 *In vivo* administration of LPS may cause a small, but not significant, reduction in mitochondrial mass and polarisation of splenic $CD8^+$ or $CD4^+$ T cells

Mice were i.p. injected with 500 ug/kg LPS, and tissues harvested 24 hours post-injection. Cells were stained according to 2.2.5 to determine mitochondrial content. $CD4^+$ and $CD8^+$ T cells were identified as in Figure 2.1. Graphs show the mean fluorescence intensity (MFI) of MTG and TMRM and the mitochondrial polarisation to mass ratios of splenic $CD8^+$ T cells **(A-C)** and $CD4^+$ T cells **(D - F)**. Data shown as mean ± SEM from 1 experiment, n = 2 mice per control group and n = 4 mice per experimental group. Dots represent individual mice. Significance was calculated using two-tailed unpaired students t-test.

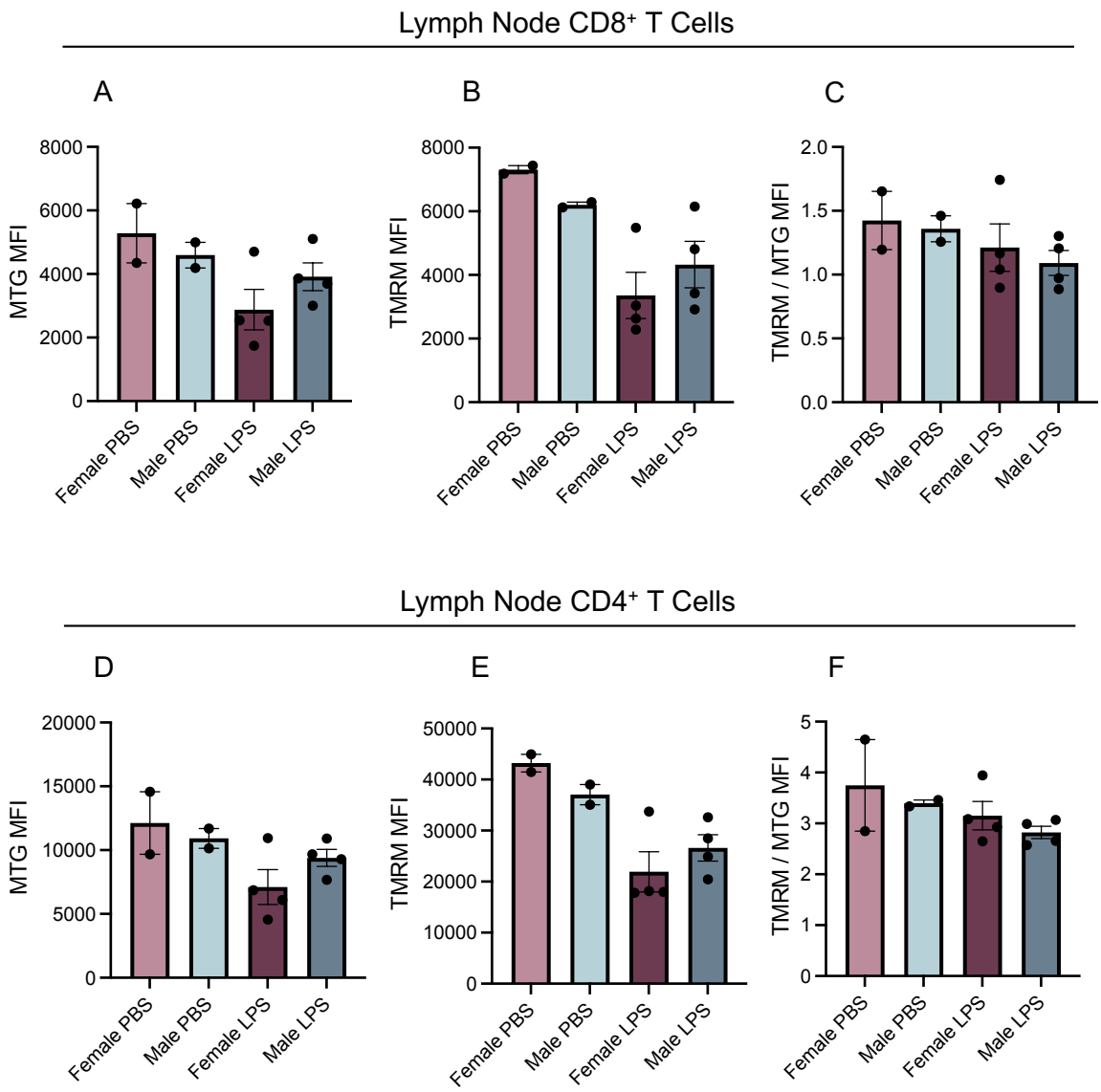

Figure 3.22 *In vivo* administration of LPS may cause a small, but not significant, reduction in mitochondrial mass and polarisation of $CD8^+$ or $CD4^+$ T cells isolated from lymph nodes

Mice were i.p. injected with 500ug/kg LPS, and tissues harvested 24 hours post-injection. Cells were stained according to 2.2.5 to determine mitochondrial content. $CD4^+$ and $CD8^+$ T cells were identified as in (Gating strategy). Graphs show the mean fluorescence intensity (MFI) of MTG and TMRM and the mitochondrial polarisation to mass ratios of $CD8^+$ T cells **(A-C)** and $CD4^+$ T cells **(D - F)** isolated from lymph nodes. Data shown as mean ± SEM from 1 experiment, n = 2 mice per control group and n = 4 mice per experimental group. Dots represent individual mice. Significance was calculated using two-tailed unpaired students t-test.

3.6 Mitochondrial associated proteins exhibit sex differences in their expression in $CD4^+$ and $CD8^+$ T cells

To determine if sex differences were apparent in the expression of proteins in $CD4^+$ and $CD8^+$ T cells, a proteomics screen was carried out on male and female $CD4^+$ and $CD8^+$ T cells, both *ex vivo* and stimulated, prepared as in 2.2.4.1. Analysis was carried out as described in 2.2.4.2. The findings show that there are sex differences in expression of a number of important mitochondrial proteins.

Proteins of note that were differentially expressed include components of the mitochondrial ATP synthase, which catalyses ATP synthesis during oxidative phosphorylation. Such proteins include Atpaf1, Atp5me, Atp5pd and Atp5f1d which represent an assembly factor for the F(1) complex, the e subunit of the F(0) complex, the d subunit of the F(0) complex, and the delta subunit of the F(1) complex of the ATP synthase, respectively. Proteins involved in mitochondrial fission, such as Mff and Fis1, and fusion, such as Opa1, Mfn1, and Mfn2 were also differentially expressed.

Other differentially expressed proteins of note include Cycs, which is a small heme protein that functions in the electron transport chain in mitochondria, Coa3, which is a member of the cytochrome c oxidase assembly factor family, and finally the important regulator of mtDNA transcription, Tfam. A summary of proteins of interest that were differentially expressed, and in which sex they were more highly expressed, can be found in Table 3.1.

Protein	Sex difference			
	$CD4^+$ T Cells ***Ex vivo***	**$CD4^+$ T Cells Stimulated**	**$CD8^+$ T Cells** ***Ex vivo***	**$CD8^+$ T Cells Stimulated**
Coa3	F > M	M > F	M > F	F > M
Cycs	M > F	M > F	F > M	M > F
Isca1	M > F	M > F	F > M	F > M
Tfam	M > F	M > F	F > M	M > F
Atpaf1	M > F	F > M	M > F	M > F
Atp5me	F > M	M > F	M > F	M > F
Atp5pd	M > F	M > F	F > M	M > F
Atp5f1d	M > F	F > M	F > M	M > F
Opa1	M > F	F > M	M > F	M > F
Mfn1	F > M	M > F	F > M	F > M
Mfn2	M > F	M > F	F > M	M > F
Mff	F > M	M > F	F > M	M > F
Fis1	M > F	M > F	F > M	M > F
Tomm22	M > F	F > M	F > M	M > F

Table 3.1 List of differentially expressed proteins of interest from proteomics analysis

Chapter 4
Discussion

4.1 Sex differences in mitochondrial mass and polarisation

The first objective of this project was to establish an understanding of the sex differences in mitochondrial mass and polarisation, as measured using Mitotracker Green (MTG) and Tetramethylrhodamine, Methyl Ester, Perchlorate (TMRM), respectively. While the results from these investigations were not significant, a trend towards higher mitochondrial mass and polarisation was observed in female $CD4^+$ and $CD8^+$ T cells isolated from both spleen and lymph nodes, although power calculations revealed that this observation is likely incidental. Greater mitochondrial mass in females would be in line with previous research indicating higher levels of mitochondrial biogenesis in females, better calcium handling and a greater resistance to oxidative stress[48]. Undoubtedly, any differences would be subtle, especially when there is no immune challenge or activation as the cellular demand for energy will not be high. It must also be acknowledged that the method of using cell dyes such as MTG and TMRM to determine mitochondrial properties only provides a relative fluorescence measurement, rather than an absolute quantification of mass or potential, and therefore a greater degree of error is to be expected. Furthermore, comparisons can only be made intra-experiment and not inter-experiment due to the highly sensitive nature of these dyes, which can be affected by the environment and changes in cell or mitochondrial volume, therefore it is essential for all samples to be kept cold throughout preparation, and for sample acquisition to occur immediately post-preparation. Acquisition should also be optimised to reduce the time elapsed between the first and last sample acquisition to reduce the likelihood that changes in dye uptake will occur due to cell death or changes in temperature.

An alternative marker that could be used to determine the mitochondrial content is citrate synthase (CS) activity. CS is a validated marker enzyme for mitochondrial density in skeletal muscle, although it has not been extensively validated in other tissues[53]. Cardiolipin content could also be used as a quantitative measure of inner mitochondrial membrane content[60]. The use of lipophilic, fluorescent cations such as TMRM, JC-1 and Rhodamine 123 remain the predominant and most accessible way to monitor mitochondrial membrane potential.

4.2 Sex differences in mitochondrial morphology

The morphological features of mitochondria can have a direct impact on their function, such as their ability to produce ATP, and in turn the host cell can affect mitochondrial shape. Mitochondrial fission and fusion are tightly regulated to ensure adequate supply of energy, as well as correct mitochondrial inheritance to daughter cells[85]. More fused, elongated mitochondria will carry out more fatty acid oxidation and oxidative phosphorylation. Whereas an increase in fission, resulting in more fragmented and rounded mitochondria, will favour aerobic glycolysis. This metabolic difference is related to the function of the cells, as activated effector T cells favour aerobic glycolysis, while memory T cells are more reliant on oxidative phosphorylation and fatty acid oxidation[86].

In this project, mitochondrial morphology was examined by utilising confocal microscopy and fluorescent dyes. Sexual dimorphism was not observed in the mitochondrial content, mitochondrial circularity, or mitochondrial branching of cells isolated from spleen or lymph nodes. However, cells isolated from the liver had a higher mitochondrial count in females compared to males, resulting in greater area, perimeter and degree of mitochondrial branching. This was perhaps unsurprising given that the liver is one of the most sexually dimorphic organs[87, 88].

It is logical that mitochondria from males and females would have an overall similar mitochondrial morphology, especially when the cells are unstimulated, because the requirement for energy at a basal state will not be very high. It would be worthwhile to investigate sex differences in mitochondrial structure following immunological stimulation, which could provide insight to the metabolism-driven skewing of immune responses between males and females. Overall, the structural differences in mitochondria that may exist between males and females are likely too subtle to be detected using the methods employed in this project, and more sensitive methods such as electron microscopy will need to be employed to definitively determine the differences.

4.3 Sex differences in activation marker expression in response to *in vivo* immune challenge

To investigate sex differences in immune activation and mitochondrial parameters following immune challenge, two *in vivo* approaches were employed. Firstly, a Poly (I:C)

regime, involving injection and tissue harvest 72 hours post-injection was trialled. No activation was observed across the males and females in either $CD4^+$ or $CD8^+$ T cell subsets isolated from spleen and lymph nodes. The failure of this regime to induce detectable immune cell activation could firstly be due to the time-point at which tissues were harvested and secondly, antigenic stimulation may not have been strong enough to activate the lymphoid cells, despite Poly (I:C) recognition by TLR3 activation on antigen presenting cells.

The second approach to be used was LPS injection with tissue harvest after 24 hours. The results indicated a higher upregulation of CD69 in females in response to *in vivo* LPS stimulation. CD25 was also slightly upregulated in response to stimulation in some of the cell subsets, but not in an obviously sexually dimorphic manner. CD44 on the other hand appeared to be downregulated with stimulation.

CD69 is a classical early marker of lymphocyte activation. Its upregulation is induced by NF-κB signalling at the beginning of an immune response. CD69 promotes proliferation and is also implicated in T cell differentiation. Specifically, CD69 promotes the differentiation of Treg cells by promoting TGF-β and IL-2 production through the JAK/STAT signalling pathway[89]. CD69 is also a marker of tissue-resident memory T cells (TRMs), the development of which is also TGF-β dependant[90]. The more pronounced upregulation of CD69 by females in response to LPS could therefore potentially indicate a greater degree of immune cell proliferation, greater Treg differentiation or a higher proportion of TRMs in females. The existing literature on sex differences in Treg populations in humans indicates a higher frequency of Treg populations in males, associated with their lower rates of autoimmune disease, but the findings are more contradictory in murine studies of organ specific Treg frequencies [5, 91, 92]. Sex differences in TRM populations have not yet been thoroughly investigated, although early evidence suggests a role for oestrogen in enhancing TRM responses, which would be in keeping with our findings[93].

CD25 is the alpha chain of the high-affinity IL-2 receptor and for this reason is one of the most prominent cellular activation markers. CD25 is important for T cell proliferation, activation induced cell death, and the actions of both regulatory (Treg) and effector (Teff) T cells[94]. CD25 expression by Tregs is linked to immune suppression and tolerance and the CD25 locus is an important region for genetic susceptibility to autoimmune diseases[94, 95]. CD25 expression was higher in females on $CD8^+$ T cells from spleen and lymph node without activation, but then decreased to match that of males following activation. There is evidence to suggest that $CD8^+$ T cells expressing lower levels of CD25 are more likely

to differentiate into functional long-lived memory cells, whereas cells expressing high levels proliferate rapidly to become short lived effector cells[96]. Therefore, higher expression of CD25 by female $CD8^+$ T cells may point to sex differences in the T cell response. Indeed, transcriptional analyses suggest that females have higher cytotoxic T cell responses *in vivo*[15].

There is growing evidence to suggest CD44 is involved in facilitating T cell migration by acting as an adhesion receptor on the surface of these migrating cells, thereby assisting in the mobilisation of effector T cells to the site of an infection or inflammation[97, 98]. CD44 is known to be upregulated following immune activation, but intriguingly, in this case the proportion of $CD8^+$ and $CD4^+$ T cells expressing CD44 generally decreased following LPS treatment. This may suggest that the cells have migrated to the site of the infection or inflammation, i.e. the injection site, and so the proportion of CD44+ cells has decreased in the spleen and lymph nodes.

4.4 Change in mitochondrial mass and polarisation in response to immune challenge

Pathogenic infections by viruses, bacteria and protozoa have all been shown to alter the mitochondrial content and metabolism of infected cells. The effects vary from pathogen to pathogen but all have evolved with the aim to modulate the mitochondria to promote survival and replication of the pathogen[99]. For example, monocytes infected with Epstein-Barr virus (EBV) show a reduction in mitochondrial content, while HIV-1 infected T cells exhibit increased mitochondrial polarisation and enhanced OXPHOS to promote viral replication[100-102]. Mitochondrial perturbations are also seen with bacterial infections. *Mycobacterium tuberculosis* infection of macrophages results in increased mitochondrial potential and ATP synthesis during the non-pathogenic phase of the disease[103].

24 hours following *in vivo* administration of LPS, mitochondrial mass and polarisation had decreased slightly across both male and female $CD4^+$ and $CD8^+$ T cells isolated from spleen and lymph nodes. This was an intriguing result since mitochondrial mass will typically increase following infection, concurrent with an increase in OXPHOS, in order to energetically support increased cell size and proliferation[104]. MitoTracker is often used to assess mitophagy because a decrease in MTG intensity, as observed in this case, can indicate the degradation of mitochondria[105]. Therefore it is possible that at the time point

the tissues were harvested, the peak of activation in response to the immunological challenge had been missed, and the mitophagy response had been triggered. Mitophagy plays an important part in the immune response, as it can attenuate activation of the inflammasome and reduce the level of apoptosis that will occur as a result of mitochondrial stress[106]. Furthermore, invading pathogens, such as viruses and bacteria can hijack host mitophagy to avoid host immune responses, but no clear mechanism has been determined[106].

The reduction in TMRM intensity indicates a reduction in mitochondrial membrane potential. TMRM intensity will decrease concurrently with a decrease in mitochondrial mass, but it may also be as a result of the opening of the mPTP, in response to factors such as increased ROS production and oxidative stress induced by the immunological challenge. Such a reduction in membrane production will result in decreased ATP production.

4.5 Sex differences in expression of mitochondrial associated proteins

Analysis of proteomics data, comparing unstimulated and *in vitro* stimulated $CD4^+$ and $CD8^+$ T cells has revealed differential expression of mitochondrial proteins such as Tfam, which regulates mtDNA replication and transcription, and is crucial for mitochondrial biogenesis[107]. Intriguingly, in three of the subsets, expression of Tfam appears to be higher in males. This is despite mitochondrial biogenesis being generally higher in females.

Mff, mitochondrial fission factor, appears to be higher in females *ex vivo* but in males when the cells are stimulated. Mff is required, alongside Fis1, to recruit Dnm1l, a GTPase essential for mitochondrial fission[107]. This increase in Mff expression could therefore indicate the cells switching to a more fragmented phenotype, which would make them more reliant on aerobic glycolysis as their energy source. The expression of other mitochondrial fission and fusion proteins did not follow a determinable pattern, perhaps unsurprising given the need for balanced expression of these proteins to maintain a dynamic mitochondrial network.

The data also highlights many other proteins which are differentially expressed between the sexes, such as Tomm22 which is an integral membrane protein of the mitochondrial outer membrane and Sod1 (superoxide dismutase) which breaks down superoxide radicals[108]. This dataset is therefore likely to represent a valuable resource in probing sex differences in $CD4^+$ and $CD8^+$ T cells when examined further.

4.6 Conclusion & Future Directions

Sex differences in the immune response, and how sex differences in mitochondrial morphology and metabolism can contribute to those responses, is a potential source of vital information relating to pathologies and their treatment. The implications of these sex differences are wide ranging and only beginning to be understood and appreciated. It is therefore pertinent to develop a clearer understanding of the landscape of sex differences. The results from this research provide preliminary evidence of sex differences in mitochondrial content in $CD4^+$ and $CD8^+$ T cells. Such differences are likely to have impacts on cellular metabolism, and therefore by extension the immune response.

The preliminary findings from this project present opportunities for further research. Assays such as the citrate synthase activity assay and cardiolipin content assay, in combination with cell dyes to measure membrane potential including TMRM, JC-1 and Rhodamine 123 could be utilised to quantifiably measure mitochondrial polarisation and total mitochondrial content. Further research could include the use of electron microscopy to definitively determine if there are structural differences in male and female mitochondria, either at baseline or in response to immunological challenge. Additionally, techniques to determine sex differences in metabolic activity, such as the Seahorse metabolic flux assay, present another avenue for investigation. With the use of these additional techniques, sex differences in mitochondrial parameters in response to *in vivo* immunological challenge could be more clearly determined. Finally, the proteomics data generated in the course of this project will likely form a useful resource for identifying key targets of further research. Such proteins that would be of interest include PPARα, PPARγ, and Pgc-1a. Pgc-1a, a major regulator of mitochondrial metabolism and biogenesis, the expression of which is promoted by oestrogen, is a clinically relevant target as it has been shown to be a factor in susceptibility to Parkinson's disease. PPARα and PPARγ regulate mitochondrial protein expression, and furthermore, their expression by T cells can influence Th1 and Th17 polarisation and could therefore also present a potential therapeutic target. Comparison of the levels of these proteins between male and female T cell subsets *ex vivo* and following *in vitro* stimulation could provide useful insight into their role in promoting sex differences.

The factors determining sex differences are likely to work in conjunction, rather than in isolation, with factors such as hormones, genes, environment (biotic and abiotic) as well as mitochondrial metabolism interacting to result in the observed sexually dimorphic phenotypes. All biological research should therefore consider biological sex as a variable,

particularly research seeking to understand the pathophysiology of the diseases, with the hope to provide more appropriate therapeutic interventions. For example, an understanding of divergent immune responses could represent a key tool in optimising vaccination strategies, as different dosing regimens in males and females may maximise efficacy while minimising side effects.

In conclusion, this research provides a foundation for further investigation into sex differences in immunity, including mitochondrial sex specificity, which represent an area of great research and therapeutic promise.

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