Testicular Immune Cells in Murine and Human Fetal Development and Implications for Male Reproductive Health

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Abstract

A considerable number of studies have investigated human and mouse adult testis immunophysiology, however a fundamental knowledge gap remains in understanding the identity and distribution patterns of immune cells in crucial phases of fetal testis development. This knowledge would facilitate a better understanding of immune cells' contribution to the earliest stages of testis development and lead to knowledge of factors that impact on immune cells to influence testis development.

This PhD research project provides knowledge of the frequency, phenotype and distribution pattern of macrophages, T cells, neutrophils (and mast cells), as all are important cell types during fetal testis development in mice, C57BL6J (embryonic ages 13.5 and 15.5 and newborn, in Chapter 2) and in human (2nd and 3rd trimesters, in Chapter 4). Briefly, this study documented for the first time the progressive emergence of neutrophils and T cells, the frequency and localisation of macrophages, T cells, neutrophils and their ratios with germ cells in the developing mice fetal testis. The findings from this study are in alignment with the timing of haematopoiesis in the murine yolk sac, fetal liver and bone marrow. Also, we demonstrated the co-localisation of macrophages and neutrophils with germ cells inside cords (in mice, C57BL6J) and peri cord area (in humans), which can imply the importance of this crosstalk for germ cell differentiation. By revealing the frequent and close cellular contacts between macrophages, T cells and neutrophils during testis development, we provided information that will be key to understanding how immune cells function during the fetal testis development and their influence on the maturation of the earliest male germ cells. The information gained from this study should be considered in developing strategies to support *in vitro* germline growth, for example, by adding immune cells into scaffolds or organoids. Moreover, the findings of this study revealed similarities and differences in the testicular immune cell population, frequency and locations between mice (C57BL6J) and humans during fetal development. In addition, this thesis provides findings on the impact of activin A levels on mouse fetal testicular macrophages using activin mutant mice (*Inhba* and *Inha*) in Chapter Three. The importance of activin A is due to its increased levels during inflammation, infection and pregnancy pathogenesis. Additionally, it is known that activin A levels significantly impact macrophages' function. This part of the thesis demonstrated that activin A levels regulate testicular macrophages frequency, distribution pattern and mRNA

levels of involved factors in their pro- and anti-inflammatory functions. The roles of macrophages, mast cells and T cells in testicular cancer have been studied for decades; however, neutrophils as a key pro-inflammatory immune cell have poorly been investigated. In Chapter Five, the study of neutrophils in samples from healthy men and patients with testicular cancers suggests their possible contribution to seminoma pathogenesis. In conclusion, the studies presented in this thesis examined testicular immune cells in fetal and newborn wild type (C57BL6J) and activin mutant mice (*Inhba* and *Inha*), human fetal testis, and human adult testicular cancer.

Zusammenfassung

In zahlreichen Studien wurde die Immunophysiologie des Hodens bei adulten Menschen und Mäusen untersucht. Eine grundlegende Wissenslücke besteht jedoch im Verständnis der Art und des Verteilungsmusters von Immunzellen in entscheidenden Phasen der fetalen Hodenentwicklung. Untersuchungen zur Beteiligung der Immunzellen an den frühesten Stadien der Hodenentwicklung und der Faktoren, die auf die Immunzellen einwirken und die damit Hodenentwicklung beeinflussen, fehlen bisher.

Dieses PhD-Forschungsprojekt liefert Erkenntnisse über die Frequenz, den Phänotyp und das Verteilungsmuster von Makrophagen, T-Zellen, neutrophilen Granulozyten und Mastzellen. Diese wurden während der fetalen Hodenentwicklung bei Mäusen (embryonaler Tag 13,5 und 15,5, Tag der Geburt) und beim Menschen (2. und 3. Trimester) detektiert (Kapitel 2 und 4). Diese Studie beschreibt zum ersten Mal die fortschreitende Besiedlung des fetalen Hodens mit neutrophilen Granulozyten und T-Zellen, die Häufigkeit und Lokalisierung von Makrophagen, T-Zellen und Neutrophilen sowie deren Verhältnis zu Keimzellen im sich entwickelnden Hoden. Diese Ergebnisse stimmen mit dem Zeitpunkt der Hämatopoese im Dottersack, der fetalen Leber und dem Knochenmark der Maus überein. Außerdem konnte die Ko-Lokalisierung von Makrophagen und Neutrophilen mit Keimzellen in den Keimschläuchen (bei Mäusen) und im Bereich um die Keimschläuche herum (beim Menschen) nachgewiesen werden, was auf eine Bedeutung für die Keimzelldifferenzierung schließen lässt. Die häufigen und engen zellulären Kontakte zwischen den Immunzelltypen während der Hodenentwicklung lassen außerdem auf die Funktion von Immunzellen während der fetalen Hodenentwicklung und den Einfluss auf die Reifung der primordialen Keimzellen schließen. Die aus dieser Studie gewonnenen Erkenntnisse können bei der Verbesserung von *in vitro* Keimzellkulturen berücksichtigt werden, z. B. durch Zugabe von Immunzellen zu Gewebekulturen oder Organoiden. Darüber hinaus konnten in dieser Studie die Ähnlichkeiten sowie die Unterschiede in der testikulären Immunzellpopulation (Häufigkeit und der Lokalisation) bei Mäusen und Menschen während der fetalen Entwicklung gezeigt werden.

Darüber hinaus lieferte diese Arbeit Erkenntnisse über den Einfluss von Aktivin A auf die fetalen Makrophagen durch die Studie von Mauslinien mit veränderter Aktivin A Bioverfügbarkeit. Aktivin A wird bei Entzündungen, Infektionen und bei der

Präeklampsie (Schwangerschaftshochdruck) vermehrt synthetisiert. Es ist bekannt, dass der Aktivin A-Spiegel die Funktion der Makrophagen erheblich beeinflusst. In diesem Teil der Arbeit konnte gezeigt werden, dass der Aktivin A-Spiegel die Häufigkeit, das Verteilungsmuster und die mRNA von testikulären Makrophagen reguliert, was wiederum Einfluss auf ihre pro- und antiinflammatorischen Funktionen hat.

Im letzten Kapitel der Arbeit (Kapitel 5) wurde die Rolle von Makrophagen, Mastzellen und T-Zellen bei Keimzelltumoren beleuchtet. Diese wird seit Jahrzehnten erforscht, aber bisher wurden die neutrophilen Granulozyten als wichtige proinflammatorische Immunzelle kaum beachtet. Kapitel 5 beschreibt die Untersuchung von Neutrophilen in Hodenbiopsien von Männern mit erhaltener Spermatogenese und von Patienten mit Keimzelltumoren (Seminomen) und weist auf ihren möglichen Beitrag zur Pathogenese des Seminoms hin.

Zusammenfassend lässt sich sagen, dass in dieser Dissertation Immunzellen in den Hoden von Wildtyp- und Aktivinmutanten-Mäusen, in menschlichen fetalen Hoden und bei Keimzelltumoren des adulten Mannes untersucht wurden. Durch diese verschiedenen Betrachtungswinkel konnte ein Beitrag zum besseren Verständnis der Pathogenese der männlichen Fortpflanzung geleistet werden.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature: 

Print Name: **Samira Hosseini**

Date: **17 June 2022**

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes **1** original paper published in a peer reviewed journals and **0** submitted publications. The core theme of the thesis is **male reproductive immunology**. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Molecular and Translational Science, Faculty of Medicine, Nursing and Health Sciences and Centre of Reproductive Health, Hudson Institute of Medical Research, Under supervision of Professor Kate L. Loveland, Professor Mark P Hedger, and at Institute for Veterinary Anatomy, Histology and Embryology, Justus Liebig University Giessen under supervision of Professor Hans-Christian Schuppe and Dr Daniela Christa Fietz.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of ***Chapter Two,*** my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*
Chapter Two	The changing landscape of immune cells in the fetal mouse testis	Accepted	Contributed to experimental design, tissue collection, analysing data and writing the first draft 65%	- Sarah C Moody and Sivanjah Indumathy, experimental design 2% - Mark P Hedger, Hans-Christian Schuppe contributed to the concept 3%, - Daniela Fietz, contributed to the concept and writing of the manuscript %10 - Kate L Loveland, contributed to the concept, experimental design, data analysis and writing of the manuscript %20

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: Samira **Date: 17 June 2022**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: Kate Loveland **Date: 18 June 2022**

List of Abbreviations

AGM	Aorta–gonad–mesonephros region
ARG1	Arginase 1
BSA	Bovine serum albumin
BM	Bone marrow
CSF1	Colony stimulating factor 1
CSF1R receptor	Colony Stimulating Factor 1
Ct	Cycle threshold
CX3CR1	Chemokine (C-X3-C motif) receptor 1
CX43	Connexin 43
CXCL	Chemokine (C-X-C motif) ligand
DAB	3,3'-Diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole
dNTPs	Deoxyribonucleotide triphosphate
DPX	Dibutylphthalate polystyrene xylene
E	Embryonic day
FLC	Fetal Leydig cell
FL	Fetal liver
GCNIS	Germ cell neoplasia in situ
GM-CSF	Granulocyte–macrophage colony-stimulating factor (CSF2)
HET	Heterozygote
HSCs	Hematopoietic stem cells
IHC	Immunohistochemistry
IF	immunofluorescence
IL	Interleukin
INHBA	inhibin beta A
INHBB	inhibin beta B
KO	Knock-out
LPS	Lipopolysaccharide
µg	Microgram
µm	Micrometre
µL Microliter	Millimolar
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MHCII	Major histocompatibility complex class II receptor
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PND	Postnatal day
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RNAseq	Ribonucleic acid sequencing
SOX9	Sry-box transcription factor 9
SRY	Sex-determining region Y
SSC	Spermatogonial stem cell
TBS	Tris-buffered saline
TGF-β	Transforming growth factor beta
WT	Wild type
YS	Yolk sac

Thesis Outline

Chapter One

Introduction

The research in this thesis addresses the growing appreciation of critical roles of immune cells and immune factors through the fetal organogenesis. Therefore, immune cells, including macrophages, T cells, mast cells and neutrophils in human and murine fetal testis were examined. Also, the hypothesis of the impact of activin A levels on immune cells in fetal murine testis was tested using two mutant mouse stains with altered activin A levels. In addition, neutrophils in adult human testicular germ cell tumours were investigated.

With the goal of orienting the reader to key background material, this literature review chapter provides an overview of several topics: fetal and adult testis structure, and fetal testis development, immunophysiology of testis development in mouse and human, immune cell subsets, testicular immune cells, and activin A. The individual data chapters which follow have individual focused background material, tailored for each topic.

1.1. Testis structure

In both mouse and human, the adult testes are ovoid and paired organs with two functions: hormone secretion and production of spermatozoa, the latter termed spermatogenesis (Wilhelm et al., 2007 and 2013; Assi et al., 2017). The general processes of spermatogenesis in mammals are presented in Figure 1.1.

The testis develops first as an abdominal organ during fetal life, and after birth enters into a cutaneous fibromuscular sac, the scrotum. Within the scrotal sac are multiple cell layers that engulf the testis. The outer layer is the tunica vaginalis, covering the anterior and lateral aspects of the testes, creating visceral and parietal layers, with the testis itself fully encapsulated by the tunica albuginea (Behre et al., 2001; Fietz and Bergmann, 2017; Sadler et al., 2018).

The human testicular parenchyma is incompletely divided by septae to form distinct testicular lobes containing a series of seminiferous tubules in the human (Kerr et al., 2006). The structure of septa does not exist in mouse testis, and seminiferous tubules run in zig-zag curves locally but concentrically as a whole along the circumference (Johnson, 1934).

The seminiferous tubules, the site of sperm production, are observed as fine coiled loops beneath the capsule. When seen in cross sections, the tubules consist of the seminiferous epithelium that consists of Sertoli cells with embedded germ cells (Nelson, 2013).

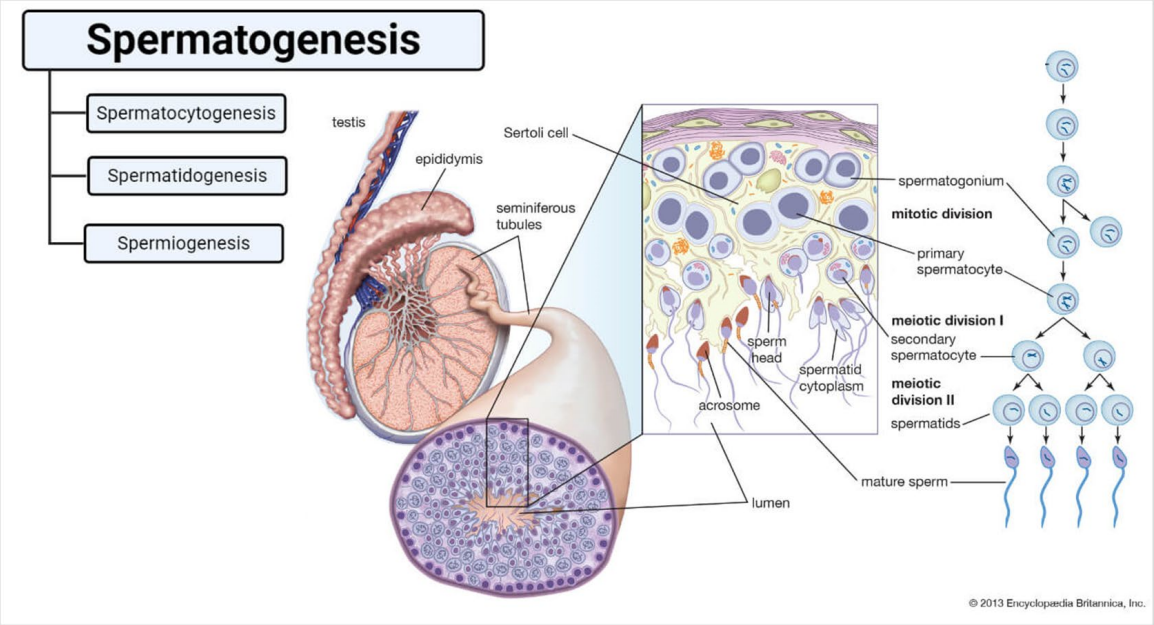

Figure 1.1. Spermatogenesis occurs within the seminiferous tubules of the adult testes. The least differentiated germline cells in adult testis are spermatogonia which contains develop within the Sertoli cells that form the seminiferous tubules. This population expands by mitosis and differentiate to different sub-types of spermatogonia (A_{pale}, A_{dark}, and B in the human). Some of these cells remain quiescent and form the spermatogonial stem cell pool (A_{dark}) while A_{pale} spermatogonia begin to differentiate and form B spermatogonia connected to their clonal siblings due to incomplete cytokinesis. B spermatogonia mature into spermatocytes, first as primary spermatocytes, characterised by an increase in volume of cytoplasm and mass of organelles. After a resting phase during which homologous chromosomes synapse and recombine, one primary spermatocyte divides into two secondary spermatocytes in the process of meiotic division I. Shortly afterwards, secondary spermatocytes divide into round spermatids as a result of meiotic division II. These haploid cells undergo the process of spermiogenesis, in which round spermatids transform into elongated spermatids, also known as testicular sperm. These are released in the lumen of the seminiferous tubules and are transported into the epididymis (Robert D. Utiger, Britannica, Anatomy & Physiology, Testis: Spermatogenesis 2020, *By courtesy of Encyclopaedia Britannica, Inc*).

The Sertoli cells support spermatogenesis by providing continuous nourishment and the epithelial scaffolding within which the germ cells mature. Sertoli cells are highly specialised cells that attach to each other through several types of junctions, including tight junctions, basal ectoplasmic specializations, desmosomes and gap junctions, to form the blood-testis barrier (BTB) that protects post-mitotic germ cells from contact with immune cells after puberty (Cheng et al., 2012; Potter et al., 2016). The BTB divides the seminiferous tubules into basal and adluminal compartments. The

adluminal compartment is an immunologically privileged site, which segregates the germ-cell antigens from the systemic circulation and potentially auto-immune T cells. The BTB also controls the passage of large molecules from the interstitial compartment, as well as regulating the passage of waste products. Therefore, the formation and maintenance of the BTB is one essential function of Sertoli cells in the production of spermatozoa (Orth et al., 1988; O'Donnell et al., 2006).

Extracellular matrix (ECM) in the testis interstitium fills the extracellular space at the cell-cell contact sites and consists of glycoproteins and polysaccharides components such as laminins, fibronectins, collagens, type IV collagen, heparan sulfate proteoglycan, entactin (Siu and Cheng, 2009). ECM forms the architecture of basement membrane and extracellular matrixes, attaches to the surface of cells to impact their trafficking (especially immune cells), and is involved in signalling pathways between peritubular and interstitial cells. The pivotal impacts of ECM remodelling in developmental processes, inflammation, tumour invasion, metastasis and cell movements are under regulation of proteases (such as MMPs), metalloprotease inhibitors and cytokines (e.g., TNFα) (Walsh et al., 2000; Siu and Cheng, 2004).

Peritubular myoid cells (PMCs) surround the seminiferous tubules, and by secreting the components of the basal lamina (together with Sertoli cells), they build a barrier that contributes to tubule integrity. The tubular wall comprises both PMCs and the basal lamina. There are also immune cells surrounding the tubules, interdigitated with the PMCs (DeFalco et al., 2014, Mossadegh-Keller and Sieweke, 2018). PMCs are contractile smooth muscle cells responsible for peristaltic contractions that push spermatozoa and testicular fluid through the tubules towards the rete testes. Therefore, the integrity of the tubular wall that includes myoid cells is critical for achieving normal spermatogenesis in adulthood (Virtanen et al., 1986; Maekawa et al., 1996; Potter and DeFalco, 2017). MPCs, based on their localisation outside of the blood-testis barrier, are in interaction with peritubular and interstitial immune cells. The function of MPCs with the blood-testis barrier and immune cells is to provide an immune-privileged microenvironment in the testis (Zhao et al., 2014; Mruk and Cheng, 2015). In normal or inflammatory conditions, PMCs are involved in regulating and shaping the testis immuno-microenvironment by secreting mediators such as TGFβ-2, MCP-1, and LIF, which can recruit inflammatory immune

cells (Li et al., 2012; Mayerhofer et al., 2013). The intertubular compartment contains groups of specialised cells, including the Leydig cells (the main producer of testosterone in adult testes), blood and lymphatic vessels, and diverse immune cells such as macrophages, dendritic cells, T cells, natural killer cells (NK), and mast cells (Hedger et al., 2006; O'Donnell et al., 2006; Heinrich and DeFalco 2020; Bhushan et al., 2020). Testis development may be broadly divided into five sequential developmental phases: fetal life, infant, pre-pubertal, puberty and adulthood. The focus of this study is on fetal development in both mouse and human. In the next sections, fetal and pre-pubertal stages of testis development are discussed.

1.2. Development of the fetal testis

The male gonads in both mouse and human contains somatic cells and germ cells. Somatic cells that migrate into the gonadal ridges present in the sexually indifferent fetus will become Sertoli, Leydig, immune and fibroblast cells (Svingen and Koopman, 2013). Mesenchymal cells form interstitial cells of the testes and the basic structure of the gonad, and sexually indifferent primordial germ cells (PGCs) migrate into and subsequently reside within the gonad that will ultimately form gametes in adult life. The early stages of testis development include the emergence of the indifferent gonads containing bipotential precursor somatic cells that can follow either the male or female fate. It follows by secreting Anti-Muellerian-Hormone by Sertoli cells due to the transcription of sex-determining region Y gene *(SRY*), on the Y chromosome, leading to a male phenotype of genitals and the formation of testicular cords in males (Wilhelm et al., 2007).

The sex-determining region governs male sex determination on the Y chromosome (SRY), a DNA-binding protein encoded by the *SRY* gene. This gene-regulatory protein/transcription factor is the critical trigger of testis development (McClelland et al., 2012; Spiller et al., 2017; Potter et al., 2017).

Sexually indifferent primordial germ cells (PGCs) are established by E6.5 in mouse. The PGC precursors arise from epiblast cells, and their specification is accrued through repression of the somatic program, reacquisition of pluripotency potential, and epigenetic re-programming of the genome (Yamaji et al., 2008). The Wolffian duct, the precursor of the male reproductive tract, is apparent by E10.5 (Hannema and Hughes 2007). Sex determination directs the bipotential gonads to develop as either

testes or ovaries; in the absence of *Sry*, the expression of *Wnt4* leads to the formation of an ovary (female gonad). In mice at E10.5, the upregulation of *Sry* expression in a portion of the gonadal supporting cell lineage leads to production of the transcription factor, Sox9, the key initiating steps that drives their differentiation into Sertoli cells. Therefore, expression of both SRY and SOX9 is necessary for development of the male gonad (Lovell-Badge and Robertson, 1990; Koopman et al., 1991; Vidal et al., 2001; Chaboissier et al., 2004; Sekido et al., 2004). Next, the nascent Sertoli cells express signalling molecules, including fibroblast growth factors (FGFs) which directly or indirectly induce differentiation of several cell types: germ cells, the testosterone-producing Leydig cells, and the peritubular myoid cells. Collectively, these reorganise to form the fetal testis cords (Palmer and Burgoyne, 1991; Colvin et al., 2001; Ross and Capel, 2005; Kim et al., 2006; depicted in Figure 1.2). At E11.5, the cords start to form around PGCs. By E12.5 the PGCs are differentiated as “male” and lose their motility (Baillie, 1964), and at this point they are now termed gonocytes.

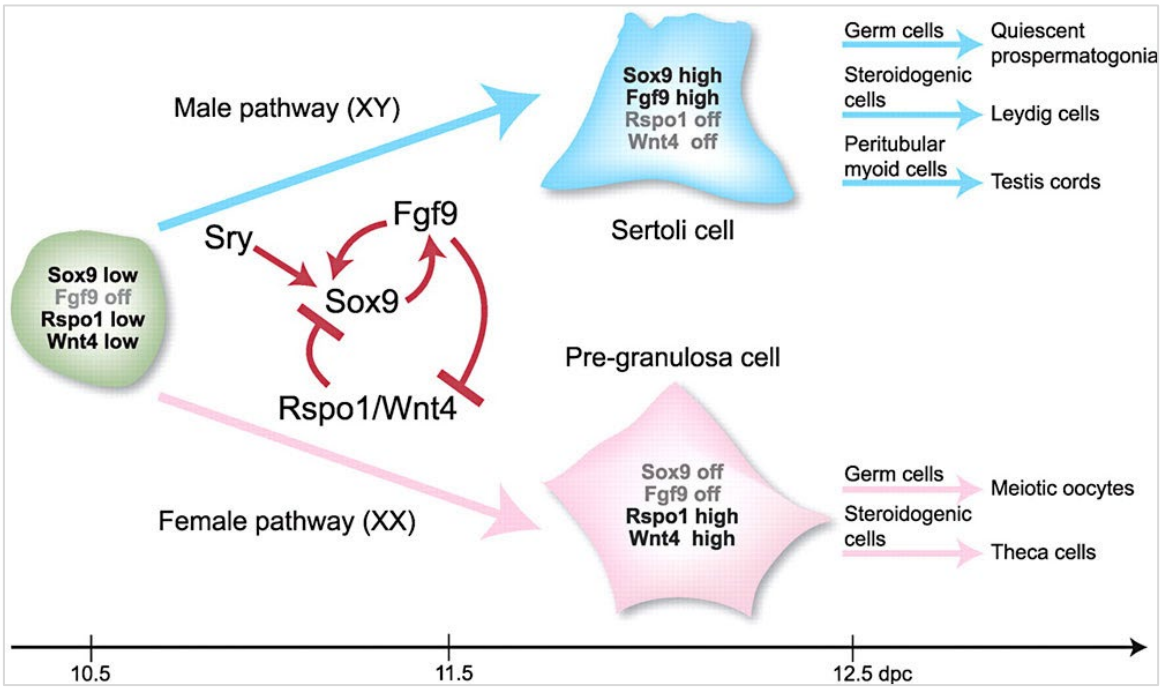

Figure 1.2. The early stages of gonad development. Schematic diagram showing differentiation of gonadal supporting cells (green) into male Sertoli cells (blue) or female pre-granulosa cells (pink). Changes in gene expression driving supporting cell differentiation and the downstream sexual differentiation of other gonadal cell types are indicated. (From Kocer et al., 2009).

Gonocytes throughout the majority of their developmental stages are structurally supported and surrounded by Sertoli cells. Regulation of cell-to-cell communication between Sertoli

cell and gonocytes is mediated by gap junctions, desmosomes, connexins and cadherins. Gonocytes differentiate into spermatogonial stem cells (SSCs) which differ between mouse and human (Figure 1.3) including the timing of fetal mitosis, quiescence, re-entry into mitosis after birth, and migration toward the basement membrane of the seminiferous cords; in the adult testis, SSCs are visible as type A spermatogonia (Culty 2009 and 2013; Guo et al., 2021). Therefore, the term of gonocyte, also called pre- or pro-spermatogonia, refers to several successive developmental stages starting from early stages of male germ cells development inside of the forming fetal testis to the time it migrates to the basement membrane of the seminiferous cord to transit a spermatogonial phenotype (Culty, 2009).

In mice, at E13.5 germ cells are situated centrally in the cords and there is a rapid increase in the gonocyte population and elongation of testis cords (Sekido et al.,2013; Wilhelm et al., 2007; Günesdogan et al., 2014). Germ cells mitotic arrest occurs gradually and in an unsynchronized manner at E12.5 and completes at E14.5 (in mouse) (Patrick et al., 2008). Therefore, between E12.5 and E15.5, gonocytes are in mitotic arrest in G0 until near birth (Cook et al., 2011).

Spermatogenesis begins in mice by 3 days after birth, and the first sperm can be detected at day 35 (Svingen and Koopman, 2013).

Generally, the temporal sequence of events that occurs in human fetal testis is similar to that which occurs mice, albeit longer (Rouiller-Fabre, et al. 2009) (Figure 1.3).

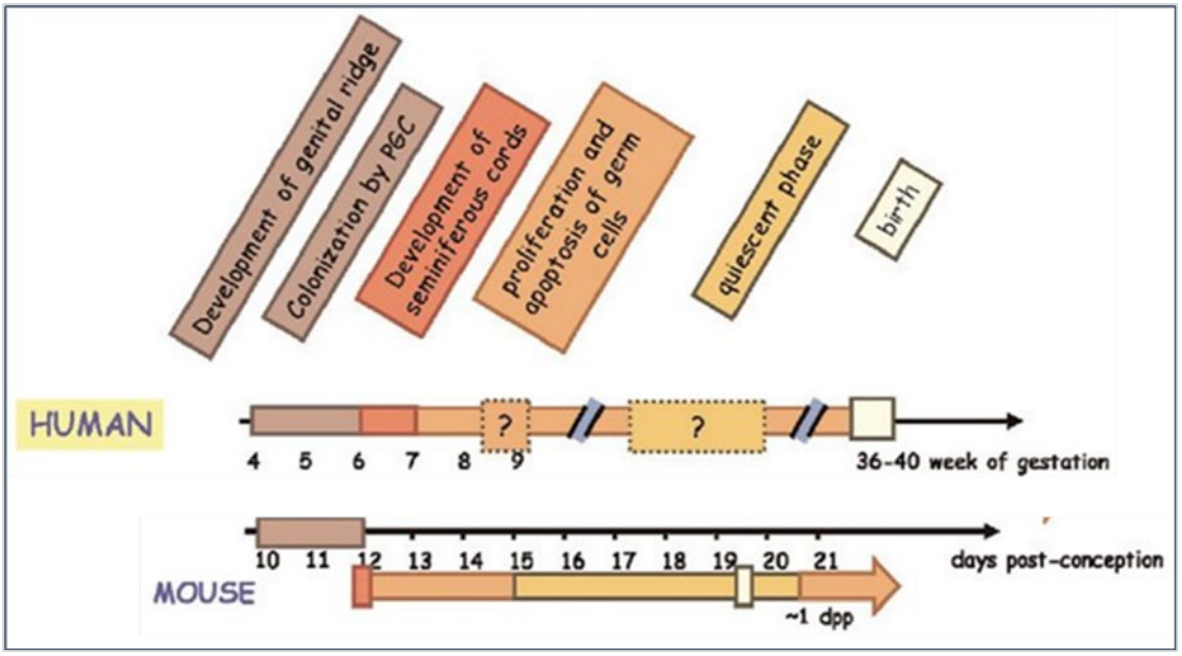

Figure. 1.3. Comparative chronology of fetal testis development in human and mouse.

In humans, primordial germ cells (PGCs), the founders of the germline, start migrating from

the stalk of yolk sac at four weeks of gestation and enter the bipotential gonad in the fifth week. The PGCs are then enclosed by the differentiating Sertoli cells to form testicular cords between 6 and 7 weeks of gestation. Mitotic divisions increase the number of germ cells during the first and second trimesters of pregnancy. In mice, gonocyte proliferation in the seminiferous cords ceases by E14.5 (Western et al., 2008), (From Rouiller-Fabre et al., 2009).

Human PGCs are derived from yolk sac progenitors that migrate (from week 4 to 5 of pregnancy) under control of stem cell factor (SCF/c-KIT) signaling to colonize in the genital ridges and surrounded by supportive cells deriving from the coelomic epithelium (Lennartsson et al., 2011). At this stage, PGC cells express specific markers including octamer binding transcription factors 3 and 4 (*OCT3/4*), receptor tyrosine kinase (*KIT*), placenta like alkaline phosphatase (*PLAP*) and *NANOG*, markers that can also be used as diagnostic markers for TGCT and GCNIS (Jørgensen et al. 1995). During normal differentiation of PGC cells around 6 gestational week (GW), *SRY* gene expression in the male embryo leads to differentiation of the indifferent genital ridges into testes (Sinclair et al., 1990). At 7 weeks, germ cells can be observed in the embryonic gonadal ridge. In this stage, genes associated with testis-determination, (such as *EMX2, CBX2, FOG2, FGF9*, and *GATA4*) are expressed in epithelial components in tissues originating from mesonephros. This expression pattern in the human fetal testis is similar to mice. In the first trimester of human gestation, primitive seminiferous cords are formed, and germ cells migrate toward the basal lamina of the seminiferous cords. Thus, in the first trimester, and throughout fetal life, human germ cells have a gonocyte phenotype. Sertoli cells and Leydig cells are observed at 9 weeks, and at 12 weeks of pregnancy, vascular endothelial cells and peritubular myoid cells have been observed. During the 13th week, there are changes in the expression of classic pluripotency markers; KIT is detectable at a relatively low level, and OCT3/4 and PLAP disappear completely (Schmahl et al., 2004; Ostrer et al., 2007; Wilhelm, et al., 2007 and 2013). Leydig cells (LC) are steroidogenic cells in the interstitium of testis that, by synthesizing androgens have a key role in both masculinisation of the testis and other secondary sex organs and spermatogenesis. In mice, there are two populations of Leydig cells with two distinct morphological and functional differences; fetal Leydig cells (FLCs) (detectable during embryonic development, show a high proportion of lipid droplets) and adult Leydig cells (ALCs) (appear postnatally and fewer lipid droplets)

(Huhtaniemi and Pelliniemi, 1992; Baker et al., 1999; Habert et al., 2001; O'Shaughnessy et al., 2000; Haider, 2004; Chen et al., 2009; Shima, 2019).

In mouse testis, FLCs are detectable in the interstitium shortly after sex determination (at E12.5), and their number reach the maximum around birth. FLCs during fetal testis development and up two weeks after birth are present but are not capable of synthesizing testosterone (Kerr et al., 1988; O'Shaughnessy et al., 2000; Shima et al., 2013; Shima, 2019). While the ALCs appear around one week after birth, their numbers increase during puberty and produce testosterone in response to stimulation by luteinizing hormone (LH). (O'Shaughnessy et al., 2000; Shima et al., 2013).

FLCs and ALCs are derived from a common pool of progenitor cells originating from the fetal gonadal surface epithelium and mesonephric mesenchymal cells (Ademi et al., 2020; Shen et al., 2021). After birth, it appears that ALCs are maintained from a perivascular and peritubular stem cell pool, which may be derived from dedifferentiation FLCs (Shima et al., 2018; Sararols et al., 2021).

1.3. Pre-pubertal development of testis

A key difference between mouse and human is the extensive pre-pubertal period in humans during which the testes and cords/seminiferous tubules do not change substantially from their appearance in neonatal phase; Mouse spermatogenesis progresses to the stage of pachytene spermatocyte in prophase I by day 17 after birth (Yuan et al., 2006), while in human the onset of spermatogenesis is between age 10-14 (Jones et al., 2013; Guo et al., 2021).

Until puberty, seminiferous "tubules" are in fact solid cords surrounded by myoid cells, fibroblasts and immune cells. These seminiferous cords are less convoluted than the tubules in the adult testis and consist of immature Sertoli and germ cells. In human newborns, the Sertoli cell is the most numerous cell type in the seminiferous cords and their cytoplasm contains abundant vimentin filaments arranged vertically relative to the basal lamina (Nistal et al., 2015). Adjacent Sertoli cells join together with extensive junctions of the occludens and adherens types. Mitotic figures are occasionally seen. In human newborn testes, germ cells comprise 4 populations: 1) fetal spermatogonia, with a well-developed nucleolus, that are in the quiescent phase, 2) A dark spermatogonia with smaller nuclei, 3) A pale spermatogonia, 4) gonocytes

which are large cells usually located in the center of the cord and are mostly positive for placental alkaline phosphatase and KIT (CD117) when examined by immunohistochemistry (Paniagua and Nistal, 1984; Nistal et al., 2015; Guo et al., 2021).

Guo et al reported that in a sample from a seven-year-old boy, the arrangement of testis cords was similar to that found in fetal samples, lacking an apparent lumen, while in samples from thirteen- and fourteen-year-old boys, the lumen was well-defined (Guo et al., 2021). Male germ cells in samples from the one and seven-year-olds exhibited early germline stem cell genes (such as *UTF1, PIWIL4, TSPAN33, GFRA1, KIT, MKI67*) indicating they are undifferentiated spermatogonia cells. But, shortly before the onset of puberty (around age 11), differentiating spermatogonia and meiotic cells began to emerge as the relative proportion of spermatogonia (as a fraction of the cellular component in the seminiferous cord) increased considerably (from ~3%–4% of total testicular cells in the infant and 7-year-old samples to ~10%–15%) (Guo et al., 2020 and 2021).

At age 13, there are more post-meiotic cells, and the onset of meiosis can be detected. Finally, the number of spermatocytes and spermatids at age 14 is similar to in the adult, indicating the successful onset of spermatogenesis (Guo et al., 2021).

In the human testis prior to age 10–11, meiotic primary spermatocytes typically undergo degeneration through a process similar to the first waves of incomplete spermatogenesis, therefore mature sperms are usually not present, although low levels of incomplete spermatogenesis are detected (Luciani et al. 1977; Paniagua and Nistal 1984, Chemes 2001). After puberty the testes enlarge markedly due to the increase in diameter of the seminiferous tubules (Jones et al., 2013).

1.4. Immunophysiology of testis development in mouse and human

The key role of the immune system is to protect the host from foreign organisms, but meiotic and post-meiotic germ cells develop after the development of immune tolerance. As a result, the antigen-specific immune response is tightly regulated in the testis, and this organ is referred to as an immune-privileged site. Since spermatocytes, spermatids and sperm could be recognized as “foreign” by the immune system, the maintenance of an immunosuppressive environment is crucial for normal testis development, ongoing spermatogenesis, and consequently male

fertility. Immune cell infiltrates can profoundly alter the cytokine milieu and may lead to reduced production of healthy sperm. The blood-testis barrier contributes to the immune-privileged environment within the seminiferous epithelium by excluding immune cells and their secreted mediators from entering the seminiferous tubules (Fijak and Meinhardt, 2006; O'Donnell et al., 2006, 2015). The following sections present a brief overview of immune cells and their relevance to testis biology, and then the three immune cell types that are relevant to immunophysiology of the testis that which are explored in this thesis in the context of the fetal testis are described.

1.4.1. General overview of immune cell subtypes, lineages and functions

The immune system provides protection of the host through a complex network of tissues, organs, cells and molecules that identify and then eliminate invading pathogens or newly developed antigens, such as cancer cells or developing germ cells. The immune system can be divided into two arms, innate and adaptive, based on the distinct responses that occur upon recognition of foreign antigens. The immune cells of the innate system recognize conserved molecular patterns expressed by pathogens (pathogen-associated molecular patterns (PAMPs), and they provide the first response to alert the specialised surveillance cells such as phagocytes. PAMPs are small molecular motifs conserved within a class of microbes that are recognized by pattern recognition receptors (PRRs) expressed by epithelial cells and immune cells, especially dendritic cells, neutrophils, and macrophages. The adaptive (also named ‘acquired’) immune system recognizes highly specific molecular patterns that are foreign to the host.

If a pathogen breaches physical barriers that would normally prevent it from entering the body, the innate immune system provides an immediate, but non-specific response. The innate immune system consists of cells in the myeloid lineage: monocytes, macrophages, neutrophils, eosinophils, basophils, and mast cells. If pathogens successfully evade the innate response, or after activation of the innate system, the adaptive immune responses become activated. This results in an immunological memory that allows for a faster and stronger response following the next exposure to the same pathogen. In the lymphoid lineage, T cells, B cells and NK cells are amongst the cellular components of the adaptive immune system that

can target and eradicate pathogens (Hoebe et al., 2004; Iwasaki et al., 2010 and 2015).

Communication between the innate and adaptive immune system arms, and their interactions with other cells in the reproductive tract, can affect male reproductive capacity. For example, antigen-presenting dendritic cells and macrophages are able to present antigens derived from post-mitotic germ cells to T cells, with the consequence that immune responses against those cells will be initiated (Zhao et al., 2014; Bhushan et al., 2020). A consequence of triggering immune responses in the testis can be induction of a pro-inflammatory condition, with immune cell recruitment to the interstitial areas, and production of high levels of autoantibodies against sperm arising if the BTB is breached. In adults, such an interruption to the immunoregulatory microenvironment of the testis, arising from infection or another inflammatory stimulus, can lead to loss of androgens and spermatogenic function, then tissue damage, ongoing autoimmunity, and finally infertility (Bozhedomov et al., 2005; Schuppe et al., 2008; Jacobo et al., 2011).

1. 4.2. Immune cell types in the testis

Amongst immune cells observed in the adult human testicular interstitium, macrophages are the most common cells, followed by lower numbers of dendritic cells (DC), mast cells and/or eosinophils, with the least frequently observed immune cell type being T cells and natural killer (NK) cells, while no B cells have been observed in normal spermatogenesis (Meinhardt and Hedger, 2011; Klein et al., 2016). The distribution and location of immune cells in different compartments of the testis is depicted in Figure 1.4. As the most abundant immune cells in the developing testis, macrophages serve key roles that influence testis growth (DeFalco et al., 2014; Fijak et al., 2017, 2018; Mossadegh-Keller et al., 2017; Lokka 2020; Wang et al., 2021).

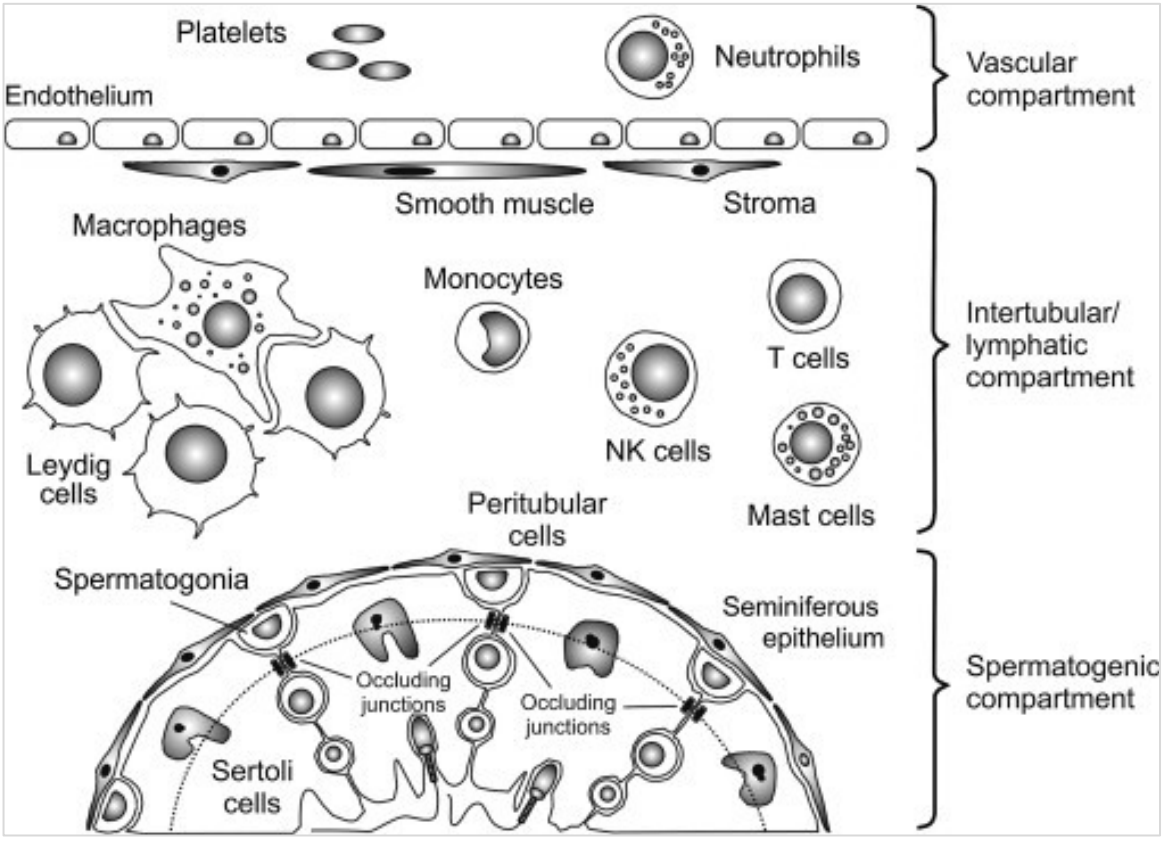

Figure 1.4. Immune cells in human adult testis. The blood–testis barrier (BTB) is formed by tight junctions between Sertoli cells. Germ cells (spermatogonia) are in close contact with Sertoli cells at all stages of their development, within the spermatogenic compartment of the testis. The intertubular, or interstitial, compartment contains Leydig cells, macrophages, dendritic cells (DCs), mast cells, T and NK cells located in the spaces between the tubules. The vascular compartment compromises neutrophils, platelets and endothelial cells (From Hedger, 2015).

1.4.2.1. Macrophages

Macrophages are professional phagocytes and can undergo polarisation depending on the local tissue environment (Stein et al., 1992; Stout et al. 2005; Murray et al., 2014; Bhushan et al., 2015; Shapouri-Moghaddam et al., 2018). They are able to recognise, engulf, and remove many potential pathogens, tumour cells, and cells undergoing programmed cell death. Macrophages also play important roles in antigen presentation, activation of the adaptive arm of the immune system, maintenance of homeostasis, tissue development, remodelling, repair and fibrosis (Gordon et al., 2010). Macrophages are able to transition between several distinct phenotypes to perform these many functions.

In general, macrophages have been divided into two main functional categories: M1 and M2 populations. M1 macrophages are potent antigen-presenting cells which

express a high level of major histocompatibility complex class II (MHC II) protein. The consequences of M1 macrophage activation are the initiation and maintenance of inflammation (Wang et al., 2014; Martinez et al., 2014). The M1 macrophage, also termed the classically-activated macrophage, is characterized by the production of high levels of pro-inflammatory cytokines, strong microbicidal properties, and high production of reactive nitrogen and oxygen intermediates, with the latter also contributing to provoke inflammation (Bhushan and Meinhardt, 2017). M2 macrophages induce and promote anti-inflammatory responses (commonly referred to as Th2-related responses), tissue remodeling, tumour promotion, and regulate the activity of pro-inflammatory and cell-mediated immunity. Therefore, M2 macrophages play important roles in reducing autoimmune responses (Metchnikoff et al., 2015; Jablonski et al., 2015).

Macrophages are most abundant immune cells in the fetal and adult testis (Mossadegh-Keller and Sieweke, 2018; Chapter Two, Three, Four and Five of this thesis). They serve vital roles to prevent initiation of immune responses and suppress immune responses to highly immunogenic spermatozoa (Hedger et al., 2015; Bhushan et al., 2015). In addition, macrophages have a central role during the initial phase of fetal testis development.

Macrophages derived from primitive yolk sac hematopoietic progenitors are detectable near developing vasculature in the fetal testis at E10.5. The macrophage population gradually increases near the coelomic surface artery and in the gonad–mesonephric vascular plexus region at the peak of *de novo* vascularisation. The phenotype of these macrophages is M2, signifying they are mainly involved in tissue remodeling and angiogenesis (Leidi et al., 2009; DeFalco et al., 2014). The role of macrophages in testis vasculature was investigated using the depletion of macrophages using a genetic cell-ablation technique. The removal of macrophages, using the *Rosa*-eGFP-Diphtheria Toxin A (*Rosa*-eGFP-DTA) system, under the control of *Cx3cr1*-Cre, led to a disorganised vasculature and aberrant testis cord formation at E13.5. This important study by DeFalco et al., (2014) demonstrated the key contribution of macrophages to fetal testis neovascularisation.

In the testis, two distinct macrophage populations, first recognized in the adult, have been characterized based on their anatomical localization. Cells in the peritubular space surrounding the seminiferous tubules are termed peritubular macrophages,

and those in the interstitial space between the tubules are interstitial macrophages (Mossadegh- Keller et al., 2017 and 2018). The interstitial macrophages engage in cell-cell contacts with Leydig cells, by thin cytoplasmic processes of Leydig cells (Bhushan et al., 2017). The latter relationship is functionally important for production of androgens by adult Leydig cells, as demonstrated when macrophages are depleted from rodent testes (Gaytan, et al. 1994).

As expected, testicular macrophages in mouse are heterogeneous and represented by different subpopulations that correspond to different stages of development and/or functional states, morphology, surface markers and gene expression (Gu et al., 2021). In the mouse fetal testis, the first origin of macrophages is primitive yolk-sac hematopoietic progenitors which give rise to gonadal–mesonephric macrophages (DeFalco et al., 2014). Recent single-cell RNA-seq analyses of fetal mouse testis have revealed that interstitial macrophages and peritubular macrophages are mainly derived from fetal-liver derived progenitors, while postnatal and adult bone marrow-derived cells likely contribute minimally to form adult testicular macrophage populations (Lokka et al., 2020; Wang et al., 2021) (Figure 1.5).

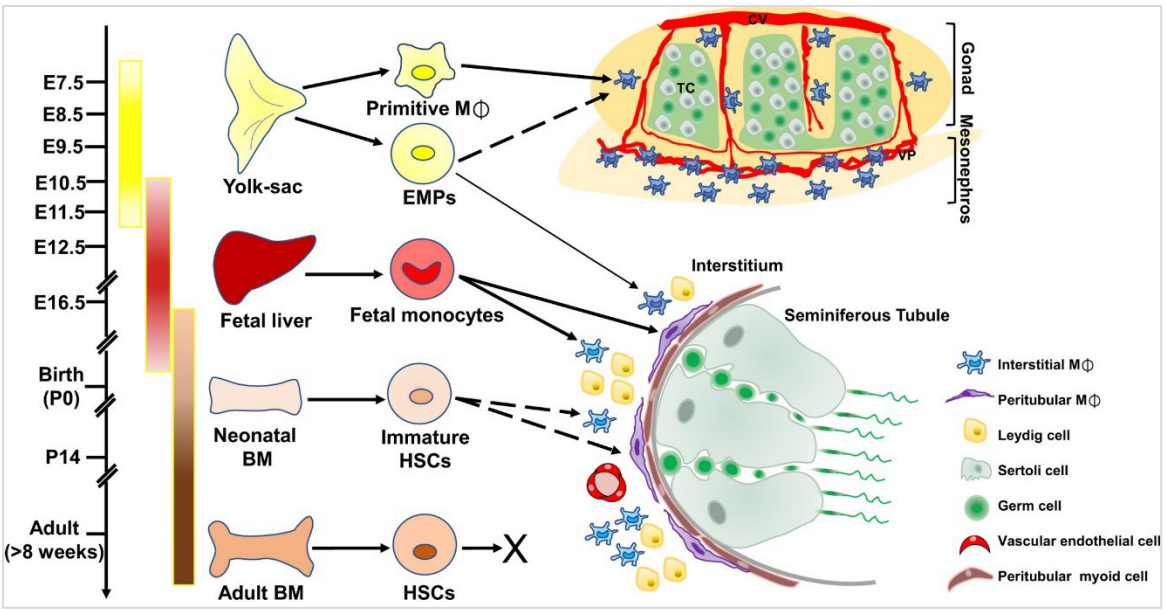

Figure 1.5. Model of testicular macrophage origins in mouse. Primitive macrophages generated in the yolk sac (E7.5) give rise to the majority of gonadal–mesonephric macrophages localized near vasculature. EMPs emerge in the yolk sac (E8.5) and exclusively contribute to a small subset of adult interstitial macrophages. Fetal liver-derived monocytes at mid-late fetal stages (E11.5-E14.5) can give rise to two testicular macrophage populations including interstitial macrophages, which are localized in the interstitial compartment in close contact with Leydig cells and blood vessels, and peritubular macrophages, which are localized to the peritubular myoid cell layer. However, adult BM-

derived monocytes do not substantially contribute to testicular macrophages. Solid arrows indicate a positive contribution and dotted arrows indicate a currently unclear relationship. BM, bone marrow; CV, coelomic vessel; EMPs, erythro-myeloid progenitors; HSCs, hematopoietic stem cells; MΦ, macrophage; TC, testis cord; VP, vascular plexus (Derived from Gu et al., 2021).

An important marker used to discriminate this heterogeneity is expression of MHC class II molecules. As antigen presenting cells, macrophages can initiate immune responses in testis. However, the majority of testicular macrophages in mouse testis showed an M2 macrophage phenotype after stimulation (Bhushan et al., 2017). Murine macrophages show reduced inflammatory and co-stimulatory activities when there is no stimulation or inflammation. In parallel with this understanding, Tung et al., demonstrated that the expression of MHC class II is restricted to macrophages located in the rete testis, and this expression is upregulated just after immunisation with testicular antigens in the presence of an adjuvant (Tung et al., 2017). Although the testis contains both subsets of macrophages, the most prevalent subpopulation in the normal state has an M2 phenotype.

Mouse fetal and postnatal testicular interstitial macrophages appear rounded and have a MCSFR^{+}/CD64 high/MHCII^{-} profile, and the peritubular macrophages are elongated and MCSFR low/ CD64 low/MHCII^{+} (Mossadegh-Keller et al., 2017. Both types of testicular macrophage populations express many macrophage-specific genes related to M2 polarisation at similar levels, for example CCL2, CCL6 and CCL12. Amongst the differences between these two populations, IL-10 (a typically immuno-suppressive cytokine) is preferentially produced by yolk sac-derived interstitial macrophages. In contrast, the peritubular macrophages express high levels of genes related to the regulation of antigen processing and presentation, such *H2.Dmb, H2.Eb1, Nlrp3, H2.K1*, and *Icam1*. They migrate to the mouse testis by around two weeks after birth, and are exclusively derived from bone marrow progenitors. These results showed their distinct developmental origins and indicate that they serve different roles in the maintenance of immune privilege (Mossadegh-Keller et al., 2017; Lokka et al., 2020; Wang et al., 2021).

Macrophages in human testis are detectable in both interstitial and peritubular sites. However, markers that discriminate between subpopulations of human testicular macrophages have to date not been conclusive, as both interstitial and peritubular macrophages express CD68, CD14 and CD163, and HLA-DR+ cells (the human

equivalent of MHCII in the mouse). Therefore, HLA-DR is not a reliable marker for identifying human testicular macrophage subpopulations, considering that dendritic cells also express HLA-DR (Pollanen and Niemi, 1987; Frungieri et al., 2002; Ponte et al., 2018).

1.4.2.2. Neutrophils

Neutrophils are the most abundant immune cells in peripheral blood circulation in the human adult. They are relatively short-lived, highly migratory cells, that also contribute to organ homeostasis (Casanova-Acebes et al, 2013 and 2018; Lok et al., 2019). Neutrophils are viewed as one of the first recruited effectors in acute inflammatory responses; they perform key roles in orchestrating a complex series of events during extracellular matrix remodeling by providing specific matrix-remodeling enzymes such as elastase, tumor necrosis factor alpha (TNF-α), interleukin-8 (IL-8): matrix metalloproteinase-9 (MMP-9), neutrophil gelatinase-associated lipocalin (NGAL) Cathepsin G and releasing exosomes (Zhu et al., 2021).

Infiltration of neutrophils in tissues can ultimately cause host tissue damage and increase the disease pathogenesis and severity (Kobayashi et al., 2009; Soehnlein and Lindblom 2010). Although the contribution of neutrophils to pathological microenvironments has been well studied, their roles in healthy organ development are not established.

Studies of testicular neutrophils are limited to examining neutrophil recruitment in the early stages of mouse fetal testis development, at E12.5 (DeFalco et al., 2014), in the adult rat testis (Lysiak et al., 2001; Sukhotnik et al., 2007; Arena et al., 2020) and in the adult human testis (Yamada et al., 2016). Considering the significant role of neutrophils in extracellular matrix remodeling, the normal frequency, and cellular contacts of testicular neutrophils cells during fetal testis development in both mouse and human were investigated, when this is a key physiological process.

1.4.2.3. T cells

T cells are the key player in orchestration and induction of adaptive immune responses. In mouse, T cell–restricted progenitors are present in the yolk-sac at E9.5 (before the sex determination at E10.5) (Yoshimoto et al., 2012). However, before E14.5, differentiated and functional T cells are not detectable in the fetal organs

(Yokota et al., 2003 and 2006), such as testis (studied at E13.5) (DeFalco et al., 2014). Generally, there are fewer studies on the first emergence, site of origin, and kinetics of T cell expansions in the testis compared to testicular macrophages. Also, the impact of T cells on testis morphogenesis and spermatogenesis is unclear. However, in mice with reduced numbers of lymphocytes mice, such as Rag1-mutants (mice that lacking the V(D)J recombination activation gene), testis morphogenesis and fertility was reported as normal (Mombaertset al., 1992; Kekalainen et al., 2015).

1.4.2.4. Mast cells

Mast cells belong to the granulocyte family or polymorphonuclear cell category of immune cells. They serve key functions related to the initiation of inflammation and allergic reactions, regulating vascular permeability, vasodilation, and leukocyte recruitment. Mast cells are long-lived cells that, after appropriate stimulation, can proliferate and also change their tissue distribution and phenotype. Therefore, they can have an important impact on host responses during infections, immune responses, persistent inflammation and tissue remodeling (Fijak and Meinhardt, 2006).

Because mast cells produce a variety of potent pro-inflammatory mediators (e.g. histamine, IL-4, Interferon-γ, TNF-α, and macrophage inflammatory proteins including MIP1α), they contribute to both adaptive and innate immune responses. Mast cells are derived from hematopoietic stem cells; upon migration into tissues, they undergo the terminal stages of differentiation in response to the destination microenvironment. Mast cells synthesise KIT, an important survival and developmental factor, which is a tyrosine kinase receptor protein. It also known as the stem cell factor receptor (SCFR), mast cell receptor, or CD117. The ligand for this receptor, KIT ligand (KITL) or stem cell factor (SCF), is produced by Sertoli cells. Local production of IL-3 and Th2– associated cytokines, including IL-4, IL-9 and KITL, by Sertoli cells and other immune cells, influence mast cells numbers and phenotype within the testis (Dvorak, 1997; Rodewald et al., 1996; Galli et al., 2005; Metz et al., 2007).

Testicular mast cells are present in the interstitium of mouse and human testis, but their frequency is higher in the human testis. Mast cell products are also involved in regulating steroidogenesis in Leydig cells (Meineke et al., 2000; Albrecht et al, 2005; Haidl et al., 2011). Since their main secretory product, the serine protease tryptase

can act as a potent mitogen for fibroblasts and peritubular cells, mast cell activation that results in secretion can cause tissue disruption such as fibrosis (Algermissen et al., 1999), sclerosis, thickening, and hyalinization of the tubules' lamina propria. In addition, an increased number of testicular mast cells has been reported in testes of men with abnormal spermatogenesis, infertility, various forms of testicular failure, and increased NOS2 (Nitric Oxide Synthase 2) expression in Leydig cells (Apa et al., 2002; Sezer et al., 2005; Welter et al., 2011; Mechlin and Kogan, 2012).

The role and function of mast cells in human adult testis has been well established, but there is not any report on mast cells in human fetal testis; therefore, in Chapter Four of this thesis, mast cells were investigated in human fetal testis sections obtained from embryos at the second and third trimesters of pregnancy.

1.5. Immunophysiology of human testicular germ cell tumors (TGCTs)

1.5.1. Characteristics of human TGCTs

Testicular germ cell tumors (TGCTs) are the most common malignancy (up to 60%) among young men (15-45 years old) and their prevalence has risen consistently over the several decades (Ghazarian et al. 2017). However, the cure and long-term survival rate following a TGCT diagnosis is above 95% for men receiving advanced treatment and surgery (Dearnaley et al., 2011; Woldu et al., 2017). TGCTs are a heterogeneous group of neoplasias divided into two main pathogenetically determined categories: Germ cell neoplasia in situ (GCNIS)-derived TGCTs (mainly post-pubertal) and non-GCNIS-derived TGCTs (mainly pre-pubertal) and the pathogenesis is different for these two groups. GCNIS-derived TGCTs consists of 89.2% of TGCT cases and non-GCNIS-derived can be found in 10.8% TGCTs (Lobo et al. 2018). Some GCNIS-related tumors are frequently characterised by a chromosome 12p amplification and include non-seminomas (i.e. embryonal carcinomas (EC), choriocarcinomas (CH), and postpubertal yolk sac tumors (YST)), seminomas (SE), nonseminomatous mixed GCTs (NSGCTs), teratomas (TE) and "burned-out" TGCTs. Non–GCNIS-related tumors commonly affect older men, lack chromosome 12p amplification, rarely appear as pure forms, but rather as mixed germ cell tumors (MGCT) and include prepubertal-type teratoma, prepubertal-type yolk sac tumor, mixed teratoma and yolk sac tumor prepubertal-type and spermatocytic tumor (Ronchi et al., 2019; Katabathina et al., 2021).

GCNIS (germ cell neoplasia *in situ*) is a TGCT precursor lesion which is considered to first arise in the seminiferous cord, develop in fetal life and expand later, due to abnormalities in primordial germ cell or gonocyte development into a spermatogonium (Skakkebaek et al., 1987; Hoei-Hansen et al., 2004; Horwich et al., 2006; Liu et al., 2019) (Figure 1.6). Therefore, an abnormality in testis development during fetal life can result in deleterious health problems such as testicular germ cell tumors (TGCT) in later life, when these cells transform into a malignant cell type.

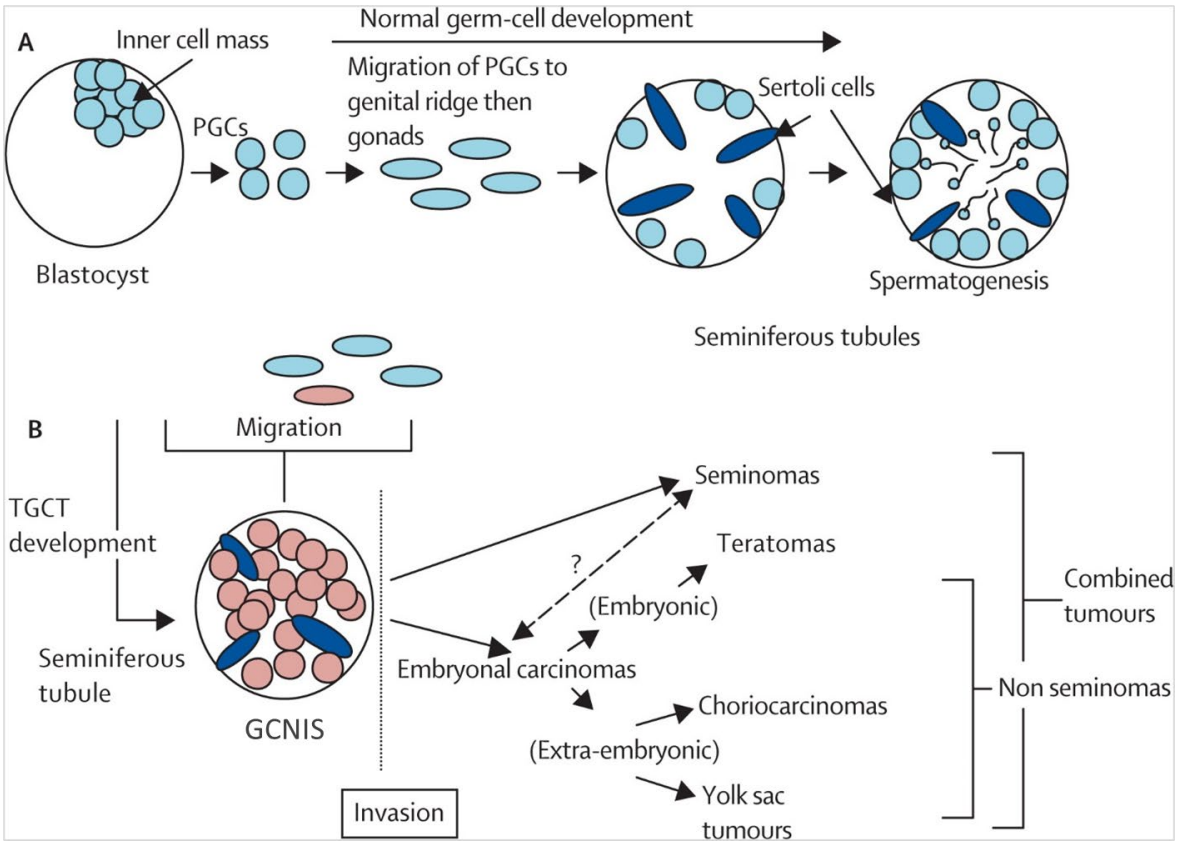

Figure 1.6. Normal and pathological germ cell development pathways. (A), The origin and histogenesis of different subpopulations of TGCT **(B)**. PGC: primary germ cell. GCNIS: (germ cell neoplasia *in situ*) unclassified (Horwich et al., 2006).

some genetic, epigenetic and microenvironmental factors that cause a halt to PGCs differentiation may transform these pluripotent cells into GCNIS. Specific abnormalities that are risk factors for the development of TGCTs consist of testicular dysgenesis syndrome (TDS) (including cryptorchidism, hypospadias, impaired spermatogenesis), family history of a TGCT, exogenous exposure to estrogen or other endocrine disrupting agents, and exposure to diagnostic radiation below the

waist (Baroni et al., 2019; Nead et al., 2020). Also, increased maternal estrogen levels from exogenous exposure or multiple endogenous hormone disrupters may result in the development of GCNIS (Holl et al., 2009).

As abnormalities in germ cell development contribute into the pathogenesis of GCNIS-related TGCTs, understanding normal germ cell development is essential to understand the pathogenesis of TGCTs (Meyts et al., 1996, 1999 and 2016; Niwa et al., 2000).

Upon puberty, intratubular GCNIS cells may be the source of pro-inflammatory cytokines (e.g., interleukin 6). The progress of intratubular GCNIS to tumour arises after increasing the number of GCNIS cells and infiltrating different types of immune cells into the testis, which transits the immune microenvironment to a “neoplastic pattern.” Followed by disruption in blood-testis barrier disruption and tumour progression. Therefore, GCNIS cells can form, with roughly equal frequency, into either seminomas or non-seminomas (Loveland et al., 2017).

Seminomas resemble an expanded population of undifferentiated gonocytes. They frequently contain lymphocytic infiltrates, suggesting that immunosuppressive conditions in the testis allow tumour growth. Non-seminomas comprise a mixture of heterogeneous tissue types that contain one or several components of embryonal carcinoma, yolk sac tumour (post-pubertal type), teratoma (post-pubertal type) or choriocarcinoma (that highly express embryonic pluripotency-related genes) (Sperger et al., 2003; Almstrup et al., 2004; Oosterhuis et al., 2005; Loveland et al., 2018).

1.5.2. Characteristics of immune cells in human TGCTs

Investigation tumor immune microenvironment (TIME) in TGCT is increasing, which includes studying infiltrating immune cells, their increase in number, and differences in the proportion of immune cells present relative to in healthy tissue (Bell et al., 1987; Hadrup et al., 2006; Berney et al., 2016) (Figure 1.7). The infiltrated immune cells in TGCT consist of $CD68^+$ macrophages, $CD3^+$ T cells, $CD20^+$ B cells and $CD56^+$ NK cells. It has been reported that the majority of $CD3^+$ T cells are cytotoxic ($CD8^+$) and/or have effector memory ($CD45RO^+$) phenotypes (Hvarness et al., 2013). TGCT in advanced stages is associated with reduced T and NK cell numbers, while Tregs (regulatory T cells), mast cells and macrophages are increased (Hvarness et al., 2013;

Klein et al., 2016; Siska et al., 2017). These findings demonstrate that the tumour microenvironment in TGCT is quite different from the microenvironment of testis in healthy conditions (Klein et al., 2016; Chovanec et al., 2018). Amongst the immune cell types present in TGCTs, neutrophils were my target for investigation of the TGCT immune microenvironment, as they have not been previously studied.

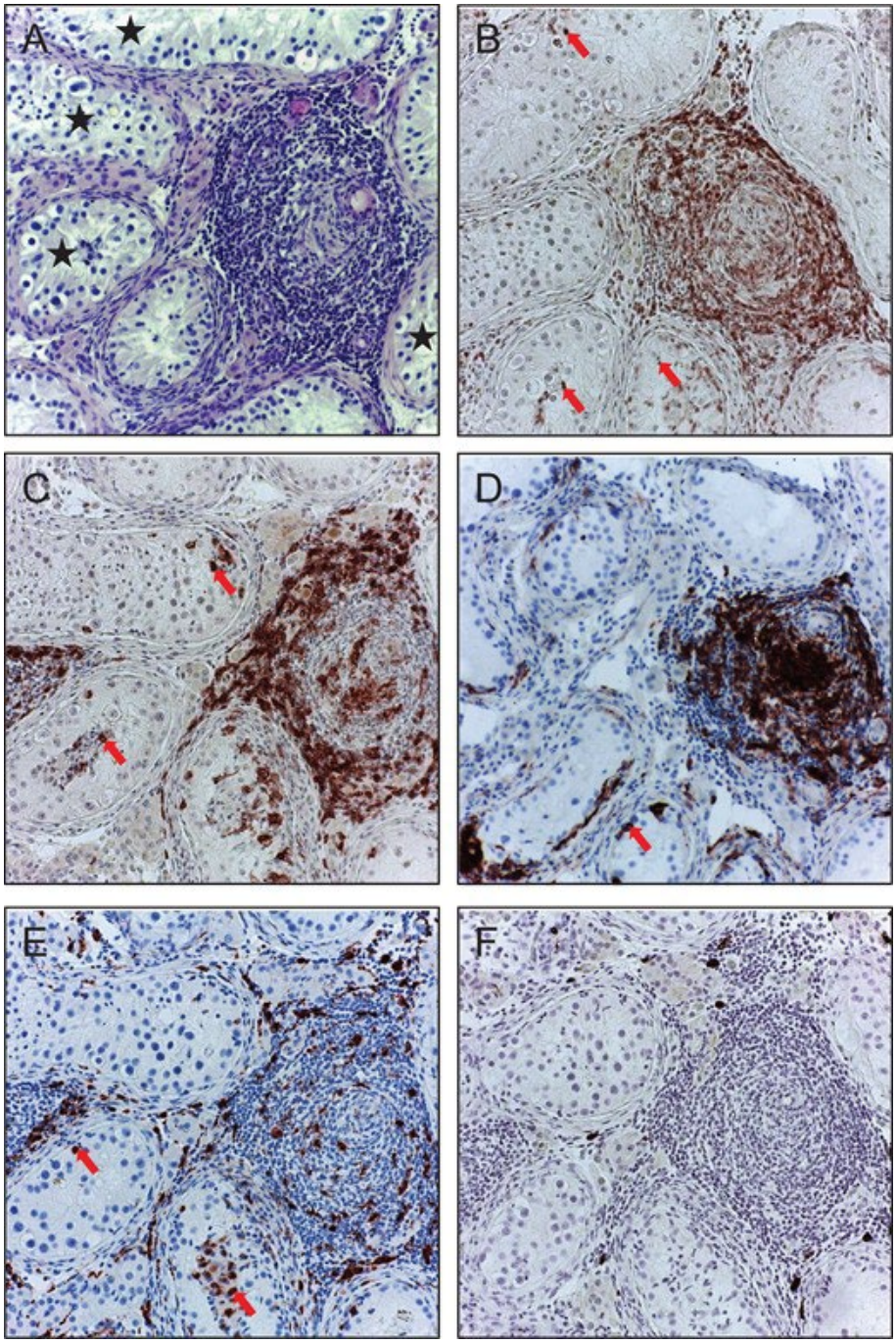

Figure 1.7. Immune cells infiltration in GCNIS. Haematoxylin and eosin staining (**A**), CD3+ T cells (**B**), CD20cy+ B cells (**C**), CD11c+ dendritic cells (**D**), CD68+ macrophages (**E**), tryptase+ mast cells (**F**). Asterisks indicate tubules containing GCNIS cells. Arrows point at intratubular immune cells. T cells (B), B cells (C) and dendritic cells (D) can be found associated with inflammatory infiltrates in GCNIS samples. Macrophages (E) and mast cells (F) have been detected within the interstitium (From Klein et al., 2016).

1.6. The Transforming Growth Factor-β Superfamily

1.6.1. Introduction to the Transforming Growth Factor-β Superfamily

The TGF-β superfamily is comprised of more than 45 members including bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) activins, inhibins, myostatin, and nodal (Hedger and de Kretser, 2013). These are multifunctional signalling proteins that regulate growth and differentiation and can act as both pro-inflammatory and anti-inflammatory mediators in various biological contexts. Therefore, genes activated by TGFβ superfamily signaling impact on inflammation, morphogenesis, adult stem cell differentiation, embryonic development, wound healing, immune regulation and cancer. They function as disulfide-linked homo- or heterodimers that bind type I and type II transmembrane receptors with an intracellular serine/threonine kinase domain. Signalling occurs via a complex set of both common and specific surface receptors, SMADs, co-receptors and regulators to perform their functions (Attisano et al., 2001; Itman et al., 2006; Beyer et al., 2013). For example, activins use two types of Type I receptors (ALK4 and ALK2). Smads 2 and 3 are the primary mediators of their intracellular signalling (SMAD, small for body size/mothers against decapentaplegic homolog), and Cripto, Inhibins and Follistatin are their regulators (López-Casillas et al., 1993; Heldin et al., 1997; Bartholin et al., 2002; Itman et al., 2009). More details on activin and inhibin structure, receptors, ligands and signaling pathways are provided in 6.3 section.

1.6.2. The roles of Transforming Growth Factor-β Family in male reproduction

TGFβ superfamily proteins have key roles in testis development during fetal life, both in human and mouse. Their receptors are present on both germ cells and somatic cells. TGFβ cytokines control development of gonocytes and spermatogonia, Leydig cell differentiation, seminiferous tubule formation, and interactions of Sertoli cells (Richards et la., 1999; Meehan et al., 2000; Li et al., 2007; Itman et al., 2006 and 2009; Young et al., 2015; Loveland et al., 2018) (Figure 1.8).

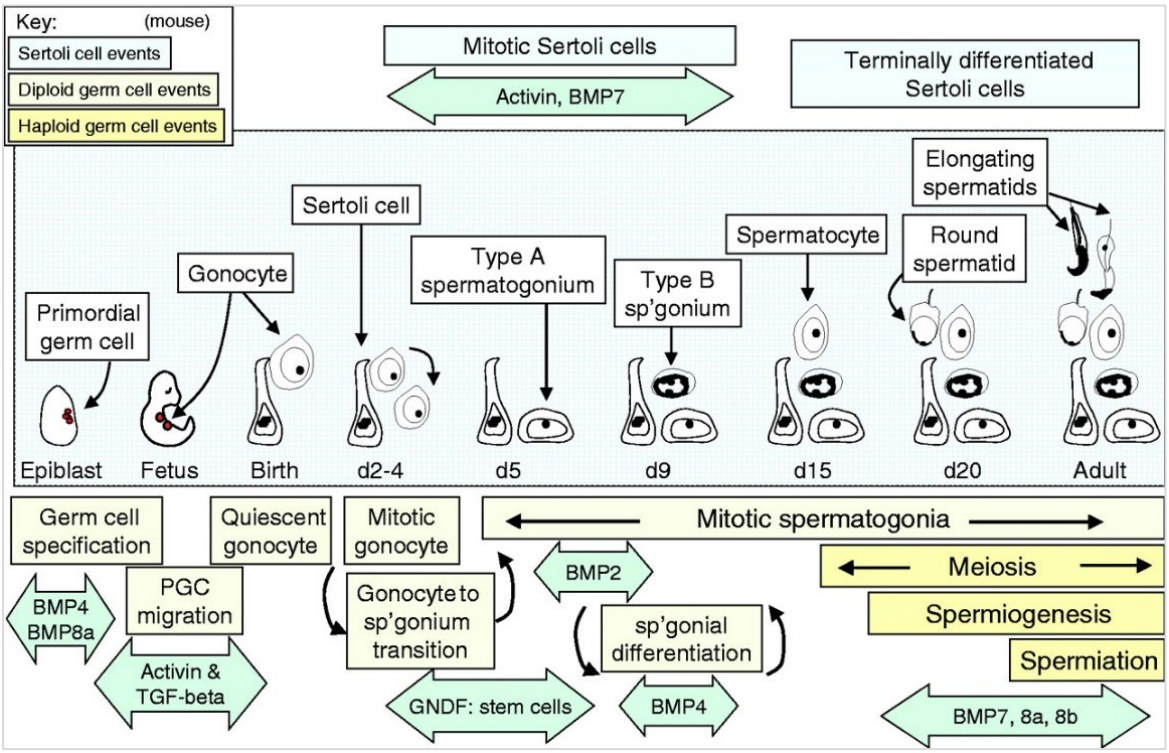

Figure 1.8. Key events in establishment of male fertility in the mouse and the roles of TGF-β superfamily in regulation of specific events of germ cell differentiation and development lead to spermatogenesis. Diagrammatic representation of key events in establishment of male fertility in the mouse. Various TGF-β superfamily ligands have been implicated in regulating spermatogenesis. Ligands known to regulate specific events of germ cell specification and development are illustrated under the diagram. Events associated with establishment of the Sertoli cell population are depicted above the diagram (From Itman et al., 2006).

Among members of this family, activin and inhibin are important for normal testicular and reproductive tract development during fetal and juvenile period. Consequently, any alteration to activin signalling may increase the risk of pathogenesis (Mithraprabhu et al., 2010; Loveland et al., 2015). For these reasons, a focus of investigations in this thesis included studying the impact of activin and inhibin on testicular development during fetal life.

1.6.3. Activin and inhibin

Activin A are dimeric growth factors that form a subfamily with the inhibins with which they share β subunits. Four mammalian genes encode 4 activin β subunits: βA, βB, βC and βE. Activin A, which is my study focus, is made from two βA subunits. Inhibin A acts as an inhibitor for activin A, and it is made of an α-subunit linked to an activin βA subunit (Figure 1.9) (encoded by the *inha* gene), (Antenos et al., 2007 and 2011; Walton et al., 2012). Activin A is a cytokine that can trigger both pro- and anti-

inflammatory responses in leukocytes. Furthermore, it is an essential mediator of tissue homeostasis and embryogenesis.

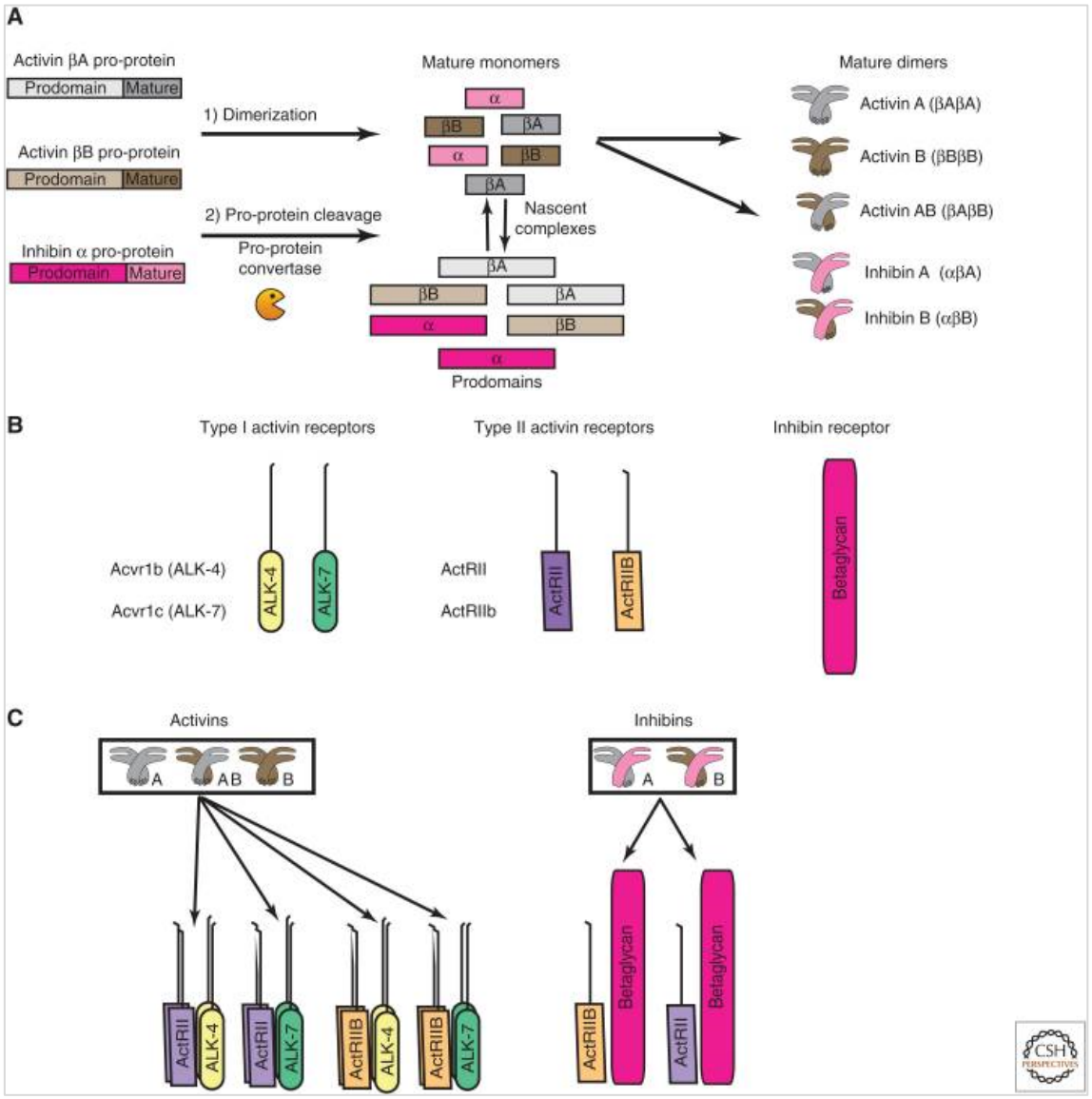

Figure 1.9. Activin and inhibin ligands and receptors. (**A**) Activin βA, activin βB, and inhibin α are synthesized as pro-proteins that comprise a prodomain and mature domain. The pro-proteins associate to form homo- or heterodimers, which are ultimately processed into activins A, B, AB, and inhibins A and B. The junctions of the pro- and mature domains are cleaved by pro-protein convertases, resulting in dimer complexes that retain the noncovalently linked prodomains. (**B**) The two types I receptors for activins are ActRIB (ALK-4) and ActRIC (ALK-7). The two type II receptors are ActRII and ActRIIB. The inhibins antagonize activin signaling by using one type II receptor and one type III TGF-β receptor, betaglycan. (**C**) The mature activin dimers bind type I and type II receptors to form active signaling complexes. Each activin dimer can bind more than one combination of type I and type II receptors with different affinities, and each type I/type II receptor combination can bind different dimers, including other members of the TGF-β family. The active signaling complex is comprised of one activin dimer, two type I, and two type II receptors. Inhibins competitively antagonize activin signaling by binding one type II receptor and betaglycan, thereby sequestering type II receptors in an inactive complex (From Namwanje and Brown, 2016).

Activin and inhibin control testis development in fetal and juvenile life, locally and through hypothalamic-pituitary– gonadal axis (Namwanje and Brown, 2016). Appropriate activin signalling is needed for normal Sertoli cell function and spermatogenesis (Figure 1.10) (Itman et al., 2009 and 2011).

The potent activin antagonist, inhibin α, is able to inhibit activin A signalling through binding to type II receptors via its β subunit. Consequently, the access of activin A to the type II receptor is blocked, intracellular Activin A dimer formation reduced, and the intracellular signalling cascade is not initiated (Feng et al., 2005; Archambeault and Yao 2010 ; Loveland et al., 2015). Activin A is produced by many cells, including hematopoietic and immune cells such as activated Th2 cells, mast cells, monocytes macrophages dendritic cells, and neutrophils (Hedger, 2015).

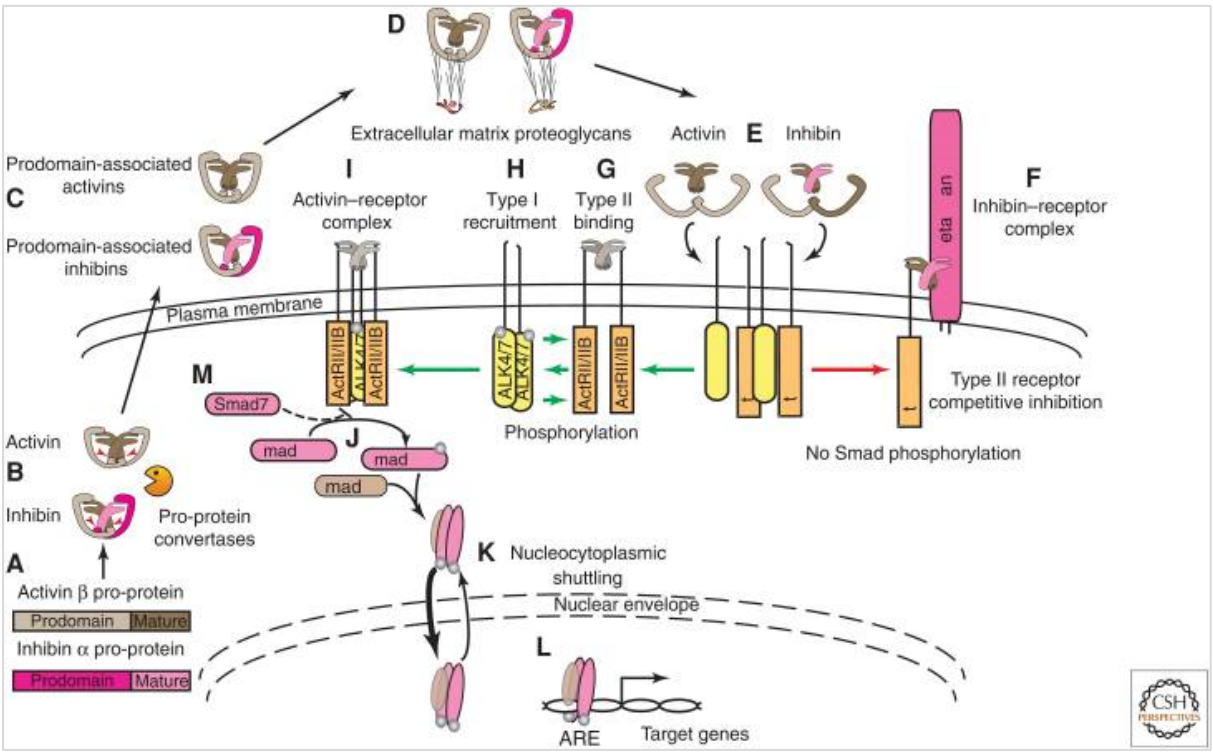

Figure 1.10. Activin and inhibin processing and signaling.
(**A**) Activin and inhibin monomers are synthesized as pro-proteins. (**B**) The pro-proteins associate as homodimers or heterodimers with their intact prodomains. Within the cell, the junctions of the pro- and mature domains (red arrowheads) are cleaved by pro-protein convertases, leaving the noncovalent interactions among the domains intact. (**C**) Prodomain-associated activins and inhibins are released from the cell. (**D**) The intact prodomains enable interactions with glycosaminoglycans on proteins within the extracellular matrix. (**E**) Activins and inhibins compete for type I and II activin receptor binding, and, on receptor binding, release their associated prodomains. (**F**) Inhibin antagonizes activin signaling through association of its single inhibin β subunit with a single type II activin receptor and the association of its single inhibin α subunit with the membrane proteoglycan, betaglycan, thereby forming an inactive inhibin–receptor complex. This complex is incapable of signal transduction and thus inhibits activin signaling by sequestering type II activin receptors (red arrow). (**G**) The activation of activin receptors requires several steps (green arrows). The

activin dimer binds two type II activin receptors, and activates type II receptor serine–threonine kinase activity. (**H**) Type II receptor binding results in recruitment and association with two type I activin receptors, ActRIB (ALK-4) or ActRIC (ALK-7), that are subsequently phosphorylated. (**I,J**) The fully assembled, hexameric ligand–receptor complex then initiates Smad-mediated signaling by phosphorylating regulatory Smad2 and/or Smad3 (Smad2 and Smad3) near their carboxyl termini, followed by association of two phosphorylated Smads with a common Smad4. (**K**) Smad complexes are in equilibrium between the cytoplasm and nucleus. Receptor signaling results in a shift in equilibrium toward the nucleus. (**L**) Binding of the Smad complex and transcription coactivators to activin-responsive elements (AREs) results in the transcription of hundreds of genes, a process that is tightly regulated by a variety of proteins that impact nucleocytoplasmic shuttling, Smad phosphorylation status, Smad degradation, and transcriptional activity. (**M**) Inhibitory Smad7 competes with Smad2 and Smad3 for activated type I receptor binding, thereby preventing Smad2 and Smad3 phosphorylation and facilitating proteasomal degradation or dephosphorylation of activin–receptor complexes. (From Namwanje and Brown, 2016).

1.6.4. The impact of activin A on macrophages

To study the influence of activin on mouse macrophages, two types of macrophages have been investigated: mouse peritoneal macrophages and the macrophage cell line RAW264.7. RAW 264.7 cells are monocyte/macrophage-like cells, an Abelson leukemia virus-transformed cell line arising in BALB/c mice. RAW 264.7 cells are a useful model for studies of macrophage behaviors and exhibit phagocytosis activities (Taciak et al., 2018). Stimulation of peritoneal macrophages and RAW264.7 cells (*in vivo* and *in vitro*) using lipopolysaccharide (LPS) and CpG, which interact with their Toll Like Receptors (TLR)2, TLR4 and TLR9, respectively, revealed this provoked a strong alteration of activin A, activin receptor type 2A and SMAD2/3 mRNA expression (Ebert et al., 2007; Wang et al., 2008; Li et al., 2013).

Data obtained from studying the outcomes of macrophage exposure to activin A have been controversial. On the one hand, Wang et al., and Ge et al reported polarization of macrophages towards a pro-inflammatory M1-like phenotype upon the treatment of resting macrophages (M0) with activin A. These investigations using RAW264.7 cells demonstrated an increase in their phagocytotic and pinocytotic activity, upregulation of transcripts encodng iNOS and IL-1β, and higher protein production of IL-1β, IL-6, and nitric oxide in culture supernatant (Wang et al., 2008; Ge et al., 2009). On the other hand, other investigations reported RAW264.7 cells skewed to favor M2-associated arginase-1 mRNA expression upon treatment by activin A (Ogawa et al., 2006). Interestingly, the expression of MHC class I and II on RAW264.7 cells and peritoneal macrophages showed two different patterns: they were upregulated in RAW264.7 cells but were unchanged in peritoneal macrophages, demonstrating

different effects on primary cells versus cell lines (Ge et al., 2009; Wang et al., 2009). Additional studies have confirmed that the impact of activin A on mouse macrophages toward either a pro-inflammatory or anti-inflammatory state depends on the origin of cells (primary or cell line), the presence of other stimulators (LPS, IFN-γ) in the microenvironment, and the status of macrophages (resting or activated) (Zhang et al., 2005; Zhou et al., 2009; Li et al., 2013). Studies on the impact of activin A on macrophages have been done on isolated macrophages (in vitro study) and not on tissue resident macrophages (in vivo). In general, the deduced understanding from these studies is that activin A has both regulatory and anti-inflammatory impacts on activated macrophages to prevent excessive responses, and pro-inflammatory effects on resting macrophages (Morianos et al., 2019).

1.6.5. Mouse models to study the effect of activin and inhibin on testis development

Activin A levels rise after sex determination in the male, but not in the female. Activin A protein levels are highest during the first week of postnatal mouse testis development, when spermatogenic differentiation first begins (with the first appearance of spermatogonial stem cells), demonstrating that activin bioactivity levels influence male germ cell maturation at the onset of spermatogenesis (Mithraprabhu et al., 2010).

Investigations of embryos of mouse models with alterd activin A levels could extends our knowledge about the importance of activin A function during fetal testis development. In accord with this hypothesis, Prof Loveland's research group has been investigating the fetal testis of a mouse model in which circulating activin A level is 100-fold increase in adult animals (fetal levels are not known), by an inhibin-α subunit gene knockout (*Inha* $^{-/-}$) (Mendis et al., 2011). They have observed that, at E15.5, *Inha* $^{-/-}$ testes display some abnormalities including frequent multinucleated germ cells inside cords, an abnormal number of germ cells outside the testis cords during fetal life, and many germ cell-depleted cords present at birth (in comparison with wild type littermates) (Loveland, unpublished). An early study using cultured rat newborn testes demonstrated that activin A affects gonocytes and impairs their differentiation into spermatogonia (Meehan et al., 2000).

One possible explanation for the existence of gonocytes outside of cords could be the suboptimal function of macrophages, which fail to phagocytose and to remove them from interstitial areas. In parallel with this possibility, RNA-seq analysis showed that in an activin A-deficient mouse model (*Inhba*$^{-/-}$) testes, more than twenty macrophage- related genes were upregulated selectively at E13.5 (and not at E15.5) in comparison with wild type littermates. Arising from observations that activin A can regulate the activation and polarization of macrophages (Sierra-Filardi et al., 2011), and in acute inflammation the level of activin A is increased (Petrakou et al., 2013), this study examined the hypothesis that elevated activin A in *Inha*$^{-/-}$ mice may alter macrophage number, distribution, recruitment and function, so that their normal function in early testis development is impaired.

Studies on mice with altered activin A bioactivity reported striking results such as detection of Sertoli cell tumours in inhibin alpha subunit (*Inha*$^{-/-}$) knockout mouse testes, that have excessive activin and failure of Sertoli cell differentiation (Matzuk et al., 1992). In contrast, proliferation and maturation of Sertoli cells are reduced in mice with low levels of activin bioactivity (*Inhba* mouse model) (Brown et al., 2000). Dias et al. showed that up-regulation of the activin A receptor subunit, ActRIIA, is associated with the progression of GCNIS cells into malignant seminomas in patient samples (Dias et al., 2008). Collectively, these results suggest that during development of testis, proliferation and differentiation of gonocytes and spermatogonial stem cells are under the control of activin, supporting the hypothesis that any change in the level of activin A may impair germ cell development and increase TGCT risk. During a normal pregnancy, levels of activin A does not vary significantly during the first and second trimesters although they are higher compared to menstrual cycle levels. After 24 gestation weeks, activin A levels rise and reaches to its maximum level at term (Bersinger et al. 2003). However, *in vivo* and *ex-vivo* studies have demonstrated that there is a significant increase in the levels of activin A in intra-amniotic inflammation and infection (triggered by both Gram-negative and Gram-positive pathogens) abnormal pregnancies such as pre-eclampsia, fetal growth restriction and gestational hypertension. For example, in pre-eclampsia, the placenta, as the major source of activin A in the maternal circulation, vascular endothelial cells and activated peripheral mononuclear cells may contribute to the raised levels of activin A

(Michel et al., 2003; Muttukrishna et al. 2004, Yndestad et al. 2009; Rosenberg et al. 2012, Torricelli et al. 2012; Park et al. 2018).

Although there are now a considerable number of studies that have investigated human and mouse adult testis immunophysiology, there is a fundamental knowledge gap in understanding the true identity and distribution of immune cells in key phases of fetal testis development, and this information would facilitate a better understanding of how immune cells contribute to the earliest stages of testis development. Therefore, the focus of this project is to study the frequency and phenotype and distribution pattern of macrophages, T cells, neutrophils and mast cells, as all are important cell types during fetal development of the testis.

1.7. Rationales and Hypotheses of this thesis

1) Although there are now a considerable number of studies that have investigated human and mouse immunophysiology in adult testis, there remains a fundamental knowledge gap concerning the identity and distribution of immune cells in key fetal testis developmental phases; this information would facilitate a better understanding of how immune cells contribute to the earliest stages of testis development. Therefore, the focus of Chapters 2 and 4 was to study the frequency, phenotype and distribution pattern of macrophages, T cells, neutrophils and mast cells, as all are important immune cell types during fetal morphogenesis. My plan is to observe the presence of immune cells in the testis at the early stages of fetal life before starting the haematopoiesis in the bone marrow (at E16.5 in mice and half gestation in humans). This finding would identify the embryonic ontogeny (yolk sac and fetal liver) of resident immune cells in the testis.

2) Macrophages are impactful immune cells on the fetal testis morphogenesis and its later function (spermatogenesis). Activin A levels impact Sertoli cell and germ cell numbers and functions during fetal testis development and the function of macrophages, as shown in studies on the adult mouse testis, resident macrophages in other mouse organs and in *in vivo*). Notably, the levels of activin A increase in some pregnant women with preeclampsia, usually after 20 weeks of pregnancy, often in the third trimester (Silver et al., 2002; Yu et al., 2012, Wong et al., 2022). Therefore, studying the impact of activin A levels (using activin A and its inhibitor mutant mice, *Inhba* and *Inha*) on the macrophages during fetal testis development can

be an informative investigation (Chapter 3) to determine the impact of preeclampsia on human fetal testis development. To achieve this, I undertook detection of macrophage number and distribution patterns and measured transcript levels of important mediators relating to macrophage phenotype and function (M1 and M2 markers); and I hypothesised detecting the significant impact of activin A levels (in KO animals) on the number and distribution pattern of macrophages and the levels of immune markers transcriptome.

3) While there are numerous investigations on the frequency and function of pro-inflammatory macrophages, T cells and mast cells in testicular cancer, there is only one report on the number of neutrophils (Yamada et al., 2016), showing their accumulation in advanced stages of testicular tumours (seminoma and non-seminoma). Therefore, in Chapter 5, the number and distribution pattern of neutrophils were targeted in testis samples from human adults with normal spermatogenesis (as controls) and with germ cell neoplasia *in situ* (GCNIS) and testicular germ cell tumour (TGCT). My hypothesis was to observe few neutrophils in normal samples but the accumulation of neutrophils (as potent pro-inflammatory cells) in the areas where other pro-inflammatory immune cells are infiltrated.

Chapter Two

The Changing Landscape of Immune Cells in the Fetal Mouse Testis

2.1. The Changing Landscape of Immune Cells in the Fetal Mouse Testis

The outcome of this chapter has been accepted into *Histochemistry and Cell Biology* on 14 June 2022

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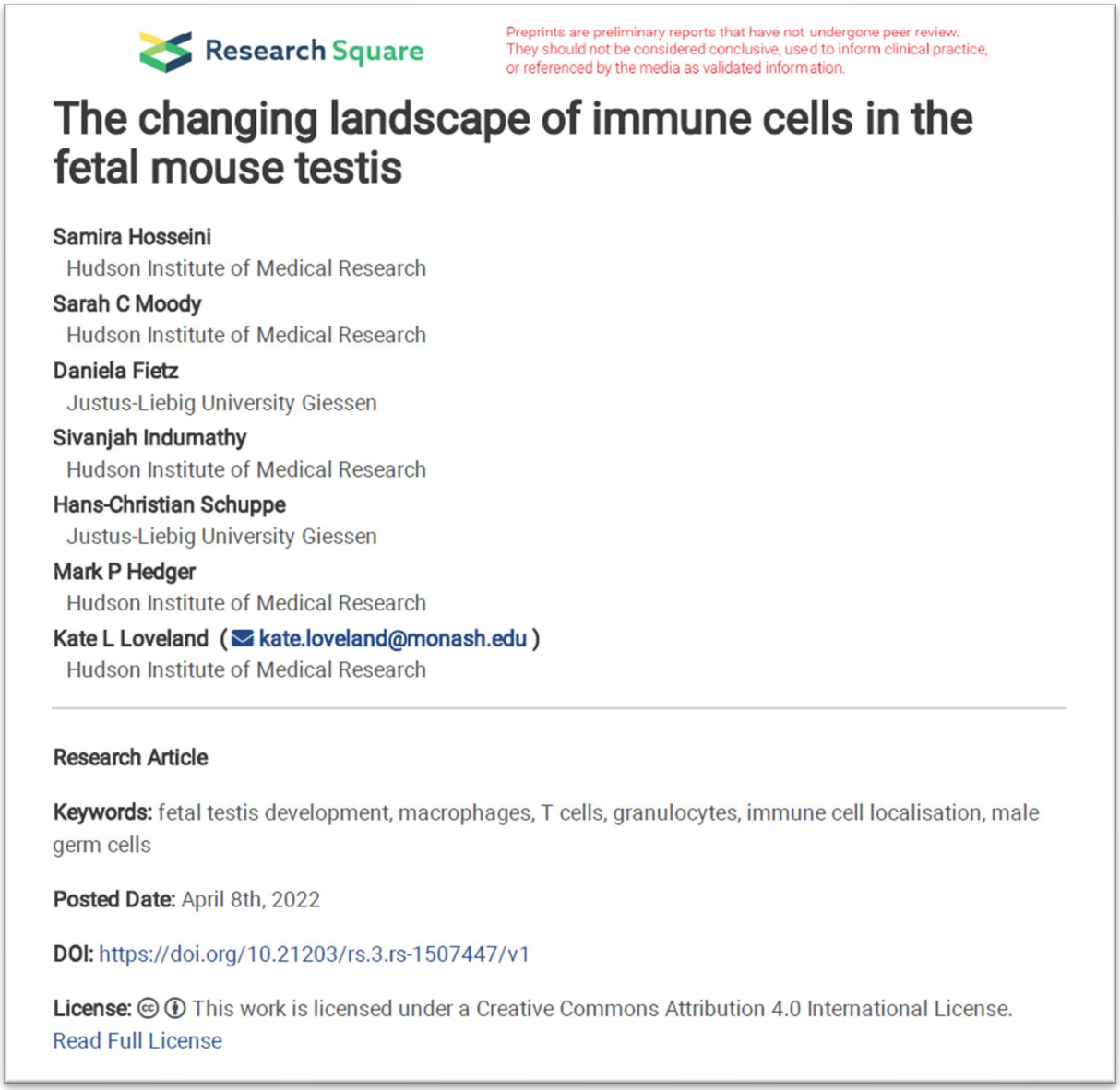
The published PDF format of this paper has not been accessible by submitting this thesis; therefore, this chapter is presented in the Word format and in the manuscript version.



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The changing landscape of immune cells in the fetal mouse testis

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Research Article

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The changing landscape of immune cells in the fetal mouse testis

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Running title: Fetal mouse testis immune populations

2.1.1. Abstract

Fetal testis growth involves cell influx and extensive remodeling. Immediately after sex determination in mouse, macrophages enable normal cord formation and removal of inappropriately positioned cells. This study provides new information about macrophages and other immune cells after cord formation in fetal testes, including their density, distribution, and close cellular contacts. C57BL6J mouse testes from embryonic day (E) 13.5 to birth (PND0), were examined using immunofluorescence, immunohistochemistry and RT-qPCR to identify macrophages (F4/80, CD206, MHCII), T cells (CD3), granulocytes/neutrophils (Ly6G) and germ cells (DDX4). F4/80^{+} cells were the most abundant, comprising 90% of CD45^{+} cells at E13.5 and declining to 65% at PND0. Changes in size, shape and markers (CD206 and MHCII) documented during this interval align with the understanding that F4/80^{+} cells have different origins during embryonic life. CD3^{+} cells and F4/80^{-}/MHCII^{+} were absent to rare until PND0. Ly6G^{+} cells were scarce at E13.5 but increased robustly by PND0 to represent half of the CD45^{+} cells. These immunofluorescence data were in accord with transcript analysis, which showed immune marker mRNAs increased with testis age. F4/80^{+} and Ly6G^{+} cells were frequently inside cords adjacent to germ cells at E13.5 and E15.5. F4/80^{+} cells were often in clusters next to other immune cells. Macrophages inside cords at E13.5 and E15.5 (F4/80^{Hi}/CD206^{+}) were different than macrophages at PND0 (F4/80^{Dim}/CD206^{-}), indicating they have distinct origins. This histological quantification coupled with transcript information identifies new cellular interactions for immune cells in fetal testis morphogenesis and highlights new avenues for studies of their functional significance.

Keywords: fetal testis development, macrophages, T cells, granulocytes, immune cell localisation, male germ cells

2.1.2. Introduction

The mouse testis first forms in fetal life, with the assignment of male fate enabled by expression of *SRY* at embryonic day (E) 10.5 in Sertoli cells (Koopman et al. 1991). This initiates the emergence of the somatic lineages central to adult testis function and commits the sexually indifferent primordial germ cells to the male fate by E12.5, when they become gonocytes (Adams et al. 2002). The fetal testis then undergoes major cellular and structural transformations, which enable it to ultimately perform two key functions in adult life: hormone secretion and production of spermatozoa (Wilhelm et al. 2007, 2013). There is increasing interest in understanding the distribution and roles of immune cells during the dynamic remodeling that characterises fetal and newborn testis development, particularly because processes that disrupt normal development may increase the risk of adult infertility and testicular neoplasia, including testicular germ cell tumors (TGCT) arising from germ cell neoplasia in situ (GCNIS) (Skakkebaek et al. 2016).

Macrophages have been of particular interest because cells in the myeloid lineage perform key roles in tissue remodeling and homeostasis. They are the most abundant immune cells in the testis at all ages (Mossadegh-Keller and Sieweke, 2018). Macrophage depletion during embryonic life demonstrated that testicular macrophages regulate testicular vascularisation and morphogenesis in the mouse (DeFalco et al. 2014). After birth, testicular macrophages play key roles in homeostasis, supporting spermatogonial differentiation, including through the production of Csf1 and promotion of Leydig cell testosterone production (DeFalco et al. 2015; Fijak et al. 2017, 2018; Mossadegh-Keller and Sieweke, 2018).

In the normal adult testis, resident macrophages are classified into two major groups: interstitial macrophages that are in close contact with Leydig cells within the interstitium, and peritubular macrophages on the seminiferous tubule surface, close to peritubular myoid cells. These are each predicted to perform different functions based on their locations and distinct expression profiles of functional markers (DeFalco et al. 2015; Mossadegh-Keller et al. 2017; Lokka et al. 2020; Indumathy et al. 2020; Wang et al. 2021). Recent investigations have defined the origins of testicular macrophages using fate mapping and single-cell RNA-sequencing (scRNAseq) analyses. The first study using fate-mapping to examine testicular macrophage emergence at the earliest stages of fetal testis development provided evidence that macrophages present in the adult testis arise from the yolk sac (DeFalco et al. 2014). A subsequent analysis

demonstrated that adult interstitial macrophages are yolk sac-derived, while peritubular macrophages emerging exclusively after birth and are bone marrow-derived (Mossadegh-Keller et al. 2017). More recent studies incorporating scRNAseq revealed that interstitial macrophages mainly originate from fetal liver-derived precursors, while peritubular macrophages develop prenatally and are detectable at birth (Lokka et al. 2020; Wang et al. 2021). The requirement of macrophages for normal testis cord formation during early fetal life was demonstrated by the outcome of their selective depletion. In addition, macrophage engulfment of germ cells and Sertoli cells outside of nascent cords was identified at E11.5 and E12.5 (DeFalco et al. 2014).

CD206, also known as mannose receptor-1 (MRC-1), is present on both immune and non-immune cells (Sheikh et al. 2000) and is used as a key marker of macrophages with an anti-inflammatory and tolerogenic phenotype. In contrast, a high level of the MHC class II molecule (MHCII) is critical for macrophage initiation of antigen-specific immune responses that can lead to inflammation (Shapouri-Moghaddam et al. 2018). In the postnatal testis, CD206 is expressed on interstitial macrophages and MHCII is exclusively expressed on peritubular macrophages (Mossadegh-Keller et al. 2017). A proposed function for MHCII on peritubular testicular macrophages is to present spermatid-derived antigens to regulatory T cells (Treg), thereby inducing cell-dependent physiological tolerance against these antigens (Smith et al. 2016; Tung et al. 2017; Mossadegh-Keller and Sieweke, 2018; Heinrich and DeFalco, 2020). Though there are conflicting reports regarding the detection of peritubular macrophages at birth (Lokka et al. 2020; Wang et al. 2021), we hypothesised that in fetal and newborn mouse testis, the dominant testicular macrophage population would be $CD206^{+}$ $MHCII^{-}$ in order to sustain an immune regulatory and tolerogenic microenvironment during testis remodeling and morphogenesis.

We were intrigued by the possibility that additional myeloid and lymphoid cells could be integral to early testis development. Based on the known functional relationships between T cells, neutrophils and macrophages during inflammation, we investigated whether T cells and neutrophils could also be present in the fetal testis. T cells ($CD3^{+}$) can have either inflammatory or immune tolerogenic functions, depending on environmental cues (Khan and Ghazanfar et al. 2018). They are relatively common in the adult testis, representing approximately 25% of all immune cells in the adult rat testis (Bhushan et al. 2020). Testicular macrophages, acting as antigen-presenting

cells, can trigger adaptive immunity, autoimmunity and inflammation in the testis; they can also present germ cell-derived antigens to the T cells under regulatory conditions to convert them to Treg (Bhushan et al. 2020; Wang et al. 2021), [b]ut information about these cells in the fetal testis is lacking.

Best understood as the first line of defense against pathogens, key cells in the granulocyte lineage include neutrophils, basophils and eosinophils as the most abundant leucocytes, that are generally best known for their role in inducing inflammatory factors (Lin and Loré, 2017). Their engagement may ultimately cause host tissue damage and increase both disease pathogenesis and severity if not cleared by the macrophages which typically restore homeostasis (Kobayashi and DeLeo, 2009; Soehnlein and Lindbom, 2010). In adults, neutrophils are abundant in circulation, and are relatively short-lived, highly migratory cells which contribute to organ homeostasis, exhibiting daily movement in and out of organs in mice (Kobayashi and DeLeo, 2009; Casanova-Acebes et al. 2013, Lok et al. 2018; Lin and Loré, 2017). In contrast, neutrophil roles in healthy organ development are not established. Most studies of testicular neutrophils have focused on the adult in rats (Lysiak et al. 2001; Sukhotnik et al. 2007; Arena et al., 2020) and humans (Yamada et al. 2016; Bolat et al. 2017), but their recruitment in the fetal mouse testis has been demonstrated at E12.5 in association with vasculature development (DeFalco et al. 2014). Neutrophils have been documented in the human testis in conditions of acute bacterial epididymo-orchitis (Schuppe and Bergmann, 2013; Fijak et al. 2018), and their presence in relatively high density in certain testicular germ cell tumors was proposed as a survival prognostic factor (Yamada et al. 2016). Beyond this, the presence of granulocytes, including neutrophils, and their function during fetal testis development in the mouse, rat and human are undocumented.

This study addresses the emergence and localisation of macrophages and granulocytes, which arise from a common myeloid progenitor cell (Rosmarin et al. 2005) and $CD3^+$ cells, during murine *in utero* development between E13.5 to birth (post-natal day 0, PND0). The frequency, localisation and close cell contacts of macrophages ($F4/80^+$ cells), T cells ($CD3^+$ cells) and neutrophils ($Ly6G^+$ cells) were documented following immunofluorescence (IF) and immunohistochemistry (IHC), including in relationship to gonocytes ($DDX4^+$ cells). This histological approach avoids the potential for under-reporting of cell types that can arise following tissue-dissociation for flow cytometry (Steinert et al 2015), and it was extended by monitoring

changes in the levels of transcripts relating to each of these cell types. The outcomes provide new ideas about what immune cells may contribute to normal mouse testis development to set the stage for adult fertility.

2.1.3. Materials and Methods

Animals

Mice were housed at the specific pathogen-free (SPF) Monash Animal Research Platform (MARP) or at the SPF Monash Medical Animal Facility in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, with a 12-hour light and 12-hour dark cycle, with food and water available *ad libitum*. All investigations conformed to the National Health and Medical Research Council/Commonwealth Scientific and Industrial Research Organisation/Animal Advisory Committee Code of Practice for Care and Use of Animals for Experimental Purposes and were approved by the Monash University Standing Committee on Ethics in Animal Experimentation. Timed matings of C57BL/6J mice were performed, with the day of plug identified as embryonic day (E) 0.5. Pregnant mice were killed by cervical dislocation, and the embryos (at E13.5, E15.5) were immediately removed from the uterus, killed, and the testes collected. Embryonic age was verified using forelimb and hindlimb development stages. At least 4 pups per age (at E13.5, E15.5 and PND0) were analysed from more than one litter for each age. Testes from three individual C57BL/6J mice were examined on PNDs 2, 7, 10, 15, 20 and adult to visualize $Ly6G^+$ cell distribution.

Tissue Preparation

Two different fixation and embedding approaches were employed. For immunohistochemistry (IHC) experiments, one testis from each mouse was immersed at room temperature (RT) in Bouin's solution 30 min for E13.5, 60 min for E15.5, and 90 min for PND0, followed by washing in 70% ethanol (3 x 1 hour). Fixed tissues were processed, embedded in paraffin, and sequential sections of 5µm thickness were collected onto Superfrost Plus microscope slides (Thermo Fisher Scientific, Waltham, Massachusetts, United States). For immunofluorescence (IF) analyses, one testis from each mouse was fixed in 4% paraformaldehyde (PFA) overnight at 4°C, then cryoprotected in phosphate-buffered saline (PBS) first with 15% (w/v) sucrose overnight at 4°C, then with 30% sucrose solution the following day. Tissues were

embedded in Tissue Tek OCT compound (Sakura Finetek, USA) then stored at -80°C. Cryosections (5µm) were placed onto Superfrost Plus microscope slides for immediate use or stored at -80°C.

Each testis was entirely serially sectioned, with 3 sections placed on each slide. Four slides corresponding to the centre of the testis were examined with each antibody, ensuring that replicate sections for each antibody were spaced 15-30 µm to prevent double-counting of individual cells. Section cross-sectional area was measured using ImageJ software, V2.1.0 (National Institutes of Health, Bethesda, Maryland, USA,); this did not differ significantly between samples within each age group (Supplementary Figure 1A). Tissue embedding and sectioning were performed by staff at the Monash Health Research Precinct node of the Monash Histology Platform, Monash University. For RNA extraction, testes at each age (n=3/age) were dissected away from the mesonephros, snap-frozen immediately on dry ice, and stored at –80°C.

Immunostaining

Immunostaining was performed using antibodies (detailed in Supplementary Table S1, including citations of prior usage) to detect all immune cells (anti-CD45), macrophages (anti-F4/80, anti-CD206, anti-MHC Class II), T cells (anti-CD3), neutrophils (anti-Ly6G), laminin (anti-laminin), and germ cells (anti-DDX4). Adult mouse spleen was used as a positive control for antibodies to immune cell markers, and adult and newborn mouse testis were used for antibodies to laminin and germ cells. Each directly conjugated antibody bound as expected to cells in appropriate locations and with appropriate morphology (e.g. size, nuclear shape). Negative controls for unconjugated primary antibodies lacked primary antibodies and were used to establish background binding of secondary antibodies.

For IHC, sections were deparaffinised and rehydrated through a series of 5-minute washes: twice each in histolene (Trajan, Australia) and 100% ethanol, then once each in 95%, 80%, 70%, 50% and 30% ethanol and deionised water. Heat-induced antigen retrieval was performed by immersing slides in Tris-EDTA buffer (10mM Tris Base, 1mM EDTA, 0.05% Triton X-100 (Sigma-Aldrich), pH 9.0); the container with slides was microwaved for 4-5 min at 800W, then 9 min at 450W, followed by cooling at RT for 30 minutes. Sections were incubated for 20 min at RT with 3% hydrogen peroxide (Merck Millipore) to quench endogenous peroxidase, followed by washes with Tris-buffered saline (TBS; 50 mM Tris-Cl, 150 mM NaCl, pH 7.5; 3 x 5 min). Sections were

next incubated in blocking solution consisting of 5% normal serum from the animal in which the secondary antibody was raised (5% in TBS) in a humid chamber at RT for 1 hr. Primary antibodies diluted in TBS with 1% bovine serum albumin (BSA; Sigma-Aldrich) were applied overnight at 4°C.

Slides were washed three times, 3 min each, between incubations at RT using TBS. Controls for non-specific secondary antibody binding in all experiments lacked primary antibody; these consistently showed no signal. Primary antibody binding was detected using a biotinylated secondary antibody in a humid chamber for 1 hr at RT. After consecutive washes (3 × 3 min), Vectastain Elite ABC kit reagents were added (Vector Laboratories, USA) according to the manufacturer's instructions. Antibody binding was detected as a brown precipitate following development with 3, 3-diaminobenzidine tetrahydrochloride (DAB) (Agilent Technologies, USA), then sections were counterstained with Harris hematoxylin (Sigma-Aldrich). Stained slides were then dehydrated through a graded ethanol series (once each in 30%, 50%, 70%, 80% and 95% ethanol for 3 min, plus twice in 100% ethanol and histosol (5 min). Finally, slides were mounted under glass coverslips using DPX mounting media (Sigma-Aldrich) and left flat overnight to air dry before imaging.

Slides for indirect and direct immunofluorescence (IF) stored at -80°C were air-dried for 20 minutes at RT and rehydrated in PBS for 10 minutes. To permeabilise the sections, 0.1% Triton X-100 (Sigma-Aldrich) in PBS was applied for 5 minutes at RT. Slides were washed in PBS (x3), followed by a two-step blocking protocol in a humid chamber at RT. First, 5% Mouse on Mouse Blocking Reagent (Vector Labs) diluted in TBS was applied to sections for 30 minutes, then the slides were washed in PBS (3 min), and finally sections were exposed to 5% normal serum (from the species of secondary antibody origin)/2% BSA/PBS for 1 hr. Blocking solution was replaced with unconjugated primary antibodies (for indirect IF) diluted in 2% BSA/0.1% Triton X-100/PBS, and slides were incubated overnight in a humid chamber at 4^{o}C. Negative control sections lacked primary antibody. The next day, slides were washed once in 0.1% Triton X-100/PBS (3 min), twice in PBS (3 min each), and appropriate conjugated secondary antibodies were applied for 1 hour at RT, with slides protected from light from this point onwards. Secondary antibodies were removed, and slides were washed as above. For direct immunofluorescence, after the blocking step, conjugated primary antibodies were added for overnight hour at 4^{o}C, slides were washed as

above, and sections were mounted under a glass coverslip using ProLong Gold Mountant (Invitrogen, USA) and stored at 4°C until scanning for image analysis (within 24 hours).

Imaging and morphometric analysis

Immunostained sections were scanned using an Olympus VS120 Slide Scanner (Olympus XM10 Monochrome Camera, 16-bit Fluorescence) and analysed using OlyVIA Software (Olympus Life Science, 2.9.1 Viewer). The minimum detection level at which no fluorescence could be seen on the negative control was established as the baseline, and signals visible above this were considered genuine; detection levels were set identically for each section treated with the same primary and secondary antibody in an individual experiment to ensure consistency. Cells with a distinct signal evident at 6000 or higher on the intensity scale were labelled as 'Hi'; cells visualised with a signal between 3000 and 6000 were identified as 'Dim'.

Immune cells were identified based on the presence of a well-defined nucleus visualised using DAPI (4′,6-diamidino-2-phenylindole; Invitrogen, D3571, 1:1000 in PBS). The position of each positive macrophage within an individual section was scored as being in the 'perimeter' area (the region between the capsule and cords) or 'interior' area (inside cords and interstitium) (Supplementary data S1B, S1C). Macrophage density was reported by adding the number of macrophages detected in the perimeter and interior areas of a section, divided by the section cross-sectional area $(\mu m)^2$, measured using ImageJ software (National Institutes of Health, USA). Analysis was initially performed by blinded counting of 3 slides by two independent operators with a variation of 5.3% for total macrophage numbers. The entire series was subsequently counted blinded, by a single operator.

RNA extraction, cDNA synthesis and quantitative RT-PCR (qRT-PCR)

Transcripts were measured by qRT-PCR in C57BL6J mouse testes at E13.5, E15.5 and PND0, from 3 independent samples per age. RNA extraction and DNase treatment using the RNeasy Mini Kit (Qiagen, Germany) was performed according to manufacturer instructions. RNA purity and concentrations were quantified on a NanoDrop™ OneC (Thermo Scientific™ Inc, USA). Complementary DNA (cDNA) was synthesised from 100 ng RNA with 500 ng oligo dTs (Promega, USA), 50 ng random primers (Promega,) and 200 Units SuperScript III Reverse Transcriptase (Thermo

Fisher Scientific Inc) per sample, according to the enzyme manufacturer's protocol. Primers designed to span exon-exon junctions in target genes (Integrated DNA Technologies, Inc. USA) are listed in Table S2. qRT-PCR was performed using SYBR-green and specific primer pairs (total 10 ng) measuring each sample in triplicate; negative controls lacking Superscript III (−RT) were included for each sample. Runs conducted on Applied Biosystems QuantStudio 6 (Thermo Fisher Scientific) in the Genomics Centre, Monash Health Translation Precinct, Clayton, AU) used the following cycling conditions: denaturation at 95 °C for 10 min; amplification for 40 cycles of 95 °C for 30 s, 62 °C for 30 s and 72 °C for 30s.

The RPLP0 housekeeping gene transcript was used to normalise transcript loading. Data were analysed using the standard curve method generated by 6 serial dilutions with 3 technical replicates using QuantStudio 6 software (ThermoFisher Scientific; version v1.3). Relative quantification of each transcript is presented as the quantity mean level. A single amplification product was detected for each target.

Statistical analysis

GraphPad Software version 9.1.2 (San Diego, USA) was used for statistical analysis and graphing. A one-way ANOVA and Tukey's multiple comparison post hoc and unpaired t-test with Welch's correction were employed to analyse differences between age groups, as indicated. Values were considered significantly different if $p < 0.05$.

2.1.4. Results

Analysis of immunofluorescence was conducted to enumerate and to categorise the localisation of the total immune cell population (identified as $CD45^+$ cells), macrophages ($F4/80^+$ cells), T cells ($CD3^+$ cells) and granulocytes ($Ly6G^+$ cells), including in relationship to male germ cells ($DDX4^+$ cells) in sections of E13.5, E15.5 and newborn mouse testes (PND0) (Figure 1). A small proportion of $Ly6G^+$ also displayed a $F4/80^{Dim}$ signal.

Testicular $F4/80^+$ cells exhibited two distinct shapes (Supplementary Figure S2). The predominant macrophage population at E13.5 consisted of relatively large and elongated cells, greater than 10 μm in diameter and featuring a round nucleus and intense F4/80 signal ($F4/80^{Hi}$, defined as visible at Fixed Scaling of 6,000 in OlyVIA Software). A population of smaller (<10 μm) macrophages displayed a lesser F4/80 signal; these were first detected at E15.5 and consisted of rounded cells with either a

rounded or polymorphic nucleus (Supplementary Figure S2). We speculated that these small, rounded F4/80^{Dim} cells with an either a band-shaped or segmented nucleus could be granulocytes.

The number of testicular immune cells increases significantly during fetal development

The density of each immune cell type per testis cross section increased significantly as the testis grew, indicating a numerical increase over time (Figure 1A). CD45^{+} cell density increased 1.74-fold from E13.5 to PND0, while F4/80^{+} cells increased 1.34-fold, and Ly6G^{+} cells a remarkable 114-fold. No CD3^{+} cells (T cells) were detected in the E13.5 testis; they were rare at E15.5 (0-1/section), and more frequent at PND0 (10-20/section) (Supplementary Figure S3). This matched the profile of the *Cd3* transcript (Figure 1A'), which was barely detectable at E13.5, but increased 2.5-fold from E15.5 to PND0. Transcript levels reinforced the immunofluorescence data, showing the same trend for *Ptprc* (encoding CD45), *Cd3* (encoding CD3) and *Ly6g* (encoding Ly6G) over this period of testis development (Figure 1A'). As stated earlier, the macrophage population transitioned from large, elongated F4/80^{Hi} cells at E13.5, to include both F4/80^{Hi} and small rounded F4/80^{Dim} macrophages at E15.5 and PND0 (Supplementary Figure 2). This difference in the F4/80 marker level was aligned with qRT-PCR data; despite a significant increase in macrophage number from E13.5 to PND0, the *Adgre1* transcript level (encoding F4/80) did not (Figure 1A'). The proportion of macrophages relative to the total immune cell number (the F4/80^{+}:CD45^{+} cell ratio) significantly decreased: from 90% at E13.5, to 84% at E15.5, and 65% at PND0 (Figure 1B and B'). On the other hand, between E15.5 to PND0 there was a massive numerical increase in other immune cell types. Detection of Ly6G^{+} cells revealed that more than half (55%) of CD45^{+} cells in the newborn testis were granulocytes (Figure 1C), and, as discussed below, were most likely neutrophils based on their appearance. The overlap between CD45^{+}/F4/80^{+} cells (65%) and CD45^{+}/Ly6G^{+} cells (55%) could be explained by a small proportion of small rounded CD45^{+} cells which expressed both F4/80 and Ly6G markers.

Changing ratios between germ and immune cell populations

Both the density of DDX4^{+} cells (germ cells) and levels of the *Ddx4* transcript decreased from E13.5 to PND0 (Figure1D and 1D´). The decreasing ratio of DDX4^{+}

: F4/80^{+} cells reflects the increasing density of macrophages and decreasing density of germ cells during this interval (Figure 1E). The transiently elevated ratio of DDX4^{+}: Ly6G^{+} cells at E15.5 arises from the inverse relationship in the density of each population during this interval (Figure 1F). Since neutrophils are absent to rare at E13.5, no calculation was performed at this timepoint.

A marked redistribution of macrophages from the testis perimeter to interior

The distribution of testicular macrophages changed markedly from E13.5 to PND0, from the perimeter into the interior of each testis section. At E13.5, 90% of F4/80^{+} cells were in the perimeter region, compared to only 26% by PND0 (Table 1 and Supplementary Figure S4).

Macrophages were also frequently observed attached to the outer layer of the testis capsule or amongst the mesothelial cells of the coelomic epithelium in the perimeter area; this was particularly evident at E13.5 and E15.5 (Figure 2).

Co-localisation of immune cells and germ cells

The appearance of close contacts between non-macrophage cells, identified as CD45^{+}/F4/80^{-} and CD45^{-}/F4/80^{-} cells, and macrophages (CD45^{+}/F4/80^{+}) was a consistent observation, with 2 to 6 cells typically identified within an individual cluster, and between 2 and 6 clusters present in each section from E15.5 and PND0 samples (Figure 3). Polymorphonuclear cells (PMNs) were detected within some clusters (Figure 3B, 3C) located in interstitial areas or around cords. The CD45 signal was dim on large and elongated F4/80^{+} cells (Figure 3D-F). The low quantity mean levels during fetal testis development of *Ptprc*, encoding CD45, compared to *Adgre1*, encoding F4/80, may reflect the low level of CD45 on F4/80^{Hi} cells (Figure 1A').

The broad distribution of F4/80^{+} cells was variously documented inside the lamina propria, adjacent to cords, inside cords and in close contact with germ cells (Figures 4 and 5). Macrophages were observed in contact with germ cells inside cords at E13.5 and E15.5 (and rarely at PND0), with between 1 and 5 in every section (Figure 4A); this suggests close contact of macrophages and germ cells inside cords may be a common phenomenon during this period of development (Supplementary Figure S5). At E13.5, apparent macrophage-engulfed germ cells were only rarely observed (between 0 and 1 per section), and these were typically close to the testis border with the mesonephros (Figure 4B1). Macrophages inside cords were observed both close

to the basement membrane and in the cord centre at E13.5 (Figure 5A2-A4 and Figure S5; between and 2 and 5 per section). Similarly, at E15.5, macrophages were in contact with germ cells near the basement membrane of cords and inside cords (Figure 5A; between and 1 and 3 per section). At PND0, there were few macrophages inside cords (Figure 5B; between and 1 and 2 per section). Whether the F4/80^{+} cells contacting the basement membrane at E13.5 and E15.5 are in transit between the cords and interstitium, and whether they form a stable population around the testis cords is unknown.

CD206^{+} macrophages are predominant at E13.5, but not at PND0

The predominance of CD206^{+} macrophages cells in the early fetal mouse testis up to E13.5 has been reported (DeFalco et al., 2014). To examine their relative frequency and position in later stages of fetal testis development, F4/80^{+}/CD206^{+} and F4/80^{+}/CD206^{-} cells were documented (Table 2). At E13.5, 81% of F4/80^{+} cells were CD206^{+}, while at PND0, this value had declined to half (49%). However, the absolute number of CD206^{+} macrophages per section increased during this interval. All F4/80^{+} cells inside cords at E13.5 were CD206^{+} (Figure 6A), at E15.5, both F4/80^{+}/CD206^{-} and F4/80^{+}/CD206^{+} were seen (Figure 6B), while at PND0, macrophages inside cords were CD206^{-} (Figure 6C-D). Therefore, at PND0, F4/80^{+}/CD206^{+} macrophages were detected across the tissue section, except for inside cords. The majority of macrophages located adjacent to cords were F4/80^{+}/CD206^{+} (Figure 6E-F). Interstitial macrophages appeared either as large and elongated F4/80^{Hi}/CD206^{+} or small, rounded F4/80^{Dim}/CD206$^{Dim/-}$ subtypes (Figure 6G).

A variety of macrophage cell-cell contacts were frequently observed in the PND0 testis; this including contact between two CD206 positive macrophages, one CD206 positive macrophage and one negative, and a cluster of CD206 positive and/or negative macrophages (Supplementary Figure S6A, Figures 6G and H). Most CD206^{+} macrophages had an elongated, rather than rounded shape (Supplementary Figure S6). The most common macrophage population in the perimeter area and in the mesonephros/epididymis of fetal and newborn testes was F4/80^{+}/CD206^{+} (Supplementary Figures S6C and S7A and B). *Cd206* transcript levels elevated modestly (1.9-fold) from E13.5 to PND0 (Figure 7A).

Absence of MHC class II^{+} macrophages in fetal and newborn testis

The transcript encoding MHC class II *(H2-eb1)* increased 3.9-fold from E13.5 to PND0 (Figure 7B). Counting of IF sections revealed that F4/80^{+}/MHCII^{+} cells were absent to rare at E13.5 but detectable by E15.5 (0-3 per section) and significantly increased by PND0 (8-16 per section). *H2-eb1* transcript (encoding MHCII) levels roughly doubled from each 13.5 to E15.5 and from E15.5 to PND0 (1.9-fold) (Figure 7B, C and Table 2). Based on size, the population of F4/80^{-}/MHCII^{+} cells could be divided into less than or equal to 10 µm, or greater than 10 µm diameter (Figure 7D and Supplementary, Figure S7C). Contact between F4/80^{-}/MHCII^{+} cells with one or more F4/80^{+} cells was a common observation at PND0 (Figure 7E,7F). Also, we identified clusters of large F4/80^{Hi}/CD206^{+}, small F4/80^{Dim}/CD206^{-} and F4/80^{-}/MHCII^{+} cells (Figure 7G). Almost all F4/80^{+} cells from E13.5 to PND0 were MHCII^{-}, however, we detected extremely rare and infrequent (0-1 per section) F4/80^{+}/CD206^{+}/MHCII^{+} cells at PND0 (Figure 7H).

CD3^{+} cells populate the newborn testis

Because almost the half the population of CD45^{+} cells in the PND0 testis were not F4/80^{+}, we reasoned that immune cell types other than macrophages must populate the fetal and newborn testis. As the rare detection of a CD3^{+} cell in the E12.5 mouse testis was previously reported (DeFalco et al. 2014), we addressed the possibility that T cells may be contributing the changing immune cell density. By immunofluorescence, the commonly used pan-T cell marker, CD3^{+}, and its encoding transcript were not detected at E13.5, although a few cells were detectable (0-1 cell/section) at E15.5 (Supplementary, Figure S3). In the newborn testis, however, CD3^{+} cells were present in the perimeter region, interstitium, and positioned adjacent to cords (Figure 8A). Contact between one or two CD3^{+} cells and F4/80^{+}, appearing as a cluster, was a frequent observation at PND0 (2 to 4 clusters/ section) (Figure 8B and 8C). CD3^{+} cells were occasionally found adjacent to the cord membrane (Figure 8D).

Ly6G^{+} cell density increases dramatically through fetal testis development

At the first stage of this investigation, we checked for the presence of neutrophils using anti-MRP14/S100A9, which marks both neutrophils and monocytes (Wang et al. 2021), and observed positive cells with high frequency in the newborn testis. We then

examined the potential presence of neutrophils, identified here as Ly6G$^+$ cells, as this cell type is commonly linked to tissue remodelling. The highly-specific Ly6G marker recognises all mouse neutrophil subtypes, but has also been reported on some macrophages (Wang et al. 2021). Their frequency was very low at E13.5 (0-3 Ly6G$^+$ cells per section) and had increased substantially by E15.5 (27-67 per section), (Figure 1A and A'). The Ly6G$^+$ population increased even further, to represent more than half of the CD45$^+$ cells, at PND0 (80-100 per section; Figure 1C). Ly6G$^+$ cells were detected in the interstitium, and were both next to and inside cords, but they were rare to absent in the perimeter region. The distribution of the increasing number of these cells at E15.5 and PND0 appeared asymmetric across the section; they were predominantly detected in the section interior and were rarely in the perimeter (Supplementary data Figure S8). This contrasts with the macrophage distribution, which shifts from the perimeter to the interior between E13.5 and PND0 (Table 1). Ly6G$^+$ cells were frequently noted inside some cords adjacent to germ cells only at PND0 (Figure 9). Ly6G$^+$ cell co-localisation with macrophages (F4/80$^+$) and/or with other immune cells (CD45$^+$ cells lacking either F4/80 or Ly6G), including as clusters adjacent to cords and/or in interstitial areas was also regularly observed (Figure 10). Some Ly6G$^+$ cells in the fetal testis showed a dim F4/80 signal. This was documented using two independent antibodies, one which was unconjugated (in-house) and the other directly conjugated (commercial), leading to our conclusion that two distinct Ly6G$^+$ cell populations are present, one lacking F4/80, and one with a low level of this protein (Figure 10F). The presence of more than 50 Ly6G+ cells per section in the newborn testis coincided with the robust elevation of *Lyg6* between E15.5 and PND0, signifying a clear answer to the question of what cell type became prevalent alongside macrophages at this age. This increase around the time of birth was followed by a significant drop in their number afterwards, with 5-10 cells per section identified at day 2, 3-5 cells at day 7 and 0-1 from day 10 to adulthood (Figure 11).

2.1.6. Discussion

Although the importance of macrophages for the initial stages of testis cord formation in the fetal testis is widely accepted, there is a gap in understanding which other immune cells contribute to testis growth after the cords first form up to shortly after birth, when spermatogenesis is initiated. In addition, macrophage origins and roles in postnatal testis growth and ongoing adult spermatogenesis in homeostatic

conditions remain key discussion points in the field (DeFalco et al. 2014; Mossadegh-Keller et al. 2017; Mossadegh-Keller and Sieweke, 2018; Lokka et al. 2020; Wang et al. 2021). The present study extends outcomes from previous investigations of fetal testis macrophages by examining their phenotype and distribution in the interval following sex determination through birth in the mouse testis. The documentation of $CD3^+$ and $Ly6G^+$ cell emergence is an important finding which highlights the dynamic nature of immune cell subpopulations in the fetal testis, revealing their close association with germ cells. The potential presence of B cells in the fetal mouse testis at E13.5 was examined previously using the B220 marker in sections, and no B cells were detected (DeFalco et al. 2014). We also checked for B cells using CD19 as a marker by IF, and no positive cells were identified at any age (data not shown). An important caveat to this study is the well-documented heterogeneity of each immune cell population, including macrophages, granulocytes and T cells, so that the detection of a single marker must be interpreted with caution. For example, T cell lineage markers have been reported on macrophages and other myeloid cells (Chávez-Galán et al. 2015), and Ly6G is found on some macrophages (Wang et al. 2021) in experiments using Flow cytometry. However, histological evaluation of the cells expressing each of these markers remains an effective approach to providing cell type identification.

The changing landscape of immune cell subpopulations in the fetal mouse testis

Quantitative analysis of sections examined using indirect immunofluorescence documented the increasing density of immune cells ($CD45^+$), highlighting changes in the relative contributions of macrophages ($F4/80^+$), T cells ($CD3^+$) and neutrophils ($Ly6G^+$) (Figure 1). While each subpopulation increases significantly in density between E13.5 and PND0, indicating their numerical elevation, the proportion of $F4/80^+$:$CD45^+$ cells decreased significantly, while $Ly6G^+$ cells were rare to absent at E13.5. Thus, while our E13.5 data agree with the earlier report that 90% of $CD45^+$ cells in the E12.5 testis are macrophages (DeFalco et al. 2014), it was surprising that, by PND0, about half of $CD45^+$ cells in the testis were $Ly6G^+$. The detection of both $Ly6G^+$ and $CD3^+$ cells in the newborn testis indicates that the immune milieu is altered dramatically by the time when spermatogenesis commences, shortly after birth.

Two phenotypes of fetal testicular macrophages

Based on a previous ontological investigation, F4/80^{Hi} macrophages detectable before E14.5 are known to be yolk-sac-derived, while F4/80^{Int} are fetal liver-derived and emerge by E16.5 (Lokka et al. 2020). In agreement with this, we detected only elongated F4/80^{Hi} macrophages at E13.5, while at E15.5 and PND0, both elongated F4/80^{Hi} and small rounded F4/80^{Dim} macrophages were noted. The dim expression of CD45 on testicular macrophages at E13.5 is consistent with previous reports (DeFalco et al. 2014; Lokka et al. 2020), while higher levels were observed at E15.5 and PND0. Adult kidney and microglia also contain two CD45^{+} macrophage subpopulations, with "intermediate" and "high" signals, which are proposed to have different origins (Lee et al. 2018; Rangaraju et al. 2018). The low level of CD45 on testicular macrophages during fetal life indicates this is not an appropriate marker for first gating in flow cytometry or cell sorting techniques to study testicular macrophages at these developmental stages. In contrast CD45 on Ly6G^{+} cells which appeared to be neutrophils was present at high and readily detectable levels.

The previous report of fetal testicular macrophages concentrated near the gonad–mesonephros boundary at E10.5 and E13.5 demonstrated that a substantial proportion of macrophages originate and enter the testis from the mesonephros (DeFalco et al. 2014). We also observed macrophages at E13.5 and E15.5 on the mesonephros side of the testis, and note that they frequently appeared to be attached to and crossing through the capsule. Intriguingly, these later profiles appeared highly similar to leukocyte extravasation (Muller, 2013). The subsequent redistribution of macrophages from perimeter to the interior region of the testis contrasts with the localisation of neutrophils. Ly6G^{+} cells are rare at E13.5 but increased from E15.5 onwards; they appeared asymmetrically distributed across the fetal testis sections at E15.5. Also, the lack of macrophages detected inside the lumen of blood vessels at E13.5, E15.5 and PND0 contrasts with the frequent observation of Ly6G^{+} cells inside vessels, particularly at PND0, a distinction that merits further careful analysis.

While confirming the previous report of F4/80^{+} polymorphonuclear cells in the fetal and newborn testis (Lokka et al. 2020), we also identified some Ly6G^{+} granulocytic cells, most likely neutrophils, that were F4/80^{+}. In agreement with previous investigations, no MHC class II^{+} macrophages were detected in the fetal testis, with only rare CD206^{+}/MHCII^{+} macrophages (0-1 per section) at PND0 (Mossadegh-Keller et al., 2017; Lokka et al. 2020; Wang et al. 2021). The main population of E13.5

macrophages, consisting of large, elongated, $CD45^{Dim}/F4/80^{Hi}/CD206^{+}/MHCII^{-}$ cells, became less prominent with the emergence of smaller, rounded $CD45^{Int}/F4/80^{Dim}/CD206^{-}/MHCII^{-}$ cells at E15.5. This may reflect the imperative for a shift from a predominantly immunosuppressive macrophage population towards one that includes more immune-reactive macrophages at birth. These findings consolidate the understanding that there are at least two distinct populations of macrophages in newborn mouse testis, as well as several other immune cell types.

Cell-cell interactions of immune cells in the fetal testis

The full functional significance of the close localisation and apparent contact between different immune cell subsets is unknown, but may include roles in fetal testis morphogenesis, immune regulation, and germ cell removal and development, all of which are integral to the foundations of male fertility. The phagocytosis of germ and Sertoli cells by macrophages in E11.5 and E12.5 mouse testes was previously demonstrated (DeFalco et al. 2014), and we also observed germ cells outside the cords apparently engulfed by macrophages at the mesonephro-testis border at E13.5. Also consistent with the report of macrophages inside testis cords at E11.5 and E12.5 (DeFalco et al. 2014), and in accord with the detection of macrophages inside cords up to two weeks after birth (Lokka et al. 2020), we found macrophages inside cords, but at a declining incidence from E13.5 to birth. In contrast, Ly6G+ cells were observed inside cords, but only at PND0. We also observed immune cells in close contact with other immune cell types from E13.5 to PND0, with between 4 and 10 immune cell clusters in each section, appearing more frequently with advanced age development. While the functional significance of these contacts is unknown, we speculate macrophages may process germ cell antigens that are subsequently exposed to regulatory T cells for induction and maintenance of immune tolerance during adult life (Tung et al. 2017; Fijak et al., 2018; O'Donnell et al. 2021) or they may produce factors, such as activins or CSF1, that influence germ and/or Sertoli cell development, as identified for the adult mouse testis (DeFalco et al. 2015). Based on others' observations from early fetal testis development (DeFalco et al. 2014; Mossadegh-Keller et al, 2017), and longstanding evidence of close structural and functional relationships between macrophages and Leydig cells in postnatal testes (Hutson, 1992; Gaytan et al. 1994; Fadel and Sarzotti, 2000) we knowledge that the important interactions between these immune cells and the developing Leydig cells

and vasculature may be highly relevant to identifying points of vulnerability that affect postnatal testis growth and function.

Testicular CD3$^+$ cells

We observed few CD3$^+$ cells at E15.5, aligned with the report of DeFalco and colleagues (DeFalco et al. 2014). This was unsurprising because T cell development occurs late in fetal life, while the first thymic T cells are detectable in murine lymph nodes at E18–E20 (Fadel and Sarzotti, 2000). Several T cells in the PND0 testis appeared to contact macrophages. However, the interaction of macrophages and T cells is not likely to be through MHC class II (*H2-Eb1*) because almost all macrophages at PND0 appeared to lack this marker. Most of the small number of MHCII$^+$ cells in PND0 testes were F4/80$^-$, indicating either that another antigen presenting molecule is present on testicular macrophages, or that antigen presentation through MHCII is not the function of T cell-macrophage interactions at PND0. The finding of T cells in the fetal testis both adjacent to cords and in interstitial regions at PND0 suggests that similar to adult macrophages (DeFalco et al. 2015; Mossadegh-Keller et al. 2017), there are at least two functionally distinct subpopulations.

Ly6G$^+$ cells in the fetal testis

Neutrophils constitute a key Ly6G$^+$ cell population that plays critical roles in antibacterial immune responses, inducing inflammation, regulation of the hematopoietic niche, and angiogenesis, but their role in morphogenesis during development is much less well-defined (McGrath et al. 2015; Christoffersson and Phillipson et al. 2018; Casanova-Acebes et al. 2018; Carnevale et al., 2020) An early report indicated that neutrophils are not detected in the adult rodent testis under normal condition but can enter from the blood supply in pathophysiological conditions (O'Bryan et al. 2000). Neutrophils have reported in mouse and rat testes with infection or testicular torsion, including in ischaemia–reperfusion models (Lysiak et al. 2001, 2004; Celebi and Paul, 2008; Michel et al. 2016; Fijak et al. 2018) and in human testicular tumors (Akhtar et al. 1996), all relating to the adult testis.

In this report we have made the remarkable identification of Ly6G$^+$ cells as the second-most frequent immune cell population in the newborn mouse testis. The only previous evaluation of neutrophils in the fetal mouse testis, conducted using flow cytometry, identified 9% of CD45$^+$ cells as neutrophils (Gr-1+ cells) at E12.5 samples (DeFalco

et al. 2014), while our analysis of tissue sections identified neutrophils as rare to absent at E13.5 (0-3 cells per section). The difference between these two observations may reflect the sensitivity of these distinct approaches. Murine fetal neutrophils are present in both the liver and bloodstream by E11.5 and expand rapidly in both compartments; neutrophil phenotypes in the E12.5 liver are consistent with early through late neutrophil maturation stage phenotypes. The initiation of haematopoiesis in the bone marrow at E18.5 (Sugiyama et al. 2011) fits with the present finding that the Ly6G$^+$ population increases in the mouse testis to compromise more than half of the CD45$^+$ population by birth. The remarkable drop in Ly6G$^+$ cell prevalence in the testis shortly after birth, and their near absence from the adult mouse testis, provide a strong indication that these cells serve a highly specific role in a particular stage of testis development. The abundance of Ly6G$^+$ cells in this period of late fetal testis development and at the onset of spermatogenesis coincides with the movement of germ cells from the cord centre to the perimeter, as they transform from gonocytes into spermatogonia. Their function may be related to a transient modification of the peri-cord environment or perhaps even the Sertoli cells, aligned with their known role in tissue remodelling (Peiseler and Kubes, 2019).

In contrast to the finding that macrophages are frequent near the mesonephros at E13.5 and E15.5, Ly6G$^+$ cells are mostly absent from this location. Their predominant location in the section interior, close to and inside vessels from E15.5 onwards, may reflect a role in vascularisation of the fetal testis, essential for normal testis development, including formation of fetal Leydig cells, smooth muscle cells and pericytes (Lysiak et al., 2001; Coveney et al. 2008; Kumar and DeFalco, 2018). It will be important to consider their mechanism of recruitment to the developing testis, particularly as signalling molecules such as CXCL12, which recruits and maintains CXCR4$^+$ neutrophils in lung (Carnevale et al., 2020), are also central to early germline development (Heckmann et al. 2018). A signalling link, such as the common potential for germ cells and neutrophils to respond to the Sertoli cell-derived products including CXCL12 and activin A, may explain why we observed neutrophils and germ cells co-located within the fetal testis.

Neutrophils have been increasingly identified as being heterogeneous in both structure and function, with a capacity for functional plasticity, and are predominantly reported in relationship to adult pathologies (Christoffersson and Phillipson, 2018; Carnevale et al. 2020). Changes in neutrophil maturation status are distinguished by

their cell and nuclear shape (Hong, 2017). Here we make the intriguing observation of Ly6G$^+$ cells with two distinct phenotypes: those with round, kidney-shaped and segmented nuclei were detected at E15.5, while at PND0 the dominant population was that with segmented nuclei. Based on their phenotypes, the Ly6G$^+$ cells appeared to represent early- and mid-developmental neutrophil stages at E15.5 and the late differentiated stage at PND0. The application of additional markers will be required to track the developmental and functional trajectory of these cells within the nascent testis.

We observed further evidence of Ly6G$^+$ cell heterogeneity in the early testis, indicating there are at least two populations: one clearly F4/80$^+$ positive and one negative/dim for F4/80. While the transcript encoding F4/80, *Adgre1*, is detectable in murine neutrophils, the protein is not normally expressed on the cell surface (Sasmono et a. 2007). However, purified Ly6G$^+$ cells express CSF-1R after overnight culture and can subsequently differentiate to form F4/80$^+$ macrophages in response to CSF-1 (Sasmono et al., 2007). It will be exciting to learn how the complex milieu of the developing testis may attract and instruct granulocyte maturation and function.

In conclusion, this study documents for the first time the progressive emergence of neutrophils and T cells, the frequency and localisation of macrophages, T cells, neutrophils and their association with germ cells in the developing fetal testis. The findings from this study are summarised in alignment with the timing of haematopoiesis in the murine yolk sac, fetal liver and bone marrow (Mikkola and Orkin, 2006; Hoeffel and Ginhoux, 2015; Mevel et al., 2019) (Figure 12). In particular, it establishes an understanding of the changing proportions of germline and immune cells that occur during fetal testis development in the mouse. Our understanding of the nature of the interactions between germline and immune cells is in its infancy. The production of Csf1 by macrophages as a modulator of spermatogonial fate (DeFalco et al, 2014) and IL-6 by seminoma cells (testicular germ cell tumours; Klein et all, 2016) are two examples of the potential cross-talk mediators between testicular immune and germ cells in different physiological states. The capacity to identify potential reciprocal signalling pathways by inspecting data from single cell RNA sequencing analyses offers a new strategy to generate hypotheses about the functional significance of the co-location of these cells in the highly dynamic fetal testis. By revealing the frequent, close cellular contacts between macrophages, T cells and neutrophils during testis development, we provide information that will be key to understanding how immune

cells function during development of the fetal testis and influence maturation of the earliest male germ cells. Information gained from this study should be considered in the development of strategies to support in vitro germline growth, for example by adding immune cells into scaffolds or organoids.

Statements and Declarations

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

All authors contributed to the intellectual content of the manuscript and approved the final version to be published. SH performed the experiments, and with KL, analysed the data, prepared the figures, and wrote the manuscript. SH and KL were responsible for the conception and design of the research. SM, SI, MH, KL interpreted the experimental results. SM, SI, DF, H-CS and MH critically reviewed the data and manuscript. All authors have reviewed the data and manuscript.

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Data availability

The data used in this study are available from the corresponding authors upon reasonable request.

Supplementary Material

Supplementary material related to this article can be found, in the online version.

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2.1.7. Figures and Figure Legends:

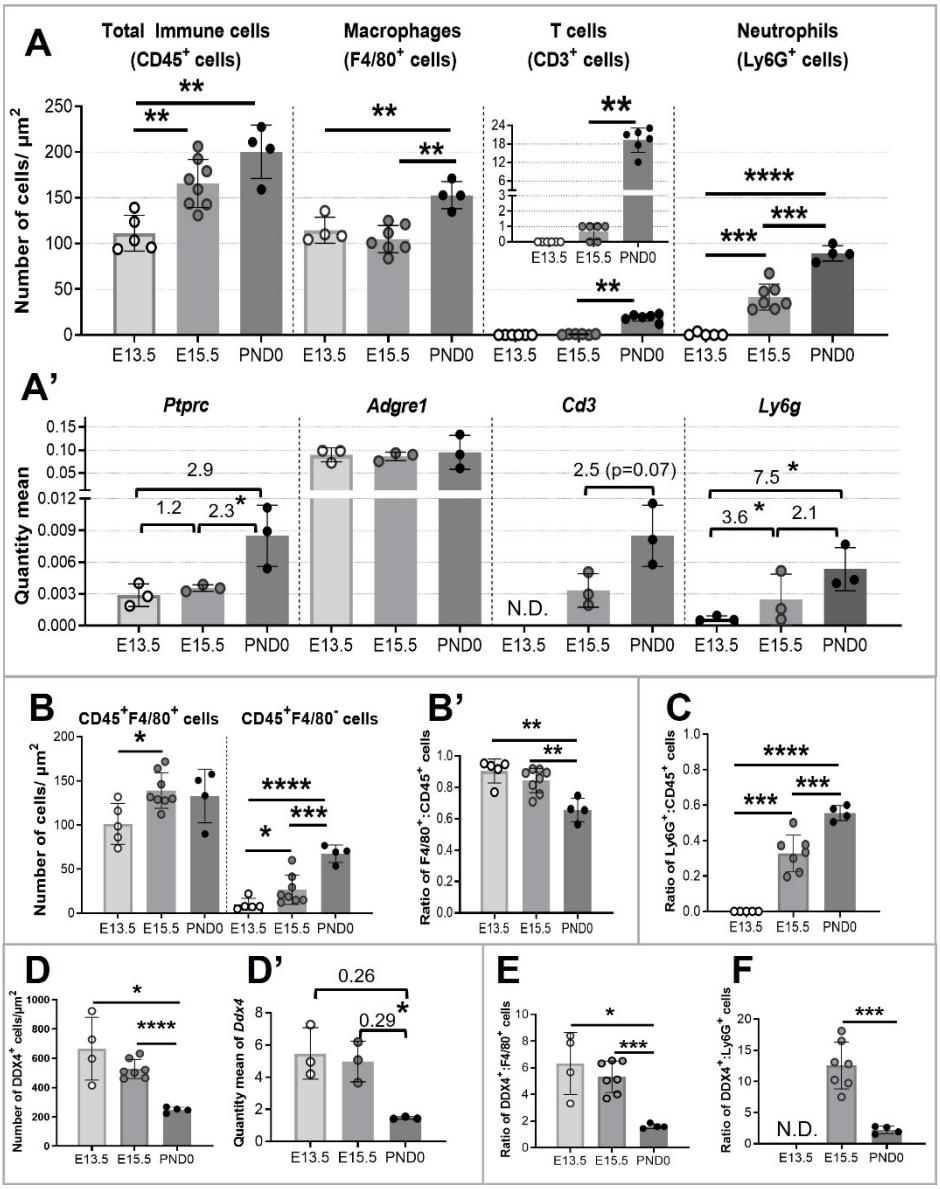


Figure 1: Quantitative changes in immune cell populations in fetal testes, E13.5 to PND0. A: The density of immune cells (CD45$^+$ cells), macrophages (F4/80$^+$ cells), T cells (CD3$^+$ cells) and neutrophils (Ly6G$^+$ cells) in E13.5, E15.5 and PND0 testes determined by counting all cells in sections labeled using immunofluorescence. **A´**: RT-qPCR results reported as quantity mean of transcripts encoding corresponding immune cell markers normalised to RPLP0, with fold-change values between age groups indicated. N.D.: Not detected. **B:** The density of CD45$^+$/F4/80$^+$ (macrophages) and CD45$^+$/F4/80$^-$ cells (non-macrophages). **B´:** The ratio of F4/80$^+$:CD45$^+$ cells. **C:** The ratio of Ly6G$^+$:CD45$^+$ cells. D: The density of germ cells (DDX4$^+$ cells) from E13.5 to PND 0. **D´**: qRT-PCR quantity mean of transcripts encoding germ cell marker *Ddx4* normalised to *Rplp0*, with fold-change values between age groups indicated. **E**: The ratio of DDX4$^+$ to F4/80$^+$. **F**: The ratio of DDX4$^+$ to Ly6G$^+$ cells (neutrophils). Circles show results from individual animals; values represent mean ± SD. Significance determined using 2-tailed unpaired t test. *: $p \leq 0.05$. **: $p \leq 0.01$. ***: $p \leq 0.001$. ****: $p \leq 0.0001$.

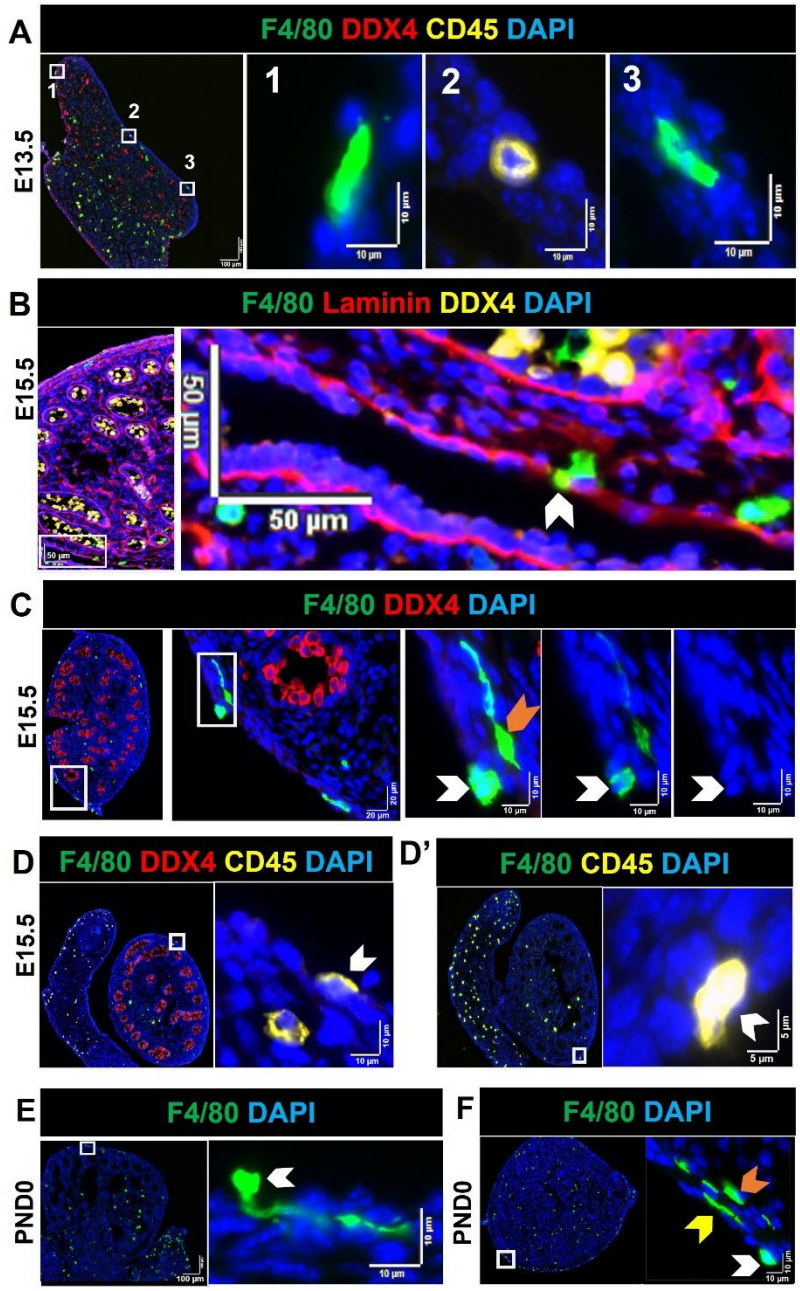


Figure 2. Immune cells attached to the outer layer of the testis capsule and amongst the mesothelial cells of the coelomic epithelium (in the perimeter region), visualised by immunofluorescence. Lower magnification images on left, with white boxes indicating regions shown in high magnification on right hand panels; dimensions as indicated. **A:** Whole E13.5 testis section **(1)** F4/80^{+} cell attached to testis capsule outer layer. **(2)** CD45^{+}/F4/80^{-} cell amongst mesothelial cells. **(3)** F4/80^{+} cell inside the capsule, amongst mesothelial cells. **B**: F4/80^{+} cell embedded in the capsule lamina (white arrow). **C:** F4/80^{+} cells attached to the capsule outer layer (white arrows) and in the perimeter (orange arrow). **D** and **D'** CD45^{+}/F4/80^{-} cells attached to the outer layer of capsule (white arrows). **E:** In PND0 testis section, F4/80^{+} cell attached to the capsule outer layer. **F:** At PND0, F4/80^{+} cells attached to the outer capsule layer (white arrow), amongst mesothelial cells (yellow arrow), and in perimeter area (orange arrow). F4/80: pan-macrophage marker, CD45: pan-immune cell marker, DDX4: pan-germ cell marker, laminin: extracellular matrix, DAPI: nuclei. Marker colours are indicated above each panel set.

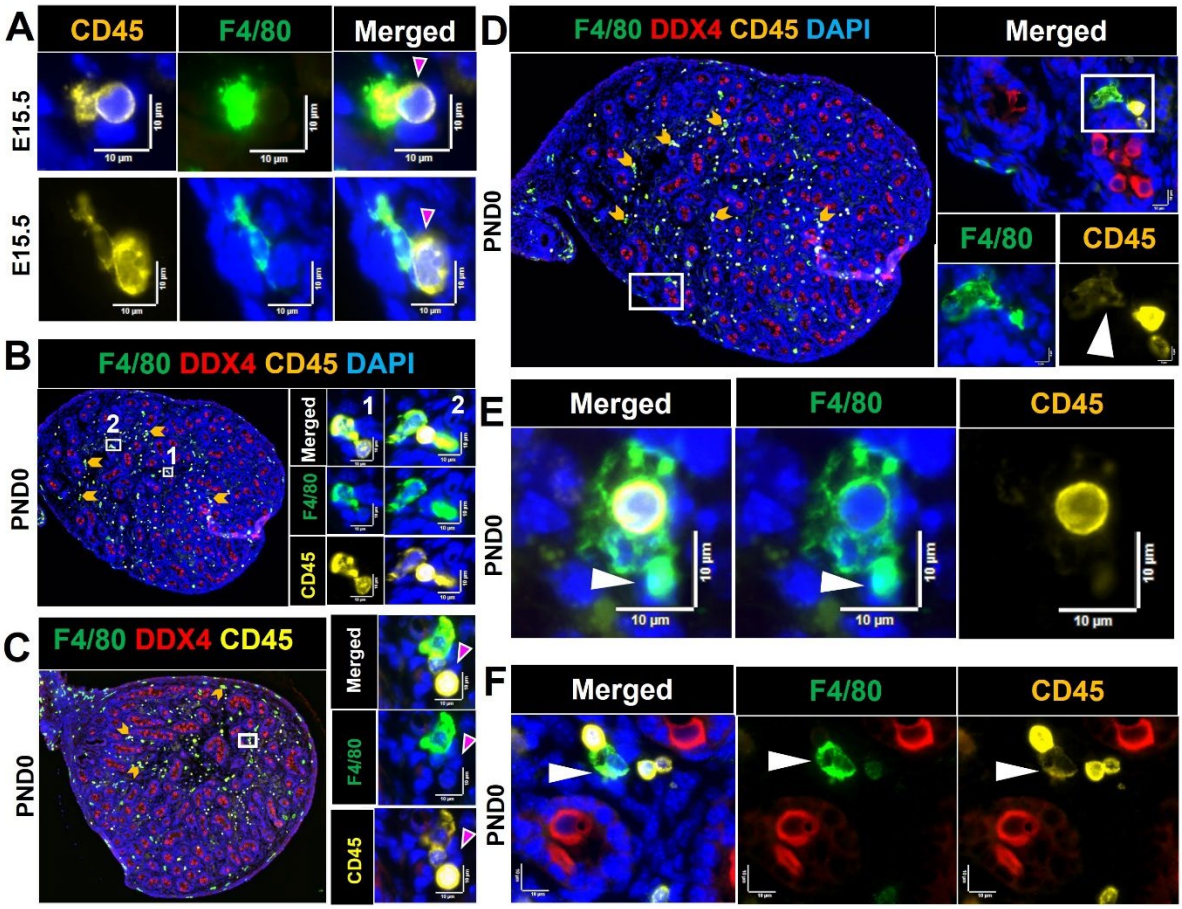


Figure 3. Contacts and co-localisation of macrophages ($CD45^+/F4/80^+$) with immune ($CD45^+/F4/80^-$) and other cells ($CD45^-$) in fetal and newborn mouse testes. A: E15.5 testis. Contact between $CD45^+/F4/80^+$ and $CD45^+/F4/80^-$ cells. Upper panels: $CD45^+/F4/80^-$ cell (pink arrow) contacting $CD45^+/F4/80^+$ cells. **B - F:** PND0 testis. **B:** (1) Co-localisation of a $CD45^+/F4/80^+$ and $CD45^+/F4/80^-$ cell; (2) two $CD45^+/F4/80^+$ cells flanking a $CD45^+/F4/80^-$ cell. **C:** A polymorphonuclear (PMN) $CD45^+$ cell flanked by a $CD45^+/F4/80^+$ and a $CD45^+/F4/80^-$ cell within a cluster of $CD45^-$ cells (e.g., pink arrow). **D:** Co-localisation of a $CD45^+/F4/80^+$, a $CD45^+/F4/80^-$ and a PMN; white arrow indicates the dim level of CD45 on $CD45^+/F4/80^+$ cell. **E:** A $CD45^+/F4/80^-$ cell engulfed by a $CD45^+/F4/80^+$ cell; white arrow indicates the very dim $CD45^+$ signal on a $CD45^+/F4/80^+$. **F:** Contact between a $CD45^+/F4/80^-$ cell with a $CD45^{Dim}F4/80^+$ cell (white arrow). White boxes in low magnification images on the left in B – D correspond to high magnification panels on the right. Marker colours are indicated above each panel set.

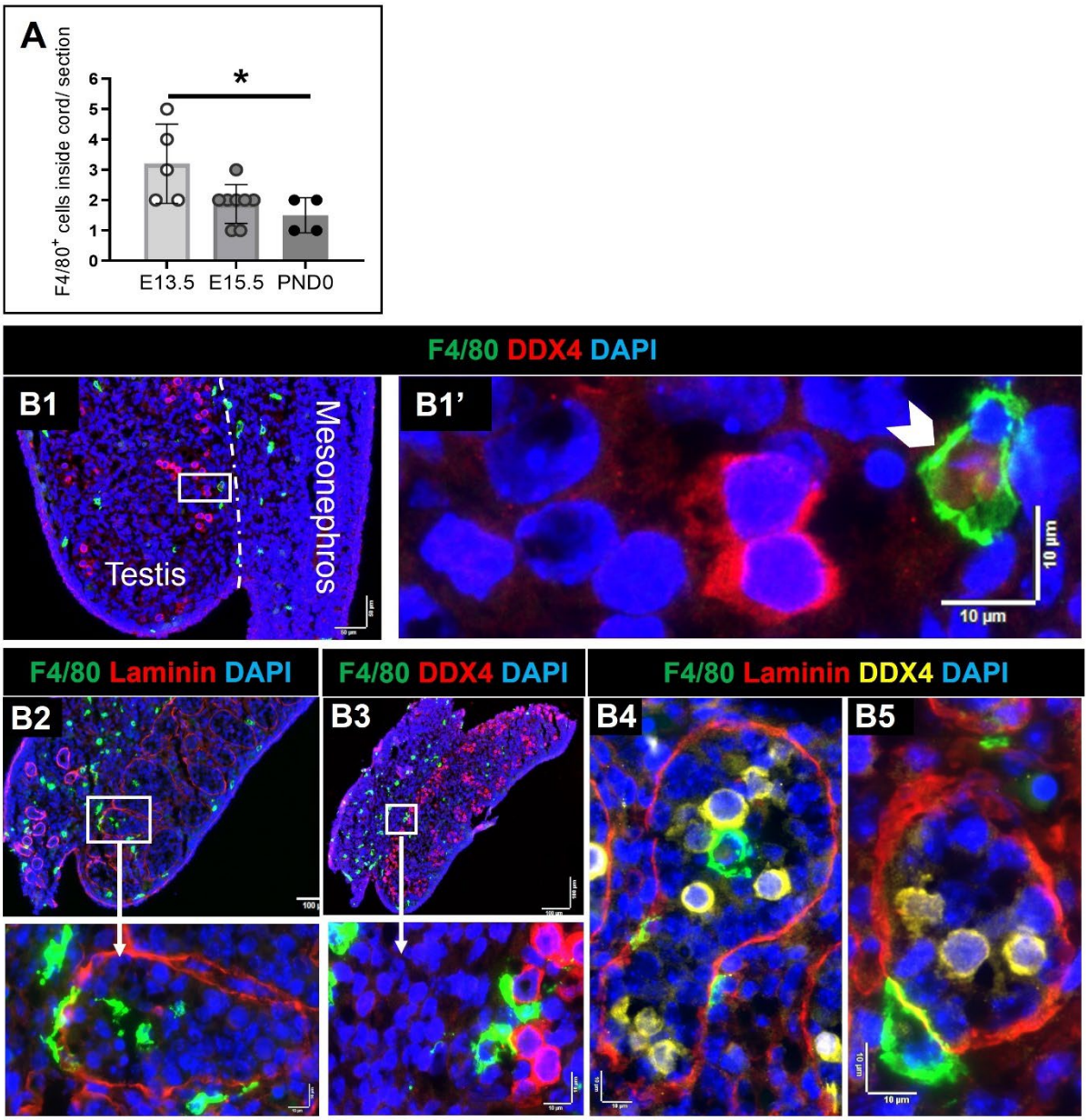


Figure 4. Proximity of macrophages (F4/80⁺) with germ cells (DDX4⁺) and cord basement membrane (laminin) in the E13.5 testis. A: The number of F4/80⁺ cells inside cords per section, from E13.5 to PND0. Each point represents the average of 3 sections per biological sample. Asterisk indicates $p \leq 0.05$. **B1:** Germ cell engulfed by a macrophage on the side of the testis adjacent to the mesonephros (white arrow). **B2 – B4:** Macrophages inside cords. **B5:** Macrophage in close contact with cord basement membrane. White boxes in high magnification panels refer to low magnification images to the right or below). Dotted white line in B1 denotes border between testis and mesonephros. Marker colours indicated above for each panel set.

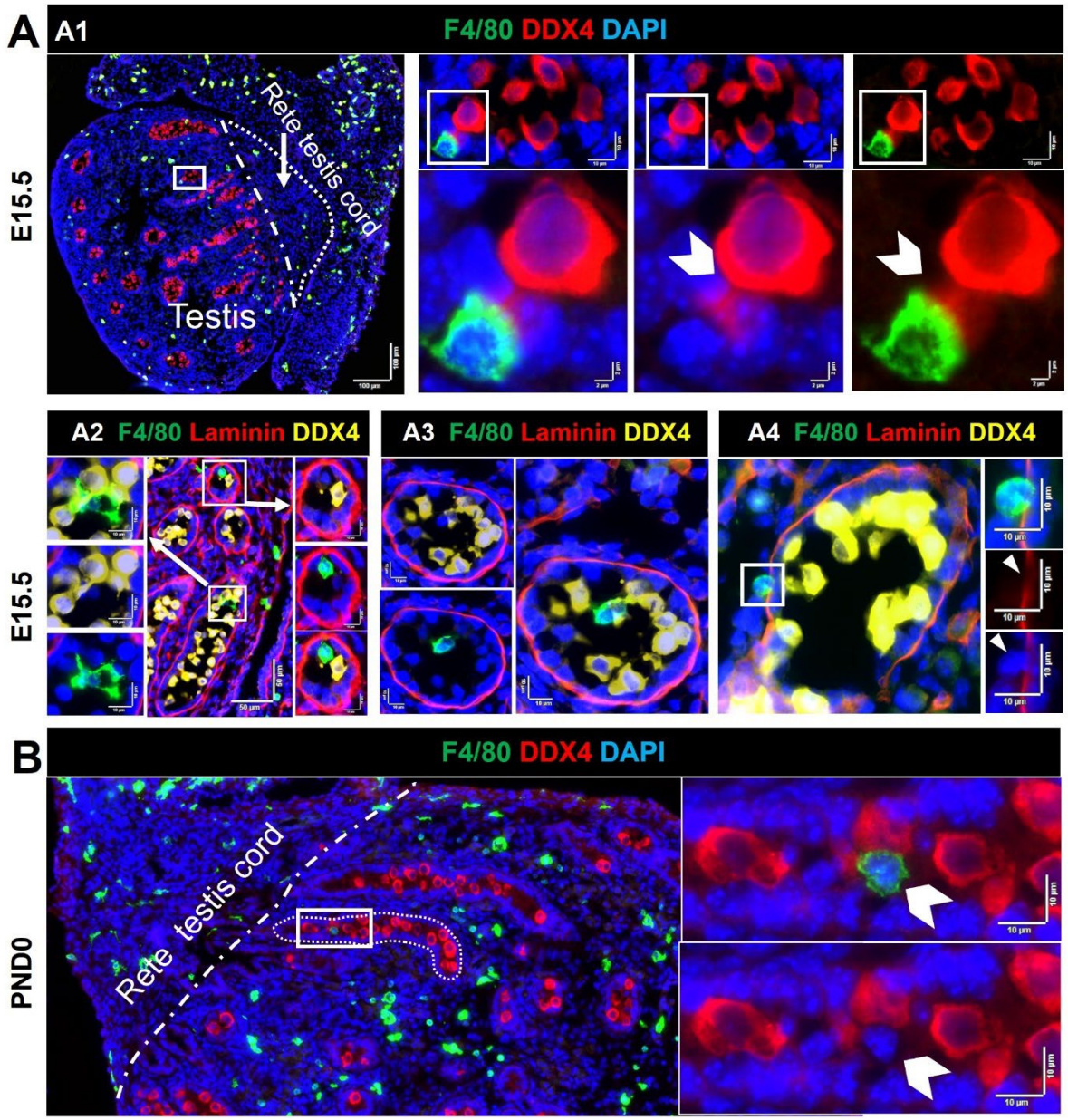


Figure 5. Proximity of macrophages (F4/80^{+}) to germ cells (DDX4^{+}) and cord basement membrane (laminin) in E15.5 and PND0 testes. A1: Germ cell in close contact with a macrophage, on the side of the testis adjacent to the mesonephros (white arrow). **A2 – A3:** Macrophages inside cords. **A4:** A macrophage positioned in the middle of the cord basement membrane (white arrow). **B:** Co-localisation of a macrophage with germ cells in cord centre (white arrow). White boxes in high magnification panels refer to adjacent low magnification images (A1-A3). Dotted white line in A1 and B denotes testis-rete cord testis border. Marker colours are indicated above each panel set.

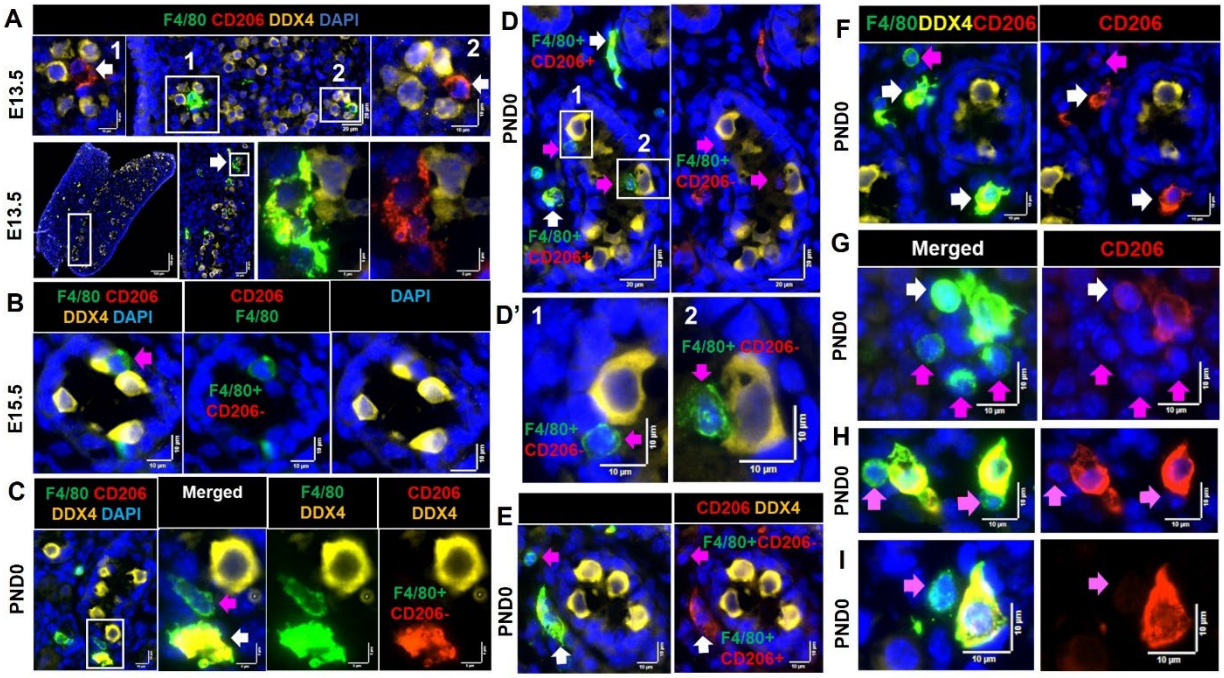


Figure 6. Features of F4/80^{Hi}/CD206^{+} and F4/80$^{Int/Dim}$/CD206^{-} cells in the E13.5 to PND0 mouse testis. A: F4/80^{Hi} cells in close contact with germ cells inside cords were CD206^{+} at E13.5 (white arrows). **B:** Small F4/80^{Int} cells in close contact with germ cells inside cords were CD206^{-} at E15.5 (pink arrows). **C** and **D**: Small F4/80^{Int}/CD206^{-} inside cords *vs* large F4/80^{Hi}/CD206^{+} cells outside cords at PND0. **E** and **F:** A small F4/80^{Int}/CD206^{-} cell in the interestituim (pink arrows) and two large F4/80^{Hi}/CD206^{+} cell in the cord perimeter area (white arrows). **G:** A cluster of F4/80^{Hi}/CD206^{+} cells (white arrow) and small F4/80^{Dim}CD206^{-} cells (pink arrows). **H:** Contact between two large F4/80^{Hi}/CD206^{+} cells with small, rounded F4/80^{Int}/CD206^{-} cells (pink arrows). **I**: Co-localisation a small F4/80^{Int}/CD206^{-} and a large F4/80^{Hi}/CD206^{+} cell. Marker colours are indicated above each panel set. White boxes on low magnification images refer to the high magnification panels.

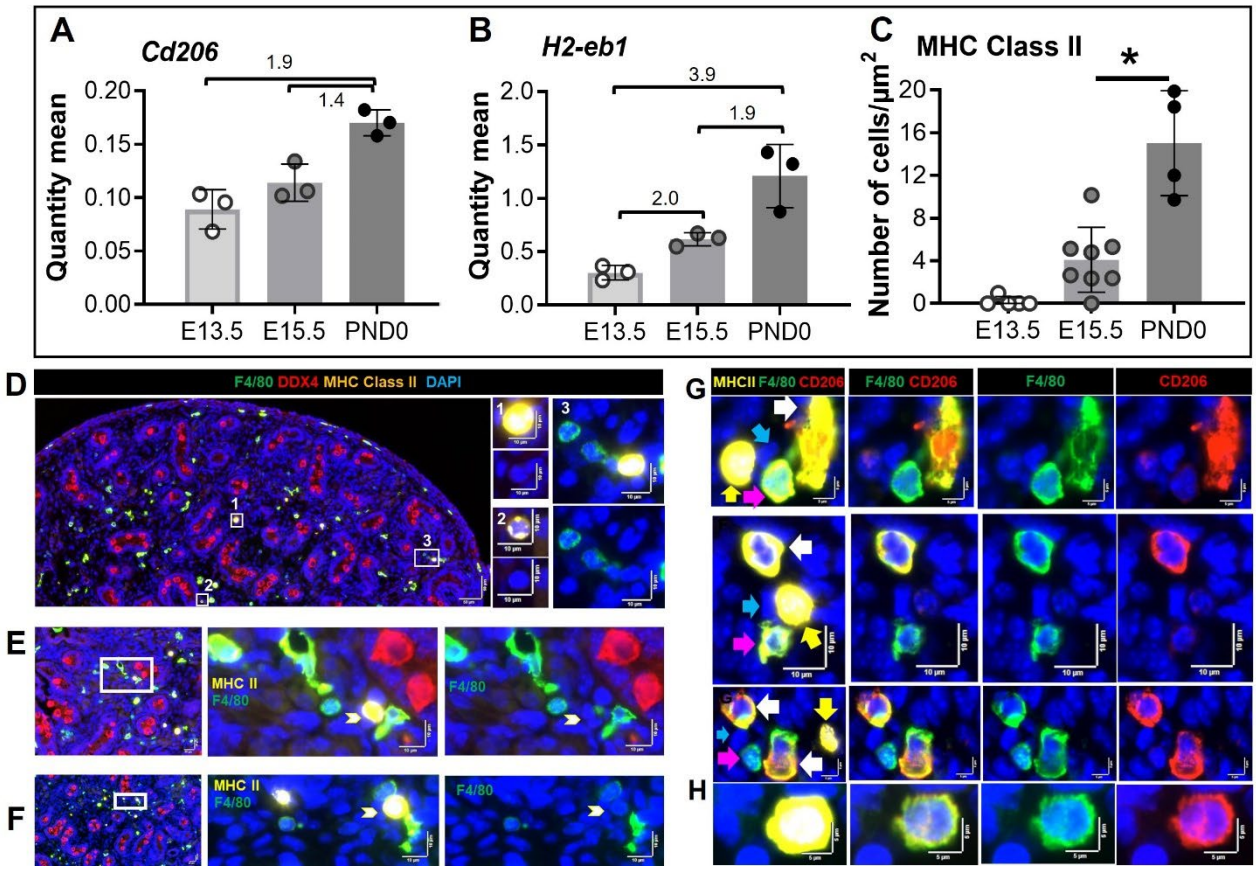


Figure 7. Features of MHC Class II⁺ cells in the newborn (PND0) mouse testis. A: *Cd206* transcript levels. **B:** *H2-eb1* (encoding MHC class II) transcript levels. RT-qPCR results reported as quantity mean of transcripts normalised to RPLP0; fold-change values between age groups indicated. **C:** The density of F4/80⁻/MHCII⁺ cells at each age, from E13.5 to PND0 testis cross sections. **D:** Large (≥10µm) (**1**) or small (≤10µm) F4/80⁻/MHCII⁺ cells (**2** and **3**). **E and F**: Contacts between small F4/80⁻/MHCII⁺ and F4/80⁺/MHCII⁻ cells. **G:** Three examples of clusters of large F4/80^{Hi}/CD206⁺ cells (white arrows), small F4/80^{Int}/CD206⁻ cells (pink arrows) and small F4/80⁻/MHCII⁺ cells (yellow arrows). Additional, unidentified cells present in clusters are indicated (blue arrows). **H**: A rare, single F4/80⁺/CD206⁺/MHCII⁺ cell. In D, E and F: White boxes on low magnification images refer to the high magnification panels. Marker colours are indicated above each panel set.

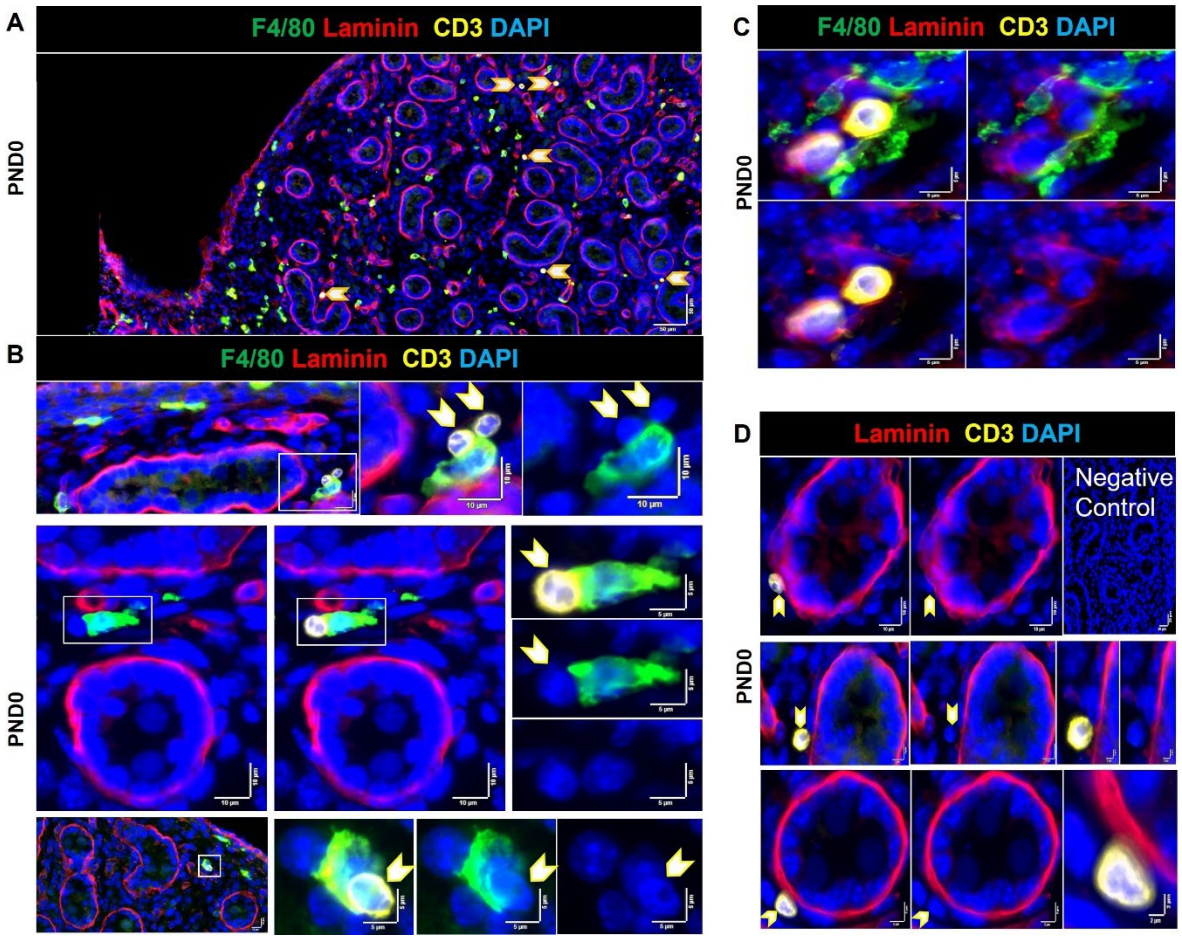


Figure 8. Location and cell-cell interactions of CD3⁺ cells (T cells) in the PND0 mouse testis. A: Testicular T cells at PND0 indicated with arrowheads. **B:** T cells appearing to be dividing. **C:** Co-localisation of macrophage (F4/80⁺) and CD3⁺ cells (arrowheads). White boxes on low magnification images refer to the high magnification panels on the right. **D**: A macrophage surrounding two CD3⁺ cells in close contact with the basement membrane (laminin⁺). Marker colours are indicated above each panel set.

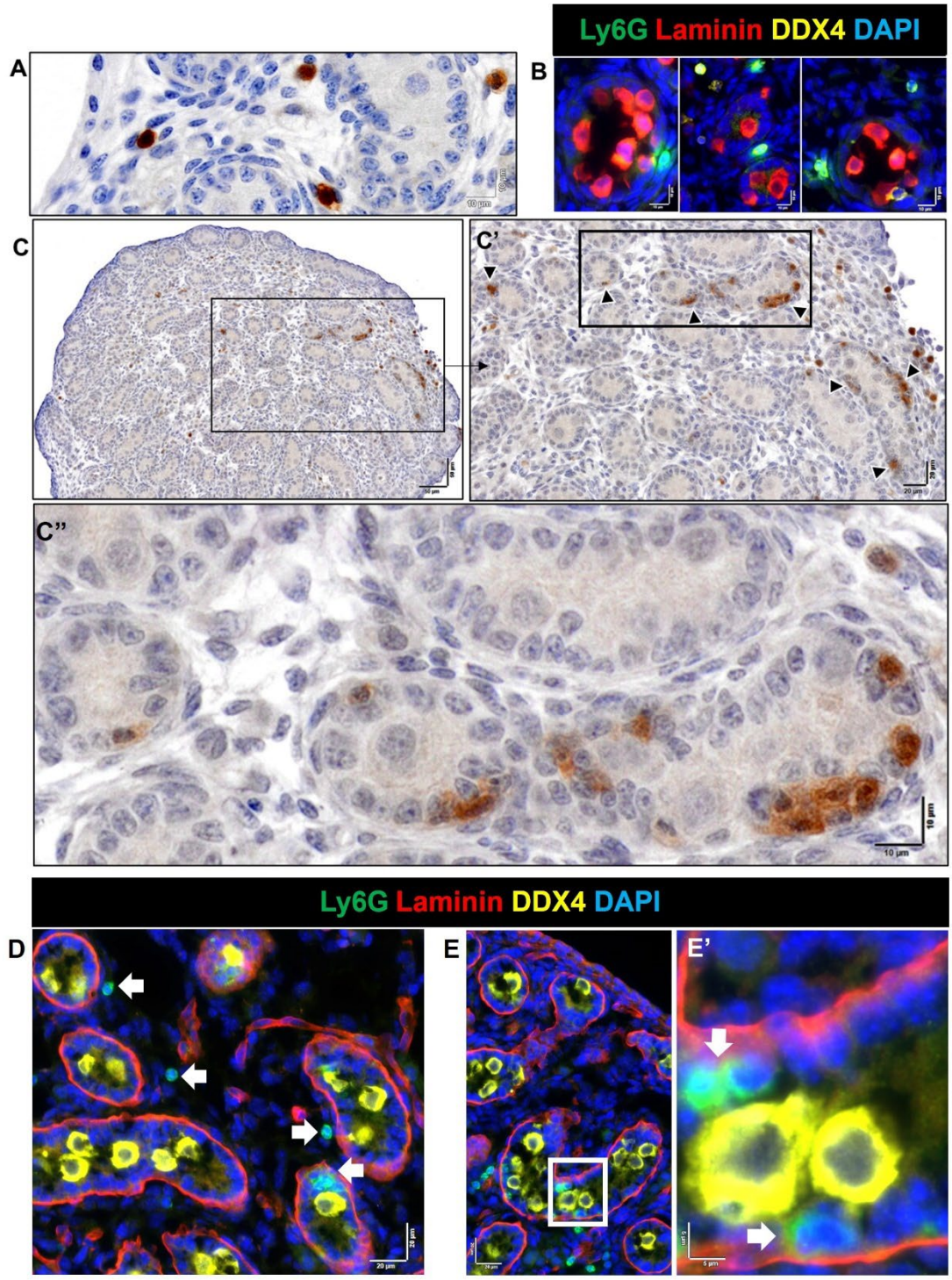


Figure 9. Localisation and cell contacts of Ly6G⁺ cells (neutrophils) in fetal and newborn mouse testes. **A:** Ly6G⁺ cells in the interstitium at PND0. **B:** Ly6G⁺ cells at cord perimeter areas at E15.5. **C:** Ly6G⁺ cells inside cords at PND0 (dark arrowheads). **D:** Several Ly6G⁺ cells in close proximity are present in the interstitium, near the cord perimeter and inside cords (white arrows) at PND0. **E:** Co-localisation of Ly6G⁺ cells and DDX4⁺ cells inside cords (white arrows) at PND0. Marker colours are indicated above each panel set. Black and white boxes on low magnification images refer to the high magnification panels.

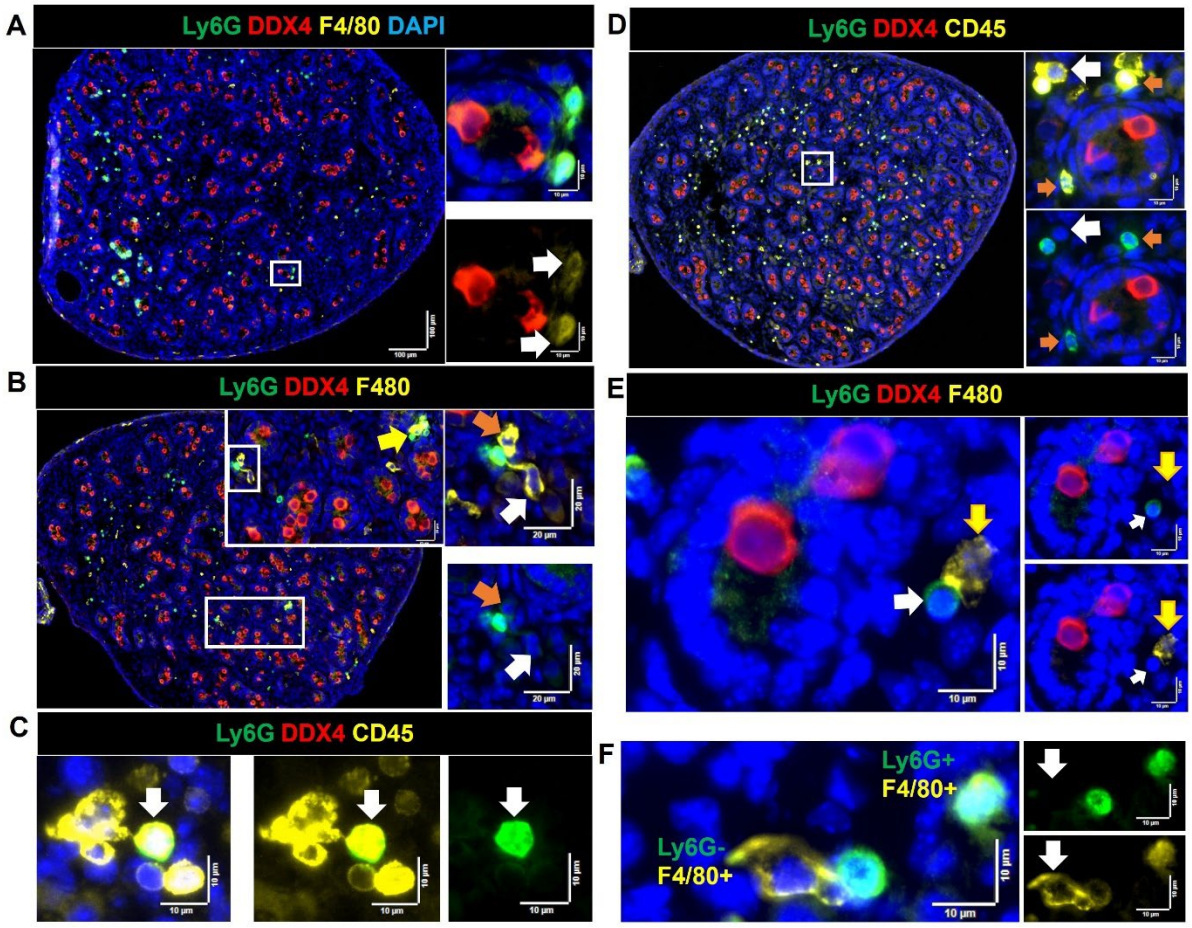


Figure 10. Interactions of Ly6G$^+$ cells (neutrophils) with other cells in the newborn mouse testis (PND0). **A:** Two Ly6G$^+$ cells in the cord perimeter area displaying a dim F4/80 signal (white arrows). **B:** A neutrophil in contact with two macrophages: one semi-rounded macrophage in the cord perimeter area (orange arrows) and another in the interstitial area (white arrows), and a grouping of Ly6G$^+$ and F4/80$^+$Ly6G$^-$ cells (yellow arrow). **C:** Co-localisation of a neutrophil (white arrow) with several CD45$^+$ cells. **D:** Co-localisation of two Ly6G$^+$ cells with CD45$^+$ cells at cord perimeter (orange arrows). Co-localisation of a CD45$^+$/Ly6G$^+$ and a CD45$^+$/Ly6G$^-$ cell in the interstitium (white arrows). **E:** A Ly6G$^+$ (white arrow) and a F4/80$^+$Ly6G$^-$ (macrophage; yellow arrow) attached to each other. **F:** Two Ly6G$^+$ cells displaying different F4/80 levels. White boxes on lower magnification images refer to the high magnification panels on the right. Marker colours are indicated above each panel set or directly on figures.

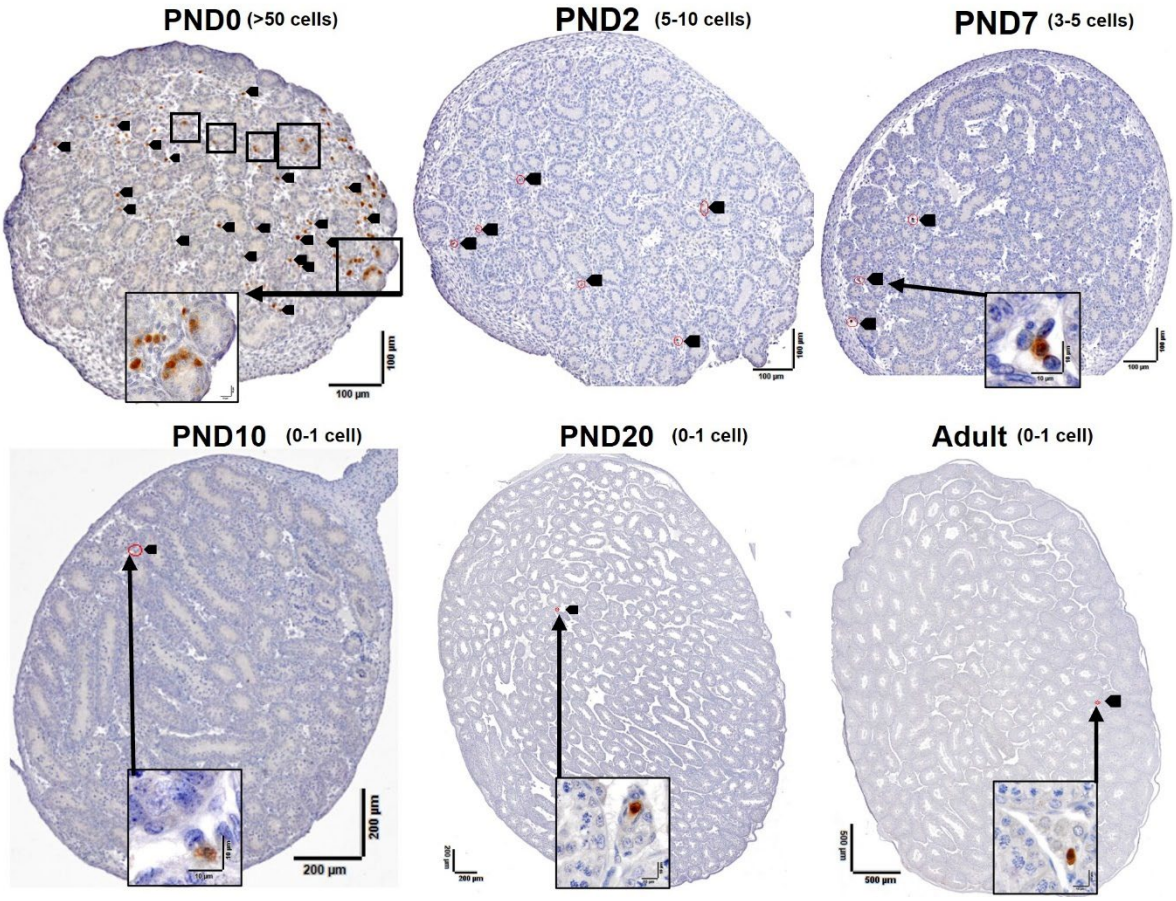


Figure 11. Ly6G$^+$ cells (neutrophils) in mouse testis sections from birth to adulthood.
Higher magnification images in black boxes show Ly6G$^+$ cells inside cords at PND0. Red circles with black arrows indicate all neutrophils detected in each section, except for the PND0 sample which had more than 50 labelled cells. The minimum and maximum number of Ly6G$^+$ cells in the central section of each of 3 testes per age testis shown in brackets. After day 10, Ly6G$^+$ cells were rare to absent.

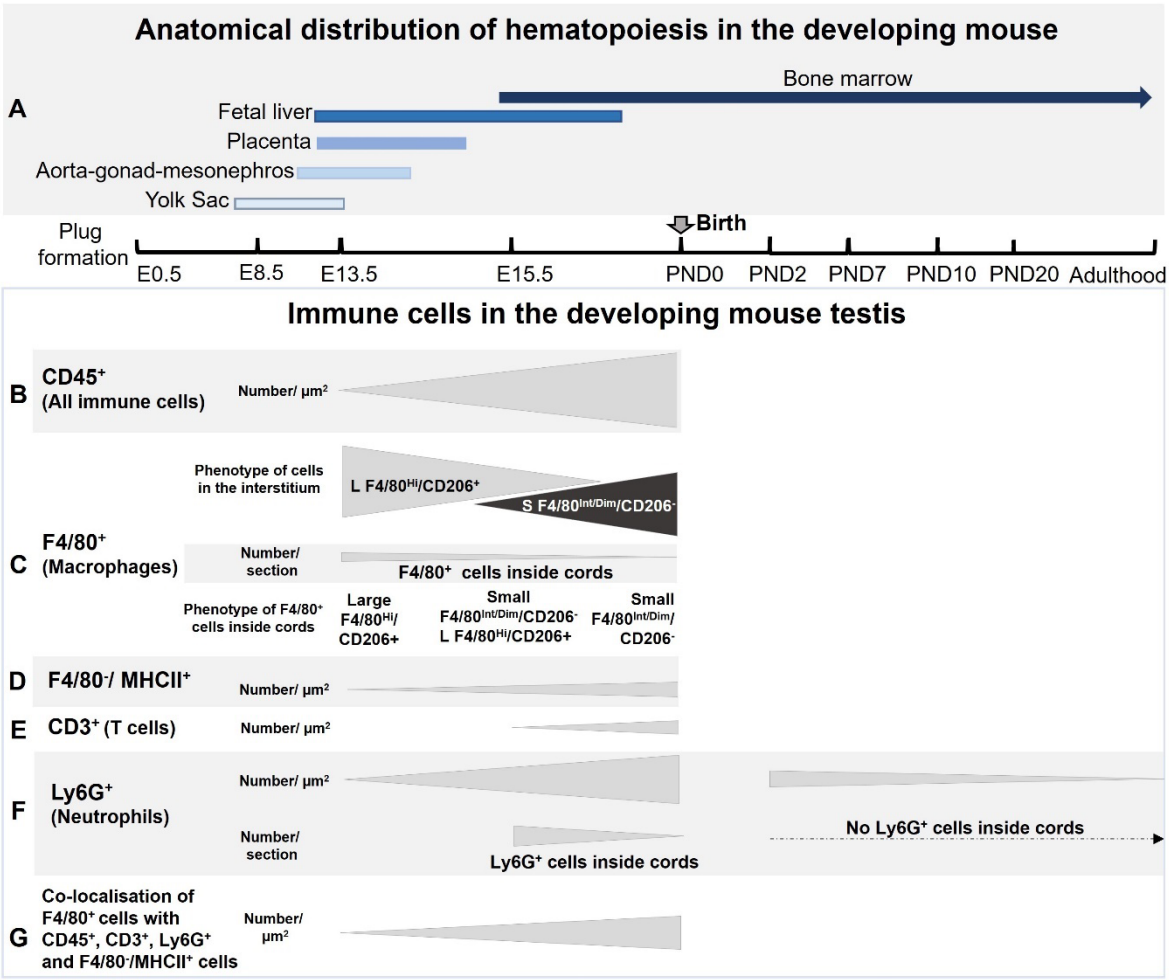
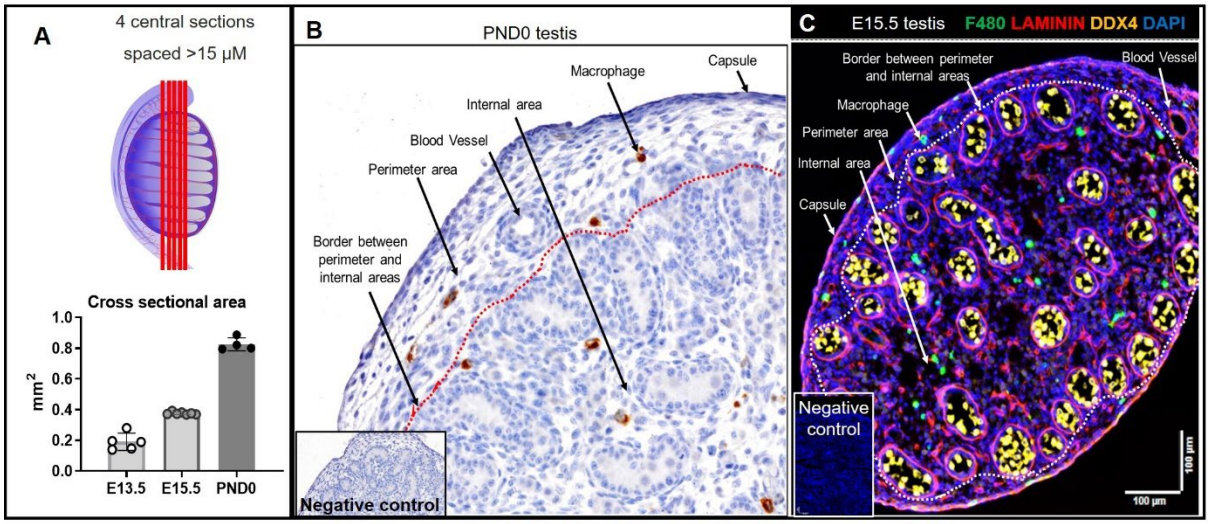
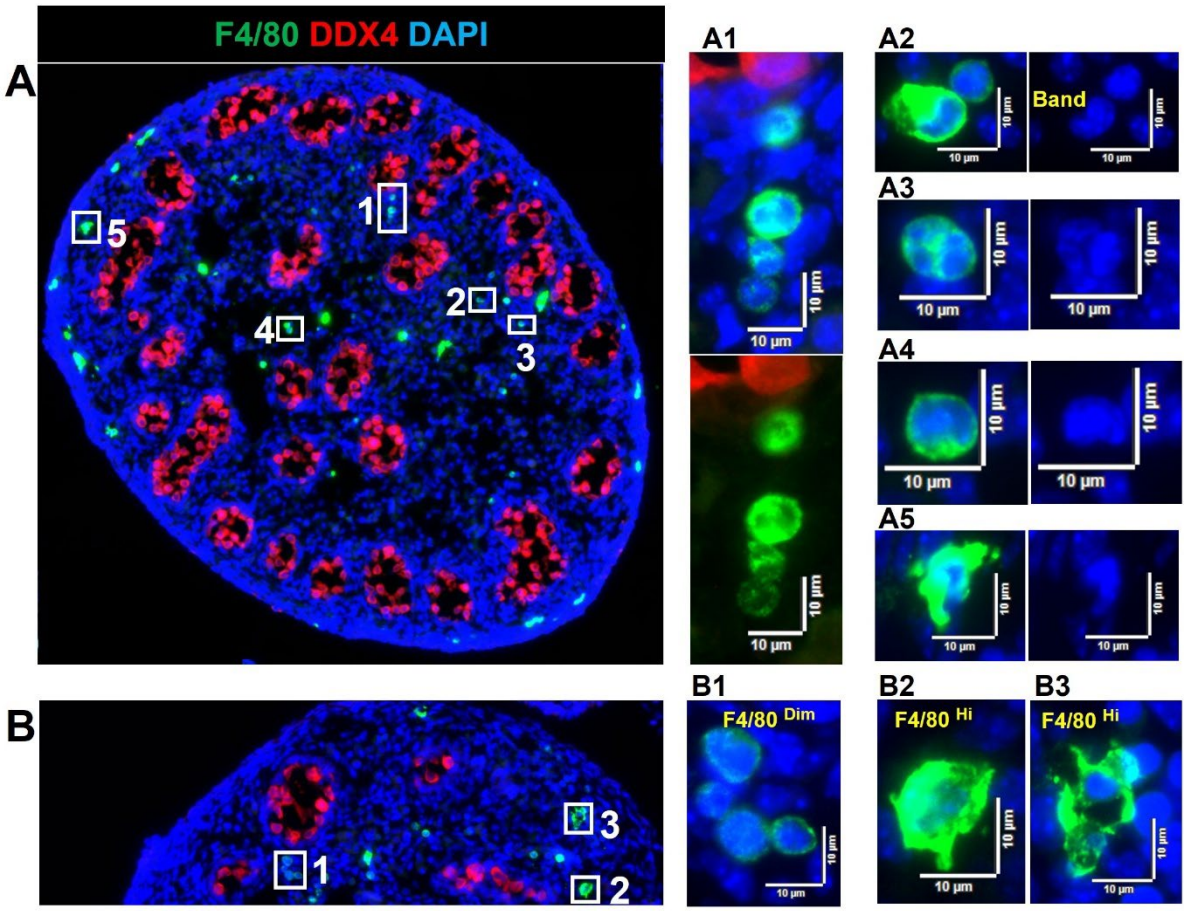


Figure 12. Anatomical distribution of hematopoiesis in the developing mouse and summary of study outcomes. A. Primitive haematopoiesis starts at E7.5 in the yolk sac. At approximately E9.5, the aorta-gonad-mesonephros region accommodates hematopoiesis, followed by haematopoiesis in placenta and fetal liver at E11.5. The bone marrow is the predominant hematopoietic tissue from about E14.5 and throughout adulthood (Mikkola and Orkin, 2006; Hoeffel and Ginhoux, 2015; Mevel et al., 2019). **B.** $CD45^+$ cell density increased from E13.5 to PND0. **C.** The phenotype of $F4/80^+$ cells in the E13.5 testis interstitium was large and $F/80^{Hi}/CD206^+$, while from E15.5, a small $F/80^{Int/Dim}/CD206^-$ population was detected. $F4/80^+$ cells inside cords at E13.5 were exclusively large and $F/80^{Hi}/CD206^+$. Macrophages inside cords were large $F/80^{Hi}/CD206^+$ and small $F/80^{Int/Dim}/CD206^-$ at E15.5, and small $F/80^{Int/Dim}/CD206^-$ at PND0. **D.** $F/80^-$/MHC class II^+ cells increased from rare/single at E13.5 to PND0. **E.** $CD3^+$ cells were not detected at E13.5. Single $CD3^+$ cells were observed at E15.5, with scattered $CD3^+$cells present at PND0. **F.** $Ly6G^+$ cells were rare at E13.5, while by PND0 they represented half of the testicular immune cell population. A lower number of $Ly6G^+$ were observed at PND7, but they were rare to absent after PND10 and in the adult testis. **G.** $F4/80^+$ cells in clusters with $CD45^+$, $CD3^+$, $Ly6G^+$, $F4/80^-$/$MHCII^+$ cells were noted with increasing frequency from E13.5 to PND0.

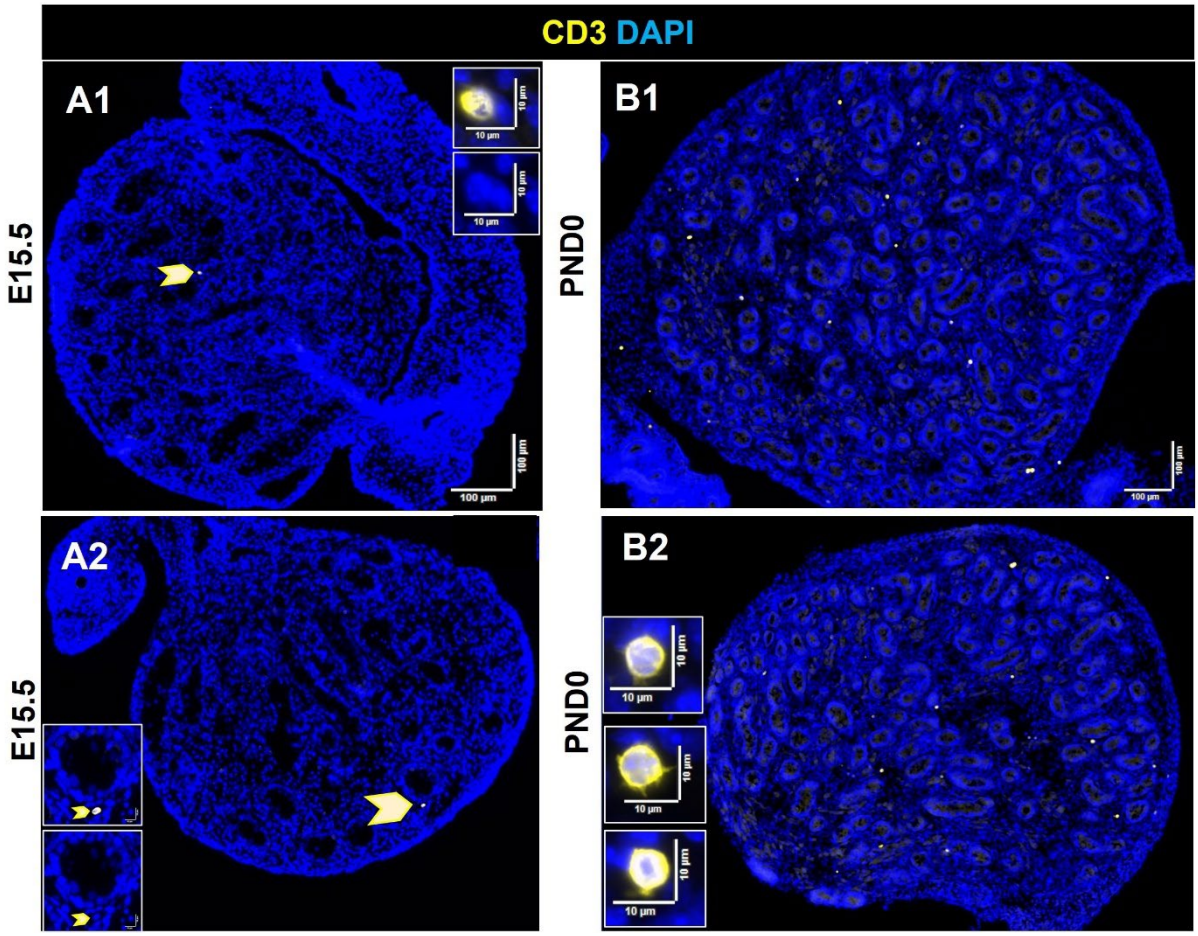
Supplementary Figure legends:



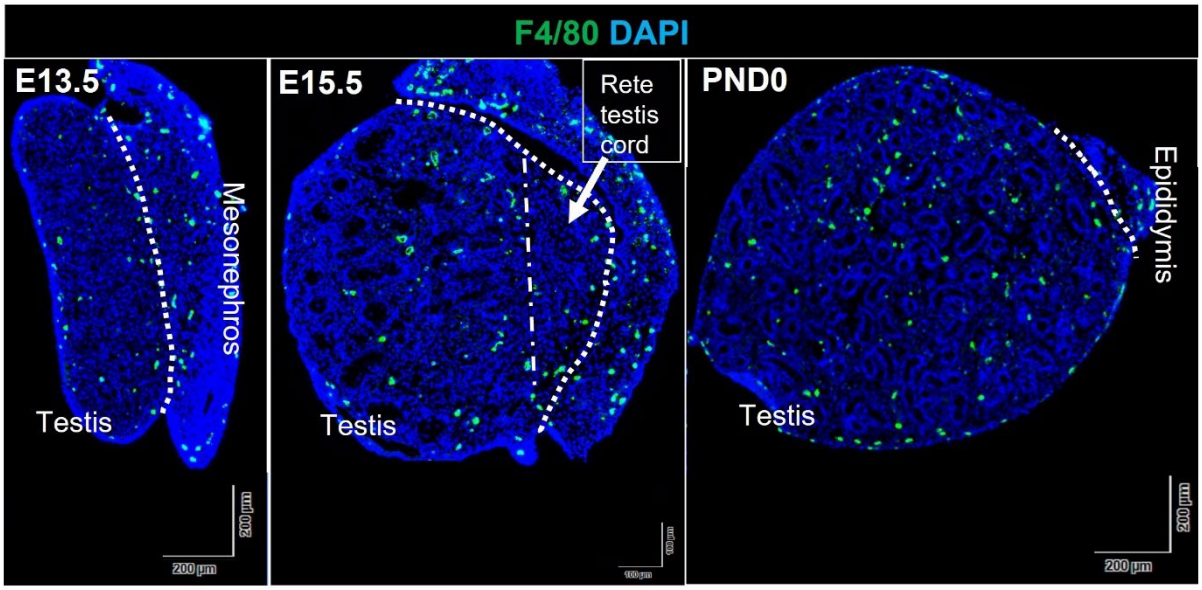
Supplementary Figure S1. Histology and approach to analysis of cell populations in sections. A. Cross sectional area (mm^2) of mouse testis sections at E13.5, E15.5 and PND0. Each data point represents the average cross-sectional area of 4 sections from the testis of an individual animal, shown with mean and SD. **B** and **C.** Delineation of perimeter and internal areas of fetal mouse testis cross sections. Two different locations of $F4/80^+$ cells are shown using **(B)** immunohistochemistry (brown stain) in PND0 testis and **(C)** immunofluorescence in E15.5 testis (macrophages (green), germ cells (yellow) and cord boundary (red)). Dotted lines denote the division between the section 'perimeter', between the testis capsule and edge of the outermost cord, and the section interior or 'internal' area. F4/80: pan-macrophage marker, DDX4: pan-germ cell marker, laminin: marks cord basement membrane. Insets in B and C show nuclear staining with hematoxylin and DAPI, respectively. Insets show lack of signal in negative control sections lacking primary antibodies.



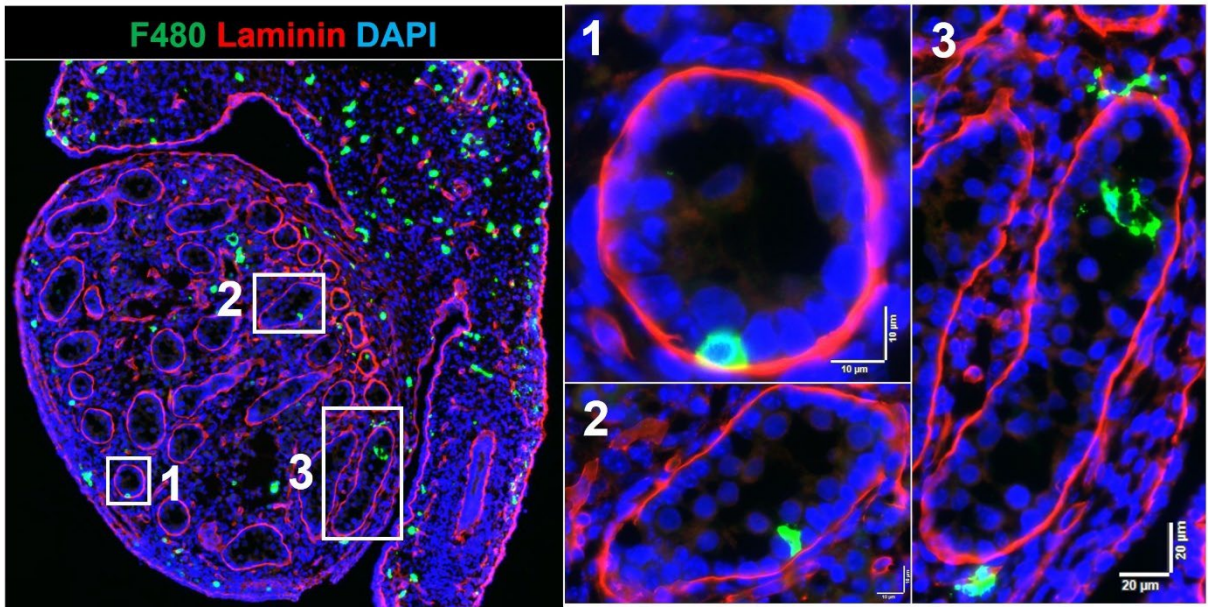
Supplementary Figure S2. Two distinct F4/80^{+} cell populations at E15.5, each exhibiting a distinctive overall size, nuclear shape and F4/80 IF signal level in E15.5 mouse testis sections. **A1** and **B1**: Small rounded F4/80^{Dim} cells. **A2**: Co-localisation of two small rounded F4/80^{+} cells with band shaped and rounded nuclei. **A3** and **A4**: Small F4/80^{+} cells with segmented and band shaped nuclei. **A5**, **B2** and **B3**: Large and elongated F4/80^{Hi}. Cells within numbered white boxes in A and B are shown in corresponding images in A.1 - A.5 and B.1 - B.3. Marker colours are indicated on image.



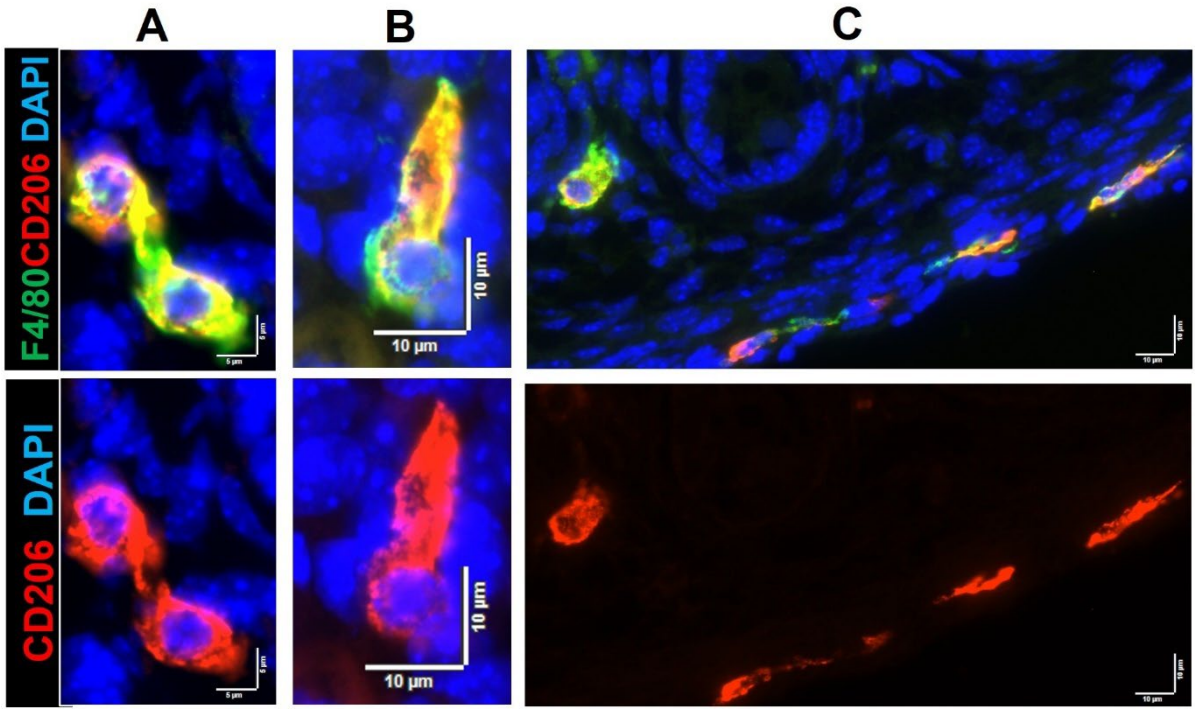
Supplementary Figure S3. A marked increase in CD3⁺ (T cell) abundance occurs between E15.5 and PND0 in the mouse testis. Arrows in **A1** and **A2** highlight the detection of a single CD3⁺ cell E15.5 testis sections from two individual animals. **B1** and **B2** illustrated detection of multiple CD3⁺ cells in two individual PND0 sections. CD3: pan-T cell marker, DAPI: nuclear stain. Insets at higher magnification show the size and shape of CD3⁺ cells. Marker colours are indicated on the figure.



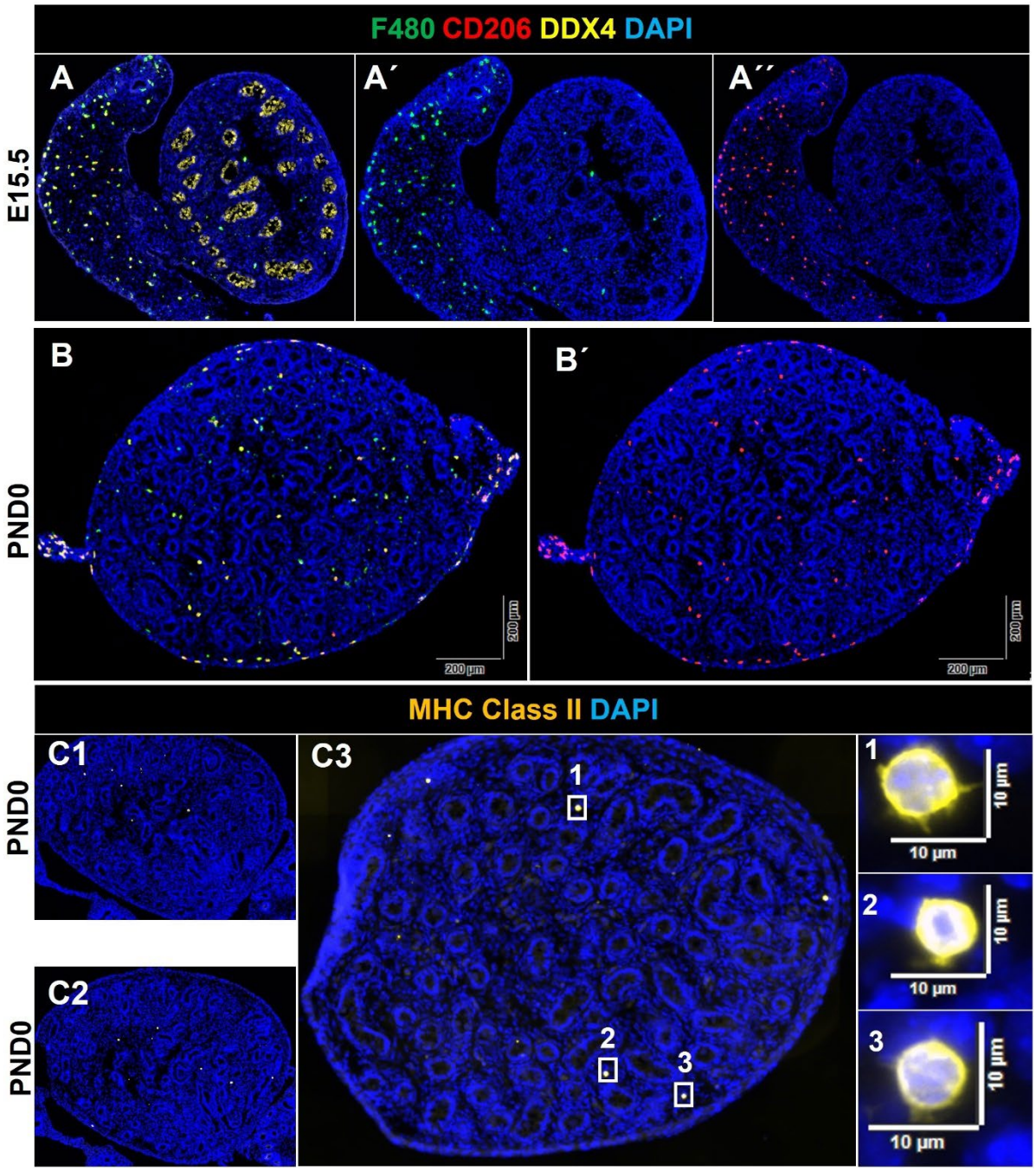
Supplementary Figure S4. A marked redistribution of macrophages (F4/80^{+}) from the testis perimeter to the interior occurs from E13.5 to PND0. Dotted lines designate the border of the testis with the mesonephros or epididymis regions. The rete testis is designated between two dotted lines on the E15.5 section. Marker colours are indicated on figure.



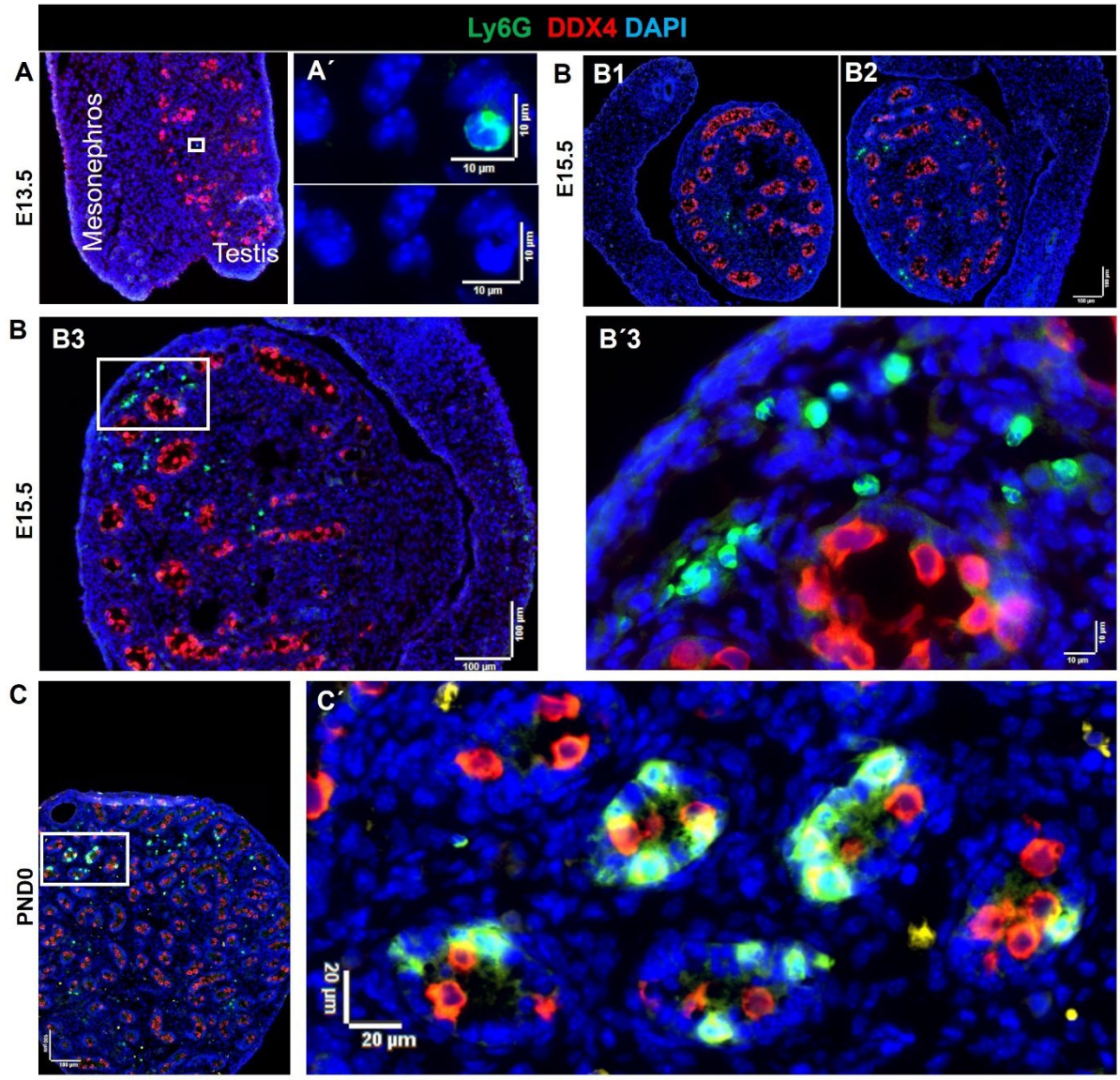
Supplementary Figure S5. F4/80^{+} cells inside E15.5 testis cords. 1, **2**: adjacent to basement membrane, and **3:** in the cord centre. Numbered white boxes on left hand low magnification image are enlarged on right hand side. Marker colours are indicated on image.



Supplementary Figure S6. F4/80^{+}/CD206^{+} interactions, shape and cell distribution patterns in PND0 mouse testis sections. **A:** Contact between two large and elongated F4/80^{Hi}/CD206^{+} cells. **B:** An elongated F4/80^{+}CD206^{+} cell. **C:** Large and elongated CD206^{+} macrophages are the predominant macrophage phenotype in the testis section perimeter. Marker colours are indicated beside each panel set.



Supplementary Figure S7. F4/80^{+}/CD206^{+} and F4/80^{-}/MHC Class II^{+} cell distribution patterns in E15.5 and PND0 mouse testis sections. A, A´, A´´: This section illustrates the significantly higher number of CD206^{+} macrophages in the mesonephros/epididymis compared to the testis at E15.5. **B and B´:** The distribution of F4/80^{+}/CD206^{+} cells across the whole testis section at PND0**. C1-3**: A low number of small, rounded F4/80^{-}/MHC Class II^{+} cells are widely distributed in the PND0 mouse testis. Each panel corresponds to an individual animal**. C3.** Numbered white boxes (1-3) in the low magnification image are shown at high magnification in the right-hand panels. Marker colours are indicated on the figure.



Supplementary Figure S8. Distribution pattern of Ly6G⁺ cells. A: This E13.5 testis section displayed a single Ly6G⁺ cell, with a band-shaped nucleus. **B1 - B3**: Asymmetric distribution of neutrophils in the testis interior at E15.5; each corresponds to an individual animal. **A´**, **B3´**: The white box on the low magnification image on the left is shown on the right in higher magnification. **C** and **C´**: Ly6G⁺ cells were frequently detected inside cords (white rectangle) and in the interstitium at PND0. Marker colours are shown above.

2.2. A hypothesis regarding macrophage recruitment from the peritoneal cavity

Macrophages were frequently observed attached to the outer layer of the testis capsule or amongst the mesothelial cells in the perimeter area; this was particularly evident at E13.5 and E15.5 (Figure 2.13). In a cross-section of a whole E13.5 embryo, the relative abundance of F4/80+ cells in the liver and connective tissue septa between organs is quite distinct from the paucity of these cells within the testis, while F4/80+ cells are readily observed in cell layers surrounding the testes, particularly on the outside surface and in connecting septa (Figure 2.14).

Figure 2.13. Detection of macrophages (F4/80+ cells) in E13.5 mouse sagittal section. F4/80+ cells are marked with a brown cytoplasmic signal in the section counterstained with hemotoxylin (blue nuclei) **A**: Position of the mouse testis. **B**. Tissues connecting the testis and other abdominal organs, and the high population of F4/80+ cells in the fetal liver. **C** and **D**: Positions of macrophages along connecting septa and at the perimeter of the testis capsule; individual cells indicated with white arrowheads. Black boxes on low magnification images refer to adjacent higher magnification panels.

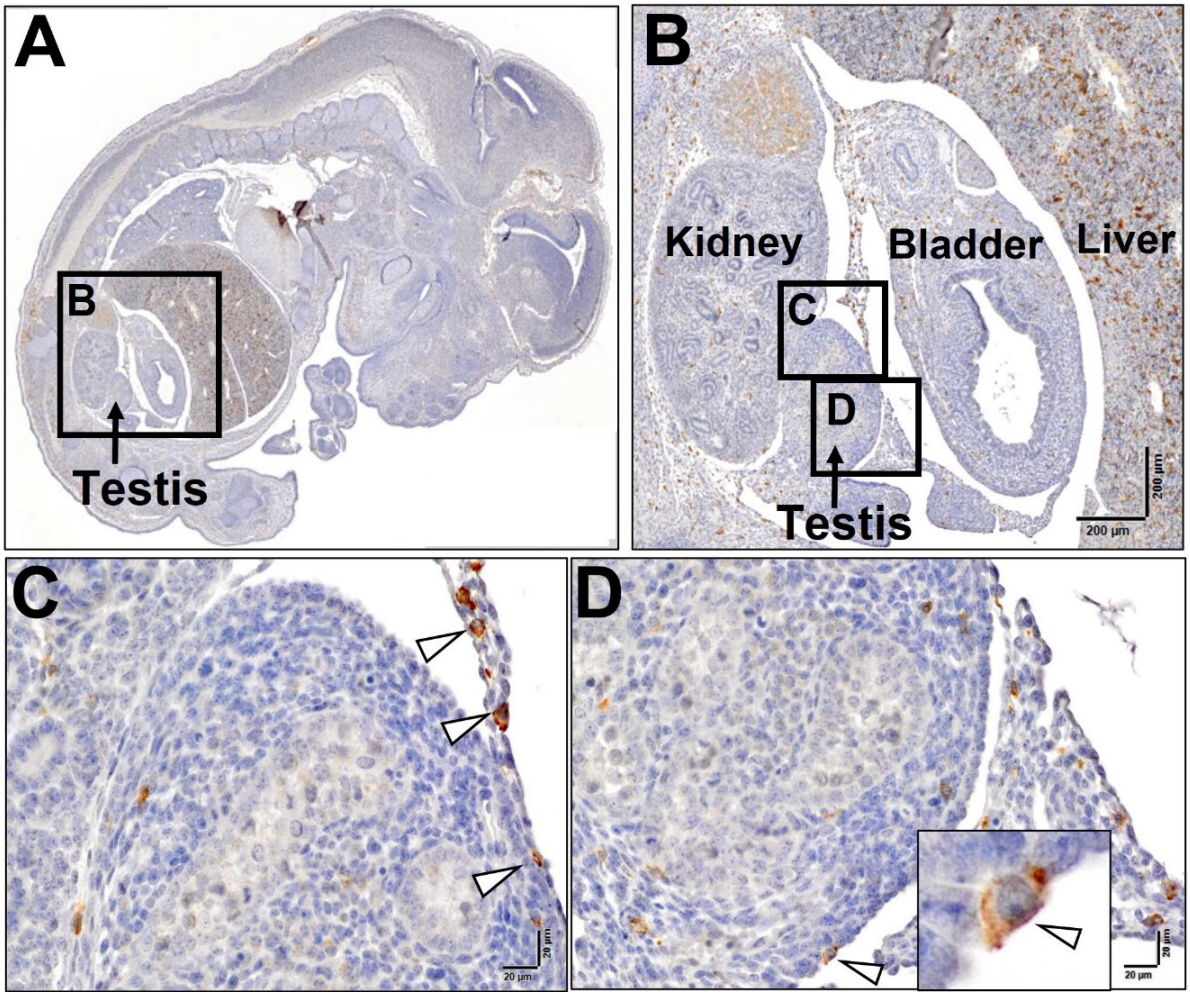
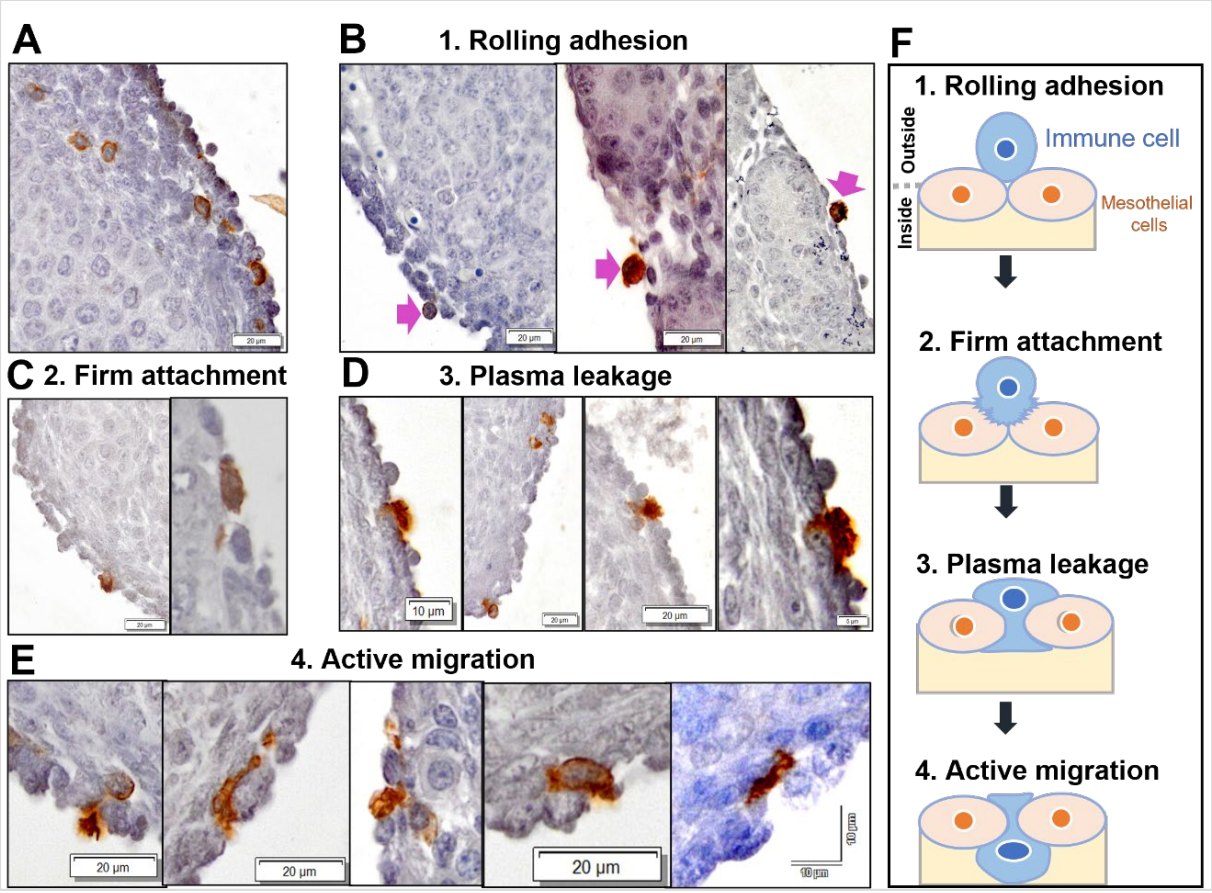


Figure 2.14. Detection of immune cells (CD45$^+$ cells or F4/80$^+$ cells) attached to the outer layer of testis capsule and/or among to the mesothelial cells of perimeter area in the fetal and newborn mouse testis sections, compared with steps in immune cell diapedesis. A: Immune cells in the perimeter area, suggesting crossing from inner side of capsule to the internal area, **B**: Rounded immune cells on the outer side of testis capsule, **C**: Elongated immune cells attached to the outer layer of capsule, **D**: Crossing immune cells through the capsule and preventing plasma leakage through mesothelial cells, **E**: Active migration, sealing endothelial cell junction and crossing the basement membrane, **F**: Stages of immune cells extravasation (also commonly known as diapedesis (Strell et al., 2008).



Based on a previous ontological investigation, F4/80^{Hi} macrophages detectable before E14.5 are known to be yolk-sac-derived, while F4/80^{Int} are fetal liver-derived and emerge by E16.5 (Lokka et al., 2020). In agreement with this, I detected only elongated F4/80^{Hi} macrophages at E13.5, while at E15.5 and PND0, both elongated F4/80^{Hi} and small rounded F4/80^{Int} macrophages were noted. The dim expression of CD45 on testicular macrophages at E13.5 is consistent with previous reports (DeFalco et al., 2014; Lokka et al., 2020), while higher levels were observed (measured here as 'intermediate') at E15.5 and PND0. Adult kidney and microglia also contain two CD45$^+$ macrophage subpopulations, with "intermediate" and "high" signals, which are proposed to have different origins (Lee et al., 2018; Rangaraju et al., 2018). I also observed macrophages at E13.5 and E15.5 on the mesonephros

side of the testis, and note that they frequently appeared to be attached to and crossing through the capsule. Intriguingly, these later profiles appeared highly similar to leukocyte extravasation (Muller, 2013). I hypothesise that a population of macrophages may be peritoneal cavity macrophages that enter the testis through the capsule. This is based on our observation of 1) direct connections between the testis and other abdominal organs including the liver, 2) the much higher frequency of F4/80^{+} cells in the liver, and 3) the lack of macrophages detected inside the lumen of blood vessels at E13.5, E15.5 and PND0. The latter point contrasts with the frequent observation of Ly6G^{+} cells inside vessels, particularly at PND0. This hypothesis is also based on previous studies on the phenotype of peritoneal macrophages, and the recruitment of immune cells from peritoneal cavity to the tissues (Cassado Ados et al., 2015; Wang and Kubes 2016; Okabe and Medzhitov, 2016; Steinert et al., 2015 and 2018). The major macrophage populations in the peritoneal cavity of mice are established prenatally and, in healthy adult animals, can be maintained independently of monocyte input. There are two peritoneal macrophage subsets with distinct origins: small peritoneal macrophages (SPMs) which are bone-marrow-derived, F4/80^{Dim}/MHCII^{high} and a pro-inflammatory phenotype (classically termed M1), and large peritoneal macrophages (LPMs) which are yolk sac-derived, capable of undergoing rapid proliferation upon inflammation, F4/80^{Hi}/MHCII^{Low} and displaying immune regulatory functions (M2; CD206^{+}) (Yona et al., 2013). In addition, the circulation of immune cells between the blood and peritoneal cavity-located organs is well established (reviewed in Davies et al., 2013).

Therefore, based on previous reports and the observation of macrophages attached to and crossing the testis capsule, I hypothesise that the large CD45Dim/F4/80Hi/CD206+/MHCII- testicular macrophages are peritoneal cavity-derived cells which enter the testis through the capsule from specific locations, which I call “gateways”.

Chapter Three

Macrophages in the Testes of Fetal and Newborn Mice with Super- and Supra-Physiological Activin A Levels

3.1. Background

Findings presented in Chapter Two demonstrate that macrophages are the most abundant immune cell type in the fetal testis after sex determination. This is an interval of rapid somatic cell population growth, when the germline becomes masculinized and stops proliferating. While the factors that govern somatic and germ cell development have been studied, there is limited knowledge about what signals influence emergence of the immune cells that lay the foundation for adult fertility. The TGF beta (TGF-β) superfamily signaling molecule, activin A, is of particular interest because of its levels in the rodent testis between embryonic day (E)13.5 and birth (PND0; discussed below and in Section 1.5 of Literature review), and its impacts on immune cell biology have been widely studied (discussed below and in Section 1.5 of Literature review).

Activin A is a member of the TGF-β superfamily that functions through two types of receptors (I and II). Activin A receptors upon ligand binding, activate their kinase activity, phosphorylate the SMAD2 and 3 and form a complex with SMAD4, then translocate to the nucleus to activate or silence target genes expression (Chapter 1, Section 5.1 of this thesis; Massagué and Chen, 2000; Namwanje and Brown, 2016).

Activin A contributes to essential biologic processes including embryonic development and morphogenesis, stem cell maintenance and differentiation, haematopoiesis, immune cell differentiation (polarisation) and cell proliferation (Rosendahl 2003; Licona et al., 2006; Wang et al; 2008; Sozzani and Musso, 2011; Rafaat et al., 2014; Hardly et al., 2015; Sainz et al; 2015). Also, many preclinical and clinical studies have highlighted crucial functions of activin A in the initiation, propagation and resolution of human microbial infections and diseases, including in cancer, in allergic disorders (atopic dermatitis and allergic asthma), autoimmune diseases (systemic lupus erythematosus, pulmonary alveolar proteinosis and rheumatoid arthritis) and in tissue fibrosis (such as lung and liver) (Kariyawasam et al., 2011; Rafaat et al., 2014; Hardly et al., 2015; Sainz et al; 2015; Chen and Dijke, 2016; Morianos et al., 2019).

Gonadal-derived activin is central to fetal testis development, governing expansion and likely coiling of the testis cords, androgen receptor and androgen target gene production, and normal spermatogenesis after puberty (Matzuk et al., 1992; Namwanje and Brown, 2016; Loveland et al, 2017). In the fetal mouse testis, Leydig cells and gonocytes are producers of testicular activin A (Meehan et al., 2000; Mendis et al., 2011; Archambeault et al., 2011).

In adult testis, interstitial macrophages are in direct contact with Leydig cells and have paracrine interactions through long microvilli of Leydig cells that are inserted into coated vesicles of the macrophage cytoplasm. In adult testes, interstitial macrophages play key roles in development, regeneration and in testosterone production by Leydig cells; therefore, due to this dependency, their numbers increase from birth to adulthood at a relatively constant ratio (digitations). The interdigitations between Leydig cells occur exclusively with testicular interstitial macrophages and have been reported in *in vitro* and *in vitro*; they do not exist between Leydig cells and other macrophage populations (Rivenson et al., 1981; Hutson, 1992, 1998 and 2006).
Activin A regulates Sertoli cell proliferation during fetal testis development (Archambeault et al., 2010; Mendis et al. 2011). Changes in levels of activin bioactivity and of its signaling mediators, Small Mothers Against Decapentaplegic (Smad) transcription factors, that occur during normal fetal testis development can influence Sertoli cell maturation, spermatogonial development, the onset of spermatogenesis, and during the first spermatogenesis wave (Barakat et al. 2008). Other cells in fetal and adult testes produce activin A including Sertoli cells, macrophages, and mast cells (Phillips et al., 2009; Archambeault et al., 2011; Barakat et al., 2012; Young et al., 2015; Hedger, 2015).
Activin A plays pivotal roles in allergies, autoimmunities and cancers specifically through regulation of immune responses. Many *in vivo* and *in vitro* studies on animal models and human samples have demonstrated impacts of activin A on the immune system function. The synthesis and secretion of activin A are stimulated in most immune cell types, including macrophages (M1 and M2, explained in Introduction Chapter, 4.2.1 section), B cells, T cell subsets (including Th2, Th9, TFH, Tr1 cells and Treg cells), DCs, NK cells, and mast cells, in response to their activation. (Cho et al., 2003; Zhang et al., 2005; Wang et al., 2008; Ogawa et al., 2008 and 2011; Huber et al., 2009; Ge et al., 2009; Morianos et al., 2019). Reflecting the many roles of activin A in the modulation of inflammation, immunity and disease, the potential of targeting activin A as a highly specific therapeutic approach has been undertaken. This includes cancers of prostate, glioblastoma, ovary, fallopian tube, endometrium, pancreatic ductal adenocarcinoma, melanoma, and it is relevant to related conditions such as cancer cachexia which is linked to cancer-related bone loss and anemia (Tsuchida et al., 2009; Rautela et al., 2018; Semitekolou et al., 2018; Ries et al., 2020).

Macrophages play significant roles during the initial phase of fetal testis development by removing germ cells and Sertoli cells remaining in the interstitium and outside the testis cords at E11.5 and E12.5 (DeFalco et al., 2014; Lokka et al., 2020; Wang et al., 2021). A recent unpublished finding in the Loveland lab identified gonocytes frequently located outside of cords in the fetal mouse testis, accompanied by a significant number of multinucleated germ cells inside the cords in *Inha* knockout (KO) mice in which activin A levels would be expected to be elevated. One explanation for this abnormality could be the mal- or poor function of macrophages, so that gonocytes are not removed from interstitial areas. Another possibility could be a change in the macrophage to germ cell ratio that results in an insufficient number of macrophages being appropriately positioned to remove germ cells from outside of cords when activin A levels are high. For that reason, we studied frequency of macrophages in activin A mutant mouse models by examining *Inha* and *Inhba* mice.
While the roles of activin A in Sertoli cell proliferation, gonocyte differentiation, and cord formation have been investigated in several studies analysing the *Inhba*$^{-/-}$ testis (lacking activin A; Meehan et al., 2000; Whiley et al., 2020), there are no reports describing of immune cells in fetal testes of *Inhba*$^{-/-}$ (KO) and *Inha*$^{-/-}$ (KO) mice. Because activin A functions affect macrophages as well as all stages of testis development and function, we studied the impact of activin A on testicular macrophage frequency, localisation and gene expression during fetal testis development in activin A mutant mice with low and high levels of activin A. For this study, testes were collected from *Inhba* and *Inha* knockout (KO, $^{-/-}$) and heterozygote (HET$^{+/-}$) mouse models as test groups in comparison to their wild type (WT) littermates at specified times after sex determination, when cords are forming and growing, and when germ cells are at different maturation stages. This reflected the stages used in previous studies on activin A effects during fetal life (Archambeault et al., 2011; Mendis et al., 2011). The ages explored in both mouse models included embryonic day (E) 13.5, when germ cells are undergoing the transition from the proliferative to the quiescent state, at E15.5 when germ cells are quiescent, and PND0 when germ cells are emerging from quiescence and preparing for movement to the cord perimeter. These findings revealed new aspects of the potential roles of activin A to influence testis biology.

3.2. Materials and Methods

Experiments were performed using mouse testicular tissue at embryonic day (E)13.5, E15.5 and post-natal day 0 (PND0), originating from KO, HET and WT *Inha* and *Inhba* mice (n=4-8 per age per genotype). At least 3 pups per age (at E13.5, E15.5 and birth) from more than one litter were analysed.

The study of testicular macrophage populations (F4/80$^+$ cells) and their localisation during fetal life were explored using immunohistochemistry (IHC).

Transcripts of CD45, CD11c, F4/80 CSF1 CX3CR1, CX3CL1, Arginase 1, CD206, IL-4 Receptor, IL-10, IL-10 receptor, CCl17/ TARC, CXCR7, MHC class II, CCR4 (CD194), CXCL12, CXCR4, Retinoic Acid Receptor Alpha, CD16, CD64, CD68, ICAM-1, TLR4 (CD284), CD38, Marco, IL-1b receptor 1, IL-1β, GM-CSF receptor, CCR2 (CD192) , CCL2 (MCP-1), MMP15, MMP23 encoding proteins expressed in immune cells were identified using the Fluidigm qRT-PCR platform in E12.5, E13.5, E14.5 and E15.5 in *Inhba* KO and WT mice, and in E13.5, E15.5 and PND0 *Inha* KO and WT mice testis.

Animals

Two mouse models were used for investigations reported in this chapter. One is the *Inhba* mouse*,* in which the gene encoding the mature βA subunit was deleted by Matzuk et al. (1995) to generate knockout mice deficient for both activin A and inhibin A. KO *Inhba* mice die at birth, so this strain is maintained by crossing heterozygous parents. Only heterozygous and wild type mice from this strain survive to adulthood and are available for research on the postnatal testis, but knockouts can be studied in fetal life and at birth. Likewise, *Inha-α* –KO mice were generated by deletion of the inhibin α-subunit *(Inha)* gene encoding the mature inhibin α subunit. Animal experiments were approved by the Monash University Animal Ethics Committee (ethics numbers MMCB/2017/41 and MMCB/2017/49/BC). Genotypes of *Inhba* and *Inha* mice were determined from tail samples by a commercial vendor (Transnetyx, TN, USA) using real-time PCR with gene-specific probes. All breeding was done using heterozygote adults. To breed heterozygote adult mice, mature heterozygote animals lacking one copy of the sequence encoding the mature region of the activin βA protein (HET *Inhba* knock-out) mated with C57BL6J mice (*Inhba* mouse model); and mice lacking one copy of the sequence encoding the mature region the alpha-inhibin protein (HET *Inha* knock-out) mated with mice C57BL6J (*Inha*

mouse model). Both mice were refreshed every 2 years through backcrossing by C56B6/J mice.

Tissue preparation

For immunohistochemistry experiments: Two different fixation and embedding approaches were employed. For IHC experiments, one testis from each mouse was immersed at room temperature (RT) in Bouin's solution 30 min for E13.5, 60 min for E15.5, and 90 min for PND0, followed by washing in 70% ethanol (3 x 1 hour). Fixed tissues were processed and embedded in paraffin. Tissue embedding and sectioning were performed by staff at MHRP node of the Monash Histology Platform, Monash University. Sequential sections of 5μm thickness were collected onto Superfrost Plus microscope slides (Thermo Fisher Scientific, Waltham, Massachusetts, USA). One testis from each mouse was divided into three regions of approximately equivalent size from apex to base along the long axis and was entirely serially sectioned, with 3 sections per slide. One slide representing the middle of each region was selected, then two slides on either slide (three slides/ testis) were also selected. Thus, three individual slides were examined with each antibody, ensuring that replicate sections for each antibody were spaced at a minimum of 50 μm to avoid double-counting of individual cells. Section cross-sectional area was measured using ImageJ software, V2.1.0 (National Institutes of Health, Bethesda, Maryland, USA).

For Fluidigm analysis: To examine transcripts in the *Inhba* and *Inhba* fetal testes (n=3), RNA was prepared from whole, paired testes (E12.5-E15.5 for *Inhba* and E13.5-PND0 *Inha*) using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Genomic DNA was eliminated using DNA-free (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's guidelines. RNA quantity was determined using Nanodrop (Thermo Scientific™ Inc, MA, USA). RNA quality in a subset of samples was assessed by Agilent 2100 Bioanalyzer, with the RNA integrity number (RIN) above 8.5 for all samples. cDNA was synthesised by reverse transcription of 300 ng (whole testes, *Inhba* age series) total RNA using Superscript III reverse transcriptase (Thermo Fisher Scientific Inc.) with random hexamer oligonucleotides (Promega), according to the enzyme manufacturer's guidelines.

Reagents

Antibodies and reagents used for immunohistochemistry are presented in Table 3.1. The F4/80 antibody was obtained from Professor Richard Kitching, Centre for Inflammatory Diseases, Monash University.

Table 3.1. Details of primary and secondary antibodies used to detect macrophages using the immunohistochemistry method.

Antibodies	Source (citations)	Cat #	Clone/ Host	Dilution
F4/80	Prof R. Kitching lab (Indumathy et al., 2020; Jones et al., 2009)	In-house	Polyclonal/ rat	1:200
CD45	Merck Millipore (Indumathy et al., 2020)	05-1416	IBL-5/25/ rat	1:100
Biotinylated Rabbit anti-rat	Dako	E0468	Polyclonal/ rabbit	1:500
Biotinylated Rabbit anti- goat	Dako	E0466	Polyclonal/ rabbit	1:500

Immunohistochemistry

Immunohistochemistry was performed using antibodies to detect the murine macrophage marker, F4/80 and the pan-leukocyte marker, CD45. Sections were deparaffinised and rehydrated through a graded ethanol series including twice each in histosol and 100% ethanol, then once each in 95%, 80% ethanol, 70% ethanol and deionised water; washing duration for all steps was 5 minutes. Heat-induced antigen retrieval was performed by immersing slides in Tris-EDTA Buffer (10mM Tris Base, 1mM EDTA, 0.05% Tween 20, pH 9.0) in a container which was then microwaved for 4-5 minutes at 800 watts and then for 9 minutes at 450 watts, followed by standing at room temperature to cool for 30 minutes. Sections were incubated with 3% hydrogen peroxide to block endogenous peroxidase for 20 minutes, followed by 3 washes of 5 minutes with TBS (Tris-buffered saline; 50 mM Tris-Cl, 150 mM NaCl, pH 7.5). Next, sections were incubated in 5% Mouse on Mouse Blocking Reagent (MOM; MKB-2213, Vector Laboratories, Burlingame, California, United States) in a humid chamber at RT for 30 minutes. Primary antibodies diluted in PB (1% BSA in PBS) and were applied overnight in a humid chamber at 4 °C. Negative controls for each slide were processed

without the primary antibody. The next day three washes (5 minutes each) were performed using TBS at RT. Primary antibody binding was detected using the biotinylated secondary antibody in a humid chamber for 1 hour at RT. After consecutive washes (3 × 5 minutes), the signal was amplified with Vectastain Elite ABC (Vector Laboratories) according to the manufacturer's instructions. Antibody binding was detected as a brown precipitate following development with 3, 3-diaminobenzidine tetrahydrochloride (DAB) (Dako, Glostrup, Denmark). Sections were counterstained with Harris hematoxylin (Sigma-Aldrich) for 50 seconds. Stained slides were then dehydrated through a graded ethanol series with once 70%, 80% and 95% ethanol (3 minutes), plus twice in 100% ethanol and histosol (5 minutes). Finally, slides were mounted under glass coverslips using DPX (Sigma-Aldrich, St. Louis, MO, USA) mounting media.

Imaging and morphometric analysis

Immunostained sections were scanned using the Olympus VS120 Slide Scanner at the Monash University Histology Platform facility based at the Monash Health Translation Precinct, then analysed using OlyVIA Software Viewer (Olympus Life Science, 2.9.1).

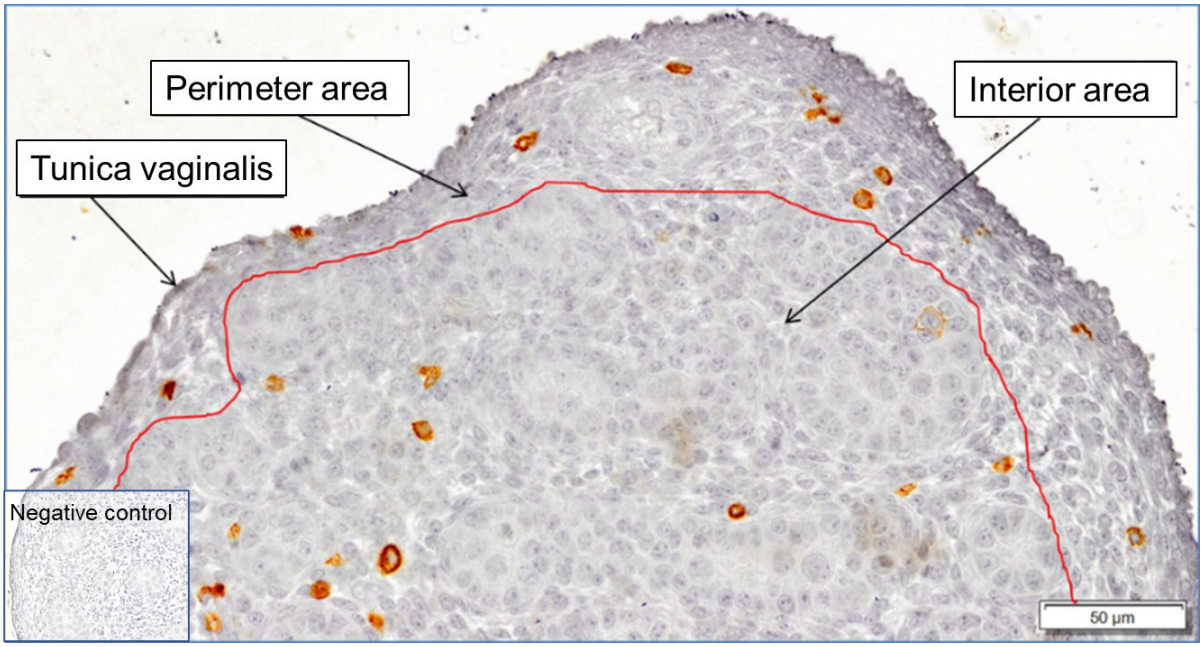
Each section was evaluated to determine whether there was a significant numerical or positional difference in macrophages between mice with higher (*Inha*$^{-/-}$ and *Inha*$^{+/-}$) and lower (*Inhba*$^{-/-}$ and *Inhba*$^{+/-}$) levels of bioactive activin A compared to in WT littermates from each strain.

For numerical evaluation of macrophages, at least three different parts of individual testes were selected. The F4/80 positive (F4/80^{+}) cells, classified as macrophages, were counted based on the presence of a brown stain and their nuclear morphology across the entire section. The cell count data were normalised to the total area of each section. Controls for non-specific secondary antibody binding were performed in all experiments by including sections without primary antibody; these specimens consistently showed no signal.

The description of the macrophage distribution pattern was based on our observation that immune cells were predominantly in the section periphery at E13.5, but at E15.5 and PND0 they were more frequently in the centre. The scanned images of individual sections were considered as 2 areas: 1) perimeter, and 2) interior (Figure 3.1). Using

this strategy, all target cells were counted, their distribution within the testis cross-section was defined, and the proportional distribution of cells within perimeter/ interior areas was calculated.

Figure 3.1. Approach to dividing testis sections into the perimeter and interior areas to quantify macrophage distribution in the mouse testis. Two different immune cell locations are shown in a PND0 knockout *Inha* mouse testis section. The perimeter area was identified as the region between the capsule and the outermost cord wall, and the interior area was defined as the rest of the space in the section, including cords and interstitial areas. Brown staining indicates F4/80+ cells. Section is counterstained with hematoxylin. Size marker and negative control section as inset are shown.



The total number of macrophages in each section was determined by adding the number of macrophages in both the perimeter and interior areas. This number was divided by the section cross-section area (μm^2) to provide an estimate of macrophage density for comparison between specimens. All slides were assessed by a single operator; a subset of three slides were independently counted to confirm accuracy (Table 3.2). The intra-operator variation was 5.3% for total macrophage numbers. The entire series was subsequently counted blinded, by a single operator.

Table 3.2. Comparison of macrophage counts in 3 individual sections from E15.5 *Inha* KO testis by two independent operators.

Operator	Number of F4/80+ cells			
	Sections	Perimeter area	Interior area	Total number of macrophages
1	Section 1	16	20	36
2		15	19	34

1	**Section 2**	14	20	34
2		18	16	34
1	**Section 3**	10	16	26
2		10	13	23

Fluidigm analyses to measure transcript levels

Samples

Whole testis samples were collected from *Inhba* strain WT and KO mice at ages E12.5, E13.5, E14.5 and E15.5, and *Inha* strain WT and KO E13.5, E15.5 and PND0 mice, with 3 animals per sample. cDNA was synthesized as described in 3.4.2 and subjected to qRT-PCR using the Fluidigm Biomark™ platform software to determine the mRNA expression profile of each transcript studied.

Primers

The transcripts encoded by 33 genes (Table 3.3) relating to immune cell function (Owen and Mohamadzadeh, 2013; Turner et al., 2014, De Falco, 2014; Palomino and Marti, 2015) were targeted for Fluidigm analysis.

Table 3.3. Transcripts measured using Fluidigm analysis**.**

Purpose	Marker/Factor	Genes	Taqman Probe number
Pan-leukocyte marker	CD45	*Cd45*	Mm01293577_m1
Pan-dendritic cell marker	CD11c	*Itgax*	Mm00498701_m1
Pan-macrophage marker	F4/80	*Adgre1*	Mm00802529_m1
Important markers on testicular macrophages	CSF1	*Csf1*	Mm00432688_m1
	CX3CR1 (fractalkine receptor)	*Cx3cr1*	Mm02620111_s1
	CX3CL1 (fractalkine)	*Cx3cl1*	Mm00436454_m1
Anti-inflammatory macrophage markers	Arginase 1	*Arg1*	Mm00475988_m1
	CD206	*MRC1*	Mm00485148_m1
	IL-4 Receptor	*Il4ra*	Mm01275139_m1
	IL-10	*Il10*	Mm00439616_m1
	IL-10 receptor	*Il10ra*	Mm00434151_m1
	CCl17/ TARC	*Ccl17*	Mm01244826_g1
	CXCR7	*Cxcr7*	Mm04931206_s1
	MHC class II	*H2-Eb1*	Mm00439221_m1
	CCR4 (CD194)	*Ccr4*	Mm00438271_m1
	CXCL12	*Cxcl12*	Mm00445553_m1
	CXCR4	*Cxcr4*	Mm00438271_m1
	Retinoic Acid Receptor Alpha	*Rara*	Mm01296312_m1
Pro-inflammatory macrophage markers	CD16	*Fcgr3*	Mm00438882_m1
	CD64	*Fcgr1*	Mm00438874_m1

	CD68	*Cd68*	Mm03047343_m1
	ICAM-1	*Icam1*	Mm00516023_m1
	TLR4 (CD284)	*Tlr4*	Mm00445273_m1
	CD38	*Cd38*	Mm00483143_m1
	Marco	*Marco*	Mm00440250_m1
	IL-1b receptor 1	*Il1r1*	Mm00434237_m1
	IL-1β	*Il1b*	Mm00434228_m1
	GM-CSF receptor	*Csf2rb*	Mm00655745_m1
Involved in polarization of macrophages	CCR2, (CD192)	*Ccr2*	Mm99999051_gH
	CCL2 (MCP-1)	*ccl2*	Mm00441242_m1
Involved in tissue remodelling	MMP15	*Mmp15*	Mm00485062_m1
	MMP23	*Mmp23*	Mm01266279_g1

Fluidigm process

Measurement of ranscript levels in whole testes from *Inhba* and *Inha* mice was conducted in separate assays using the Fluidigm Biomark™ HD system (96×96, and 48x48 Dynamic Array Integrated Fluidic Circuits (IFCs), respectively). The Fluidigm Biomark experiment was performed using Taqman Assays with primers listed in Table 3.3 and conducted by staff at the Genomics Centre, MHTP Medical Genomics Facility, Hudson Institute of Medical Research.

Fluidigm analysis

Raw data were analysed with Fluidigm Real-Time PCR Analysis Software (Version 4.1.2). The data generated were exported to a Microsoft Excel spreadsheet, and fold-changes were calculated for each gene. The calculation of correlation coefficients (R^2=0.87) for two candidate housekeeping/reference transcripts (*Canx* and *Mapk1*) and standard curve shows good linearity (Livak and Schmittgen, 2001). Negative control assays were confirmed by samples lacking reverse transcriptase, which were below the detection limit.

Two reference genes, *Canx* and *Mapk1* (van den Bergen et al. 2009), were detected at the same level per input RNA in testes at all ages and genotypes tested. The R^2 value of > 0.87 obtained from both reference genes for each sample indicates they have a high positive correlation; thus, the average of both was used to normalize gene expression data using the ΔΔCT method (Livak and Schmittgen 2001). Next, the ΔΔCT of the WT of each genotype/age group was used for normalising the KO values.

Statistical analysis

Statistical analyses and graphing were performed using GraphPad Software (San Diego, CA, USA, 8.2.1). The t-test was employed to analyze significant differences between values from the two mutant mouse strains *(Inhba* and *Inha*). Values were considered significantly different if $p < 0.05$.

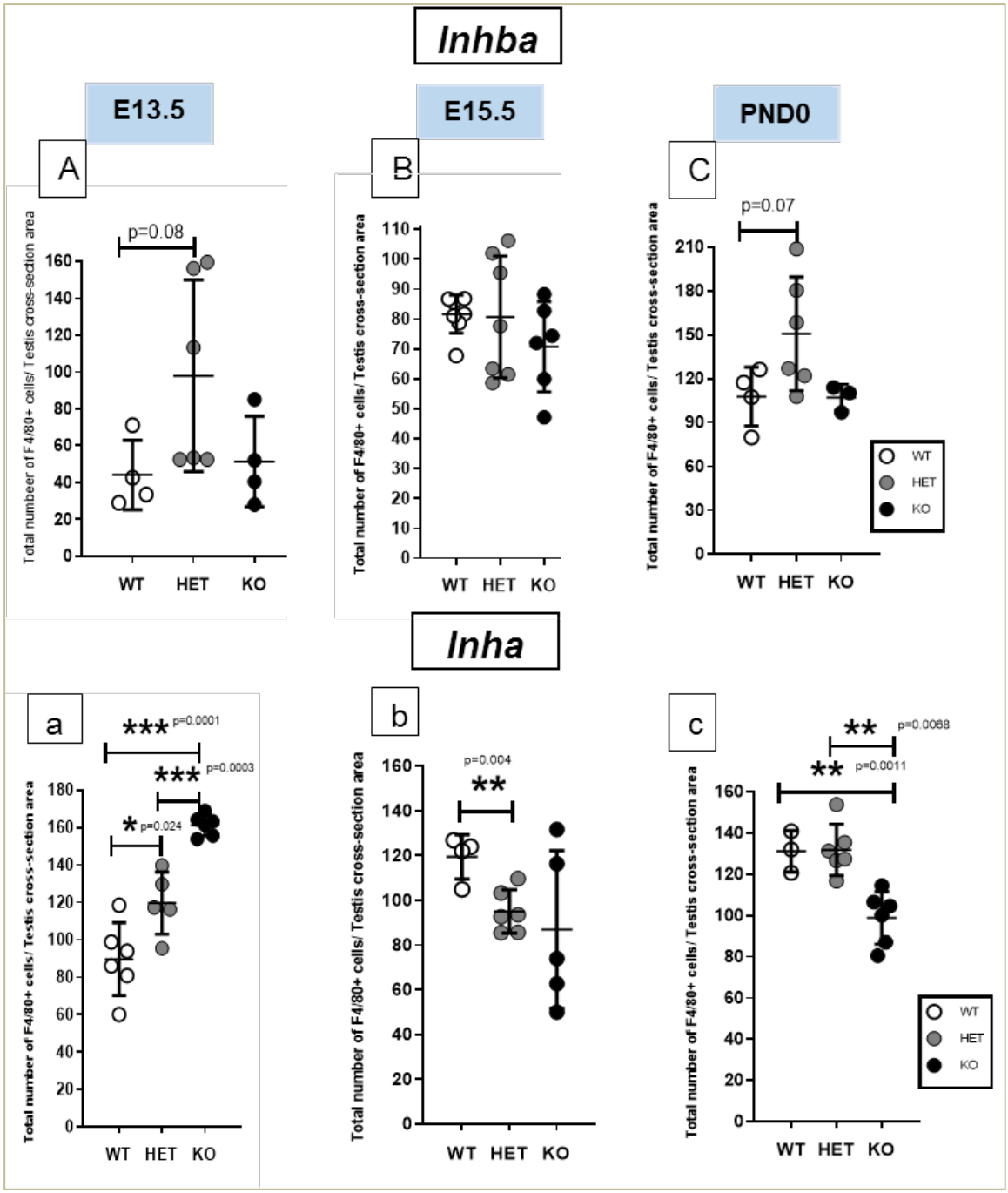
3.3. Results

Determination of macrophage density and distribution in *Inhba* and *Inha* fetal and newborn testes using immunohistochemical detection

3.3.1. Total density of F4/80+ cells in *Inhba* and *Inha* mouse testes at E13.5, E15.5 and PND0: Comparisons within and between strains

In this initial analysis of *Inhba* mouse testis sections, there was no significant difference identified in macrophage density between samples at the same age, however the density was significantly increased in some HET samples compared to WT sections at both E13.5 and PND0 (Figures 2A-C). In the testis sections of *Inha* KO animals (Figure 3.2, Panels a-c), the total number of macrophages per cross-sectional area at E13.5 was significantly higher than in WT and HET animals, while at PND0 the macrophage population density was significantly lower in the KO compared to WT and HET samples. Moreover, in the HET *Inha* mouse testis sections, the density of macrophages at E13.5 was significantly higher than in those of WT testes, and lower than in KO sections, at E15.5 was significantly lower than WT samples, and at PND0 was significantly higher than KO sections (Figures 3.2a, b and c).

Figure 3.2. The density of macrophages (F4/80$^+$ cells) in *Inhba* and *Inha* mouse testis sections at E13.5 (A, a), E15.5 (B, b) and PND0 (C, c). WT (open circles); HET (grey circles); KO (black circle). Each dot shows the value obtained from counting three sections from one individual testis sample; lines show mean and SD. Analysis was performed by Mann-Whitney U test, two-tailed. *= p<0.05, **= p <0.01, ***= p<0.001. Each value was represented following normalisation to the sample cross-sectional area (μm^2).



The next comparison examined macrophage density in WT *Inhba* and *Inha* mouse testis samples to determine whether the background strain makes a difference. Despite the fact that these mice have been backcrossed onto a C57BL6/J background for over a decade because they were originally derived from a 129S6/SvEv background (Matzuk et al., 1995). In addition, embryos and pups during their fetal life were exposed

to a HET placenta and mother with altered activin A levels. As a result, it was important to check whether WTs in each model were identical to WTs of another model.

Also, to study the contrasting impact of activin A deficiency and elevation on macrophage density, HET and KO *Inhba* and *Inha* mouse testis sample values were graphed together for ready comparison in Figure 3.3, with histological images shown in Figures 3.4 and 3.5. The density of macrophages in WT and KO *Inha* mouse testis sections was significantly higher compared to WT and KO *Inhba* mice, and there was no difference in density between the HET samples from both mouse strains at E13.5 (Figure 3.3A). At E15.5, the density of macrophages in WT *Inha* mouse testis sections compared to WT *Inhba* samples was significantly higher, and there was no difference in HET samples. The macrophage of density was increased in some KO *Inha* samples compared to KO *Inhba* sections at this age (Figure 3.3B). There was no significant difference in the total density of macrophages in WT, HET and KO *Inha* mouse testis sections compared to *Inhba* samples at PND0 (Figure 3.3C).

Figure 3.3. Comparison of macrophages (F4/80$^+$ cells) density in *Inhba* and *Inha* mouse testis sections at E13.5 (A), E15.5 (B) and PND0 (C). WT: open circles; HET: grey circles; KO: black circle. Each dot shows one individual sample; lines show mean and SD. Analysis was performed by Mann-Whitney U test, two-tailed, *= p<0.05, **= p<0.01, ***= p<0.001. Each value was represented following normalisation to the sample cross-sectional area (μm^2). <0.01, ***= p<0.001.

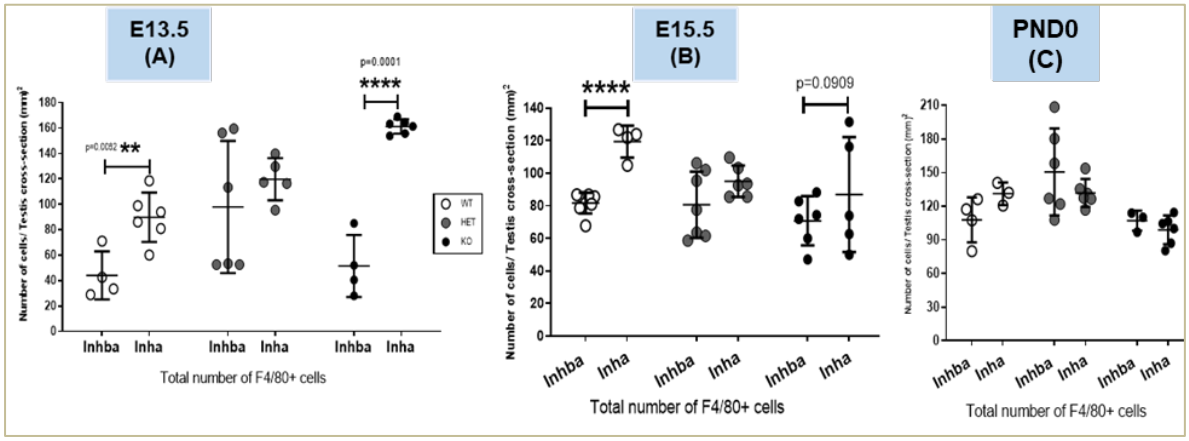


Figure 3. 4. Comparison of F4/80^{+} cells in WT *Inha* and *Inhba* testes at E15.5. The total number of macrophages in WT *Inha* mouse testis sections compared to WT *Inhba* samples was significantly higher. Black arrowheads show all macrophages in interior areas on testis. WT: wild type.

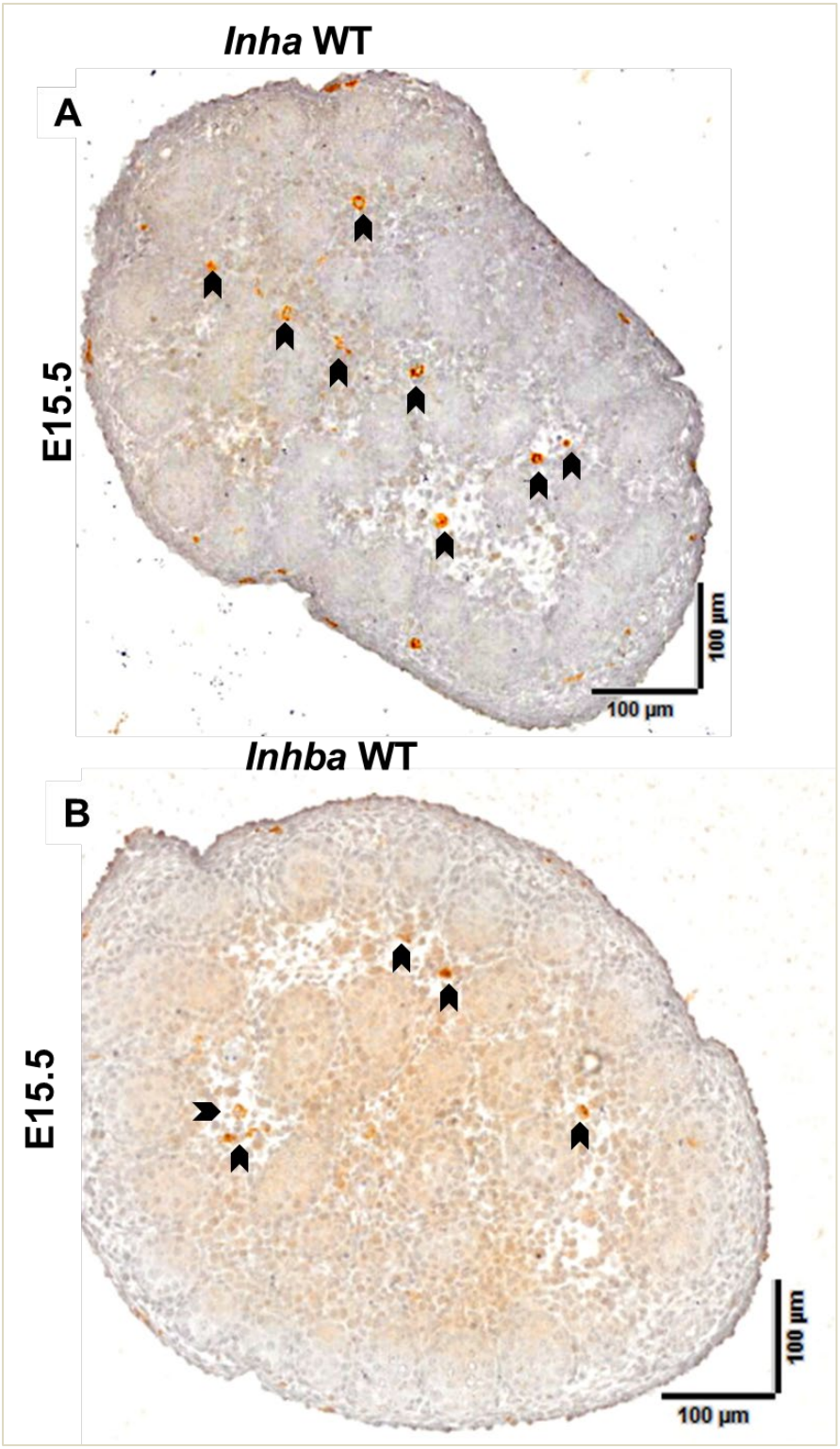
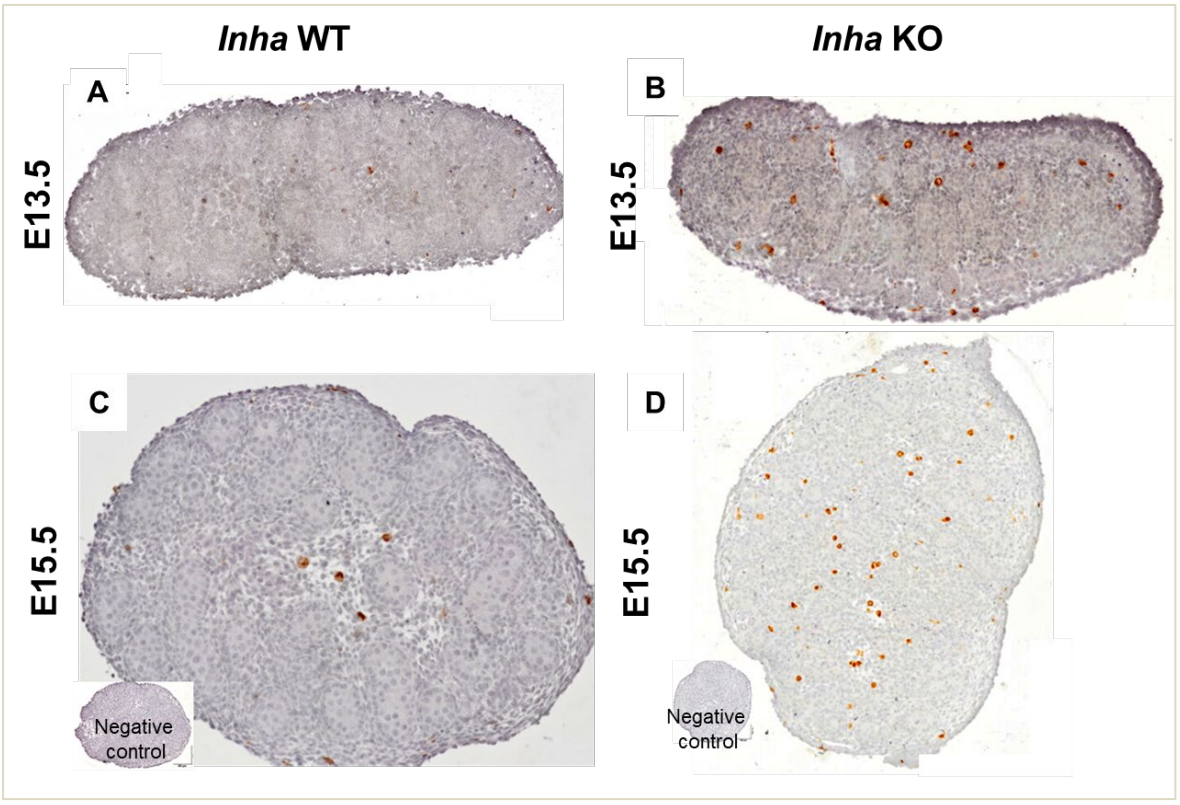


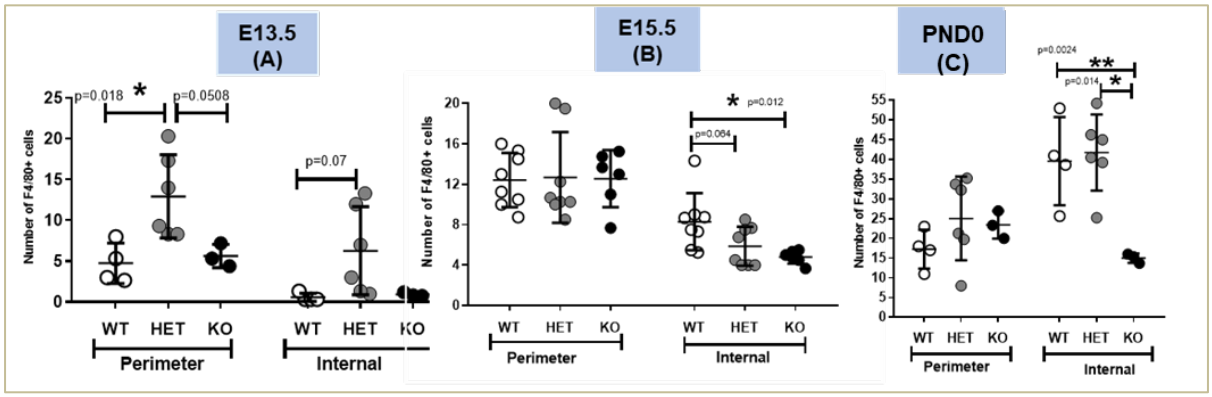
Figure 3.5. Comparison of F4/80^{+} cells in *Inha* WT and KO mouse testes at E13.5 (A and B) and E15.5 (C and D). The macrophage number was higher in KO *Inha* samples compared to WT *Inha* sections. Each panel corresponds to an individual animal**.** Insets show lack of signal in negative control sections lacking primary antibody.



3.3.2. F4/80^{+} cell counts in perimeter and interior areas in E13.5, E15.5, and in PND0 *Inhba* and *Inha* mouse testis sections

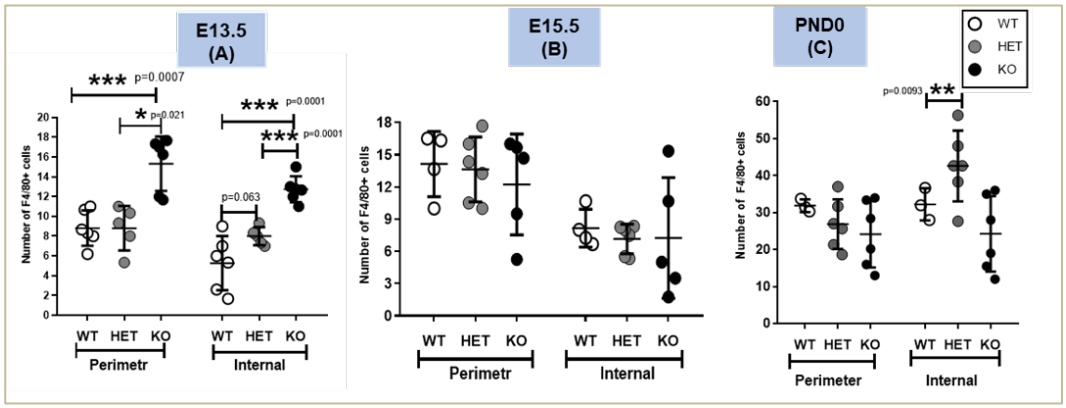
The distribution pattern of testicular macrophages during fetal life was determined by comparing their numbers in perimeter and interior areas in WT, KO and HET *Inha* and *Inhba* mouse testis sections (Figures 3.6- 3.9). The number of macrophages in the perimeter area did not differ between WT and KO sections but was significantly higher in HET samples at E13.5. There was no significant difference in macrophage numbers in the interior area between any samples, however the number was generally higher in most HET samples (Figure 3.6A). The number of macrophages in the perimeter area did not differ between WT, HET and KO sections, at E15.5. In contrast, macrophage number was significantly lower in interior area of the *Inhba* KO samples compared to the WT sections (Figure 3.6B). Significantly fewer macrophages were detected in the interior area of the KO testis sections compared to WT and HET samples in the *Inhba* mouse testis at PND0 (Figure 3.6C).

Figure 3.6. F4/80$^+$ cell counts in perimeter and interior areas in *Inhba* mouse testes at E13.5 (A), E15.5 (B) and PND0 (C). WT: open circles; HET: grey circles; KO: black circle. Each dot shows one individual sample; lines show mean and SD. Analysis was performed by Mann-Whitney U test, two-tailed, *= p<0.05, **= p<0.01, ***= p<0.001.



The number of macrophages in perimeter and interior areas of the KO testis sections was significantly higher compared to the HET and WT samples at E13.5 (Figure 3.7 A). There were no significant differences in the number of macrophages in perimeter and interior areas in the WT, HET and KO *Inha* mouse testis sections at E15.5 (Figure 3.7 B). The number of macrophages in the perimeter area did not differ between the WT, HET and KO *Inha* mouse testis sections, at PND0, but there was significantly higher number of macrophages in the HET samples compared to the WT sections (Figure 3.7 C).

Figure 3.7. F4/80$^+$ cell counts in perimeter and interior areas in *Inha* mouse testes at E13.5 (A), E15.5 (B) and PND0 (C). WT: open circles; HET: grey circles; KO: black circle. Each dot shows one individual sample; lines show mean and SD. Analysis was performed by Mann-Whitney U test, two-tailed, *= p<0.05, **= p<0.01, ***= p<0.001.



To study the impact of activin A on the distribution pattern of testicular macrophages during testis development, the density of macrophages in perimeter and interior areas in *Inhba* and *Inha* mice testis sections was compared (Figure 3.8). There was a significantly higher density of macrophages in perimeter and interior areas in the KO

and WT *Inha* mouse testis sections compared to *Inhba* mice at E13.5 Figure (3.8A). There was a significantly higher density of macrophages in interior area in the KO *Inha* mouse testis sections compared to *Inhba* mice at 15.5. There were no significant differences in the density of macrophages in perimeter area (3.8A). There was significantly higher density of macrophages in perimeter area in the WT *Inha* mouse testis sections compared to *Inhba* mice at PND0. There were no significant differences in the density of macrophages in interior area (3.8A).

Figure 3.8. **Comparing of F4/80⁺ cells in *Inhba* and *Inha* mouse testes at E13.5 (A), E15.5 (B) and PND0 (C)**. WT: open circles; HET: grey circles; KO: black circle. Each dot shows one individual sample; lines show mean and SD. Analysis was performed by Mann-Whitney U test, two-tailed, *= p<0.05, **= p<0.01, ***= p<0.001.

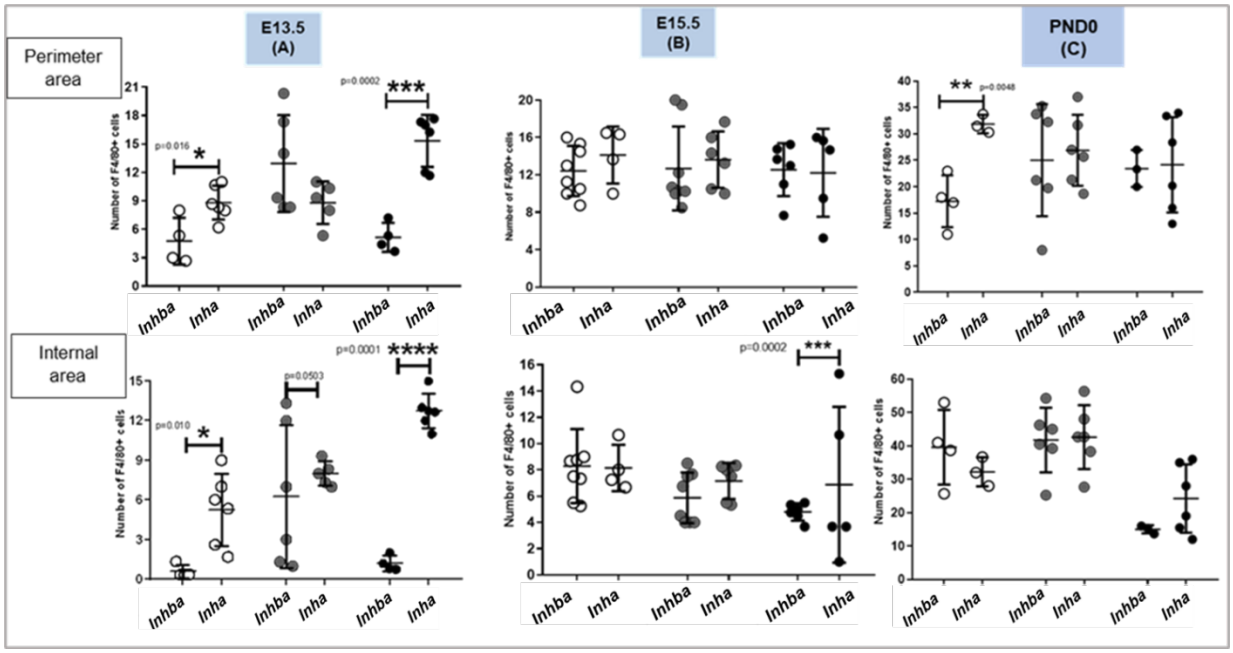
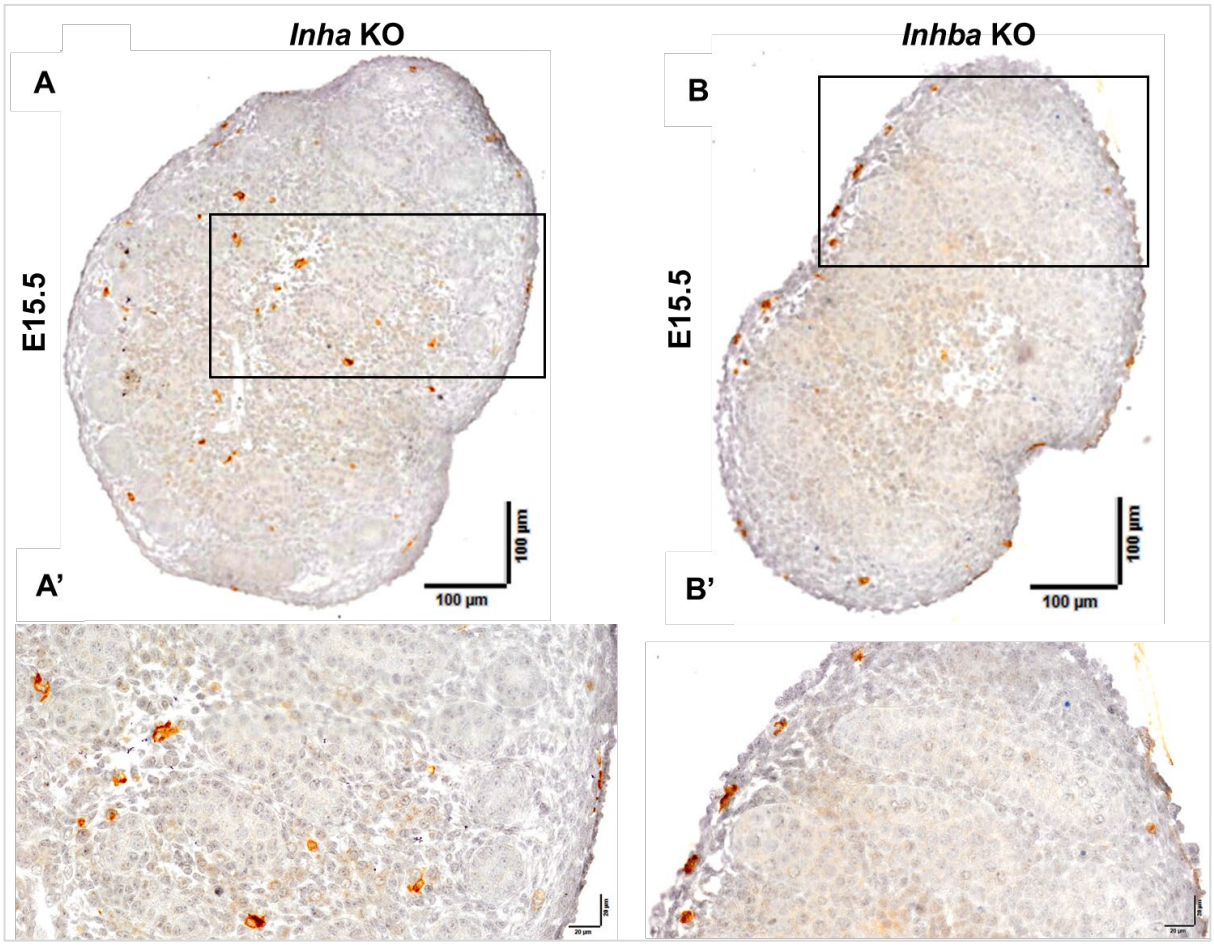
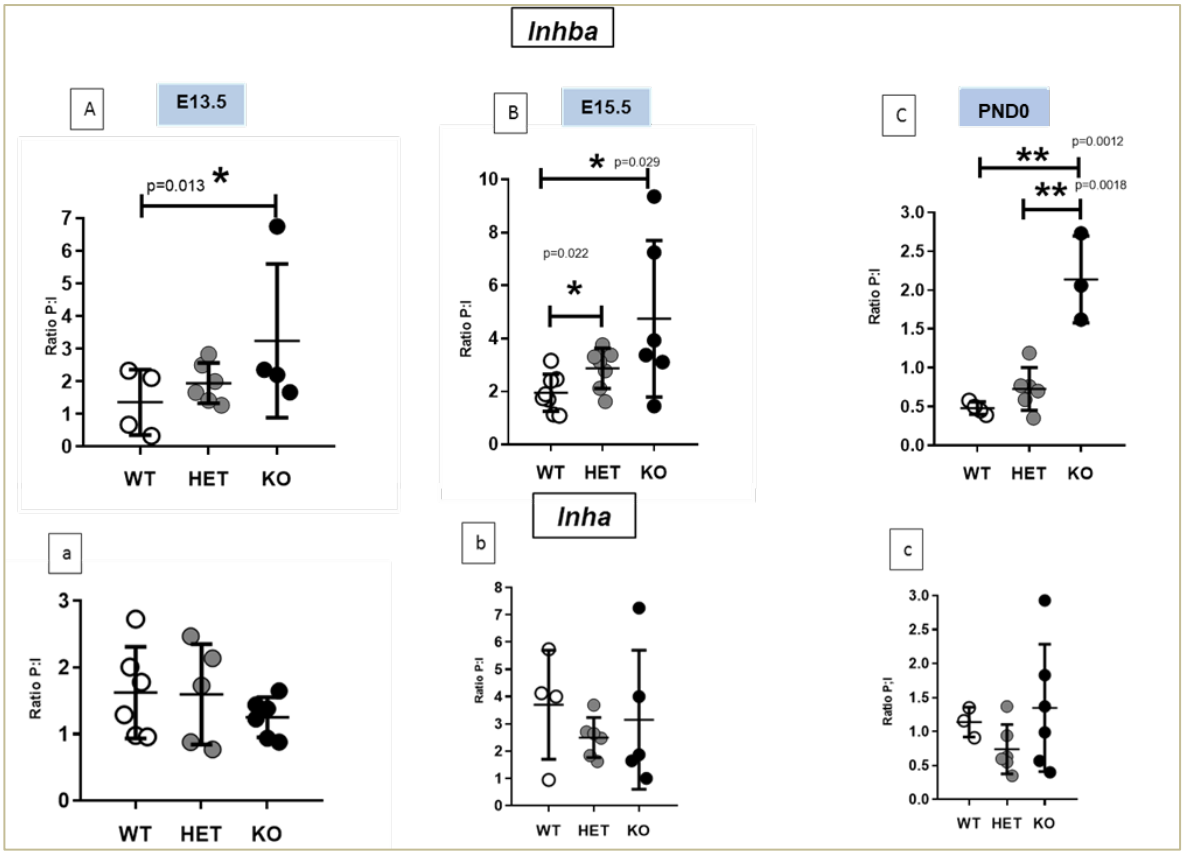


Figure 3.9. Comparison of F4/80^{+} cells in KO *Inha vs Inhba* testes at E15.5 using IHC method. There was significantly higher density of macrophages in perimeter and interior areas in the *Inha* KO mouse testis sections compared to *Inhba* KO mouse at E15.5. Black boxes in high magnification panels refer to adjacent low magnification images (A' and B')



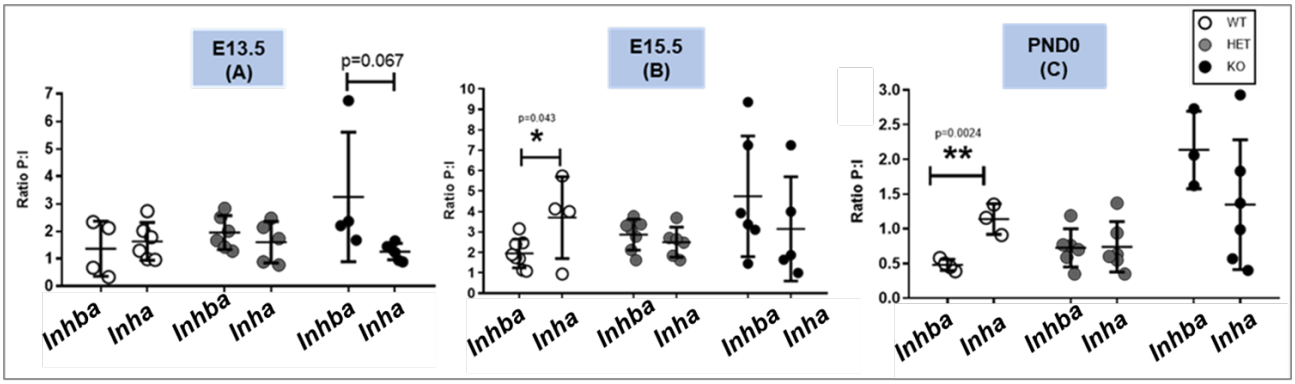
The P: I macrophage ratio in the KO *Inhba* mouse testis was significantly higher at E13.5 compared to WT mice (Figure 3.10 A). The P: I macrophage ratio at E15.5 was significantly lower in sections of WT *Inhba* testes *vs* the HET and KO samples (Figure 3.10 B). The P: I macrophage ratio was significantly higher at PND0 in KO *Inhba* testes compared to HET and KO samples (Figure 3.10 C). There was no significant difference in the P: I macrophage ratio in in WT, HET and KO *Inha* mouse testis sections at E13.5, E15.5 and PND0 (Figures 3.10 a,b and c).

Figure 3.10. **Comparison of macrophage distribution between perimeter and interior areas (P: I macrophage ratio) in *Inhba* and *Inha* mouse testis sections at E13.5 (A, a), E15.5 (B, b) and PND0 (C, c)**. WT: open circles; HET: grey circles; KO: black circle. Values plotted represent the ratio of F480+ cells in perimeter versus interior areas (Ratio P: I). Each dot shows one individual sample; lines show mean and SD. Analysis was performed by Mann-Whitney U test, two-tailed, *= p<0.05, **= p<0.01, ***= p<0.001.



There were no significant differences in P: I macrophage ratio in *Inhba* WT, HET and KO mouse testis sections compared to *Inha* samples at E13.5 (Figure 3.11A). This ratio was significantly higher in *Inha* WT mouse testis sections compared to *Inhba* samples at E15.5, though there were no significant differences in HET or KO samples (Figure 3.11B). The P: I macrophage ratio at PND0 was significantly higher in *Inha* WT mouse testis sections compared to *Inhba* samples, and there were no significant differences in the HET and KO samples (Figure 3.11C).

Figure 3.11. P: I macrophage ratio in ***Inhba*** **and** ***Inha*** **mouse testis sections at E13.5 (A), E15.5 (B) and PND0 (C).** WT: open circles; HET: grey circles; KO: black circle. Each dot shows one individual sample; lines show mean and SD. Analysis was performed by Mann-Whitney U test, two-tailed, *= p<0.05, **= p<0.01, ***= p<0.001.



3.3.3. Analysis of transcripts encoding immune cell markers in fetal and newborn *Inhba* and *Inha* WT and KO mouse testes

Levels of transcripts encoding a broad selection of markers linked with immune cells, and with macrophage functional properties in particular, were measured in whole testes collected from both mouse strains. The outcomes are presented in a summary Table 3.4 and are described below individually (3.3.4.1). Their significance is addressed in the Discussion section

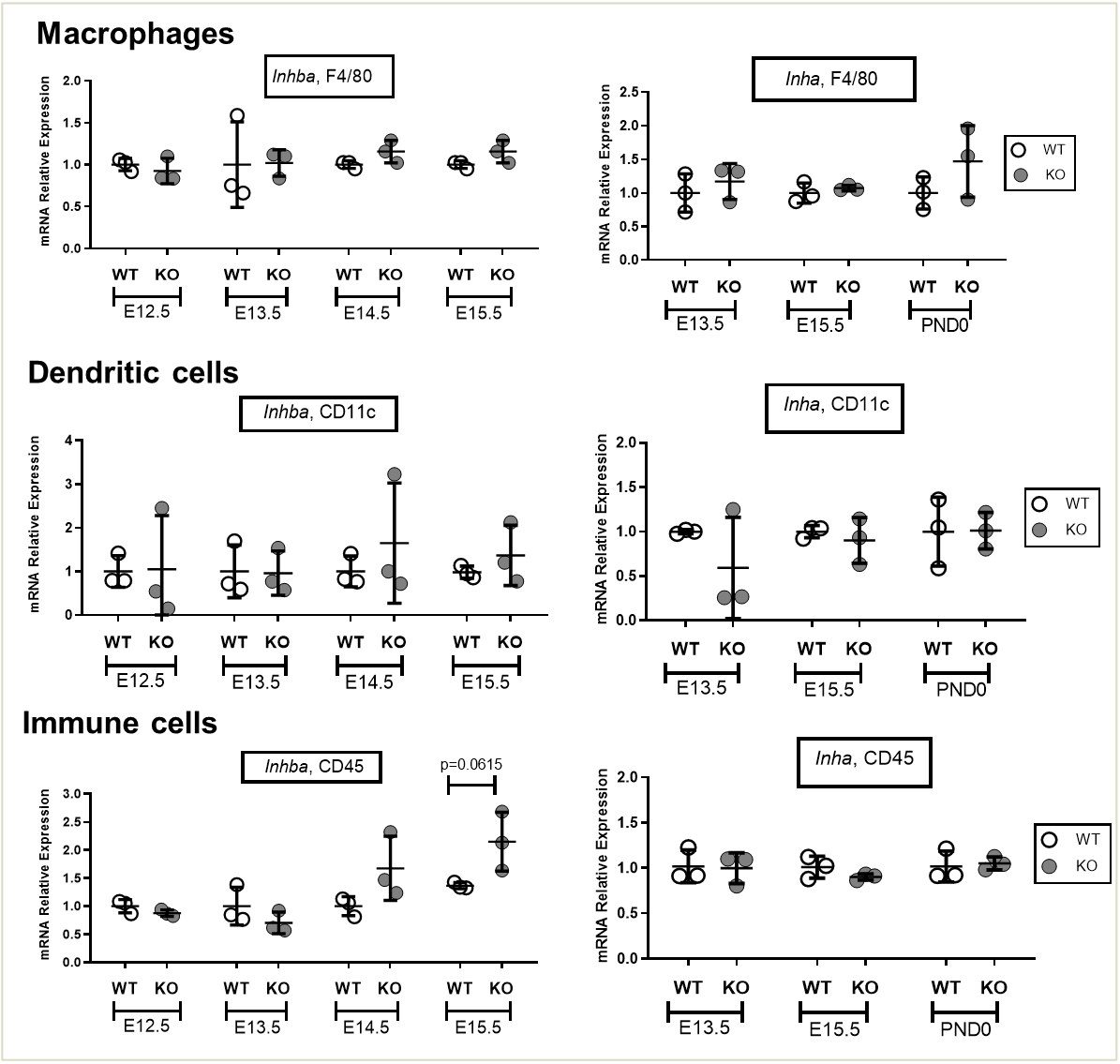
Measurement of mRNAs encoding a pan-macrophage marker (F4/80), a pan-dendritic cell marker (CD11c), and a pan-immune cell marker (CD45) was undertaken (Figure 3.12). This identified no significant differences between KO and WT samples from *Inhba* and *Inha* mouse testes at each individual age. Therefore, any changes identified in level of transcripts encoding immune markers/factors in *Inhba* and *Inha* KO mouse testes were assumed to be associated with a generally altered phenotype and function of immune cells in the testis, rather than relate to their relative frequency.

Table 3.4. Summary of the levels of mRNAs encoding in *Inhba* and *Inha* knockout mouse (KO, $^{-/-}$) testes compared to those in wild type (WT, $^{+/+}$). littermates from the Fluidigm analysis. Transcripts were investigated using Fluidigm analysis (qRT/PCR). The peach columns: RNA sequencing data (count per million) of testis somatic cells from Whiley et al., 2020. The remaining columns represent outcomes from Fluidigm analysis of whole testes from indicated strains and ages. Statistical significance was measured using unpaired T-test, two-tailed, *= p<0.05, **= p <0.01, ***= p<0.001. Additional notation used to denote a considerable, but non-significant, difference, c= p≥ 0.1 and <0.05. L= lower in KO *vs* WT, H= higher in KO *vs* WT. Yellow highlight indicates opposite responses between the two genotypes.

Purpose	Marker/Factor	*Inhba*		*Inhba*				*Inha*		
		E13.5	E15.5	E12.5	E13.5	E14.5	E15.5	E13.5	E15.5	PND0
Pan-Leukocyte marker	CD45	*H					cH			
Pan-Dendritic cell marker	CD11c									
Pan-macrophage marker	F4/80									
Important markers on testicular macrophages	CSF1							**H		
	CX3CR1	*H			*L					
	CX3CL1		***L		*L	*L	***L	**H	*H	
Anti-inflammatory macrophage markers/ mediators	Arginase 1			* L				*L		cH
	CD206	*H	**H							
	IL-4 Receptor	**H	***H		**H			*L		
	IL-10	*H			-					
	IL-10 receptor		*H			*H	*H			cL
	CCl17/ TARC	*L	***L		*L	**L	*L	***H	**H	*H
	CXCR7		cH		cL			*H		
	CCR4 (CD194)	-	-					cL		
	CXCL12		*H							
	CXCR4	*H		cH			cH	*L		
	CD16	**H	*H							

Pro-inflammatory macrophage markers/ mediators	CD64									
	CD68									[c]H
	MHC class II				*L	*L	*L		**H	
	ICAM-1	***** H		*H	[c]L					
	TLR4 (CD284)		**H							
	CD38	**H				*H	*H			
	Marco	-	-			[c]H			[c]L	
	IL-1b receptor 1				**L	[c]H				
	IL-1β	[c]H	[c]H						[c]H	
	Retinoic Acid Receptor Alpha							*L		
	GM-CSF receptor	**H					[c]H			
Involved in macrophage polarisation	CCR2, (CD192)	[c]H					[c]H			
	CCL2 (MCP-1)					*H		**H	[c]H	
Involved in tissue remodelling	MMP2				[c]L		[c]L	*H		
	MMP15	**H	****H	[c]L		[c]H	*H		[c]L	
	MMP23	**H								

Figure 3.12. Levels of transcripts encoding F4/80, CD11c and CD45 in *Inhba* and *Inha* WT and KO mouse testes. WT: open circles; KO: black circle. Each dot shows one individual sample; lines are at mean and SD; statistical significance was measured using an unpaired T-test, two-tailed, *= p<0.05. Transcript levels are shown normalized to *Canx* and *Mapk1*.



The next two sections present data are categorised in relationship to macrophages with either anti-inflammatory (M2) or pro-inflammatory (M1) phenotypes (for explanations on M1 and M2 phenotypes of macrophages please refer to Introduction Chapter, 4.2.1). The function of individual markers and factors are described.

3.3.3.1. Anti-inflammatory markers/ factors associated with an M2 phenotype

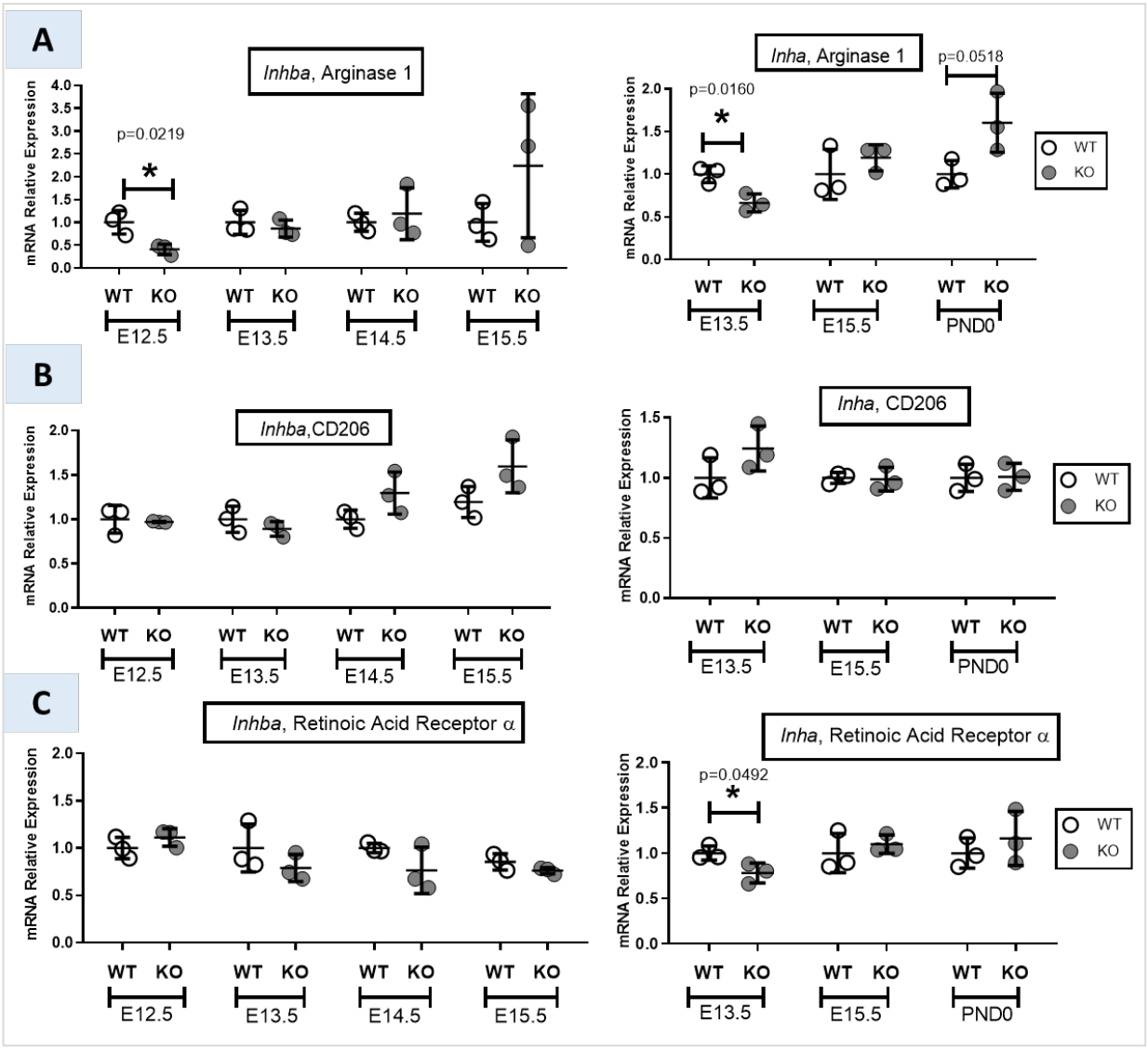
Arginase 1: Type-I arginase (Arg-1), is an enzyme involved in l-arginine/nitric oxide (NO) metabolism, and it is regarded as one of the key markers of the M2 phenotype.

Activation of arginase 1 blocks iNOS activity and therefore inhibits inflammation and fibrosis (Yang and Ming, 2014). We detected a significantly lower mRNA *Arg-1* level in both the *Inhba* KO at E12.5 and in *Inha* KO at E13.5 (Figure 3.13 A).

CD206:CD206 also known as macrophage mannose receptor 1, (C-type mannose receptor 1; MRC1) is a scavenger receptor expressed predominantly by most resident tissue macrophages and dendritic cells. The protein functions in endocytosis and phagocytosis, thereby playing important roles in immune homeostasis. It is considered as an M2 marker for its functional roles in regulating inflammation (Azad et al., 2014). We did not detect any significant differences between *Inhba* and *Inha* KO and WT samples (Figure 3.13 B).

Retinoic Acid Receptor alpha (α) (Rara): Retinoic acid (RA) is a morphogen derived from retinol (vitamin A) produced by a number of cell types, including macrophages and dendritic cells. RA is transcriptional activator that binds to intracellular retinoic acid receptors (RARs) and regulates gene expression via RA-responsive elements. Three RAR isoforms have been described: RARα, -β, and –γ. RA plays crucial roles in biological process including cell growth, cell proliferation, differentiation, organogenesis and reproduction. Also, RA plays key roles in immunomodulatory and anti-inflammatory activities for equilibrating immunity and tolerance. RA modulates inflammation by promoting M2 macrophage polarisation, enhancing the differentiation of Foxp3+ inducible regulatory T cells (iTregs), inhibiting the differentiation of naive T cells into Th17 cells, promoting effector T cell responses during infection or autoimmune diseases, and decreasing pro-inflammatory cytokines while at the same time increasing anti-inflammatory proteins such as IL-10 (Oliveira et al., 2018; Yu et al., 2017; Kim, 2011). Thus, RA plays roles in immune homeostasis in the steady state, but also activates pathogenic T cells in conditions of inflammation (Raverdeau and Mills, 2014). We identified a significantly lower mRNA *Rarα* level in the *Inha* KO at E13.5, and no changes in the *Inhba* KO compared to the WT samples (Figure 3.13 C). This outcome may reflect a change in immune cells or in another cell type, as RARs are not specific markers for immune cells.

Figure 3.13. The levels of mRNAs encoding Arginase 1, CD206 and Retinoic Acid Receptor α in *Inhba* and *Inha* WT and KO mouse testes. WT: open circles; KO: black circle. Each dot shows one individual sample; lines are at mean and SD; statistical significance was measured using unpaired T-test, two-tailed, *= p<0.05.

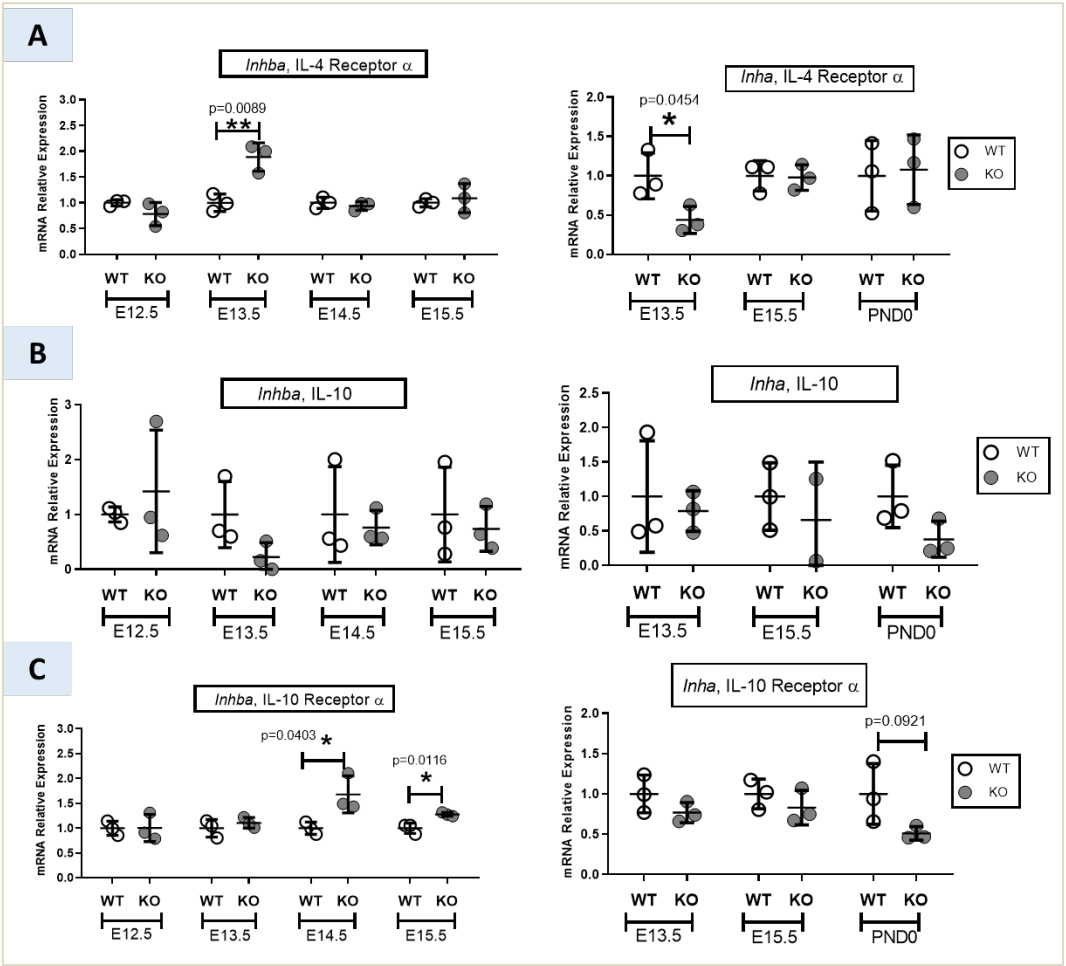


Interleukin 4 Receptor Alpha (IL4 Rα): One of the crucial events for the generation of a Th2-dominated early immune response and alternatively activated macrophages (M2) is binding of the Interleukin 4 (Il4) cytokine to Interleukin 4 receptor alpha (IL4Rα) (Weng et al., 2018; Ferrante et al., 2013). Interestingly, we identified that the level of *IL4Rα* transcript was changed in relationship to the expected levels of activin A bioactivity; in *Inhba* KO, the *IL4Rα* mRNA was significantly higher, and in *Inha* KO it was lower, indicating that activin A reduces levels of this transcript. In other samples there was not any significant change (Figure 3.14 A).

Interleukin 10 (IL10): IL10 is an anti-inflammatory cytokine that plays essential roles in control of immune responses and resolution of inflammation (Ip et al., 2017). We did not detect any significant differences between *Inhba* and *Inha* KO and WT samples (Figure 3.14 B).

Interleukin 10 Receptor Alpha (IL10 Rα): IL10 Rα mediates the immunosuppressive signal of interleukin 10 which inhibits the synthesis of pro-inflammatory cytokines (Riley et al., 1999). There was a significantly higher level of *IL10 Rα* mRNA in *Inhba* KO samples compared to the WT at E14.5 and E15.5, while it was considerably lower in *Inha* KO PND0 compared to WT samples (Figure 3.14 C).

Figure 3.14. The levels of mRNAs encoding Interleukin 4 receptor alpha (IL4Rα) (A), Interleukin 10 (IL10) (B) and Interleukin 10 receptor alpha (IL10Rα) (C)in *Inhba* and *Inha* WT and KO mouse testes. WT: open circles; KO: black circle. Each dot shows one individual sample; lines are at mean and SD; statistical significance was measured using unpaired T-test, two-tailed, *= p<0.05, **= p <0.01.

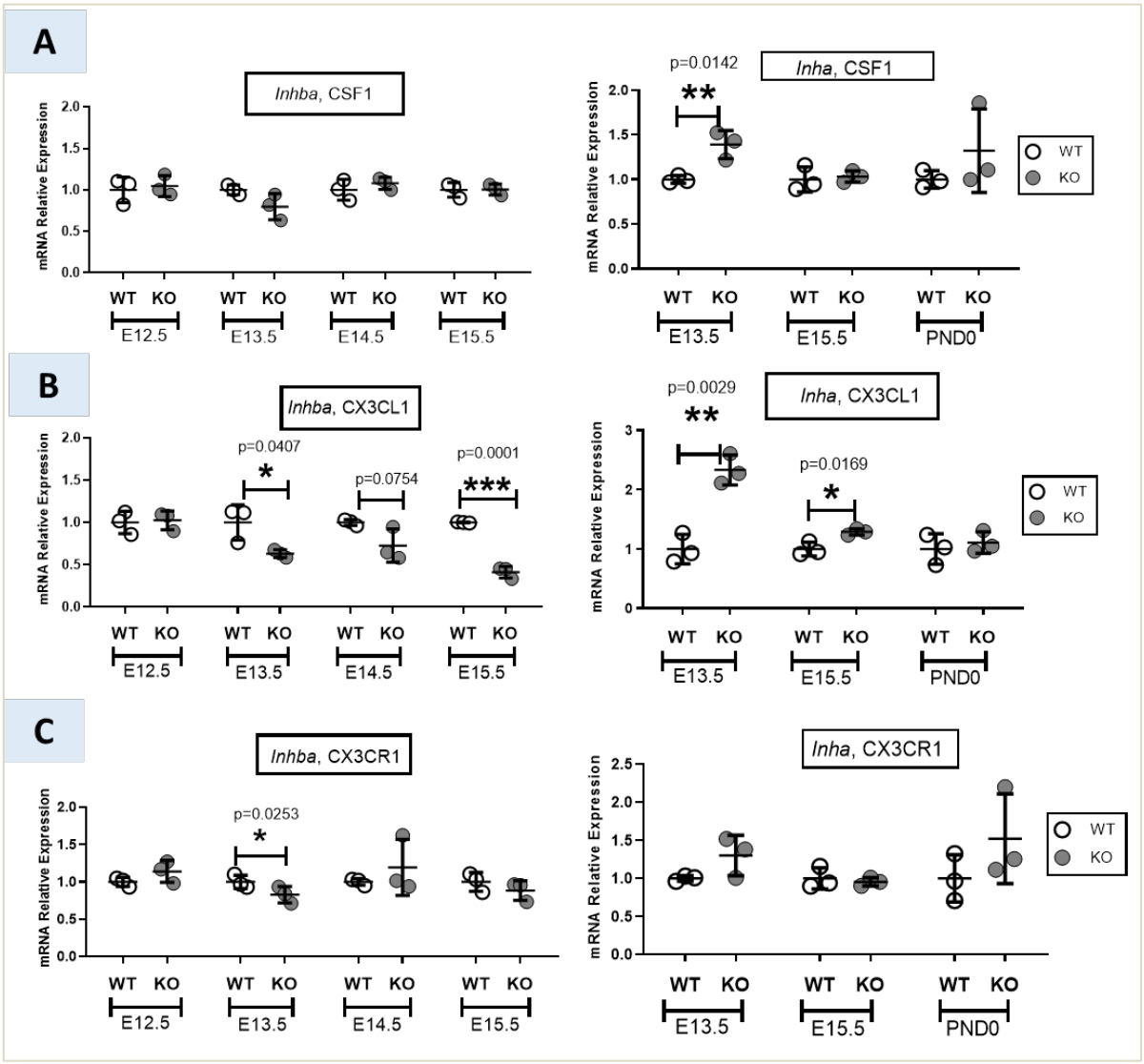


Colony stimulating factor 1 (CSF1): CSF1, also known as macrophage colony-stimulating factor (M-CSF), is a secreted cytokine which causes differentiation of hematopoietic stem cells into macrophages. In addition, CSF-1 regulates an alternative macrophage activation state involved in organ development and repair (Jones et al., 2013). An important function of CSF1 in the testis is to promote proliferation of spermatogonial stem cells (Kokkinaki et al., 2009; Oatley et al., 2009). In adult mouse testis, interstitial macrophages contain a relatively higher levels of CSF1 protein, while peritubular macrophages have lower levels (DeFalco et al., 2015; Mossadegh-Keller, 2017). Although there were not any significant differences in the level of *Csf1* mRNA in *Inhba* samples, a significantly higher level was detected in *Inha* KO mouse testes at E13.5 (Figure 3.15A).

CX3CL1: CX3CL1 (also known as fractalkine) is a cellular adhesion molecule that attracts and activates monocytes and macrophages (Habasque et al., 2003). The two known testicular macrophage subtypes, interstitial and peritubular, both express CX3CL1 receptor, (CX3CR1) (DeFalco et al., 2015; Mossadegh-Keller and Sieweke, 2018). Our data showed the *Cxcl1* mRNA levels changed in relationship to the expected levels of activin A bioactivity; there was a significantly lower level of *Cxcl1* mRNA in *Inhba* KO mouse testes at E13.5 and E15.5, while there was a significant increase in *Inha* KO testes at E13.5 and E15.5 (Figure 3.15 B).

The Fractalkine (CX3CL1) receptor (CX3CR1): CX3CR1 is widely expressed on interstitial and peritubular testicular macrophages. The transient ablation of testicular macrophage populations using a Cx3Cr1-Cre; Rosa-iDTR system resulted in decreased spermatogonial differentiation, which highlights the key roles of macrophages in the normal testis morphogenesis and function (DeFalco et al., 2014 and 2015). We detected significantly a lower mRNA *Cx3cr1* level in the *Inhba* KO at E13.5, and there were not any significant differences in the relative levels detected'in *Inha* samples at E13.5, E15.5 and PND0 (Figure 3.15 C).

Figure 3.15. The levels of mRNAs encoding colony stimulating factor 1 (CSF1) (A), CX3CL1 (B) and CX3CR1 (C) in *Inhba* and *Inha* WT and KO mouse testes. WT: open circles; KO: black circle. Each dot shows one individual sample; lines are at mean and SD; statistical significance was measured using unpaired T-test, two-tailed, *= p<0.05, **= p <0.01., ***= p<0.001.



CCL17: Chemokine (C-C motif) ligand 17 (CCL17) (also known as thymus and activation regulated chemokine (TARC) is a small cytokine produced by M2 macrophages; it prevents alternatively activated macrophages from producing an inflammatory response, (Saeki et al., 2006). Our data showed that the level of *Ccl17* mRNA is regulated by activin A levels. It was significantly lower in *Inhba* KO at E13.5, E14.5 and E15.5, and significantly higher in *Inha* KO at E13.5, E15.5 and PND0, compared to their respective WT samples (Figure 3. 16A). It is interesting that production of CCL17 in Sertoli cells has been documented in fetal and newborn mouse testes (Loveland lab, unpublished) and in human adult testes with GCNIS (Szarek et al., 2019).

CCR4: C-C chemokine receptor type 4 (also known as CD194) is a receptor for CCL2 (MCP-1), CCL4 (MIP-1), CCL5 (RANTES), CCL17 (TARC) and CCL22 (Yoshie and Matsushima, 2015). We did not detect any significant differences between *Inhba* and *Inha* KO and WT samples with the exception of *Inha* KO samples at E15.5, when the *Ccr4* mRNA level was lower compared to in the WT samples (Figure 3. 16B). Our own RNAseq data and Fluidigm values indicate the transcript is present at very low levels, hence some samples did not show detectable signal levels.

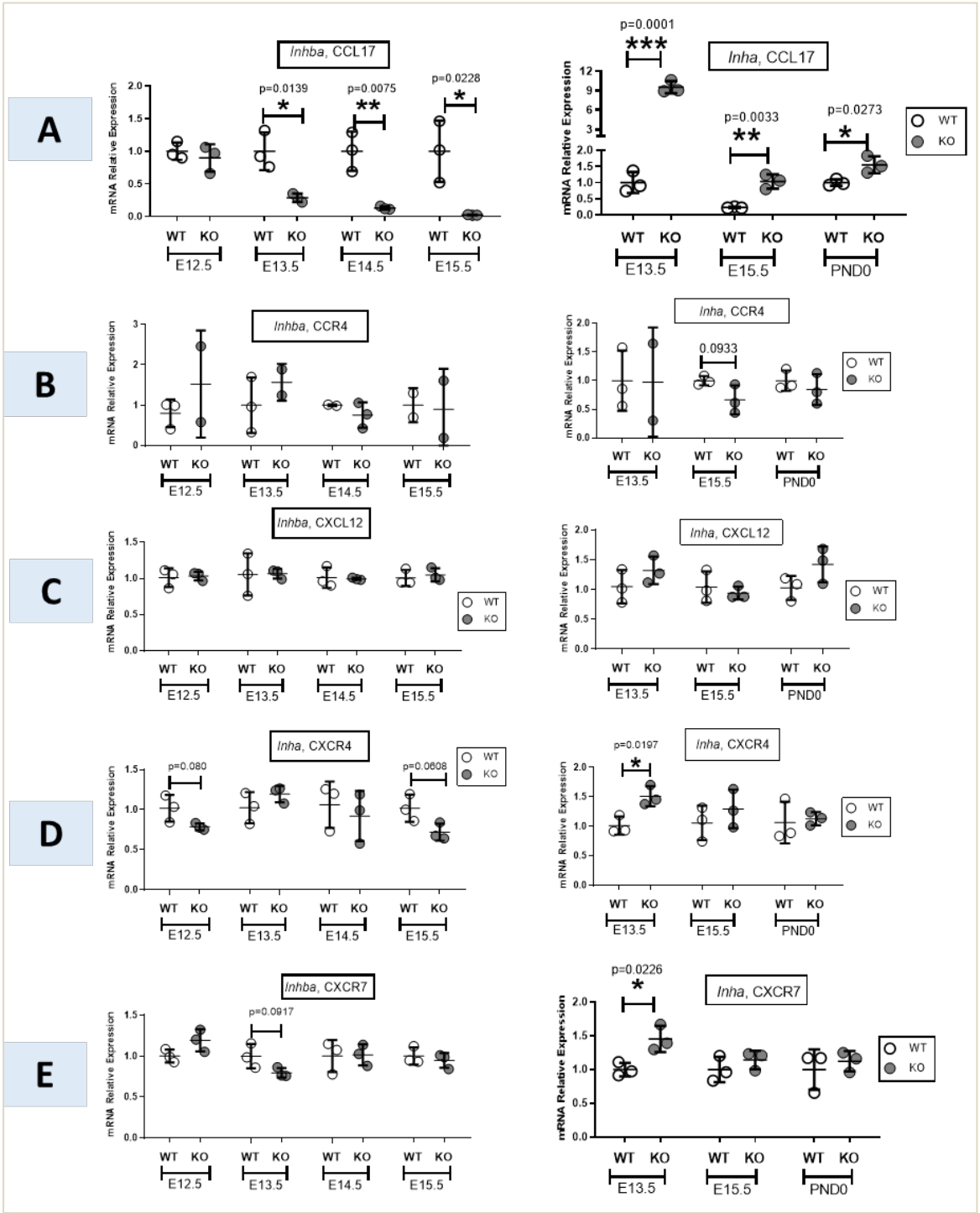
CXCL12 and CXCR4: Signalling by the C-X-C motif chemokine 12 (CXCL12), also known as stromal cell-derived factor 1 (SDF1), via its receptor (CXCR4) plays crucial roles in the homing and retention of hematopoietic stem and progenitor cells (HSPCs). In physiological conditions, low numbers of (HSPCs) constantly circulate from the bone marrow to the blood and back. The CXCL12/CXCR4 axis functions in homeostatic processes instead of the inflammation pathways, and is associated with embryonic development (Döring et al., 2014). CXCL12 has an alternative receptor CXCR7, and the pro-inflammatory chemokine macrophage migration inhibitory factor (MIF) is an important second ligand for CXCR4. CXCR4 is expressed on spermatogonia and Sertoli cells of mouse testis (Yoon et al., 2009; Kanatsu-Shinohara et al., 2012).

We did not detect any significant differences in *Cxcl12* mRNA levels between *Inhba* and *Inha* KO and WT samples. However, there was a considerably higher level of *Cxcr4* in *Inhba* KO testes at each E12.5 and E15.5, and a significantly lower level in *Inha* KO at E13.5 compared to the WT samples (Figure 3.16C and D), indicating that activin A suppresses levels of this transcript.

CXCR7: CXCR7 is an alternative receptor subunit for CXCL12 with 10-fold higher affinity compared with CXCR4. It is involved in cell survival, adhesion and recruitment of macrophages. CXCR7 regulates CXCL12 availability and concentration, and acts as decoy receptor, reducing acute CXCL12/CXCR4 signalling (Sun et al., 2010; Naumann et al., 2010; Yoon et al., 2009).

We detected an interesting trend of opposite *Cxcr7* mRNA levels between *Inhba* and *Inha* KO samples at E13.5, so that it was considerably lower in *Inhba* KO, while significantly higher in *Inha* KO (Figure 3.16E).

Figure 3.16. The levels of mRNAs encoding CCL17 (A), CCR4 (B), CXCL12 (C), CXCR7 (D) and CXCR4 (E) in *Inhba* and *Inha* WT and KO mouse testes. WT: open circles; KO: black circle. Each dot shows one individual sample; lines are at mean and SD; statistical significance was measured using unpaired T-test, two-tailed, *= p<0.05, **= p <0.01., ***= p<0.001.



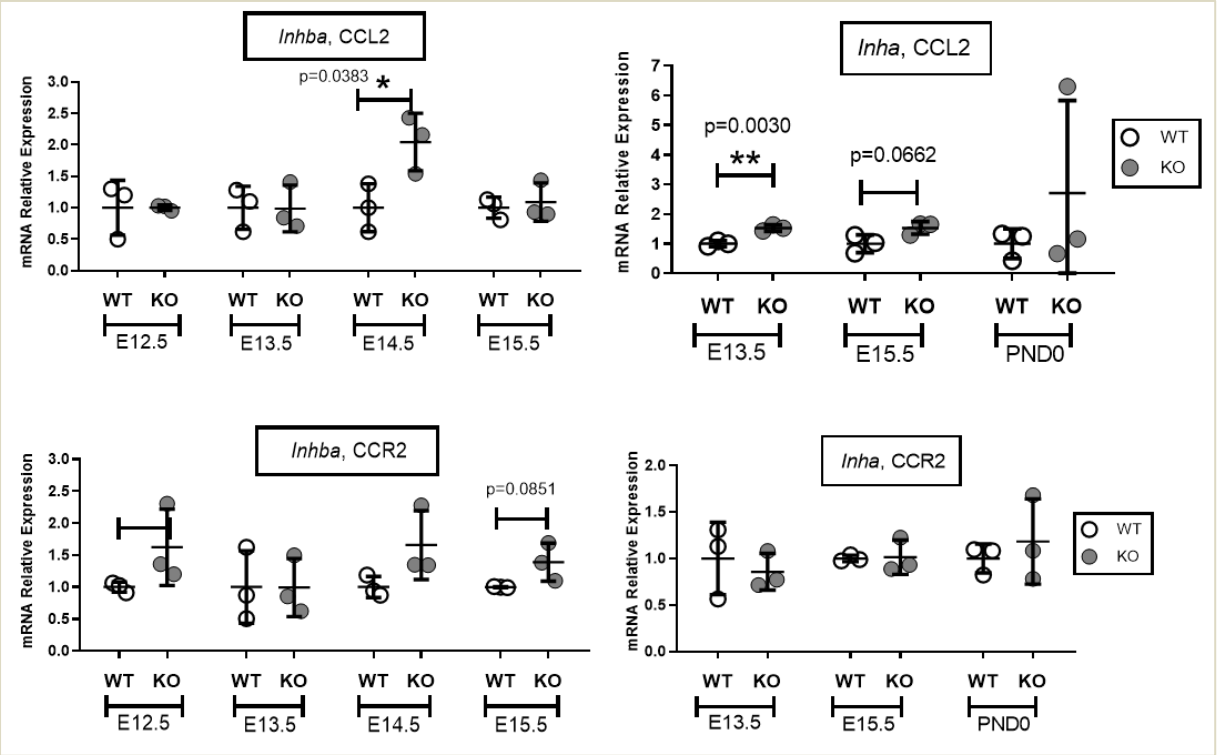
CCL2 (MCP-1) and CCR2: The chemokine (C-C motif) ligand 2 (CCL2), first named as monocyte chemoattractant protein 1 (MCP1), recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or

infection. Both CCR2 and CCR4 cell surface receptors bind CCL2 (Craig et al., 2006). CCR2 is expressed on macrophages, Th1 cells, Th17 cells, and activated microglia; in the latter, its expression is critical for their capacity to cross the blood-brain barrier (BBB). CCR2 has both pro-inflammatory (mediated by APC and T cells) and anti-inflammatory (mediated by regulatory T cells) effects (Zhang et al., 2010). CCL2 is the first chemokine identified in the testis was which is implicated in regulation of the large testicular macrophage population (Aubry et al., 2000)

CCL2 is one of the key chemokines that regulates macrophage polarisation, recruitment and infiltration during inflammation (Deshmane at al., 2009). M1/M2 polarisation of macrophages involves CCR2 signalling; CCL2 blockade leads to enhanced expression of M1 polarisation-associated genes and cytokines, and diminished expression of M2-associated markers in macrophages. Therefore, the CCL2/CCR2 axis regulates macrophage polarisation by influencing the expression of functionally relevant and polarisation-associated genes and down-modulating pro-inflammatory cytokine (TNF-α, IL-6) production. Activin A controls the expression of the CCL2/CCR2 pair in macrophages, as activin A increases CCR2 expression but inhibits the acquisition of CCL2 expression by M-CSF-polarised macrophages *in vitro* (Sierra-Filardi et al., 2014; Carson et al., 2017).

CCR2- macrophages are a tissue-resident population exclusively replenished through local proliferation, whereas CCR2+ macrophages are maintained through monocyte recruitment and proliferation. Moreover, CCR2- and CCR2+ macrophages have distinct functional properties (Bajpai et al., 2018). We detected a significantly higher *Ccl2* level in both the *Inhba* KO at E14.5 and in *Inha* KO testes at E13.5. We did not detect any significant differences in the *Ccr2* mRNA level between *Inhba* and *Inha* KO and WT samples at any age (Figure 3.17).

Figure 3.17. The levels of mRNAs encoding CCL2 (A) and CCR2 (B) in *Inhba* and *Inha* WT and KO mouse testes. WT: open circles; KO: black circle. Each dot shows one individual sample; lines are at mean and SD; statistical significance was measured using unpaired T-test, two-tailed, *= p<0.05, **= p <0.01.



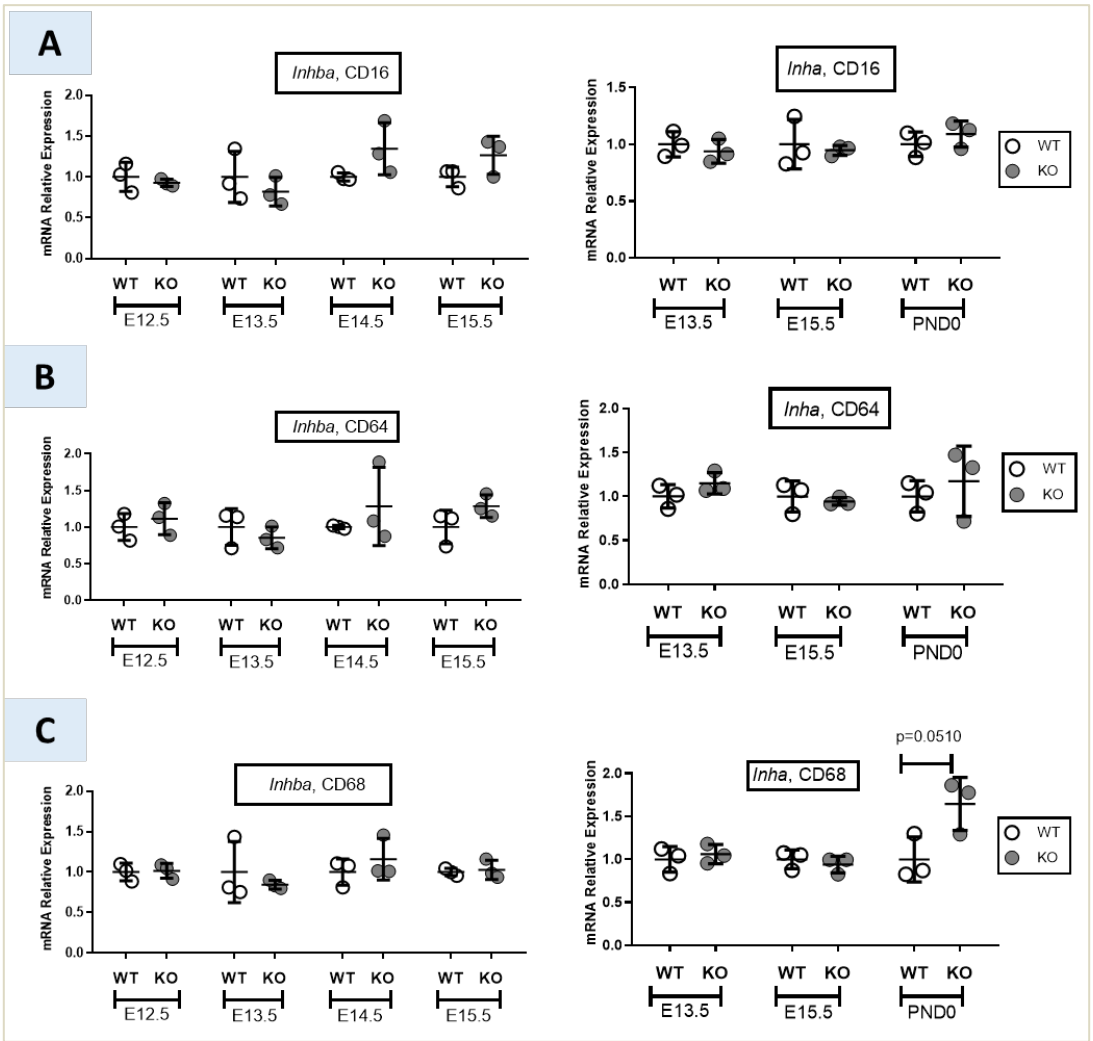
3.3.3.2. Pro-inflammatory Markers/ Factors Associated with an M1 Phenotype

CD16 (FcγRIII) and CD64 (FcγRI): CD16 and CD64 are transmembrane glycoproteins belonging to the large immunoglobulin (Ig) superfamily that bind the monomeric IgGs IgG1 and IgG4 with low and high affinity, respectively' (Lu et al., 2015). CD16 is involved in antibody-dependent cellular cytotoxicity (ADCC). It is expressed at moderate levels on granulocytes and tissue macrophages, and it is present in subsets of monocytes, eosinophils, and dendritic cells. CD64 is constitutively found on only macrophages and monocytes, but treatment of polymorphonuclear leukocytes with pro-inflammatory cytokines such as IFNγ and G-CSF can induce CD64 expression on these cells (Nimmerjahn F and Ravetch J, 2006).

CD68/ Macrosialin: CD68 is a scavenger receptor protein highly expressed by circulating macrophages, and tissue macrophages. It functions to clear cellular debris, promote phagocytosis, and mediate the recruitment and activation of macrophages. CD68 is mainly located in the endosomal/lysosomal compartment but can rapidly

shuttle to the cell surface where it binds oxidatively modified low-density lipoprotein (oxLDL), phosphatidylserine on apoptotic cells. CD68 is not involved in binding bacterial/viral pathogens, innate, inflammatory or humoral immune responses, although it may potentially be involved in antigen processing and presentation (Chistiakov et al., 2017). No significant differences in levels of mRNAs encoding CD16, CD64 and CD68 were measured between KO and WT samples from *Inhba* and *Inha* mouse testes (Figure 3.18 A-C), however the level of CD68 trended higher in the PND0 *Inha* KO.

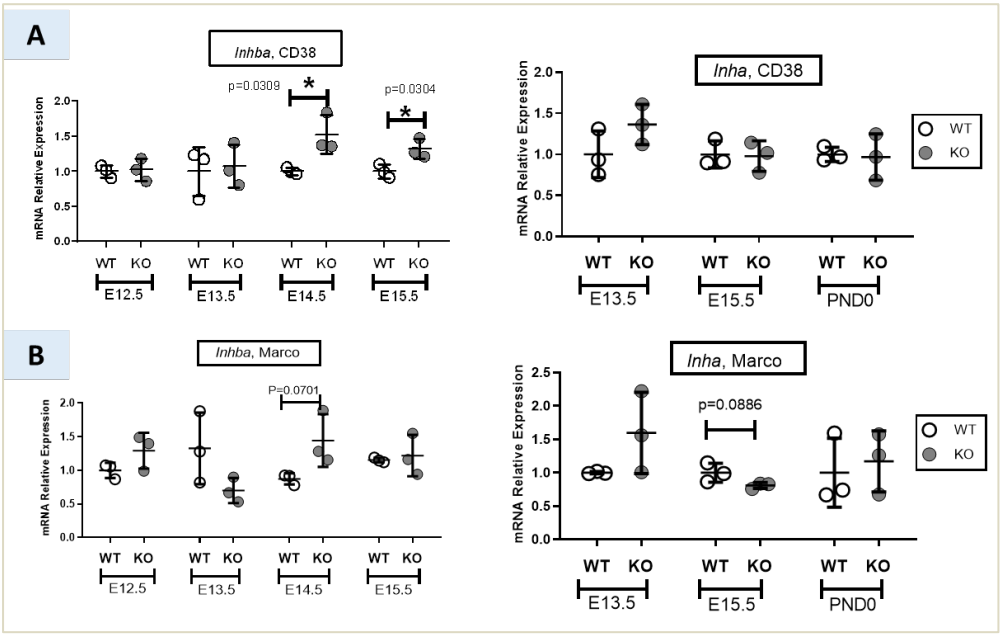
Figure 3.18. The levels of mRNAs encoding CD16 (A, CD64 (B) and CD68 (C) in *Inhba* and *Inha* WT and KO mouse testes. WT: open circles; KO: black circle. Each dot shows one individual sample; lines are at mean and SD; statistical significance was measured using unpaired T-test, two-tailed, *= p<0.05.



CD38: CD38 (also known as cyclic ADP ribose hydrolase) is found on the surface of many immune cells, including macrophages, T and B lymphocytes, and natural killer cells. CD38 functions in cell adhesion (mediates a selectin-like binding to endothelial cells), modulating signal transduction and calcium signalling, associated with impaired immune responses (Kumagai et al., 1995). Furthermore, CD38 is expressed by most (71%) M1 macrophages *(*in *vitro* and in *vivo*), with close to a 30-fold higher level compared to resting macrophages (M0). Therefore, the use of CD38 provides an advantage over the classical iNOS, Arginase-1 and CD206 phenotype markers to discriminate between M1, M2 and M0 macrophages (Jablonski et al., 2015).
We detected significantly higher mRNA *Cd38* levels in both the *Inhba* KO at E14.5 and E15.5 samples, but no changes in the *Inha* KO compared to the WT samples (Figure 3.19 A).

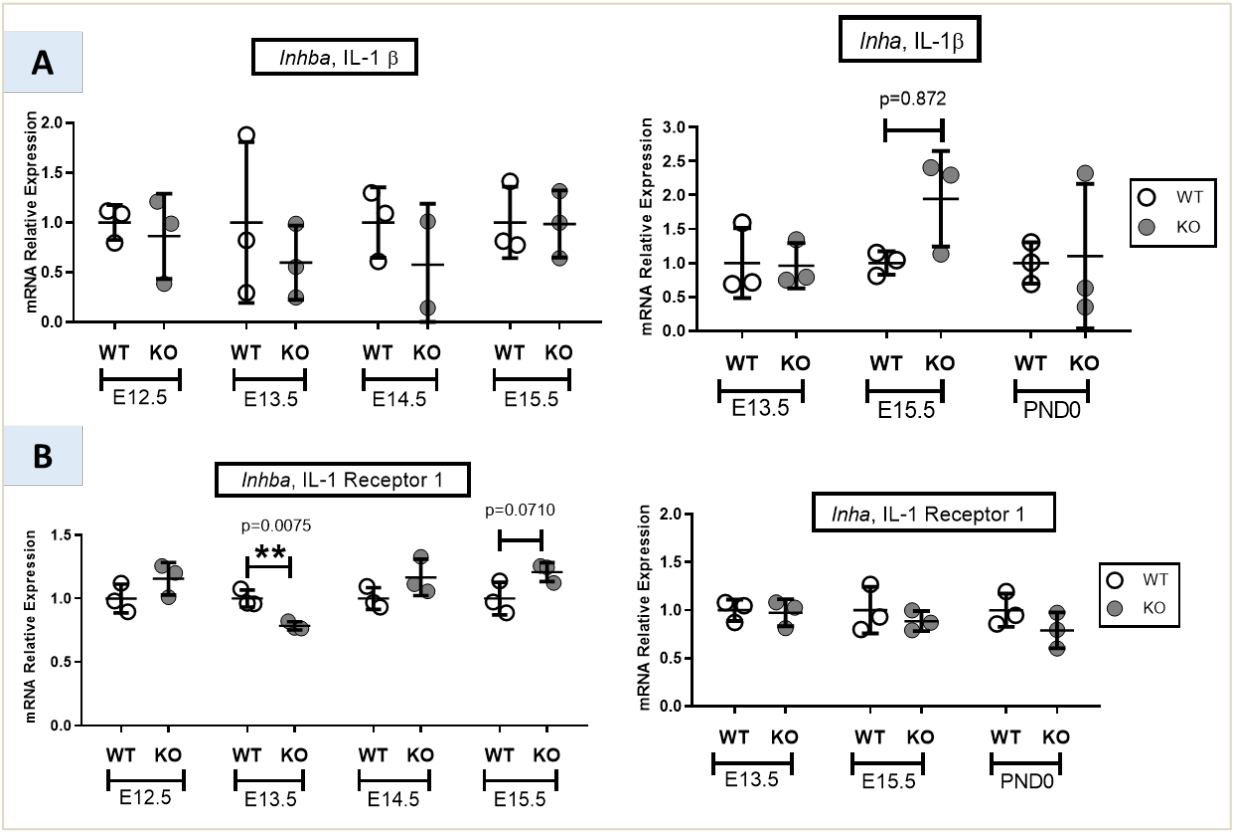
Marco: Macrophage receptor with collagenous structure (Marco) is found on particular subsets of macrophages and dendritic cells present in the marginal zone of the spleen, the medullary lymph nodes, and liver. Marco acts as a scavenger receptor and is highly expressed on testicular macrophages (Kangas et al., 1999; Mukhopadhyay et al., 2011; Sun et al., 2017; Mossadegh-Keller and Sieweke, 2018).
Detection of mRNAs encoding Marco showed no significant differences between KO and WT samples from *Inhba* and *Inha* mouse testes (Figure 3.19 B).

Figure 3.19. The levels of mRNAs encoding CD38 (A), and Marco (B) in *Inhba* and *Inha* WT and KO mouse testes. WT: open circles; KO: black circle. Each dot shows one individual sample; lines are at mean and SD; statistical significance was measured using unpaired T-test, two-tailed, *= p<0.05, **= p <0.01.



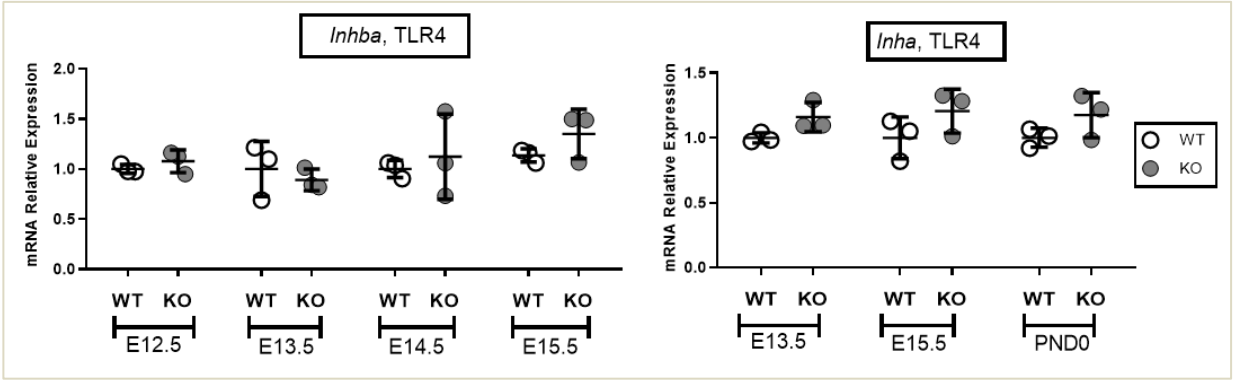
IL-1β and Type 1 IL-1 Receptor 1 (IL-1R1): The production and effects of IL-1β and its receptors are controlled at many levels. IL-1β is synthesised as a precursor form protein only after stimulation following exposure of innate immune cells (macrophages and dendritic cells) to alarmins (Damage-associated molecular patterns produced by damaged or dead/dying cells), such as lipopolysaccharide (LPS). This is in contrast to IL-1α, which is widely produced by many cell types (Contassot et al., 2012). IL-1β is a crucial mediator of inflammatory responses, playing an important part in the development of pathologic conditions that eventually lead to chronic inflammation. IL-1β is synthesised as a precursor inactive protein, which needs to be correctly localised and cleaved (by caspase-1) to be secreted as a mature protein. Type 1 IL-1 Receptor 1 (IL-1R1) is a receptor for interleukin 1 alpha (IL1A), interleukin 1 beta (IL1B), and interleukin 1 receptor antagonist (IL1RA), and hence functions as an important mediator in many cytokine -induced immune and inflammatory responses (Boraschi et al., 2018). The mRNAs encoding IL-1β and Type 1 IL-1 Receptor 1 (IL-1R1) showed no significant differences in levels between KO and WT samples from *Inhba* and *Inha* mouse testes (Figure 3.20 A and B).

Figure 3.20. The levels of mRNAs encoding IL-1β (A) and Type 1 IL-1 Receptor 1 (IL-1R1 (B) in *Inhba* and *Inha* WT and KO mouse testes. WT: open circles; KO: black circle. Each dot shows one individual sample; lines are at mean and SD; statistical significance was measured using unpaired T-test, two-tailed, *= p<0.05.



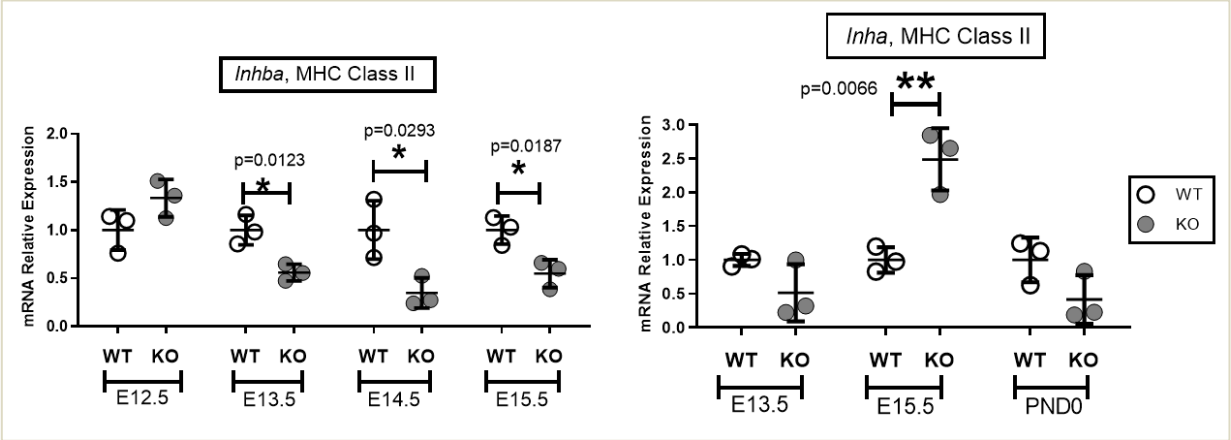
TLR4 (CD284): Toll-like receptor 4 (TLR4; also known as CD284) belongs to the family of highly conserved pattern recognition receptors (PRRs) that recognise conserved pathogen-associated molecular patterns (PAMPs). Activation of PRRs represents the first line of defense against infections by inducing a pro-inflammatory response. TLR4 activation leads to inflammatory cytokine production, which is responsible for activating the innate immune system, and thus TLR4 plays pivotal roles as amplifier of the inflammatory response (Molteni et al., 2016; Vaure and Liu, 2014). No significant differences in *TLR4* levels were measured between KO and WT samples from *Inhba* and *Inha* mouse testes (Figure 3.21).

Figure 3.21. The levels of mRNAs encoding Toll-like receptor 4 (TLR4) in *Inhba* and *Inha* WT and KO mouse testes. WT: open circles; KO: black circle. Each dot shows one individual sample; lines are at mean and SD; statistical significance was measured using unpaired T-test, two-tailed, *= p<0.05.



MHC class II (H2-Eb1): MHC class II molecules (MHCII) are a class of major histocompatibility complex (MHC) molecules normally found only on antigen-presenting cells such as dendritic cells, mononuclear phagocytes, some endothelial cells, thymic epithelial cells, and B cells. The antigens presented by class II peptides are derived from extracellular proteins, rather than those that are cytosolic and presented by MHC class I. Extracellular proteins that are endocytosed, digested in lysosomes and then loaded on an MHCII molecule are important in initiating immune responses. MHCII interacts mainly with immune cells, like T helper cells ($CD4^+$) to regulate how T cells respond to an infection (Jones et al., 2006; Roche and Furuta., 2015). We identified a significantly lower mRNA *MHCII (H2-Eb1)* level in the *Inhba* KO at E13.5, E14.5 and E15.5 samples, and higher level in the *Inha* KO at E15.5 compared to the WT samples (Figure 3.22).

Figure 3.22. The levels of mRNAs encoding MHC class II (H2-Eb1) in *Inhba* and *Inha* WT and KO mouse testes. WT: open circles; KO: black circle. Each dot shows one individual sample; lines are at mean and SD; statistical significance was measured using unpaired T-test, two-tailed, *= p<0.05, **= p <0.01.



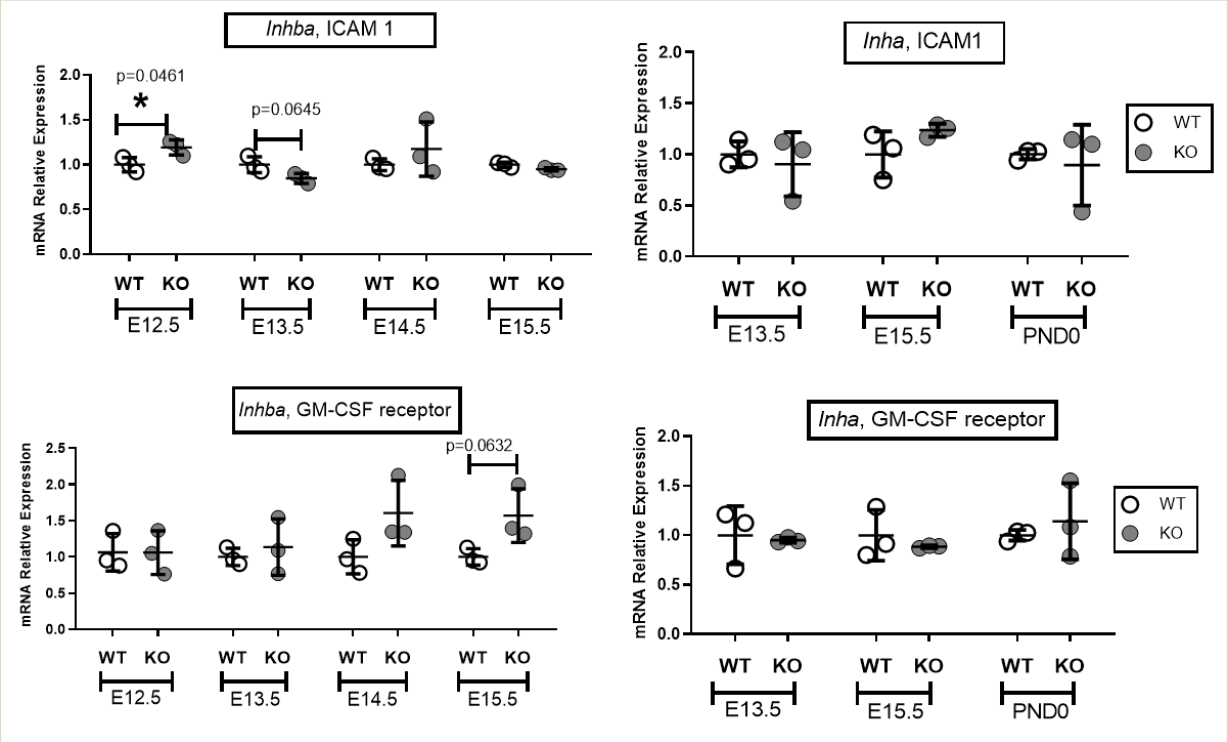
ICAM1: ICAM 1 (Intercellular Adhesion Molecule 1) is a highly glycosylated cell surface receptor present on leukocytes and endothelial cells. It binds to the leukocyte adhesion protein LFA-1. ICAM1 is the primary endothelial cell adhesion molecule mediating the firm adhesion of leukocytes (cell-cell adhesion) and subsequent leukocyte transmigration (extravasation) (Ramos et al., 2014; Martinelli et al., 2009). These functions demonstrate that pro-inflammatory cytokines increase ICAM1 expression on macrophages and endothelial cells (Hubbard and Giardina, 2000). ICAM1 is involved in regulating junction restructuring events during spermatogenesis. (Xiao et al., 2013). We identified a significantly lower *ICAM1* mRNA level in the *Inhba* KO at E12.5, and no significant differences between KO and WT samples from *Inha* mouse testes (Figure 3.23 A).

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) Receptor: GM-CSF is a pluripotent cytokine produced by many cells, which regulates hematopoiesis, and influences the survival, proliferation, differentiation, and functional activity of many myeloid cells, innate and adaptive immunity (Hercus et al., 2012). Also, GM-CSF induces an M1 phenotype in *in vivo* and *in vitro* microenvironment (Fleetwood et al., 2007).

Binding of GM-CSF to the GM-CSF receptor, also named GM-CSFR, CSF-2R or CD116, stimulates the production of murine white blood cells (Nicola and Metcalf 1985). GM-CSFR signalling does not determine monocyte recruitment and

differentiation, however, it plays vital roles in fine-tuning and governing macrophages with a high level of MHCII. M-CSFR and GM-CSF receptor signalling are opposing forces in the tumour microenvironment, regulating the MHCII low and MHCII high tumor-associated macrophage (TAM) populations, respectively. While M-CSFR predominantly regulates the differentiation and M2- like properties of MHCII low, GM-CSFR fine-tunes the M1-like MHCII phenotype (Van Overmeire et al., 2016). Detection of mRNAs encoding GM-CSF receptor revealed no significant differences between KO and WT samples from *Inhba* and *Inha* mouse testes (Figure 3.23 B).

Figure 3.23. The levels of mRNAs encoding ICAM1 and GM-CSF receptor in *Inhba* and *Inha* WT and KO mouse testes. WT: open circles; KO: black circle. Each dot shows one individual sample; lines are at mean and SD; statistical significance was measured using unpaired T-test, two-tailed, *= p<0.05.



Matrix metalloproteinases (**MMPs):** Matrix metalloproteinases (MMPs) form a family of enzymes that mediate various functions in tissue destruction, remodeling, embryonic development, reproduction and immune responses by hydrolysing components of the extracellular matrix under physiological and pathological conditions. Most MMPs are secreted as inactive pro-proteins which are activated when cleaved by extracellular proteinases (Visse et al., 2003). Three different MMPs were selected for analysis

because RNA seq data from the Loveland lab (unpublished data) indicated that they are regulated in some testis samples by activin A levels.

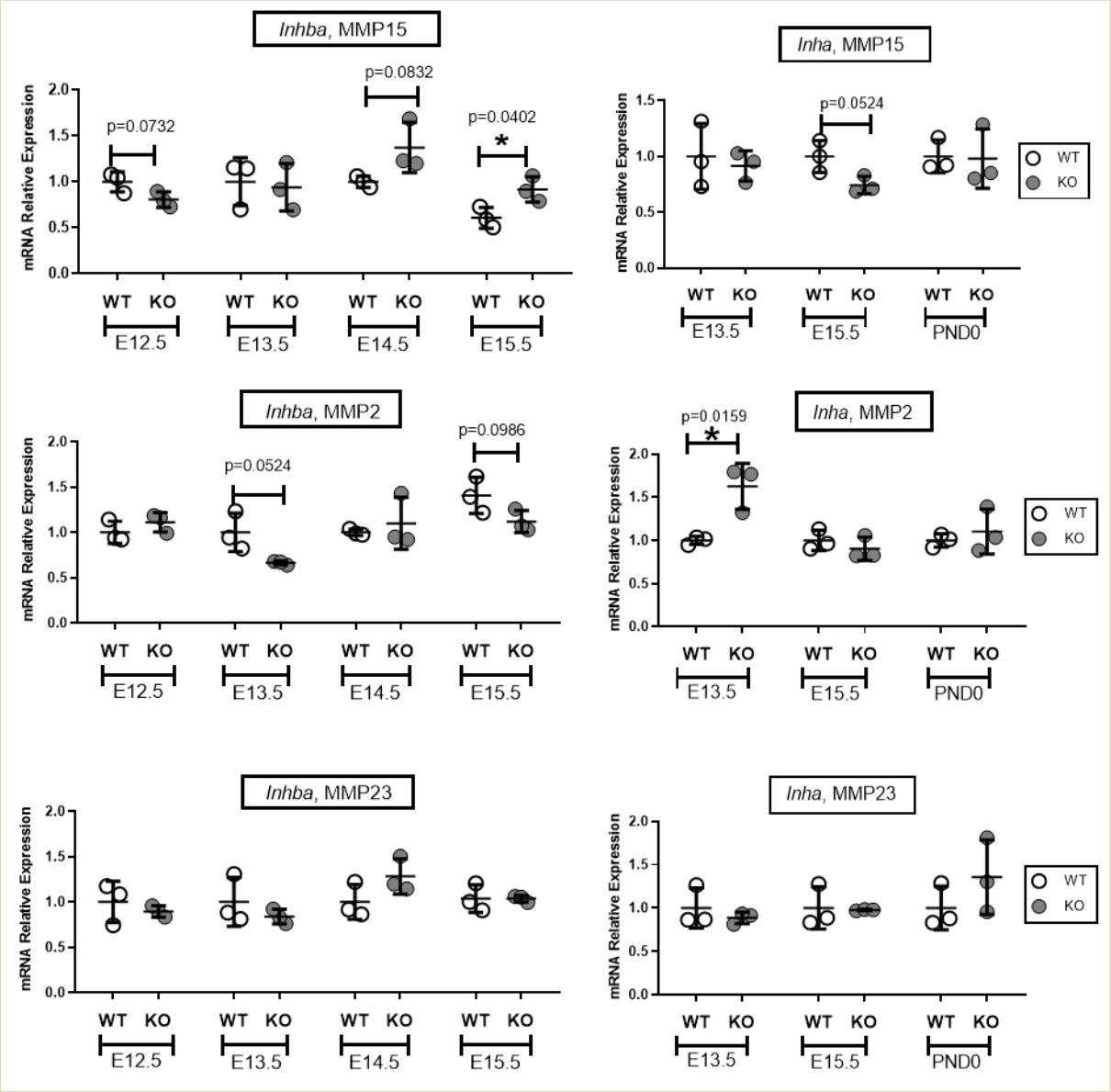
MMP2: Matrix metallopeptidase 2 (MMP2; also known as gelatinase A) is the most ubiquitous metalloproteinase in this enzyme family, which is produced throughout the body to form spaces between cells. MMP-2 has a wide range of substrates which include collagen, elastin, endothelin, fibroblast growth factor, MMP-9, MMP-13, plasminogen, and TGF-β, indicating widespread roles of MMP-2 (Ma et al., 2015). MMP2 is involved in diverse cellular functions such as blood vessel formation, tissue remodeling, repair and regeneration. One significant function of MMP2 is to cleave type IV collagen, a major structural component of basement membranes (Malemud, 2017). Our data showed an inverse trend for the *MMP2* mRNA level between *Inhba* and *Inha* KO mouse testes compared to the WT samples. There was a considerably lower level (p=0.0524) of *MMP2* mRNA in *Inhba* KO mouse testes at E13.5 and E15.5, while there was a significant increase in *Inha* KO at E13.5 (Figure 3.24 A).

MMP15: MMP15 is involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodelling. MMP15 is a member of the membrane-type MMP (MT-MMP) subfamily which is anchored to the extracellular membrane by either a transmembrane domain or glycophosphatidylinositol linkage, suggesting that this protein is expressed at the cell surface rather than secreted in a soluble form (Lemaître and D'Armiento, 2006).

Our data showed an reciprocal trend for the *MMP15* mRNA levels, between *Inhba* and *Inha* KO mouse testes, compared to the WT samples. There was a significantly higher level of *MMP15* mRNA in *Inhba* KO mouse testes at E15.5, and a considerable (p=0.0524) decrease in *Inha* KO at E15.5 (Figure 3.24 B).

MMP23: MMP23 is a member of the matrix metalloprotease family of zinc- and calcium-dependent endopeptidases which have been reported at high levels in mouse macrophages (Huang et al., 2012). Detection of mRNAs encoding *MMP23* showed no significant differences between KO and WT samples from *Inhba* and *Inha* mouse testes (Figure 3-24 C).

Figure 3.24. The levels of mRNAs encoding MMP2, MMP15 and MMP23 in *Inhba* and *Inha* WT and KO mouse testes. WT: open circles; KO: black circle. Each dot shows one individual sample; lines are at mean and SD; statistical significance was measured using unpaired T-test, two-tailed, *= p<0.05.



3.4. Discussion

The results of this study have demonstrated that macrophages in the testes of KO and HET *Inhba* mice accumulate at the testis perimeter at E13.5, E15.5 and PND0; this indicates that in the testis of mice (at E13.5-PND0) with lower activin A levels, macrophages may have impaired migration from the perimeter areas into the interior regions. This is supported by measurements of transcripts encoding important chemokine and chemokine receptors involved in macrophage recruitment, including CXCL1, CCL17, CXCR7 and ICAM-1, each of which displayed transcript levels that

were significantly lower in mice with lower levels of activin. These outcomes are summarised in Table 3.4. The levels of *CXCL1* and *CCL17* transcripts were significantly lower in *Inhba* KO at E13.5 compared to the WT testes, while in *Inha* KO E13.5 testes, these transcripts were significantly higher compared to in WT samples. *CXCR7* and *ICAM-1* transcript levels were considerably lower in *Inhba* KO mice at E13.5 compared to the WTs (Table 3.4). Thus, these results collectively indicate that transcripts which encode proteins required for macrophage trafficking, may be involved into movement of macrophages to the area where testis cords are growing in fetal life, and they are positively regulated by activin A.

It is important to note that the density of macrophages in *Inha* KO mouse testes was higher than WT only at E13.5 and not at E15.5 and PND0. One possible explanation could be that other factors mediate recruitment of macrophages to the testis so that only a specific number arrive, but that activin A serves a specific role in controlling macrophage functions inside the testis. Also, the activation of other compensating factors to the regulated population of macrophages in the testis should be considered; this general concept has been considered to govern immune cell physiology in many contexts (Rankin and Artis 2018). Another possibility could relate to the differing origins of testicular macrophages. This study demonstrated that activin A levels affected synthesis of immune factors in the testis at E13.5, but not at E15.5 or PND0. Of relevance, several studies have recently shown that testicular macrophages before E14.5 are exclusively yolk-sac derived, while from E15.5 onwards, fetal-derived monocytes enter the testis (DeFalco et al., 2014, Lokka et al., 2020; Wang et al., 2021). Another study of peritoneal macrophages identified one of the two population of macrophages resident in the peritoneal cavity, the large peritoneal macrophages, or LPMs type~~,~~ as yolk-sac derived (Cassado et al., 2015). The critical roles of activin A in maturation and proliferation of peritoneal macrophages has been shown through both *in vitro* and *in vivo* studies in adults (Zhang et al., 2017; Wang et al., 2008). Therefore, we suggest that activin A is a pivotal in determining the behaviour of yolk-sac-derived macrophages which reside in the testis at E13.5, however, the fetal liver-derived monocytes which enter the testis later are not affected by activin A in such a robust manner. The recent published study by our group showed in the adult mouse testis with a super-physiological activin A level (HET *Inha*), selectively one population of testicular macrophages ($F4/80^{+}/CD206^{+}/MHCII^{-}$) was significantly increased (Indumathy et al., 2020). The phenotype of this macrophage population is aligned with

large peritoneal cavity macrophages, or LPMs type, which are yolk-sac derived (Cassado et al., 2015). Therefore, based on well-approved impact of activin A on regulation of peritoneal cavity macrophages, and also the recruitment of macrophages resident in the peritoneal cavity to injured and inflamed peritoneal organs (Zhou et al., 2016; Wang et al., 2009; Wang and Kubes, 2016; Rehermann, 2017; Steinert et al., 2018), we proposed that high levels of activin A can impact recruitment of peritoneal macrophages into the testis at adulthood.

Previous studies have identified *CX3CR1, CCL17, CCR4, IL-4 and IL-10* as activin A target genes (Yamashita et. al., 1993; Sierra-Filardi et al., 2011; Petrakou et al., 2013; Hardy et al., 2015; Antsiferova et al., 2017; Tousa et al., 2017; Szarek et al., 2019; Whiley et al., 2020). In looking for immune cell regulatory factors/ mediators, we detected lower *CX3CR1, CX3CL1, CCL17 and CXCR7* mRNA levels and higher *CD45*, *IL-4 receptor, IL-10R and CCR4* mRNA levels in *Inhba* KO mouse testes. Transcripts encoding CD11c, F4/80, CD206, CSF1, IL-10, CCR4 and CXCL12 exhibited no significant differences between KO and WT samples in *Inhba* mice. The measurement of mRNAs encoding F4/80, CD11c, CD45 and CD206 also revealed no significant differences between KO and WT samples from *Inha* mouse testes.

For markers that we did not find any significant differences in KO and WT mice, we suggest future studies to examine their protein levels as mRNA levels do not accurately reflect the amount of protein, and different activin A levels may impact translation, post translation or turnover of proteins.

Of great interest, the dose-dependent effect of activin A on transcript levels was observed in both animal models for the following genes: *CX3CL1, IL-4R, IL-10 receptor, CCL17, CXCR7* and *CXCR4*.

In considering the impact of activin A on transcripts encoding proteins commonly identified as inflammatory mediators/ factors, we detected significantly or considerably lower *MHCII* and higher *CD38, Marco, IL-1 receptor, CCR4* mRNA levels in *Inhba* KO testes. However, the mRNAs encoding CD16, CD64, CD68, TLR4, IL-1β - *Retinoic Acid Receptor alpha (α) (RARα)* displayed no differences between KO and WT samples from *Inhba* mice. In *Inha* testes, we detected significantly or considerably lower *Marco, RARα* levels and higher *CD68, MHCII, IL-1 receptor* levels in KO mouse testes. The mRNAs encoding CD16, CD64, ICAM-1, TLR4, CD38, IL-1β, GM-CSFR receptor showed no differences between KO and WT samples from *Inha* mouse testes.

Of particular interest, the data also demonstrated a dose-dependency between activin A and *MHCII mRNA* levels. This is important because MHCII levels are critical to the phenotype and function of testicular and other macrophages. MHCII is mainly expressed on antigen-presenting cells, such as macrophages, B cells and DCs, where it stably binds and presents fragments of exogenous antigen to T cells. The demonstration that MHCII expression is directly influenced by activin A levels indicates that activin A might promote the ability of macrophages to present exogenous antigens. We know from previous studies and data in Chapter Two of this thesis that macrophages are the most abundant antigen presenting cell in the testis during fetal life and at birth (Mossadegh-Keller et al., 2017; Wang et al., 2021). Thus, up-regulation of MHCII by activin A in postnatal life could be associated with an increased capacity to present meiotic germ cell antigens (MGCA) that are expressed on sperm by testicular macrophages to T cells resident in the testis (Tung et al., 2017). The presentation of MGCA can cause immune responses and consequently inflammation and malignancy. Autoimmune responses to MGCA are detected in 3%–12% of men with spontaneous infertility, a factor in 5% of infertile couples (Turek et al., 1994). In agreement with the finding presented in this chapter, another study also showed that activin A promotes MHCII expression on the surface of mouse macrophage cell line, RAW264.7 cells, thus inducing their activation, but without any significant changes in cell proliferation (Ge et al., 2009). Thus, the findings in this study provides evidence that up-regulation of MHCII expression is another example of a direct role for activin A in the regulation of primary macrophage activation in the early phase of inflammation. In the context of the fetal testis, this may be important for the remodeling events which are essential for normal cord formation and removal of cells that are inappropriately located. We are aware that this study was limited to measuring MHCII transcripts; it will be important to document levels of the protein on the surface of macrophages.

Ccl2 mRNA levels were higher, either significantly or trending, in both *Inhba* and *Inha* KO testes, however there were no differences in the *Ccr2* mRNA level. CCL2 (MCP-1) and CCR2 (receptor that bind CCL2) are involved in the recruitment of monocytes, memory T cells and dendritic cells to inflammation sites; they are expressed on macrophages, Th1 cells, Th17 cells (Craig et al., 2006). CCR2 has both pro-inflammatory (mediated by APC and T cells) and anti-inflammatory (mediated by regulatory T cells) effects (Zhang et al., 2010). CCL2 was the first chemokine identified in the testis to be implicated in regulation of the large testicular macrophage population

(Aubry et al., 2000). Key roles for the CCL2/CCR2 axis in macrophage polarisation have been identified in more general contexts (Deshmane at al., 2009). Activin A exposure *(in vitro*) elevated CCR2 expression but prevented the acquisition of CCL2 in M-CSF-polarised macrophages (Sierra-Filardi et al., 2014; Carson et al., 2017). In this study, the absence of any significant differences in the *Ccr2* mRNA level between *Inhba* and *Inha* KO and WT samples at any age could be related to the phenotype of fetal testicular macrophages. $CCR2^-$ and $CCR2^+$ macrophages have distinct functional and ontogeny features. $CCR2^-$ macrophages are a tissue-resident population exclusively replenished through local proliferation, whereas $CCR2^+$ macrophages are maintained through monocyte recruitment and proliferation. (Bajpai et al., 2018). As discussed earlier, fetal testicular macrophages are both yolk-sac and fetal liver derived, therefore we cannot reach a conclusion about the impact of activin A on *Ccr2* levels at this point, however there is a possibility that testicular macrophages during fetal development are $CCR2^-$.

Based on these outcomes, I conclude that activin A regulates transcript levels encoding many of the immune factors and functional markers selected for investigation. However, this regulation does not shift the fetal testis to either a pro-inflammatory or suppressive immune microenvironment. On the other hand, many parallel and compensatory mediators in the immune system work together to provide a functional balance that supports normal development or homeostasis, and the outcomes suggest that activin A may contribute to this outcome. For example, in the *Inhba* KO samples lacking activin A, mRNA levels encoding both regulatory mediators (IL-4 and IL-10 receptors) and inflammatory markers (CD38 and Marco) showed an increase. In the same manner, in fetal *Inha* KO mouse testes, the opposite outcome was measured for two critical mediators of macrophage activation, MHCII and Marco.

Chapter Four

The Emergence and Distribution of Immune Cell Populations in the Fetal Human Testis

4.1. Introduction

The adult human testis is both a reproductive and endocrine organ, performing the two functions of hormone secretion and sperm production (de Kretser et al., 2015). The human testis first develops during fetal life as an abdominal organ, and enters the scrotum after birth (Behre et al., 2001; Sadler et al., 2011). In the following months, diverse somatic cells collectively build a niche that is permissive for driving and maintaining masculine features and that sustains decades of spermatogenesis. Amongst the somatic cells contributing to testis development and function are immune cells. These initially represent a small fraction of the total fetal testis cell number but increase proportionately as the immune system develops and the testis grows (Hedger et al., 2015; Dutta et al., 2021). Knowledge of how immune cells contribute to organ development in the human is limited, and this is particularly true for the fetal testis. Thus, the objective of this study is to identify the emergence and identity of immune cell populations in the human fetal testis.

The human fetal testis first forms after sex determination (at 4-6 gestational weeks [GW]). Testis cords form (starting around 6-7GW) as Sertoli cells proliferate and surround the fetal male germ cells (also termed gonocytes), while simultaneously diverse interstitial somatic cell types reorganise to establish the lymphatic and blood vessels and to commence steroidogenesis. By 14-18GW, the germ cells transition into a quiescent state, forming spermatogonial precursors that will underpin lifelong spermatogenesis, while the somatic cell population continues to expand (Li et al., 2017; Guo et al., 2021).

In the adult testis of both humans and rodents, the most abundant immune cell type are macrophages; these reside both in the interstitial spaces between testis tubules, in close opposition to Leydig cells (termed ‘interstitial macrophages’), and surrounding the testis cords where they are interdigitated amongst the peritubular myoid cells (termed ‘peritubular macrophages’). In each position, they exhibit a range of markers, indicative of their functional diversity (DeFalco et al., 2015; Mossadegh-Keller et al., 2017 and 18; Indumathy et al., 2021). The close physical relationship between Leydig cells and macrophages in the interstitium is important for maintenance of testosterone production by Leydig cells (Gaytan et al., 1994 and 1995). Adult testicular macrophages perform the crucial regulatory functions of maintaining the immune-privileged microenvironment in the presence of post-mitotic germ cell ‘neo-antigens’ and delivering pro-inflammatory activity in response to infection (Bhushan and

Meinhardt, 2017; Bhushan et al. 2020). Present in the adult testis at a lower frequency are dendritic cells (DC), mast cells and/or eosinophils, with T cells and natural killer (NK) cells in even lower abundance; B cells are rarely observed in a healthy testis (Hedger et al., 2015; Klein et al. 2016, Bhushan et al. 2020). How these cell populations are established during testis development gained interest due to important findings that indicate certain immune cell products influence not only development of the testis but also ongoing spermatogenesis in adults (DeFalco et al., 2015). In the fetal mouse testis, macrophages were shown to be of particular importance for normal cord formation (DeFalco et al., 2014) and, together with Leydig cells, to influence development of the spermatogonial stem cell niche in the adult testis (Heinrich and DeFalco, 2020).

The two arms of the immune cell network, innate (including myeloid-derived cells, monocytes, macrophages and dendritic cells, and neutrophils) and adaptive (e.g., lymphocytes, T and B cells), develop in fetal life. The early concept that tissue resident macrophages originate from bone marrow hematopoietic stem cells (HSCs) and are replenished by circulating blood monocytes (Van Furth and Cohn, 1968) has been superseded through knowledge gained from studies in mice (reviewed in Ginhoux and Jung, 2014). The development and function of each lineage are complex and not simply linear, as previously understood, and should be considered independently for each organ. It is now known that tissue resident macrophages in both mouse and human arise in embryonic life; because this precedes full development of the adaptive immune system, their role in organ development has been deduced but remains to be established (Miah et al., 2021). These cells, arising independent of HSCs, are long-lived and capable of self-renewal (Bhushan and Meinhardt, 2017). Investigations of testicular macrophage ontogeny in mice identified that these are either yolk sac-derived (interstitial macrophages) or fetal liver-derived (peritubular macrophages) (DeFalco et al., 2014; Lokka et al., 2020; Wang et al., 2021; Meinhardt et al., 2022). To date, all studies of testicular macrophages in the human have used testis samples from adult men, and similar to observations in mouse, these have identified macrophages as the most abundant immune cells in the testis.

Parallel data on the emergence of cells in the human fetal testes are not available, despite their likely importance in the key steps of testis growth that determine adult fertility.

Human fetal haematopoiesis consists of several waves, with the earliest red blood cells and immune cells originating from the yolk sac between 3 and 4GW, and then the aorta–gonad–mesonephros (AGM) developing as the site of haematopoiesis. Haematopoiesis has begun in the fetal liver at 10-12GW and this organ is the major source of blood cells by 20-24GW. The last wave of haematopoiesis arises in fetal bone marrow. Fetal bone marrow is the dominant site of haematopoiesis after mid-gestation to birth (Holt and Jones, 2000; Travnickova et al. 2015; Bian et al., 2020). The generated immune cells are seeded in tissues and have specific functions and phenotypes based on their ontogeny and tissues microenvironment (De Kleer et al., 2014).

The trajectory of human immune cell formation and development in the human thymus (collected at fetal (7-17GW) and postnatal/adult (3 month to ~35 years) ages) was recently revealed though single cell RNA sequencing (scRNA-seq) (Park et al., 2020). These findings identified macrophages as the forerunner monocyte population, with dendritic cells emerging around 12GW. A similar approach documented the emergence of haematopoietic cell lineages from stem cells and multipotent progenitors in other human fetal organs (liver, skin, kidney and yolk sac) (Popescu et al., 2019).

T cells play key roles in the adaptive immune response, and their contributions in human adult testis normal and pathological conditions have been studied (Cecilia et al., 2013; Klein et al., 2016; Gong et al., 2021). Different to the adult, knowledge of T cells in the human fetal testis is lacking (Xiaowei et al., 2021).

Arising from the myeloid lineage, neutrophils in adults serve potent innate immune roles relating to the induction of inflammation, tissue damage and increasing disease pathogenesis and severity (Kobayashi et al., 2009; Soehnlein and Lindblom 2010). In contrast, their roles in fetal tissue morphogenesis are not established. Analysis of human adult testicular pathologies have identified neutrophils in acute bacterial epididymo-orchitis (Schuppe and Bergmann 2013; Fijak et al., 2018) and their accumulations in testicular germ cell tumors (TGCTs) were proposed as a prognostic factor for overall survival (Yamada et al., 2016). Studies of testicular neutrophils are limited to investigating the early stages of mouse fetal testis at embryonic age E12.5 (DeFalco et al., 2014), E13.5 to adult mouse testis (own unpublished data), the adult rat testis (Lysiak et al., 2001; Sukhotnik et al., 2007; Arena et al., 2020) and a single

adult human testis study (Yamada et al., 2016). To date, the presence of neutrophils in human fetal testis is undocumented.

Mast cells, derived from HSCs, function predominantly in the initiation of inflammation by regulating vascular permeability, vasodilation, and leukocyte recruitment. These long-lived cells can, after appropriate stimulation, proliferate and change their tissue distribution and phenotype, undergoing terminal differentiation in their destination microenvironment. In this manner, mast cells contribute to both innate and adaptive immune responses, persistent inflammation and tissue remodelling (Fijak et al., 2006). Within the mouse and human testis, mast cells are present throughout the interstitium, where their products contribute to the homeostatic regulation of Leydig cell steroidogenesis. Mast cell accumulation and actions are linked to pathologies involving fibrosis and hyalinisation around the seminiferous tubules (Fijak and Meinhardt, 2006), as well as to various forms of testicular failure and infertility (Roaiah et al. 2007; Hedger 2015; Loveland et al., 2017; Mayerhofer et al. 2018). Whether mast cells are present in the human fetal testis is currently unknown.

The availability of scRNA-seq datasets to unveil the developmental trajectory of human immune cells has provided the opportunity to enumerate and functionally analyse these diverse and inter-related cell types in the context of organ development. To begin to understand their particular function during periods of dynamic growth in the fetal human testis, a critical first step is to gain knowledge of immune cell distribution within the testis.

In the present study, the frequency and localisation of immune cells ($CD45^+$), including macrophages ($CD68^+$), T cells ($CD3^+$), mast cells ($tryptase^+$ and $chymase^+$ cells) and neutrophils ($CD66b^+$) in the human fetal testis have been assessed using immunohistochemical methods on relatively rare archival samples that span from the second trimester through the third trimester of pregnancy. In addition, scRNA-seq data from published analysis of human fetal testes (Guo et al., 2021) was used to interrogate the potential presence of immune cell population and sub-populations (macrophages, dendritic cells, NK cells, T cells, B cells, and mast cells) in earlier stages of testis development. This combined approach to study human fetal testis samples tracks the emergence of key immune cell populations, documenting population size and position within the growing organ, and providing new information about how immune cells contribute to the earliest stages of human testis development.

4.2. Materials and Methods

Samples

Human fetal testes fixed in 4% paraformaldehyde (PFA) were obtained from archival paraffin material collected during the routine paedopathological autopsy of spontaneously aborted/stillborn fetuses between 14 and 41GW. Some had portions of epididymis attached. Specimens were provided by Prof Davor Jezek from Zagreb University and Dr Ewa Rajpert-De Meyts from the University of Copenhagen. Samples were from 14/15-20 (n=10), 21-24 (n=5) and 25 (n=2), 33-34 (n=2) and 41 (n=1). All investigations conformed to the appropriate Ethics Committee, in the Department Histology and Embryology, University of Zagreb and the University of Copenhagen.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using antibodies to detect all immune cells (anti-CD45), macrophages (anti-CD68), T cells (anti-CD3) mast cells (anti-tryptase and chymase) and neutrophils (anti-CD66b). Primary and secondary antibodies used are listed in Table 4.1.

In brief, sections of 5 μm were placed on glass slides, deparaffinised and rehydrated through washes in histolene (Trajan Scientific Australia; 2 x 5 min), then in decreasing concentrations of ethanol (100%, 2 x 5 min, then 95%, 80%, 70%, 50% and 30% for 3 min each) and placed in deionised water for 3 min. Heat-induced antigen retrieval was performed by immersing slides in Citrate Buffer (pH 6.0) (Sigma-Aldrich). Sections were incubated with 3% hydrogen peroxide (Merck Millipore) for 25 min at RT, followed by washing in Tris-buffered saline (TBS; 50 mM Tris-Cl, 150 mM NaCl, pH 7.5) containing 0.1% Triton X-100 for 3 min then 2 x 3 min with TBS. Sections were next incubated in blocking solution consisting of 5% bovine serum albumin (BSA, Sigma-Aldrich) in TBS in a humid chamber at RT for 1 hr. Primary antibodies diluted in 1.5% BSA/0.1% Triton X-100/TBS were applied in a humid chamber overnight at 4 °C. Consecutive sections incubated with washing buffer and lacking primary antibody were used as negative controls.

Slides were washed with washing buffer (0.1% Triton X-100/TBS) for 3 min then 2 x 3 min with TBS, and biotinylated secondary antibodies were added in a humid chamber for 1 hr at RT. After three consecutive washes (1 x 3 min 0.1% Triton X-100/TBS then 2 x 3 min TBS), Vectastain Elite ABC kit reagents were added according to the manufacturer's instructions (Vector Laboratories) for 30 min at RT followed by three

further washes, as above. Antibody binding was identified as a brown precipitate using 3, 3-diaminobenzidine tetrahydrochloride (DAB) (Agilent Technologies). Next, sections were counterstained with Harris hematoxylin (Sigma-Aldrich). Stained slides were washed and dehydrated through a graded ethanol series in 30%, 50%, 70%, 80%, 95% ethanol (each 1 x 3 min) and washed with 100% ethanol and histosol (each 2 x 5 min). Sections were mounted using DPX (Sigma-Aldrich) under glass coverslips.

Immunofluorescence

For indirect immunofluorescence (IF), testis sections were deparaffinised and dehydrated, and heat-induced antigen retrieval was performed as above. Then, slides were permeabilised using 0.1% Triton X-100/PBS for 5 min at RT. Slides were washed in PBS (3 x 3 min), then blocking solution (5% donkey normal serum, in 10% BSA/PBS) was applied to each section for 1 hr at RT. Blocking solution was replaced with unconjugated primary antibodies (anti-CD68, anti-CD45 and anti-Laminin) diluted in 5% BSA/0.1% Triton X-100/PBS, and slides were incubated overnight in a humid chamber at 4^{o}C. Negative control sections lacked primary antibody. Primary and secondary antibodies are listed in Table 1. The next day, slides were washed once in 0.1% Triton X-100/PBS (3 min), twice in PBS (3 min each), and appropriate conjugated secondary antibodies were applied for 1 hour at RT, with slides protected from light from this point onwards. Then, slides were washed as mentioned above. DAPI (4′,6-diamidino-2-phenylindole; Invitrogen, D3571, 1:500 in PBS) was used to visualise the nucleus with incubation at RT for 15 min. Washed slides were mounted under a glass coverslip using ProLong Gold Mountant (Invitrogen) and stored at RT for 30 min followed 4°C until scanning for image analysis (within 4-6 hours).

Table 4.1. Primary and secondary antibodies used for immunohistochemistry and immunofluorescence.

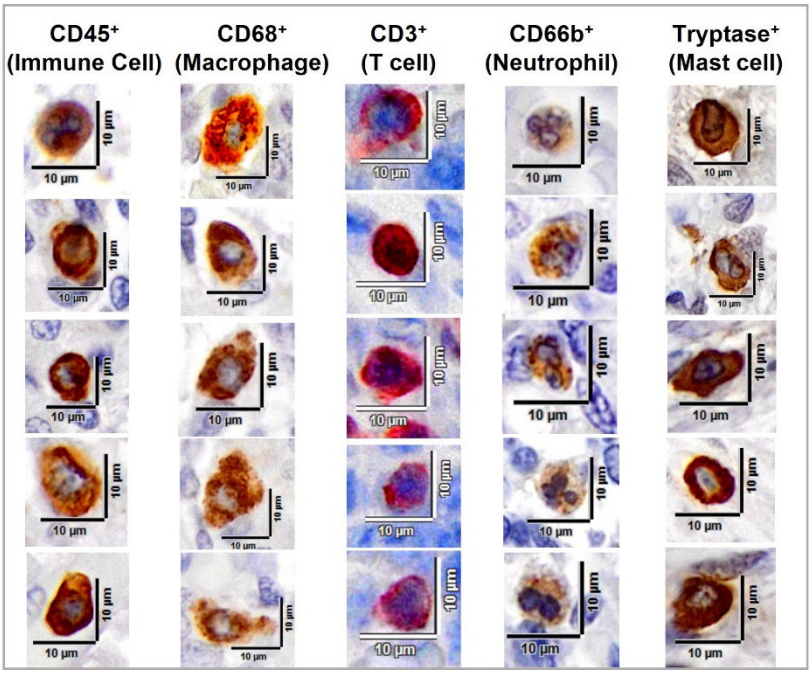
Antibodies	Company	Cat #	Clone	Raised in	Dilution
Anti-CD45	DAKO	M0701	2B11+ PD7/26	Mouse	1:250
Anti-CD3	DAKO	0452	Polyclonal	Mouse	1:150
Anti-CD68	DAKO	M0876	PG-M1	Mouse	1:250
Anti-Tryptase	DAKO	7052	AA1	Mouse	1:200
Anti-Tryptase	Abcam	Ab2378	AA1	Mouse	1:8000
Anti-Chymase	Abcam	Ab233103	Polyclonal	Mouse	1:200
Anti-CD66b	BioLegend	G10F5	G10F5	Mouse	1:200

Anti-Laminin	Sigma-Aldrich	L9393	Polyclonal	Rabbit	1:250
Rabbit anti-mouse	DAKO	E0468	Polyclonal	Rabbit	1:500
Goat-anti mouse	Dianova	115-035-003	Polyclonal	Goat	1:400
Donkey anti-Rabbit	Invitrogen	A21202	Polyclonal	Donkey	1:500
Donkey anti-Mouse	Invitrogen	A31572	Polyclonal	Donkey	1:500

Imaging and morphometric analysis

Immunostained human fetal testis sections were scanned by Monash Histology Platform staff using an Olympus VS120 Slide Scanner and analysed using OlyVIA Software (Olympus Life Science, 2.9.1 Viewer). Immune cells were identified based on the presence of a well-defined nucleus (Figure 4.S1) and marker staining. The localisation of immune cells within each section was either defined as in the 'perimeter' (the region between the capsule and the outermost edge of cords) or 'interior' area (the more central region of each section, containing the interstitium and cords). To identify background staining and exclude cells with non-specific signals from counting, the IHC or IF signals visible in negative control samples lacking primary antibody were subtracted from signals visible in the positive sample.

Figure 4.S1. Shape and size of immune cells in fetal human testes during second and third trimesters of pregnancy. Immunohistochemical signal (brown, cytoplasm) using antibodies to specific markers, with Harris hematoxylin counter stain (blue, nucleus). Dimensions as indicated.



Cell type identification and clustering using Seurat

To analyse publicly available single cell RNA sequencing (scRNA-seq) data relating to the human fetal testis (Guo et al., 2021), the Seurat (Butler et al., 2018; Stuart et al., 2019) pipeline (http://satijalab.org/seurat/, R package, v.4.0) was employed. Datasets retrieved from the Gene Expression Omnibus (GEO) repository consisted of 10X Chromium datasets (Guo et al., 2021) for GW6, 7, 8, 12, 15, 16) in market exchange (MEX) format (Accessions GSE143356). The raw unique molecular identifiers (UMI) count tables were loaded into R using the Read10X function for the 10X data.

Individual Seurat objects were created for all ages. Seurat objects of GW6, 7, and 8 ('embryonic') and GW12, 15, 16 ('fetal') were filtered, merged, and log-normalised with the default settings as described in the original study. Next, cells were normalised to the total UMI read count as well as mitochondrial read percentage (http://satijalab.org/seurat/). Cell clustering and UMAP analyses were performed based on the statistically significant principal components. All software (R Studio) and packages were publicly available from CRAN (Seurat). All graphs were generated by the Seurat package in R.

4.3. Results

This study used a unique collection of human fetal testis sections spanning the second and third trimesters of pregnancy to identify the location and gauge the relative frequency of specific immune cell populations using immunohistochemistry; their presence in epididymis was also noted if this tissue was in the section. A set of markers known to work reliably on PFA-fixed samples was used to provide a broad representation of key immune cell types, i.e., pan-leukocytes (CD45), $CD68^+$ cells (macrophages), $CD3^+$ cells (T cells), tryptase and $chymase^+$ cells (mast cells), and $CD66b^+$ cells (neutrophils). A summary of these outcomes is provided in Table 4.2.

Table 4.2. Semi-quantitative description of immune cell numbers and distributions during the second and third trimesters of pregnancy in human fetal testis sections. Absent: -, rare (1-5 cells/section): +, frequent (5-20 cells/section): ++, abundant (>20 cells/section): +++.

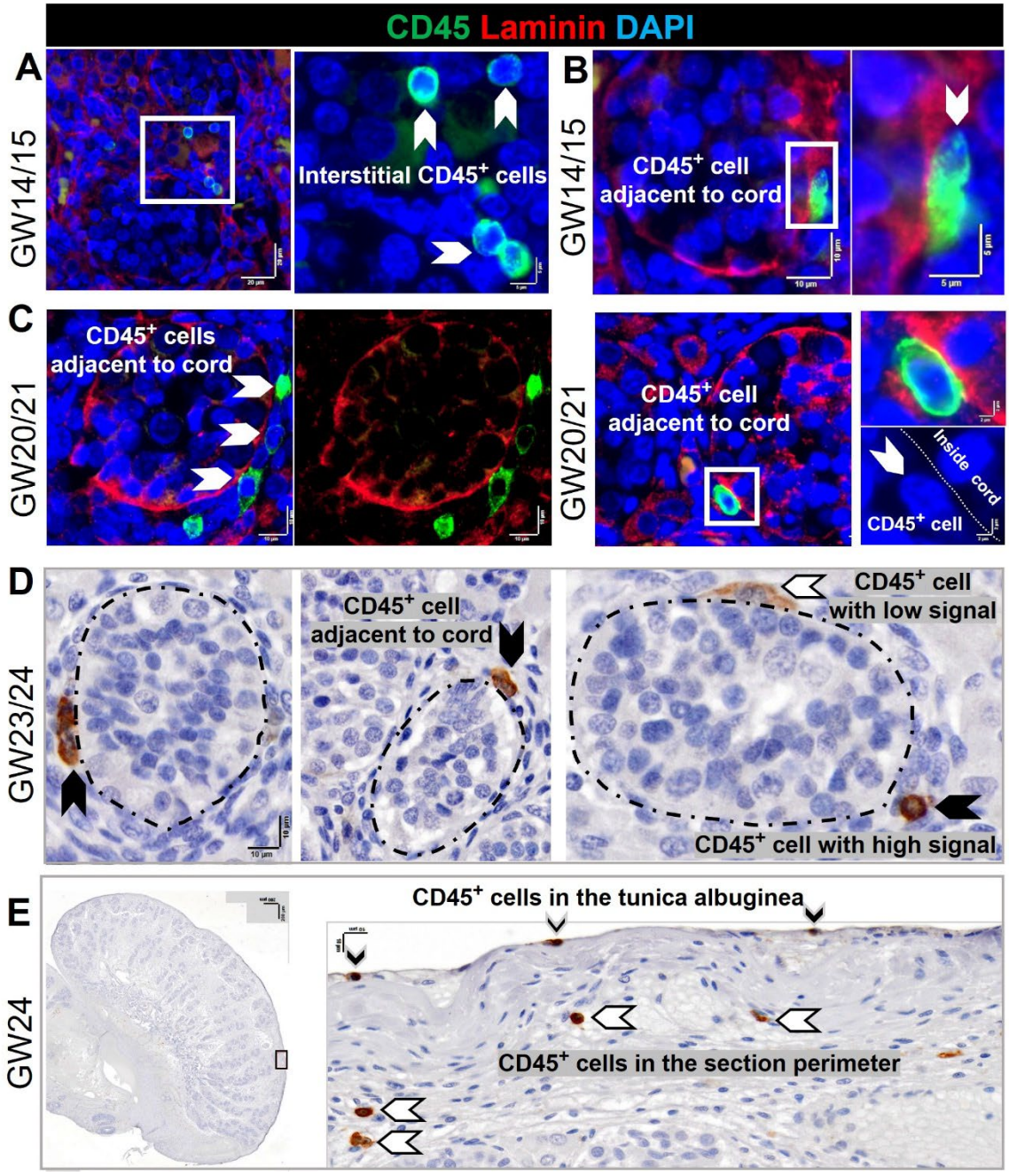
Immune cell type	Pregnancy trimester	Interstitium	Perimeter (Tunica albuginea)	Inside vessels	Cord perimeter	Inside cords

CD45$^+$	2nd	++	+	+	++	-
	3rd	+++	++	++	+++	-
CD68$^+$	2nd	++	++	-/+	++	-
	3rd	+++	+++	-/+	+++	-
CD3$^+$	2nd	-/+	-	+	-/+	-
	3rd	+/++	-	++	+	-/+
CD66b$^+$	2nd	-	-	+	-	-
	3rd	+	-	++	-/+	-
Tryptase$^+$	2nd	-	+	-	-	-
	3rd	-	++	-	-	-
Chymase$^+$	2nd	-	-	-	-	-
	3rd	-	-	-	-	-

To add important knowledge on immune cells subpopulations, we analysed a published scRNA-seq dataset obtained from human fetal testis samples at GW6, 7, 8, 12, 15 and 16, thus spanning the first to the early second trimester of pregnancy (Guo et al., 2021). This method allowed us to evaluate the number of cells expressing transcripts encoding macrophage markers (CD68, CD14, CD206, MHC Class II, CD163, CD64, CD80, CD86, CD115), T cell markers (CD3E, CD3G, CD3D, CD4, CD8A and B, CD25, FOXP3, CTLA-4, CD28), mast cell markers (tryptase and chymase), neutrophil markers (CD33 and CD66b), B cell markers (CD19 and CD20), dendritic cell (DCs) markers (CD1C, CD141, CD11C, CD303), NK cell markers (CD56, CD94, CD16, NKG2A/CD159a) and additional markers such as CD45 (pan-leukocyte), Ki-67 (proliferation), PD-1 and PD-L1 (immune checkpoints involved in regulating the immune system's response and promoting self-tolerance).

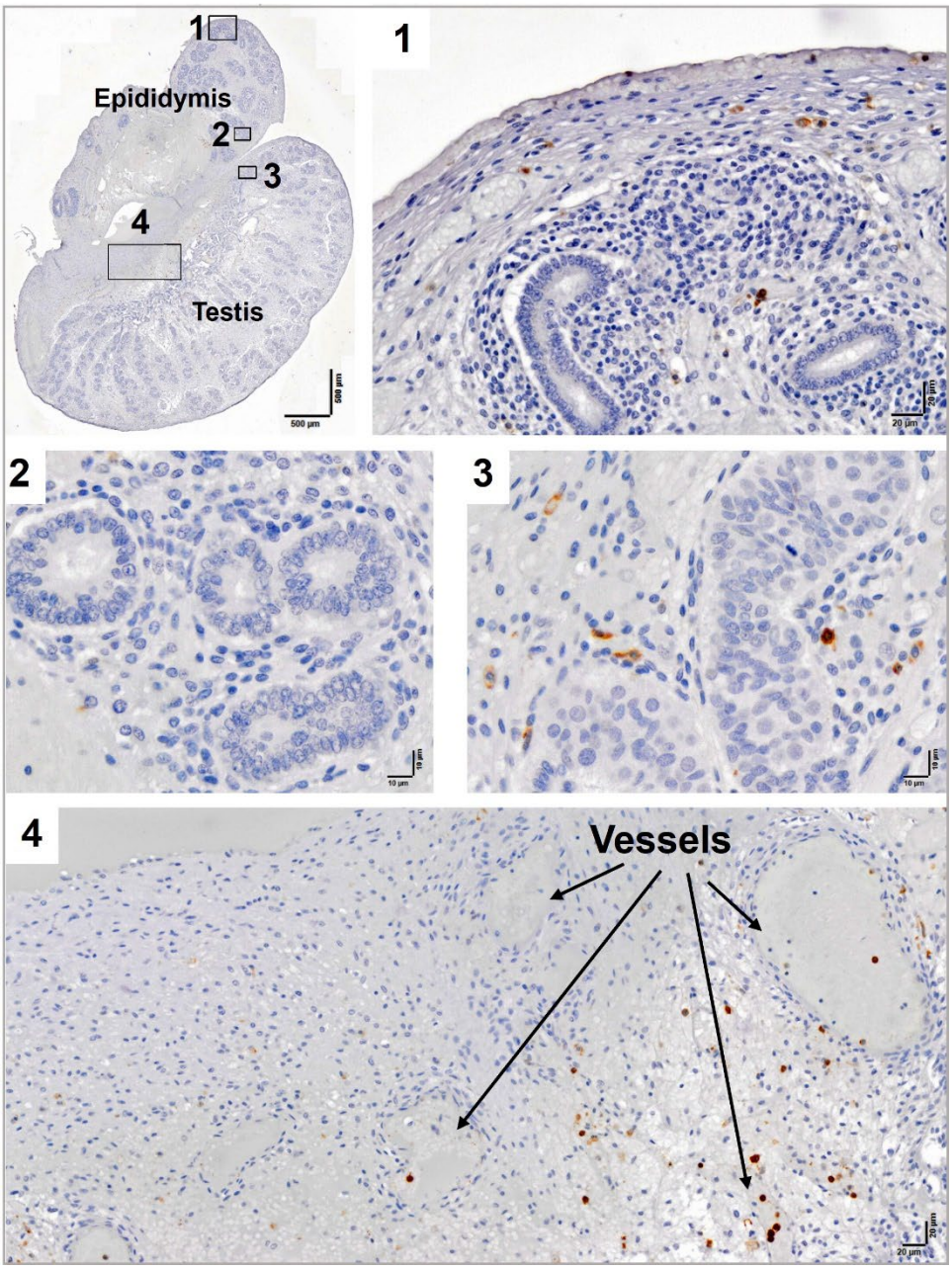
CD45, also known as leucocyte common antigen (LCA) or T200, is a transmembrane protein present on all differentiated immune cell types (Kaplan et al., 1990). Detection of CD45 as a pan-leucocyte marker yielded an initial overview of the distribution and abundance of immune cells at each stage in the human fetal testis. During the second and third trimesters, CD45$^+$ cells were consistently abundant in the testis interstitium, frequent in the cord perimeter adjacent to the cord wall (Figure 4.1A-D), and frequent in the tunica albuginea at all ages (Figure 4.1E). Moreover, CD45$^+$ cells were abundant inside and around the testicular vasculature and in the rete testis.

Figure 4.1. CD45$^+$ cells in human fetal testis sections in cord perimeter and interstitial areas. **A-C:** CD45$^+$ cells are identified using immunofluorescence (IF) in the interstitium (**A**), and adjacent to cords at GW 14/15 (**B**) and at GW20/21 (**C**). Marker colours indicated above for IF panels A-C; nuclear staining with DAPI. Dotted white line indicates cord perimeter (**D)** CD45$^+$ cells shown close to the cord membrane displaying with two levels of signal: strong (black arrows) and weak (white arrow). Dotted black lines indicate cord perimeters. (**E)** CD45$^+$ cells in the tunica albuginea (black arrows), and in the section perimeter area (white arrows) between the testis capsule and edge of the outermost cord; dotted black lines indicate cord perimeters D, E: Nuclear staining with hemotoxylin. Black and white boxes on low magnification images refer to adjacent high magnification panels. Each panel represents an individual sample.



Relative to the testis, CD45^{+} cells were more frequently observed during second and third trimesters in the epididymis compared to the testis, but they were absent from areas close to epididymal ducts (Figure 4. 2).

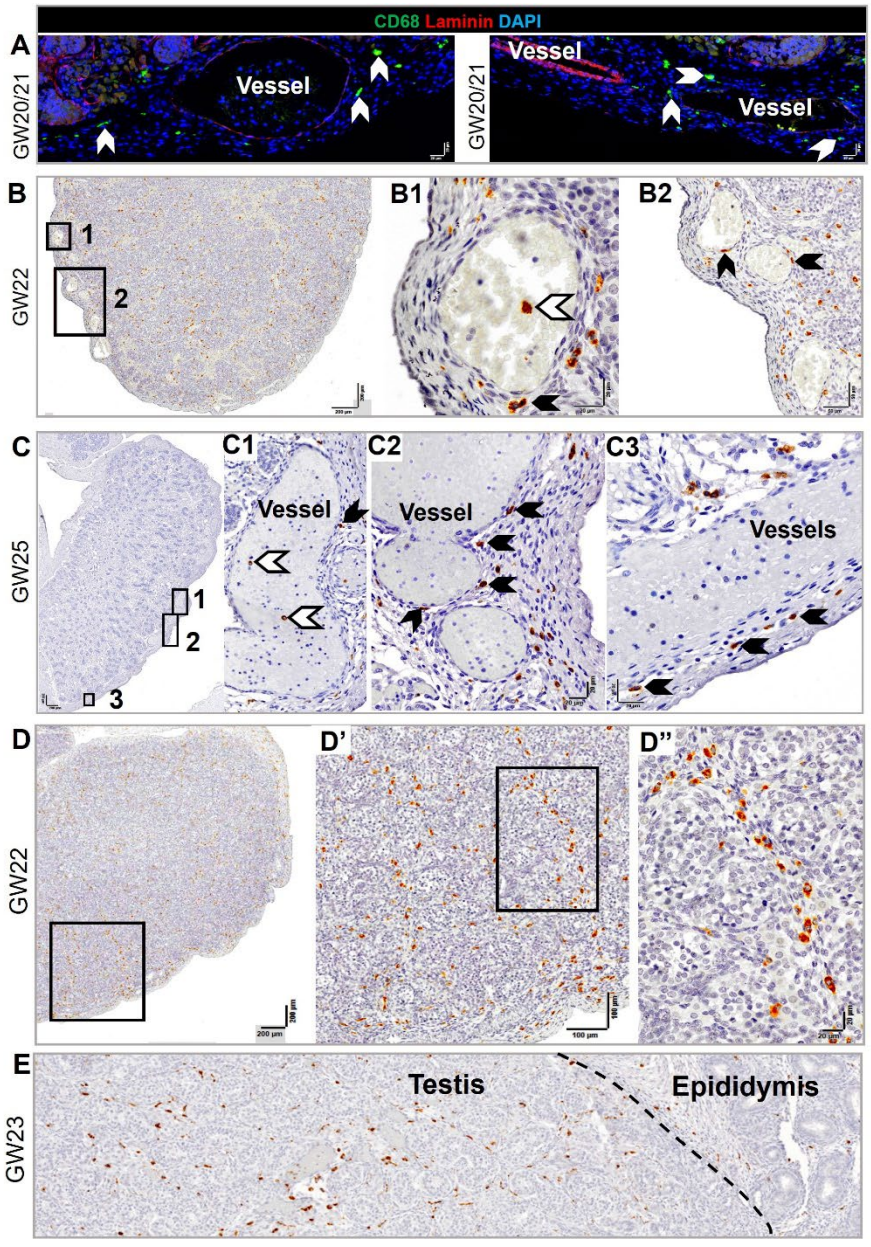
4.2. Detection of CD45^{+} cells in fetal human testis and epididymis section at GW24. CD45^{+} cells (brown) are visible in epididymis (**1** and **2**), testis (**3**), and vasculature area (**4**) of the testis. Black boxes on low magnification images refer to the higher magnification panels.



CD68, also known as microsialin, is a cell surface protein conventionally used to identify cells in the monocyte lineage, and most commonly associated with macrophages. CD68^{+} cells were abundant in the interstitium, perimeter area,

especially around vasculature, but extremely rare to absent inside vessels (Figure 4.3 A-C). Vessels were identified based on their rounded appearance in cross section, formed by elongated cells with elongated nuclei (endothelial cells) and often with red blood cells inside. After GW22, accumulation pattern of macrophages and their locations were shaped a stream appearance in the testis (Figure 4.3D). After GW23, macrophages were populated all around the testis (Figure 4.3E).

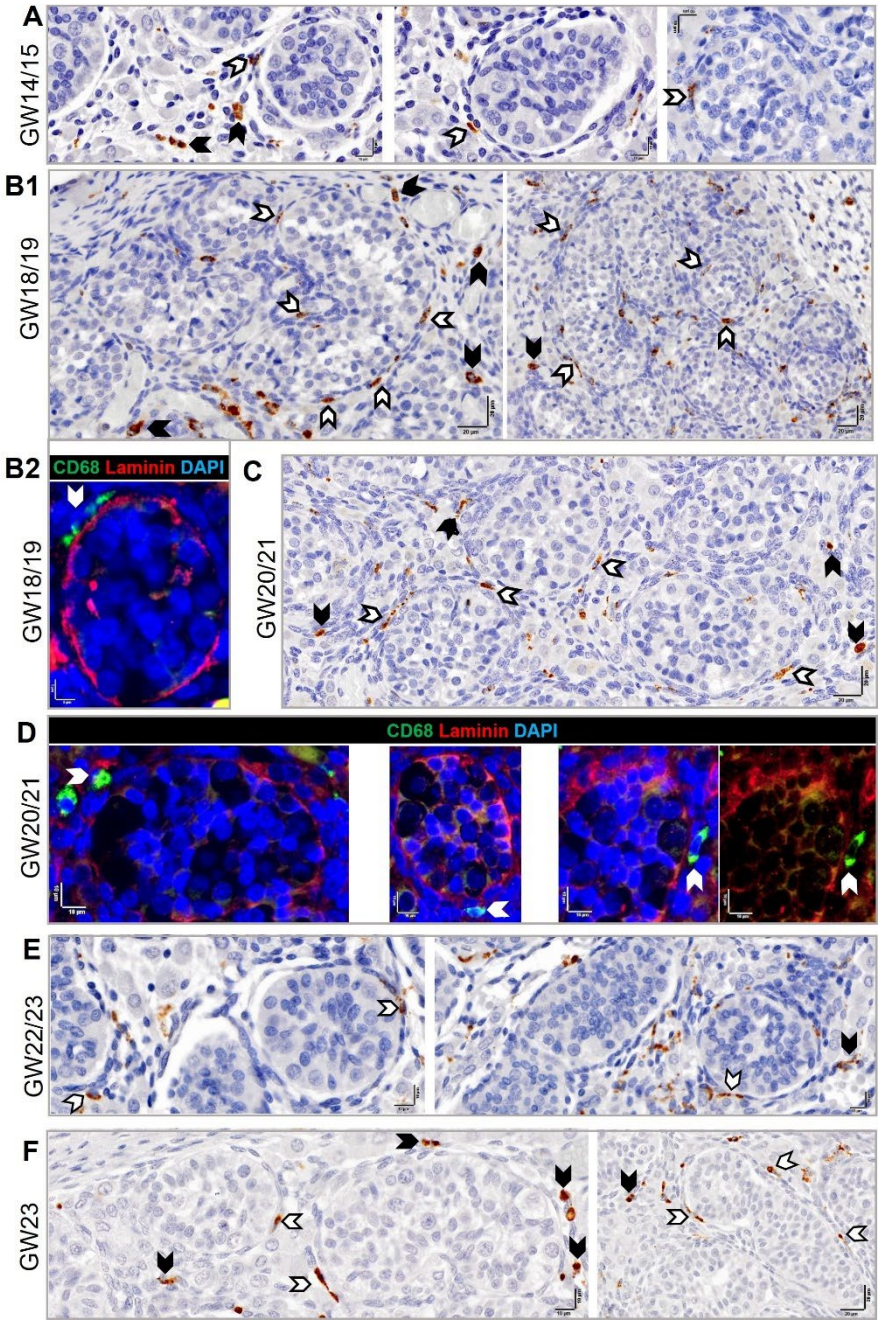
Figure 4.3. Overall patterns of CD68^{+} cells (macrophages) distribution in human fetal testis sections. (A): CD68^{+} cells were not detected using IF inside vessels at GW20/21 but were frequently present in surrounding cell layers (white arrows). Marker colours are indicated above IF images. (**B- E):** IHC images. (**B)** and **(C):** Numbered black boxes in the left-hand panel in low magnification images are shown at higher magnification in right-hand panels. Macrophages were extremely rare inside vessels (white arrows) (**B1** and **C1**), but frequent around vessels (black arrows), at GW22 (**B2)** and 25 (**C2** and **C3**). **D**: Macrophages in the



interstitium at GW22 appearing in aligned (**D'** and **D**'' provide increasingly higher magnifications). **E**: Macrophages were frequent at all locations in the testis at GW23. Nuclear staining with DAPI in A; hematoxylin in B, C, D and E.

Few macrophages were detectable adjacent to the cords at GW14/15, but they were more abundant at GW23 and afterwards (Figure 4.4).

Figure 4.4. CD68^{+} cell (macrophage) distribution in cord perimeter and interstitium of human fetal testis sections. CD68^{+} cells in perimeter area (white arrows) and interstitium and around vessels (black arrows) at GW14/15 (**A**), at GW18/19 (**B1** and **B2**), at GW20/21 (**C** and **D**), at GW22/23 (**E**) and at GW23 (**F**). IF marker colours are indicated on images. Nuclear staining with hematoxylin in insets in A, B1, C, E and F, and with DAPI in B2 and D.



Investigating CD68⁺ cells in the epididymis revealed they are frequent in different parts of epididymis including caput and cauda but absent in close proximity of ducts (Figure 4.5 and 4.6).

Figure 4.5. CD68^{+} cell (macrophage) distribution patterns shown using IHC in human fetal epididymis sections. Macrophages were frequently observed in the epididymis at GW20/21(**A)** and GW24/25 **(B).** Macrophages were present in the cord perimeter area and adjacent to the testis cord lamina propria but were not identified close to the epididymal ducts. Numbered black boxes in the left-hand low magnification image panels are shown at higher magnification in the right-hand panels. Dimensions as indicated.

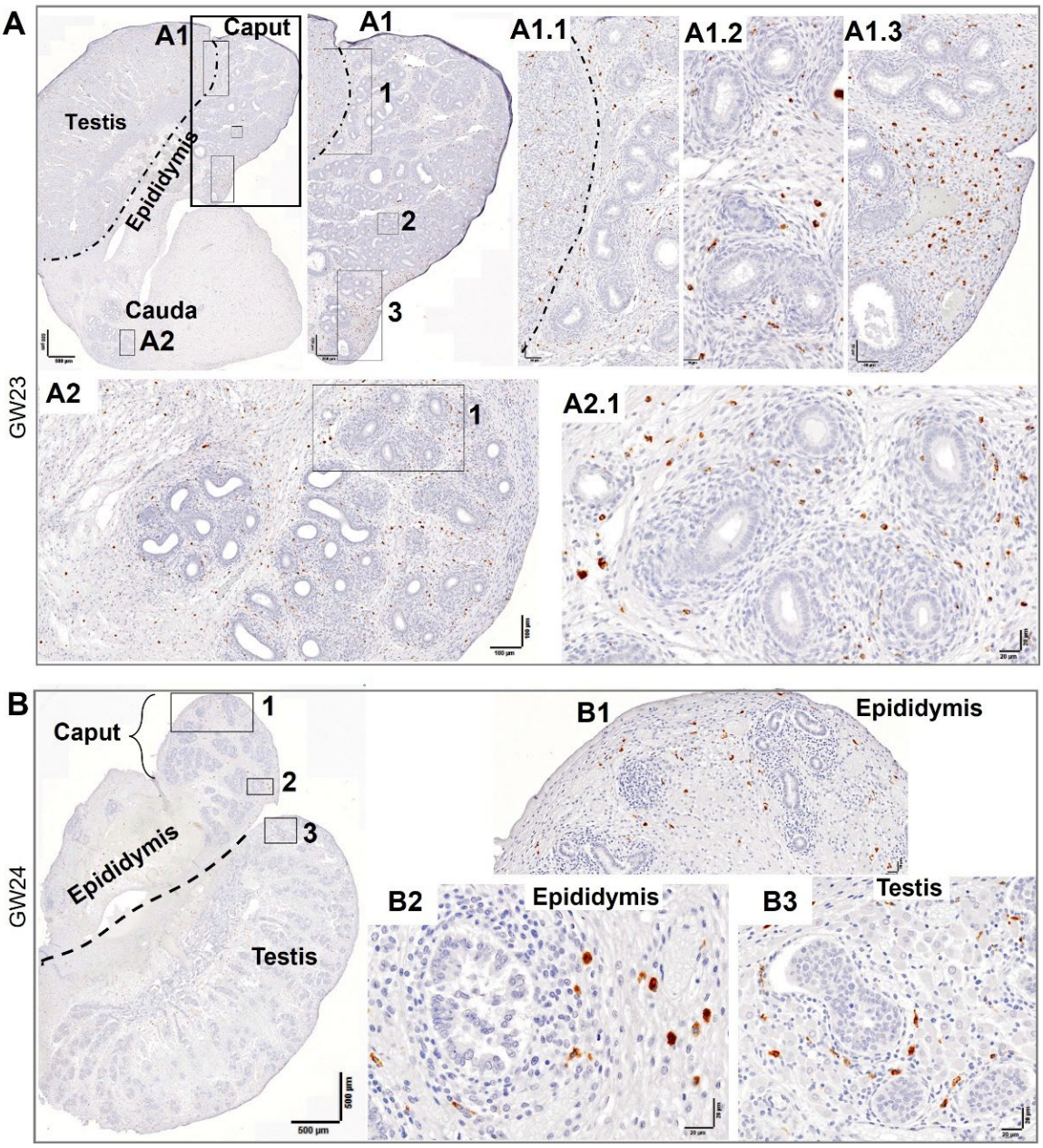
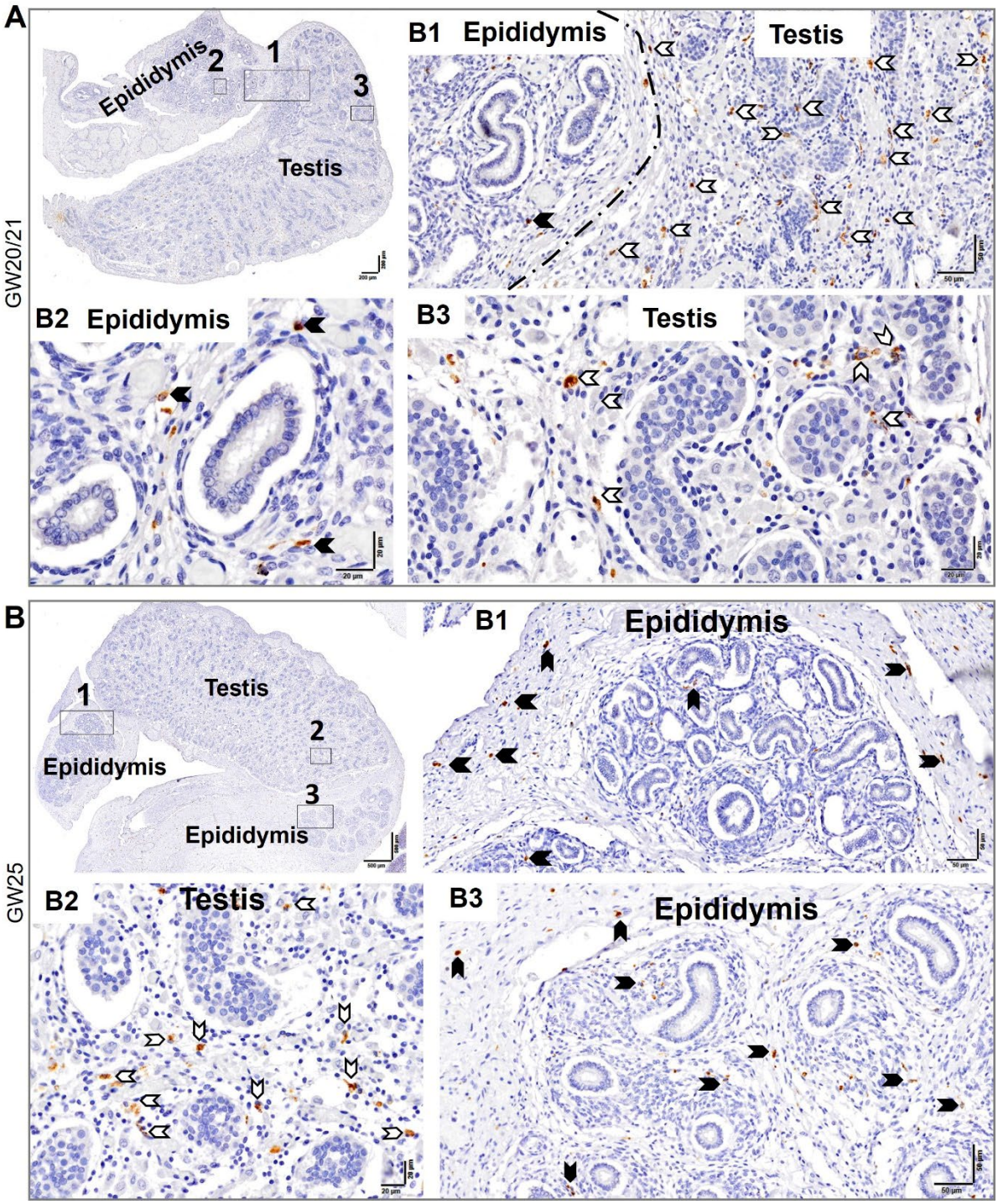


Figure 4.6. CD68$^+$ cells (macrophages) in human fetal epididymis and testis. CD68$^+$ cells on epididymis (black arrows) and testis (white arrows)) at GW20/21 (**A**) and GW25 (**B**). Dimensions as indicated.



In human fetal testis, CD3$^+$ cells were rare at the second trimester and frequent at the third trimester in interestituim. The majority of CD3$^+$ cells were located in the close proximity of blood vessels or inside the vessels (Figure 4.7). The presence of 2-3 CD3+ cells next to each other in the interstitium close to the cords was also noted in

some samples (Figure 4.7C). $CD3^+$ cells were noticeable inside and around blood vessels in the testis (Figure 4.7D). In a vessel in the testis at GW25, T cells were extremely frequent, we assume that it could be a lymph vessel (Figure 4.7E). Detection of $CD3^+$ cells around of cords was documented (Figure 4.8).

Figure 4.7. Detection of $CD3^+$ cells (T cells) in human fetal testis. A: $CD3^+$ cells were adjacent to the red blood cells in the interstitial area. **B:** T cells in the interior. **C:** Detectable T cells in interior area of the testis (black arrows), and T cells attached to each other around the cord (**C'**) at GW19/20. **D:** T cells in the margin of blood vessels and inside blood vessels, at GW19/20. **E:** T cells inside blood vessels (**E1** and **E2**) and in a lymph vessel (**E3**). CD3: pan-T cell marker. Dimensions as indicated.

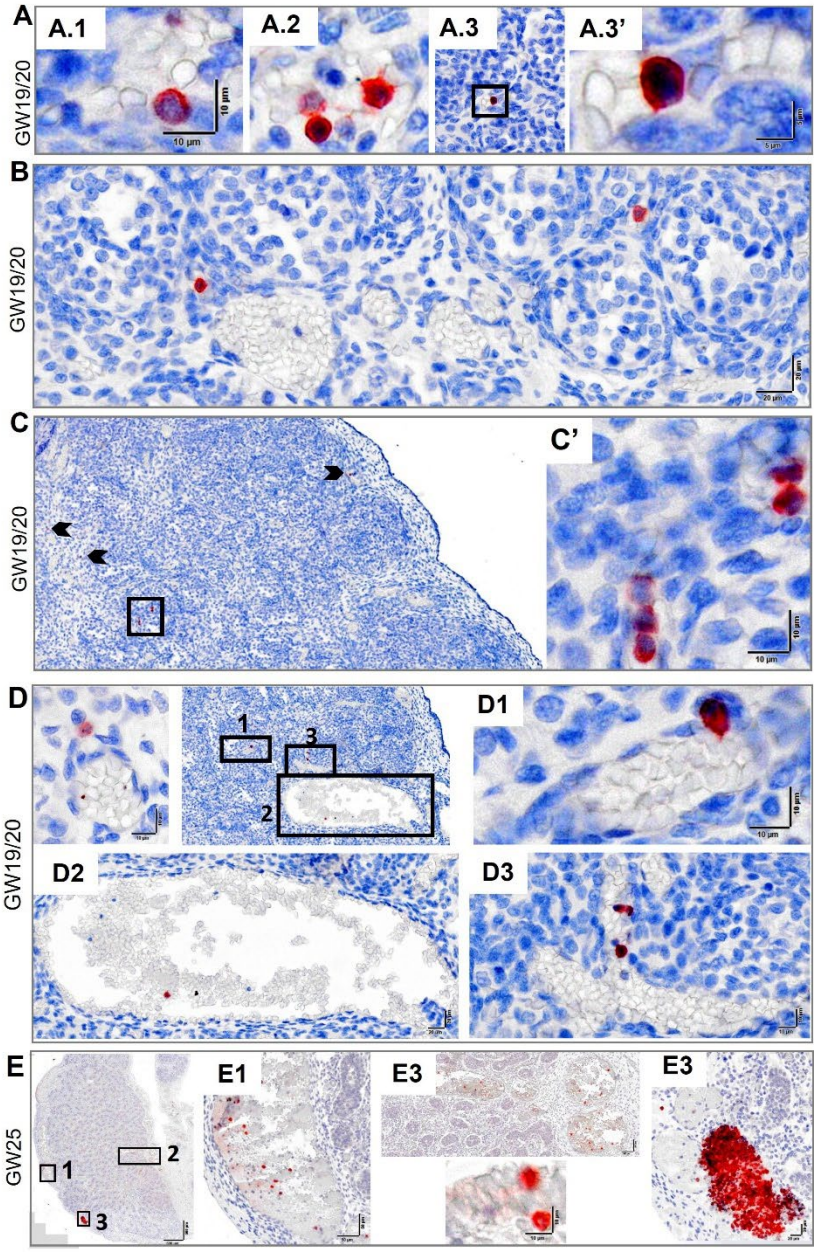
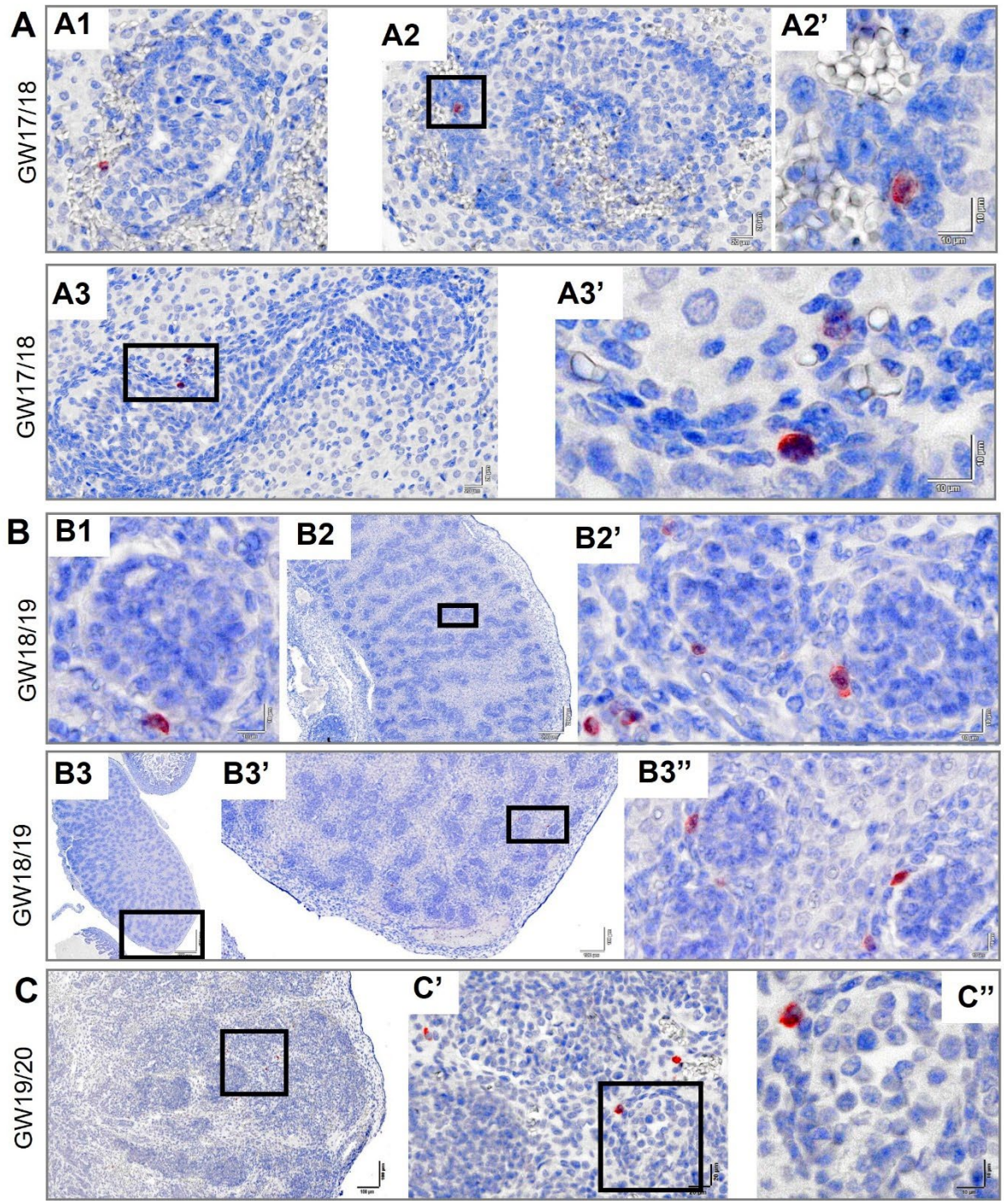
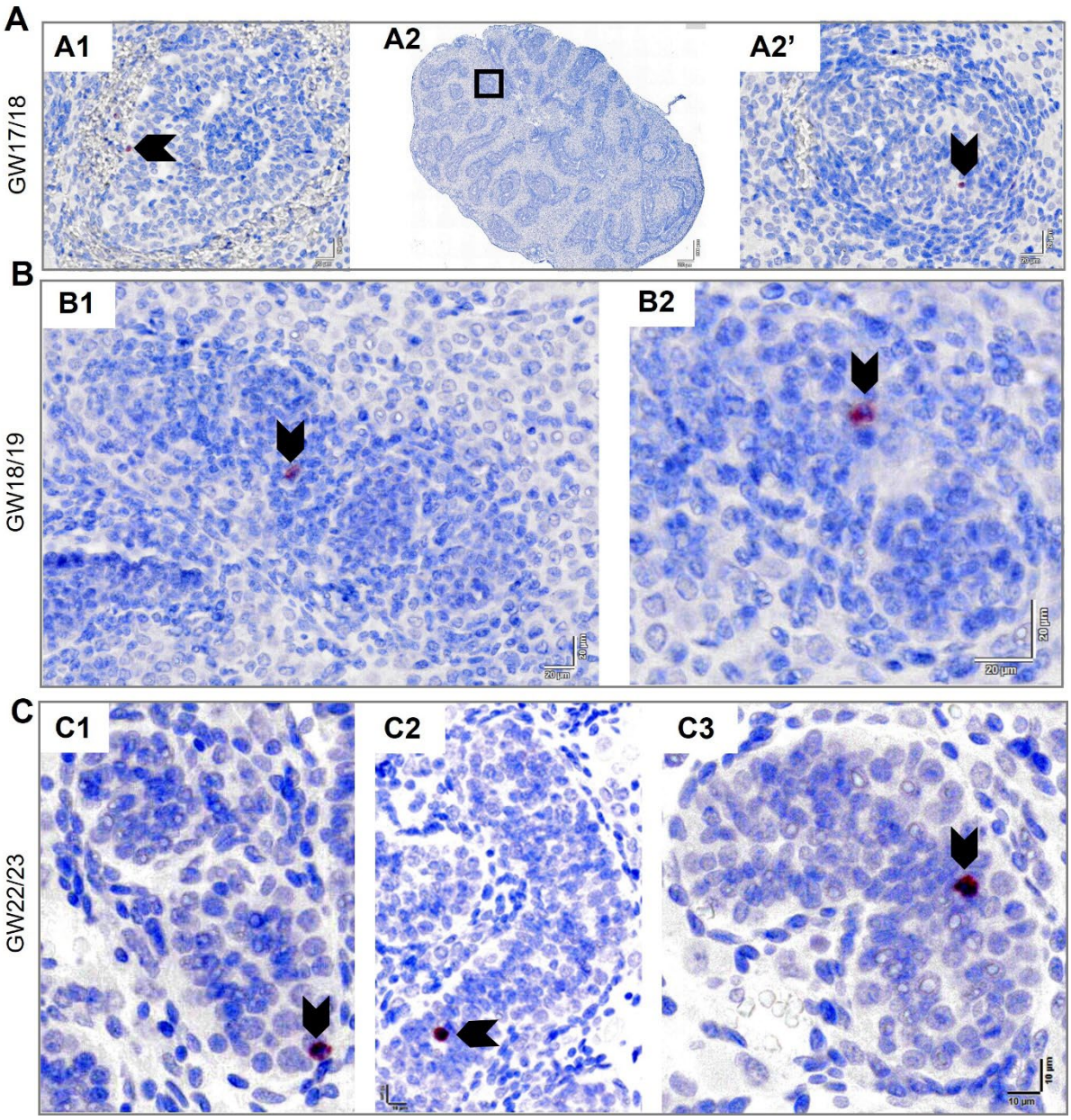


Figure 4.8. CD3$^+$ cells (T cells) in cord perimeter areas of human fetal testis. CD3$^+$ cells detected using IHC were visible adjacent to the testis cords at GW17/18 (**A**), GW18/19 (**B**) and GW19/20 (**C**). Each panel represents an individual sample. CD3: pan-T cell marker. Numbered black boxes in the lower magnification images are shown at higher magnification in the right-hand panels. Dimensions as indicated.



We identified few numbers of CD3$^+$ cells inside the cords in each individual samples. These CD3$^+$ cells inside the cord were either in periphery or interior regions of cords (Figure 4.9). In addition, a small number of CD3$^+$ cells were detected in tunica albuginea and rete testis.

Figure 4.9. CD3$^+$ cells (T cells) inside cords in human fetal testis. CD3$^+$ cells were detected inside testis cords of at GW17/18 (**A**), at GW18/19 (**B**) and GW22/23 (**C**). Each panel represents an individual sample. The numbered black box in the low magnification image is shown at higher magnification in the right-hand panel. CD3$^+$ cells are indicated using black arrows.



CD66b$^+$ cells were extremely rare in the testis interstitium and not found in the epididymis from GW17 to GW25, but individual CD66b$^+$ cells were observed inside the testis blood vessels. At GW41, CD66b$^+$ cells were numerous around vessels in the epididymal side of the testis and detected in the testicular interstitium (Figure 4.10). Also, at GW41, neutrophils were abundant in the caput of epididymis, but absent in the corpus and cauda (Figure 4.11).

Figure 4.10. CD66b$^+$ cells (neutrophils) in human fetal testis. C66b$^+$ cells were generally rare. CD66b$^+$ cells are indicated using black boxes and arrows at GW17/18 (**A**), at GW19/20 (**B**) and GW25 (**C**). C66b$^+$ are shown in higher magnifications in panels A and C. At GW41, CD66b$^+$ cells were present in the testis section interior, and they were most frequently seen around blood vessels and in the epididymis side of testis (**D**). Numbered black boxes in low magnification images are shown in higher magnification panels. Dimensions as indicated.

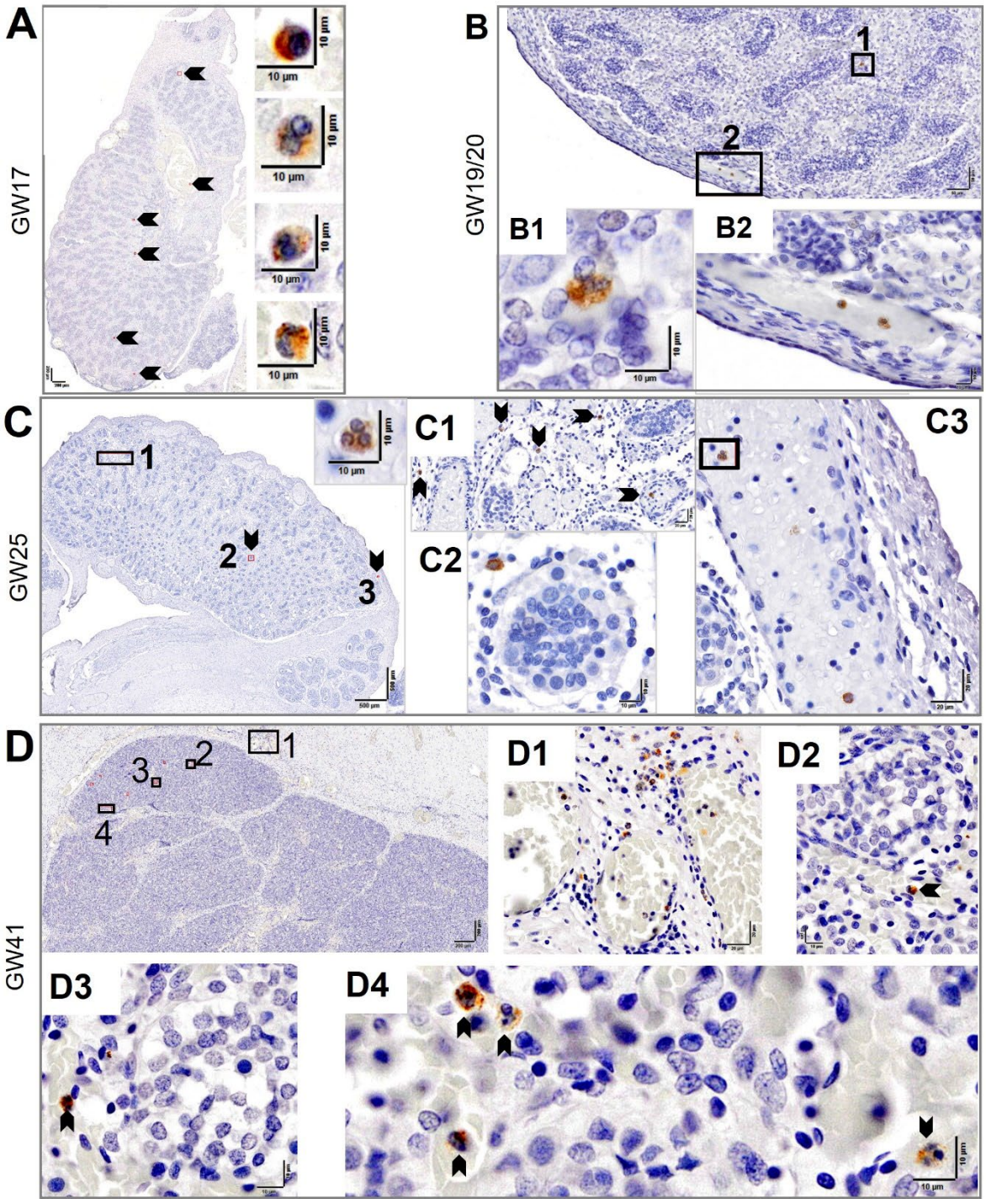
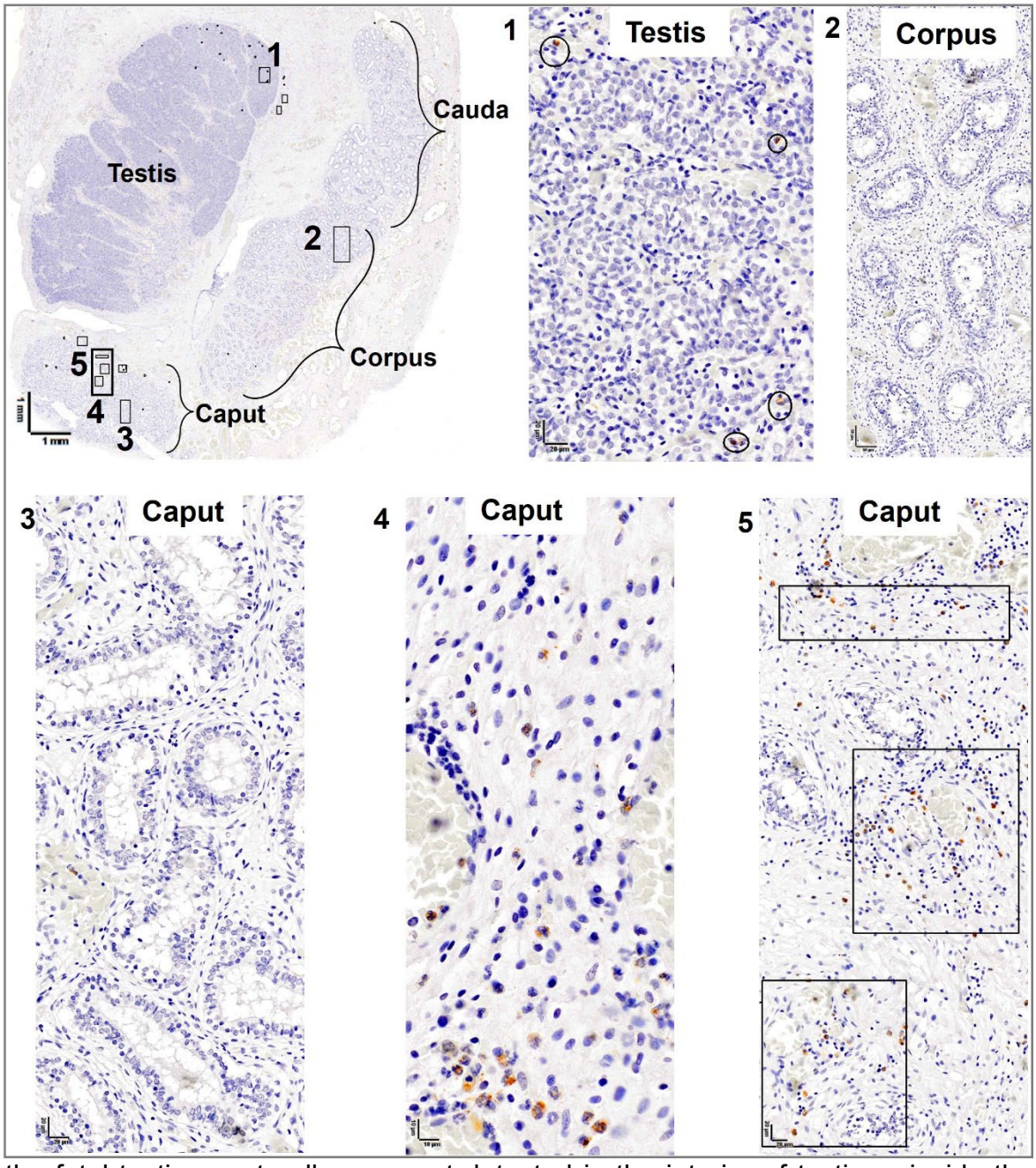


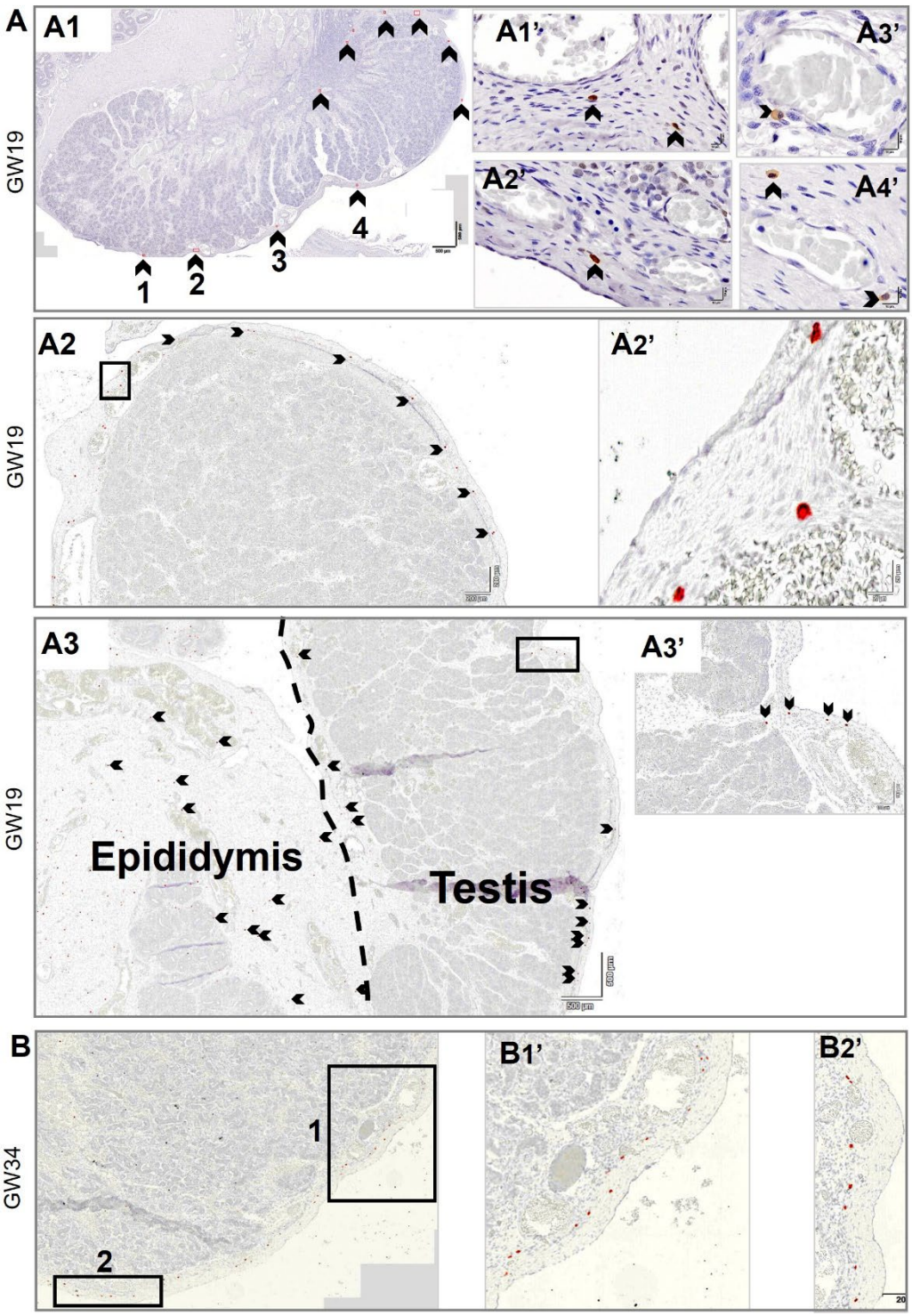
Figure 4. 11. CD66b^{+} cells (neutrophils) in human fetal epididymis and testis at GW41. **1:** Detectable CD66b^{+} cells were shown using black circles in the testis. **2:** In the corpus of epididymis CD66^{+} cells were absent. **3**: Absence of neutrophils in the side of caput which is near to corpus. **4** and **5:** Neutrophils were numerous around blood vessels or epididymis Caput; black boxes show the accumulation of neutrophils around vessels. CD66b: pan-neutrophil marker. Dimensions as indicated.



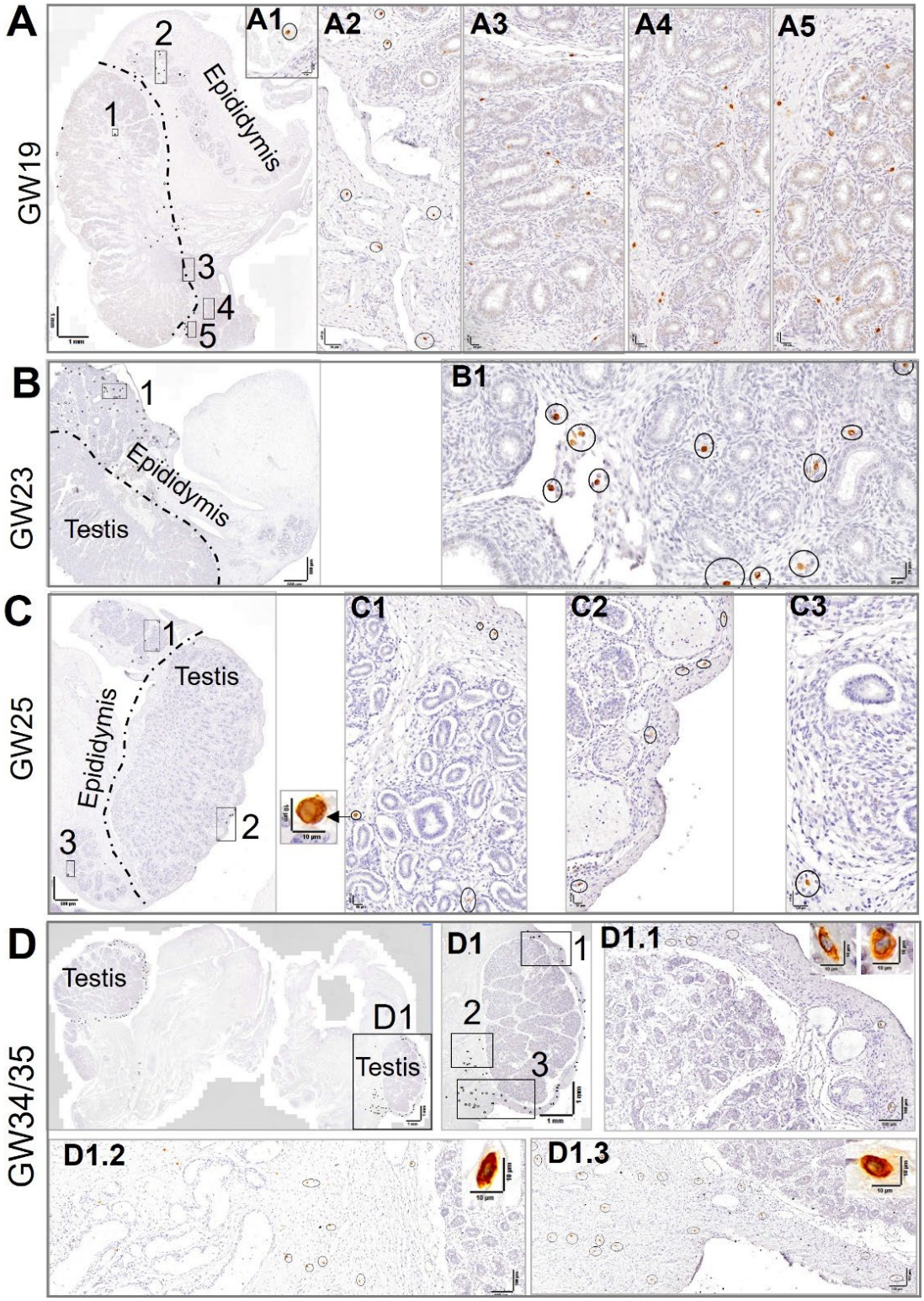
In the fetal testis, mast cells were not detected in the interior of testis or inside the blood vessels during the second and third trimesters. Mast cells were only detectable in perimeter areas close to the capsule, around vessels, but were frequent on the epididymis side of testis (Figure 4.12 and 4.13). Comparison of number of mast cells

in the testis and epididymis revealed that these cells were much more abundant and distributed in the epididymis.

Figure 4.12. Tryptase$^+$ mast cells in human fetal testis. (A1) and **(A2):** Tryptase$^+$ mast cells in the testis were detectable only in the perimeter region between the capsule and the outermost cords. (**A3):** Although mast cells were absent from the interestituim, they were numerous in the epididymis side of testis. (**B**) At GW34, mast cells were around the testis perimeter. Arrowheads indicate tryptase$^+$ cells. Numbered black boxes in the low magnification images are shown in higher magnification panels. Dimensions as indicated.



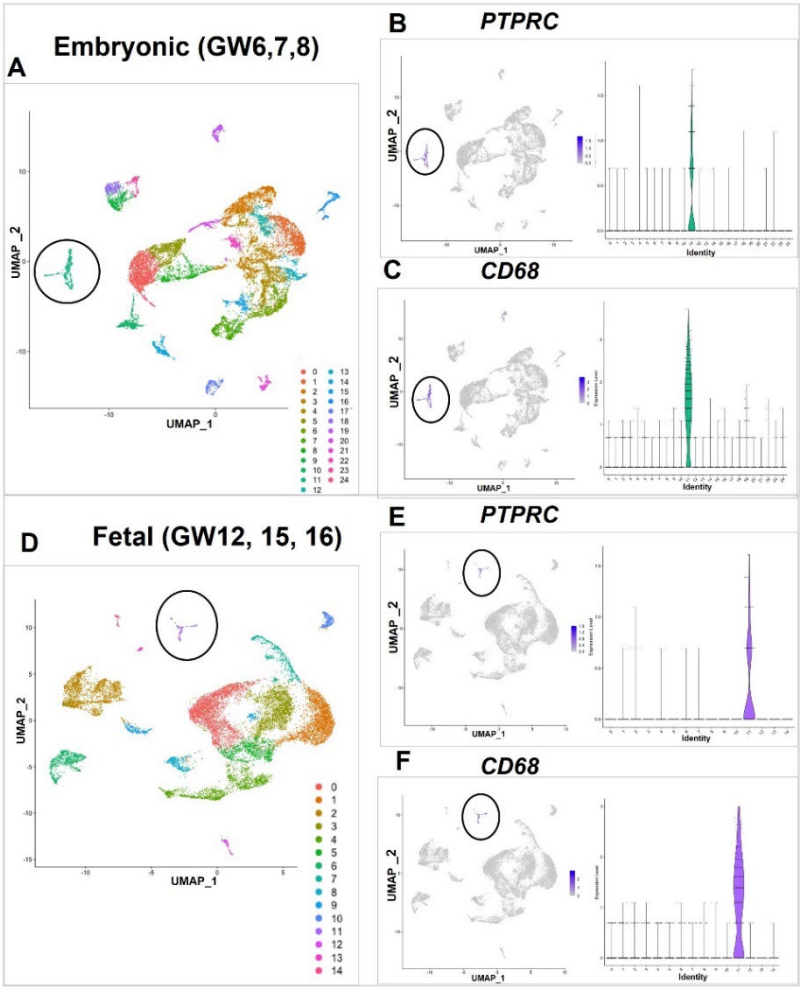
4.13. Tryptase$^+$ mast cells in human fetal epididymis and testis. In the testis, tryptase$^+$ mast cells were only detectable in perimeter area, absent in the interestituim of testis, rare in the rete cord testis, numerous in the epididymis side of testis and abundant in the epididymis. Dimensions as indicated.



4.3.1. Single Cell RNA-sequencing data analyses

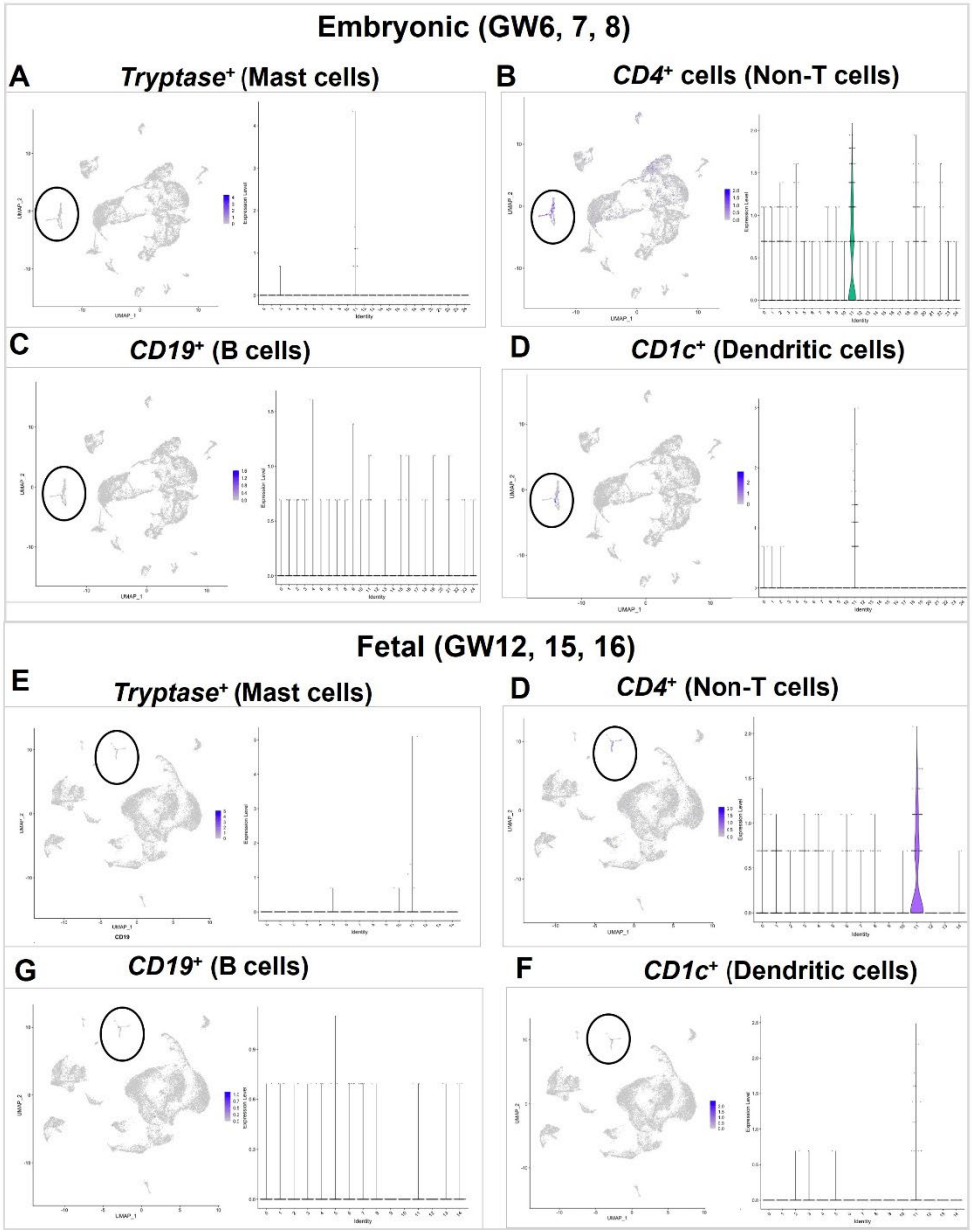
Analysis of published scRNA-seq data (Guo et al., 2021) obtained from human embryonic (GW6, 7, 8) and fetal (GW12, 15, 16) testes identified a single cluster, cluster 11, in both age groups that was comprised of cells expressing *PTPRC*. Identification of cells in the *CD45*$^{+}$ cluster as immune cells was verified by the profiles of other markers (Figures 4.14B and E) which were barely or not detected in all other clusters. In both age groups, the number of cells expressing *CD68* in this cluster was much higher (visualised on UMAP and violin plots) than were those expressing other immune cell markers.

Figure 4. 14. **Transcript data from human embryonic and fetal testis single cell-RNA sequencing (scRNA-seq) (Guo et al., 2021) illustrates profiles of *PTPRC* (CD45) and *CD68*.** (**A, D**). Cell clusters (UMAP) were generated by combining single-cell transcriptome data from embryonic human testes samples at gestation weeks (GW) 6, 7 and 8 and fetal human samples at GW12, 15 and 16 (n = 1 for each age). (**B, E**). Cluster 11 of immune cells expressing *PTPRC* (encoding CD45) transcript in embryonic and fetal samples is circled (**B, F**). UMAPs (**A, D**) and violin plots for (**B, C, E, F**) CD68 (macrophage marker) in embryonic and fetal samples. Individual points represent a single cell. Black circles identify immune cell clusters. Colour intensity scales indicate relative transcript levels. Violin plots illustrate immune cell marker levels within individual cells in each cluster (n = 1 for each age).



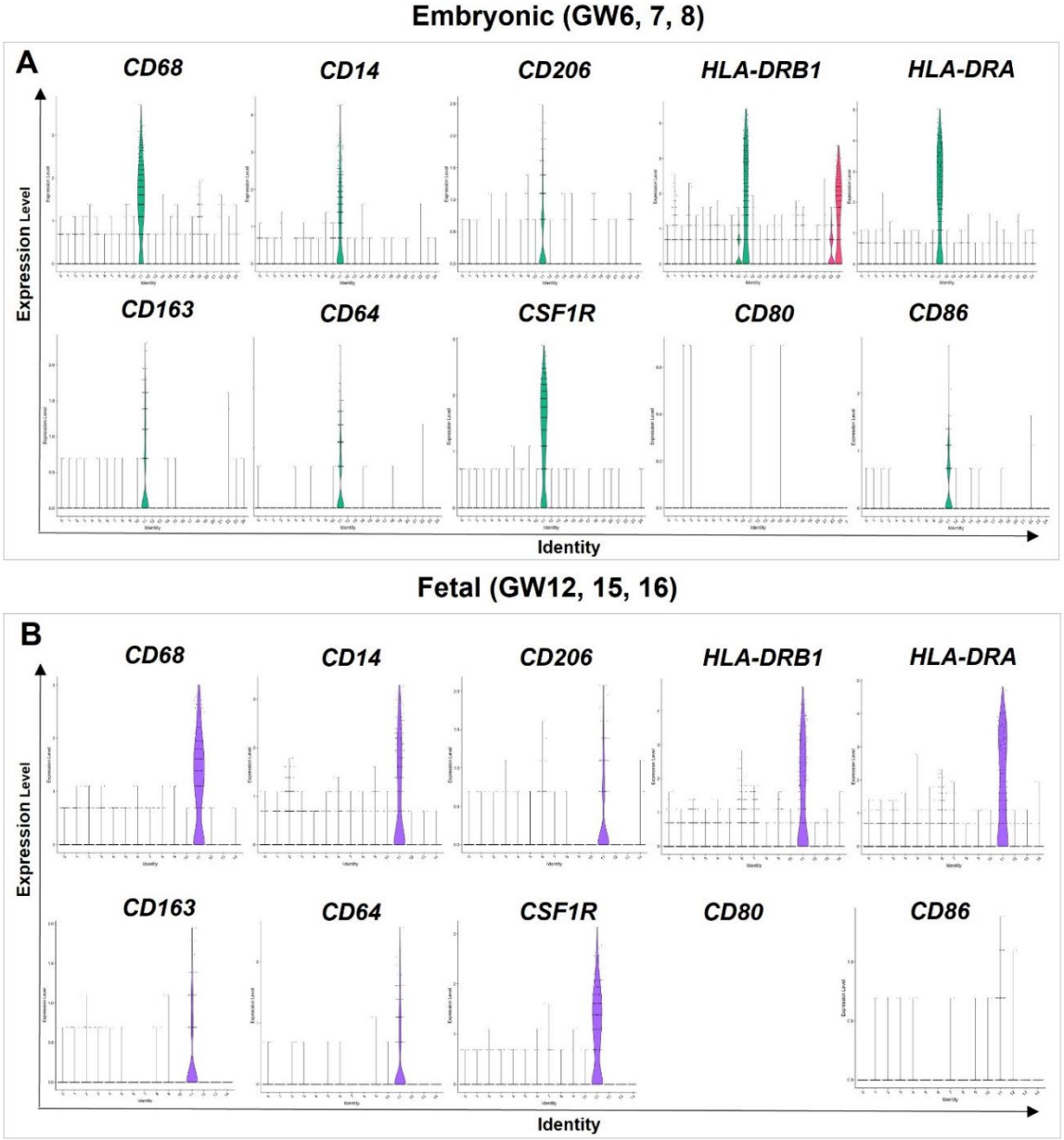
CD4 was the next most frequently detected marker, while *TPSAB1* (encoding mast cell tryptase) and *CD1C* (dendritic cells) were barely detected but registered most frequently in this *CD45*$^{+}$ cluster. Classical markers/genes for other cell types were not detected, including *NKG2A/CD159A* (NK cell), *CD66B* (neutrophils) and *CD3* (T cells) CD19 (B cells) (Figures 4.15, 4.17 and 4.18).

Figure 4.15. **Transcript data from human embryonic and fetal testis single cell-RNA sequencing (scRNA-seq) (Guo et al., 2021) illustrates profiles of *TRYPTASE, CD4, CD19* and *CD1C*.** *TRYPTASE,* expressed by mast cells (**A** and **E**); *CD4,* expressed by macrophages (**B** and **D**); *CD19* expressed by B cells; (**C** and **G**), *CD1C*, expressed by dendritic cells (**D** and **F**). Individual points represent a single cell. Black circles indicate immune cell clusters. Colour intensity indicates relative transcript level, displayed on adjacent scales. Violin plots represent immune cell marker levels within individual cells in each cluster (n = 1 for each age).



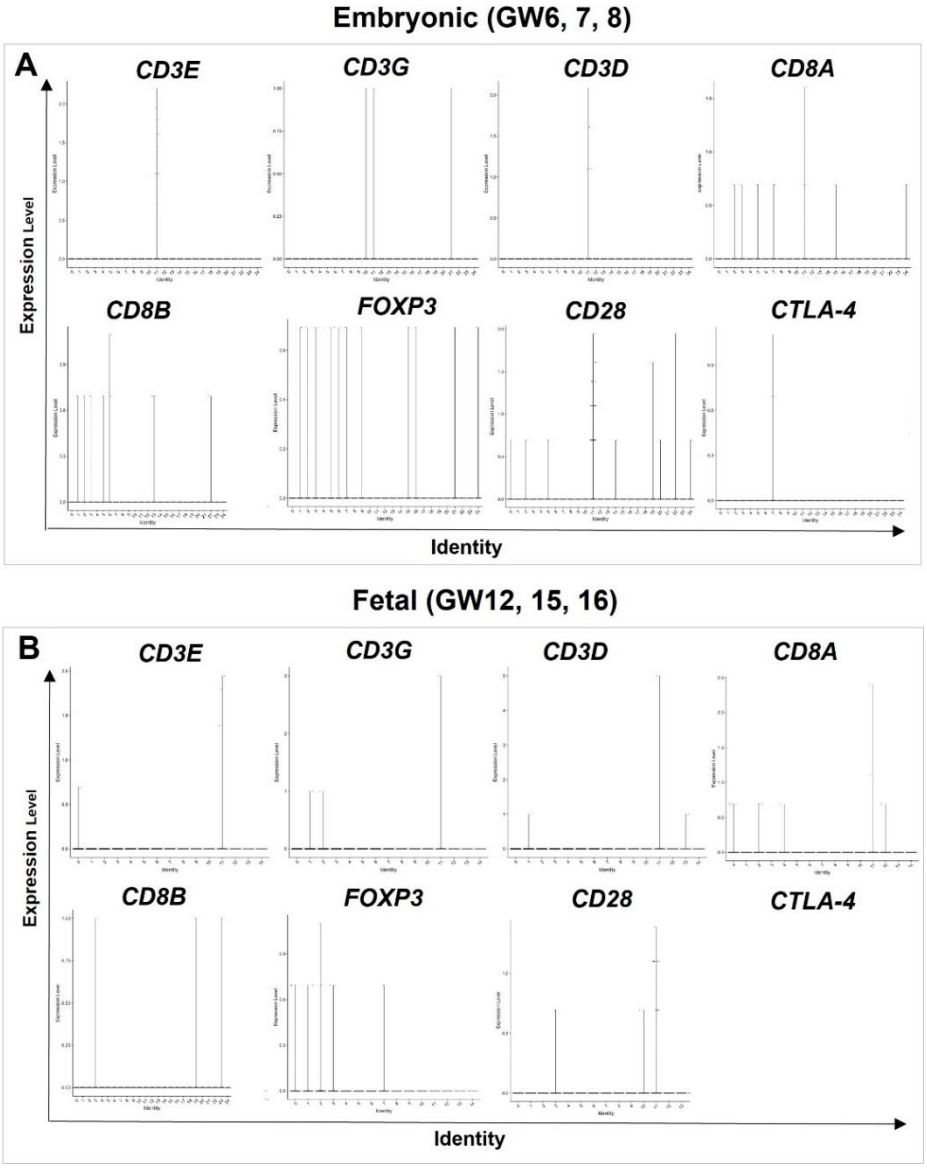
The levels of *HLA-DRB1, HLADRA and CSF1R* transcripts were higher than *CD206, CD163, CD64* and *CD86*, while the *CD80* level was extremely low for embryonic samples and undetectable for fetal ones (Figure 4.16).

Figure 4.16. **Transcript data from human embryonic and fetal testis single cell-RNA sequencing (scRNA-seq) (Guo et al., 2021) illustrates profiles of *CD68, CD14, CD206, HLA-DRB1, HLA-DRA, CD163, CD64, CSF1R, CD80* and *CD86.*** Single cell-RNA sequencing (scRNA-seq) data analysis for the human embryonic (gestation week 6, 7 and 8) (**A**) and fetal (gestation week 12, 15 and 16) (**B**) testis samples. Violin plots represent immune cell marker levels within individual cells in each cluster. The cluster number 11 is consisted of immune cells in both age groups. Violin plots for macrophages encoding CD68, CD14, CD206, HLA-DRB1, HLA-DRA, CD163, CD64, CSF1R, CD80 and CD86 were presented. Individual points represent a single cell. Green and purple colour intensity indicates relative transcript level, displayed on adjacent scales (n = 1 for each age).



To study T cells, we used CD3, CD8 and CD4 markers. Although there were few cells encoding *CD3E, CD3D, CD3G* and *CD8A* and *CD B* in the testes of embryonic and fetal samples, there were significantly higher numbers of *CD4*$^{+}$ cells (Figures 4.16 and 4.17). These *CD4* cells were most likely macrophages and not T cells because cells expressining *CD3* were rare and macrophages also express CD4 (Filion et al., 1990; Zhen et al., 2014). There were only detectable and very low levels of expression of the *CD28* transcript, but *FOXP3* and *CTLA-4* transcripts were almost undetectable (Figure 4.17).

4.17. Transcript data from human embryonic and fetal testis single cell-RNA sequencing (scRNA-seq) (Guo et al., 2021) illustrates profiles of *CD3E, CD3G, CD3D, CD8A, CD8B, FOXP3, CD28 and CTLA-4.* Violin plots represent T cell marker levels within individual cells in each cluster. The cluster number 11 is consisted of immune cells in both age groups. Violin plots for T cells expressing CD3E, CD3G, CD3D, CD8A, CD8B, FOXP3, CD28 and CTLA-4 were presented. Individual points represent a single cell. Green and purple colour intensity indicates relative transcript level, displayed on adjacent scales (n = 1 for each age).



There were few cells expressing the mRNA encoding TRYPTASE, while a higher number of cells expressed the *CD1C* transcript (dendritic cell marker) (Figures 4.18 and 4.19). We also used *CD141* (conventional DCs), *CD11C* (myeloid DCs) and *CD303* (plasmacytoid DCs) to define DCs (Kassianos et al., 2010; Heidkamp et al., 2016) that confirmed the *CD11c* is a more specific marker to detect DCs, as it was only presented on cluster 11 (Figures 4.18 and 4.19). The number of cells (B cells) expressing *CD19* was very low, and no cells contained CD*20* (Figures 4.15C and G and 4.18B and 4.19B). In both age groups, cells expressing *CD66b* were rare (Figures 4.18C and 4.19C).

As markers of NK cells, we examined *CD56*, *CD94* and *NKG2A/CD159a* and *CD16* (Cianga et al., 2021; Fogel et al., 2013). There were few cells expressing *CD56*, *CD94* and *NKG2A/CD159a*, leading to the conclusion that there no NK cells were present at these ages. A small number of cells expressing *CD16* were most likely macrophages because this marker expresses on macrophages as well (Kapellos et al., 2019) (Figures 4.18D and 4.19D).

The population of cells expressing *PD-1* mRNA in older samples (GW12, 14 and 15) was drastically higher than in embryonic samples (GW6, 7 and 8), but there were few cells expressing PD-L1 in both age groups (Figures 4.18E .and 19E). Few cells in cluster 11 contained the *MKI67* transcript (Figures 4.18F and 4.19F).

Figure 4.18. Transcript data from human embryonic testis single cell-RNA sequencing (scRNA-seq) (Guo et al., 2021) illustrates profiles of *CD141, CD11C, CD303, CD20, CD66B, CD56, CD94, NKG2A/CD159A, CD16, PD-1, PD-L1* and *MKI67.* Violin plots represent immune cell marker levels within individual cells in each cluster. The cluster number 11 is consisted of immune cells in both age groups. Violin plots for Dendritic cells expressing CD141, CD11C, CD303 (**A**), B cells expressing CD20 (**B**), neutrophils expressing CD33 and CD66B (**C**), NK cells expressing CD56, CD94, NKG2A/CD159A, CD16 (**D**) and immune check points PD-1 and PD-L1 (**E**) and proliferation marker MKI67 (**F**) were presented. Individual points represent a single cell. Green and purple colour intensity indicates relative transcript level, displayed on adjacent scales. On all graphs vertical lines show expression levels and horizontal lines represent identity (n = 1 for each age).

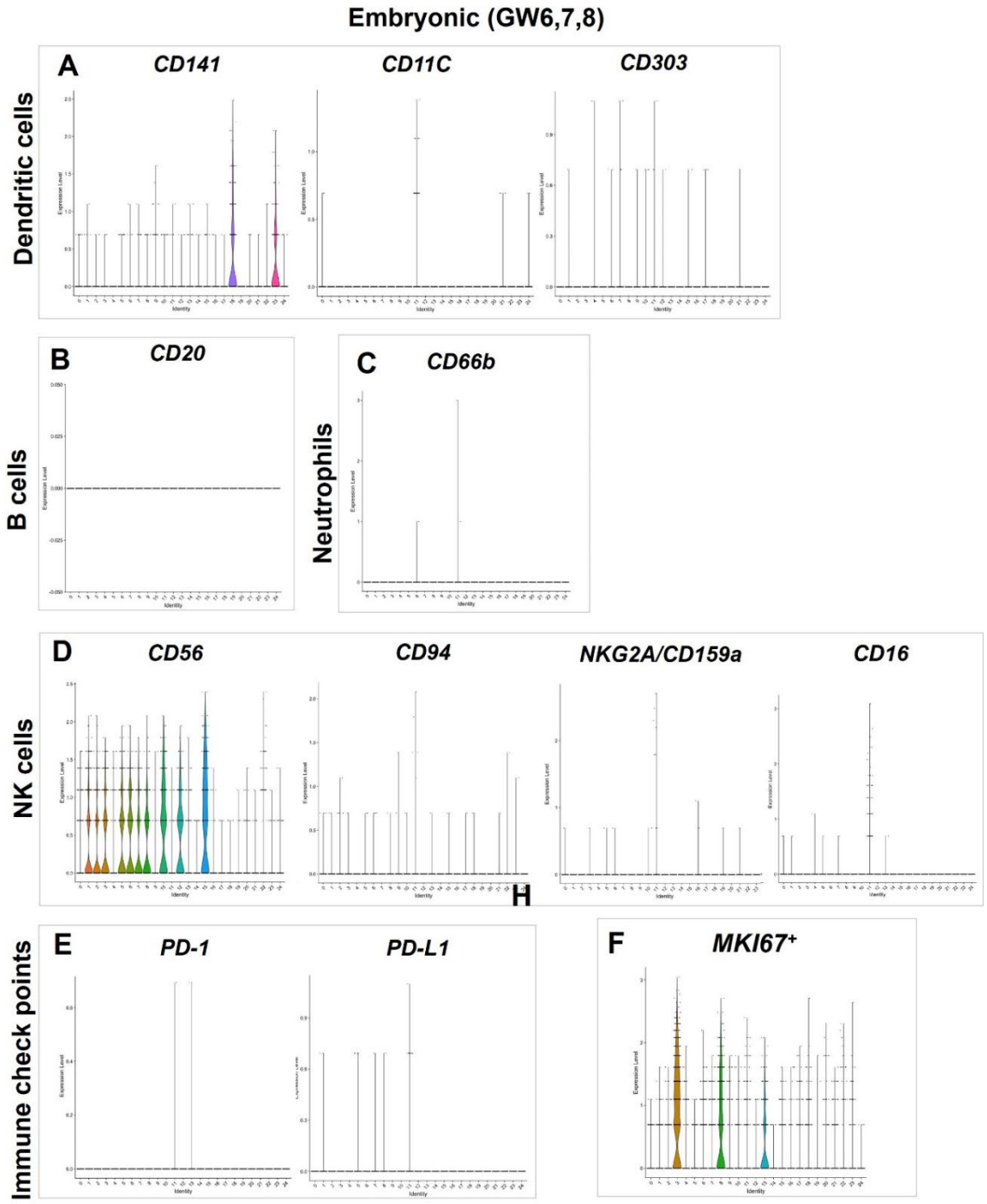
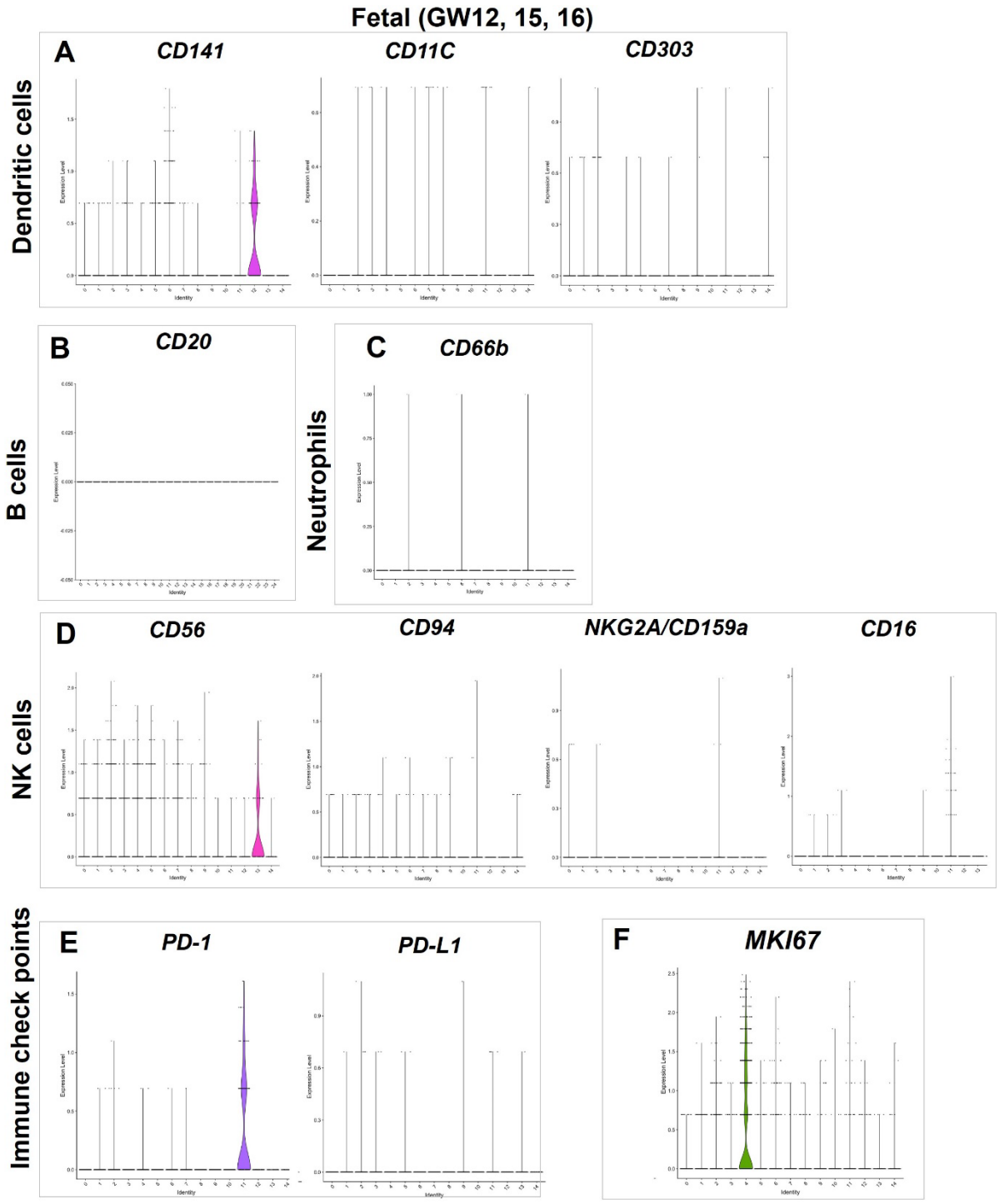


Figure 4.19. Transcript data from human fetal testis single cell-RNA sequencing (scRNA-seq) (Guo et al., 2021) illustrates profiles of *CD141, CD11C, CD303, CD20, CD66B, CD56, CD94, NKG2A/CD159A, CD16, PD-1, PD-L1* and *MKI67.* Violin plots represent immune cell marker levels within individual cells in each cluster. The cluster number 11 is consisted of immune cells in both age groups. Violin plots for Dendritic cells expressing CD141, CD11C, CD303 (**A**), B cells expressing CD20 (**B**), neutrophils expressing CD33 and CD66B (**C**), NK cells expressing CD56, CD94, NKG2A/CD159A, CD16 (**D**) and immune check points PD-1 and PD-L1 (**E**) and proliferation marker MKI67 (**F**) were presented. Individual points represent a single cell. Green and purple colour intensity indicates relative transcript level, displayed on adjacent scales. On all graphs vertical lines show expression levels and horizontal lines represent identity (n = 1 for each age).



4.2. Discussion

The main focus of studies examining the impact of environmental and pharmaceutical exposure on general aspects of human testis development and function has been germ cell development, using approaches including xenografting, *in vitro* testicular organoid culture, and single-cell analysis (Lambrot et al., 2006; Houmard et al., 2009; Cowan et al., 2010; Mitchell et al., 2010; Rouiller-Fabre et al., 2014; Kilcoyne et al., 2019; Yuan et al., 2020; Guo et al., 2021; Sararols et al., 2021). However, there is little knowledge about the identity of testicular immune cells during fetal morphogenesis, particularly since human samples are rarely available. This study presents the first systematic examination of key immune cell types that emerge during the second and third trimesters of human pregnancy using immunohistochemistry on archival materials. This is extended by inclusion of the newly available information regarding human fetal testicular immune cells which can be accessed through scRNA-seq data published by Guo et al. and Sararols et al., (Guo et al., 2021; Sararols et al., 2021). Although the main analyses presented in these reports focused on germ, Sertoli, Leydig and myoid cells, we have extracted information on immune cells from the raw data made available by Guo et al. and present it here in combination with the results derived from analysis of tissue sections.

As single cell RNA-seq data were generated from fetuses at GW6-8, immune cells were exclusively yolk sac-derived, in contrast to data generated from immunohistochemistry, which also includes bone marrow-derived immune cells.

Gestational age refers to the length of pregnancy, calculated from the first day after the last menstrual period, while the true fetal age is defined by the time of conception (i.e., post-coital week, PCW). In this study, fetal age for both histological and transcript samples is reported as gestational week, or GW. In both age groups analysed using single cell RNA-seq (embryonic: GW6, 7, 8, and fetal: GW12, 15, 16), cluster number 11 consisted of as a well-defined population of cells containing the *PTPRC* transcript, encoding CD45. Abundant $CD45^{+}$ cells were detected by IHC in the testis (in the interstitium, inside and around vessels, around and adjacent to cords, and in the rete testis), but these were less frequent in the epididymis. Recent studies on diverse human and mouse tissues have demonstrated that most tissue resident immune cells in the steady state are derived from embryonic progenitors (Hoeffel et al., 2012; De Kleer et al., 2014; Hoeffel and Ginhoux, 2015; Lokka et al., 2020; Wang et al., 2021).

Thus, a description of immune cells present during fetal testis development can shed light on the origin of immune cells resident in the adult human testis.

CD68$^+$ cells (macrophages)

The originally dominant concept that tissue-resident macrophages are monocyte-derived has been challenged by depletion experiments in adult mice which demonstrated the diverse origins of macrophages in different tissues. In mice, microglia, Langerhans cells, alveolar macrophages and Kupffer cells first originate from yolk sac progenitors, followed by the emergence of fetal liver-derived monocytes. Macrophages in the heart, pancreas, gut, and dermis initially originate from fetal liver monocytes, but these are substantially or almost entirely replaced by bone marrow-derived monocytes later in life (Wolf et al., 2019). Therefore, this report describing macrophages in the early second trimester of pregnancy can provide evidence of the embryonic ontogeny for human testicular macrophages. While CD68$^+$ macrophages were observed to be relatively abundant in the perimeter, interstitium, and around vessels in the fetal testis, they were extremely rare or absent within blood vessels. The density of macrophages adjacent to seminiferous cords increased from GW14/15 to GW41. CD68$^+$ cells were also observed in relatively high numbers in the interstitium of the caput and cauda epididymis but were rarely observed close to the ducts. In scRNA-seq data from both embryonic and fetal testes, the abundance of macrophage marker transcripts *CD68, CD14* and *CSF1R* in immune cells indicates the prevalence of this cell type in the fetal testis.

The scRNA-seq dataset also allowed exploration of the potential function status of macrophages in the fetal testis. HLA-DR proteins on macrophages are involved in antigen presentation to T cells, permissive for either inducing an immune response or suppressing T cell activation (Bright and Munro, 1981). Fetal liver-derived macrophages (*CD14* cells) at 6–17PCW exhibit high *HLA-DR* levels on monocyte/macrophage progenitors (Popescu et al., 2019). scRNA-seq data confirmed that, in both fetal and embryonic testes, there was a high number of cells expressing *HLA-DR* and *CD14* transcripts. Cells expressing *HLA-DRA* and *HLA-DRB1* (predominantly found on M1 macrophages) were more abundant than cells expressing *CD206* and *CD163* (predominant on macrophages with an M2 phenotype). In the mouse fetal testis, the MHC class II (E2-eb1, the homologous mouse protein of HLA-DR in human) marker protein is not detected on testicular macrophages before birth

(Mossadegh-Keller et al., 2017; Lokka et al., 2020; Chapter Three of this thesis). Although *HLA-DR* is also present in B cells and dendritic cells, we are confident that its presence represents detection of macrophage transcripts in these early testis ages, as the numbers of B cells (indicated by *CD19* and *CD20*) and dendritic cells (represented by *CD1c*) were low during the second trimester. This provides a point of contrast between mouse and human fetal testes regarding immune cell composition. Additional investigations are needed to evaluate the phenotypic and functional similarities and differences between mice and humans regarding MHC class II (HLA-DR) expression on macrophages.

HLA-DR proteins require CD80 and CD86 as costimulatory molecules to present antigens to T cells, in order to regulate their activation status (Halliday et al., 2020). However, in human fetal testis samples, the number of cells expressing the *CD86* transcript was low and *CD80* was undetectable, as shown by scRNA-seq. This suggests macrophages before GW16 may not be proficient antigen presenters.

$CD3^+$ cells (T cells)

Colonisation of T lymphocyte progenitors in the human thymus takes place during the 8^{th} GW, and the first mature T cells are detectable at 13GW in the thymus (Stites and Pavia 1979; Haynes et al., 1989). Cells expressing *CD3* are present in the human fetal liver from GW7- 8, and those with *CD8* mRNA are detectable at 12–14PCW (Lobach and Haynes, 1987; Haynes and Heinly, 1995; Farley et al., 2013; Popescu et al., 2019). T cell ($CD3^+$ and $CD4^+CD25^+$ cells) spreading from the thymus to the periphery starts rapidly after the end of 13GW. $CD3^+$ T cells are observed in the peripheral blood at 15–16GW. The presence of $CD3^+$ cells in the thymus and periphery occurs concordant with the appearance of Tregs ($CD4^+CD25^+$ cells), indicating a tight control loop of fetal immune responses (Darrasse-Jeze et al., 2005). $CD3^+$ cells and Tregs are detectable in the fetal spleen, liver, bone marrow and intestinal mucosa between 14.5 and 16GWs (Hayward, 1983; Spencer et al., 1986; Royo et al., 1987; Darrasse-Jeze et al., 2005).

Therefore, the detection of a low number of cells expressing T cells transcripts in human embryonic and fetal testis agrees with previous studies. From 14GW onwards, $CD4^+CD25^+$ cells in fetal organs are functional and express intracellular cytotoxic T-lymphocyte-associated antigen 4 (*CTLA-4*) and *Foxp3* mRNA (Darrasse-Jeze et al., 2005). Fetal Tregs have been of interest as an *in vitro* study demonstrated that human

fetal CD4$^+$ cells preferentially polarized into Tregs after stimulation against non-inherited maternal alloantigens. This immune suppression is critical during pregnancy, and interestingly, the regulated immune response against maternal alloantigens continues even during adulthood (Mold et al., 2008).

The current study demonstrates that CD3$^+$ cells are rare and scattered in the interstitium, in the proximity of or inside blood vessels, and around the cords during the second and third trimesters of pregnancy in the human fetal testis; CD3$^+$ cells are rarely observed inside cords. Although there were a considerable number of cells containing the *CD4* transcripts in both age groups, we considered these cells belonged to the macrophage population and not T cells, as the number of cells expressing *CD3* (*CD3, E, D and G*), *CD8* (*CD8a and B*), and other Treg markers (*FOXP3, CD25, CTLA-4*) was very low. Although all monocytes and macrophages express *CD4*, the number of transcripts per cell is lower than for T cells (Filion et al., 1990; Zhen et al., 2014).

Tryptase$^+$ and chymase$^+$ cells (mast cells)

Tissue-resident mast cells in adult human tissues are typically categorised as either mucosal mast cells or connective tissue mast cells. Studies on mice have demonstrated that mucosal mast cells (e.g., detectable in the intestine and lung) are T cell-dependent for activation, can induce inflammation, and exclusively originate from bone marrow-derived progenitors. In contrast, connective tissue mast cells (detectable in blood vessels, *heart*, and *skin*) are seeded during fetal morphogenesis, are exclusively derived from embryonic progenitors (mainly fetal liver), and are maintained at a steady-state (Kitamura et al., 1977; Heng et al., 2008; Dwyer et al., 2016; Li et al., 2018).

In the human fetus, tryptase-expressing mast cells are present in the yolk sac at 4 - 7PCW and fetal liver at 7- 8PCW (Popescu et al., 2019). Later, but before birth, these cells distribute through the bloodstream to organs, forming local pools of self-renewing mast cell progenitors that are sustained through adulthood. However, the predominant source of mast cell progenitors in adults are derived from bone marrow (Valent et al., 2020).

In the second and third trimester human fetal testis samples, tryptase$^+$ cells were exclusively located in the tissue section perimeter, the tunica albuginea. Mast cells were absent in the testicular interstitium and rare in the rete testis. In addition, tryptase$^+$ cells were frequent in testis regions close to the epididymis, and they were abundant

in the epididymis. The low number of tryptase$^+$ cells (mast cells) in the testis was confirmed by scRNA-seq data showing few cells expressing *TRYPTASE* mRNA. The absence of chymase$^+$ cells was consistent from immunostaining and scRNA-seq data analysis. Thus, we conclude that the testis and epididymis regions which contain high amounts of connective tissue are populated by embryonically derived mast cells, while we speculate that mast cells in the interior of the testis after birth would be recruited from the bone marrow.

CD66b$^+$ cells (neutrophils)

Neutrophilic myeloid cells are the yolk sac blood islands and fetal liver (Ohls et al., 1995). Most neutrophils emerge during haematopoiesis in the bone marrow, their major source during fetal development (Koenig et al., 2019). At 10-11PCWs, neutrophils are detectable within the human clavicular marrow cavity for the first time (Charbord et al., 1996; Lawrence et al., 2018). Next, neutrophil precursors circulate in the peripheral blood, which are mixed with mature neutrophils at 14-16PCWs. This timeline explains why CD66b$^+$ neutrophils were rare to absent in the fetal testis and epididymis before GW41, in agreement with scRNA-seq data. At GW41, few CD66b$^+$ cells were detected inside the testis, but they were numerous around vessels at the epididymal side and in the caput epididymis, and absent from the corpus and cauda epididymis.

Other immune cell types: what is yet to learn?

At 7 PCW, immature DCs are detected in the fetal liver and non-lymphoid organs such as skin and kidney (Popescu et al., 2019). Functional DCs are detectable in human skin and liver by 13GW which are understood mainly to function to induce immune suppression by interaction with activating Tregs (Ledford, 2017). In scRNA-seq, only few cells expressing *CD1C, CD141* and *CD303* transcripts were found.

B cells are first present in the human fetal liver at 8PCW, however, their frequency is very low before PCW12, but increases thereafter (Popescu et al., 2019). In human fetal and embryonic testis scRNA-seq samples, *CD19,* and *CD20* transcripts were extremely rare and absent, respectively. NK cell precursors originate in the yolk sac and fetal liver; upon populating target organs, they differentiate *in s*itu and acquire tissue-related gene-expression profiles. (Popescu et al., 2019). The number of cells expressing relevant markers *CD56, CD94,* and *NKG2A/CD159A* mRNA in scRNA-seq

datasets was very low, suggesting NK cells do not reach the fetal tests earlier than 16GW. Future experiments could examine the trajectory of B cells, DC and NK cell population of the tests in the 2nd and 3rd trimesters of pregnancy using immunohistochemistry.

PD-1 and its ligand, PD-L1, are expressed on activated immune cells (T cells, B cells, monocytes and macrophages) and non-haematopoietic cells. PD-1 acts on T cells to induce Treg development, which then suppress immune responses to chronic inflammation and inhibit immune responses against autoantigens. The roles of PD-1 during human fetal development have mainly been studied in relationship to the feto-maternal interface, revealing its involvement in induction of tolerance (Qin et al., 2019). Although the PD-1 transcript is rarely detected on unstimulated primary T cells (Yamazaki et al., 2002), scRNA-seq data revealed an immune cell population (cluster 11) expressing the *PD-1* transcript in fetal (GW12, 14, 15), but not embryonic (GW6, 7, 8) testes. However, only a small number of cells expressed PD-L1 in both age groups. Further investigations are required to understand the function of high levels of *PD-1* mRNA expression on immune cells at GW12, 15 and 16, and whether this is sustained as the testis matures further.

Conclusion

This analysis of various immune cell populations in the embryonic and fetal human testis combined immunohistochemistry and immunofluorescence to localize specific cells,and examined transcript levels and cellular distribution using scRNA-seq datasets (Guo et al. 2021). The findings of this analysis were in alignment with the timing of haematopoiesis in the human yolk sac, fetal liver and bone marrow.

The data revealed that, as identified for the fetal mouse testis, macrophages are the most abundant immune cell type in the developing human testis. However, the high levels of *HLA-DR* transcript compared to *CD206* was not predicted, because the fetal mouse testis showed the opposite pattern (Chapter Two). Detection of high levels of *HLA-DR* mRNA in human fetal testis requires further investigations to confirm both levels of its encoded protein on macrophages and its function in these cells. Although HLA-DR functions in antigen presentation to T cells, both IHC and scRNAseq data indicated that T cells are scarce. In addition, other immune cells involved in extracellular matrix and remodeling such as mast cells and neutrophils were rare to absent, which implies this is the key and exclusive role of macrophages during testis

organogenesis. The absence of B cells was aligned with our prediction and its rare presence in the adult human testis. In contrast, we anticipated detection of higher levels of *FOXP3*, *CTLA-4*, markers of Treg function in relation to creating an immune regulatory microenvironment. The interesting localisation of $CD45^+$ cells and macrophages in the cord perimeter areas suggest these cells may contribute to processes including germ cell differentiation and cord formation. Differences in macrophages and $tryptase^+$ mast cell numbers recorded between testis and epididymis indicate these tissues have a different immunephysiology, which has been demonstrated in adult tissues.

In conclusion, this study provides a clear basis for furFurther studies to understand the function of tissue-resident immune cells with embryonic ontogeny in the human fetal testis.

Chapter Five

Characterisation of Neutrophils in Samples of Germ Cell Neoplasia in Situ and Human Testicular Germ Cell Tumour

5. Characterisation of Neutrophils in Human Testicular Germ Cell Tumour Samples and Germ Cell Neoplasia *in Situ*

5.1. Introduction

Testicular germ cell tumours (TGCTs) are the most frequent malignancy in young adult men. They have a relatively good prognosis due to good primary treatment options (surgery, chemotherapy, radiation therapy) but also frequently relapse and exhibit metastatic behaviour (Kobayashi et al., 2013; Chovanec et al., 2016; Lobo et al., 2020). The majority of TGCTs identified are classified as seminoma or non-seminoma (embryonic carcinoma, yolk-sac tumour and teratocarcinoma), both arising from the same pre-invasive tumour precursor, germ cell neoplasia in situ (GCNIS) (Berney et al., 2016). GCNIS develops from undifferentiated primordial germ cells, which are unable to progress to the differentiation stage after embryonic stem cell-like cells, so that expressing pluripotency markers such as Oct3/4, NANOG and PlAP is observed. Residing within the seminiferous tubules with an intact tubular wall, these cells can remain quiescent for long time periods or become invasive due to unknown reasons (Rajpert-De Meyts et al, 2016; Loveland et al., 2017).

Sperm disorders are another frequent male reproductive pathology, including defects in the quantity of sperm (too few (oligozoospermia) or none (azoospermia)), quality of sperm (such as abnormal motility or morphology) or sperm emission (because of retrograde ejaculation). Most men attending fertility clinics are presented with azoospermia, either obstructive or non-obstructive with the latter caused by impaired spermatogenesis which can have a wide range of causes, including endocrine, genetic, genitourinary disorders, obstructive disorders (leading to obstructive azoospermia), or exposure to heat, drugs (e.g., anabolic steroids) or toxins (Rebar, 2020).

Immune responses have two effects: increasing inflammation and reducing inflammation. Therefore, some immune cells mediate pro-inflammatory activities, whereas others serve to control and reduce inflammation and promote healing by anti-inflammatory cascades. At the site of inflammation, activated and recruited monocytes, tissue-resident macrophages, neutrophils, lymphocytes (natural killer cells [NK cells], T cells, and B cells), and mast cells mediate local responses to tissue damage or infection and release chemokines and growth factors (IL-1β, IL-6, IL-8,

IL-12, TNF-α, IFN-γ, GM-CSF) that promote the inflammatory cascade, and attract more neutrophils and monocytes to the inflamed site (Chen et al., 2018). The anti-inflammatory responses are mainly triggered by T regulatory cells (Tregs) and T helper 2 (Th2) cytokines (TGF-β, IL-4, IL-10, IL-11, and IL-13). However, other immune cells also can be a source of anti-inflammatory mediators depending on the microenvironment (Zhang and An, 2009).

Neutrophils are another potent innate immune responder that are best known for their role in inducing inflammatory factors. Their recruitment can ultimately cause host tissue damage and increase the disease pathogenesis and severity if not cleared by the macrophages which would typically restore homeostasis (Kobayashi et al., 2009; Soehnlein and Lindblom 2010). Neutrophils are abundant in circulation, relatively short-lived, highly migratory cells, and are now understood to contribute to organ homeostasis, including through their daily movement into and out of organs in adult mice (Casanova-Acebes et al., 2013 and 2018; Lok et al., 2019).

In all TGCT forms, from GCNIS to seminoma / non-seminoma, immune cells are more frequently observed in the diseased testis compared to the normal adult human testis (Hvarness et al., 2013; Klein et al., 2016; Rajpert-De Meyts et al, 2016; Renner et al., 2017; Siska et al., 2017; Loveland et al., 2017). There were the massive infiltration and accumulation of macrophages, T cells, B cells, dendritic cells and mast cells in the biopsies of men with TGCTs, but less Tregs in seminoma compared to other TGCT types, however, Treg cells increased with advanced seminoma stages. Interestingly, although they reported that mast cells in GCNIS were significantly increased compared to healthy testes, mast cells were absent from seminoma samples. These findings demonstrated that testicular tumours are different compared to the healthy adult human testis, and the authors proposed tumour types “shape” their own immunological microenvironment (Klein et al., 2016; Siska et al., 2017).

The potential importance of neutrophils in the human testis is indicated by their density in TGCTs. An increased number of tumor-infiltrating neutrophils detected in non-seminomatous germ cell tumour samples was identified as a potential prognostic factor for overall survival in patients that may enable discrimination between different testicular tumour stages (Yamada et al., 2016; Siska et al., 2017).

Considering the role of neutrophils in the induction and amplification of inflammation, damage and immune responses, and their absence in the normal adult human testis

(Hedger, 2015), it is important to understand the mechanisms involved in their recruitment and activation as this is essential to elucidate the impact of neutrophils in pro-carcinogenic and suppression anti-tumour pathways.

We hypothesised, that beside other immune cells that showed increasing number in testicular failure (i.e., impaired spermatogenesis) and TGCTs as shown by Klein et al. (2016), also neutrophils may have significant changes in distribution and frequency comparing normal testis tissue, impaired spermatogenesis and TGCT samples. This study could aid in increasing knowledge about the immune milieu in distinct pathological manifestations. To address the hypothesis that neutrophil distribution and frequency could reveal possible functional roles, I examined the neutrophil number, position and distribution pattern in testis sections from adult men with intact and impaired spermatogenesis with TGCT.

5.2. Materials and Experimental Methods

Samples

Testicular biopsies, obtained from men undergoing andrological workup for infertility or taken during surgery for testicular cancer, were used in this study (approved by the ethics committee of the Medical Faculty of the Justus Liebig University Giessen; Ref. No. 26/11 and 152/16). Samples (n = 5 / group) were classified by histological work-up (performed at JLU) as follows: intact spermatogenesis (NSP), hypospermatogenesis associated with focal inflammatory infiltrates (HYP+ LY), Sertoli cell only (SCO), germ cell neoplasia *in situ* (GCNIS), GCNIS associated with lymphocytic infiltration (GCNIS + LY), and seminoma. Bouin's fixed paraffin-embedded 5-µm thick testis sections on slides were shipped from Justus-Liebig University, where they form part of the larger screening study, to Monash University.

Immunohistochemistry

IHC was performed on human testis sections using antibodies to detect leukocytes (CD45), macrophages ($CD68^+$ cells), T cells (CD3), tryptase (mast cells), chymase (mast cells) and neutrophils ($CD66b^+$ cells), to characterise their relative frequency, and patterns of distribution. The main objective of this study was not to study macrophages and mast cells, but immunostaining for $CD45^+$, $CD68^+$, $tryptase^+$ and $chymase^+$ cells was done to use those cells as a guide to identify areas with immune cells present, and to examine their comparative distribution.

Primary and secondary antibodies used are listed in Table 5.1.

Table 5.1. Primary and secondary antibodies used for immunohistochemistry.

Antibodies	Company	Cat #	Clone	Raised in	Dilution
Anti-CD45	DAKO	M0701	2B11+ PD7/26	Mouse	1:400
Anti-CD68	DAKO	M0876	PG-M1	Mouse	1:500
Anti-Tryptase	Abcam	Ab2378	AA1	Mouse	1:8000
Anti-Chymase	Abcam	Ab233103	Polyclonal	Rabbit	1:500
Anti-CD66b	BioLegend	G10F5	G10F5	Mouse	1:500
Rabbit anti-mouse	DAKO	E0468	Polyclonal	Rabbit	1:500
Goat-anti mouse	Dianova	115-035-003	Polyclonal	Goat	1:400
Donkey anti-Rabbit	Invitrogen	A21202	Polyclonal	Donkey	1:500
Donkey anti-Mouse	Invitrogen	A31572	Polyclonal	Donkey	1:500

In brief, sections of 5 µm (cut in the Institute for Veterinary Anatomy, Histology and Embryology, Justus Liebig University of Giessen (JLU) Giessen University) were placed on glass slides, deparaffinised and rehydrated through a standard series of washes in histolene (Grade HDS, VIC, Australia) for 2 x 5 min, then in decreasing concentrations of ethanol (100% for 2 x 5 min, 95%, 80%, 70%, 50% and 30%, for 3 min each) and placed in deionised water for 3 min. Heat-induced antigen retrieval was performed by immersing slides in Citrate Buffer (Sigma-Aldrich), pH 6.0); the container with slides was microwaved for 4-5 min at 800W, then 9 min at 450W, followed by cooling at RT for 30 minutes. Sections were incubated with 3% hydrogen peroxide (Merck Millipore) for 15 min at RT, followed by washing buffer for 3 min (Tris-buffered saline; TBS; 50 mM Tris-Cl, 150 mM NaCl, pH 7.5 containing 0.1% Triton X-100) and 2 x 3 min with TBS. Sections were next incubated in blocking solution consisting of 5% bovine serum albumin (BSA, Sigma-Aldrich) in TBS in a humid chamber at RT for 1 hr. Primary antibodies were diluted in 1.5% BSA/ 0.1% Triton X-100/TBS and applied in a humid chamber overnight at 4 °C. Consecutive sections incubated with washing buffer and no primary antibody were used as negative controls, which consistently showed no signal.

Slides were washed with washing buffer (0.1% Triton X-100/TBS) for 3 min and 2 x 3 min with TBS, and biotinylated secondary antibodies were added in a humid chamber for 1 hr at RT. After consecutive three washes (1 x 3 min 0.1% Triton X-100/TBS and 2 x 3 min TBS), Vectastain Elite ABC kit reagents were added according to the manufacturer's instructions (Vector Laboratories) for 30 min in RT followed by three times washing (1 x 3 0.1% Triton X-100/TBS and 2 x 3 TBS). Antibody binding was

identified as a brown precipitate using 3, 3-diaminobenzidine tetrahydrochloride (DAB) (Agilent Technologies, Santa Clara, United States). Next, sections were counterstained with Harris hematoxylin (Sigma-Aldrich). Stained slides were then washed and dehydrated through a graded ethanol series in 30%, 50%, 70%, 80%, 95% ethanol 1 x 3 min and washed with 100% ethanol and histosol 2 x 5 min. Slides were mounted by using DPX (Sigma-Aldrich) under glass coverslips.

Imaging and morphometric analysis

Immunostained human adult testis sections were scanned by Monash Histology Platform staff using an Olympus VS120 Slide Scanner and analysed using OlyVIA Software (Olympus Life Science, 2.9.1 Viewer). Immune cells were identified based on the presence of a well-defined nucleus and marker staining.

Histological evaluation of testicular sections

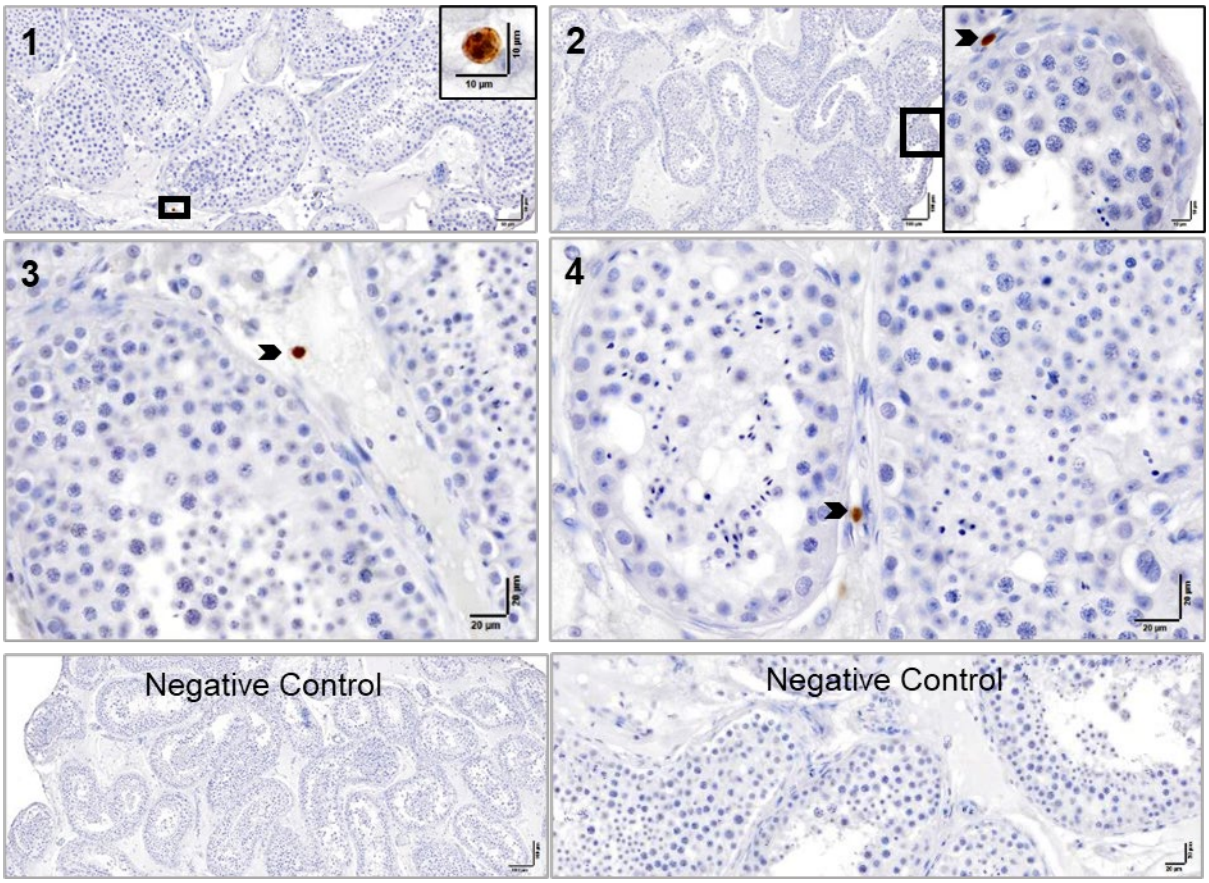
The overall descriptive histopathological assessment was combined with a score count based on a standard semi-quantitative scoring of the degree of interstitial immune cell infiltration (Klein et al., 2016).

5.3. Results

Neutrophils in men with normal spermatogenesis

$CD66b^{+}$ neutrophils were extremely rare in testis sections of men with normal spermatogenesis; if present, there were single cells localised in the interstitium, peritubular areas (Figure 5.1), and vessels. Vessels were identified based on their rounded appearance in cross section, formed by elongated cells with elongated nuclei (endothelial cells) and often with red blood cells inside. Neutrophils were never observed inside seminiferous tubules in normal patient samples.

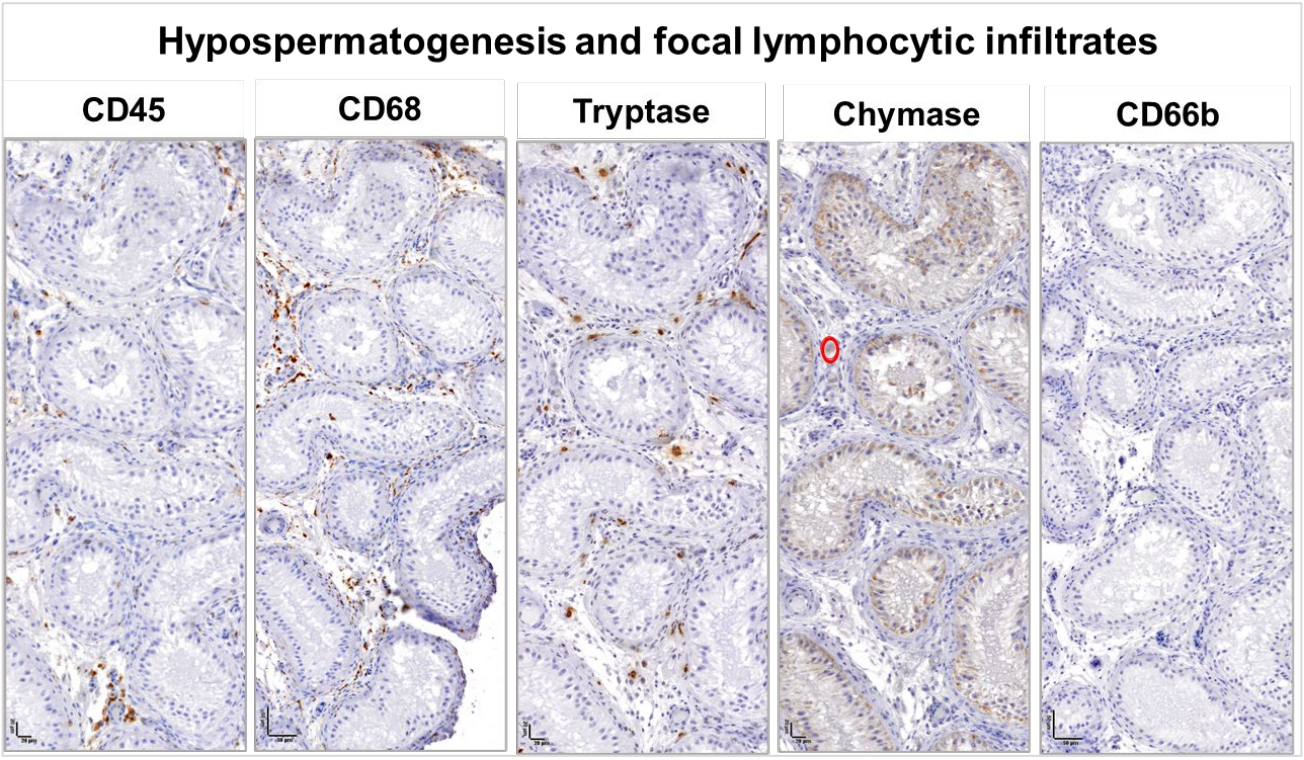
Figure 5.1. Neutrophil (CD66b$^+$ cell) distribution patterns in testis sections with normal spermatogenesis. All detectable neutrophils in each panel are indicated using arrowheads. The black boxes in the low magnification image are shown at high magnification in the right-hand panels. Each panel represents an individual sample. The inset shows a stained cell. The negative controls are shown below.



Neutrophils in patient samples with hypospermatogenesis with focal lymphocytic infiltrates

CD45$^+$, CD68$^+$, tryptase$^+$ and chymase$^+$ cells in hypospermatogenesis and focal lymphocytic infiltrates samples were scattered, while neutrophils (CD66b$^+$ cells) were extremely rare to absent in all samples examined (Figure 5.2).

Figure 5.2. Representative images of immune cell distribution patterns in serial sections from a patient with hypospermatogenesis and focal lymphocytic infiltrates. Neutrophils were absent from this section. The only chymase-positive cell detected in the interstitium is shown using a red circle.



Neutrophils in patient samples with Sertoli-cell-only (SCO) testes

Neutrophils were extremely rare to absent in the interstitium of SCO samples but if detected were found inside vessels. Despite evidence of a massive infiltration of CD45$^+$, macrophages and tryptase$^+$ cells, the number of chymase$^+$ (mast) cells was much higher in this pathology compared to the others (Figures 4.3 and 5.4).

Figure 5.3. Immune cell distribution patterns in serial sections from a patient with SCO. CD45$^+$ cells, macrophages and tryptase$^+$ cells were scattered, and there were single neutrophils (shown using red cycles). However, the number of chymase$^+$ mast cells was much higher in this pathology compared to other malignancies (shown using arrows).

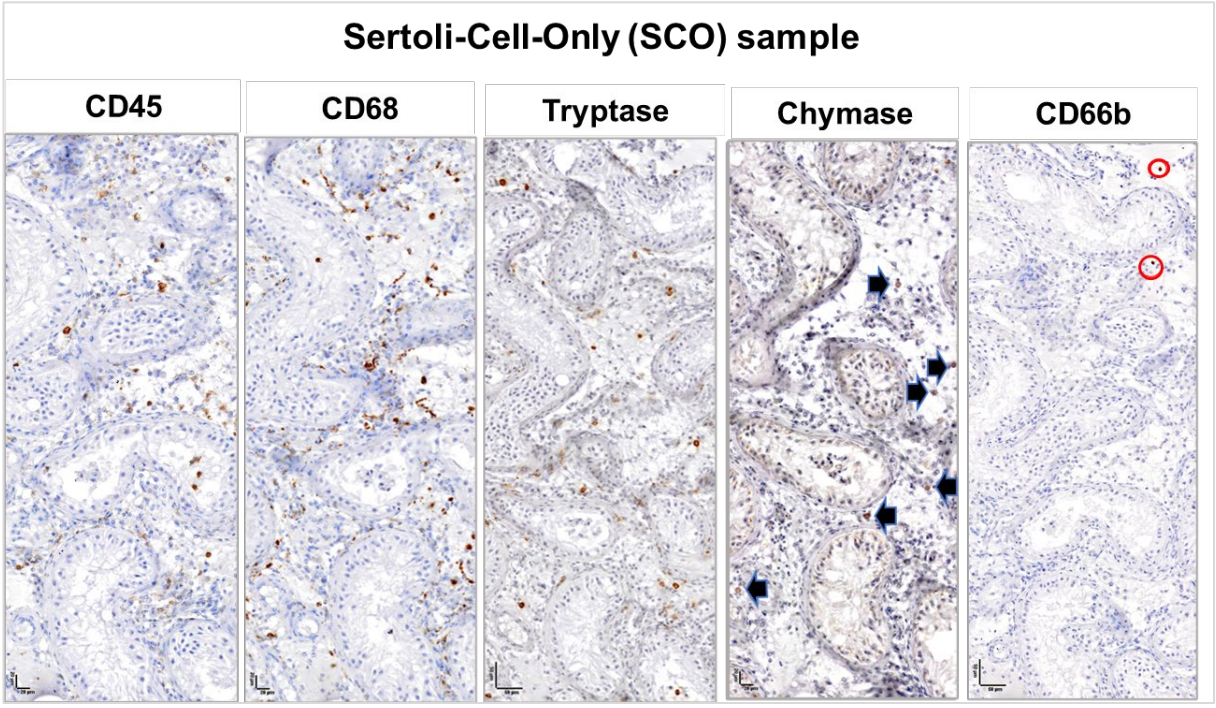
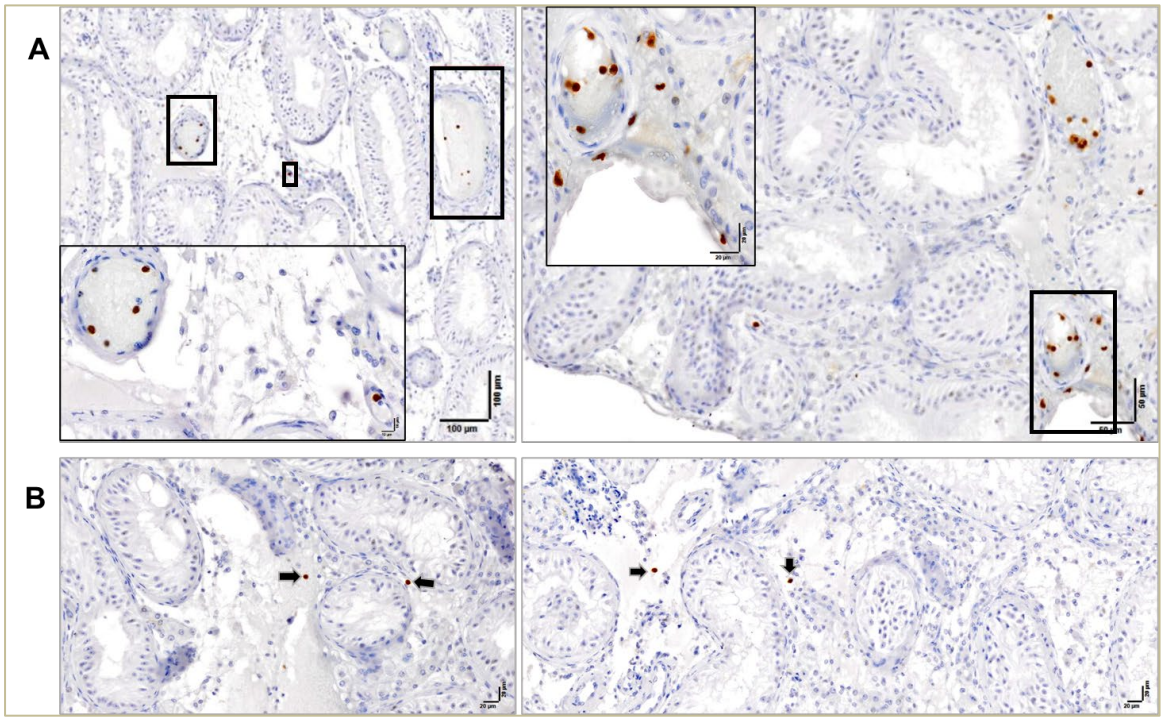


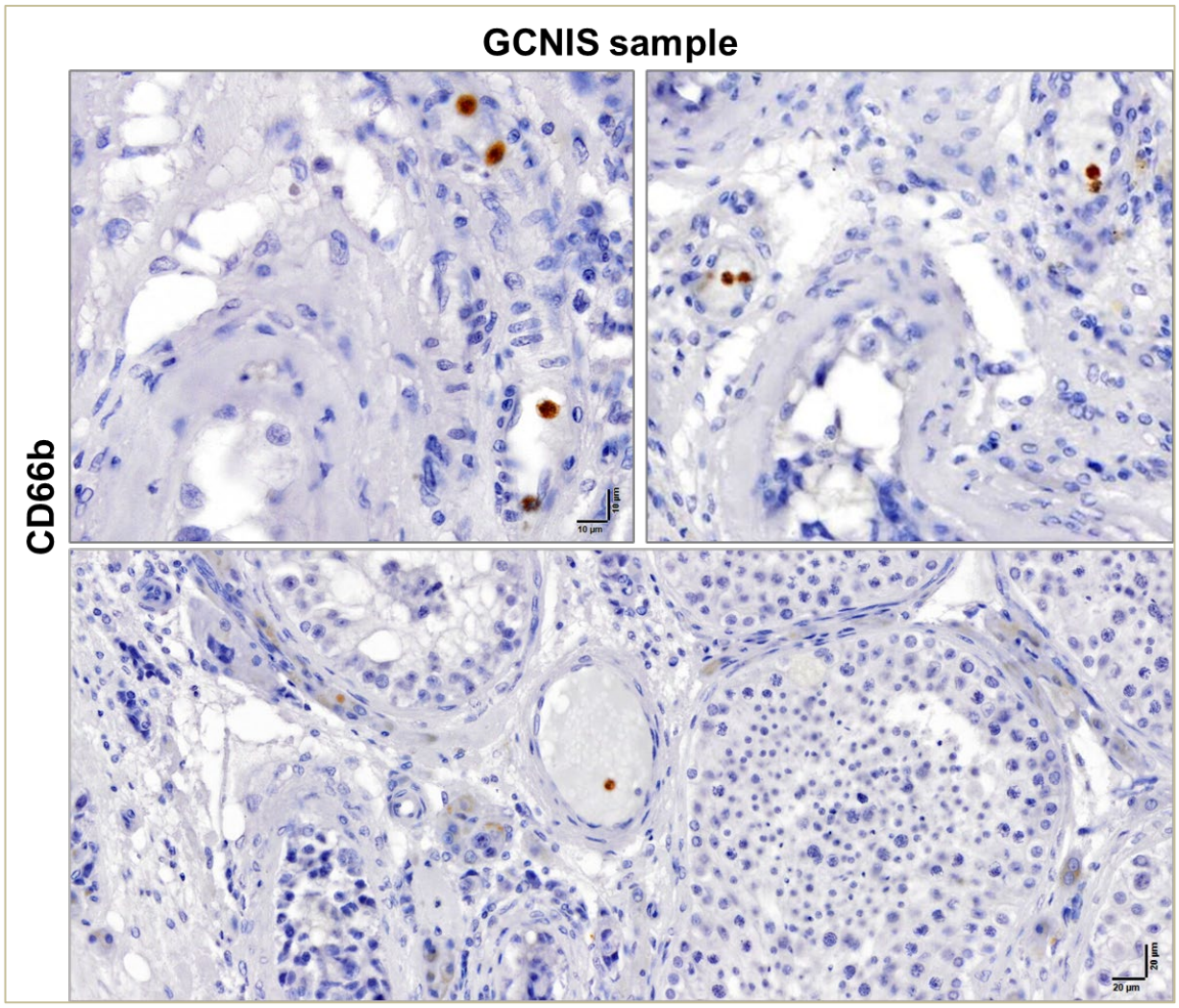
Figure 5.4. Detailed description of CD66b$^+$ cells (neutrophils) in different locations of SCO. Neutrophils were frequently observed inside vessels (**A**) but were rare in the interstitium (**B**). All detectable neutrophils are indicated in boxes (also shown enlarged) or with arrows.



Neutrophils in patient samples with GCNIS

Neutrophils were rare in the interstitium of GCNIS samples but were visible inside vessels (Figure 5.5).

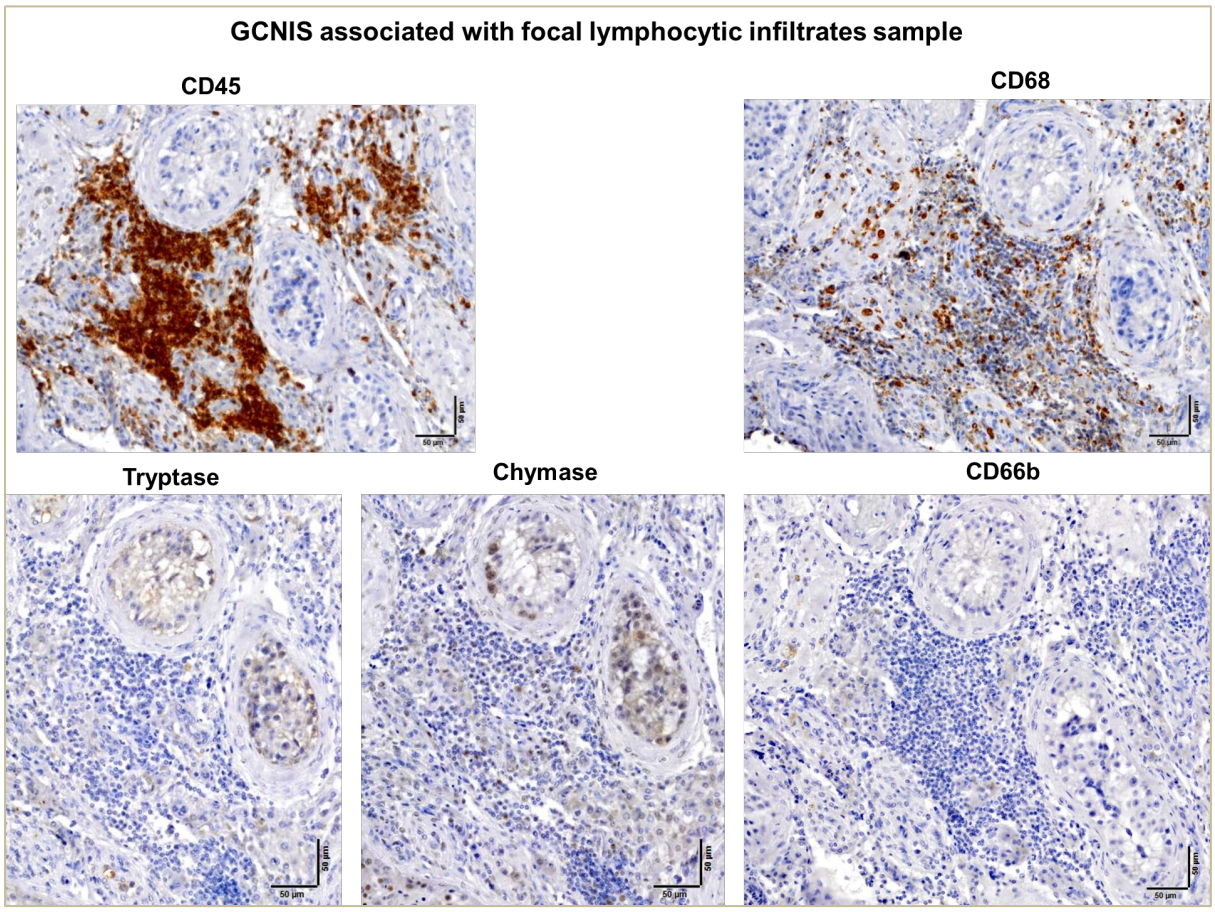
Figure 5.5. Neutrophil distribution patterns in sections obtained from patients with GCNIS. Neutrophils were absent in the interstitium and were only detectable inside vessels.



Neutrophils in patient samples exhibiting GCNIS associated with focal lymphocytic infiltrates

Neutrophils were rare to absent in the lymphoid infiltrated regions, and interstitium of GCNIS associated with focal lymphocytic infiltrates samples (Figure 5.6).

Figure 5.6. Immune cell distribution patterns in serial sections obtained from a patient with GCNIS associated with focal lymphocytic infiltrates. Neutrophils were absent from lymphoid infiltrated regions.



Neutrophils in patient samples with seminoma

Seminoma samples displayed a high level of heterogeneity in terms of remaining testicular structure and number of neutrophils, especially in sections from the central region of the tumour samples. $CD66^{+}$ neutrophils varied from absent to rare (single cells) in the interstitium in one sample (Figure 5.7) and scattered to sparse infiltrates in other samples (Figure 5.8).

Figure 5.7. Immune cell infiltration in a seminoma tumour sample. Neutrophils were absent or present as single cells in areas of massive leukocyte and macrophage infiltration ($CD45^+$ and $CD68^+$ cells). All detectable neutrophils ($CD66b^+$ cells) were shown using black boxes. The black boxes in the low magnification image are shown at high magnification.

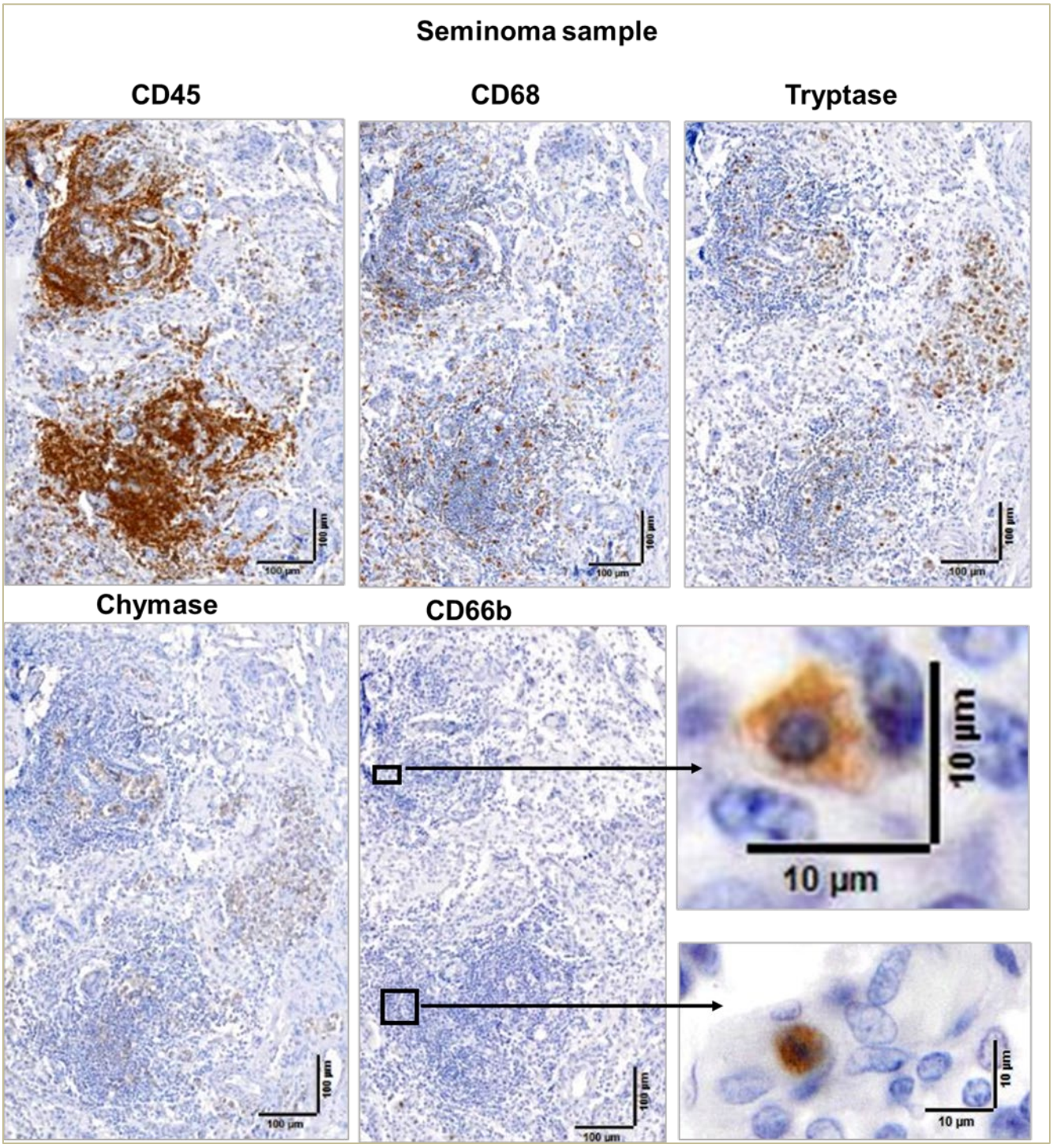
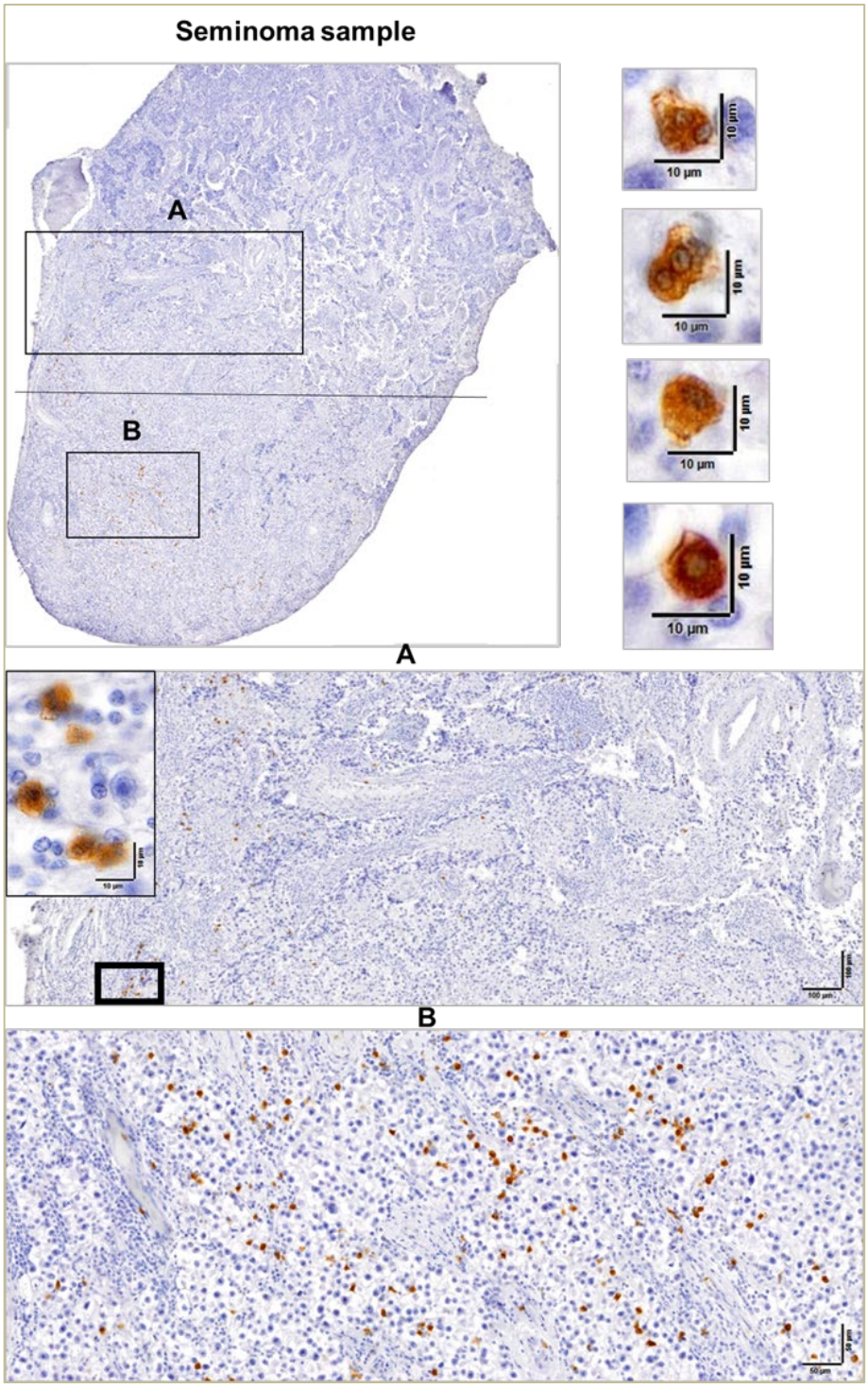


Figure 5.8. CD66b^{+} cell (Neutrophil) distribution patterns in a tumour section obtained from a patient with seminoma. The section shows two parts: the top part (A) in which the seminoma is accompanied by high amounts of connective tissue and tubular shadows and the bottom part (B) presenting full-blown seminoma. **A**: The number of neutrophils in top part is much lower than the bottom part. **B**: No tubules are left here, neutrophils were much frequent than in the top region of the section. The black boxes in the low magnification image are shown at high magnification panels. The four panels on the right-hand side show neutrophils detected in the section.



5.4. Discussion

Studies of tissue-resident neutrophils have recently focussed on tumour sites, with tumour-associated neutrophils (TANs) being detected in malignancies such as breast cancer, hepatocellular cancer, gastric carcinoma, oesophageal cancer, lung cancer etc. (Taucher et al., 2021; Kwantwi et al., 2021). Two phenotypes of TANs are engaged in the tumour microenvironment and tumour cell growth, including N1 TANs (also described as anti-carcinogenic), which exert an antitumor activity, and N2 TANs (pro-carcinogenic) stimulating immunosuppression, tumour growth, angiogenesis and metastasis (Fridlender et al., 2009).

Mishalian et al. used two mouse models and human tumour samples, and demonstrated a clear link between TANs, Treg recruitment to the tumour site, and impairment of anti-tumour immunity (Mishalian et al., 2014). They induced tumours in BALB/c mice using AB12, a murine malignant mesothelioma cell line, derived from an asbestos-induced tumour, and used Lewis lung carcinoma (LLC) cell line to establish tumours in C57BL/6 mice. In addition, two human lung tumour samples and adjacent normal lung tissues were studied. Using RT-PCR and ELISA analyses, this study demonstrated that murine TANs secrete significant amounts of CCL17, a T-reg chemoattractant at a level higher than that secreted by circulating or splenic neutrophils. The level of increase paralleled the progression of tumour development. In addition, in *vitro* and *in vivo* migration assays showed recruitment of T-regs was regulated by TANs, and this was inhibited with anti-CCL17 monoclonal antibodies. This role of TANs was confirmed by systemic neutrophil depletion in tumour-bearing mice using an anti-Ly6G monoclonal antibody; neutrophil depletion led to reduced Treg recruitment and, consequently, decreased tumour growth (from 11.3% ± 2.7% of the lung in the control mice to 4.3% ± 1.8% of the lung in mice depleted of neutrophils. This finding in mice was evaluated in two human lung adenocarcinoma tumour samples using flow cytometry, which revealed a small but apparent population of TANs ($CD66b^+/CD15^+$ cells) were $CCL17^+$, while there was no CCL17 expression on neutrophils from adjacent normal lung tissues. Therefore, they concluded that TANs affect tumour growth by suppressing the anti-tumour function of immune cells via recruiting Tregs (Mishalian et al., 2014).

More studies have shown poor prognosis with a high number of TANs and/or Neutrophil-to-Lymphocyte Ratio (NLR) in tumour sites (Cupp et al., 2020). Thus, TAN counts and NLR can be regarded as biomarkers. Also, therapeutic strategies to target

TANs (e.g., targeting the CCL20, CXCL8/CXCR1/CXCR2 axis) have been suggested because of the pivotal role of TANs in stimulating tumour progression (Masucci et al., 2019; Kwantwi et al., 2021).

There are only two reports on TANs in TGCT (Yamada et al., 2016; Siska et al., 2017). Yamada et al., studied the prognostic value of $CD66b^+$ tumour-infiltrating neutrophils ($CD66b^+$ TINs) in 102 patients who underwent orchiectomy for TGCT. They found a significant correlation between high density of TINs and tumour diameter ≥ 10 cm, presence of nodal/distant metastasis, S stage, diagnosis of non-seminomatous germ cell tumour, and presence of venous invasion. However, TIN density was an independent prognostic factor for overall survival (Yamada et al., 2016).

Siska et al. studied infiltration of immune cells in TGCT samples at stage I (localized disease), stage II (lymph node metastases), and stage III (disseminated metastases). Their quantitative immunohistochemistry and gene-expression profiling showed advanced TGCT stage was associated with increased neutrophils. Also, gene expression profile of cancer/testis antigen and neutrophils correlated with recurrence free survival; the patients with low neutrophil gene expression score showed a 100% recurrence free survival, whereas 63% of patients with a high neutrophil score were recurrence free only two years after diagnosis. This report suggested infiltrated neutrophils act as pro-tumour immune cells which also are involved in impairment of antitumor immunity (Siska et al., 2017).

In this research project, we studied the number and distribution patterns of neutrophils ($CD66b^+$ cells) in one testis section each from adult human men with normal spermatogenesis and with diverse testicular pathologies (n=5 in each group). Our data showed neutrophils were rare in the interstitium and peritubular areas of the testes of men with normal spermatogenesis, hypospermatogenesis with focal lymphocytic infiltrates, SCO, GCNIS, and GCNIS associated with focal lymphocytic infiltrates. Also, neutrophils were absent in the lymphoid-infiltrated regions in patients' samples. However, the number of neutrophils was higher in some seminoma samples than in other pathologies. Neutrophils were never detected inside seminiferous tubules of sections from all groups.

These findings shed light on the possible contribution of neutrophils, as a pro-inflammatory cell type, in the pathogenesis of human seminoma. Due to high inter-individual variations, evidence would need to be supported by analysing more

samples. So far, our findings do not hint at a causative correlation of neutrophil number and human seminoma pathogenesis.

It will also be useful to examine the levels and distribution of key cytokines, chemokines and chemokine receptors known to be involved in the activation and recruitment of neutrophils, such as CXCL8, IL-8, IL-18, CXCR1 (receptor of IL-8, GCP2, NAP2), and CXCR2 (receptor of GRO, NAP2, IL-8 and ENA78) (Tecchio et al., 2014; Capucetti et al., 2020). Studying cytokines produced by neutrophils is of importance because of the ability of these cells to selectively recruit neutrophils, monocytes, DCs, NK cells, and T-helper type 1 (Th1) and Th17 cells into sites of injury, and thereby amplifying infiltration of immune cells (Scapini et al., 2000; Sadik et al., 2011). However, many chemokines, chemokine receptors and products of neutrophils (such as IL-8, GROα, MIG, IP-10, and I-TAC, MCP-1, MIP-1αand MIP-1β) are also produced by other immune cells, therefore finding a direct link between neutrophils and such mediators will require use of appropriate methologies (Kasama et al., 2005).

Our suggested approach for studying neutrophils in the human adult testis is IHC and IF, and not flow cytometry or qRT-PCR. Because we demonstrated that, in all samples, $CD66b^+$ cells were detected in vessels, techniques that assess the whole or disrupted tissues such as flow cytometry and qRT-PCR could not yield a reliable representation of neutrophil distribution or functionality, because the detected $CD66b^+$ cells/ mRNA (CEACAM8) might not be located inside the testicular parenchyma.

Chapter Six

General Discussion

The following sections provide a summary of the key discoveries and discuss their significance.

6.1. Chapters Two and Four: Immune cells in fetal human and mouse testis

6.1.1 Main findings

The findings presented in Chapter Two demonstrate the dynamic ratio between germ cells, macrophages and neutrophils. This overall finding is supported by documenting the appearance of T cells ($CD3^+$) in the testis at E15.5, detection of neutrophils ($Ly6G^+$) in E13.5 testis followed by their increasing number at E15.5 and PND0; they become half of the testicular immune cell population in newborn pups (from E13.5 to PND0, fold-change: 114). I provided descriptive and quantitative reports of neutrophils and macrophages inside cords, (next to germ cells), and of the density of male germ cells ($DDX4^+$), macrophages ($F4/80^+$), T cells, neutrophils, non-macrophages MHC class II^+ cells from E13.5 to PND0. I demonstrated that most $F4/80^+$ cells are $CD206^+$ at E13.5 (81%), reducing to 60% at E15.5 and 50% by PND0. These results demonstrate the dynamic numerical ratio between germ cells, macrophages and neutrophils.

Results presented in Chapter Four provide evidence that the number of macrophages ($CD68^+$), T cells ($CD3^+$), neutrophils ($CD66b^+$) and mast cells ($tryptase^+$) in the human fetal testis increases with gestational age, similar to what was observed in the mouse. I documented the localisation of $CD68^+$ cells, which are abundant in the interstitium, perimeter area, especially around vasculature, but are extremely rare to absent inside vessels; also, few macrophages are detectable adjacent to the cords at GW14/15, but they are more abundant at GW23 and afterwards. Studying $CD3^+$ revealed that T cells are likely to be rare at the second trimester and frequent at the third trimester in the interstitium; these cells are rarely detected inside cords. $CD66b^+$ cells are extremely rare, and $chymase^+$ mast cells are absent from the testis interstitium. $Tryptase^+$ mast cells displayed a specific distribution pattern, as they are only detectable in the testis perimeter areas, close to the capsule and around vessels, but they are not detected in the testis interior or inside blood vessels. Aanalyzing published scRNA-seq data (Guo et al., 2021) obtained from human embryonic (GW6, 7, 8) and fetal (GW12, 15, 16) testes identifies a higher number of cells expressing *CD68* than those expressing other immune cell markers, *TPSAB1* (encoding mast cell tryptase) and *CD1C* (dendritic cells) are barely detected, and cells expressing

NKG2A/CD159A (NK cell marker), *CD66B* (neutrophils marker) and *CD3* (T cell marker). Signal encoding the *CD19* (B cell marker) is extremely rare to absent.

6.1.2 Main conclusions

Neutrophil and T cell presence, frequency and roles in inducing immune regulation, immune responses and inflammation in the postnatal testis have been established, but their existence and frequency during fetal testis development is undocumented in mouse, rat and human. However, macrophages have been studied in mice, particularly shortly after sex determination, when they have been shown to serve significant functions in matrix and organ remodelling during fetal testis development. This thesis quantifies for the first time the frequency and localisation of macrophages, T cells and neutrophils during the later time points in fetal testis development in mice and humans. It reports the close apposition of immune cells and seminiferous cords, in both mice and humans, during fetal testis development, suggesting that such close contact might be essential for normal germ cell development or cord formation. Also, in mice, ratios between germ cells and macrophages, germ cells and neutrophils, and their close cellular contacts, are documented. These results may be important for *in vivo* studies and suggest there may be practical benefits to adding immune cells into germ cell cultures, because immune cells may contribute to normal male germ cell survival or differentiation.

6.1.3 Strengths and limitations

This study was enabled by the fortunate access to rare tissue samples of human fetal testis and epididymis obtained from embryos collected during the second and third trimesters of pregnancy. These were used to determine the location and gauge the relative frequency of specific immune cell populations; however, more samples from the third trimester provide a more detailed understanding of the fetal testicular immune cell composition. These are most probably bone marrow-derived, as fetal bone marrow is the dominant site of haematopoiesis after mid-gestation to birth (Holt and Jones, 2000; Travnickova et al. 2015; Bian et al., 2020). Understanding the ontogeny of tissue resident immune cells can provide information about their phenotype, specific markers and self-renewal capacity.

I analysed a published scRNAseq dataset obtained from human fetal testis samples at GW6, 7, 8, 12, 15 and 16, that spans the first to the early second trimester of

pregnancy (Guo et al., 2021). This approach allowed us to evaluate the number of cells expressing transcripts encoding macrophage markers (CD68, CD14, CD206, MHC Class II, CD163, CD64, CD80, CD86, CD115), T cell markers (CD3E, CD3G, CD3D, CD4, CD8A, CD25, FOXP3, CTLA-4, CD28), mast cell markers (tryptase and chymase), neutrophil markers (CD33 and CD66b), B cell markers (CD19 and CD20), dendritic cell (DCs) markers (CD1C, CD141, CD11C, CD303), NK cell markers (CD56, CD94, CD16, NKG2A/CD159a) and additional markers such as CD45 (pan-leukocyte), Ki-67 (proliferation), PD-1 and PD-L1. Having more samples at each trimester (Ganti, 2022) and from the third trimester gestation weeks (GW 25 to 40) could provide a clearer picture of the immune cell populations and function in human testis during fetal development. Although statisticians suggest studying at least 30 human samples is required to reach a conclusive outcome, ethical and practical limitations in accessing human fetal tissues are a fact.

We recommend multi-colour immunofluorescence staining on macrophages and $CD45^{+}$ cells located in cord perimeter areas (adjacent to the cord membrane), to discover their phenotype and possible function. For this purpose, suggested markers are HLA-DR, CD206, IL-10, CSF-1 (macrophage markers), (Mossadegh-Keller et al., 2017; Bhushan et al., 2020), SOX9 (Sertoli cells) and CSF-1R and DDX4/ VASA (germ cells) to reveal which cells inside cords, either germ or Sertoli cells, are in close association with peri-cord immune cells.

6.1.4 Future directions

The findings of this PhD thesis highlighted several knowledge gaps in areas for future investigations. I propose it would be valuable to identify the phenotype of fetal testicular immune cells, and specific receptors and ligands involved in the interaction of macrophages (CCR2, CCL2, MHCII, CD40L, CD80/86, CXCL1, CXCL9, CXCL10, CCL17, CCL22, IL-10R, PD-1) with T cells (CD40, CD28, CCR3, CCR4, IL-10, PD-1R) and neutrophils (Ly6G, S100A9). This may reveal the impact of different phenotype of macrophages and its interactions in the developing testis (Dayer, 2003; Herrero-Cervera et al., 2022). It is as yet known what the phenotype of fetal testicular T cells is after the activation due to interaction by macrophages; this could be addressed using markers involved in polarization of T cells to either pro-inflammatory or anti-inflammatory phenotypes (Th1: T-bet, STAT1; Th2: GATA3, STAT6; Th17: RORS, STAT3; Treg: FOXP3; Tfh: BCL-6, STAT3) (Zhang et al., 2013) and would

broaden our understanding of immunophysiology of developing testis. I documented presence of macrophages and neutrophils (in mouse testis) and T cells (in human testis) inside cords. There is a need to finding out the importance and function of immune cells inside cords at different stages of germ cell differentiation. An additional area requiring further study is the mechanisms involved in moving immune cells inside or outside of testis cords; this could have clinical implications, as there are immune cells inside seminiferous tubules in human adult testis cancer (Bhushan et al., 2020). However, for the mentioned implication, it is necessary to consider differences between fetal and adult testis immune microenvironment.

6.2. Chapter Three: Impact of activin A levels on testicular macrophages in fetal and newborn mice

6.2.1 Main findings

Data generated in Chapter Three demonstrates that in the testes of mice with high activin A bioactivity levels (KO *Inha*), the total macrophage numbers per cross-sectional area are significantly higher compared to WT and KO mice with low activin A bioactivity levels (*Inhba*) at both E13.5 and E15.5. Also, the data show mRNA levels of certain immune factors involved in regulating macrophage function and phenotype are affected by activin A levels in the fetal mouse testis. I identified a reciprocal dose-dependent effect of activin A levels on *CX3CL1, IL-4* and *IL-10 receptors, MHC class II, CCL17, CXCR7, CXCR4* and *Marco* transcripts.

6.2.2 Main conclusions

I concluded that activin A plays key roles in establishing the population, phenotype and distribution pattern of fetal testicular macrophages in mice. However, our Fluidigm platform data indicated that the activin A bioactivity levels did not shift fetal testis macrophages to either a pro-inflammatory or anti-immune microenvironment (at the mRNA levels).

6.2.3 Strengths and limitations

Studying Sertoli and germ cell proliferation in the fetal mouse testis in the activin A mutant mouse model, *Inhba*, provided novel findings on the impact of activin A levels on establishing the balance between Sertoli and germ cell number (Mendis et al., 2011, reviewed in Barakat et al., 2012). My research provides knowledge of the

impact of activin A levels on macrophages, as an important cell type for testis morphogenesis and spermatogenesis (DeFalco et al., 2014, Bhushan et al., 2020; Lokka et al., 2021).

Our Fluidigm data is informative for revealing the association between activin A levels and mRNA levels for 33 immune factors/ mediators in *Inhba* and *Inha* strains. However, we should consider that for this study the whole testis was used, and some markers, including chemokines and chemokine receptors, are not exclusive to macrophages and are expressed on other immune cells (such as DCs, mast cells and T cells) and non-immune cells (e.g., endothelial cells).

6.2.4 Future directions

I studied the impact of activin A levels (using *Inhba* and *Inha* mouse models) on macrophage phenotype (M1 and M2) at the mRNA level, and suggest that future studies should be performed to validate these findings. This would involve detection of CD206, MHCII, CD80, CD86, CD163, and other markers (Sierra-Filardi et al., 2011) in the protein levels using IHC, IF and flow cytometry techniques. Activin A levels increase in inflammatory status and also in preeclampsia; based on this knowledge, I propose to study testicular immune cells in embryos aborted due to preeclampsia, intra-amniotic infection, or spontaneous preterm delivery, and to compare the results with testicular immune cells in *Inha* mouse testis, as in both situations there can be an elevation in the activin A bioactivity levels (Muttukrishna et al. 2004, Rosenberg et al. 2012, Torricelli et al. 2012; Park et al. 2018).

6.3. Chapter Five: Neutrophils in testicular cancer

6.3.1 Main findings

Investigating testis samples from human adults with normal spermatogenesis and with germ cell neoplasia *in situ* (GCNIS) and human testicular germ cell tumour (TGCT) disclosed neutrophils are rare in the interstitium and peritubular areas of the testes of men with normal spermatogenesis, hypospermatogenesis with focal lymphocytic infiltrates, SCO, GCNIS, and GCNIS associated with focal lymphocytic infiltrates. Moreover, neutrophils are absent inside the seminiferous tubule of sections from all groups. Although I expected to detect accumulation of neutrophils in lymphoid-infiltrated regions in patients' samples, neutrophils were absent. However,

the number of neutrophils is higher in some seminoma samples than in other pathologies.

6.3.2 Main conclusions

I detected a massive infiltration of neutrophils in some seminoma samples, while these cells were rare in others. As expected, the histological variations between samples makes it challenging to reach a clear conclusion using just 5 samples per group.

6.3.3 Strengths and limitations

Samples from patients with testicular cancer were well-defined in terms of the category of their malignancy. The low number of samples (N=5) was a limitation for this study. Yamada et al., (2016) reported a prognostic value of $CD66b^{+}$ tumour-infiltrating neutrophils in testicular germ cell tumour by studying 102 patients (Low value in 81 patients and high in 21 patients). Also, Siska et al., (2017) reported a higher number of neutrophils in non-seminoma samples (N=24) compared to seminoma (N=11) (Yamada et al., 2016; Siska et al., 2017). Therefore, having more samples, at least 30 (Ganti, 2022), can improve the confidence of a conclusion to suggest a value, pattern or diagnostic tool for infiltrated neutrophils.

My original research plan for studying immune cells in samples of the adult human testis with GCNIS and TGCT was to investigate fresh collected human samples in JLU, (Germany) by flow cytometry, qRT-PCR, IHC and IF. That plan would provide an opportunity to determine the different phenotypes of infiltrated immune cells, and to perform experiments which could delineate the chemokines and chemokine receptors involved in their recruitment. However, due to the pandemic and border closures I was unable to travel, so the only available samples for this project were Bouin's fixed paraffin-embedded sections on slides which were sent from Justus Liebig University Giessen (Germany) to Melbourne. Therefore, I could not conduct any further investigations for Chapter Five.

6.3.4 Future directions

Neutrophils are abundant in solid tumours (investigated in 14 different cancer types), and their density correlates with metastasis at lymph node sites, tumour grade and tumour progression (Fridlender et al., 2015; Shaul et al., 2016; Wu et al.,

2020). In the early stages of cancer, tumour associated neutrophils (TANs) locate at the margin of the tumour site, but they can massively infiltrate into the centre of the tumour in the late stage. Therefore, regulation of recruitment of TANs impacts tumour progression or controls its growth. In addition, studying and determining the function and phenotype of TANs can improve the diagnosis and treatment approaches (Fridlender et al., 2009 and 2015).

Two populations of neutrophils are found in human tumours named N1 and N2. N1 neutrophils have anti-tumour activity against tumour cell proliferation and metastasis. N1 neutrophils function through antibody-dependent cellular cytotoxicity (ADCC), activation T cells, B cells, NK cells and DCs and the production of reactive oxygen species, which are cytotoxic to tumour cells. The N1 TANs are characterised by high levels of IL-12, TNFα, CCL3, CXCL9, CXCL10, ICAM-1 and low levels of the arginase axis. Therefore, N1 subpopulation exerts a potent anti-tumour activity mainly by releasing pro-inflammatory or immunostimulatory cytokines while failitating recruitment and activation of CD8+ T cells with strong ADCC function. In contrast, N2 neutrophils have pro-tumour activity and strong immunosuppressive impacts on anti-tumour responses. N2 cells promote, directly or indirectly, tumour growth, as well as facilitate invasion and tumour cell metastasis by remodelling extracellular matrix (using MMP9) and promoting angiogenesis. N2 neutrophils are characterised by upregulation of hepatocyte growth factor, oncostatin M, reactive oxygen species, reactive nitrogen species, matrix metalloproteinase (MMPs), neutrophil elastase, and chemokines, including CCL2, CCL4, CCL8, CCL12, and CCL17, and CXCL1, CXCL2, IL-8/CXCL8 and CXCL16 (Kasama et al., 2005; Tecchio et al., 2014; Shaul et al., 2016; Capucetti et al., 2020).

Moreover, there is a correlation between CCL17 expression by neutrophils and Treg cell populations in the tumour (Mishalian et al., 2014). Briefly, N2 neutrophils at progressive stages of cancer recruit Tregs into tumours via secretion of CCL17. CCL17 is a chemoattractant of Tregs. Tregs as strong immune-suppressive cells, which enhance anti-tumour immunity and lead to increased tumour growth. While N1 neutrophils downregulate the expression of CCL17. The levels of CCL17 progressively increase during tumour development. The demonstrative finding showed depletion of neutrophils or using suppressive/ blocking antibodies for

neutrophils, Tregs and CCL17 significantly decreased the population of Tregs in the tumour site and consequently the tumour growth.

In conclusion, due to accumulating evidence supporting the presence of neutrophils in tumour milieu and their dual role in cancers, I suggest detecting N1 and N2 through mentioned markers and studying their function in seminoma samples with neutrophil infiltration. The proposed study could be beneficial for understanding the role of neutrophils in the initiation and/ or progress of this malignancy. The recommended approach could be investigating the neutrophil number, cytokines (IL-8, GROα, MIG, IP-10, I-TAC, MCP-1, MIP-1α, MIP-1β) and chemokine receptors (CXCL8, IL-8, IL-18, CXCR1 receptors of IL-8, GCP2, NAP2; CXCR2 receptor of GRO, NAP2, IL-8 and ENA78).

6.4 Final Conclusion

These findings have an impact on the field because of identifying the composition and distribution of specific testicular immune cell types in fetal to newborn (at E13.5, E15.5 and PND0) wild type mice (C57BL6J), in mutant mice with lower and higher levels of activin A protein (*Inhba* and *Inha* strains; at E13.5, E15.5 and PND0), in human embryos, and in adult human testes, including those with testicular cancer. One project in this thesis is presented as an accepted manuscript (Chapter 2), and three are presented as traditional thesis chapters (Chapters 3, 4 and 5).

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Publications during enrolment

Journal articles

Hosseini S, Moody S, Daniela Fietz, Indumathy S, Schuppe H-Ch, Hedger M, Loveland K. *The changing landscape of immune cells in the fetal mouse testis and their interactions with germ cells*. **Histochemistry and Cell Biology**, Accepted, https://doi.org/10.21203/rs.3.rs-1507447/v1

Conference proceedings

Hosseini S, Moody S, Loveland K. *T cells Frequency, Localisation and Interactions during Fetal Mouse Testis Development.* **Australian Society for Reproductive Biology, 21-24 Nov 2021** (online). Poster

Hosseini S, Jezek D, Fietz D, Schuppe H-Ch, Hedger M, Loveland K. *Delineation of Macrophages, T Cells and Mast Cells during Human Fetal Testis Development.* **European Testis Workshop (ETW), May 30th - June 3rd, 2021** (online). Poster/ oral presentation

Hossein S, Whiley P, Fietz D, Schuppe H-Ch, Hedger M, Loveland K. *Study Frequency, Location and Interaction of Macrophages and Neutrophils during Fetal Testis Development in Mouse*. **46th American Society for Andrology (ASA,) 21-26 April 2021** (online), Poster/ Oral presentation

Hossein S, Indumathy S, Fietz D, Schuppe H-Ch, Hedger M, Loveland K. *High activin A levels do change testicular macrophage numbers during fetal testis development*. **59th ASMR National Scientific Conference, 18-19 November 2020** (online), Oral presentation

Hossein S, Indumathy S, Fietz D, Schuppe H-Ch, Hedger M, Loveland K. *The Impact of Activin A on Testicular Macrophages during Testis Development*. **11th Annual ASMR Victorian Student Research Symposium, 17 November 2020** (online), Poster/ oral presentation

Biniwale S, Wijayarathna R, Indumathy S, **Hosseini S**, et al., Heger M. *Enumerating macrophages in the mouse testis using classical histological and immunohistochemical techniques.* **Endocrine Society of Australia and the Society for Reproductive,** Poster

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.