Effect of \textit{C1q} Null Mutation on Mammary Gland Development and Breast Cancer Susceptibility

A Thesis Submitted for the Degree of Doctor of Philosophy by

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In loving memory of my mother, whose role in my life was, and remains, immense.

I dedicate this thesis to you, Mak,

FATIMAH ABDULLAH

13 April 1949 – 22 December 2016
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Summary

The complement protein C1q promotes rapid macrophage-mediated clearance of dying cells and tolerance to self antigens. mRNA encoding C1q is a key gene upregulated during mammary gland regression, and we hypothesise that C1q complement protein promotes mammary gland tumourigenesis through induction of tolerance to tumour antigens. We have investigated the role of C1q in normal mammary gland development, hormone-mediated regression, and in tumour development using two different mammary tumour mouse models together with C1q−/− mice. In the absence of C1q, mammary gland development proceeds normally during puberty, with a similar abundance of terminal end buds and rate of epithelial cell proliferation at 6 weeks of age compared to wildtype C1q+/+ mice. However, deficiency in C1q perturbed mammary gland regression, with 45% increased number of ductal branch points 24 hours following progesterone receptor antagonist RU486 (also known as mifepristone)-induced epithelial cell apoptosis in C1q−/− mice compared to C1q+/+ mice (p=0.027, n=7-11). There was a reduction of macrophage abundance (p=0.002, n=6-7) and a 3.5-fold increase of TUNEL positive apoptotic cells (p=0.011, n=5-6) in the ductal epithelium of C1q−/− mice compared to C1q+/+ mice 24 hours following RU486 administration. To investigate the role of C1q in mammary tumour development, MMTV-PyMT transgenic mice were crossed with C1q−/− mice and monitored weekly from 6 weeks by palpation to determine tumour latency, and mammary tumours dissected to assess total tumour burden. Lack of C1q did not affect development of mammary hyperplasia at 10 weeks, however development of palpable tumours was increased by 1 week in PyMT+/C1q−/− mice compared to PyMT+/C1q+/+ mice (p=0.012, n=43-45). The number of tumours was significantly reduced in 15-week old PyMT+/C1q−/− mice compared to PyMT+/C1q+/+ mice (p=0.028, n=15-16). There was also a significant reduction in the total tumour burden in PyMT+/C1q−/− mice compared to PyMT+/C1q+/+ mice at 15 weeks of age (p=0.036, n=15-16). The frequency of tumours that had progressed to carcinoma stage was also reduced in PyMT+/C1q−/− mice at 15 weeks of age (p=0.05, n=11-15) and 18 weeks of age (p=0.003, n=28-29). Carcinogen-induced mammary tumourigenesis was also investigated in mice administered 7,12-dimethylbenz[a]anthracene (DMBA) by oral gavage for 6 weeks. DMBA-treated C1q−/− mice were highly resistant to mammary tumourigenesis, with tumours detected in only 2 of 20 mice over the 30 week monitoring period, compared to 10 of 20 DMBA-treated C1q+/+ mice (p=0.02). T lymphocytes were skewed to the CD4+ subset, with a significant increase in IFNG-producing CD4+ T cells in the mammary gland draining lymph nodes in DMBA-treated C1q−/− mice (p=0.014, n=6-10). These findings suggest that C1q promotes tissue remodeling and clearance of dying cells during mammary gland regression, and increases mammary cancer susceptibility and tumour progression.
Declaration

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

In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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........................................
Siti Mariam Noor Din
December 2017
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Noor Din
Presentations at Scientific Meetings

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2013

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Siti M Noor Din, Danielle J Glynn, Leigh J Hodson, Sarah A Robertson, Wendy V Ingman. “Null mutation in C1q impairs tumour development in MMTV-PyMT mice”, Gordon Research Conference on Mammary Gland Biology, 8th-14th June 2013, Stoweflake Resort and Conference Centre, Stowe, Vermont, United States. (Poster)

2012

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Siti M Noor Din, Lachlan M Moldenhauer, Mark DeNichilo, Sarah A Robertson, Wendy V Ingman. “The carcinogen DMBA increases the proportion of IL10-producing CD4 and CD8 T cells in the mammary gland draining lymph node”, The Queen Elizabeth Hospital Research Day Conference, Adelaide, Australia, 12th October 2012, Basil Hetzel Institute, Adelaide, South Australia, Australia. (Poster)

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2011

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Siti M Noor Din, Lachlan M Moldenhauer, Sarah A Robertson, Wendy V Ingman. “Defining the immune response to carcinogen challenge in the mammary gland”, Faculty of Health Sciences Postgraduate Research Conference, 25th August 2011, National Wine Centre, Adelaide, South Australia, Australia. (Poster)

Siti M Noor Din, Lachlan M Moldenhauer, Sarah A Robertson, Wendy V Ingman. “Defining the immune response to carcinogen challenge in the mammary gland”, Australian Society for Medical Research (ASMR) Scientific Meeting, 8th June 2011, Adelaide Exhibition and Convention Centre, Adelaide, South Australia, Australia. (Poster)
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>allophycocyanin-Cy7</td>
</tr>
<tr>
<td>APAF1</td>
<td>apoptotic protease activating factor 1</td>
</tr>
<tr>
<td>ASR</td>
<td>age-standardised rate</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium</td>
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<td>CLN</td>
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</tr>
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<td>DR</td>
<td>death receptor</td>
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<tr>
<td>E&lt;sub&gt;2&lt;/sub&gt;</td>
<td>estradiol</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>FLIP</td>
<td>FADD-like apoptosis regulator</td>
</tr>
<tr>
<td>FDCs</td>
<td>follicular dendritic cells</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>hydrogen peroxidase</td>
</tr>
<tr>
<td>HI-FBS</td>
<td>heat inactivated-fetal bovine serum</td>
</tr>
<tr>
<td>HMGB1</td>
<td>high mobility group box chromosomal protein 1</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IFNA</td>
<td>interferon alpha</td>
</tr>
<tr>
<td>IFNG</td>
<td>interferon gamma</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>IL4</td>
<td>interleukin-4</td>
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<td>interleukin-6</td>
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<td>IL10</td>
<td>interleukin-10</td>
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<tr>
<td>IL12</td>
<td>interleukin-12</td>
</tr>
<tr>
<td>IL13</td>
<td>interleukin-13</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>iNOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LRP1</td>
<td>low-density lipoprotein receptor-related protein 1</td>
</tr>
<tr>
<td>lysoPC</td>
<td>lipid lysophosphatidylcholine</td>
</tr>
<tr>
<td>MAC</td>
<td>membrane attack complex</td>
</tr>
<tr>
<td>MASP</td>
<td>ficolin-C1s serine proteases complex</td>
</tr>
<tr>
<td>MBL</td>
<td>mannose-binding lectin</td>
</tr>
<tr>
<td>MCA</td>
<td>methylcholanthrene</td>
</tr>
<tr>
<td>MDSCs</td>
<td>myeloid-derived suppressor cells</td>
</tr>
<tr>
<td>MG DLN</td>
<td>mammary gland draining lymph nodes</td>
</tr>
<tr>
<td>MHC I</td>
<td>major histocompatibility complex class I</td>
</tr>
<tr>
<td>MHC II</td>
<td>major histocompatibility complex class II</td>
</tr>
<tr>
<td>MLKL</td>
<td>mixed lineage kinase domain-like</td>
</tr>
<tr>
<td>MMS</td>
<td>methyl methanesulfonate</td>
</tr>
<tr>
<td>MMTV</td>
<td>mouse mammary tumour virus</td>
</tr>
<tr>
<td>MOMP</td>
<td>mitochondrial outer membrane permeabilization</td>
</tr>
<tr>
<td>MTORC1</td>
<td>mechanistic target of rapamycin complex 1</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NBF</td>
<td>neutral buffered formalin</td>
</tr>
<tr>
<td>NK cells</td>
<td>natural killer cells</td>
</tr>
<tr>
<td>NKT cells</td>
<td>natural killer T cells</td>
</tr>
<tr>
<td>NMU</td>
<td>N-methyl-N-nitrosourea</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PALN</td>
<td>para-aortic lymph nodes</td>
</tr>
<tr>
<td>PAMPs</td>
<td>pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>pDCs</td>
<td>plasmacytoid dendritic cells</td>
</tr>
<tr>
<td>PE</td>
<td>phycoethrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>peridinin-chlorophyll-protein complex</td>
</tr>
<tr>
<td>PerCP-Cy5.5</td>
<td>peridinin-chlorophyll-protein complex Cy5.5</td>
</tr>
<tr>
<td>P4</td>
<td>progesterone</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered solution</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMN-MDSCs</td>
<td>neutrophil-like myeloid-derived suppressor cells</td>
</tr>
<tr>
<td>PRRs</td>
<td>pattern recognition receptors</td>
</tr>
<tr>
<td>PtdSer</td>
<td>phosphatidylerine</td>
</tr>
<tr>
<td>PTX3</td>
<td>pentraxin</td>
</tr>
<tr>
<td>P/S</td>
<td>penicillin/streptomycin</td>
</tr>
<tr>
<td>PyMT</td>
<td>polyomavirus middle T antigen</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RIPK1</td>
<td>receptor-interacting serine/threonine protein kinase 1</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RU486</td>
<td>progesterone receptor antagonist mifepristone</td>
</tr>
<tr>
<td>S1P</td>
<td>sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>SRP</td>
<td>serum amyloid P-component</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>T1D</td>
<td>type 1 diabetes mellitus</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate-EDTA</td>
</tr>
<tr>
<td>TAMs</td>
<td>tumour-associated macrophages</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TEB</td>
<td>terminal end bud</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;</td>
<td>T helper</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;1</td>
<td>T helper 1</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;2</td>
<td>T helper 2</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TNFA</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR-associated death domain</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>T&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>T regulatory cells</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling</td>
</tr>
<tr>
<td>TWEAK</td>
<td>TNF-related weak inducer of apoptosis</td>
</tr>
<tr>
<td>V(D)J</td>
<td>variable (V), diversity (D), and joining (J) gene segments</td>
</tr>
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</table>
Chapter 1 - Literature Review
1.1 Introduction

Breast cancer is a major health burden worldwide, with an estimated 1.38 million new cases per year (Eccles et al. 2013). In Australian women, it is reported that breast cancer accounts for 27% of all new cancer diagnoses (Table 1.1) and 1 in 8 women will develop the disease before the age of 85 (AIHW 2014, AIHW 2015). A number of factors are associated with increased risk of breast cancer including increasing age, family history, genetic predisposition, early menarche and older age at menopause, nulliparity, pregnancy after the age of 35 and not breastfeeding (Daudt et al. 1996, McPherson et al. 2000, Key et al. 2001, Hanf and Hanf 2014). A number of these established risk factors such as early age at menarche, late age at menopause, nulliparity, and not breastfeeding are linked to increased total number of menstrual cycles (Ewertz and Duffy 1988, Kampert et al. 1988, Henderson and Bernstein 1991, Clavel-Chapelon 2002, Chavez-MacGregor et al. 2005). The relationship between menstrual cycling and breast cancer risk is most clearly illustrated by the findings that the more years a woman is menstrual cycling, the greater her lifetime breast cancer risk (Key et al. 2001). Furthermore, for each year earlier a young girl enters menarche, there is a 5% increase in lifetime breast cancer risk and for each year older a woman enters menopause, there is a 3.5% increased risk (Clamp et al. 2002).

Changes in the circulating concentration of progesterone and estrogen over the course of the ovarian cycle cause the mammary gland to undergo development and regression. These morphological changes in the mammary gland are similar to those which occur over the course of pregnancy, lactation and involution, although they are far less extensive (Schedin et al. 2000, Fata et al. 2001, Navarrete et al. 2005, Strange et al. 2007). The regression phase, which occurs principally at the proestrus phase of the estrous cycle in rats and mice and the menstrual phase in women, is particularly susceptible to mammary gland tumour initiation (Nagasawa et al. 1976, Lindsey et al. 1981, Ratko and Beattie 1985). However, the underlying mechanisms that lead to the increased breast cancer risk during the regression phase are yet to be elucidated.

The immune system is not only able to recognise and destroy invading foreign pathogens, it also recognises and eliminates any host cells that can potentially become cancerous (Janeway and Medzhitov 2002, Swann and Smyth 2007, Chaplin 2010, Kumar et al. 2011). This is termed “immunosurveillance”, and is a mechanism that involves both the innate and adaptive arms of the immune system, acting in concert to effectively mount defence responses to combat tumour development (Dranoff 2004, Swann and Smyth 2007). The innate and adaptive immune responses are carried out by effector functions of different cell types including B and T lymphocytes,

Macrophages are well-appreciated as one of the major cell components of the immune system; these cells are involved in phagocytosing dying cells and debris, maintaining tissue homeostasis, and modulating the adaptive immune response through antigen processing and presentation (Elhelu 1983, de Visser et al. 2006, Gordon and Martinez 2010, Murray and Wynn 2011, Biswas and Mantovani 2012). In addition, investigations on mammary gland morphogenesis also implicate macrophages in providing support for epithelial cell proliferation and differentiation, phagocytosing dying epithelial cells and promoting tissue remodelling at different stages of mammary gland development including puberty, ovarian-cycling, pregnancy and post-partum mammary involution (Cecchini et al. 1994, Pollard and Hennighausen 1994, Gouon-Evans et al. 2000, Fata et al. 2001, Gouon-Evans et al. 2002, O'Brien et al. 2012, Hodson et al. 2013). These discoveries therefore establish a new paradigm of the developmental roles of macrophages in the mammary gland, adding another layer of complexity to paradigms based on macrophage roles in the immune system. This also demonstrates the high versatility of macrophages in directing a diverse range of biological processes. However, the dependence of the mammary gland on macrophage-mediated morphogenesis may affect the microenvironment of the tissue, potentially diverting macrophages and the broader immune system from appropriately responding to tumour initiation.

Programmed cell death (PCD) is one of the key mechanisms in maintaining normal tissue homeostasis (Savill and Fadok 2000, Elliott and Ravichandran 2010, Nagata and Tanaka 2017). The three major cell death modes include apoptosis, necroptosis and autophagy; each of these cell death modes are initiated by diverse and distinct ranges of stimuli, involve specific mechanisms and events, and are characterised by unique morphological features (Krysko et al. 2006, Inoue and Tani 2014, Nunes et al. 2014). Accumulated data from previous studies has highlighted the importance of cell death modes and their distinct immunological outcomes, which in turn indirectly determine immune responses being executed, to be either immunogenic or tolerogenic (Krysko et al. 2006, Nagata and Tanaka 2017). Inefficient or aberrant cell death machinery is associated with development of autoimmune diseases, immunodeficiencies, and tumours (Han et al. 2011). Indeed, a major hallmark of cancer involves defective programmed cell death which enables tumour cells to sustain proliferative signalling (Hanahan and Weinberg 2011). As the mammary gland exhibits active cellular turnover and tissue remodelling, not only occurring during puberty, pregnancy and involution, but also over the course of the ovarian cycle (Fata et al. 2001), this raises the question of the role of cell death and the ensuing immunological responses in tumour development in this tissue.
C1q is one of the components of the complement cascade. Six C1q molecules complex with two modular serine proteases C1r and C1s to form an inactive C1 that circulates as a large (∼790-kDa) serum molecular complex (Noris and Remuzzi 2013, Garcia et al. 2016). Engagement of C1q to a complex antigen-antibody molecule initiates activation of the classical pathway, which subsequently elicits a tightly regulated series of inflammatory responses, opsonisation, phagocytosis and induction of the adaptive arm of the immune response (Merle et al. 2015). Complement C1q interacts with macrophages to facilitate rapid phagocytosis of dying cells and promotion of tolerance to self-antigens (Roos et al. 2004). C1q is produced primarily by macrophages, and binds to apoptotic cells through recognition of apoptotic cell-associated molecular patterns (Kim et al. 2003). Significantly, deficiency in C1q is associated with the generation of the autoimmune disease systemic lupus erythematosus (Walport et al. 1998, Sontheimer et al. 2005), demonstrating the essential role of C1q in maintaining normal tissue homeostasis and preventing autoimmunity. The mRNA encoding C1q is increased in abundance during mammary gland involution (Clarkson et al. 2004, Stein et al. 2004), and may be similarly upregulated during ovarian cycle-associated regression.

The underlying mechanisms that lead to increased susceptibility of the breast to cancer due to menstrual cycling and the regression phase of the cycle are still poorly understood. Here, we review the current understanding of the cellular and molecular mechanisms that underpin mammary gland development during the menstrual cycle, the innate and adaptive elements of the immune system including effector functions of macrophages both in immunity and mammary morphogenesis and programmed cell death, the involvement of immune system factor C1q in macrophage-mediated phagocytosis, and how these factors might affect breast cancer risk.

1.2 Mammary Gland
The mammary gland is a unique organ that undergoes the majority of its development postnatally, with proliferation, differentiation and tissue remodelling occurring during puberty, pregnancy, lactation and involution (Van Nguyen and Pollard 2002, Russo and Russo 2004, Watson 2006). There is a complex interplay of both intracellular and extracellular signals within the different cell types in the mammary gland which direct histological and morphological changes during pubertal and adult life (Humphreys et al. 1997, Watson 2009, Brisken and Ataca 2015, Inman et al. 2015, Shamir and Ewald 2015).
1.2.1 Ovarian cycle-associated changes in the mammary gland

Cellular proliferation, differentiation and apoptosis in the non-pregnant mammary gland have been observed in both humans and mice. Full characterisation of nulliparous murine mammary glands during the estrous cycle demonstrated that the morphological, cellular and molecular changes that occur are heavily influenced by cyclical production of ovarian estrogen and progesterone (Walmer et al. 1992, Schedin et al. 2000, Fata et al. 2001). These changes are also observed in the human mammary epithelium during the menstrual cycle (Pike et al. 1993, Nazario et al. 1995, Navarrete et al. 2005). Analysis of the cellular content of vaginal smears from adult female mice can be used to group the mice into four different stages of the estrous cycle; proestrus, estrus, metestrus and diestrus. The concentration of circulating estradiol in the mouse peaks around the time of ovulation, which occurs at the onset of the estrus stage, and circulating progesterone concentration rises during metestrus and diestrus (Fig. 1.1) (Walmer et al. 1992, Fata et al. 2001, Chua et al. 2010). Active ductal outgrowth and alveolar expansion are observed during the estrous cycle (Fata et al. 2001). Following ovulation, the rise in progesterone is associated with an increased number of developing alveolar buds (Fata et al. 2001). This is termed the development phase (Chua et al. 2010), and occurs in the metestrus and diestrus phase in rats and mice, and in the luteal phase in humans (Nazario et al. 1995, Fata et al. 2001). If a pregnancy occurs, these buds will mature and further develop into milk-secreting elements to support lactation. If pregnancy does not occur, the fall in progesterone triggers apoptosis of the newly formed alveolar buds and the gland is remodelled back to its basic architecture (Fata et al. 2001). This is termed the regression phase (Chua et al. 2010) and occurs principally at the proestrus phase in rats and mice (Fata et al. 2001), and during the menstrual phase in humans (Fig. 1.1) (Navarrete et al. 2005).

1.2.2 Regression phase and mammary gland tumour susceptibility

Chemical carcinogen models, which have been used for the past 50 years, have greatly contributed to our understanding of the factors influencing susceptibility to breast cancer and mechanisms controlling mammary tumour initiation and development. Polycyclic hydrocarbons (i.e. 7, 12-dimethylbenz[a]anthracene (DMBA) and nitrosoureas (i.e. methylnitrosourea –MNU; ethylnitrosourea – ENU) are chemical carcinogens utilised in mouse models to study mammary cancer initiation (Clarke 1996, Russo and Russo 1996, Medina 2007, Perse et al. 2009). Other families of chemicals such as urethane also have been used to induce mammary tumours (Medina 1974). A number of studies using these models have shown the capability of different stages of the estrous cycle to affect susceptibility to tumour initiation and the subsequent incidence of mammary...

An early investigation of tumour initiation using DMBA found an increase in the mean number of tumours in rats administered DMBA at diestrus, compared to other stages of the estrous cycle (Young et al. 1970). This finding was difficult to interpret due to the 16 hour delay in maximal DMBA-DNA adduct formation in the mammary gland, which then resulted in formation of active metabolites and subsequent binding of DNA at the time of peak circulating estradiol concentration (Nagasawa et al. 1976, Janss and Ben 1978). Interestingly, it was also shown that a carcinogen challenge at the peak of estradiol secretion during proestrus was not only associated with a shortened tumour latency, but also increased mean number of mammary tumours per rat than those challenged at diestrus (Lindsey et al. 1981). However, despite the shorter tumour latency, the tumours that developed had a slower rate of growth (Lindsey et al. 1981).

Female Sprague-Dawley rats aged 50-days old administered a single intravenous injection of chemical carcinogen DMBA at proestrus exhibited a significant increase in the incidence of progressive mammary tumours, which was accompanied by increased tumour growth rate and increased total tumour burden per rat compared to rats administered DMBA at diestrus (Nagasawa and Yanai 1974, Nagasawa et al. 1976). However, these effects were not observed in 90-day-old rats, indicating an age-dependence of carcinogen-induced mammary tumourigenesis (Nagasawa and Yanai 1974, Nagasawa et al. 1976). The basis of this greater susceptibility of younger rats to tumour formation was suggested to be due to an elevated rate of DNA synthesis of the terminal end buds (TEBs) occurring at this age compared to normal subtending ducts (Russo and Russo 1980, Williams and Daniel 1983). These highly hormone-responsive cells in the TEB undergo rapid DNA synthesis and division, and therefore are believed to pose a higher oncogenic risk than quiescent cells (Bertram and Heidelberger 1974, Janss and Ben 1978).

Other studies demonstrated no correlation of mammary tumour incidence with the rate of proliferation (Lindsey et al. 1981, Ratko and Beattie 1985, Anderson et al. 1991, Nandi et al. 1995). Instead, the authors suggested that the susceptibility of the mammary gland to tumour initiation, and subsequent nature of the tumour induced, was most heavily influenced by the prevailing hormonal milieu at carcinogen delivery. One study reported that virgin female Buffalo rats exhibited a shorter tumour latency of 83.6 days when administered with 5 mg dose of NMU at proestrus, compared to 104.4 days and 91.4 days at diestrus and estrus groups respectively, when administered with the same carcinogen (Lindsey et al. 1981). The mean number of tumours per rat was also significantly
increased in rats treated at proestrus compared to the other estrous cycle stages (Lindsey et al. 1981).

Similarly, another study using virgin female Sprague-Dawley rats also found rats injected with NMU intravenously at proestrus exhibited shortened mean mammary gland tumour latency, increased incidence and increased tumour growth rate compared to rats injected at the diestrus phase (Ratko and Beattie 1985). However, in 1994, Rivera and colleagues observed female Sprague-Dawley rats injected intraperitoneally with NMU at estrus showed an increased tumour incidence (95.2%) compared to those received at proestrus (71.4%) and diestrus (77.4%) (Rivera et al. 1994). This was suggested to be the result of high circulating estradiol and prolactin concentration, resulting in a profound number of DNA alkylation sites, which led to DNA damage in the mammary epithelial cells. It was suggested that subsequent DNA replication containing these adducts ultimately resulted in increased susceptibility for somatic mutation at estrus (Ratko et al. 1988, Rivera et al. 1994).

Although these studies have reported different effects of estrous cycle phases on mammary gland susceptibility to tumour induction, the majority of the studies suggest that mammary tumours are more likely to arise when chemical carcinogens are administered during proestrus. These effects have been largely attributed to elevated circulating estradiol. However, a mechanistic relationship between estradiol and tumour susceptibility at proestrus has not been established. In addition to elevated estradiol, the proestrus phase is also associated with declining progesterone, characterised by cell death and tissue remodelling as part of mammary gland regression, which may also contribute to increased susceptibility to tumour development. Moreover, roles of different cell lineages within the mammary gland during the regression phase as well as in response to carcinogen administration are not clear. Therefore, examination on the microenvironment of the regression phase and how this environment might affect the mammary gland function during tumourigenesis is warranted.

1.3 Immune system

The immune system is composed of a network of cells, lymphoid organs, humoral factors and cytokines that are highly regulated to collectively protect the body from a wide range of harmful factors including invading pathogenic bacteria and viruses, as well as internal threats such as mutated cells (Parkin and Cohen 2001, Medina 2016). Fundamentally, the immune system is comprised of two main parts; termed the innate arm and the adaptive arm of the immune system. These two parts to the immune system are extremely complex, with many of the components of
each arm working inter-dependently, interacting with each other to discriminate “self” from “non-self” to effectively maintain tissue homeostasis (Swann and Smyth 2007, Medina 2016).

1.3.1 Innate and adaptive arms

Broadly defined, the innate arm of the immune system is comprised of different key effectors including the phagocytic system, the complement system, the acute-phase response, and cellular components including granulocytes, mast cells, macrophages, dendritic cells, and natural killer cells (Dranoff 2004, Medina 2016). It is commonly known as the non-specific arm of the immune system, with each component of innate immunity playing integral roles to effectively and rapidly perform protective functions against invading pathogens, infectious agents, or non-self proteins and cells. Hence, it constitutes the first line of host immune surveillance (Parkin and Cohen 2001, Chaplin 2010). Macrophages and neutrophils recognise pathogens or their components upon engagement of the pattern recognition receptors (PRRs) that bind pathogen-associated molecular patterns (PAMPs), leading to subsequent cellular responses associated with innate immunity. Some of the products of the acute-phase response and complement system direct antimicrobial activities, and others enhance opsonisation to assist macrophage-mediated phagocytosis of cellular debris, apoptotic cells and immune complexes, thus maintaining cell homeostasis (Medina 2016).

In contrast to the innate arm, the adaptive immune response is specific, consisting of specialised cell types including B and T lymphocytes and antigen-presenting cells (APCs) (Alberts et al. 2002, Michaud et al. 2015). Mature lymphocytes in the central lymphoid organ migrate to the peripheral lymphoid organs via the blood before being activated through cross-presentation of foreign antigens or class I or class II alleles of major histocompatibility complex (MHC) by APCs to become effector lymphocytes. These lymphocytes carry out different functions including activating humoral and cell-mediated immune responses, assisting cytotoxicity and producing different immunostimulatory and immunomodulatory cytokines that collectively protect against bacteria, viruses and mutated cells (Janeway et al. 2001, Alberts et al. 2002, de Visser et al. 2006).

The innate immune response not only contributes to the induction of the adaptive response, but can also dictate and control adaptive immunity (Iwasaki and Medzhitov 2015). Recognition of PAMPs by PRRs as part of innate immunity primes activation of long-lasting adaptive responses including lymphocyte differentiation and production of antibodies and cytokines (Iwasaki and Medzhitov 2015). The type of cytokines released by bystander cells during the innate response can differentially modulate the adaptive response to virus-infected cells and to tumours. For example, immune cells exposed to IL4 can become skewed to exert a Th2-dominated immune response and may impair
efficient Th$_1$ response (Marcenaro et al. 2005, Smyth et al. 2007). A number of mouse studies have demonstrated the capacity of components of the innate complement cascade to direct B and T lymphocyte-mediated immunity, illustrating the complement system as a bridge between innate and adaptive immunity (Carroll 2000, Carroll 2004, Dunkelberger and Song 2010).

1.3.2 Tumour immune responses

Crosstalk between innate and adaptive immunity enables effective execution of tumour immune surveillance (Smyth et al. 2007, Swann and Smyth 2007, Pandya et al. 2016). However, compelling evidence documents a dual capacity of the immune system to not only provide protection against cancer, but also to promote tumour growth through a tumour-sculpting process of immunoediting (Fig. 1.2) (Dunn et al. 2002, Kim et al. 2007, Swann and Smyth 2007, Pandya et al. 2016). The cancer immunoediting concept underlies tumour evasion and growth in spite of a fully functional immune system, and is comprised of three divided phases, termed elimination, equilibrium and escape (Fig. 1.2) (Dunn et al. 2004). The elimination phase represents the initial theory of immune surveillance, in which the immune system identifies tumour antigens on the surface of nascent transformed cells, leading to elimination before they become cancerous. Partial or incomplete elimination lead to immune pressure that selects tumour cells with reduced immunogenicity. This characteristic enables them to persist and subsequently become resistant to immune attack in the equilibrium phase. During this phase, tumour cells can either remain dormant or undergo immune selection and acquire further genetic changes by chance or mediated by immune-induced inflammation (Grivennikov et al. 2010), resulting in tumour variants that enable the tumour cells to resist or suppress anti-tumour immunity. Subsequently, tumour cells exit the equilibrium state and transit to the escape phase to progressively become overt clinical cancer (Swann and Smyth 2007, Pandya et al. 2016).

Tumourigenesis is a multistep process. Hanahan and Weinberg (2011) have described the hallmarks of cancer, which are (1) resistance to cell death, (2) sustained proliferative signalling, (3) evasion of growth suppression, (4) activation of invasion and metastasis, (5) induction of replicative immortality, (6) angiogenesis, (7) reprogramming of energy metabolism, and (8) evasion of destructive immune attack (Hanahan and Weinberg 2011). Although considerable understanding has been generated on how tumour cells successfully acquire these capabilities, the process is still far from being fully understood. Studies have documented different key aspects of the immune system that are exploited by tumour cells to enable tumour growth and progression (Vinay et al. 2015). For instance, tumour-derived CD4$^+$CD25$^+$FOXP3$^+$ regulatory T cells (T$_{reg}$) mediate immune suppression within the tumour environment (Gasparoto et al. 2010, Jacobs et al. 2012), and
activation of immune-induced inflammation is increasingly recognised to facilitate tumour initiation, angiogenesis and metastasis (Grivennikov et al. 2010). In addition, previous studies have demonstrated the ability of tumour cells to exploit apoptotic machinery in deletion of tumour-specific cytotoxic T lymphocytes (CTLs) (Bogen 1996, Lauritzen et al. 1998). Engagement of tumour cells to the T cell receptor in the absence of co-stimulatory molecules can promote anergy or tolerance, significantly assisting tumour evasion (Staveley-O'Carroll et al. 1998, Foell et al. 2007).

Taken together, these findings demonstrate the diversity of different factors that facilitate tumour development and progression. Given that tumour cells utilise different strategies to evade immune attack, modulation of the immune system may therefore offer a strategy to potentially suppress cancer development.

1.3.3 Macrophages

Macrophages are highly versatile cells of the immune system that exhibit remarkable plasticity, and are capable of directing a diverse range of biological events including tissue development, homeostasis and immunity (Ingman et al. 2006, Pollard 2009, Murray and Wynn 2011, Care et al. 2013, Wynn et al. 2013). Their precursors, monocytes, are rapidly recruited to tissues in response to injury or infection, where they undergo a series of differentiation steps to become tissue-resident macrophages. Previous studies have revealed a tremendous heterogeneity in tissue-resident macrophage populations, highlighting the phenotypic variation of macrophages in different tissue microenvironments (Davies et al. 2013). These different macrophage subtypes perform multiple functions to promote development, maintain homeostasis, and contribute to immunosurveillance. For example, resident liver macrophages (known as Kupffer cells) mediate effective clearance of microorganisms, cell debris and aged erythrocytes, while motile liver macrophages are involved in immune surveillance (Klein et al. 2007, Ganz 2012). In the central nervous system, tissue-resident macrophages known as microglia play specific and unique roles, including promoting neuronal integrity, assisting in clearance of cell debris, contributing to matrix and protein deposits, and regulating synaptic remodelling (Paolicelli et al. 2011, London et al. 2013).

Macrophages are well-described as the “big eaters” of the immune system as they phagocytose dying cells and apoptotic bodies, a process integral to the resolution of inflammation. As professional antigen presenting cells (APCs), they process antigens and present peptides to T cells to initiate adaptive immune responses (Shi and Pamer 2011). In addition, numerous studies have documented the flexibility of macrophages to switch from one functional phenotype to another depending on the different stimuli they receive from the local microenvironment (Stout and Suttles 2004, Stout et al.
2005, Hagemann et al. 2008, Mosser and Edwards 2008, Mylonas et al. 2009). The underlying mechanism that drives macrophage switching to activate different functional phenotypes remains unclear and far from fully elucidated. However, there is substantial data to suggest that macrophage activation status and its functional implications can be attributed to the “M1” versus “M2” activation and polarisation program in response to different conditions within the tissue microenvironment (Italiani and Boraschi 2014).

1.3.3.1 Macrophage activation and polarisation

The conceptual framework of macrophage activation and polarisation is often broadly discussed based on the “M1/M2” macrophage paradigm (Mantovani et al. 2002, Biswas and Mantovani 2010). The “M1” and “M2” activation states, also known as classic and alternative macrophage activation respectively, was initially proposed to be linked to two different subsets of T helper (T_H) cells producing either interferon-gamma (IFNG) or interleukin-4 (IL4), as the macrophages exhibit differential responses to the cytokines secreted by these T cell subsets (Mosmann et al. 1986, Mills et al. 2000). Release of pathogen or danger-associated molecular patterns (PAMPs or DAMPs) and IFNG, produced by the Th1 T cell subset, are some of the major stimuli governing “M1” macrophage activation (Mantovani et al. 2004, Benoit et al. 2008). Previous studies have demonstrated the protective roles of the “M1” macrophage subset in tumourigenesis, which includes activation of tumour-killing mechanisms and disruption of suppressive activities of other macrophage subsets including tumour-associated macrophages (TAMs), regulatory macrophages, immature myeloid cells, myeloid-derived suppressor cells, and the “M2” macrophage subset. “M1” macrophages also amplify Th1 responses to provide a positive feedback loop in the anti-tumour immune response (de Visser et al. 2005, Nardin and Abastado 2008, Andreu et al. 2010, Biswas and Mantovani 2010, Yang et al. 2011).

Classically activated “M1” macrophages are characterised by the expression of inducible nitric oxide synthase (iNOS), the production of reactive oxygen species (ROS), and synthesis of pro-inflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-12 (IL12). These characteristics confer the ability of “M1” macrophages to be key immune cells in promoting a strong immune response (Qian and Pollard 2010). However, it is important to note that “M1” macrophage responses not only can suppress tumour development, but continuing production of inflammatory cytokines can also drive tumour progression (Mantovani et al. 2002, Cook and Hagemann 2013). In addition, excessive or unresolved activity of activated “M1” macrophages has been associated with the pathogenesis of several inflammatory conditions including atherosclerosis, diabetes and
glomerulonephritis and tissue damage (Valledor et al. 2010, Mantovani et al. 2013, Wynn et al. 2013).

The alternative “M2” macrophage activation state is generally defined by a number of markers including arginase-1, expression of chitinase-3-Like Protein 3 or Ym1, CD206, CD163, Egr2 and c-Myc, and secretion of anti-inflammatory cytokines IL-10 and IL-1ra (Jablonski et al. 2015, Roszer 2015). Macrophage exposure to IL4, produced by Th2 cells, eosinophils, basophils, and macrophages, drives “M2” activation and polarisation (Mantovani et al. 2004). Macrophages can also commit to “M2” macrophage activity when activated by the combined stimuli of either immune complex and Toll-like receptor (TLR) ligands, or IL-10 together with glucocorticoids, leading to the sub-classification of the “M2” activation program to be “M2a”, “M2b” and “M2c”, which all perform similar consequent responses (Mantovani et al. 2004, Martinez and Gordon 2014).

In general, alternatively activated “M2” macrophages are associated with Th2 type immune responses, are involved in the suppression of Th1-mediated inflammation, and promote tissue remodelling and wound healing (Heusinkveld et al. 2011, Heusinkveld and van der Burg 2011, Murray and Wynn 2011). Thus, the “M2” macrophage subtype possesses the ability to promote tumour development compared to the “M1” macrophages. Indeed, Mantovani and colleagues built a strong case that tumour-associated macrophages (TAMs) should be viewed as “M2”-polarised (Mantovani et al. 2002). TAMs isolated from established tumours are found to exhibit characteristics of the “M2”-like phenotypes (Biswas and Mantovani 2010, Ojalvo et al. 2010). Consistently, IL4 and IL13 activities are associated with tumour promotion, which also simultaneously promote “M2” macrophage polarisation (Terabe et al. 2000, Sinha et al. 2005, Gocheva et al. 2010). A more recent study has revealed “M2a” and “M2c”-activated macrophages to be associated with lung cancer invasion and tumour growth, whilst the “M1” macrophages demonstrate capabilities to suppress proliferation and cell viability of A549 cancer cells, simultaneously reducing angiogenesis and inducing apoptosis of lung cancer cells (Yuan et al. 2015).

However, given the heterogeneity of the tumour microenvironment and the complex dynamics of tumour progression, it has become clear that the view of TAMs to be entirely “M2”-polarised is an over-simplification (Quatromoni and Eruslanov 2012). Instead, the modulation of function in tumour-inhibiting or tumour-promoting macrophages is critically tuned by the balance of activatory and inhibitory signals they receive from the tissue microenvironment (Hagemann et al. 2006). Hence there may be the possibility that both populations co-exist with mixed phenotypes. Although it is undeniable that the conceptual framework of “M1/M2” macrophage activation and polarisation has
contributed to a better understanding of macrophage activity in response to different stimuli in vitro, diverse and complex macrophage functions and roles are increasingly recognised in various developmental processes, both in health and disease states (Mosser and Edwards 2008, Cook and Hagemann 2013). There is growing evidence that indicates a larger spectrum of macrophage activation and polarisation states exist, with overlapping “M1” and “M2” signalling pathways. This has led to the realisation that the macrophage activation program is complex and the “M1/M2” classification is insufficient to fit in all macrophage phenotypes, hence, calling for a revised and comprehensive classification system (Martinez and Gordon 2014). Recently published transcriptome analysis of human macrophages has revealed at least nine different macrophage activation programs (Xue et al. 2014). Although this study is limited by a number of technical issues and the complexity in genetic variability and dynamic transitions of macrophage responses in different physiological and pathological environments (Natoli and Monticelli 2014), it expands the “M1/M2” paradigm to a spectrum model of macrophage activation and polarisation, which bring us a step closer to fully understanding the regulation and function of macrophages.

1.3.3.2 Macrophages in mammary gland development and function

In addition to phagocytic and immune functions previously discussed, macrophages are also implicated in developmental process including mammary gland development and function (Cecchini et al. 1994, Pollard and Hennighausen 1994, Gouon-Evans et al. 2000, Chua et al. 2010). Immunohistochemical analysis using anti-F4/80 antibody in developing mouse mammary tissue has demonstrated recruitment of macrophages to the stroma surrounding terminal end buds (TEB) during puberty (Cecchini et al. 1994, Gouon-Evans et al. 2000, Gouon-Evans et al. 2002), and that these cells are also in close proximity to alveoli during pregnancy and lactation (Pollard and Hennighausen 1994). Mice lacking macrophage-colony-stimulating factor-1 (CSF1), which is a cytokine essential to the differentiation and survival of macrophages, exhibit dramatically reduced macrophage recruitment to the stroma surrounding the TEBs, and severe impairment of TEB formation and adolescent ductal outgrowth (Nathan 1987, Pollard and Hennighausen 1994, Gouon-Evans et al. 2000). These reproductive defects can be rescued by administration of exogenous human recombinant CSF1 to null mutant mice daily or delivery of a CSF1 transgene expressed specifically in the mammary gland, demonstrating the essential role of CSF1-regulated macrophages in directing appropriate ductal elongation and branching during mammary gland development (Cecchini et al. 1994, Gouon-Evans et al. 2000, Van Nguyen and Pollard 2002).

Macrophages are spatially and temporally distributed in the non-pregnant cycling mammary gland over the course of the ovarian cycle (Chua et al. 2010). Hormonal changes during the cycle cause
fluctuations in macrophage abundance, in which the higher abundance is observed during the metestrus and diestrus phases and is lower at proestrus and estrus. Macrophage ablation at precise developmental stages of the cycle using an acute macrophage depletion mouse model (Cd11b-dtr) has demonstrated roles for macrophages in promoting epithelial cell proliferation and alveolar bud development. During the regression phase, macrophages phagocytose dying epithelium and promote tissue remodelling to return the gland to its basic architecture (Chua et al. 2010).

Macrophages are also essential in promoting epithelial apoptosis and adipocyte repopulation during mammary gland involution (O'Brien et al. 2012). Rapid removal of apoptotic cells by macrophage-mediated phagocytosis is necessary in promoting tolerance to self-antigens and avoiding autoimmunity (Roos et al. 2004), which are essential for normal mammary gland function. These findings demonstrate the key roles of macrophages in ensuring normal mammary gland development during puberty, ovarian cycling, pregnancy and involution, where their activity may affect immune responses to tumour formation. However, the underlying cellular and molecular mechanisms of how these cells regulate mammary morphogenesis and tumourigenesis are not clearly understood.

1.3.4 Tolerance and autoimmunity

Many of the effector mechanisms of the immune response are potent and able to damage the host’s own tissues. Therefore, both the innate and adaptive arms of the immune system work synergistically not only to provide an effective host defence mechanism, but simultaneously maintain tolerance towards self-antigens (Chaplin 2010). Failure to maintain self-tolerance is often associated with development of autoimmune diseases such as systemic lupus erythematosus (SLE), thyroiditis, rheumatoid arthritis, and type 1 diabetes mellitus (Mackay 2000, Anderson and Mackay 2015). The immune system employs sophisticated, tightly regulated mechanisms to establish tolerance both in the central and peripheral lymphoid organs (Goodnow et al. 2005). These regulatory mechanisms include the generation of immune repertoires during B and T-cell differentiation in the central lymphoid tissues, and deletion and death of self-reactive lymphocytes by apoptosis (Goodnow et al. 2005, Nagata and Tanaka 2017). Weakly self-reactive lymphocytes may undergo further V(D)J (variable (V), diversity (D), and joining (J) gene segments) recombination or somatic hypermutation to induce receptor rearrangement and modifications to achieve tolerance (Luning Prak et al. 2011). Alternatively, the self-reactive lymphocytes can intrinsically undergo biochemical and gene-expression changes, known as clonal anergy. If there is any ‘leakiness’ of these mechanisms, the self-reactive lymphocytes will be extrinsically regulated, particularly through the limitation of essential growth factors, immunogenic costimuli, pro-inflammatory mediators and suppression by regulatory
T cells (T<sub>regs</sub>) (Goodnow et al. 2005). Previously, it was poorly understood how T<sub>regs</sub> come into play in maintaining tolerance. T<sub>regs</sub> were initially characterised as CD4<sup>+</sup> CD25<sup>+</sup> T cells, as depletion of this subset has been shown to induce multiple autoimmune disorders (Baecher-Allan and Hafler 2006). However, further analysis later revealed T<sub>reg</sub> cells also express Foxp3, a transcription factor essential for the suppressive phenotype of this T cell subset (Sakaguchi et al. 2010). FOXP3<sup>+</sup> T<sub>reg</sub> cells can avoid aberrant self-tolerance by regulating intrinsic and extrinsic immunoregulatory mechanisms and promoting suppression through a CTLA-4-dependent suppression mechanism (Sakaguchi et al. 2009, Yadav et al. 2013). However, the suppressive function of FOXP3<sup>+</sup> T<sub>reg</sub> cells is quite controversial due to the capacity of these lymphocytes to skew the immune system to favour tumour growth and progression through suppression of anti-tumour immune responses accompanied by promotion of angiogenesis (Sakaguchi et al. 2010, Facciabene et al. 2012). Indeed, accumulated data has demonstrated FOXP3<sup>+</sup> T<sub>reg</sub> cell infiltration in various stages of tumour progression (Woo et al. 2001, Curiel et al. 2004, Bates et al. 2006, Zou 2006, Facciabene et al. 2012), illustrating the dual roles of immune cell FOXP3<sup>+</sup> T<sub>reg</sub> both in tolerance and tumourigenesis.

Apoptosis is also instrumental in the establishment of self-tolerance (Vives-Pi et al. 2015, Nagata and Tanaka 2017). In the central lymphoid organs such as the thymus, thymocytes that express a high affinity for self-peptides or MHC molecules, or possess non-reactive T cell receptors (TCRs), will be selectively eliminated by apoptosis, induced by the activity of pro-apoptotic Bcl-2 family member Bim (Bouillet et al. 2002, Nagata and Tanaka 2017). This in turn drives T cell differentiation and maturation to produce pools of T cells that are more tolerogenic to self-antigens and able to elicit immune responses to foreign pathogens. In contrast, defective apoptosis during central tolerance leads to an autoimmune T lymphocyte population, which has been documented in various animal models to be one of the key players in the induction of autoimmune diseases (Van Parijs and Abbas 1998, Dornmair et al. 2003, Jiang and Chess 2009, Lee et al. 2012). For instance, autoimmune T cells mediate development of multiple sclerosis lesions by targeting myelin self-antigens, although more recent data suggest other effector cells also play important roles during different developmental stages of the disease (Stinissen et al. 1997, McFarland and Martin 2007, Severson and Hafler 2010). In addition, pathogenic T cell responses to insulin-producing β-cells in the pancreas lead to the autoimmune disease insulin-dependent diabetes mellitus, commonly known as type 1 diabetes mellitus (T1D) (Xu et al. 2013, Vives-Pi et al. 2015). Interestingly, various studies have also discovered the resulting autoimmune reaction in T1D to be associated with an excessive rate of β-cell apoptosis and defective efferocytosis (Trudeau et al. 2000, Mathis et al. 2001, Roggli et al. 2010). While the underlying mechanism is still unclear, the defective apoptotic machinery may
lead to secondary necrosis, which subsequently modulates the immune response to be more immunogenic rather than tolerogenic, illustrated by activated APCs, inflammation, and loss of self-tolerance (Vives-Pi et al. 2015).

1.4 Programmed cell death (PCD)

Programmed cell death (PCD) is an important homeostatic mechanism in all multicellular organisms. It serves an important role in various biological and physiological events including morphogenesis, cell cycle maturation, tissue remodelling and elimination of harmful cells (Bursch et al. 2000, Broker et al. 2005, Krysko et al. 2008, Sun and Peng 2009, Fuchs and Steller 2011). Dysregulated PCD associated with imbalanced cell proliferation and cell death is not only known as one of the cancer hallmarks, but it can also contribute to a number of developmental abnormalities and human disorders such as autoimmune diseases and AIDS (acquired immunodeficiency syndrome) (Thompson 1995, Baehrecke 2002, Xu et al. 2014, Zhang et al. 2016). Hence, it is vital to understand how the cell death execution machinery works, particularly the signalling pathways and key regulators involved, and how these define its immunological consequences, both in health and disease.

1.4.1 Mechanisms of programmed cell death

Previously, it was believed that all programmed cell death occurs only through the process of apoptosis (type 1 PCD) (Schwartz et al. 1993). However, recent developments in technical approaches have documented evidence of a number of different pathways associated with programmed cell death. In addition to apoptosis, these pathways include autophagy (type 2 PCD) and necroptosis (type 3 PCD) (Mizushima 2007, Peter 2011, Inoue and Tani 2014, Chan et al. 2015, Pasparakis and Vandenabeele 2015). These three are the major forms of PCD, characterised by distinct key initiators, specific mechanism of actions, and morphological differences (Fig. 1.3) (Krysko et al. 2008, Mizushima et al. 2008, Sun and Peng 2009, Peter 2011, Inoue and Tani 2014, Nunes et al. 2014). It is important to note that although each cell death mode is initiated by specific key molecules, not all cells induced by a similar stimulus will respond in a similar cell death mode (Elmore 2007). Hence, this indicates a real complexity and intricate interaction and possible crosstalk in all three PCD execution machineries, which can result in different immunological consequences.

Apoptosis is well-described as a highly orchestrated form of PCD (Elmore 2007, Nagata and Tanaka 2017). It is an energy-dependent cell death mode that can be induced through extrinsic and intrinsic signalling pathways, with an additional mechanism utilising T-cell mediated cytotoxicity and the perforin/granzyme pathway (Fig 1.4) (Elmore 2007, Voskoboinik et al. 2015). All three pathways are
regulated by caspases, a family of cysteine proteases, which converge on the same execution pathway, which is the executioner caspase 3. The extrinsic pathway is initiated through transmembrane receptor-mediated interactions, characterised by a transduction of apoptotic signals upon engagement of death receptors such as TNF receptor 1 (TNFR1), TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 or Fas to its corresponding ligands, TNFR, TRAIL and FasL (Ashkenazi and Dixit 1998, Suliman et al. 2001, Elmore 2007). Subsequent cascades involve the recruitment of cytoplasmic adaptor proteins such as Fas-associated death domain (FADD) and TNFR-associated death domain (TRADD) to their receptor death domains, leading to auto-catalytic activation of procaspase-8 to caspase 8. Caspase 8 cleaves the downstream effector caspase 3, which in turn induces apoptosis (Fig 1.4). The pathway can be inhibited by FADD-like apoptosis regulator (also known as FLIP) as this molecule can bind to procaspase 8, preventing its cleavage (Cuda et al. 2016).

In contrast to the extrinsic pathway of apoptosis, the intrinsic pathway is a non-receptor-mediated pathway. The extrinsic pathway is initiated by external stimuli such as DNA damaging agents, toxins, viral infection, withdrawal of growth factors, or absence of certain cytokines which lead to activation of intracellular apoptotic signals (Nagata and Tanaka 2017). These signals in turn induce activation of pro-apoptotic BAK and BAX that trigger mitochondrial outer membrane permeabilization (MOMP) and subsequently cause release of cytochrome c from mitochondrion into the cytosol (Fig. 1.4). Cytochrome c engages with apoptotic protease activating factor 1 (APAF1), resulting in apoptosome assembly, which in turn activates caspase 9. Activated caspase 9 induces activity of caspase 3, leading to apoptosis (Elmore 2007, Cuda et al. 2016, Ichim and Tait 2016). The intrinsic pathway is regulated by the members of the Bcl-2 family, and as such the commitment or abortion of apoptosis is heavily dependent on the balance between pro-apoptotic and anti-apoptotic Bcl-2 proteins (Ichim and Tait 2016).

On the other hand, the perforin/granzyme pathway has become the key pathway to mediate target cell death by cytotoxic lymphocytes such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (Fig. 1.4) (Elmore 2007). Upon induction of this pathway by cytotoxic lymphocytes, perforin forms large transmembrane pores on the surface membrane of target cell in the presence of Ca2+, enabling diffusion of granzymes into the cytosol. Pore-like membrane lesions can also form on cells targeted by antibody-dependent degranulation of NK cells. Subsequently, granzymes initiate apoptosis of the target cell (Voskoboinik et al. 2015). The loss of perforin is associated with the termination of granule-dependent target cell death in mice, illustrating the synergy of perforin and granzymes to execute apoptosis (Voskoboinik et al. 2015).
Execution of apoptosis is a tightly controlled process which includes shrinking of cytoplasm, extensive blebbing of the plasma membrane, chromatin margination at the nuclear membrane, and fragmentation of cells into smaller bodies (Fig. 1.3) (Kerr et al. 1972, Elmore 2007). The cell membrane integrity is maintained, hence all of these events are enclosed within an intact membrane. Scavenger cells such as macrophages rapidly phagocytose these apoptotic bodies and cells to ensure their intracellular contents including proteolytic enzymes, cationic proteins and oxidising molecules are not released into the surrounding tissues, a process called efferocytosis (Silva 2010, Elliott et al. 2017). This rapid removal by surrounding macrophages not only prevents secondary necrosis, but also inhibits the production of inflammatory cytokines by the engulfing cells. This is a very coordinated process to avoid induction of local inflammatory responses, and promotes immune tolerance to self-antigens, thus preventing autoimmunity (Saraste and Pulkki 2000, Savill and Fadok 2000, Kurosaka et al. 2003, Elmore 2007).

Cells undergoing necroptosis differ markedly from apoptosis (Fig. 1.3) (Nagata and Tanaka 2017). Necroptosis is a degenerative phenomenon, generally initiated by TNFA, FasL, or TRAIL, microbial infections and ischemic injury (Inoue and Tani 2014). It is characterised by highly ordered morphological changes including cell swelling, formation of cytoplasmic blebs and vacuoles, and distended endoplasmic reticulum. Mitochondria and lysosomes are ruptured, ribosomes are disaggregated, organelle membranes are disrupted and eventually these lead to lysing of the cell membrane (Kerr et al. 1972, Majno and Joris 1995, Trump et al. 1997). Lysis of the injured cells causes their intracellular contents to be released into the surrounding tissue, sending chemotactic signals to recruit inflammatory cells that amplify local inflammation (Krysko et al. 2008). The high mobility group box chromosomal protein 1 (HMGB1) molecules and DAMPs released by necrotic cells associate with DNA or immune complexes to activate dendritic cells and macrophages, in turn inducing secretion of pro-inflammatory cytokines. These events subsequently lead to inflammation and generation of rejective immune responses (Sims et al. 2010). These downstream events closely resemble the “classic” unregulated necrosis, however necroptosis cell death is discovered to be driven by distinct molecular pathways and is less inflammatory compared to necrosis. The upstream pathways are caspase-independent (Weinlich et al. 2017) and are mediated by a number of key regulators including Fas ligand (FasL), tumor necrosis factor (TNF) and TNF-related apoptosis-inducing ligand (TRAIL), receptor-interacting serine/threonine protein kinase 1 (RIPK1), and RIPK3 and its substrate MLKL (mixed lineage kinase domain-like) (Holler et al. 2000, Linkermann and Green 2014, Vanden Berghe et al. 2014). RIPK1, RIPK3 and MLKL form a complex known as the necrosome which plays a vital role in executing necroptosis (Pasparakis and Vandenabeele 2015).
Another form of PCD is autophagic cell death (Mizushima 2007, Lu and Walsh 2012), which is implicated in response to nutrient starvation and involved in a number of developmental processes and human diseases (Jiang and Mizushima 2014, Yin et al. 2016). Previous studies have reported roles of autophagy in the immune system, particularly in the clearance of intracellular pathogens and T cell activation and proliferation (Li et al. 2006, Lu and Walsh 2012). The term “autophagy” is derived from the Greek meaning “eating of self”, characterised by a conserved process that begins with the transient formation of a phagophore, which subsequently expands to engulf cytosolic components such as dysfunctional organelles, protein aggregates and ribosomes, and then matures to become an autophagosome (Fig. 1.3) (Mizushima 2007, Glick et al. 2010). This autophagosome docks and fuses with the lysosome or vacuole, forming an autolysosome, where the cargo degradation and recycling occur assisted by resident hydrolases (Yin et al. 2016). These processes through autophagic cell death machinery are complex and are regulated by the mechanistic target of rapamycin complex 1 (MTORC1), phosphatases and guanosine triphosphatases (GTPases) (Elmore 2007, Yin et al. 2016).

### 1.4.2 Secondary necrosis as an alternative outcome of apoptosis

Apoptosis is an active, genetically controlled process, characterised by profound cellular morphological changes occurring within an intact membrane. Modifications in cell surface molecules and release of a number of soluble factors by apoptotic cells including triphosphate nucleotides, the chemokine CX3CL1, sphingosine-1-phosphate (S1P) and lipid lysophosphatidylcholine (lysoPC) prompt a “find-me” signal, which facilitates the recruitment of phagocytes (Ravichandran 2010, Elliott et al. 2017). During this phase, the apoptotic cells begin to transduce multiple “eat-me” signals such as the exposure of the plasma membrane inner leaflet phospholipid phosphatidylserine (PtdSer) and calreticulin, modifications of glycosylation patterns, and alteration of intercellular adhesion molecule 1 (ICAM-1) epitopes, to initiate efferocytosis by macrophages (Fig. 1.4) (Ravichandran 2010, Segawa and Nagata 2015). Efferocytosis is vital not only to ensure effective clearance of apoptotic cells and bodies, but also to prevent leakage of intracellular contents that can ultimately contribute to inflammation and autoimmunity (Korns et al. 2011). Therefore, the efferocytic ability of macrophages may play a key role in indirectly shaping the mode of cell death and the ensuing immunological consequences.

Since the early studies of apoptosis by Kerr et al. (1972), the phagocytic clearance of apoptotic cells and cellular debris (efferocytosis) has been predominantly described as the natural outcome of the apoptotic cascade (Kerr et al. 1972). However, given the heterogeneity of the cellular microenvironment during apoptosis, rapid macrophage-mediated phagocytosis can be disrupted and
consequently, apoptotic cells may succumb to secondary necrosis as the alternative outcome of the complete apoptotic program (Silva 2010) (Fig. 1.4). Indeed, in vivo studies in mice with defects in macrophage efferocytic capability have observed both inefficient clearance of apoptotic cells and an accumulation of secondary necrotic cells (Scott et al. 2001, Cohen et al. 2002). Previous mouse studies utilising combined treatments of endotoxin lipopolysaccharide (LPS) and phosphatidylserine-containing liposome, known to inhibit macrophage phagocytosis of apoptotic cells (Fadok et al. 1992), showed elevated level of necrosis which is believed to be secondary to apoptosis due to the ineffective phagocytic clearance mechanism (Medan et al. 2002). Apoptotic cells that are not cleared by phagocytosis become secondary necrotic cells which share many similar features of primary necrotic cells and necroptotic cells (Kryska et al. 2006, Vanden Berghe et al. 2010). Indeed, a comparative study of necroptosis, necrosis and secondary necrosis using high-resolution time-lapse imaging reveals that these three necrotic cell death modes converge on identical subcellular events including lysosomal membrane permeabilisation, oxidative burst, mitochondrial hyperpolarisation and plasma membrane permeabilisation, occurring with different kinetics (Vanden Berghe et al. 2010). Oxidative burst and subsequent leakage of intracellular contents lead to release of damage-associated molecular patterns (DAMPs) and nucleosomes associated with HMGB1 into the extracellular space, inducing production of inflammatory cytokines. Similar to necrosis and necroptosis, secondary necrosis also triggers inflammation (Sims et al. 2010).

Taken together, these different cell death pathways determine the nature of the subsequent immune responses initiated, and can result in either immune tolerance or localised inflammation. Given the importance of efferocytosis during cell death, modulation of macrophage efferocytic ability and subsequent secondary necrosis may have relevant medical implications to potentially treating disorders associated with chronic inflammation, autoimmunity and reducing susceptibility to tumour initiation.

1.4.3 Cell death during mammary involution and regression phase

Proliferation and apoptosis of mammary epithelial cells are observed during the estrous cycle of rats and mice (Andres and Strange 1999, Schedin et al. 2000, Fata et al. 2001) and the menstrual cycle in humans (Ferguson and Anderson 1981, Olsson et al. 1996, Navarrete et al. 2005) and are critical for normal mammary gland development and maintenance of homeostasis. These processes occur under the influence of fluctuating ovarian hormones and growth factors as well as changes in cell survival signals. The process of menstrual cycle-associated regression shares similar mechanisms to mammary gland involution – both involve apoptosis of alveolar epithelium and tissue remodelling to return the gland to its basic architecture (Fata et al. 2001, Watson and Kreuzaler 2011). Whilst
both regression and involution are equally important in mammary gland tissue homeostasis, much effort has been directed towards defining the complex process of mammary gland involution (Clarkson et al. 2004, Stein et al. 2007, Sutherland et al. 2007, Watson 2009, O’Brien et al. 2012, Sargeant et al. 2014). Less well understood is the process of mammary gland regression during the menstrual cycle.

Studies on apoptosis of mammary epithelial cells commonly utilise a forced weaning involution model which includes two phases; (1) early, localised and reversible changes that are associated with cell death that occurs in response to physical stress on epithelial cells and accumulation of unsecreted milk protein, and (2) a more global and irreversible response of cell death due to detachment from the extracellular matrix (Lund et al. 1996, Li et al. 1997, Green and Streuli 2004, Watson 2006). The first phase, which lasts approximately 48 hours and can be reversed through re-introduction of suckling pups, is associated with elevated levels of pro-apoptotic factors and downregulated survival factors (Green and Streuli 2004). Studies using knockout mouse models of p53, Stat3, Tgfβ3, c/ebpβ and Vdr exhibited a delayed involution process along with reduced apoptosis during this first phase, indicating the roles of these genes and transcription factors in the cell death associated with mammary involution (Stein et al. 2007). In response to accumulated milk in the mammary gland, signalling pathways including the Tnf/ death receptor pathway, the Lif-receptor/Jak-Stat pathway, and the Vitamin (D)3 receptor pathway are activated, inducing apoptosis either through reduction of survival factor pAkt or direct induction of apoptosis (Stein et al. 2007).

On the other hand, the non-reversible, second phase is associated with a more global apoptosis and tissue remodelling, and requires different systemic factors and proteases (Green and Lund 2005, Stein et al. 2007). Although the multi-step process of mammary involution is tightly regulated, the rapid and very extensive tissue remodelling creates a highly reactive microenvironment, which shows characteristics similar to those of wound healing and tumour development (van Kempen et al. 2003, Clarkson et al. 2004, Schedin et al. 2007). Any impairment or deregulation of the mammary involution machineries or the cell death pathways may become a stimulus for tumour initiation and development. Indeed, resistance to apoptosis is one of the hallmarks of cancer (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011). In addition, a number of studies have also demonstrated a relationship of delayed involution with an increased risk of breast cancer and mammary tumour growth and progression (Ackler et al. 2002, Milanese et al. 2006, Radisky and Hartmann 2009).
Clearly, cell death pathways play essential roles in directing normal mammary gland involution. Ovarian cycle-associated regression shares similar features to involution, such as induction of apoptosis and tissue remodelling, and it is proposed that any alterations and deregulation of these pathways at this phase may also affect breast cancer risk. Indeed, previous studies utilising rats challenged with chemical carcinogens at proestrus are associated with increased risk of breast cancer (Nagasawa and Yanai 1974, Nagasawa et al. 1976, Lindsey et al. 1981, Ratko and Beattie 1985). A full investigation on the cell death pathways during the regression phase will provide insight on how this can be potentially modulated to prevent breast cancer.

1.4.4 Detection of apoptosis

Given the importance of apoptosis in homeostasis, cellular development, and pathogenesis of different diseases (O'Reilly and Strasser 1999, Elliott and Ravichandran 2010, Nagata et al. 2010), tremendous progress has been made in the past decade in developing appropriate tools to detect and study apoptosis. Archana and colleagues have reviewed multiple techniques available to detect apoptotic cells including the advantages and limitations of these techniques (Archana et al. 2013). These include light microscopy and electron microscopy that can assess morphological and subcellular changes during apoptosis, gel electrophoresis, flow cytometry, in situ-end labelling method and immunochemical detection of apoptotic cells. The detection and quantification of apoptosis in breast tissue through morphological assessment using light microscopy or electron microscopy examine the presence of nuclear fragmentation, chromatin aggregation, cell shrinkage and blebbing of the plasma membrane (Cohen et al. 1992, Kerr et al. 1994, Elmore 2007). Although this method provides a useful outcome, it is technically challenging and time consuming. The TUNEL assay (terminal deoxynucleotidyl transferase mediated dUTP nick end labelling), one of the in situ-end labelling methods, has been developed to simplify the process of identifying apoptotic cells. It is a sensitive approach, relying on the incorporation of labelled nucleotides on to the end of both double- and single-stranded DNA fragments that occur during apoptosis (Gavrieli et al. 1992, Mundle et al. 1995). However, for years, there has been a debate about the sensitivity and accuracy of this assay due to problems associated with inappropriately labelled necrotic cells identified by the assay as apoptotic. In addition, the specificity and sensitivity of TUNEL assay may also be affected depending on the fixative used, the pre-treatment steps and the concentration of terminal transferase enzyme (Mundle et al. 1995, Archana et al. 2013). Improvements have been made and the new assay should only identify cells in the last stage of apoptosis if performed correctly (Negoescu et al. 1996, Negoescu et al. 1998). Apoptosis can be a rapid process, with dying cells cleared by phagocytic macrophages. This presents a considerable challenge to investigating apoptosis in the...
mammary gland, as the TUNEL assay can only give a snapshot of the whole process and does not identify those cells which have already been removed from the tissue.

DNA fragmentation in cells undergoing apoptosis can also be studied using the gel electrophoresis technique (Oberhammer et al. 1993, Higuchi 2004, Shaker and Melake 2012). Although this technique offers precise determination of cell death and DNA damage, it is often tedious, involving multiple steps and generally providing qualitative results rather than quantitative (Archana et al. 2013). Hence, flow cytometry has been developed to provide another alternative approach in accurately quantifying apoptosis and distinguishing apoptotic from non-apoptotic cells. It enables simultaneous analysis of the physical and chemical characteristics of each cell, hence it is a powerful method to examine various aspects of apoptosis induced by different apoptotic agents (Archana et al. 2013). Immunohistochemical analysis, on the other hand, utilises antibodies against different key apoptotic markers such as caspases 3, p53, Annexin V, and M30 (Archana et al. 2013). It is a useful method, enabling examination of apoptosis at the single cell level and at cell-to-cell contact sites. However, the current direct and indirect immunohistochemical detection methods are quite time consuming due to the multiple steps involved. This may lower the sensitivity, thus, leading to poor outcomes. In addition, a careful selection of reagents is important to eliminate background staining (Archana et al. 2013, Hofman and Taylor 2013).

Utilisation of different detection techniques not only enables quantitative and qualitative analysis of apoptosis in different experimental conditions, but these can also be used to validate observations obtained with other approaches. Hence, employment of these different detection techniques allows extensive investigation of various stages and key markers of apoptosis, which ultimately leads to a better understanding of apoptotic cell death mode.

1.5 Complement System
The complement system is primarily considered an important effector of both innate and antibody-mediated acquired immune system responses. It was discovered many years ago from some of the earliest observations of complement activity – a heat-sensitive component of serum that ‘complemented’ the ability of antibodies to kill bacteria (Janeway et al. 2001). Complement plays a major role in innate immunity by generating opsonins, the molecules that ‘tag’ apoptotic bodies to enhance the ability of macrophages and neutrophils to phagocytose them. Complement also stimulates production of anaphylatoxins, peptides that induce local and systemic inflammatory responses, and complement can directly kill pathogens (Janeway et al. 2001). Complement can be activated through three different pathways; the classical, mannose-binding lectin, and alternative
pathways (Fig. 1.5). The initiation of each pathway involves distinct recognition events and components, but these converge such that the later stages of all three pathways utilise the same components (Fig. 1.5) (Fujita et al. 2004, Zipfel and Skerka 2009, Dunkelberger and Song 2010).

1.5.1 The complement pathways

Activation of the classical complement system predominantly begins with formation of the antibody-antigen complex (immune complex); thus this pathway is a major effector pathway of the humoral adaptive immune response. The formation of this complex induces conformational changes in the Fc region of immunoglobulin, allowing complement protein C1 to bind. C1 is a complex of three different proteins, containing six identical subunits of the C1q subunit, combined with two subunits of both C1r and C1s. C1q binding to the antibody-antigen complex subsequently causes C1s to be enzymatically active. The next events of this pathway include cleavage of complement components C4 and C2 by C1s esterase, leading to formation of C4b2a complex known as the classical pathway C3 convertase (Janeway et al. 2001). This enzyme cleaves the next component in the pathway, C3, to generate C3a and C3b (Fig. 1.5).

The mannose-binding lectin pathway is part of the innate immune defence as it is activated without the presence of antibody. This pathway is activated by binding of the mannose-binding lectin (MBL) or ficolin-C1s serine proteases complex (MASPs) to terminal mannose residues of proteins and common carbohydrate structures found on microorganisms (Matsushita and Fujita 1992, Lu et al. 2002, Fujita et al. 2004). The binding of these MASPs sequentially cleaves the classical component pathway components C4 and C2 to form C4b2a, the classical pathway C3 convertase, on the surface of the bacterium. Thus, the lectin pathway converges with the classical pathway at the activation of C3 (Janeway et al. 2001, Zipfel and Skerka 2009, Dunkelberger and Song 2010).

The alternative pathway of the complement system is initiated by almost any foreign substance. This pathway is not triggered by antibody; therefore it is an effector arm of innate immune defence. Components of the alternative pathway include serum factor B and plasma protease factor D which both react to C3b and C3bB respectively to form C3 convertase (Janeway et al. 2001). These three distinct pathways illustrate the complexity and flexibility of complement networks, making it one of the key players in the immune system.

1.5.2 The terminal pathway

Early stages of complement activation involve a sequential cascade of events which produce successive components. The activation of one component activates its enzymatic function that triggers the activation of the next component in the sequence, amplifying a relatively small initiating
As previously mentioned, the initiation of each pathway involves unique events and components, but the later stages use the same components. C3 cleavage plays a critical step in the later stage of each complement pathway. C3 convertases cleave C3 into smaller fragments, leading to a cascade in production of C5b, C6, C7, and C9 components that terminally form a membrane attack complex (MAC) in the membrane of the injured or apoptotic cells (Petry et al. 2001, Walport 2001, Kim et al. 2003, Zipfel and Skerka 2009, Dunkelberger and Song 2010). The deposition of MAC causes the apoptotic cells to lyse, forming apoptotic bodies that will be rapidly phagocytosed by macrophages, hence promoting tolerance to self-antigens and avoiding autoimmunity (Roos et al. 2004).

1.5.3 Complement C1q as regulator of inflammation and autoimmunity

C1q plays a critical role in initiating classic macrophage-mediated phagocytosis. Structurally, C1q is a 460 kDa hexameric molecule which is composed of six distinct subunits; each subunit has six A (233 residues; 34kDa), B (226 residues; 32 kDa) and C (217 residues; 27kDa) chains. The globular head regions of C1q are capable of binding to a broad range of molecular structures and ligands including apoptotic cells, antibody-antigen complexes and receptors such as calreticulin-CD91 and integrin αβ1 (Sellar et al. 1991, Kishore and Reid 2000, Kishore et al. 2004). Binding of C1q, the recognition component of C1, to antibody-antigen complexes (immune complexes) activates the C1q-associated proteases, C1r and C1s, to activate the events that ultimately lead to formation of MAC in the membrane of the injured or apoptotic cells (Fig. 1.5) (Petry et al. 2001, Walport 2001, Kim et al. 2003). If these apoptotic cells are not efficiently cleared, the programmed cell death pathway continues into secondary necrosis, which resembles necrosis (Fig. 1.4) (Sims et al. 2010). The necrotic cells send danger signals such as high mobility group box chromosomal protein 1 (HMGB1) to induce antigen presenting cells, particularly “M1” macrophages to phagocytose the cells. This is followed by migration of the macrophages to the lymph node and presentation of antigens to T cells. Rather than promoting tolerance, these events generate rejective adaptive immune responses (Sims et al. 2010, Tang et al. 2011).

The classical complement system is also activated when C1q binds to various ligands and receptors including soluble secreted C-reactive protein (CRP), serum amyloid P-component (SAP), and pentraxin (PTX3), and transmembrane receptors such as CD35, CD91, and CD93 (C1qRp) (Tarr and Eggleton 2005, Lu et al. 2008, Duus et al. 2010). C1q and PTX3 both can bind to apoptotic cells, but each interacts at different domains. Interestingly, PTX3 can inhibit C1q-induced complement activation by decreasing C1q deposition and hence affecting the removal of apoptotic cells by APCs (Baruah et al. 2006). C1q interacts with calreticulin (CRT, also known as cC1qR) and is subsequently
associated with CD91 to promote apoptotic cell ingestion by macrophages (Ogden et al. 2001). Opsonisation by C1q and mannose-binding lectin enhance apoptotic cell uptake not only by macrophages, but also by dendritic cells (Nauta et al. 2004). Taken together, direct and indirect interaction of C1q and these ligands and receptors exert different consequences, hence suggesting a broader regulatory function of C1q.

Hereditary deficiency of complement components is linked to increased susceptibility to development of autoimmune disease (Botto and Walport 2002, Sturfelt and Truedsson 2005, Lewis and Botto 2006). In particular, autoimmune diseases have been associated with a deficiency in the early components of the complement pathways such as C1q and C4 (Meyer et al. 1985, Bowness et al. 1994, Pickering et al. 2000, Yang et al. 2004). Genetic deficiency in C1q has long been associated with the development of the autoimmune disease systemic lupus erythematosus (SLE), with impaired clearance of apoptotic cells and nearly 100% penetrance (Walport et al. 1998, Botto and Walport 2002, Sontheimer et al. 2005). Mice genetically deficient in C1q exhibit an increase of apoptotic bodies and development of glomerulonephritis (Botto et al. 1998). These findings suggest that autoimmunity in the setting of C1q deficiency could be due to a reduced ability to opsonise apoptotic bodies and inefficient clearance of dying cells, leading to inappropriate immune system responses and the development of self-reactive immune cells.

1.5.4 Complement and tumourigenesis

The complement system has been well-described as an innate effector against tumour cells. A number of studies have reported complement activation in cancer patients (McConnell et al. 1978, Niculescu et al. 1992, Lucas et al. 1996, Bu et al. 2007). In vitro studies using cancer cell lines have reported increased C5 deposition and generation of active product C5a in lung cancer cells compared to the non-malignant bronchial epithelial cells (Corrales et al. 2012). Elevated complement activity is also detected in bodily fluids of cancer patients (Nishioka et al. 1976, Maness and Orengo 1977, Ytting et al. 2004, Bjorge et al. 2005). However, dating back as early as 1975, studies have also demonstrated the promotion of cancer growth by complement activation (Shearer et al. 1975). The ability of complement to enhance tumour growth in mouse models has been described including the capacity of complement component C5a to recruit neutrophil-like myeloid-derived suppressor cells (PMN-MDSCs) to tumours, followed by production of reactive oxygen species (ROS) that can inhibit the anti-tumour CD8+ T cell response (Markiewski et al. 2008). Blockade of C5a receptor resulted in a reduction in myeloid-derived suppressor cells and immunomodulators ARG1, CTLA-4, IL6, IL10, LAG3, and PDL1 (B7H1) (Corrales et al. 2012), and impairment of tumour growth, suggesting C5a contributes to lung and cervical cancer (Markiewski et al. 2008). In addition, a
reduction in tumour growth is observed in mice deficient in C3, suggesting the presence of complement component C3 can promote tumour progression (Markiewski and Lambris 2009). Selective inhibition of complement factor C5a impaired ovarian cancer in mice (Nunez-Cruz et al. 2012). A number of studies have reported reduced risk of cancer incidence including breast, endometrial and ovarian cancer in patients with autoimmune disease (Hemminki et al. 2008, Smitten et al. 2008, Hemminki et al. 2012). SLE patients genetically deficient in C1q have a substantially decreased risk of developing breast and endometrial cancer (Bernatsky et al. 2013). Hence, these findings implicate complement in tumourigenesis.

The roles of complement in tumourigenesis might be complicated, but it is entirely consistent with the cancer editing theory. This cancer immunoediting process involves three sequential phases; elimination (immune surveillance), equilibrium, and escape (Fig. 1.2) (Swann and Smyth 2007, Pandya et al. 2016). Mutated cells may avoid elimination by taking advantage of complement-mediated recruitment of suppressor cells and inhibition of anti-tumour CD8+ T cell responses. If tumour cells survive the elimination phase, they enter an equilibrium state during which surviving cells remain dormant but may acquire genetic and epigenetic abnormalities (Schreiber et al. 2011). These findings propose a new perspective of the complex roles of complement in cancer development and suggest there may be opportunities to reduce cancer risk through targeted anti-complement therapies.

1.6 Conclusion

There is substantial evidence that implicates macrophages in development and remodelling of the mammary gland during puberty, pregnancy, lactation and involution. Macrophages act as key effector cells in promoting development and regression of the mammary gland during the ovarian cycle, where their activity might affect immune responses to tumour formation. Rapid removal of dying epithelial cells by macrophages during mammary gland regression may be facilitated by complement C1q opsonisation which promotes self-tolerance and prevents autoimmunity. In the presence of C1q, rejective immune responses are not initiated, which may result in an immune environment skewed in favour of tumourigenesis. Subsequently, the risk of breast cancer increases with the regression phase of each menstrual cycle. However, whether the immune system limits or promotes tumourigenesis appears to depend on the balance between “M1” and “M2” macrophage phenotypes. The significance of this is still poorly understood in the setting of normal mammary gland tissue homeostasis and the implications for mammary tumour development. Moreover, the role of C1q in normal mammary gland development and in shaping macrophage phenotypes and downstream immune responses is unknown. Further studies that dissect the role of C1q in
macrophage function, ovarian cycle-associated mammary gland regression, and in mammary tumour initiation, development and progression could assist development of therapeutic interventions to reduce breast cancer risk in women.
1.7 Hypotheses and Aims

We hypothesise that C1q is a key complement protein that promotes rapid macrophage-mediated phagocytosis during the regression phase of the ovarian cycle, but as a consequence also promotes an immune tolerant, pro-tumourigenic environment. Therefore, if C1q-mediated phagocytosis is disrupted, apoptotic cells undergo secondary necrosis, which generates rejective immune responses that better protect the tissue from tumourigenesis. It is hypothesised that mice genetically deficient in C1q will exhibit impaired clearance of dying cells in the mammary gland, an anti-tumourigenic immune environment, and reduced tumour initiation and development.

The experiments described in this thesis address the following aims:

1. To explore the roles of C1q in normal mammary gland development and function.
2. To investigate the effect of C1q deficiency on the mammary gland immune microenvironment and cancer risk using the carcinogen DMBA model.
3. To investigate the effect of C1q deficiency on the development of mammary gland tumours using transgenic cancer model MMTV-PyMT.
Table 1.1 Estimated 20 most commonly diagnosed cancers in Australian women, 2014.

Breast cancer is the most common invasive cancer and is the leading cause of cancer related death in Australian women (adapted from Cancer in Australia: an overview 2014).

<table>
<thead>
<tr>
<th>Site/type</th>
<th>Cases</th>
<th>Percent</th>
<th>ASR^</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>15,270</td>
<td>27.4</td>
<td>114.5</td>
</tr>
<tr>
<td>Colorectal</td>
<td>7,340</td>
<td>13</td>
<td>51.5</td>
</tr>
<tr>
<td>Melanoma of skin</td>
<td>5,210</td>
<td>9</td>
<td>39.4</td>
</tr>
<tr>
<td>Lung</td>
<td>4,720</td>
<td>8</td>
<td>33.2</td>
</tr>
<tr>
<td>Uterus</td>
<td>2,490</td>
<td>4</td>
<td>17.9</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>2,430</td>
<td>4</td>
<td>17.9</td>
</tr>
<tr>
<td>Thyroid</td>
<td>1,890</td>
<td>3</td>
<td>15.4</td>
</tr>
<tr>
<td>Leukaemia</td>
<td>1,440</td>
<td>3</td>
<td>10.4</td>
</tr>
<tr>
<td>Ovary</td>
<td>1,430</td>
<td>3</td>
<td>10.5</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1,410</td>
<td>3</td>
<td>9.7</td>
</tr>
<tr>
<td>Unknown primary site</td>
<td>1,210</td>
<td>2</td>
<td>7.8</td>
</tr>
<tr>
<td>Head and neck</td>
<td>1,160</td>
<td>2</td>
<td>8.4</td>
</tr>
<tr>
<td>Kidney</td>
<td>1,060</td>
<td>2</td>
<td>7.8</td>
</tr>
<tr>
<td>Cervix</td>
<td>865</td>
<td>2</td>
<td>7.0</td>
</tr>
<tr>
<td>Stomach</td>
<td>785</td>
<td>1</td>
<td>5.4</td>
</tr>
<tr>
<td>Brain</td>
<td>740</td>
<td>1</td>
<td>4.3</td>
</tr>
<tr>
<td>Myeloma</td>
<td>700</td>
<td>1</td>
<td>4.9</td>
</tr>
<tr>
<td>Bladder</td>
<td>675</td>
<td>1</td>
<td>4.5</td>
</tr>
<tr>
<td>Myelodysplastic syndrome</td>
<td>490</td>
<td>1</td>
<td>3.3</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>455</td>
<td>1</td>
<td>3.1</td>
</tr>
<tr>
<td><strong>All cancers combined</strong></td>
<td><strong>55,660</strong></td>
<td><strong>100</strong></td>
<td><strong>406.2</strong></td>
</tr>
</tbody>
</table>

* Includes cancer coded in ICD-10 as C00-C97, D45, D46, D47.1 and D47.3 with the exception of those C44 codes that indicate a basal or squamous cell carcinoma of the skin

^ ASR, age-standardised rate. The rates were standardised to the Australian population as at 30 June 2001 and are expressed per 100,000 population
The mammary gland undergoes development and regression over the course of the mouse estrous cycle. Circulating progesterone concentration peaks at diestrus, and circulating estradiol concentration is highest at estrus. The rise in progesterone promotes ductal epithelial cell proliferation and differentiation into alveolar buds during metestrus and diestrus. Following the fall in circulating progesterone, alveolar buds undergo apoptosis and the gland remolds to its basic architecture. E₂, estradiol; P₄, serum progesterone.
Figure 1.2 Schematic illustration of the immunoediting theory incorporating different roles of the immune system in tumourigenesis

Illustration of the fundamental roles of the immune system that both protect the host against tumour and promote tumour growth. Tumour suppression mechanisms are facilitated by effector functions of different immune cells including T cells, natural killer cells (NK cells), natural killer cells (NKT cells), gamma-delta T cells (γδ T cells), and macrophages. This is known as the elimination phase of the cancer immunoediting process during tumour immunosurveillance. Partial elimination of mutated cells results in selection of cells to transit to the equilibrium phase, where they remain dormant or undergo further genetic modifications enabling them to acquire abilities to become resistant to escape anti-tumour immune responses.
Transformed

Innate and adaptive immunity

CD4+ T cell  "M1" macrophage
CD8+ T cell  NKT cell
NK cell  γδ T cell

Evasion mediated by immunosuppressive mechanisms and immunological tolerance

Genetic instability and/or immune selection

Tumour persistence

Protection

ELIMINATION  EQUILIBRIUM  ESCAPE

Tumour cells resistant to immunosurveillance

Normal

Tumour cells

Noor Din
Figure 1.3 Schematic representation of three major forms of programmed cell death

The three major pathways of cell death comprising apoptosis, necroptosis and autophagy. These pathways are triggered by a plethora of stimuli and are characterised by tightly regulated and specific mechanisms of action and morphological differences, hence leading to distinct immune responses initiated. Key morphologic features of apoptosis include membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation and generation of membrane-enclosed fragments called apoptotic bodies. These apoptotic bodies are efficiently eliminated by phagocytes such as macrophages. In contrast to apoptosis, cells that die through necroptosis lose membrane integrity, releasing their intracellular contents, hence inducing inflammation. Autophagy, commonly triggered by nutrient starvation, leads to recycling of the internal materials of the cell. DAMPs, damage-associated molecular patterns; FasL, Fas ligand; HDAC, histone deacetylase; HMGB1, high mobility group box chromosomal protein 1; TNFA, tumour necrosis factor alpha; TRAIL, TNF-related apoptosis-inducing ligand.
Noor Din

Viable cell

Membrane blebbing

Cellular shrinkage

TNFA, FasL, or TRAIL, infectious pathogens

Further cell shrinkage

Nuclear compaction (pyknosis)

Apoptotic bodies

“Eat-me”, “Find-me” signals

Nuclear fragmentation (karyorrhexis)

APOPTOSIS

Cell swelling

Influx of fluid

Leakage of intracellular contents (DAMPs)

APOPTOSIS

Viable cell

Nucleus

Nutrient starvation, HDAC inhibitors, hypoxia, infectious pathogens

TNFA, FasL, or TRAIL, microbial infections, ischemic injury

Cell membrane

Autophagosome

1. Phagophore

Cytoplasmic components

2. Autophagosome

Docking and fusion

3. Lysosome

4. Autolysosome

Cytoplasm

Leakage of intracellular contents (DAMPs)

HMGB1

HMGB1

NECROPTOSIS

 bahia

Autophagy

 (Adapted from Nunes 2014, Inoue and Tani 2014)
Figure 1.4 Schematic overview of apoptotic events and the outcomes

The pathways of apoptosis comprising the extrinsic, intrinsic, and perforin/granzymes pathways. Each pathway requires induction by specific stimuli, followed by a cascade of energy-dependent molecular events including activation of initiator caspases (caspase 8, 9, 10) and executioner caspase 3. The extrinsic and intrinsic pathways can be negatively regulated by FLIP and molecules of Bcl-2 family, respectively. Complement component C1q can directly bridge apoptotic epithelial cells to macrophages by recognising surface “eat-me” signals expressed such as phospholipid phosphatidylserine (PtdSer) and calreticulin (CRT), or indirectly through antigen-antibody complex to promote rapid clearance. Macrophage-mediated phagocytosis efficiently eliminates dying cells and apoptotic bodies without inducing inflammation. Alternatively, in the absence of scavenger cells, apoptosis may progress to autolytic secondary necrosis as another alternative of the natural outcome of the complete apoptotic program, characterised by cellular disintegration and release of intracellular components. Ag, antigen; APAF1, apoptotic protease activating factor 1; Ca$_2^+$, calcium; C1qR, C1q receptor; FLIP; FADD-like apoptosis regulator; Ig, immunoglobulin; MOMP, mitochondrial outer membrane permeabilization.
**Death ligands**

**Death receptors**

**Adaptor proteins**

**FLIP**

**Caspase 8**

**Caspase 9**

**Caspase 10**

**Caspase 3**

**Caspase 10**

**Caspase 9**

**Cytochrome c**

**Apoptosome**

**Bak**

**Bax**

**Bcl-2 family members**

**Mitochondrial changes**

**MOMP**

**DNA damaging agents, toxins, viral infection etc.**

**Cytotoxic lymphocyte**

**Perforin pore**

**Perforin/Granzyme pathway**

**Granzymes**

**Cytotoxic**

**Macrophage**

**Macrophage-mediated apoptosis**

**No clearance, delayed uptake by scavenger cells**

**Release of intracellular contents**

**Secondary necrosis**

(Adapted from Elmore 2007, Silva 2010, Merle et al. 2015)
The complement system comprises the classical, mannose-binding lectin (MB-lectin), and alternative pathways. All three pathways are activated by unique events and distinct stimuli, which subsequently converge towards the activation of component C3. Cleavage of C3 generates smaller fragments including C3a and C3b, leading to formation of the C5 convertase. C5 convertase cleaves C5 to become split products C5a and C5b. Sequentially, C5b associated with C6, C7, C8 and C9 to assemble the membrane attack complex (MAC). Terminal formation of MAC pores results in cell lysis, subsequently forming apoptotic bodies that will be rapidly phagocytosed by macrophages.
(Adapted from Janeway et al. 2001)
Chapter 2 - Materials and Methods
2.1 Animals and General Procedures

2.1.1 Mice
All animal experiments were approved by the University of Adelaide Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition, 2004). All mice were maintained in specific pathogen-free conditions with controlled light (12 hours light, 12 hours dark cycle) and temperature at the Laboratory Animal Services, University of Adelaide Medical School. Throughout the study, food and water were provided ad libitum.

2.1.2 Mouse models

2.1.2.1 C57BL/6 mice
Adult virgin female C57BL/6 mice (8-10 weeks of age) were obtained from Laboratory Animal Services, University of Adelaide.

2.1.2.2 C1q null mutant mice
C1q null mice were originally generated by homologous recombination disrupting the coding region of the first exon of the C1qa gene with a neomycin-resistance cassette (Botto et al. 1998). A colony of C1q null mutant mice on a C57BL/6 background were established from 3 breeder pairs kindly provided by Professor Michael Brown (Experimental Therapeutics Laboratory, Hanson Institute, Adelaide). C1q \(-/-\) mice are fully viable, fertile and show no overt signs of impaired mammary gland development, lactation and involution as they are able to feed successive litters (unpublished observation). C1q \(-/-\) mice exhibit an increased susceptibility to development of autoimmunity (Lu et al. 2008) and accelerated rejection to skin and cardiac allografts (Csencsits et al. 2008). These are consistent with the role of C1q in promoting self-tolerance. Mice deficient in C1q also exhibit increased abundance of antigen specific CD8-T cells (Baruah et al. 2010).

2.1.2.3 Mammary cancer mouse models
These studies employed a chemical carcinogen model and transgenic model to investigate mammary tumour susceptibility, development and progression. 7,12-Dimethylbenz[a]anthracene (DMBA) is a chemical carcinogen that causes DNA damage and tumour formation (Medina and Warner 1976). Weekly administration of 1 mg of DMBA for six weeks by oral gavage results in tumours in 50% of C57BL/6 mice with an average latency of 12 weeks (Ip and Asch 2000).
The polyomavirus middle T antigen (PyMT) is an oncogene, driven by the mouse mammary tumour virus (MMTV) promoter resulting in mammary gland tumours with 100% penetrance and average latency of 12 weeks in C57BL/6 mice. MMTV-PyMT mice on a FVB inbred background were kindly provided by Professor William Muller (Guy et al. 1992) and were back-crossed for 12 successive generations onto an inbred C57BL/6 background.

2.1.2.4 Generation of PyMT\(^+\)/C1q\(^-\) mouse cohort
Male PyMT\(^+\) (C57BL/6 strain) mice were mated with C1q\(^-\) females (C57BL/6 strain). Pups were weaned at 21 days, and genotyped. Male offspring with the genotype of PyMT\(^+\)/C1q\(^+/\) were mated with C1q\(^-\) females to generate the PyMT\(^+\)/C1q\(^-\) mice used in this study.

2.1.2.5 Mammary gland regression model
An ovariectomised hormone replaced mouse model was used to investigate the role of C1q during the regression phase of the ovarian cycle. This model was developed by Hodson et al. (2013) to mimic the development and regression that occurs in the mammary gland during the ovarian cycle. In the model, early alveolar development is induced with administration of exogenous progesterone and estradiol, and alveolar regression is induced through administration of exogenous progesterone and estradiol followed by a progesterone receptor antagonist. The hormone replacement regimen to induce regression is illustrated in Figure 2.1.

To induce alveolar regression, 12 week-old mice were ovariectomised (as described in 2.1.4) and allowed to recover from surgery for 7 days. Mice were then administered a combination of 17β-estradiol, E\(_2\) (1 µg) (Sigma-Aldrich, St Louis, USA) and progesterone, P\(_4\) (1 mg) (Sigma-Aldrich, St Louis, USA) in 100 µL sesame oil (Sigma-Aldrich, St Louis, USA). This combination of exogenous progesterone and estradiol was administered subcutaneously once per day for 3 days, and promotes mammary gland alveolar development (Aupperlee and Haslam 2007). Twenty four hours after the final injection of progesterone and estradiol, mice were injected subcutaneously with the progesterone receptor antagonist RU486 (also known as mifepristone) (Sigma-Aldrich, St Louis, USA) at a concentration of 200µg in 100 µL sesame oil. RU486 blocks progesterone signalling. This RU486 dose has been previously used to investigate the effect of progesterone withdrawal on the uterus in a mouse model of menstruation (Morison et al. 2008). In the present studies, mammary glands dissected just prior to RU486 administration are referred to as the “0 h” mouse cohort (Fig. 2.1). Mammary glands dissected 24 hours and 48 hours following RU486 administration and are referred to as “24 h” and “48 h” mouse cohorts respectively.
2.1.3 Matings
Female mice were housed with a stud male of proven fertility. Mice were checked each morning between 0900 h and 1100 h for the presence of a copulatory plug. The morning a plug was found was regarded as day 1 post-coitum.

All experiments involving pregnant mice utilized females between 10 and 12 weeks of age at the time of mating.

2.1.4 Surgery
All surgical instruments were sterilized by autoclaving or submerging in 70% ethanol prior to use. For anaesthesia, mice were administered 1-3% isoflurane as an inhalant (Veterinary Companies of Australia, Sydney, Australia) delivered in 1-3% oxygen (up to 5% for initial induction) using a precision vaporizer. Surgical incisions were closed using 9 mm MikRon® wound clips (Becton Dickinson, Sparks, USA). When mice regained consciousness during the post-operative period, they were injected subcutaneously with buprenorphine analgesia (Temgesic; Intervet, Schering-Plough, Victoria, Australia) at 0.8 µg/10 g of body weight.

2.1.4.1 Ovariectomy
Mice were anaesthetized under fluorothane anaesthesia and placed in ventral recumbency with tail towards surgeon. The toe of the hindlimb was pinched firmly between the surgeon’s fingernails and if there was no response to the toe pinch, medium-deep anaesthesia was inferred. The dorsal mid-lumbar region was shaved and swabbed with 70% ethanol. A small longitudinal dorsal incision (1-1.5 cm) was made, followed by a retroperitoneal incision above the ovarian fat pad. Using forceps, the periovarian fat was gently grasped to lift and exteriorize the ovary. The ovary was removed through the incision and the remaining reproductive tract tissue was returned to the peritoneal cavity. Skin incisions were closed with stainless steel wound clips.

2.1.5 Estrous cycle tracking
Estrous cycles in adult mice were tracked by vaginal smearing, as described previously (Allen 1922, Snell 1956). Vaginal smears were conducted daily between 0800 h and 1100 h. In brief, the vagina was flushed with a pipette containing 20 µL of phosphate buffered solution (PBS) and the recovered fluid was placed on a glass slide with a coverslip. The cellular contents were analysed under a phase contrast microscope to determine estrous cycle stage (Table 2.1). Mice were tracked for a minimum of one full 4- to 5-day normal estrous cycle before being utilised for experiments at proestrus.
2.1.6 **Injection of estradiol and progesterone**

17β-estradiol (E2; Sigma-Aldrich, St Louis, USA) and progesterone (P4; Sigma-Aldrich, St Louis, USA) were each dissolved in absolute ethanol and diluted in sesame oil (Sigma-Aldrich, St Louis, USA) to obtain the required concentrations (20 µg/ml and 20 mg/ml respectively). These two hormone preparations were combined in a ratio 1:1 just prior to injection, such that injection of 100 µL contained 1 µg of E2 and 1 mg of P4.

2.1.7 **Bromodeoxyuridine (BrdU) administration**

Mice were administered intraperitoneally with 100 µL of 10 mg/mL BrdU (Sigma-Aldrich, St Louis, USA) 1 h prior to sacrifice. The BrdU In-Situ Detection Kit (BD Pharmingen, San Jose, USA), containing a biotinylated anti-BrdU antibody was used according to the manufacturer’s instructions to identify proliferating cells in paraffin-embedded tissues (Refer 2.3.2.5).

2.1.8 **DMBA administration**

Wildtype and C1q−/− mice were given 1 mg of 7,12-dimethylbenz[a]anthracene (DMBA) (Sigma-Aldrich, St Louis, USA) in sesame oil or 100 µl of sesame oil control (Sigma-Aldrich, St Louis, USA) on a weekly basis for six weeks by oral gavage (Fig. 2.2). Chemical carcinogen DMBA is a prototypical polycyclic aromatic hydrocarbon (PAH) that has been commonly used and well-described in mice as a model of mammary tumour development (Ip and Asch 2000). Administration of 1 mg dose of DMBA weekly for 6 weeks by oral gavage to female mice has been shown to induce development of mammary tumours (Ip and Asch 2000, Currier et al. 2005).

2.1.9 **Genotyping**

Genotyping was performed by polymerase chain reaction (PCR) amplification using allele-specific primers and genomic DNA extracted from mouse tail biopsies.

2.1.9.1 **Tail digestion**

Approximately 0.5 cm of tail tip was snipped from newly weaned mice (3 weeks old) and placed in sterile 1.5 mL Eppendorf tube. The tissue was digested in 0.5 mL of digestion buffer (17 mM Tris, 17 mM EDTA, 170 mM NaCl, 0.85% SDS) with 100 µg of Proteinase K (Sigma-Aldrich, St Louis, USA) and incubated at 55°C for 4-5 hours. Five µL of extracted genomic DNA was added into 95 µL of sterile water and heated to 95°C to inactivate Proteinase K. Extracted genomic DNA was stored at 4°C until PCR analysis was performed.
2.1.9.2 **PCR conditions for C1q genotyping**

Detection of the C1q allele was performed using C1q primers mC1qA/5' + (F) 5'-GGGGCCTGTGATGCCAGAGC-3', mC1qIN/2- (R) 5'-TAACCATTGCCCTCCAGGATGG-3' and neo3' (R) 5'-GGGATCGCAATACAAAGAC-3' (band size, 360 base pairs wildtype and 160 base pairs knockout) (Fig. 2.3). PCR reaction conditions were 95°C for 3 minutes and 94°C for 1 minute, followed by 30 cycles of 60°C for 30 seconds, 72°C for 1 minute and 70°C for 10 minutes. The PCR reaction (25 µL) contained 2.5 µL 10x DNA polymerase reaction buffer, 0.5 µL 10mM dNTPs, 2.5 µL 25mM MgCl₂, 0.5 µL of mC1qA/5' 100 ng/mL, 0.5 µL mC1qIN/2 100 ng/mL, 0.75 µL neo3' primer (Geneworks Pty Ltd, SA, Australia), 0.125 µL *Taq polymerase* (Fisher Biotec, WA, Australia), 15.1 µL sterile miliQ water and 2 µL extracted DNA.

2.1.9.3 **PCR conditions for PyMT genotyping**

Detection of the MMTV-PyMT allele was performed using MMTV-PyMT primers MMTV 490 (F) 5'-CGTCCAGAAAACCACAGTCA-3' and MMTV 685 (R) 5'-CCGCTCGTACCTTATCCTTC-3' (band size, 195 base pairs) (Fig. 2.4). PCR reaction conditions were 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and 72°C for 7 minutes. The PCR reaction (25 µL) contained 2.5 µL 10x DNA polymerase reaction buffer, 2.5 µL 25mM MgCl₂, 0.5 µL 10mM dNTPs, 1.25 µL for each reverse and forward primer (Geneworks Pty Ltd, SA, Australia), 0.125 µL *Taq polymerase* (Fisher Biotec, WA, Australia), 16 µL milliQ H₂O and 2 µL extracted DNA.

2.1.9.4 **Detection of PyMT and C1q PCR products**

Gel electrophoresis was performed to detect PCR products of DNA encoding C1q (Fig. 2.3) and PyMT (Fig. 2.4) genotyping. PCR products were separated on a 2% agarose gel (Promega, WI, USA) with 1x loading buffer for 40 minutes at 80 volts. Agarose was dissolved in tris-acetate-EDTA (TAE) buffer mixed with 0.01% GelRed™ (Biotium, Hayward, CA, USA) as intercalating agents to detect DNA. Images of the gels were photographed using Gel Doc™ EZ Imager (BioRad Laboratories Inc., Hercules, CA, USA) under UV light.

2.1.10 **Tumour detection by palpation**

Mice were gently but firmly restrained by grasping the scruff of the neck between the thumb and fore-finger, and the base of the tail with the little finger. The mouse was held vertically upright to identify any tumour from the first mammary gland to the tenth mammary gland by gently and firmly palpating the mammary gland against the rib cage (1st – 3rd pairs of glands) or between the thumb.
and fore-finger (4th and 5th pairs of glands). \( \text{PyMT}^+/\text{wildtype} \) and \( \text{PyMT}^+/\text{C1q}^- \) mice were monitored weekly from 6 weeks to 18 weeks by palpation to determine tumour latency.

### 2.1.11 Tumour burden

Tumours in \( \text{PyMT}^+/\text{wildtype} \) and \( \text{PyMT}^+/\text{C1q}^- \) mice were weighed at the time of tissue collection, analysed and data presented as primary mammary tumour weight, number of tumours and total tumour burden. Primary mammary tumour weight was defined as the mammary tumour with the highest tumour weight at time of dissection. Total tumour burden was calculated as the sum of total measured mammary tumour weight per single animal.

### 2.2 Flow Cytometric Analysis for Identification and Quantification of Immune Cells Populations

Mammary glands, mammary gland draining lymph nodes (inguinal lymph nodes), para-aortic lymph nodes, cervical lymph nodes and spleens were collected following cervical dislocation.

#### 2.2.1 Mammary gland collection

For experiments in Chapter 4, the inguinal lymph nodes were dissected from the fourth pair of mammary glands and the glands were minced finely and digested in collagenase type 1A (10 µL) and dispase II (0.98 U/mg) (Roche Diagnostics, Mannheim, Germany) for 60 minutes in a rotating incubator at 37°C. Digested glands were then filtered through a nylon 70 µm cell strainer (BD Falcon, San Jose, USA) to produce a single cell suspension and then centrifuged at 300 x g, at 4°C for 9 minutes to pellet cells. The supernatant was discarded and cells were resuspended in FACS buffer (1x PBS/0.1% BSA/0.05 sodium azide) (BSA, bovine serum albumin, sodium azide; Sigma Aldrich, St Louis, USA).

For experiments in Chapter 5, the first, second, third and fifth pair mammary glands were dissected, followed by the series of processing steps described above.

#### 2.2.2 Lymph nodes collection

Lymph nodes were dissected and placed into complete RPMI1640 (Life Technologies, Victoria, Australia) with 10% of heat inactivated-fetal bovine serum (HI-FBS), penicillin and streptomycin antibiotics (P/S). Lymphocytes were dispersed by manually grinding of the tissues between the frosted ends of two sterile microscope slides. A single cell suspension was obtained by filtration through a nylon 70 µm cell strainer (BD Falcon, San Jose, USA), the cells were then centrifuged and resuspended in RPMI1640 + 10%HI-FBS (+P/S). Cell viability was assessed using trypan blue.
staining and cells were counted using a haemocytometer. Cells were resuspended at 1 x 10^7 cell/mL for stimulation in culture.

2.2.3 Spleen collection

Spleens were macerated through a nylon 70 µm cell strainer (BD Falcon, San Jose, USA) into RPMI1640 + 10%HI-FBS (+P/S). This was done by placing the cell strainer on top of a 50 mL Falcon tube (sitting on ice) and grinding with the sterile plunger taken from a 3 mL syringe. Cells were washed through the filter several times with 10 mL each of RPMI1640 + 10%HI-FBS (+P/S), followed by centrifugation at 450 x g for 5 minutes. The supernatant was removed and red blood cells (RBC) were lysed using 2 mL ACK lysis buffer (ammonium chloride 8.29 g, potassium bicarbonate 1.0 g, Na₂EDTA 37.2 mg, milli-Q water, pH 7.4). Red blood cells were lysed by gently pipetting cells up and down for 2 minutes. Approximately 15 mL of RPMI1640 + 10%HI-FBS (+P/S) was added, followed by centrifugation at 450 x g for 5 minutes to pellet cells. The supernatant was discarded and cells were resuspended in RPMI1640 + 10%HI-FBS (+P/S). Cell viability was assessed using trypan blue staining and cells counted using a hemocytometer. Cells were resuspended to 1 x 10^7 cell/ml for stimulation in culture. Each spleen yielded approximately 1-2 x 10^8 splenocytes with over 90% viability.

2.2.4 Cell stimulation

Lymphocytes and splenocytes were stimulated in a U-shaped 96 well polystyrene culture plate (BD Falcon, San Jose, USA) with a combination of phorbol 12-myristate 13-acetate (PMA) (5 ng/mL) (Sigma-Aldrich, St Louis, USA), ionomycin (500 ng/mL) (Sigma-Aldrich, St Louis, USA) and GolgiPlug (10 µg/mL) (BD Biosciences, San Jose, USA), which contains Brefeldin A to prevent cytokine release. Cell stimulation was performed at 37°C for 4 hours in 5% CO₂. Unstimulated cells were similarly incubated, but without PMA and ionomycin.

2.2.5 Labelling of single cell suspensions

2.2.5.1 Cell surface markers

For experiments in Chapter 4, single cell suspensions from mammary glands were labelled with 50 µL of FACS buffer containing 0.5 µg each of allophycocyanin-Cy7 (APC-Cy7)-conjugated anti-CD45, phycoethrin (PE)-conjugated anti-F4/80, allophycocyanin (APC)-conjugated anti-MHC Class II and biotinylated anti-MCA1322B for 30 minutes at 4°C in the dark (Table 2.2). Cells were then washed twice with 1 mL of FACS buffer, followed by centrifugation. Samples with biotinylated anti-MCA1322B were then labelled with peridinin-chlorophyll-protein complex (PerCP)-conjugated
streptavidin 30 minutes at 4°C in the dark, followed by two washes with FACS buffer. Data were analysed using FACS Canto II (BD Biosciences, San Jose, USA) using FACS Diva software (version 7.0, BD Biosciences). Viability dye 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, USA) (2 µL at 200 µg/mL to a 300 µL of cell suspension) was added to labelled cell suspensions prior to flow cytometric analysis to enable exclusion of non-viable cells from analysis. Gates were applied to the forward/side scatter dotplots to exclude cell debris.

For experiments in Chapter 5, single cell suspensions from pooled first, second, third and fifth pair mammary glands were labelled with 50 µL of FACS buffer containing 0.5 µg each allophtocyanin-Cy7 (APC-Cy7)-conjugated anti-CD45, fluorescein isothiocyanate (FITC)-conjugated anti-CD3, allophtocyanin (APC)-conjugated anti-CD4, phycoethrin (PE)-conjugated anti-CD8, phycoethrin (PE)-conjugated anti-F4/80, and allophtocyanin (APC)-conjugated anti-MHC Class II for 30 minutes at 4°C in the dark (Table 2.2). This antibody labelling was followed by a series of steps described above.

2.2.5.2 Intracellular cytokines

Following cell stimulation, cells were transferred from the plates to FACS tubes (BD Falcon, San Jose, USA) and 1 mL of FACS buffer was added to each tube, followed by centrifugation at 400 x g for 5 minutes to pellet cells. Culture supernatant was discarded. Fc receptors were blocked by resuspending cells in 50 µL of FACS buffer containing 0.5 µg anti-Fc-γIIIR antibody (CD16/CD32, Fc Block, BD Pharmingen, BD Biosciences) (Table 2.2) for 20 minutes at 4°C. Following the blocking step, cells were washed twice with 1 mL of FACS buffer and centrifuged.

Surface markers (CD3, CD4 and CD8) were labelled by adding 50 µL of FACS buffer containing 0.5 µg each of fluorescein isothiocyanate (FITC)-conjugated anti-CD3, phycoethrin (PE)-conjugated anti-CD4 and phycoethrin (PE)-conjugated anti-CD8 to appropriate tubes (Table 2.2). Cells were incubated for 30 minutes at 4°C in the dark. Cells were then washed with twice with 1 mL of FACS buffer and centrifuged at 450 x g for 5 minutes at 4°C.

Cells were fixed and permeabilised by adding 100 µL of 1 x Fix/Perm (eBioscience) buffer to each tube, followed by 30 minutes incubation in the dark at 4°C. Cells were washed twice with 1 mL of 1 x Perm buffer (eBioscience) and centrifuged at 450 x g for 5 minutes at 4°C. Cells were labelled with intracellular cytokine monoclonal antibodies (mAbs) by adding 50 µL of 1 x Perm buffer containing 0.5 µg each of allophtocyanin-Cy7 (APC-Cy7)-conjugated anti-interferon gamma (IFNG) and peridinin-chlorophyll-protein complex Cy5.5 (PerCP-Cy5.5)-conjugated anti-interleukin-10 (IL10) (Table 2.2) and then incubated for 30 minutes at 4°C in the dark. Isotype controls were labelled with
APC-Cy7-conjugated rat IgG1 and PerCP-Cy5.5-conjugated rat IgG2b (Table 2.2). Cells were washed twice with 1 mL of 1 x Perm buffer and centrifuged at 450 x g for 5 minutes at 4°C. Cells were resuspended in FACS buffer and ready for flow cytometric analysis. Data were analysed using FACS Canto II (BD Biosciences, San Jose, USA) using FACS Diva software (version 7.0, BD Biosciences). Gates were applied to the forward/side scatter dotplots to exclude cell debris.

2.3 Immunohistochemistry

2.3.1 Tissue collection, embedding and sectioning

2.3.1.1 Fresh frozen tissues
Tumours dissected from mice at autopsy were embedded in Tissue-Tek OCT compound (Sakura Fintek, Torrance, USA) and stored at -80°C. Tissue was sectioned at 6 µm using a Leica CM 1850 cryostat (Leica Microsystems, NSW, Australia) with disposable microtome blades (Feather, Osaka, Japan). The tissue sections then mounted onto SuperFrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany). Slides were allowed to dry for approximately 1 h before being stained or stored with desiccant (silica gel; Sigma-Aldrich, St. Louis, USA) at -80°C.

2.3.1.2 Paraffin tissues
Mammary glands, tumours and lungs were collected and fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS (pH 7.4) for 24 h at 4°C. Tissue was washed three times in PBS over the following two days, and late on the second day, tissue was transferred to a 70% ethanol solution for storage at 4 °C until tissue processing. Tissue was processed and embedded using the Leica TP1020 Tissue Processor (Leica Microsystems) involving the following dehydration and embedding protocol; 30 minutes 75% ethanol, 30 minutes 85% ethanol, 30 minutes 90% ethanol, 2 x 30 minutes absolute ethanol (ANALAR), 2 x 30 minutes 100% Xylene (Ajax Finechem), 2 x 30 minutes paraffin wax (Ajax Finechem) under vacuum conditions. Tissue was embedded immediately into paraffin blocks and stored at room temperature for sectioning. Using Leica Rotary Microtome (Leica Microsystems), paraffin-embedded blocks were cut into 5 µm sections and fixed onto SuperFrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany) using a 45°C water bath. Slides were dried overnight at 37°C. Blocks and sections were stored at room temperature prior to staining.

2.3.2 Immunohistochemistry Protocols

2.3.2.1 F4/80 staining of paraffin embedded tissues
Tissue sections were dewaxed in Safsolv (Ajax Finechem) and rehydrated through graduated dilutions of ethanol (2 x 5 minutes 100%, 1 x 3 minutes 90%, 1 x 3 minutes 70% and 1 x 3 minutes...
50% ethanol) followed by 1 minute in milliQ water. Endogenous peroxidase activity was quenched by incubating sections with freshly prepared 1.5% hydrogen peroxide solution (Sigma-Aldrich) in methanol (Sigma-Aldrich; solution contained 10 mL hydrogen peroxide, 100 mL methanol, 90 mL water) for 15 minutes. Sections were washed with milliQ water (3 x 3 minutes). Sections were blocked with 15% normal rabbit serum (NRS) in PBS for 30 minutes at 37°C, and then were washed twice with PBS for 5 minutes each. Following this, sections were incubated with rat anti-mouse F4/80 antibody (eBioscience) diluted at 1:100 in PBS containing 1.5% NRS overnight at 4°C in a humidified chamber. Sections were washed in PBS (3 x 5 minutes) before being incubated with biotinylated secondary antibody at a dilution of 1:600 for 40 minutes at room temperature (biotinylated rabbit anti-rat IgG; Vector Laboratories, Burlingame, USA). Sections were treated with streptavidin-conjugated horseradish peroxidase (HRP; Vectastain ABC kit, Vector Laboratories, Burlingame, USA) and detection was performed using 3, 3′-diaminobenzidine (DAB; SigmaFast tablets, Sigma) according to the manufacturer’s instructions. Tissue sections were counterstained with haematoxylin (Sigma-Aldrich, St Louis, USA) for approximately 30 seconds, before rinsing in milliQ water. The sections were then mounted with coverslips using DPX mountant (Merck, Darmstadt, Germany).

2.3.2.2 Haematoxylin and eosin staining
Tissue sections were stained with haematoxylin (Sigma-Aldrich, St Louis, USA) for 30 seconds. To enhance the blue nuclear stain, sections were incubated in tap water (high Ca^{2+} solution) for 2 minutes, dipped in ammonia water for 5-10 seconds before being stained with eosin (diluted 1:2 in 70% ethanol; Sigma-Aldrich, St Louis, USA) for 30 seconds and rinsed in milliQ water prior to dehydration, clearing and mounting as described above.

2.3.2.3 Carmine alum staining
Wholemounts were fixed in Carnoy’s fixative (2-4 hours) and stained in carmine alum solution overnight according to the published method (http://mammary.nih.gov/tools/histological/Histology).

2.3.2.4 Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining
Paraffin-embedded mammary gland sections were stained using the DeadEnd™ Colorimetric TUNEL System (Promega, Wisconsin, USA) according to the manufacturer’s instructions to detect TUNEL-stained apoptotic cells. Tissue sections were dewaxed in Xylene (Ajax Finechem) and rehydrated through graduated dilutions of ethanol, (100%, 95%, 85%, 70%, 50%), 3 minutes each
time. Sections were immersed in 0.85% NaCl for 5 minutes at room temperature, before washing with PBS for 5 minutes. Sections were refixed in neutral buffered formalin (NBF) for 15 minutes and then washed twice in PBS for 5 minutes each time. Following this, tissue sections were incubated with Proteinase K solution for 15 minutes and before washing in PBS for 5 minutes. Tissue sections were refixed again in NBF for 5 minutes and were washed twice in PBS for 5 minutes each time at room temperature. DNase treated tissue was used as a positive control and incubated with 50/50 dilution of Dnase (1.5 mg/mL) in PBS for 10 minutes, followed by three washes in milliQ water (3 minutes each) and 5 minutes in PBS. All tissue sections were treated with equilibration buffer for 5 minutes. Positive tissue sections and DNase control sections were incubated in TdT reaction mix containing rTDT enzyme and biotinylated nucleotide mix, whilst the negative control tissue was incubated with reaction mix without rTdT enzyme, for 60 minutes at 37°C. All tissue sections were immersed in SSC buffer for 15 minutes at room temperature, before washing in PBS containing 0.1% Triton® X-100 + 5 mg/mL BSA (bovine serum albumin) (3 x 5 minutes). Sections were washed in PBS for minutes, followed by incubation in 0.3% hydrogen peroxide (H₂O₂) for 5 minutes. Sections were treated with streptavidin-conjugated horseradish peroxidase (HRP) and detection was performed using 3, 3′-diaminobenzidine (DAB). Tissue sections were counterstained with haematoxylin (Sigma-Aldrich, St Louis, USA) for 30 seconds, before rinsing in milliQ water. Sections were then dehydrated through a graduated increase in ethanol concentration and cleared in two changes of Xylene (Ajax Finechem) for 5 minutes each. The sections were coverslipped using microscopy entallen (ProSciTech, Queensland, Australia).

2.3.2.5 **Bromodeoxyuridine (BrdU) staining**

Epithelial cell proliferation was identified by BrdU immunostaining of paraffin-embedded mammary glands. Sections were stained for BrdU incorporation into DNA using a BrdU In-Situ Detection Kit (BD Pharmingen) according to the manufacturer’s instructions. Tissue sections were dewaxed in Safsolv (Ajax Finechem) and rehydrated through graduated dilutions of ethanol (2 x 5 minutes xylene, 2 x 3 minutes 100% ethanol and 1 x 3 minutes 95% ethanol). Endogenous peroxidase activity was blocked by incubating sections for 10 minutes in 3% hydrogen peroxide (H₂O₂) in PBS. Sections were washed in PBS (3 x 5 minutes) before being incubated with BD™ Retrievagen A solution for 10 minutes at 89°C to unmask antigenic sites. Sections were allowed to cool down to room temperature for 20 minutes and then were washed three times with PBS for 5 minutes each, followed by incubation with the biotinylated anti-BrdU antibody for 1 h in a humidified chamber. Sections were washed three times with PBS for 2 minutes each and then treated with streptavidin-conjugated horseradish peroxidase for 30 minutes at room temperature. Detection was performed using the
DAB (diaminobenzidine) substrate system. Sections then were washed three times in milliQ water, 2 minutes each time. Tissue sections were counterstained with haematoxylin (Sigma-Aldrich, St Louis, USA) for 30 seconds, before rinsing in milliQ water. Sections were then dehydrated through a graduated increase in ethanol concentration and cleared in two changes of Safsolv (Ajax Finechem) for 5 minutes each. The sections were mounted with coverslips using DPX mountant (Merck, Darmrsadt, Germany). Tissue sections were counterstained with haematoxylin.

2.3.3 Image capture and cell quantification

Images of the carmine alum-stained wholemount mammary glands were taken using a light microscope (Leica MZ16FA stereo microscope; Adelaide Microscopy). Analysis was conducted by a researcher blinded to mouse genotype. Photographed wholemounts were analysed using ImageJ to quantify terminal end buds (TEBs), secondary branching, and ductal elongation (Chapter 3). Ductal elongation was calculated by drawing a straight line from the nipple end to the furthest end of the duct. Quantification of secondary branching was done by selecting five primary ducts randomly within the middle of the gland, close to the lymph node for each mouse and the number of secondary branches along a known length of primary duct were counted. The number of terminal end buds (TEBs), the active proliferative components of the duct that face an open expanse of the fat pad, was manually counted.

Images of the stained paraffin-embedded tissue sections were captured using a Hamamatsu Protonics Nanozoomer 1.0 (Hamamatsu, Shizuoka, Japan). Analysis was conducted by a researcher blinded to mouse genotype. Epithelial cell proliferation was quantified by BrdU immunostaining. The number of BrdU-positive cells was expressed as number of positive cells per area of epithelium in terminal end buds (TEBs). The abundance of TUNEL positive cells was identified using the DeadEnd™ Colorimetric TUNEL System. The number of TUNEL-positive cells was expressed as number of positive cells per area of ductal epithelium.

To quantify extent of alveolar development using H&E-stained sections at proestrus, epithelial regions were categorised as ductal or alveolar. The classification system was described previously (Fata et al. 2001, Chua et al. 2010), whereby ductal epithelium was defined as a single epithelial cell layer and alveolar epithelium defined as clusters of epithelial structures containing alveolar lumens. The numbers of ductal and alveolar epithelial structures were counted manually for each mouse. The total number of alveolar buds was expressed as percent of total epithelial structures (ductal and alveolar combined). Alveolar development in day 18 post coitus tissues was calculated as areas of alveolar clusters and expressed as a percentage of total gland area.
F4/80 positive cells found around the neck of TEBs, within the stroma of glands from 6-week old mice were manually counted. For day 18 post coitus, F4/80 positive cells were localised in direct contact with alveolar epithelium (Pollard and Hennighausen 1994). The abundance of F4/80 positive cells in mammary glands of mice at proestrus and in the regression mouse model was quantified in five randomly selected areas of ductal stroma at 40x magnification. Only F4/80 positive cells with visible haematoxylin-stained nuclei were included and the assessment was done by a researcher blinded to genotype. Data were averaged to give a single value per mouse for statistical analysis.

A blinded analysis of photographed PyMT\(^+\)/C1q \(^+/+\) and PyMT\(^+\)/C1q \(^{-/-}\) wholemounts, collected at 10 weeks of age, was performed to quantify the number of hyperplastic areas and total area of hyperplasia within a whole mammary gland. The region of hyperplasia was manually traced in the captured images using ImageJ software and expressed as a percent of total area of mammary gland.

In the primary mammary tumours from 15-week old and 18 week-old of age PyMT\(^+\)/C1q \(^+/+\) and PyMT\(^+\)/C1q \(^{-/-}\) mice, F4/80 cells were localised within the tumour, mammary fat pad, stroma and connective tissue. Macrophage abundance in early carcinoma and late carcinomas in 18 week old mice were analysed.

Clinical features of H&E-stained primary mammary tumour sections from PyMT\(^+\)/C1q \(^+/+\) and PyMT\(^+\)/C1q \(^{-/-}\) mice were analysed by a veterinary pathologist (Dr. Lucy Woolford, The University of Adelaide) blinded to genotype. The primary tumour of each mouse was assessed for the parameters of tumour grade, cytological atypia, tumour necrosis, and inflammation. Tumours were graded based on criteria stipulated by a previous study in the literature (Lin et al. 2003). For each parameter, the tumour was classified into the category that best described the clinical feature. The clinical features that defined the classification for each pathological parameter are shown in Table 2.3. Data for each pathological parameter were expressed as percent proportions in PyMT\(^+\)/C1q \(^+/+\) and PyMT\(^+\)/C1q \(^{-/-}\) mice.

H&E-stained lung sections from PyMT\(^+\)/C1q \(^+/+\) and PyMT\(^+\)/C1q \(^{-/-}\) mice were analysed by a veterinary pathologist (Dr. Lucy Woolford, The University of Adelaide) blinded to genotype for the presence of pulmonary metastasis. Data were expressed as percent proportions in PyMT\(^+\)/wildtype and PyMT\(^+\)/C1q \(^{-/-}\) mice.
2.4 Quantitative Real-Time PCR

2.4.1 RNA extraction

Lungs were dissected under RNase-free conditions, snap frozen in liquid nitrogen and stored at -80°C until processing. Total lung RNA was extracted in 1 mL Trizol (Invitrogen, Carlsbad, USA) to tubes containing 1.4 mm ceramic beads (Geneworks, Adelaide, Australia). Tissues were homogenised using the PowerLyzer™ 24 Homogenizer (MO Bio Laboratories) with speed 3500 rpm, 2 x 30 seconds and 1 x 30 seconds. Homogenized samples were incubated for 5 minutes at room temperature to allow complete dissociation of the nucleoprotein complex. Approximately 0.2 mL of chloroform (Unilab, Ajax Finechem, Tarenpoint, Australia) was added and tubes were vigorously mixed by hand for 15 seconds, followed by another incubation for 2-3 minutes at room temperature. Samples were centrifuged at 12,000 x g for 15 minutes at 4°C, separating the mixtures into a lower red phenol-chloroform phase, an interphase and a colourless upper aqueous phase. The aqueous phase of the samples was removed by angling the tube at 45° and pipetting the solution out. Approximately 0.5 mL of 100% isopropanol (Sigma-Aldrich) was added to the aqueous phase and samples were incubated overnight for optimal precipitation. Samples were pelleted at 12,000 x g for 5 minutes at 4°C and supernatant discarded. The pellet was then washed with 1 mL of 75% ethanol, followed by a brief vortex and centrifugation at 7500 x g for 5 minutes at 4°C. Excess ethanol was removed and the RNA pellet was air-dried for 5-10 minutes before being resuspended in 50 µL of RNAse-free milliQ water. Samples were treated with DNase to remove contaminating DNA using AMBION DNase Treatment and Removal DNA-free kit (Life Technologies, Applied Biosystems, Carlsbad, USA) according to the manufacturer's instructions. Extracted RNA was quantified using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, USA).

2.4.2 Reverse transcription and cDNA generation

Each RNA sample was reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. A total of 1-2 µg of RNA was prepared for each sample to be reverse transcribed. Briefly, 1 µL of 10 mM dNTPs was added to tubes containing RNA, followed by 1 µL of random primers. Mixtures were briefly vortexed to collect reagents at the bottom of the tubes. Mixtures were incubated at 65°C for 5 minutes, followed by incubation on ice for at least 1 minute. Reverse transcription master mix (4 µL of 5 x first strand buffer, 1 µL of 0.1 M DTT, 1 µL of Superscript III RT) was added to each sample and incubated at 25°C for 5 minutes, and then 50°C for 60 minutes. The reverse transcription reaction was stopped by heating the samples at 70°C for 15 minutes. For quantitative real-time PCR, cDNA was diluted in milliQ water to a final concentration of 20 ng/µL and stored at -20°C.

Noor Din 73
2.4.3 Quantitative real-time PCR

Detection of the PyMT transgene in lung cDNA samples was performed using primer sets MMTV-PyMT; (F) 5’-GAGCGAGGAACTGAGCAGAG-3’ and (R) 3’-CTTAGCGGCGACTGGTACGC-5’ (product size, 195 base pairs) with the housekeeping gene L19 primers sets; (F) 5’-CTGAAGGCTCAAAGGGAATGTG and (R) 3’-GGACAGAGTGCTTGATGATCC (product size, 194 base pairs) on 96-well plates. An ABI Prism 7000 Sequence Detection System (Applied Biosystems) and a 2 x SYBR Green master mix (7900HT Fast Real-Time PCR System, Applied Biosystems, NSW, Australia) were used for this qRT-PCR according to the manufacturer’s instructions. Serial dilutions of primer sets with a positive control sample cDNA were conducted to determine the optimal primer concentration. Each reaction (20 µL) contained 1 µL of cDNA and 0.0625 µM of RpL19 primer sets or 0.05 µM of MMTV-PyMT primer sets. The efficiency of MMTV-PyMT primer sets was determined by regression analysis across cDNA serial dilution. The negative control included in each reaction was milliQ water substituted for cDNA (non-template control) to show that primers did not produce primer dimers as evidenced by no amplification.

The cycling parameters were: 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and then 60°C for 1 minute. Amplification plots for each sample were obtained and cycle threshold (Ct) limits were determined using the ABI 7000 software package (Applied Biosystems). All samples were amplified in triplicates for each primer set. The CT values were normalised relative to the housekeeping gene, RpL19 and were calibrated using the 2-(ΔΔCT) quantitation method (Livak and Schmittgen 2001). All samples for each primer set were expressed relative to the mRNA content of control tissue.

2.5 Statistical Analysis

Statistical analysis to compare parameters of tumour development in the 10, 15 and 18 week-old PyMT+/C1q+/- and PyMT+/C1q-/- mice, presented in Chapter 5, was conducted by statistician Dr Stuart Howell (Data Management & Analysis Centre, University of Adelaide). The analyses were undertaken using SAS v9.3 (SAS Institute Inc., Cary, NC, USA). All tests conducted by Dr Howell were two-tailed and significance was set at the 5% alpha level.

The analysis of hyperplastic areas of mammary gland wholemounts from 10-week old mice and number of tumours, total tumour burden and primary tumour weight of mammary tumours from 15-week and 18-week old mice were assessed using the Wilcoxon (Mann-Whitney) test. This was a subgroup analysis - analysed separately for each age group. The rationale for this was that the tumours were assessed at different time points and it is likely that age would influence the outcomes.
Hence, a direct comparison of animals of differing age would produce biased results. Data are presented as means ± standard error of mean (SEM).

Data analysis of primary mammary tumours for tumour grade, cytological atypia, inflammation, and tumour necrosis, as well as the presence of lung metastases in H&E-stained sections, were assessed using the Fisher's Exact test. Each of these parameters were scored by veterinary pathologist Dr Lucy Woolford, blinded to mouse genotype, who classified each parameter on distinct clinical features. The histological parameters that define each score are outlined in Section 2.3.3 and Table 2.3. The statistical test analyses the frequency of scores for each parameter occurring in the entire cohort of PyMT+/C1q−/− mice, compared to PyMT+/C1q+/+ mice. As such, the analysis produces a result for the cohort as a whole, rather than comparing the frequency of one score versus another. Therefore, the results are presented as percent proportions of each score in PyMT+/C1q+/+ and PyMT+/C1q−/− mice, with differences between the groups as a whole cohort. As a result of structural zeros arising from differences in the age at assessment, subgroup analyses were performed so that mice aged 15 and 18 weeks were assessed separately.

All other statistical analyses were performed using SPSS software, version 20.0 for Windows (SPSS, Chicago, IL, USA). Data were assessed for normal distribution using the same software. Within analyses, Dixon’s Q test was used for identification and rejection of outliers. Independent samples T test and general linear model test were used and presented as the mean ± standard error of mean (SEM) to determine statistical significance between the different genotype groups. Kaplan Meier's survival analyses were performed to determine differences in latency to first palpable tumour in the PyMT and DMBA mouse models of mammary cancer. The difference between groups was considered statistically significant if *p<0.05. If more than two groups were compared, different letters (a, b, c, d) were used to indicate a statistical difference between the groups. If there was no statistical difference between the groups, all were assigned the same letter (for example: a).
Figure 2.1 Hormone-treatment regime used in the mouse model to induce regression in the mammary gland

Ovariectomised wildtype C57BL/6 and C1q−/− mice treated with estradiol (E₂) and progesterone (P₄) daily for 3 days. This hormone injection protocol induces alveolar development. Twenty four hours following the final injection of estradiol and progesterone, the mice were injected with RU486 to induce alveolar regression. Mammary glands dissected just prior to RU486 administration are referred to as the 0 hour cohort. Mammary glands dissected 24 hours and 48 hours after RU486 administration are referred as 24 h and 48 h cohorts respectively. OVX, ovariectomised.
Figure 2.2 DMBA administration by oral gavage

Wildtype C57BL/6 and C1q−/− mice administered with 1 mg dose of DMBA weekly for 6 weeks by oral gavage. Tissues including mammary gland, mammary gland draining lymph nodes (inguinal lymph nodes), para-aortic lymph nodes, cervical lymph nodes and spleen were harvested 24 hours following the sixth DMBA dose.
Figure 2.3 Genotyping C1q transgene by PCR

Each lane represents DNA from each mouse amplified by C1q primers, yielding PCR products of 360 bp (wildtype) and 160 bp (null mutant). WT, wildtype; bp, base pairs.

Figure 2.4 Genotyping PyMT transgene by PCR

Male PyMT$^+$ (C57BL/6 strain) mice were mated with C1q$^-$ females (C57BL/6 strain). Their male PyMT$^+$/C1q$^+$ offspring were mated with C1q$^-$ females to generate the PyMT$^+$/C1q$^-$ mice used in this study. All pups were weaned at 21 days, and genotyped. Each lane represents DNA from each mouse amplified by MMTV primers, yielding PCR product of 195 bp. Neg, negative; bp, base pairs.
### Table 2.1 Classification of estrous cycle stages by cell morphology in vaginal smears

<table>
<thead>
<tr>
<th>Stage of cycle</th>
<th>Epithelial cells</th>
<th>Cornified epithelial cells</th>
<th>Leukocytes</th>
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</thead>
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<tr>
<td>Proestrus</td>
<td>+ to ++</td>
<td>0 to +</td>
<td>0</td>
</tr>
<tr>
<td>Estrus</td>
<td>0 to +</td>
<td>++ to +++</td>
<td>0</td>
</tr>
<tr>
<td>Metestrus</td>
<td>0 to +</td>
<td>clumps + to ++</td>
<td>++ to +++</td>
</tr>
<tr>
<td>Diestrus</td>
<td>0</td>
<td>- -</td>
<td>- -</td>
</tr>
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</table>

Cell density: 0 = none, + = few, ++ = moderate, +++ = heavy, - - = low number of cells
Table 2.2 Monoclonal antibodies used in flow cytometric analysis

<table>
<thead>
<tr>
<th>mAb</th>
<th>Conjugate</th>
<th>Clone</th>
<th>Isotype</th>
<th>Alternative names</th>
<th>Source</th>
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<tr>
<td>CD45</td>
<td>APC-Cy7</td>
<td>30-F11</td>
<td>Rat (LOU) IgG2b</td>
<td>leukocyte common antigen (LCA), Ly-5, T200</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>F4/80</td>
<td>PE</td>
<td>BM8</td>
<td>Rat IgG2a</td>
<td>pan macrophage marker</td>
<td>eBioscience</td>
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<tr>
<td>MHC Class II</td>
<td>APC</td>
<td>Mf/114.15.2</td>
<td>Rat IgG2b</td>
<td>MHC II, IA, IE, I-A/E, IA/IE</td>
<td>eBioscience</td>
</tr>
<tr>
<td>MCA1322B</td>
<td>biotin</td>
<td>2F8</td>
<td>Rat IgG2b</td>
<td>scavenger receptor type I/II</td>
<td>Serotec</td>
</tr>
<tr>
<td>streptavidin</td>
<td>PerCP</td>
<td></td>
<td>sav-perCP</td>
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<td>BD Biosciences</td>
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<tr>
<td>CD3</td>
<td>FITC</td>
<td>17A2</td>
<td>Rat (SD) IgG2b</td>
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<td>PE</td>
<td>RM4-5</td>
<td>Rat IgG2a</td>
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<td>BD Biosciences</td>
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<tr>
<td>CD4</td>
<td>APC</td>
<td>RM4-5</td>
<td>Rat IgG2a, κ</td>
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<td>CD8a</td>
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<td>Ly-2, Lyt-2</td>
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<td>XMG1.2</td>
<td>Rat IgG1</td>
<td>IFN-gamma, interferon gamma type II interferon</td>
<td>BD Biosciences</td>
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<tr>
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<td>PerCP-Cy5.5</td>
<td>JES5-16E3</td>
<td>Rat IgG2b</td>
<td>interleukin-10</td>
<td>eBioscience</td>
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<tr>
<td>CD16/CD32</td>
<td>2.4G2</td>
<td></td>
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<td>FcγRIII/FcγRII, Fcgr3/Fcgr2</td>
<td>BD Biosciences</td>
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</table>

**Isotype controls**

<p>| | | | | | |</p>
<table>
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<tr>
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<td>eB149/10H5</td>
<td></td>
<td>Rat IgG2b</td>
<td></td>
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**Table 2.3 Scoring system of pathological parameters for PyMT tumours**

<table>
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<tr>
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<th>Adenoma</th>
<th>Early carcinoma</th>
<th>Late carcinoma</th>
</tr>
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<tbody>
<tr>
<td>Tumour grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytological atypia</td>
<td>None or minimal</td>
<td>Mild</td>
<td>Moderate</td>
<td>Marked</td>
</tr>
<tr>
<td>Inflammation</td>
<td>None</td>
<td>Mild, focal</td>
<td>Mild, multifocal</td>
<td>Moderate, focal, multifocal</td>
</tr>
<tr>
<td>Tumour necrosis</td>
<td>No necrosis</td>
<td>Presence of necrosis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 3 – Effect of C1q Null Mutation on Mammary Gland Development
3.1 Introduction

C1q is one of the powerful effector proteins of the complement system. It activates the classical pathway to exert a number of functions including clearance of pathogens and modulation of immunological and inflammatory processes (Nauta et al. 2004, Bohlson et al. 2007, Ricklin et al. 2010, Galvan et al. 2012). Numerous in vitro and in vivo studies show a prominent role of C1q in the clearance of apoptotic cells (Korb and Ahearn 1997, Botto et al. 1998, Taylor et al. 2000, Ogden et al. 2001, Vandivier et al. 2002). C1q opsonisation of apoptotic cells triggers macrophage-mediated phagocytosis by directly binding to macrophage-expressed CD91 (also known as LRP-1) (Ogden et al. 2001), or indirectly through forming complexes with immunoglobulins to activate the complement cascade and C3 deposition (Kim et al. 2003, Ogden et al. 2005). Deficiency in C1q is associated with the development of systemic lupus erythematosus autoimmune disease (SLE) in humans as a consequence of inefficient clearance of apoptotic cells (Kallel-Sellami et al. 2008, Rahman and Isenberg 2008, Roumenina et al. 2011). C1q has also been shown to have direct interactions with SLE immune complexes to inhibit IFNA production by plasmacytoid dendritic cells (pDCs), assisted by monocytes (Santer et al. 2010). Recent investigations on C1q-macrophage interactions revealed C1q as one of the key modulators in directing macrophage polarisation and inhibiting inflammasome activation (Benoit et al. 2012, Galvan et al. 2012).

Whilst the roles of C1q in the immune response are well-characterised, there is still a lack of understanding of complement C1q involvement in normal mammary gland development. A previous investigation on local complement gene expression in human breast tissue revealed complement C4 mRNA expressed in mammary ductal epithelial cells in non-neoplastic quiescent breast tissue (Laufer et al. 1999). There was also an increase of C3 and C4 complement gene expression observed in the human mammary gland during pregnancy (Laufer et al. 1999). Although this study did not investigate C1q gene expression, it raises the question of potential complement roles in mammary gland development. Furthermore, an unbiased whole genome study that described gene expression changes during mouse mammary gland involution reported C1q upregulation in the involuting mammary gland (Stein et al. 2004). Consistently, Clarkson and colleagues demonstrated elevated C1q gene expression in mammary glands of C57BL/6 mice within 12 hours of forced weaning (Clarkson et al. 2004), coinciding with transcriptional activation of different apoptotic-related genes observed such as caspase 9 effector, Apaf1, and Bax, and death receptor ligands including Tnf (TNF-α), Tnfsf6 (Fas ligand), Tnfsf10 (TRAIL), and Tnfsf12 (TWEAK). These findings suggest a role for C1q in clearance of apoptotic cells during involution, which drives the tissue to tolerance and reduces inflammation as part of a healthy process to return the mammary gland to a non-pregnant state.
state. Furthermore, Clarkson and colleagues also observed an upregulation in the monocyte/macrophage markers Lrp, Csf1r, and Cd14, illustrating the recruitment of phagocytic macrophages to the involuting mammary gland to effectively clear dying cells (Clarkson et al. 2004). Hence, this is likely to suppress the generation of harmful adaptive immune responses, allowing for effective tissue remodelling and damage prevention.

C1q null mice have no overt sign of impaired mammary gland development, lactation or involution, as they are able to feed successive litters (unpublished observations). However, the impact of C1q null mutation on mammary gland development has not been formally investigated. Closer examination may reveal subtle but important differences in mammary gland development in the absence of C1q. Hence, in this chapter, a series of experiments was undertaken to fully evaluate the role of C1q in normal mammary gland development and function using C1q null mutant mice. We hypothesise that C1q promotes rapid macrophage-mediated phagocytosis of dying epithelial cells during the regression phase of the ovarian cycle. This study seeks to investigate the crosstalk between C1q and macrophages and to understand the roles of C1q in regulation of normal mammary gland development and function. In this study, we investigated the overall effect of null mutation in C1q on mammary gland development during puberty, the ovarian cycle, and pregnancy using C1q−/− and wildtype C1q+/+ mice (both on a C57BL/6 background).

3.2 Results

3.2.1 Effect of C1q null mutation on mammary gland development during puberty

To determine the effect of null mutation in C1q on mammary gland development during puberty, the fourth pair mammary glands from wildtype C1q+/+ and C1q null female mice were dissected at 6 weeks of age. Both genotypes exhibited basic primary epithelial ducts with some secondary branches and very little alveolar budding (Fig. 3.1A, C). Ductal elongation was assessed by measuring the length between the nipple to the furthest end of the duct in carmine alum stained wholemounts (Fig. 3.1A, C, double-headed arrows indicated). There was no difference in ductal length in C1q−/− mice when compared to wildtype mice (13.1±0.9 mm in C1q+/+ versus 13.3±1.2 mm in C1q−/− mice, n=8-9) (Fig 3.1B). Terminal end buds (TEBs), the most active and proliferative component of the ducts, were evident from the carmine alum-stained wholemount glands (Fig. 3.1A, C, single-headed arrows indicated). C1q−/− mice exhibited similar number of TEBs compared to wildtype mice (11±3 in C1q+/+ versus 15±1 in C1q−/− mice, n=8) (Fig. 3.1D), suggesting a normal structural development of the mammary glands in C1q−/− mice during puberty.
Epithelial cell proliferation in pubertal mice was quantified by BrdU incorporation in TEBs. BrdU positive cells (Fig. 3.2A-B, arrows indicated) represent the proliferating cells in the TEBs of both genotypes. There was no difference in the percentage of ductal epithelial cells undergoing proliferation between $C1q^{+/+}$ and $C1q^{-/-}$ null mice ($2626\pm386$ in $C1q^{+/+}$ versus $2671\pm249$ BrdU positive cells/mm$^2$ in $C1q^{-/-}$ mice, n=8) (Fig. 3.2D).

The effect of $C1q$ null mutation on macrophage abundance during puberty was determined by F4/80 antibody immunostaining of the mammary glands of pubertal $C1q^{+/+}$ and $C1q^{-/-}$ mice. F4/80 positive cells were found to be localised within the stroma of the neck of the TEBs (Fig. 3.3A-B, arrows indicative F4/80 positive macrophages). The negative control showed no F4/80 positive staining (Fig. 3.3C) There was no difference in macrophage abundance between wildtype and $C1q^{-/-}$ mice during puberty ($2720\pm426$ in $C1q^{+/+}$ versus $2356\pm369$ macrophages/mm$^2$ in $C1q^{-/-}$ mice, n=6) (Fig. 3.4).
Figure 3.1 Effect of C1q null mutation on ductal elongation and the number of terminal end buds (TEBs) during puberty

Ductal length was measured from the nipple to the furthest end of the duct (A, C), double-headed arrow indicated. Both wildtype C1q +/+ and C1q −/− mice showed similar ductal elongation (B). Carmine alum stained mammary gland wholemount of 6-week old female mice showed development of mammary gland from nipple end to open expanse of fat pad, single-headed arrows indicated terminal end buds (TEBs) (A, C). Both genotypes showed similar number of TEBs (D), n=6-8 per genotype. Data shown as a mean±SEM of ductal length (mm) and TEB numbers. Data were evaluated using independent samples T test. LN, lymph node.
Figure 3.2 Effect of C1q null mutation on epithelial cell proliferation during puberty

Representative images of BrdU antibody staining indicative of proliferating cells found in terminal end buds of 6-week old C1q $^{+/+}$ (A) and C1q $^{-/-}$ (B) female mice. Negative control shows no BrdU positive staining (C). No difference was observed in proliferation of epithelial cells between C1q $^{+/+}$ and C1q $^{-/-}$ mice (D), n=8 per genotype. Data shown as a mean±SEM of BrdU positive cells/mm$^2$. Data were evaluated using independent samples T test.
Figure 3.3 Effect of C1q null mutation on macrophage abundance during puberty

Representative images of F4/80 antibody staining indicative of macrophage positive cells found within stroma surrounding terminal end buds of 6-week old C1q $^{+/+}$ (A) and C1q $^-/(B)$ female mice, taken at 20X magnification. F4/80 positive staining is also shown at 40X magnification in inset with examples of F4/80 positive macrophages identified by arrows. Negative control shows no F4/80 positive staining (C).
Figure 3.4 Effect of C1q null mutation on macrophage abundance during puberty

Quantification of F4/80 positive macrophages found within stroma surrounding terminal end buds of 6-week old C1q $^{+/+}$ and C1q $^{-/-}$ female mice, n=6 per genotype. Data shown as a mean±SEM of F4/80 positive cells/mm$^2$. Data were evaluated using independent samples T test.
3.2.2 Effect of C1q null mutation on mammary gland regression during the proestrus phase of the ovarian cycle

Previous studies have shown that the morphology of mammary epithelial ducts and the abundance of macrophages changes across the estrous cycle in mice and the menstrual cycle in women (Andres and Strange 1999, Fata et al. 2001, Navarrete et al. 2005, Chua et al. 2010, Hodson et al. 2013). The proestrus phase of the mouse estrous cycle is associated with mammary gland regression and tissue remodelling to return the mammary gland to its basic architecture (Fata et al. 2001, Chua et al. 2010). Mammary gland structure and macrophage abundance during the proestrus phase was investigated in C1q +/+ and C1q −/− female mice. Estrous cycles were tracked for a minimum of 2 weeks by daily cytological analysis of vaginal smears. Mice were euthanized at proestrus and the fourth abdominal pair of mammary glands were dissected.

Carmine alum-stained wholemount mammary glands from C1q +/+ (Fig. 3.5A) and C1q −/− (Fig. 3.5B) mice exhibited basic ductal structures with minimal alveolar budding. The extent of branching was analysed by quantifying the branch points along a known length of primary ducts. There was no difference observed in the number of branch points in C1q +/+ when compared to C1q −/− mice at proestrus phase (2±0.1 C1q +/+ versus 2±0.1 branch points/mm C1q −/− mice, n=7-10) (Fig. 3.5C).

Paraffin embedded sections of mammary gland were stained with haematoxylin and eosin to identify percent alveolar epithelium per total epithelium (Fig. 3.5D-E). There was no difference in the percent of alveolar epithelium between genotypes (7.9±1.2% C1q +/+ versus 9.3±1.4% alveolar epithelium C1q −/− mice, n=7-9) (Fig. 3.5F). F4/80 staining was conducted to identify macrophage abundance and location between C1q +/+ (Fig. 3.6A) and C1q −/− mice (Fig. 3.6B) during proestrus. No positive staining was observed in the negative control (Fig. 3.6C). There was a significant reduction in the macrophage number within ductal stroma of C1q −/− mice compared to C1q +/+ mice (1874±175 C1q +/+ versus 1363±71 F4/80 positive cells/mm² C1q −/−, p=0.032, n=7-10) (Fig. 3.6D).

During mammary gland regression, epithelial cells undergo apoptosis. However, it is important to note that TUNEL positive apoptotic cells are infrequently observed in the cycling mouse mammary gland likely due to the rapid phagocytosis of these cells by macrophages (Fata et al. 2001, Chua et al. 2010). As anticipated, TUNEL positive apoptotic cells were observed infrequently in C1q +/+ (Fig. 3.7A) and C1q −/− mice (Fig. 3.7B) during proestrus. No positive TUNEL staining was observed in the negative control (Fig. 3.7C). No difference was observed in the density of TUNEL positive apoptotic cells within ductal stroma of C1q −/− mice compared to C1q +/+ mice at proestrus (740±196 C1q −/− versus 617±197 TUNEL positive cells/mm² C1q +/+ , n=4-6) (Fig. 3.7D).
Figure 3.5 Effect of C1q null mutation on ductal branching and alveolar development during the proestrus phase of the ovarian cycle

Representative carmine alum stained wholemount (A, B) and H&E stained images (D, E) of mammary glands of C1q \(^{+/+}\) (A, D) and C1q \(^{-/-}\) (B, E) adult female mice at the proestrus phase of the ovarian cycle. Arrows indicated alveolar epithelium (D, E). No difference in the number of branch points (C) (n=7-10) or percent of alveolar epithelium per total epithelium (F) (n=7-9) was observed between genotypes. Data shown as a mean±SEM of branch points/mm and percent of alveolar epithelium. Data were evaluated using independent samples T test.
Figure 3.6 Effect of C1q null mutation on macrophage abundance during the proestrus phase of the ovarian cycle

Representative images of F4/80 antibody staining indicative of macrophage positive cells (arrows) found within stroma surrounding mammary epithelial ducts of adult female C1q +/- (A) and C1q -/- (B) mice at the proestrus phase of the ovarian cycle, taken at 40X magnification. Negative control shows no F4/80 positive staining (C). Macrophage number was significantly reduced in C1q -/- compared to C1q +/- mice (D), n=7-11 per genotype. Data shown as a mean±SEM. Asterisk (*) indicates statistical significance (p<0.05) between C1q +/- and C1q -/- mice. Data were evaluated using independent samples T test.
Representative images of TUNEL positive staining indicative of apoptotic cells found within epithelium surrounding mammary epithelial ducts of adult female $C1q^{+/+}$ (A) and $C1q^{-/-}$ (B) mice at the proestrus phase of the ovarian cycle, taken at 40X magnification. Negative control shows no TUNEL positive staining (C). Abundance of TUNEL positive cells was not affected by genotype (D), n=4-6 per genotype. Data shown as a mean±SEM of TUNEL positive apoptotic cells/mm$^2$. Data were evaluated using independent samples T test.
3.2.3 Effect of C1q null mutation on hormone-mediated mammary gland regression

During mammary gland regression at the proestrus phase of the mouse estrous cycle, which is triggered by declining progesterone, it is hypothesised that C1q might play a role in facilitating rapid macrophage-mediated phagocytosis. However a difference in abundance of apoptotic cells at proestrus in C1q null mice was not observed (Section 3.2.2). This might be due to the variations in circulating estrogen and progesterone that occur between mice, and the difficulty in collecting mice at the precise point of the proestrus phase at which progesterone is declining. Macrophage-mediated phagocytosis of apoptotic epithelial cells is very rapid and highly coordinate by a complex signalling network (Savill et al. 1993, Elmore 2007, Ouyang et al. 2012). Therefore, variations in circulating hormones and the precise point of the estrous cycle at which the mice were dissected might be the reason for the lack of difference observed in apoptotic cells between C1q +/− and C1q −/− mice.

In the following experiments, we therefore utilised a hormone-induced mammary gland regression model in order to standardise the hormonal environment in the cycling mammary gland. The hormone replacement regimen was described earlier (Fig. 2.1) and is reported to mimic the morphological changes that occur across the mouse ovarian cycle (Hodson et al. 2013). As anticipated, the 0 h mouse cohort illustrated the induction of early alveolar development due to the administration of estradiol and progesterone daily for 3 days. Carmine alum-stained wholemount of wildtype C1q +/− (Fig. 3.8A) and C1q −/− (Fig. 3.8B) mice showed evidence of epithelial ducts with alveolar budding (arrows indicated), confirming that exogenous administration of estrogen and progesterone successfully induced early alveolar bud development in the mammary glands from both C1q +/− and C1q −/− mice. These wholemount images were further analysed for the number of ductal branch points by randomly selecting five primary ducts within the lymph node area and the numbers of secondary branches along a known length of primary duct were counted. There was no difference in the number of ductal branch points between these two genotypes at 0 h time point (3.1±0.1 C1q +/− versus 3.2±0.2 C1q −/− branch points/mm, n=7-11) (Fig. 3.8G).

Mammary glands from C1q +/− mice 24 h following administration of progesterone antagonist RU486 were showing signs of regression (Fig. 3.8C), demonstrated quantitatively by reduced ductal branch points compared to C1q +/− mice dissected at 0 h (Fig. 3.8G). However, regression appeared to be delayed in C1q −/− mice based on the wholemount images (Fig. 3.8D). This was confirmed quantitatively, as the 30% reduction in branch points between 0 h and 24 h in wildtype mice did not occur in C1q −/− mice, resulting in a significant difference in branching at 24 h between wildtype mice and C1q −/− mice (2.2±0.1 C1q +/− versus 3.2±0.5 C1q −/− branch points/mm, p=0.027, n=7-11) (Figure
Both genotypes exhibited a similar extent of ductal branching at 48 h following RU486 administration (2.5±0.1 C1q +/+ versus 2.6±0.1 C1q −/− branch points/mm, n=7-11) (Fig. 3.8E-G).

Paraffin embedded sections of C1q +/+ and C1q −/− female mice were stained with macrophage specific F4/80 antibody to quantify macrophage abundance. Previous studies reported that macrophages were localised in direct contact with ductal epithelium, alveolar epithelium, in the connective tissue and also found within the adipose tissue (Schwertfeger et al. 2006, Chua et al. 2010). F4/80-stained sections of both genotypes at 0 h illustrated recruitment of macrophages to the ductal stroma upon stimulation with progesterone and estradiol (Fig. 3.8E-G). There was no difference observed in macrophage abundance in the ductal stroma of wildtype mice compared to C1q −/− mice at 0 h time point (1620±176 C1q +/+ versus 1890±143 C1q −/− F4/80 positive cells/mm², n=8-9) (Fig. 3.10). Macrophages were also identified in C1q +/+ and C1q −/− mice treated with RU486 at 24 h and 48 h (Fig 3.9C-F). In wildtype mice, macrophage abundance was increased by 50% in 24 h compared to 0 h mouse cohorts (1620±176 versus 2535±106 F4/80 positive cells/mm² respectively, p=0.005, n=6-9) (Fig. 3.10). This increase was not observed in C1q −/− mice at 24 h, and there was a significant reduction of macrophage abundance in C1q −/− mice compared to wildtype mice at this time point (2535±106 C1q +/+ versus 1480±61 C1q −/− F4/80 positive cells/mm², p=0.002, n=6-7) (Fig. 3.10). Both genotypes demonstrated a similar abundance of macrophages at 48 h following RU486 administration (920±150 C1q +/+ versus 1088±153 C1q −/− F4/80 positive cells/mm², n=8-10) (Fig. 3.10).

Macrophages phagocytose dying epithelium and promote tissue remodelling during the regression phase. TUNEL staining was conducted to identify dying cells in hormone-treated C1q +/+ and C1q −/− mice at 0 h, 24 h and 48 h of the treatment protocol (Fig. 3.11A-F). TUNEL positive apoptotic cells were detected infrequently, similar to that observed in the cycling mouse mammary gland (Fig. 3.7) (Fata et al. 2001, Chua et al. 2010). Quantification of the abundance of TUNEL positive apoptotic epithelial cells revealed there was a significant increase in TUNEL positive cells between 0 h and 24 h in C1q −/− mice (p=0.013), which was not seen in wildtype mice (Fig. 3.12). Compared to wildtype mice, a 3.5-fold increase of TUNEL positive cells in the ductal epithelium of C1q −/− mice was observed at the 24 h time point following RU486 administration (260±78 C1q +/+ versus 924±277 C1q −/− TUNEL positive cells/mm², p=0.011, n=5-6) (Fig. 3.12).
Figure 3.8 Effect of C1q null mutation on ductal branching in mammary glands of ovariectomised hormone-treated mice

Representative carmine alum-stained wholmount images of mammary glands of ovariectomised and hormone-treated adult female C1q +/+ (A, C, E) and C1q −/− (B, D, F) mice. The mice were treated for 3 days with estradiol and progesterone and mammary glands collected on the fourth day for the 0 h mouse cohort (A, B). The other mouse cohorts were treated for 3 days with estradiol and progesterone, followed by RU486 (mifepristone), and mammary glands dissected at 24 h (C, D) and 48 h (E, F) following RU486 administration. Alveolar buds indicated by arrows. Data shown as a mean±SEM of branch points/mm (G), n=7-11 per group. Different letters (a, b) indicate statistical significance (p<0.05) between C1q +/+ and C1q −/− mice. Data were evaluated using general linear model test. LN, lymph node.
Figure 3.9 Effect of C1q null mutation on macrophage abundance in mammary glands of ovariectomised hormone-treated mice

Representative images of F4/80 antibody staining indicative of macrophage positive cells found within stroma surrounding epithelial ducts of ovariectomised and hormone-treated adult female C1q ++ (A, C, E) and C1q −/− (B, D, F) mice, taken at 20X magnification. 40X magnification shown in inset, with examples of F4/80 positive macrophages identified by arrows. The mice were treated for 3 days with estradiol and progesterone and mammary glands collected on the fourth day for the 0 h mouse cohort (A, B). The other mouse cohorts were treated for 3 days with estradiol and progesterone, followed by RU486 (mifepristone), and mammary glands dissected at 24 h (C, D) and 48 h (E, F) following RU486 administration.
Figure 3.10 Effect of C1q null mutation on macrophage abundance in mammary glands of ovariectomised hormone-treated mice

Quantification of F4/80 positive macrophages in the stroma surrounding epithelial ducts in ovariectomised hormone-treated adult female mice. The mice were treated for 3 days with estradiol and progesterone and mammary glands collected on the fourth day for the 0 h mouse cohort. The other mouse cohorts were treated for 3 days with estradiol and progesterone, followed by RU486 (mifepristone), and mammary glands dissected at 24 h and 48 h following RU486 administration. The number of F4/80 positive cells were calculated and expressed as macrophage/mm², n=6-10 per group. Data shown as a mean±SEM of F4/80 positive cells/mm². Different letters (a, b, c, d) indicate statistical significance (p<0.05) between groups. Data were evaluated using general linear model test.
Figure 3.11 Effect of C1q null mutation on abundance of TUNEL positive cells in mammary glands of ovariectomised hormone-treated mice

Representative images of TUNEL positive staining indicative of apoptotic cells found within epithelium surrounding mammary epithelial ducts in the mammary glands of ovariectomised and hormone-treated adult female C1q +/+ (A, C, E) and C1q -/- (B, D, F) mice, taken at 40X magnification. The mice were treated for 3 days with estradiol and progesterone and mammary glands collected on the fourth day for the 0 h mouse cohort (A, B). The other mouse cohorts were treated for 3 days with estradiol and progesterone, followed by RU486 (mifepristone), and mammary glands dissected at 24 h (C, D) and 48 h (E, F) following RU486 administration. TUNEL positive cells identified by arrows.
Figure 3.12 Effect of C1q null mutation on abundance of TUNEL positive cells in mammary glands of ovariectomised hormone-treated mice

Quantification of TUNEL positive cells in the stroma surrounding epithelial ducts in ovariectomised hormone-treated adult female mice. The mice were treated for 3 days with estradiol and progesterone and mammary glands collected on the fourth day for the 0 h mouse cohort. The other mouse cohorts were treated for 3 days with estradiol and progesterone, followed by RU486 (mifepristone), and mammary glands dissected at 24 h and 48 h following RU486 administration. Data shown as a mean±SEM of TUNEL positive apoptotic cells/mm², n=4-9 per group. Different letters (a, b) indicate statistical significance (p<0.05) between C1q +/- and C1q -/- mice. Data were evaluated using general linear model test.
3.2.4 Effect of C1q null mutation on mammary gland development and function during late pregnancy

To examine the role of C1q in pregnancy, virgin wildtype C1q +/+ and C1q −/− mice were housed with proven-fertile stud wildtype and C1q +/+ males respectively and checked each morning between 9:00 and 11:00 h for the presence of a vaginal copulatory plug (2:1 female to male ratio). On day 18 post-coitus, mice were killed by cervical dislocation and the fourth mammary glands from wildtype C1q +/+ and C1q −/− mice were collected and processed for immunohistochemistry analysis. Epithelial ducts with extensive alveolar budding were clearly evident as observed in wildtype (Fig. 3.13A) and C1q −/− mice (Fig. 3.13B) wholemounts. The effect of null mutation in C1q on alveolar development during pregnancy was investigated by manually quantifying the percentage area of alveolar clusters per mm² area of the wildtype (Fig. 3.13C) and C1q −/− (Fig. 3.13D) stained sections. There was no difference in the percentage of area containing alveolar clusters within the gland area between these two genotypes (25.2±2.4% C1q +/+ versus 26.7±1.7% alveolar cluster/mm² C1q −/−, n=8-9) (Fig. 3.13E).

To investigate the effect of C1q null mutation on macrophage abundance, the paraffin-embedded gland sections of wildtype C1q +/+ (Fig. 3.14A) and C1q −/− (Fig. 3.14B) mice were stained with macrophage specific F4/80 antibody. Macrophages were found in direct contact with the alveolar epithelium within the stroma (Fig. 3.14A-B) (Chua et al. 2010). There was no difference found in macrophage abundance within ductal stroma between wildtype and C1q −/− mice (427±54 C1q +/+ versus 554±91 F4.80 positive cells/mm² C1q −/−, n=8-9) (Fig. 3.14D).
Figure 3.13 Effect of C1q null mutation on alveolar development at day 18 post-coitus

Representative images of carmine alum-stained wholemounts of day 18 post-coitus mammary gland from C1q +/+ (A) and C1q −/− (B) mice. Stained sections from wildtype female C1q +/+ (C) and C1q −/− (D) mice showed similar alveolar development (E), n=8-9. Data shown as a mean±SEM of % alveolar epithelium/mm². Data were evaluated using independent samples T test.
Figure 3.14 Effect on C1q null mutation on macrophage abundance at day 18 post-coitus

Representative images of F4/80 staining indicative of macrophage positive cells (arrows) in direct contact with alveolar epithelium, within stroma of wildtype C1q⁺/⁺ (A) and C1q⁻/⁻ (B) mammary glands. Negative control shows no F4/80 positive staining (C). No difference was observed in macrophage abundance between C1q⁺/⁺ and C1q⁻/⁻ female mice (D), n=8-9. Data shown as a mean±SEM of F4/80 positive cells/mm². Data were evaluated using independent samples T test.
3.3 Discussion

C1q complement protein has been identified in the acute phase response during mammary gland involution (Stein et al. 2004), where we hypothesise that it has a role in opsonisation of dying epithelium and promotion of tolerance to self-antigens. C1q opsonisation of apoptotic epithelial cells leads to phagocytosis mediated by macrophages (Kim et al. 2003, Roos et al. 2004, Bottcher et al. 2006). These events are part of an immune defence mechanism, essentially to minimise tissue injury and promote tissue repair and normal mammary gland function (Eckersall 2000, Stein et al. 2004). However, it is now recognised that macrophages act not only as one of the key effectors in immunosurveillance, but also are implicated in mammary gland development (Cecchini et al. 1994, Pollard and Hennighausen 1994, Gouon-Evans et al. 2000, Gouon-Evans et al. 2002, Chua et al. 2010). These findings then raised a question of the role of C1q in mammary gland development.

To the best of our knowledge, this is the first study that has investigated effects of C1q null mutation on mammary gland development. By using C1q null mutant mice, here we describe the roles of C1q in the context of macrophage function in normal mammary gland development during puberty, ovarian cycling and pregnancy. The experiments presented in this chapter suggest that mammary gland development during puberty and pregnancy are not affected by C1q deficiency. Due to time constraints, the role of C1q was not investigated in clearance of dying cells during involution. However, utilisation of ovariectomised hormone-replaced mice revealed delayed mammary gland regression and an increase of apoptotic epithelial cells at the 24 h time point following induction of regression by RU486 administration as a consequence of C1q deficiency. Furthermore, C1q null mice also exhibited a reduced macrophage abundance at this time point. Hence, our results suggest that C1q plays critical roles during mammary gland regression, potentially through C1q-macrophage crosstalk to eliminate apoptotic bodies.

3.3.1 Deficiency in C1q does not affect developmental stages of mammary gland

The mammary gland is a unique organ as it undergoes the majority of its development postnatally. The stages of development include pubertal development, pregnancy, lactation and involution (Fata et al. 2001, Watson and Khaled 2008, Reed and Schwertfeger 2010, Hodson et al. 2013). At birth, the mammary gland consists of a hormonally responsive epithelium that is organised into a rudimentary network of ducts and remains largely quiescent until the onset of puberty (Sternlicht 2006, McNally and Martin 2011). Puberty is one of the major stages of mammary gland development. During puberty, there is a rapid outgrowth of ductal epithelium into the open expanse of the fat pad under the influence of hormones and growth factors (Ruan and Kleinberg 1999, Gallego et al. 2001, Trott et al. 2008, Macias and Hinck 2012). Pubertal development of the mammary gland is
associated with immune cell macrophages (Ingman et al. 2006). Here, we demonstrate normal pubertal mammary gland development in the absence of C1q. Ductal elongation, which indicates how far the gland has developed across the mammary fat pad, is not affected by C1q null mutation. In addition, these C1q−/− mice also exhibited a similar number of terminal end buds and proliferating epithelial cells compared to C1q+/- mice. Therefore, deficiency in C1q does not appear to affect mammary gland development during puberty.

Tissue remodelling and cellular turnover in the adult mammary gland not only actively occur during puberty, pregnancy and involution, but also over the course of the ovarian cycle. During this period, the naturally cycling mammary gland rotates through a sequence of development and regression every 4-5 days in mice and every 28 days in women (Schedin et al. 2000, Fata et al. 2001, Navarrete et al. 2005, Strange et al. 2007). Mammary regression, which is triggered by declining circulating progesterone due to breakdown of the ovarian corpus luteum (Hodson et al. 2013), occurs principally at the prooestrus phase in rats and mice, and the menstrual phase in humans (Fata et al. 2001). Our observation on mouse mammary gland regression at prooestrus found that C1q null mutation does not affect ductal branching and abundance of alveolar epithelium at this phase. Although there was a significant reduction in the macrophage density within ductal epithelium of C1q−/− mice, epithelial cell death was not affected. These findings suggest normal mammary gland development during prooestrus. However, there are some limitations in comparing the effect of progesterone decline in naturally cycling mice of different genotypes. Macrophages are important regulators of ovarian function and their activity and phenotypes are ovarian cycle stage dependent (Wu et al. 2004, Chua et al. 2010, Hodson et al. 2013). Hence, the reduced macrophage abundance observed in the absence of C1q may potentially exert subtle effects on mammary gland function. It is also essential to highlight that as macrophage-mediated phagocytosis is very rapid, it may be difficult to identify the time point of the natural estrous cycle at which C1q may be exerting a fairly subtle effect on phagocytosis. Natural variations in circulating concentration of estrogen and progesterone occur between mice, and this would make it difficult to accurately assess and standardise the precise stage of the ovarian cycle in mice at which progesterone is declining. This difficulty was overcome by using a standardised hormone environment in ovariectomised mice administered exogenous hormones, which is discussed in more detail in Section 3.2.3.

Pregnancy is a very complex process, marked with distinct immunological changes not only in the maternal but also fetal tissues. Data accumulated over the years have shown critical roles of the complement system in ensuring a normal and healthy pregnancy (Baines et al. 1974, Denny et al. 2013, Regal et al. 2015). A functioning complement system is also essential to maintain host defence.
to protect both the fetus and mother from infection (Regal et al. 2015). The placenta and semi-allogenic fetal tissue that both express histocompatibility paternal antigens are considered foreign by the maternal immune system. Hence, they present as a potential target for complement-mediated immune attack (Tedesco et al. 1993, Derzsy et al. 2010). Indeed, previous studies have demonstrated that pregnancy is associated with increased complement activation, with complement products including C1q, C3, C4, C5, C6, and C9 found to be deposited on the placental tissues (Faulk et al. 1980, Weir 1981, Tedesco et al. 1990, Bulla et al. 2008). An in vitro study of mouse trophoblasts observed C3 involvement in phagocytic activity, suggesting C3 has a role in trophoblast invasion of the decidua and endometrial blood vessels (Albieri et al. 1999).

C1q has been shown to be produced by trophoblasts and decidual endothelial cells (DECs), as well as human decidual stroma and synthesised invasive extravillous trophoblasts (Bulla et al. 2008, Agostinis et al. 2010). Thus, these indicate C1q plays a role at the fetal-maternal interface during pregnancy, such that it is likely to regulate differentiation of trophoblast and stromal cell lineage differentiation during the early stages of pregnancy (Madhukaran et al. 2015). C1q is also associated with different pregnancy complications. Mice lacking C1q on a C57BL/6 background exhibit key characteristics of preeclampsia such as hypertension, albuminuria, decreased placental vascular endothelial growth factor, and abnormal invasion of trophoblasts (Singh et al. 2011). These mice were also unable to clear apoptotic trophoblast cells, which in turn might lead to abnormal placentaion (Botto et al. 1998). Many of these studies documented the role of C1q in pregnancy outcomes and fetal-maternal tissue. However, none looked at the effect of C1q null mutation on development of the mammary gland during pregnancy. It should be noted that we did not see the effect of C1q null mutation on the previously documented pregnancy outcomes, despite studying mice on the same background strain, C57BL/6. However, it is possible that there were subtle effects of C1q deficiency on pregnancy which we did not specifically study. The reason for this difference in research findings is not clear, however, it suggests that other factors within the specific environment of the animal house or subtle genetic differences may affect the phenotypic outcome of C1q deficiency.

In the context of mammary gland development during pregnancy, we showed normal gland development, demonstrated by the lack of difference in the percentage of alveolar epithelium in mice lacking C1q. The epithelium undergoes extensive proliferation to form numerous secondary branches and early alveolar budding that then develop into milk-secreting elements during pregnancy. Under the continued presence of prolactin and other lactation-promoting hormones, these epithelial cells further differentiate to produce high quantities of milk to meet the increasing...
demand during lactation (Fata et al. 2001, Watson and Khaled 2008). In this study, C1q null mice were able to feed successive litters, hence suggesting a normal mammary gland development during pregnancy and lactation.

Previous work utilised the macrophage colony-stimulating factor 1 (CSF-1) deficient mouse model to demonstrate the essential role of macrophages during pregnancy (Pollard and Hennighausen 1994), as the mammary glands of these mice exhibited premature alveolar growth and milk protein expression. Depletion of macrophages in C1q null mice led to infertility due to failure in embryo implantation and impaired progesterone synthesis (Care et al. 2013). These studies highlight the contributions of macrophages to the sequence of reproductive events from early pregnancy to lactation. However, our studies suggest that C1q is not essential for macrophage-mediated mammary gland development during pregnancy. Our findings confirm that the mammary gland developmental stages including puberty, pregnancy and proestrus stage of estrous cycle are not affected by C1q null mutation. Therefore, any observed effects of C1q deficiency on mammary gland tumourigenesis in adult mice is not secondary to perturbed mammary gland development, which will be further investigated in Chapter 5 and 6.

3.3.2 Deficiency in C1q affects regression stages of mammary gland

The utilisation of a hormone-replacement model enables us to standardise analysis of the effect of C1q null mutation in the cycling mammary gland. This model mimics both the early alveolar development that occurs in the presence of rising progesterone, and the alveolar regression that occurs as progesterone falls (Hodson et al. 2013). We observed normal ductal branching, macrophage abundance and epithelial cell death in both C1q+/+ and C1q−/− mice stimulated with estrogen and progesterone, hence confirming hormone-induced mammary gland development in C1q deficient mice. This suggests that any abnormalities that occurred during the hormone-induced regression phase in C1q null mice was not a consequence of dysregulated mammary gland development prior to administration of progesterone antagonist RU486.

During the regression phase (proestrus in mice, menstrual phase in humans), apoptosis of the newly formed alveolar buds occurs, and the gland is remodelled back to its basic architecture (Fata et al. 2001). A role for macrophages is apparent during this phase as these cells are observed at high density within the regressing alveolar buds, in which they phagocytose dying epithelium and promote tissue remodelling (Chua et al. 2010, Hodson et al. 2013). In this study, we investigated the effect of C1q null mutation on the regression phase at two different time points; 24 h and 48 h following administration of RU486. Macrophage-mediated phagocytosis during apoptosis is a very rapid and
involves a highly orchestrated series of intercellular events coordinated by a complex signalling network (Kerr et al. 1972, Elmore 2007, Ouyang et al. 2012). The first 24 hour timeframe captures the early apoptotic epithelial cells, and is interpreted to represent the early process of macrophage-mediated phagocytosis. The second timeframe, 48 hours after RU486 administration, on the other hand, may represent the later phase of the apoptosis process.

In the absence of C1q, we observed an increase in the ductal branch points and TUNEL positive apoptotic cells within the ductal epithelium and reduced macrophage abundance at 24 h post-RU486 administration. These, however, were not seen at the 48 h time point, suggesting the effects of C1q null mutation on mammary gland regression is rapid and time-restricted. These temporal effects are an indication of a very high rate of cellular turnover during the regression phase, supporting a previous study investigating tissue remodelling and cellular turnover in the adult mammary gland (Fata et al. 2001).

Previous studies have demonstrated C1q to be a chemoattractant for monocytes and immature dendritic cells (Oiki and Okada 1988, Kuna et al. 1996, Vegh et al. 2006), and recently, Vogel and colleagues reported the migration capacities of macrophages towards a number of chemoattractants including CCL2, CCL5, CXCL10, CXCL12, and C1q (Vogel et al. 2014). Interestingly, C1q was shown to be the most powerful attractant for “M2” macrophages (Vogel et al. 2014), thus suggesting that the reduced macrophage abundance at 24 h time point within the ductal epithelium of C1q−/− mice may be a direct effect of C1q deficiency. Our finding also raises a question of potential macrophage migration to the mammary gland draining lymph nodes to promote a T helper cell-mediated immune response, which remains unclear due to limited knowledge of C1q roles in macrophage migration. Therefore, further studies dissecting macrophage abundance in the mammary gland draining lymph node would be informative to elucidate C1q roles in mediating macrophage migration.

There was an increase in the epithelial cell death observed in the C1q−/− mice at the 24 h time point, suggesting impaired phagocytosis by macrophages. Whether this effect was due to the lack of C1q that lead to an inefficient apoptotic machinery or reduced abundance of macrophages present to phagocytose dying epithelial cells, is not clear. Presence of these persistent apoptotic cells within the environment, without rapid clearance by macrophages, will pose risks to the mammary gland. These uncleared apoptotic cells might acquire necrotic properties that may lead to pro-inflammatory immune responses, as well as to the development of autoimmune disease (Kerr et al. 1972, Gregory and Devitt 2004, Krysko et al. 2006, Elmore 2007). Moreover, aberrant dead cell clearance is often
associated with pathobiology of many diseases (Elliott and Ravichandran 2010, Nagata et al. 2010, Green et al. 2016). Further studies to explore the impact of C1q in tolerance to self and non-self antigens in the mammary gland are required, and could utilise test antigens such as ovalbumin, or could model inflammatory processes such as mastitis.

Apoptosis is a very dynamic and highly regulated mechanism, thus the side effects or the immunological consequences of impaired cell death are not straightforward as it might be perceived. This, then may provide an explanation to the different responses observed in the mammary gland regression at proestrus. There was a similar effect of reduced macrophage density within ductal epithelium, as seen in the 24 h time point of the ovariectomised and hormone-treated mammary gland, but no effects of C1q null mutation observed on the ductal branching and epithelial cell death. One possible explanation could be that the effects we have seen at the proestrus phase are the results of hormone fluctuations across the ovarian cycle, leading to a less disrupted apoptotic machinery during mammary gland regression.

Together, these findings suggest delayed mammary gland regression due to null mutation in C1q. However, with this limited data, it is not possible to conclude whether this effect was solely due to the lack of C1q or due to a combined effects of C1q-macrophage interactions.

### 3.3.3 Limitations and future research directions

This study is limited by the little understanding of the regulation of C1q in the mammary gland. Further studies are required to investigate the underlying mechanisms of how C1q-macrophage crosstalk may affect the mammary gland and breast cancer susceptibility. It has become increasingly clear that the regulation of macrophage function and response can affect multistage tumourigenesis and pathogenesis of several inflammatory conditions (Biswas and Mantovani 2010, Valledor et al. 2010, Mantovani et al. 2013, Wynn et al. 2013). In addition, given that previous studies documented C1q as one of the key modulators in directing macrophage polarisation (Benoit et al. 2012, Galvan et al. 2012), further assessment of the “M1” and “M2” macrophage activation and polarisation states is required to provide a better insight into the basis of C1q-macrophage crosstalk in the mammary gland. Future experiments may utilise different novel markers of the “M1” macrophages including expression of IL12\textsuperscript{high}, IL23\textsuperscript{high} and IL10\textsuperscript{low} phenotype, inducible nitric oxide synthase (iNOS), production of reactive oxygen species (ROS), and pro-inflammatory cytokines such as tumour necrosis factor (TNF) (Qian and Pollard 2010, Locati et al. 2013). On the other hand, the “M2” macrophage state can be examined based on the IL10\textsuperscript{high}, IL12\textsuperscript{low} and IL23\textsuperscript{low} phenotype and expression of arginase-1, chitinase-3-Like Protein 3 or Ym1, CD206, CD163, Egr2 and c-Myc. Future
investigation may also utilise chemokine profiles to identify the activated states of macrophages as
the “M1” macrophages are generally characterised by Th1 cell-attracting chemokines such as
CXCL9 and CXCL10, whereas the “M2” macrophages are expressed chemokines CCL17, CCL22

In this series of experiments, we did not study mammary gland involution. Mammary involution is a
highly orchestrated process by which a fully functional, lactating mammary gland regresses to return
the gland to a quiescent, resting non-pregnant state (Watson 2006, Watson and Kreuzaler 2011).
Therefore, future studies may look at mammary involution in the absence of C1q to further our
understanding of the underlying C1q-macrophage crosstalk in the regression associated with
involution. Different models can be used to study the effect of C1q null mutation in mammary
involution including allowing the gland to naturally involute or inducing involution through forced
weaning. Teat sealing and weaning with concurrent pregnancy may be employed to further define
mammary involution in the absence of C1q.

Dying cells are usually rapidly taken up by phagocytes before chromosomal fragmentation (Savill et
al. 1993). Therefore, TUNEL-positivity for DNA fragmentation may not be the best cell death marker
to capture all dying epithelial cells as this is a late stage marker for cell death. However, TUNEL-
positivity was chosen from a broad range of apoptosis-detecting methods due to its availability,
reliability, specificity, and suitability to be used in paraffin-embedded tissues. The steps involved in
the TUNEL assay are user-friendly and highly reproducible. Hence, it is arguably the most standard
and common technique employed to identify nuclei containing fragmented DNA, enabling
quantification of apoptotic cells in histological tissue sections (Oberhaus 2003, Prochazkova et al.
2003, Loo 2011). Previous studies documented the lack of TUNEL assay sensitivity due to problems
associated with false-positively stained necrotic cells (Ansari et al. 1993, Didenko and Hornsby
1996). These findings highlight the importance of future studies that utilise combined detection
methods and a variety of death markers to distinguish stages of apoptosis and types of cell death
following induction of mammary gland regression. For instance, immunofluorescent staining using
Annexin V can be employed to detect externalisation of membrane phosphatidylserine (PtdSer).
Translocation of the membrane PtdSer from the inner to the outer surface of the plasma membrane
is one of the early key events of apoptosis (Ravichandran 2010, Segawa and Nagata 2015). Hence,
Annexin V that has high affinity for PtdSer, commonly serves as the sensitive marker detecting early
cell death. Simultaneous staining of Annexin V with vital dye such as propidium iodide (PI) can
discriminate cells undergoing different apoptotic phases in a mixed population using flow cytometry
(Vermes et al. 1995). Viable cells with intact membranes will exclude PI, whereas the membranes
of dead cells will stay permeable to PI, hence cells undergoing early apoptosis are Annexin V positive and PI negative, and cells that are in late apoptosis or undergoing necrosis are most likely to be Annexin V and PI positive. A number of biomarkers can also be used to distinguish apoptosis and necrosis. Molecular changes including lysosomal membrane permeabilisation and mitochondrial depolarisation during apoptosis can be detected using LysoTracker and tetramethylrosamine, respectively. Given that the activation of caspase enzymes is one of the distinctive features of the early stages of apoptosis (Fig. 1.4), plasma caspase-3 activity and caspase-cleaved fragment of cytokeratin 18 (CK18) can be indicative of early apoptotic cell death. Necrosis, on the other hand, can be identified using markers such as micro-RNA 122, full-length cytokeratin 18 (FK18), and high-mobility group box 1 (HMGB1) protein (Yang et al. 2014).

Employment of different detection techniques and markers will enable quantification and qualitative analysis of different cell death pathways. Simultaneously, it will provide information of the apoptotic or necrotic properties of the dying cells, hence generating further understanding on C1q-macrophage interactions during cell death in the mammary gland. Localisation of C1q in the mammary gland can be examined using anti-C1q antibody.

### 3.4 Conclusion

In summary, the findings reported in this chapter shed light on likely C1q-macrophage interactions that regulate mammary gland development and function. We have revealed that despite lacking C1q, the mammary gland develops normally, which is an indication that any effects of C1q null mutation on mammary gland tumourigenesis are not secondary to perturbed mammary gland development. We have also identified a role for C1q in mammary gland regression, and we propose that macrophage functions during the regression phase of the ovarian cycle may affect the ability of the immune system to protect the mammary gland from tumour formation. Our findings raise the question of significance of rapid phagocytosis for induction of tolerance during mammary gland regression, which is beneficial for mammary gland homeostasis. However, tolerance may not be ideal for protection against tumourigenesis. Hence, the role of C1q in mammary gland tumour development is further explored in the following chapter by utilising a tumourigenesis model with the carcinogenic agent DMBA.
Chapter 4 – Defining the Immune Response to DMBA Carcinogen Challenge in C1q Null Mutant Mice
4.1 Introduction

Components of both the innate and adaptive immune system participate in tumour immunosurveillance through the elimination of damaged or mutated cells, hence protecting the body from cancer (Diefenbach et al. 2000, Smyth et al. 2000, Girardi et al. 2001, Murphy et al. 2003, Yusuf et al. 2008). The innate immune response is the first line of defence against cancer by recognising molecular patterns found in cancer cells as opposed to the antigen-specific immune responses of adaptive immunity (de Visser et al. 2006). When a carcinogenic injury occurs, sentinel macrophages and mast cells undergo activation and release inflammatory mediators such as cytokines, chemokines, and bioactive mediators to recruit leukocytes to the site of injury. At the same time, antigen presenting cells (APCs) such as macrophages and dendritic cells take up antigens (including tumour antigens) and migrate to the draining lymph nodes and present the antigens to adaptive immune cells to stimulate naive, tumour-specific CD4+ and CD8+ T lymphocytes (de Visser et al. 2006, Johansson et al. 2008). Effector cells, including natural killer cells (NK cells) and gamma-delta T cells (γδ T cells) are capable of specifically recognising and killing tumour cells (Girardi et al. 2001, Gasser and Raulet 2006, Berzins and Ritchie 2014, Silva-Santos et al. 2015). T lymphocytes and NK cells secrete cytokines such as interferon gamma (IFNG) to modulate the immune response towards protection against tumour formation (Ikeda et al. 2002, Dunn et al. 2005). IFNG is a pro-inflammatory cytokine, originally characterised for its antiviral activities (Belardelli 1995, Gattoni et al. 2006). It is well known for its capabilities in activating innate and adaptive immune responses, modulating macrophage effector function, and inducing production of cytokines (Gattoni et al. 2006).

The complement system, one of the innate effectors of the immune response, also plays critical roles in tumour immunosurveillance. A number of studies have observed deposition of complement components at different tumour sites including breast, papillary thyroid, colorectal, and ovarian carcinoma (Markiewski and Lambris 2009, Stover 2010, Pio et al. 2014), suggesting the importance of complement activation in tumour protection. The complement system has been shown to control tumour development by promoting inflammation and exerting direct cytotoxic effects on tumour cells (Markiewski and Lambris 2009, Meyer et al. 2014, Taylor and Lindorfer 2014, Melis et al. 2015). Innate and adaptive immune responses, however, not only protect the body against tumour development but also have the capacity to promote tumour growth (Shankaran et al. 2001, Dunn et al. 2002). There is growing recognition that inflammatory responses can nurture developing malignancies in tissues before overt tumours are established through the release of reactive oxygen species (ROS) and reactive nitrogen intermediates (Kulkarni et al. 1993), which both have the capacity to induce DNA damage and genomic instability (Coussens and Werb 2002, Dunn et al.)
These observations demonstrate the remarkable complexity of the immune system; not only can it induce an anti-tumourigenic response, but it can also promote a pro-tumourigenic response depending on the signals it receives.

In addition to diverse roles in tumour initiation, studies have demonstrated that the immune system has important roles in mammary gland development and function. As previously mentioned, macrophages are implicated in the normal functioning of the mammary gland, hence the involvement of macrophages might affect the immune response to DNA damage in this tissue. The ability of the immune system to detect and eliminate pre-cancerous DNA-damaged cells might be impaired, or, conversely, inflammation might be enhanced, increasing the susceptibility of the breast to tumour formation.

An as yet unpublished study in our laboratory investigated the effect of C1q on mammary gland tumourigenesis, using C1q null mutant and wildtype control mice administered the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA). This study showed that C1q deficiency resulted in almost complete protection of the mammary gland against tumour formation (p=0.02) (Fig. 4.1) (Hodson, unpubl). This suggests a role for C1q in promoting mammary gland tumourigenesis. However, the underlying mechanism is yet to be elucidated. To address this, we challenged wildtype and C1q \(-/-\) mice with 1 mg dose of carcinogen DMBA weekly for six weeks, which was the same protocol used in the previous unpublished study to induce mammary tumour formation. The types of immune cells and how they are activated in the non-pregnant adult mammary gland, the mammary gland draining lymph nodes, para-aortic lymph nodes, cervical lymph nodes and the spleen in response to carcinogen-induced DNA damage were defined in wildtype mice and compared to C1q \(-/-\) mice. Comparison of immune responses in different tissues are essential to dissect the specific effects of C1q on mammary gland and mammary gland draining lymph nodes challenged with DMBA. This is the first step to understanding how the immune response to the carcinogen DMBA and the complement protein C1q affect a woman’s risk of developing breast cancer.
Figure 4.1 Effect of C1q null mutation on susceptibility to DMBA-induced mammary tumourigenesis

Six week-old C1q $^{+/+}$ and C1q $^{-/-}$ female mice were administered 1 mg DMBA in sesame oil by oral gavage once a week for 6 weeks and then monitored weekly by palpation to detect mammary tumour development. DMBA-treated C1q $^{-/-}$ mice were highly resistant to mammary tumourigenesis, with tumours detected in only 2 of 20 mice over the 30 weeks monitoring period, compared to 10 of 20 DMBA-treated control mice, n=20 females per group, p=0.02 (Hodson, unpubl). Data were evaluated using Kaplan-Meier test. Asterisk (*) indicates statistical significance (p<0.05) between both genotypes.
4.2 Results

Different sets of antibodies (Table 2.2) were used to define the immune response to DMBA carcinogen challenge in the wildtype and C1q−/− mice in the mammary gland, mammary gland draining lymph nodes (MG DLN), para-aortic lymph nodes (PALN), cervical lymph nodes (CLN) and spleen by flow cytometry. To comprehensively evaluate the effect of DMBA administration on T cell phenotype, a study was conducted to compare T cell phenotypes in untreated, vehicle-treated, and DMBA-treated C1q+/− and C1q−/− mice. The rationale for this was that C1q+/− and C1q−/− mice might have different T cell phenotypic profiles in the absence of any treatment, which might then affect T cell responses to DMBA. Furthermore, administration of vehicle (sesame oil) might cause changes in T cell phenotype in C1q−/− mice and/or C1q+/− mice, in which case, any differences between DMBA-treated C1q+/− and C1q−/− mice might be an artefact of sesame oil administration, not specifically DMBA.

4.2.1 Effect of C1q null mutation on CD3+ T lymphocyte populations

T lymphocytes have been a subject of great interest in different types of neoplasia such as melanoma, cervical, ovarian, gastric cancer (Zhang et al. 2003, Piras et al. 2005, Piersma et al. 2007, Lee et al. 2008), and also in breast cancer (Kuroda et al. 2005, Macchetti et al. 2006, Sheu et al. 2008). It was previously demonstrated that C1q deficiency affects the cytokine profile of antigen-specific T lymphocytes in mice immunised intraperitoneally with DNP-KLH (DNP, 2,4-dinitrophenyl; KLH, keyhole limpet hemocyanin) in alum (Cutler et al. 1998). However, the effects of C1q null mutation on T lymphocytes and phenotypes in carcinogen DMBA-challenged mice have never been investigated. Therefore, to determine the effect of carcinogen DMBA-challenge on T cell abundance in wildtype C1q+/− and C1q−/− mice, single cell suspensions of mammary gland draining lymph nodes, para-aortic lymph nodes, cervical lymph nodes and spleen were labelled with CD3, CD4 and CD8 antibodies for flow cytometry (Table 2.2). CD3 monoclonal antibody reacts with T cell receptor-associated CD3 complex expressed on thymocytes, mature T lymphocytes and natural killer T cells. Data was collected using FACS Diva software (BD Biosciences, San Jose, CA). Gates were applied to the forward scatter/side scatter density plots to exclude all debris and dead cells. An additional gate was applied to the CD3 histogram to identify CD3+ T cells (Figure 4.2A). Additional gates were also applied to CD4 (Fig. 4.2B) and CD8 (Fig. 4.2D) dotplots to identify CD4+ T helper and CD8+ T cytotoxic cells, respectively.

CD3+ T cells were found to be most prevalent in the mammary gland draining lymph nodes (70.4%), followed by para-aortic lymph nodes (65.8%), cervical lymph nodes (57.6%) and were least prevalent in the spleen (34.9%) (data not shown). In the mammary gland draining lymph nodes, the proportion
of CD3+ T cells was approximately the same regardless of any treatment in both C1q +/+ and C1q −/− mice. There was no significant difference observed in the percent of CD3+ T cell population in DMBA-treated C1q −/− when compared to DMBA-treated C1q +/+ mice (n=8-10 per group) (Fig. 4.3A). The untreated control C1q +/+ mice exhibited a similar proportion of CD3+ T cells as the vehicle control C1q −/− mice in the para-aortic lymph nodes, whilst a significant increase of CD3+ T cell population was found in C1q +/+ mice when challenged with DMBA compared to vehicle control mouse cohort (73.7±1.4% DMBA-treated C1q +/+ versus 58.4±3.2% vehicle control C1q +/+ mice, p=0.006, n=8-10) (Fig. 4.3B). This effect was not seen in C1q −/− mutant mice as the vehicle control C1q −/− and DMBA-treated C1q −/− mouse cohorts produced the same proportion of CD3+ T cells (66.6±5.3% vehicle control C1q −/− versus 65.6±2.1% DMBA-treated C1q −/− mice, n=8-10) (Fig. 4.3B). There was no significant difference in the CD3+ T cell population observed in DMBA-treated C1q +/+ and DMBA-treated C1q −/− mice in this tissue (Fig. 4.3B).

In the cervical lymph nodes, there was no effect of DMBA treatment observed in C1q −/− compared to vehicle control C1q −/− mice (Fig. 4.3C). Wildtype C1q +/+ mice, on the other hand, exhibited an increased percent of CD3+ T cell proportion due to DMBA treatment when compared to vehicle control wildtype mouse (68.2±1.8% DMBA-treated C1q +/+ versus 50.1±3.6% vehicle control C1q +/+ mice, p=0.007, n=8-10) (Fig. 4.3C). The DMBA-treated C1q +/+ and DMBA-treated C1q −/− mice demonstrated no significant difference in the CD3+ T cell population in this tissue (Fig. 4.3C).

The CD3+ T cell population in the spleen was approximately the same in each treatment group and in both genotypes (Fig. 4.3D), suggesting the splenic CD3+ T cell population was not affected by DMBA treatment. However, it was apparent that the CD3+ T cell population in the spleen was relatively low in both C1q −/− and C1q +/+ mice, regardless of treatment, compared to the other three tissues (Figure 4.3).

In summary of these complex results, there was no significant difference in the percentage of CD3+ T cell populations in all controls and DMBA-treated cohorts in the mammary gland draining lymph nodes. In the para-aortic lymph nodes, the CD3+ T cell population was found to be similar between both genotypes in the DMBA-treated cohort. A significant increase of CD3+ T cell population observed in the DMBA-treated C1q +/+ when compared to untreated and vehicle control C1q +/+ mice. This effect was not seen in C1q −/− mice. In the cervical lymph nodes, there was no significant difference in the percentage of CD3+ T cell population in all controls and DMBA-treated cohort between both genotypes. However, the CD3+ T cell population was increased in C1q +/+ mice challenged with DMBA compared to the untreated and vehicle control C1q +/+ mice. The percentage
of CD3+ T cell population in the spleen was approximately the same in vehicle control and DMBA-treated groups and in both genotypes, the only difference observed was in the untreated control cohort between both genotypes. The CD3+ T cell population in the spleen was relatively low in both C1q−/− and C1q+/* mice (about 25%-40%), regardless of treatments compared to the other three tissues. Overall, there were relatively minor differences between abundance of CD3+ T cells in C1q+/* compared to C1q−/− mice.
Figure 4.2 Gating strategy for analysis of CD3+ T cell markers

T cells were analysed by flow cytometry using the following gating strategy. The CD3 positive population (A) was selected and analysed for co-expression of CD4 (B) or CD8 (D). The CD4+ and CD8+ T cell populations were then analysed for percent of these producing cytokine IL10 or IFNG or double positive T cells (C, E).
Figure 4.3 Effect of C1q null mutation on abundance of CD3+ T cell population in lymph nodes and spleen in control and DMBA-treated mice

Female wildtype C1q +/+ and C1q −/− female mice received six doses of DMBA. Control mice received none (untreated) or sesame oil as vehicle control, with tissues dissected 24 hours following the final dose. The percentage of CD3+ T cell population was assessed in the mammary gland draining lymph nodes (A), para-aortic lymph nodes (B), cervical lymph nodes (C) and spleen (D) by flow cytometry. LN, lymph nodes. Data shown as a mean±SEM of n=8-10 per group. Different letters (a, b, c) indicate statistical significance (p<0.05) between groups. Data were evaluated using general linear model test.
4.2.2 Effect of C1q null mutation on CD3+ T lymphocyte phenotypes

In the mammary gland draining lymph nodes, there was no difference in the CD4 to CD8 ratio observed in either genotype in the untreated control or vehicle control groups (Fig. 4.4A). In comparison to untreated control and vehicle control C1q +/+ mice, DMBA-treated C1q +/- mice showed no significant difference in the ratio of CD4 to CD8 in the mammary gland draining lymph nodes (Fig. 4.4A). The ratio of CD4 to CD8 was found to be significantly increased in DMBA-treated C1q -/- compared to DMBA-treated C1q +/- mice by about 70% (1.1±0.1 DMBA-treated C1q +/- versus 1.9±0.2 DMBA-treated C1q -/- mice, p<0.001, n=8-10) (Fig. 4.4A), due to combination effects of greater abundance in CD4+ T cells and reduced percent of CD8+ T cells. This suggests a skewed T cell population towards greater abundance of CD4 T cells and lower abundance of CD8 T cells in C1q -/- mice compared to C1q +/- mice in response to carcinogen DMBA challenge.

In the para-aortic lymph nodes, the DMBA-treated C1q +/- mice exhibited an increase in the ratio of CD4 to CD8 of 70% when compared to vehicle control C1q +/- mice (1.2±0.1 DMBA-treated versus 0.7±0.04 vehicle control C1q +/- mice, p=0.008, n=8-10) (Fig. 4.4B). A similar response was observed in C1q -/- mice as the ratio of CD4 to CD8 increased in DMBA-treated C1q -/- compared to vehicle control C1q -/- mice (1.5±0.1 DMBA-treated versus 1.0±0.02 vehicle control C1q -/- mice, p=0.001) (Fig. 4.4B). In C1q -/- mice, there was a 25% increase in the ratio of CD4 to CD8 compared to C1q +/- mice following DMBA treatment (1.2±0.1 DMBA-treated C1q +/- versus 1.5±0.1 DMBA-treated C1q -/- mice, p=0.017, n=8-10) (Fig. 4.4B), indicating a differential T cell response to DMBA challenge in the para-aortic lymph nodes due to C1q null mutation.

In the cervical lymph nodes, DMBA treatment led to a significant increase in the CD4 to CD8 ratio observed in the C1q -/- mice compared to vehicle control of the same genotype (1.1±0.04 vehicle control C1q -/- versus 1.7±0.1 DMBA-treated C1q -/- mice, p=0.001, n=8-10), but this effect was not seen in the wildtype C1q +/- mouse cohort (Fig. 4.4C). Administration of carcinogen DMBA also led to an increased CD4 to CD8 ratio in the C1q +/- compared to C1q +/- mice in the cervical lymph nodes (1.7±0.1 DMBA-treated C1q +/- versus 1.1±0.1 DMBA-treated C1q +/- mice, p=0.001, n=8-10) (Fig. 4.4C), consistent with the effect we observed in the mammary gland draining lymph nodes (Fig. 4.4A) and para-aortic lymph nodes (Fig.4.4B).

In the spleen, there was no difference in the CD4 to CD8 ratio in the DMBA-treated C1q -/- compared to DMBA-treated C1q +/- mice (Fig. 4.4D). However, the administration of DMBA caused the T cell phenotype of both genotypes skewed to CD4 T helper cell population as there was a significant increase of CD4 to CD8 ratio in the DMBA-treated mice (1.2±0.03 vehicle control C1q -/- versus
1.9±0.08 DMBA-treated $C1q^{-/-}$ mice, $p<0.001$, $n=8-10$, (1.1±0.1 vehicle control $C1q^{+/+}$ versus 1.6±0.1 DMBA-treated $C1q^{+/+}$ mice, $p=0.013$, $n=8-10$) (Fig. 4.4D). The increase of CD4 to CD8 ratio was primarily due to a combined effect of upregulated CD4$^+$ T cell and downregulated CD8$^+$ T cell levels.

In summary of these complex results, in the mammary gland draining lymph nodes, the six DMBA administrations resulted in a significant increase in the ratio of CD4 to CD8 in $C1q^{-/-}$ compared $C1q^{+/+}$ mice ($p<0.001$). Similarly, this effect was observed in the para-aortic lymph nodes ($p=0.017$). There was no significant difference observed in the ratio of CD4 to CD8 in the cervical lymph nodes and spleen in DMBA-treated $C1q^{+/+}$ compared to DMBA-treated $C1q^{-/-}$ mice. Overall, these findings suggest a differential T cell response to DMBA challenge in the mammary gland draining lymph nodes and para-aortic lymph nodes due to $C1q$ null mutation and a skewed T cell population towards greater abundance of CD4 T cells and lower abundance of CD8 T cell in $C1q^{-/-}$ mice. These findings, together with our previous unpublished study demonstrating reduced tumour development in DMBA-treated $C1q$ null mice, suggest a role for the CD4$^+$ T cell subset in protecting $C1q$ deficient mice from DMBA-induced mammary tumourigenesis.
Figure 4.4 Effect of C1q null mutation on abundance of CD4+ and CD8+ T cell populations in lymph nodes and spleen in control and DMBA-treated mice

Female wildtype C1q +/+ and C1q −/− female mice received six doses of DMBA. Control mice received none (untreated) or sesame oil as vehicle control, with tissues dissected 24 hours following the final dose. Of the CD3+ population, the ratio of CD4+ to CD8+ T cells was assessed in the mammary gland draining lymph nodes (A), para-aortic lymph nodes (B), cervical lymph nodes (C) and spleen (D) were assessed by flow cytometry. LN, lymph nodes. Data shown as a mean±SEM of n=8-10 per group. Different letters (a, b, c) indicate statistical significance (p<0.05) between groups. Data were evaluated using general linear model test.
4.2.3 Intracellular cytokine detection by fluorescence-activated flow cytometry

Simultaneous analysis of cell surface markers and signalling proteins using flow cytometry can be conducted to characterise the nature of signalling responses within a defined cell population. This is possible due to the powerful technique of staining both extracellular surface markers and intracellular cytokines on the same cells. *Ex vivo* cells do not normally express sufficient quantities of cytokines for detection by flow cytometry; therefore, detection of intracellular cytokine by flow cytometry involves *in vitro* stimulation using polyclonal activators phorbol 12-myristate 13-acetate (PMA) and ionomycin (Jung *et al.* 1993, Nylander and Kalies 1999, Caraher *et al.* 2000, Pala *et al.* 2000, Baran *et al.* 2001, Freer and Rindi 2013) with the presence of a protein secretion inhibitor to trap the cytokines within the cells. In this study, we utilised GolgiPLug (10µg/mL) (BD Biosciences), which contains Brefeldin A (BFA) to inhibit intracellular transport of proteins, hence cytokines produced during activation are retained inside the cell (Ferrick *et al.* 1995, Openshaw *et al.* 1995, Picker *et al.* 1995).

Prior to analysis of the effect of C1q null mutation on T cell cytokine production, T cells isolated from lymph nodes and spleen were assessed for the impact of *ex vivo* stimulation by PMA and ionomycin on the cell surface expression of CD4 and CD8, and the effect of stimulation on cytokine production. This was done to determine whether the stimulation protocol had an effect on T cell phenotype. The unstimulated control sample also contained BFA. Unstimulated cells cultured without PMA and ionomycin were the control to show the level of residual cytokine synthesis from *in vivo* activation. Isotype controls were fluorochrome-conjugated isotype control antibodies used at matching concentrations to detect non-specific binding to cells due to the class of the mouse monoclonal antibody. Cell stimulation was performed at 37°C for 4 hours in a 5% CO₂ incubator, followed by staining of surface markers, fixation, permeabilisation and staining of intracellular cytokines with fluorochrome-conjugated antibodies.

Figure 4.5 and Figure 4.6 show data from *in vitro* CD4⁺ and CD8⁺ T cell stimulation, respectively, in the untreated control mouse cohort of both genotypes. In both of the negative controls, no label control and IgG1 APC-Cy7 isotype-matched control of irrelevant specificity, there was no detectable presence of IFNG⁺ cells (Fig. 4.5A, B). When analysing the effect of PMA/ionomycin *in vitro* stimulation, the double-labelling of unstimulated cells with anti-CD4 and anti-IFNG exhibited no expression of IFNG (Fig. 4.5C), unlike the stimulated cells (Fig. 4.5D). Intracellular IFNG production in CD4⁺ T cells was observed following PMA/ionomycin *in vitro* stimulation compared to the unstimulated cells in the mammary gland draining lymph nodes (Fig 4.5E). Likewise, the same effect was observed in the para-aortic lymph nodes (Fig. 4.5F) and the cervical lymph nodes (Fig. 4.5G).
Activated splenocytes showed quite an obvious increase of IFNG production in CD4+ T cells particularly in the C1q +/+ mice when compared to the unstimulated cells (Fig. 4.5H). A similar observation was also found for the CD8+ T cells as the in vitro stimulation with PMA/ionomycin with the continued presence of Brefeldin A in both genotypes, leading to a profound increase of cytokine IFNG in the mammary gland draining lymph nodes (Fig. 4.6E), para-aortic lymph nodes (Fig. 4.6F), cervical lymph nodes (Fig. 4.6G) and spleen (Fig. 4.6H). These findings, therefore showed that polyclonal activators PMA/ionomycin are able to activate T cells to produce a detectable level of cytokine production in both genotypes, supporting the previous studies found in the literature.
Figure 4.5 Effect of PMA and ionomycin stimulation on intracellular IFNG production in CD4+ T cells in lymph nodes and spleen in C1q+/+ and C1q−/− untreated mice

Representative dotplots of unlabelled cells as the negative control (A), cells labelled with rat IgG1 APC-Cy7 as the isotype-matched control (B), unstimulated cells cultured without PMA and ionomycin labelled with anti-IFNG (C), and cells stimulated with PMA and ionomycin labelled with anti-IFNG showed presence of CD4+ lymphocytes expressing IFNG (D). Differential responses of cytokine IFNG production in the unstimulated cells cultured without PMA and ionomycin and cells cultured with PMA and ionomycin were observed in the mammary gland draining lymph nodes (E), para-aortic lymph nodes (F), cervical lymph nodes (G) and spleen (H) in both genotypes. Data shown as a mean±SEM of n=8 per group. Different letters (a, b, c) indicate statistical significance (p<0.05) between groups. Data were evaluated using general linear model test.
Figure 4.6 Effect of PMA and ionomycin stimulation on intracellular IFNG production in CD8\(^+\) T cells in lymph nodes and spleen in C1q\(^{+/+}\) and C1q\(^{-/-}\) untreated mice

Representative dotplots of unlabelled cells as the negative control (A), cells labelled with rat IgG1 APC-Cy7 as the isotype-matched control (B), unstimulated cells cultured without PMA and ionomycin labelled with anti-IFNG (C), and cells stimulated with PMA and ionomycin labelled with anti-IFNG showed presence of CD8\(^+\) lymphocytes expressing IFNG (D). Differential responses of cytokine IFNG production in the unstimulated cells cultured without PMA and ionomycin and cells cultured with PMA and ionomycin were observed in the mammary gland draining lymph nodes (E), para-aortic lymph nodes (F), cervical lymph nodes (G) and spleen (H) in both genotypes. Data shown as a mean±SEM of n=8 per group. Different letters (a, b, c) indicate statistical significance (p<0.05) between groups. Data were evaluated using general linear model test.
4.2.4 Effect of PMA/ionomycin in vitro stimulation on CD4 and CD8 expression

Previously, analysis of cytokine production within a specific target cell has been possible with different techniques used including the Enzyme-Linked ImmunoSpot assay (ELISPOT), immunochemistry, limiting dilution analysis, single cell PCR, and in situ hybridisation (Carter and Swain 1997, Pala et al. 2000). However, these techniques require high technical proficiency and involve tedious data collection and analysis. The emergence of multicolour flow cytometry technology has enabled a better and improved analytic technique in which single cells can be simultaneously analysed for several parameters including size, granularity, expression of surface markers, and intracellular cytokines and proteins (Jung et al. 1993, Vikingsson et al. 1994, Prussin and Metcalfe 1995, Carter and Swain 1997). It is not only a highly specific and sensitive technique, multicolour flow cytometry is also able to rapidly analyse a large, heterogeneous population of target cells at a time (Picot et al. 2012). The flow cytometric analysis of T-cell cytokine production involves a few basic principles; (1) in vitro stimulation of T cells with PMA/ionomycin with a continued presence of protein transport inhibitor to prevent cytokine release, (2) fixation and permeabilisation, (3) intracellular staining, followed by flow cytometry analysis (Jung et al. 1993).

Cell activation with PMA and ionomycin has been shown to cause a downregulation of CD4+ T cell surface expression (Petersen et al. 1992, Ito et al. 1997, Pala et al. 2000, Baran et al. 2001). This was reported in human CD4+ T cell clone HA1.7 that was previously stimulated with an increasing concentration of PMA and ionomycin for 4 hours in the presence of 0, 2, 10 or 50 µM monensin. A progressive downregulation of CD4+ cell surface expression was observed as the PMA doses increased not only in the absence of monensin, but also in its presence (Pala et al. 2000). In vitro stimulation of human CD4+ T cell clone AC1.1 with PMA and ionomycin also caused a reduction of CD4+ T cell expression from 99% in the resting cells, to 13.2% by the end of 6 hours stimulation. In addition, activation with these agents also altered cell surface morphology and caused a dramatic downregulation of CD4 cell surface expression on stimulated peripheral blood mononuclear cells (PBMCs) isolated from healthy donors (Baran et al. 2001). We therefore investigated the effect of PMA/ionomycin stimulation on CD4 cell surface expression on mouse lymphocytes. When comparing the unstimulated and stimulated cells of C1q +/+ and C1q −/− mice, there was no significant difference observed in the percent of CD4+ T cells either in the untreated control (Fig. 4.7A, B), vehicle control (Fig. 4.7C, D) or DMBA-treated (Fig. 4.7E, F) mouse cohorts. We further analysed the effect of PMA/ionomycin on CD8 cell surface expression on lymphocytes. Similarly, the C1q +/+ and C1q −/− mice showed no significant difference in the percentage of CD8 cell surface expression...
in the unstimulated compared to stimulated cells in all tissues regardless of the treatments (Fig. 4.8A-F).

These findings confirmed that any observed CD4 and CD8 T cell abundance in this study was the real effect of DMBA challenge in both genotypes, not due to the effect of the PMA/ionomycin *in vitro* stimulation on the expression of these cell surface markers.
Figure 4.7 Effect of PMA/ionomycin in vitro stimulation on CD4 expression by T cells isolated from lymph nodes and spleen of C1q \( ^{+/+} \) and C1q \( ^{-/-} \) untreated, vehicle-treated and DMBA-treated mice.

Percent CD4\(^+\) expressing CD3\(^+\) T cells without in vitro stimulation compared to PMA/ionomycin in vitro-stimulation in lymph nodes and spleen from C1q \( ^{+/+} \) (A, C, E) and C1q \( ^{-/-} \) (B, D, F) female mice. The mice were either untreated (A, B), vehicle-treated (C, D), or DMBA-treated (E, F). Data shown as a mean±SEM of n=8-10 per group. Data were evaluated using independent student T test, no significant difference was observed for all groups.
Figure 4.8 Effect of PMA/ionomycin in vitro stimulation on CD8 expression by T cells isolated from lymph nodes and spleen of C1q^{+/+} and C1q^{-/-} untreated, vehicle-treated and DMBA-treated mice.

Percent CD8^{+} expressing CD3^{+} T cells without in vitro stimulation compared to PMA/ionomycin in vitro-stimulation in lymph nodes and spleen from C1q^{+/+} (A, C, E) and C1q^{-/-} (B, D, F) female mice. The mice were either untreated (A, B), vehicle-treated (C, D), or DMBA-treated (E, F). Data shown as a mean±SEM of n=8-10 per group. Data were evaluated using independent student T test, no significant difference was observed for all groups.
4.2.5 Effect of C1q null mutation on IFNG-producing T cells following DMBA administration

A previous study reported a significant reduction of IFNG production but equal level of interleukin-4 cytokine (IL4) secretion by primed T lymphocytes in C1q−/− mice compared with control mice (Cutler et al. 1998). However, at present, little is known about the effect of DMBA challenge on cytokine production by T cells in the C1q+/+ and C1q−/− mice. To investigate abundance of IFNG-producing T cells in C1q null mice, mammary gland draining lymph nodes (inguinal), para-aortic lymph nodes, cervical lymph nodes and spleens were harvested 24 hours after the sixth DMBA administration. The tissues were enzymatically digested to a single cell suspensions and stimulated *in vitro* with PMA/ionomycin for 4 hours. Cells were subsequently labelled with CD3, CD4 and CD8 antibodies and subjected to intracellular staining for IFNG. We have shown previously that there was no effect of PMA/ionomycin stimulation on CD4 and CD8 cell surface expressions on mouse lymphocytes. Hence, the findings observed in these experiments were the real effects of carcinogen DMBA challenge to both genotypes.

The full data set of all treatment groups, genotypes and tissues is shown in Table 4.1. The analysis also investigated the percentage of IL10-producing CD4+ and CD8+ T cell populations following DMBA administration. The experimental design has been validated and it was confirmed that the antibody did detect IL10 successfully. However, there was a very low percentage of IL10 positive events detected in both T cell subsets in both genotypes across all four tissues (Table 4.1), leading to difficulties in comparing and interpreting the IL10 cytokine profile between the wildtype C1q+/+ and C1q−/− mice when challenged with the carcinogen DMBA. Therefore, this was not analysed further. In addition, analyses of the mean fluorescent intensity for IL10 and IFNG (Table 4.1) were also performed to investigate any shift in the fluorescent intensity of these phenotypic markers. There were no significant differences detected between both genotypes.

In the untreated mammary gland draining lymph nodes of both genotypes, there was less than 1% of IFNG-producing CD4+ T cells (0.8±0.1% untreated control C1q+/+ versus 0.5±0.03% untreated control C1q−/− mice, n=8) (Fig. 4.9A) (Table 4.1), illustrating the basal level of this cytokine produced from *in vivo* activation, in resting and unchallenged immune microenvironment. When comparing the effect of DMBA administration between C1q+/+ and C1q−/− mice, there was a significant increase of IFNG-producing CD4+ T cells observed in the C1q−/− mice (2.2±0.3% DMBA-treated C1q+/+ versus 6.9±1.9% DMBA-treated C1q−/− mice, p=0.014, n=6-10) (Fig. 4.9A, Table 4.1). This finding may be due to the skewed T cell population to CD4+ T cell subset, previously observed in the mammary gland draining lymph nodes (Fig 4.4A), which then suggests IFNG-producing CD4 T helper cells might help mediate the reduced susceptibility to mammary tumourigenesis in the absence of C1q.
In the para-aortic lymph nodes, the percent of IFNG-producing CD4+ T cells was approximately the same for the untreated and vehicle control mice, not only in the C1q +/- mice, but also in the C1q -/- mouse cohort (Fig. 4.9B). There was no significant difference in the IFNG-producing CD4+ T cells in response to DMBA challenge in C1q +/- mice when compared to the vehicle control or untreated control. C1q -/- mice exhibited a non-significant trend of increased IFNG-producing CD4+ T cells in response to DMBA treatment compared to untreated control C1q -/- (p=0.057) and vehicle control C1q -/- (p=0.06) mouse cohorts (Fig. 4.9B). Approximately 5% of CD4+ T cells produced IFNG in response to six DMBA administrations in both genotypes (Fig. 4.9B, Table 4.1).

When analysing the level of IFNG-producing CD4+ T cells in the cervical lymph nodes, the abundance of these cells was relatively lower than the other three tissues regardless of the treatments. The highest level observed was only about 2.5% in the DMBA-treated C1q +/- mice (Fig. 4.9C), whilst in the spleen, untreated control C1q +/- mice already exhibited approximately 6% of IFNG-producing CD4+ T cells (Fig. 4.9D, Table 4.1). IFNG-producing CD4+ T cells in C1q -/- spleen was lower compared to C1q +/- mice regardless of treatment group (Fig. 4.9D). There was also a trend towards a reduction in the IFNG-producing CD4+ T cells in response to DMBA challenge in C1q -/- compared to C1q +/- mice in the spleen (p=0.077) (Fig. 4.9D), suggesting a role for IFNG cytokine in C1q -/- mice during early mammary tumorigenesis.

Investigation of the level of IFNG-producing CD8+ T cell subset revealed a greater percentage of IFNG compared to CD4+ T cell in untreated control C1q +/- mice for all lymph nodes and spleen tissues (Fig. 4.10A-D). This is quite interesting as the abundance of the CD8+ T cell subset was previously found to be relatively lower than the CD4+ T cell subset (Fig. 4.4). The mammary gland draining lymph nodes and para-aortic lymph nodes of the untreated control C1q +/- mice recorded about 5% and 7% IFNG-producing CD8+ T cells, respectively (Fig. 4.10A, B, Table 4.1). Whilst in the untreated cervical lymph nodes and spleen, higher percentages of IFNG-producing CD8+ T cells were observed (7.8±2.0% and 11.8±3.3% respectively) (Fig. 4.10C, D, Table 4.1). In contrast, all tissues of untreated control C1q -/- mice exhibited a lower percentage of IFNG-producing CD8+ T cell subset with approximately 3-4% recorded. The spleens of C1q -/- mice exhibited a reduced level of IFNG-producing CD8+ T cell subset compared to untreated control C1q +/- mice, hence raising a question of in vivo IFNG production in the absence of C1q (11.8±3.3 untreated C1q +/- versus 4.1±1.0 untreated C1q -/-, p=0.034, n=10) (Fig. 4.10D, Table 4.1).

There was no effect of treatments on IFNG-producing CD8+ T cells in the mammary gland draining lymph nodes as the vehicle control and DMBA-treated mouse cohorts showed no difference to the
untreated control mouse cohorts (Fig. 4.10A). A similar effect was observed in the para-aortic lymph nodes (Fig. 4.10B) and spleen (Fig. 4.10D), but in the cervical lymph nodes, IFNG-producing CD8+ T cells were reduced in DMBA-treated C1q +/- when compared to vehicle control C1q +/- mice (5.4±1.0% versus 10.8±0.8% respectively, p=0.02, n=10) (Fig. 4.10C, Table 4.1). There was no overt effect of DMBA treatment on the IFNG-producing CD8+ T cells in all tissues of C1q +/- and C1q +/- mice (Fig.4.10A-D).

In summary, these results demonstrate that in the mammary gland draining lymph nodes, there was less than 1% of IFNG-producing CD4+ T cells in the untreated mouse cohort of both genotypes, indicative of the basal level of IFNG produced from in vivo activation, in resting and unchallenged immune microenvironment. There was a significant increase of IFNG-producing CD4+ T cells observed in the DMBA-treated C1q +/- compared to DMBA-treated C1q -/- mice (p=0.014). In the para-aortic lymph nodes, the IFNG-producing CD4+ T cell abundance was unaffected in both genotypes following six administrations of DMBA, a similar effect observed in the cervical lymph nodes and spleen. The percentage of IFNG-producing CD4+ T cells in the cervical lymph nodes was relatively lower than the other three tissues regardless of the treatments. In the spleen, there was a trend towards a reduction in the IFNG-producing CD4+ T cell population in response to DMBA challenge in C1q -/- compared to C1q +/- mice (p=0.077). With regards to the CD8+ T cell population, in the mammary gland draining lymph nodes, there was no significant difference observed in the percentage of IFNG-producing CD8+ T cell population in all controls and DMBA-treated cohort for both genotypes. Similarly, there was no effect of treatments on IFNG-producing CD8+ T cell population in the para-aortic lymph nodes. In the cervical lymph nodes, the abundance of IFNG-producing CD8+ T cells was significantly reduced in DMBA-treated C1q +/- compared to vehicle control C1q +/- mice (p=0.02). Overall, these findings reveal no overt effect of DMBA challenge on the IFNG-producing CD8+ T cell populations, however they do demonstrate phenotypic activation of CD4+ T cells and suggest a key role of IFNG in protecting the mammary gland from tumour initiation and development in the absence of C1q.
Table 4.1 Phenotype of CD4+ and CD8+ T cells in control and DMBA-treated C1q ++/− and C1q −/− mice

The percentage of IL10-producing CD4+ and CD8+ T cells and IFNG-producing CD4+ and CD8+ T cells in the mammary gland draining lymph nodes (MG DLN), para-aortic lymph nodes (PALN), cervical lymph nodes (CLN) and spleen. Mean fluorescent intensity (MFI) was measured to demonstrate any shift in fluorescent intensity of IL10 and IFNG in the T cell population. Data shown as a mean±SEM of n=7-10 females per group. Data were evaluated using general linear model test. Asterisks (*) indicate statistical significance (p<0.05) between both genotypes.

<table>
<thead>
<tr>
<th></th>
<th>Untreated control</th>
<th>Vehicle control</th>
<th>DMBA-treated</th>
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<tbody>
<tr>
<td></td>
<td>C1q ++</td>
<td>C1q −/−</td>
<td>C1q ++</td>
</tr>
<tr>
<td>MG DLN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% IL10</td>
<td>0.02 ± 0.03</td>
<td>0.09 ± 0.02</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>MF IL10</td>
<td>878 ± 89</td>
<td>751 ± 67</td>
<td>627 ± 292</td>
</tr>
<tr>
<td>% IFNG</td>
<td>5.1 ± 0.9</td>
<td>2.7 ± 0.4</td>
<td>8.5 ± 0.5</td>
</tr>
<tr>
<td>MF IFNG</td>
<td>480 ± 38</td>
<td>522 ± 37</td>
<td>522 ± 7</td>
</tr>
<tr>
<td>PALN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% IL10</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>MF IL10</td>
<td>629 ± 202</td>
<td>916 ± 349</td>
<td>639 ± 203</td>
</tr>
<tr>
<td>% IFNG</td>
<td>1.7 ± 0.4</td>
<td>1.0 ± 0.1</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>MF IFNG</td>
<td>544 ± 42</td>
<td>627 ± 54</td>
<td>681 ± 22</td>
</tr>
<tr>
<td>CLN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% IL10</td>
<td>0.01 ± 0.01</td>
<td>0.05 ± 0.02</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>MF IL10</td>
<td>724 ± 223</td>
<td>577 ± 102</td>
<td>543 ± 51</td>
</tr>
<tr>
<td>% IFNG</td>
<td>7.3 ± 1.5</td>
<td>3.3 ± 0.5</td>
<td>8.9 ± 0.6</td>
</tr>
<tr>
<td>MF IFNG</td>
<td>472 ± 31</td>
<td>537 ± 25</td>
<td>537 ± 11</td>
</tr>
<tr>
<td>SLEEN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% IL10</td>
<td>0.01 ± 0.01</td>
<td>0.02 ± 0.04</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>MF IL10</td>
<td>323 ± 646</td>
<td>646 ± 60</td>
<td>673 ± 123</td>
</tr>
<tr>
<td>% IFNG</td>
<td>1.6 ± 0.4</td>
<td>0.8 ± 0.1</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>MF IFNG</td>
<td>690 ± 34</td>
<td>612 ± 43</td>
<td>694 ± 12</td>
</tr>
<tr>
<td>SLEEN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% IL10</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>0.11 ± 0.01</td>
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<tr>
<td>MF IL10</td>
<td>273 ± 509</td>
<td>1259 ± 630</td>
<td>469 ± 86</td>
</tr>
<tr>
<td>% IFNG</td>
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<td>7.4 ± 1.0</td>
</tr>
<tr>
<td>MF IFNG</td>
<td>814 ± 37</td>
<td>612 ± 32</td>
<td>821 ± 13</td>
</tr>
<tr>
<td>SLEEN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% IL10</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>MF IL10</td>
<td>339 ± 53</td>
<td>227 ± 11</td>
<td>900 ± 487</td>
</tr>
<tr>
<td>% IFNG</td>
<td>11.8 ± 3.3</td>
<td>4.1 ± 1.0</td>
<td>17.1 ± 1.2</td>
</tr>
<tr>
<td>MF IFNG</td>
<td>549 ± 25</td>
<td>561 ± 27</td>
<td>623 ± 11</td>
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</table>
Figure 4.9 Effect of C1q null mutation on abundance of IFNG-producing CD4+ T cells in lymph nodes and spleen in control and DMBA-treated mice

Wildtype C1q +/+ and C1q −/− female mice received six doses of DMBA. Control mice received none (untreated) or sesame oil as vehicle control, with tissues dissected 24 hours following the final dose. The percentage of IFNG production in the CD3+CD4+ T cell population was assessed in the mammary gland draining lymph nodes (A), para-aortic lymph nodes (B), cervical lymph nodes (C) and spleen (D) by flow cytometry. LN, lymph nodes. Data shown as a mean±SEM of n=6-10 females per group. Different letters (a, b, c) indicate statistical significance (p<0.05) between groups. Data were evaluated using general linear model test.

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Figure 4.10 Effect of C1q null mutation on abundance of IFNG-producing CD8+ T cells in lymph nodes and spleen in control and DMBA-treated mice

Wildtype C1q +/+ and C1q −/− female mice received six doses of DMBA. Control mice received none (untreated) or sesame oil as vehicle control, with tissues dissected 24 hours following the final dose. The percentage of IFNG production in the CD3+CD8+ T cell population was assessed in the mammary gland draining lymph nodes (A), para-aortic lymph nodes (B), cervical lymph nodes (C) and spleen (D) by flow cytometry. LN, lymph nodes. Data shown as a mean±SEM of n=7-10 per group. Different letters (a, b, c, d) indicate statistical significance (p<0.05) between groups. Data were evaluated using general linear model test.
4.2.6 Effect of C1q null mutation on F4/80+ macrophage population and phenotypes in the mammary gland

Previous investigations focused on the phenotype of T lymphocytes and their cytokine production within the mammary gland draining lymph nodes, para-aortic and cervical lymph nodes and spleen in response to carcinogen DMBA challenge in absence of C1q. These may not define the whole process of the immune response to DMBA challenge. Hence, we also investigated the effect of DMBA administration on the immune cell population in the mammary gland from both C1q+/+ and C1q−/− mice.

To determine the effect of carcinogen DMBA challenge on the abundance and phenotypes of CD45+ population in C1q+/+ and C1q null mutant mice, the fourth pair mammary glands were dissected 24 hours after the final dose of DMBA. The glands were enzymatically digested and stained with CD45, F4/80 and MHC Class II antibodies (Table 2.2). Data was collected using FACS Diva software (BD Biosciences, San Jose, CA). Viability dye DAPI (Vector Laboratories, Burlingame, USA) was used to identify and exclude dead cells. A gate was applied to the CD45 histogram to identify cells positive for CD45+ leukocytes (Fig. 4.11A). Additional gates were applied to CD45+/F4/80+ density plot to identify cells positive for F4/80 (Fig. 4.11B). Macrophage specific F4/80+ cells were then phenotyped for MHC Class II (Fig. 4.11C) and Macrophage Scavenger Receptor 1 (MSR1) (MCA1322B) cell surface expressions (Fig. 4.11D). Using splenocytes, it was confirmed that the antibody did detect MSR1 successfully, and MSR1 was in abundance (Fig. 4.11E), however within the mammary gland only a very low number of macrophage cells were identified as positive for MSR1. Whether this was truly indicative of reduced MSR1 expression on macrophages in the mammary gland in response to DMBA challenge, it is hard to interpret and remains unclear due to a very low number of positive events. Therefore, this was not analysed further.

The population of CD45+ leukocytes in the vehicle control mice did not differ significantly to untreated control mice in either genotype (Fig. 4.12A). Following DMBA treatment, the population of CD45+ leukocytes in the mammary gland was reduced in C1q−/− mice compared to untreated control C1q−/− (p=0.002) and vehicle control C1q−/− mice (p=0.002) (Fig. 4.12A). DMBA-treated C1q−/− mice also exhibited a significant reduction in CD45+ leukocytes population compared to DMBA-treated C1q+/+ mice (33.9±3.1% DMBA-treated C1q+/+ versus 8.7±2.0% DMBA-treated C1q−/− mice, p=0.024, n=8-10) (Fig. 4.12A, Table 4.2).

The prevalence of macrophages in the mammary gland was analysed using the macrophage specific antibody F4/80 (Table 2.2). There was no difference in the macrophage proportion in both genotypes
in response to DMBA challenge (Fig. 4.12B). However, when the macrophage populations were analysed for MHC Class II expression, MHCII activation marker expression on macrophages was significantly reduced in the DMBA-treated C1q−/− compared to wildtype mice (50.4±3.4 DMBA-treated C1q+/- versus 35.0±5.0% DMBA-treated C1q−/− mice, p=0.036, n=10) (Fig. 4.12C, Table 4.2). However, there was no significant difference in the mean fluorescence intensity of MHC Class II in any of the groups (Table 4.2). Overall, these findings indicate a striking reduction in the abundance of CD45+ leukocyte population and MHCII phenotypic expression on macrophages in the mammary gland of DMBA-treated C1q null mice compared to similarly treated C1q+/- mice.
Figure 4.11 Gating strategy for analysis of CD45+ leukocytes in the mammary gland

CD45+ leukocytes were analysed by flow cytometry using the following gating strategy. Viability dye DAPI was used to identify dead cells which were excluded before the CD45 gate was applied. CD45+ population (A) was analysed to identify cells positive for F4/80 (B). The F4/80 positive population was analysed for MHC Class II and Macrophage Scavenger Receptor 1 (MCA1322B) positivity to quantify CD45+ F4/80+ cells expressing MHC Class II (C) or MSR1 (D). Moderate and high MSR1 expression on F4/80 positive splenocytes as the positive control (E).
Wildtype C1q $^{+/+}$ and C1q $^{-/-}$ female mice received six doses of DMBA. Control mice received none (untreated) or sesame oil as vehicle control, with mammary glands dissected 24 hours following the final dose. Cells were labelled with CD45 (A), F4/80 (B) and MHC Class II (C) antibodies. Data shown as a mean±SEM of n=7-10 per group. Different letters (a, b, c, d) indicate statistical significance (p<0.05) between groups. Data were evaluated using general linear model test.
Wildtype $C1q^{+/+}$ and $C1q^{-/-}$ female mice received six doses of DMBA. Control mice received none (untreated) or sesame oil as vehicle control. Cells were labelled with CD45, F4/80 and MHC Class II antibodies. Mean fluorescent intensity (MFI) was measured to quantify fluorescent intensity of surface marker MHCII in the F4/80$^+$ macrophage population. Viability dye DAPI was used to identify and exclude dead cells. Data shown as a mean±SEM of n=7-10 females per group. Data were evaluated using general linear model test. Asterisks (*) indicate statistical significance (p<0.05) between both genotypes.

<table>
<thead>
<tr>
<th>DAPI$^+$</th>
<th>Untreated control</th>
<th>Vehicle control</th>
<th>DMBA-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C1q^{+/+}$</td>
<td>$C1q^{-/-}$</td>
<td>$C1q^{+/+}$</td>
</tr>
<tr>
<td>% CD45$^+$</td>
<td>42.9 ± 7.3</td>
<td>40.7 ± 3.7</td>
<td>61.2 ± 4.6</td>
</tr>
<tr>
<td>% F4/80$^+$</td>
<td>30.6 ± 5.8</td>
<td>36.4 ± 6.2</td>
<td>13.8 ± 2.0</td>
</tr>
<tr>
<td>% MHCII$^+$</td>
<td>63.2 ± 2.5</td>
<td>65.5 ± 1.7</td>
<td>45.5 ± 4.1</td>
</tr>
<tr>
<td>MFI MHCII</td>
<td>5734 ± 388</td>
<td>5623 ± 581</td>
<td>5389 ± 387</td>
</tr>
</tbody>
</table>
4.3 Discussion

We have previously discovered that mammary tumour susceptibility was reduced in C1q null mutant mice administered 7,12-dimethylbenz[a]anthracene (DMBA) compared to wildtype mice (Hodson unpubl, p=0.02) (Fig. 4.1). In this study, wildtype C1q +/+ and C1q −/− female mice received DMBA by oral gavage for 6 weeks and were monitored weekly thereafter for palpable mammary tumours. Half the wildtype control mice developed mammary gland tumours. In comparison, of the twenty C1q null mutant mice administered DMBA, only two developed tumours (Fig. 4.1) (p=0.02). This finding suggests a new paradigm wherein C1q expression in the normal wildtype mammary gland increases the risk of tumourigenesis. In Chapter 3, we demonstrated that null mutation in C1q does not perturb mammary gland developmental stages during puberty, pregnancy, and proestrus but delays the regression phase in the hormone-induced regression mouse model. In addition, we observed a reduction in the macrophage number in C1q −/− mice at proestrus and 24 h following administration of progesterone antagonist RU486. Given the involvement of macrophages and C1q in the phagocytosis of apoptotic bodies, there is a possibility that macrophages associated with normal developmental events may impair tumour immunosurveillance. Alternatively, C1q-macrophage crosstalk might promote an inflammatory microenvironment leading to either increased susceptibility of the mammary gland to DNA damage or an anti-tumour environment that better protects the tissue from tumourigenesis.

The roles of C1q complement protein in the immune response to DMBA-induced mammary gland tumours have not been previously investigated. Further investigation on the effects of C1q null mutation in mammary gland tumourigenesis is therefore necessary to elucidate the roles of C1q in mammary gland tumourigenesis. Here, we broadly characterise the induction of an immune response to multiple doses of chemical carcinogen 7,12-dimethylbenz[a]anthracene (DMBA), and compare the responses between the mammary gland, mammary gland draining lymph nodes (inguinal), para-aortic lymph nodes, cervical lymph nodes, and spleen in C1q +/+ and C1q −/− mice.

4.3.1 Immune responses to chemical carcinogen DMBA challenge

The carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) has been widely used in cancer research (Russo and Russo 1978, Ip and Asch 2000, Thompson and Singh 2000, Li et al. 2002). DMBA metabolises in vivo to reactive intermediates that have carcinogenic effects (Baskaran et al. 2010), therefore it is an excellent model to investigate the early stages of tumourigenesis. Administration of DMBA by oral gavage causes chromosomal damage leading to mammary tumours in 50% C57BL/6 mice with an average latency of 12 weeks (Ip and Asch 2000). Mice challenged with DMBA also have been found to develop lymphomas, ovarian cancer, and skin cancer (Qing et al. 1997, Nicol et
al. 2004), however at a much lower frequency than mammary gland tumours. Therefore, this method of tumour induction takes advantage of the mammary gland’s high susceptibility to cancer compared to other tissues.

We demonstrated a differential immune response to chemical carcinogen DMBA challenge in both C1q \(^+/+\) and C1q \(^{-/-}\) mice in different tissues including mammary gland draining lymph nodes, para-aortic lymph nodes, cervical lymph nodes, spleen and mammary gland tissue. However, whether the immune responses we observed in both genotypes in this study is due to the carcinogen DMBA itself or due to the DNA damage induced by DMBA is indistinguishable. This could be elucidated by comparing the immune response profiles induced by other DNA-damaging conditions and agents such as ultraviolet light, gamma-radiation, cisplatin, ara-C and methyl methanesulfonate (MMS) (Chang et al. 2002, Park et al. 2002, Heinloth et al. 2003, Gasser et al. 2005).

Cytokines play crucial roles in all immune responses. Different cytokine profiles activated at the onset of an immune response can determine the subsequent pathological and physiological consequences (Turner et al. 2014, Wynn 2015). In this study, we utilised the intracellular cytokine staining to detect cytokine levels in wildtype and C1q \(^{-/-}\) mice challenged with DMBA. This approach involves permeabilisation of cell membranes and antibodies are used to detect secreted cytokines within cells by flow cytometry. This is a powerful approach as it does not only enable quantitation of cytokines but also identifies which specific cells are producing the cytokine of interest. However, measurement of cytokines in vivo is difficult as resting cells do not normally express detectable cytokines and cytokines have a very short half-life (Sullivan et al. 2000, O'Shea and Murray 2008). Hence, detection of cytokines is less feasible without in vitro stimulation. We have shown that the in vitro stimulation by stimulants like PMA and ionomycin can activate lymphocytes to effectively produce measurable amount of cytokines in samples derived from in vivo sources. In comparison to the DMBA-treated mouse cohort, the cohort of untreated mice stimulated with PMA and ionomycin was included to be representative of naive immune cell subtype abundance and activation state. Thus, the differential immune responses observed to PMA/ionomycin stimulation and DMBA was predicted. This strategy enables us to explore the immune response in wildtype and C1q \(^{-/-}\) mice challenged with DMBA and confirm that any differences seen were the real effect of carcinogen DMBA challenge in both genotypes.

Natural fluctuations in immune cells in the mammary gland occur over the course of the estrous cycle (Chua et al. 2010, Hodson et al. 2013), and it is important to consider the findings presented here in the context of these normal developmental processes. These normal developmental
fluctuations in macrophages and potentially other immune system cells across the cycle complicate the analysis of the development of DMBA-induced immune responses. The stage of estrous cycle of mice utilised in this study could not be controlled due to multiple administrations of DMBA. Therefore, comparisons between DMBA-treated and control-treated mice of both genotypes are essential to dissect the specific effects of DMBA on the immune cell environment.

4.3.2 Deficiency in C1q affects T cell populations following chemical carcinogen DMBA challenge

Previous studies reported that T cell lymphocytes are critical in adaptive immunosurveillance against cancer cells (Girardi et al. 2001, Shankaran et al. 2001, de Visser et al. 2006). Lymphocyte-deficient mice have been shown to develop higher frequency of lower bowel carcinoma compared with congeneric wild-type mice (Shankaran et al. 2001). Our present findings observed fluctuations in CD3+ T cell abundance in all tissues of both C1q ++ and C1q -- mice following six administrations of DMBA. Further investigation on T cell CD4 and CD8 subsets revealed a significant increase in the CD4+ to CD8+ ratio, not only in the mammary gland draining lymph nodes, but also in the para-aortic and cervical lymph nodes of DMBA-treated C1q -- mice. This is due to both upregulation of CD4+ and downregulation of CD8+ expression in the CD3+ T cell population.

The effector functions of CD4+ T cells are highly dependent on the host immune and tumour microenvironment (Zamarron and Chen 2011, Sun and Zhang 2014). A previous study has observed a protective role of CD4+ T lymphocytes in tumour immunity as CD4+ T cell deficiency in methylcholanthrene-initiated sarcomas enhances tumour development (Koebel et al. 2007). In addition, a subset of CD4+ T cells expressing the cell surface marker CD25 and the master transcription factor Foxp3 (CD4+CD25+Foxp3+) known as inducible regulatory T cells (Treg) has shown capacity to suppress the activation of anti-cancer cell immunity, thus promoting tumour growth and progression (Woo et al. 2001, Chen et al. 2003, Curiel et al. 2004). However, in this study, we were not able to determine the identity of the CD4+ T cells present. They may be T helper cells, or alternatively inducible regulatory T cells (Treg). CD4+ T helper cells can either trigger a T helper 1 (Th1) response or T helper 2 (Th2) response depending on the cytokine microenvironment and the specific interactions between the T cell receptor and MHC-peptide complex and other co-receptors (Constant and Bottomly 1997). A Th1 response results in inflammation and enhanced macrophage-mediated killing, while a Th2 response acts on B cells to promote antibody formation (Abbas et al. 1996). Th2-CD4+ T lymphocytes have been demonstrated to be indirectly involved in tumour promotion by regulating the phenotype and effector function of tumour-associated macrophages (DeNardo et al. 2009).
Further investigation of cytokine production following DMBA administration reveals an activation of CD4+ and CD8+ T cell subsets, represented by production of IFNG in all tissues of wildtype C1q+/+ and C1q-/- mouse cohorts. Overall we found a higher proportion of CD4+ T cells present in all tissues, but this T cell subset produced less IFNG. In comparison, although the CD8+ T cell subset was less prevalent within the environment of all tissues, there was a high proportion of them producing IFNG. Although in this study we observed no significant difference in IFNG-producing CD8+ T cells, a number of studies have shown activated CD8+ T cells infiltrated both within the tumour and in the peritumoral stroma in early stage colorectal cancer patients, and within breast tumours, melanoma and ovarian cancer, to be associated with a positive prognostic value with better patient survival (Zhang et al. 2003, Pages et al. 2005, Galon et al. 2006, Azimi et al. 2012, Gajewski et al. 2013, Ali et al. 2014). In vivo studies also demonstrate the anti-tumour properties of CD8+ T cells, when combined with natural killer cells, in controlling tumour growth (Shanker et al. 2007, Shanker et al. 2010).

Interestingly, there was a significant increase of IFNG-producing CD4+ T cells specifically in the mammary gland draining lymph nodes of DMBA-treated C1q null mice. This increased percentage of IFNG-producing CD4+ T cells may indicate a key role of IFNG in protecting the mammary gland from tumour initiation and development in the absence of C1q. Indeed, IFNG exerts many functions including activating innate and adaptive immune responses, inhibiting cell proliferation, inducing apoptosis, and also playing important roles in tumour immunosurveillance (Boehm et al. 1997, Stark et al. 1998, Szabo et al. 2003, Dunn et al. 2006). Mice treated with neutralizing monoclonal antibody to IFNG showed faster growth of transplanted immunogenic sarcomas than in control mice (Dighe et al. 1994). In addition, IFNG receptor-deficient mice exhibit increased tumour growth and tumour burden when induced with different doses of the chemical carcinogen methylcholanthrene (MCA). Similar effects were observed when these mice were bred onto a background deficient in the p53 tumor-suppressor gene, with a broader spectrum of tumour types developed compared to mice lacking p53 alone (Kaplan et al. 1998). Anti-tumour features of IFNG are not only observed in tumour recognition and elimination, but IFNG also appears to play important roles in preventing tumour metastasis (Street et al. 2001). These findings suggest a role for T lymphocytes, specifically the CD4+ T cell subset and the cytokine IFNG in regulation of DMBA-induced mammary tumourigenesis. Future studies may look at the phenotypes of these CD4+ T cells and further investigate the crosstalk between CD4, IFNG and complement C1q within the mammary gland and in a breast cancer setting.

In this study, we were particularly interested in exploring the roles of CD4+ and CD8+ T lymphocytes in C1q-/- mice challenged with carcinogen DMBA, as the first step in defining how the immune
system responds to mammary tumour initiation in the absence of C1q. Future studies may further expand the knowledge generated in this study by exploring roles of different types of immune cells such as natural killer cells (NK cells), gamma-delta T cells and dendritic cells in the mammary gland tumourigenesis. These cells have been well-described as potent effector cells in the tumour immunosurveillance (Berzins and Ritchie 2014, Silva-Santos et al. 2015). Previous studies utilising various mouse cancer models have demonstrated different protective roles of gamma-delta T cells including tumour recognition through the gamma-delta T cell receptor (TCR), IFNG-mediated anti-tumour response and tumour eradication (Liu et al. 2008, Lanca et al. 2013, Silva-Santos et al. 2015). Activation of NK cells has been associated with cytotoxic-mediated tumour killing and production of different cytokines like IFNG and TNFA to trigger anti-tumour adaptive immune response (Waldhauer and Steinle 2008, Berzins and Ritchie 2014, Marcus et al. 2014, Wang et al. 2015). Tumour evasion and immune suppression have been shown in previous studies to be secondary to dysfunctional dendritic cells (Pinzon-Charry et al. 2005, Hanks 2016). Thus, further investigation using different cell surface markers such as TCR gamma-delta, NK and CD11b and CD11c antibodies to detect gamma-delta T cells, NK cells and dendritic cells, respectively will further characterise the impact of complement C1q on the immune response profile to carcinogen DMBA. Discoveries in these areas may provide opportunities to selectively manipulate the immune response to prevent breast cancer.

4.3.3 Deficiency in C1q affects CD45+ cell population and MHC Class II expression on macrophages in the mammary gland following chemical carcinogen DMBA challenge

Apoptotic bodies tagged with C1q are internalised by macrophage-mediated phagocytosis, processed, and expressed as MHC Class II molecules on the professional antigen presenting cells (APCs). These peptides are presented to CD4+ T cells to induce appropriate immune responses (Robinson and Delvig 2002). Our present results demonstrate a reduction of CD45+ immune cells in the mammary gland of mice deficient C1q, following multiple doses of DMBA. Despite reduced abundance of CD45+ immune cells, the percent of these that were F4/80+ macrophages was similar in both genotypes. Of these macrophages, MHC Class II expression was reduced following DMBA administration in the mammary gland of C1q−/− mice. The null mutation of C1q may affect the ability of macrophages to internalise and process foreign antigens, as other studies have reported a capability of C1q to impact on the function of antigen presenting cells and T cells in vitro and in vivo (Baruah et al. 2006, Peng et al. 2006, Peng et al. 2008, Baruah et al. 2009). Moreover, van Moonfort and colleagues have demonstrated a crucial role of C1q in enhancing uptake and MHC class I presentation of peptides derived from circulating immune-complexed antigen by APC to CD8+ T
cells (van Montfoort et al. 2007). These findings may explain the reduced expression of the class II MHC on these macrophages. Downregulation of MHC Class II and defective antigen-processing machinery are associated with tumour escape from immunosurveillance, hence leading to growth and progression (Anichini et al. 2006, Poschke et al. 2011, Thibodeau et al. 2012).

Alternatively, the reduction of MHC Class II on macrophages also suggests a migration of these activated APCs from the mammary gland to the mammary gland draining lymph nodes, which then promotes T helper cell-mediated immune responses that effectively clear tumour cells by direct tumour killing (Quezada et al. 2010), or assisting anti-tumour activity of CD8+ T lymphocytes (Hadrup et al. 2013).

Taken together, these findings suggest synergistic effects of antigen presentation, CD4+ T lymphocytes and IFNG action in mammary tumour immunosurveillance in the absence of C1q. However, the exact mechanism is yet to be understood. Clear understanding of these interactions will be useful in paving the way for development of new immunotherapeutic interventions for breast cancer.

4.3.4 Complement directs innate and adaptive immune responses

It is now becoming clear that the complement system not only modulates innate immune responses, but also directs adaptive immune responses (Kolev et al. 2014). There is emerging evidence that complement receptors are present on almost all immune cells, including B and T cells, suggesting broad complement functions and actions in both immune compartments. Interaction between complement receptor 2 (CR2, CD21), which is primarily present on B cells and follicular dendritic cells (FDCs) in humans and mice, and complement fragment C3d can dictate B cell activation, tolerance and memory and regulate antigen handling by FDCs (Carroll 2000, Carroll and Isenman 2012). C3-deficient mice exhibit impaired B and T cell function, and when infected with influenza virus, these mice failed to clear the virus efficiently, leading to higher mortality compared to wildtype controls (Kopf et al. 2002, Fernandez Gonzalez et al. 2008).

Clear roles for complement in modulating T cell function during homeostasis and disease remain elusive. However, recent studies have revealed direct and indirect effects of complement proteins on T lymphocyte activation (Elkon and Santer 2012, Clarke and Tenner 2014, Clarke et al. 2015). C1q opsonisation facilitates macrophage-mediated phagocytosis which in turn indirectly activates the production of macrophage anti-inflammatory cytokines such as IL27 and IL10, and decreases the production of inflammatory cytokines such as TNFA (Benoit et al. 2012). Within primary human cells in vitro, C1q complement protein has been shown to suppress macrophage- and immature
dendritic cell-mediated T cell activation (Teh et al. 2011, Clarke et al. 2015). It is unclear whether the C1q null mutation in mice and humans resulting in autoimmune disease is due to direct or indirect effects on T cell function (Botto et al. 1998, Botto and Walport 2002).

On the basis of these findings, complement appears to play distinct roles in shaping both innate and adaptive immune responses. Although our present results show significant differences in T cell immune responses and antigen presentation to DMBA challenge in the absence of C1q, the underlying mechanism is unclear. We propose that absence of C1q may impair the apoptotic machinery which then activates T lymphocytes to generate an adaptive immune response that better protects the mammary gland from tumour initiation and development. Future studies may include a longer timeframe to investigate the effect of C1q null mutation on mammary tumour development using this carcinogen DMBA mouse model.

4.4 Conclusion and future directions
This study explores the complex role of the immune system in both protecting the mammary gland against tumourgenesis and nurturing tumour progression. Our findings of fluctuations of T cell subsets, leukocyte CD45+ population, IFNG production, and MHC Class II expression on macrophages demonstrate the involvement of the innate and adaptive arm of the immune response during carcinogen challenge. However, the definitive roles of C1q in response to the DMBA-induced mammary tumour initiation are yet to be fully understood. Future studies need to address this by utilising directed approaches looking at innate and adaptive immune responses to DMBA in the absence of C1q. The prevalent CD4+ T cells need to be analysed in greater detail to further dissect the specific identity of the CD4+ T cell population affected by C1q deficiency. These experiments will provide greater insight into the complement immune networks that constitute tumour immunosurveillance and tissue development in the mammary gland.
Chapter 5 – Effect of C1q Null Mutation on Mammary Gland Tumourigenesis in MMTV-PyMT mice
5.1 Introduction

The complement system is well-described as an effector of innate immunity that acts as a first line defense against mutated self-cells and to eliminate foreign elements. Complement-associated functions range from causing direct cell lysis, promoting development of humoral and adaptive immunity, and controlling a number of immunological and inflammatory processes to help maintain homeostasis (Ricklin et al. 2010). There is growing evidence that suggests a dual role of complement in the tumour microenvironment. Through its activated complement proteins such as C3, C3a, C4, C5, and the membrane attack complex (MAC), complement creates an inflammatory state within the tumour microenvironment which may actually protect established tumours and enhance their growth (Niculescu et al. 1992, de Visser et al. 2004, Ytting et al. 2004, Bjorge et al. 2005). Complement component C5a generated by the activated complement system has been reported to assist tumour growth by recruiting and activating myeloid-derived suppressor cells (MDSCs) in cervical, lung, and ovarian cancers (Corrales et al. 2012, Nunez-Cruz et al. 2012). Studies in mice and humans report impaired tumour development in ovarian, lung and cervical cancer when the action of complement C5 was inhibited (Markiewski et al. 2008, Nunez-Cruz et al. 2012), supporting previous findings of complement roles in tumourigenesis. C3-deficient mice demonstrate a reduction in tumour growth, suggesting potential roles of C3 in promoting tumour progression (Markiewski and Lambris 2009). Together, these findings suggest complex roles of complement in immunity and in the tumour microenvironment.

Whilst the functional roles of complement products such as C3a and C5a in the immune system and tumour microenvironment are relatively well-defined in the literature (Mastellos and Lambris 2002, Vivanco and Sawyers 2002, Venkatesha et al. 2005, Markiewski et al. 2008, Markiewski et al. 2009, Rutkowski et al. 2010, Rutkowski et al. 2010), little is known about complement protein C1q in tumourigenesis. Deficiency in C1q is known to be an important susceptibility factor for the autoimmune disease systemic lupus erythematosus (SLE) (Walport et al. 1998), suggesting a role for C1q in maintenance of self-tolerance. Interestingly, SLE patients who are genetically deficient in C1q have a substantially decreased risk of developing breast and endometrial cancer (Bernatsky et al. 2013), suggesting for the presence of C1q increases cancer susceptibility.

C1q is a complement protein that promotes rapid macrophage-mediated clearance of dying cells and tolerance to self-antigens (Roos et al. 2004). We previously showed that C1q is necessary for expedient mammary gland regression, and affects the abundance of IFNG-producing T cells activated in response to carcinogen challenge. This study seeks to investigate the causal role of C1q in mammary tumourigenesis, by utilising the transgenic mouse mammary tumour virus polyoma...
middle T (MMTV-PyMT) model. Unlike the carcinogen DMBA model utilised in the previous chapter which is best employed to study tumour initiation, the MMTV-PyMT model enables study of tumour progression and metastasis. In this model, the PyMT transgene initiates mammary epithelial cell hyperplasia and the resulting tumours spontaneously progress to metastatic disease, recapitulating the multistage progression of human breast cancer (Qiu et al. 2004). This model is closely aligned with the basal epithelial subtype of human breast disease (Lim et al. 2010). In this study, we introduced the C1q null mutation into the MMTV-PyMT model and established mouse lines whereby expression of the tumour protein PyMT on the C57BL/6 background was made homozygous or heterozygous for a recessive null mutation in the C1q gene. The effect of C1q deficiency on mammary tumour latency, development, and progression was analysed.

5.2 Results

5.2.1 Effect of C1q null mutation on early mammary hyperplasia
Transgenic mice carrying MMTV-PyMT develop mammary gland tumours with 100% penetrance, with an average latency of 12 weeks in C57BL/6 mice (Lin et al. 2003). Therefore, this model can be used to study tumour progression. However, it is essential to investigate the early onset of mammary tumourigenesis in order to fully characterise the impact of C1q on breast cancer. These studies analysed the effect of C1q null mutation on tumour development in PyMT transgenic mice at 10, 15 and 18 weeks of age. A summary of the analysis of tumour development at each of these time points is presented in Table 5.1 and Table 5.2.

To examine the effect of C1q null mutation on tumour initiation in PyMT transgenic mice, PyMT+/C1q+/- and PyMT+/C1q−/− mice were monitored weekly from 6 weeks by palpation to determine tumour latency. At 10 weeks, mice were killed and the fourth pairs of mammary glands were collected and stained with carmine alum. As seen in the carmine alum wholemounts, the development of mammary tumours in PyMT+/C1q+/- and PyMT+/C1q−/− mice showed a comparable pattern; the hyperplastic lesion consisted of a few apposed foci developing firstly around the main collecting duct of the nipple area (open arrows indicated) (Fig. 5.1A, C) (lower magnification). Examination of the PyMT mammary glands at younger age often observed a single focus developed on the ducts emanating from the nipple prior to tumour formation (Lin et al. 2001, Lin et al. 2003). Both genotypes also exhibited growth of multiple small nodules on the distant ducts (white arrows indicated) (Fig. 5.1B, D) (higher magnification). The mammary hyperplastic areas were quantified in a number of different ways, including quantifying the total hyperplasia area, total hyperplasia area/gland area, the largest hyperplastic area, the number of distinct hyperplastic areas, and the number of hyperplastic...
areas/gland area (Table 5.1). There was no significant difference in the percent of hyperplastic area per total gland area within the mammary glands of 10 weeks of age $PyMT^+\backslash C1q^{+/+}$ and $PyMT^+\backslash C1q^{-/-}$ mice (Fig. 5.1E). There was also no difference in the number of distinct hyperplastic areas within the whole mammary glands between these genotypes (Fig. 5.1F), suggesting that absence of C1q does not affect mammary tumour hyperplasia.

Leukocyte infiltration is one of the key events during early tumourigenesis, which can contribute to protection against tumour growth (Dunn et al. 2004, Dushyanthen et al. 2015). To further investigate whether the lack of C1q in PyMT transgenic mice altered immune cells infiltration in early mammary tumourigenesis, the abundance and phenotypes of immune cells in the mammary glands at 10 weeks of age $PyMT^+\backslash C1q^{+/+}$ and $PyMT^+\backslash C1q^{-/-}$ mice were quantified. The first, second, third, and fifth pairs of mammary glands were collected and analysed by flow cytometry. The mammary glands were enzymatically digested and stained with CD45, F4/80, MHC Class II, and lymphocyte markers CD3, CD4 and CD8 (Table 2.2). Data was collected using FACS Diva software (BD Biosciences, San Jose, CA). Viability dye DAPI (Vector Laboratories, Burlingame, USA) was used to identify dead cells. Cells positive for DAPI were not viable and therefore excluded. The abundance of F4/80+ macrophages in the mammary glands of $PyMT^+\backslash C1q^{+/+}$ and $PyMT^+\backslash C1q^{-/-}$ mice was similar (Fig. 5.2A), however there was a significant reduction in the percentage of MHC Class II positive macrophages, in $PyMT^+\backslash C1q^{-/-}$ compared to $PyMT^+\backslash C1q^{+/+}$ mice (49±3% $PyMT^+\backslash C1q^{-/-}$ versus 59±3% $PyMT^+\backslash C1q^{+/+}$, p=0.039, n=10) (Fig. 5.2B).

The effect of null mutation in C1q was further explored in leukocyte and T lymphocyte cell populations. There was no significant difference observed in the percent of CD45+ leukocytes in the mammary glands at 10 weeks of age $PyMT^+\backslash C1q^{-/-}$ compared to $PyMT^+\backslash C1q^{+/+}$ mice (Fig. 5.3A). Of these CD45+ cells, approximately 20% were positive for CD3 (Fig. 5.3B). Similarly, there were no detectable differences in CD4+ or CD8+ T cell populations or the mean fluorescence intensity of CD3+ populations in mammary glands of $PyMT^+\backslash C1q^{-/-}$ mice compared to $PyMT^+\backslash C1q^{+/+}$ mice (Table 5.3).

Overall, these findings suggest that C1q null mutation does not affect early mammary tumourigenesis in the PyMT transgenic mouse model. However, the reduction of MHC Class II expression on macrophages in the absence of C1q suggests a potential role for C1q in antigen presentation during initiation of mammary tumourigenesis.
Table 5.1 Summary of statistics to assess the effect of C1q null mutation on early mammary gland tumourigenesis in PyMT transgenic mice

Data shown as a mean±SEM for each variable of both genotypes.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Genotype</th>
<th>n=14</th>
<th>n=13</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total hyperplastic area (mm²)</td>
<td>PyMT+/C1q ++</td>
<td>12.53±10.55</td>
<td>10.02±5.38</td>
<td>0.943</td>
</tr>
<tr>
<td>Total hyperplastic area/gland area (%)</td>
<td>PyMT+/C1q ++</td>
<td>10.07±7.69</td>
<td>8.8±5.56</td>
<td>0.867</td>
</tr>
<tr>
<td>Largest hyperplastic area (mm²)</td>
<td>PyMT+/C1q ++</td>
<td>9.97±9.19</td>
<td>8.42±4.46</td>
<td>0.755</td>
</tr>
<tr>
<td>Largest hyperplastic area/gland area (%)</td>
<td>PyMT+/C1q ++</td>
<td>7.96±6.63</td>
<td>7.41±4.47</td>
<td>0.943</td>
</tr>
<tr>
<td>No. hyperplastic areas</td>
<td>PyMT+/C1q ++</td>
<td>4.36±3.46</td>
<td>4.00±3.96</td>
<td>0.438</td>
</tr>
<tr>
<td>No. hyperplastic areas/gland area (mm²)</td>
<td>PyMT+/C1q ++</td>
<td>0.04±0.03</td>
<td>0.03±0.04</td>
<td>0.364</td>
</tr>
</tbody>
</table>
Table 5.2 Analysis of 15 weeks and 18 weeks of age \( PyMT^+/C1q^{+/+} \) and \( PyMT^+/C1q^{+-} \) mammary tumours

Data shown as a mean±SEM for tumour number, tumour burden and primary tumour weight.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Genotype</th>
<th>n=16 ( PyMT^+/C1q^{+/+} )</th>
<th>n=15 ( PyMT^+/C1q^{+-} )</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour number</td>
<td>4.75±0.5</td>
<td>3.2±0.4</td>
<td></td>
<td>0.028</td>
</tr>
<tr>
<td>Tumour burden</td>
<td>0.18±0.03</td>
<td>0.08±0.01</td>
<td></td>
<td>0.036</td>
</tr>
<tr>
<td>Primary tumour weight</td>
<td>0.08±0.06</td>
<td>0.04±0.02</td>
<td></td>
<td>0.079</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>Genotype</th>
<th>n=29 ( PyMT^+/C1q^{+/+} )</th>
<th>n=29 ( PyMT^+/C1q^{+-} )</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour number</td>
<td>5.79±1.74</td>
<td>4.79±2.09</td>
<td></td>
<td>0.094</td>
</tr>
<tr>
<td>Tumour burden (g)</td>
<td>0.37±0.36</td>
<td>0.23±0.21</td>
<td></td>
<td>0.071</td>
</tr>
<tr>
<td>Primary tumour weight (g)</td>
<td>0.18±0.27</td>
<td>0.11±0.15</td>
<td></td>
<td>0.492</td>
</tr>
</tbody>
</table>
Figure 5.1 Effect of C1q null mutation on early mammary gland tumourigenesis in PyMT transgenic mice

Representative carmine alum stained wholemounts of 10-week old PyMT+/C1q ++ (A, low magnification) (B, higher magnification) and PyMT+/C1q −/− (C, low magnification) (D, higher magnification) female mice. Hyperplastic areas (open arrows indicated) first started in the nipple area, typically consist of multiple alveolar formed on the duct that connects to the main collecting duct. Growth of multiple small nodules is seen along more distal ducts (white arrows indicated). LN, the mammary gland draining lymph nodes. The mammary hyperplastic areas were analysed by quantifying the percent of hyperplastic area per total gland area and the number of distinct hyperplastic areas. No difference was observed in the percent of hyperplastic area per total gland area between both genotypes (E) (n=13-14). Number of hyperplastic areas per gland was counted for each genotype (F), there was no significant difference observed. Data shown as a mean±SEM and were assessed using the Wilcoxon (Mann-Whitney) test. All tests were two-tailed and significance was assessed at the 5% alpha level.
Figure 5.2 Effect of C1q null mutation on macrophage abundance and phenotype in 10-week old PyMT transgenic mice

The first, second, third and fifth pair mammary glands of 10-week old PyMT+/C1q+/+ and PyMT+/C1q−/− female mice were harvested, enzymatically digested and labelled with macrophage marker F4/80 and MHC Class II. Viability dye DAPI was used to identify dead cells. There was no difference in the percent of macrophage population in the mammary glands of both genotypes (A). However, the percent of MHC Class II expression on macrophages was significantly reduced in PyMT+/C1q−/− compared to PyMT+/C1q+/+ mice (B) (p=0.039, n=10). Data shown as a mean±SEM and were assessed using independent student T test. Asterisk (*) indicates statistical significance (p<0.05) between both genotypes.
Figure 5.3 Effect of C1q null mutation on leukocyte CD45+ and lymphocyte CD3+ populations in 10-week old PyMT transgenic mice

The first, second, third and fifth pair mammary glands of 10-week old PyMT+/C1q+/+ and PyMT+/C1q−/− female mice were harvested, enzymatically digested and labelled with leukocyte marker CD45 and lymphocyte marker CD3. Viability dye DAPI was used to identify dead cells. There was no difference in the percent of CD45+ (A) and CD3+ T cell populations (B) in the mammary glands of both genotypes (n=10). Data shown as a mean±SEM and were evaluated using independent student T test.
Table 5.3 Effect of C1q null mutation on percentage of leukocyte CD45+ and lymphocyte CD3+, CD4+ and CD8+ populations in 10-week old PyMT transgenic mice

The percentage of leukocyte CD45+, lymphocyte CD3+ and T cell subset CD4+ and CD8+ populations within the 10-week mammary glands of PyMT+/C1q +/+ and PyMT+/C1q -/- female mice. Mean fluorescent intensity (MFI) was measured to demonstrate any shift in fluorescent intensity of cell surface marker CD3. Viability dye DAPI was used to identify dead cells. Data shown as a mean±SEM and were assessed using independent student T test.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>n=10 PyMT+/C1q +/+</th>
<th>n=10 PyMT+/C1q -/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% CD45+</td>
<td>14.9 ± 2.7</td>
<td>22.4 ± 3.8</td>
</tr>
<tr>
<td>% CD3+</td>
<td>20.9 ± 1.4</td>
<td>20.3 ± 1.2</td>
</tr>
<tr>
<td>MFI CD3</td>
<td>381.5 ± 30.3</td>
<td>360.1 ± 10.3</td>
</tr>
<tr>
<td>% CD4+</td>
<td>1.6 ± 0.8</td>
<td>4.9 ± 2.6</td>
</tr>
<tr>
<td>% CD8+</td>
<td>1.0 ± 0.6</td>
<td>0.6 ± 0.4</td>
</tr>
</tbody>
</table>
5.2.2 Effect of C1q null mutation on mammary tumour latency, development and progression

The previous investigation of mammary glands collected from 10 week-old PyMT\(^+/-\) and PyMT\(^+/-\) C1q\(^+/-\) mice observed hyperplastic lesions, with no palpable tumours being detected. In separate cohorts of mice, mice were monitored and data collected at 15 and 18 weeks of age to investigate mammary tumour latency and tumour progression in the PyMT transgenic mouse model. There was a slight but significant increase in mammary gland tumour-free survival (Fig. 5.4A) and mean tumour latency (Fig. 5.4B) in PyMT\(^+/-\) C1q\(^+/-\) compared to PyMT\(^+/-\) C1q\(^+/-\) mice (14.3±0.3 weeks vs 13.5±0.3 weeks respectively; p=0.012, Kaplan-Meier test, n=43-45).

To investigate the effect of null mutation in C1q on mammary tumour development in PyMT transgenic mice, PyMT\(^+/-\) C1q\(^+/-\) and PyMT\(^+/-\) C1q\(^+/-\) female mice were sacrificed at 15 weeks or 18 weeks and the tumours were dissected, weighed, and processed for analysis. Previous studies have demonstrated that the transgenic PyMT mice at older age (>14 weeks) exhibit primary mammary tumour progression to malignant stages as well as development of secondary tumours and onset of pulmonary metastasis (Lin et al. 2001, Lin et al. 2003). Given that the C1q null mutation does not affect early mammary tumourigenesis in the PyMT transgenic mouse model, demonstrated previously in Section 5.2.1 utilising 10-week old mice, the two different age cohorts of 15-week and 18-week old mice were then chosen to further explore a role for C1q at different stages of mammary tumourigenesis. These two sets of experiments were also chosen to enable investigation on the effect of null mutation in C1q on the mammary tumour transition to malignancy and progression to pulmonary metastasis. There was a significant reduction in the tumour number in PyMT\(^+/-\) C1q\(^+/-\) compared to PyMT\(^+/-\) C1q\(^+/-\) mice at 15 weeks of age (3.2±0.4 versus 4.8±0.5 respectively, p=0.028, n=15-16) (Fig. 5.5A, Table 5.2). This effect was not observed at the 18 week time point (n=28-29) (Fig. 5.5A, Table 5.2). Although there was no significant difference observed in the primary tumour weight in both genotypes at 15 and 18 weeks of age (Fig. 5.5B, Table 5.2), the total tumour burden was significantly reduced by 50% in the PyMT\(^+/-\) C1q\(^+/-\) mice at 15 weeks (0.2±0.03g PyMT\(^+/-\) C1q\(^+/-\) versus 0.1±0.01g PyMT\(^+/-\) C1q\(^+/-\) mice, p=0.036, n=15-16), but not in the 18 week cohort (Fig. 5.5C, Table 5.2).

To further investigate whether the absence of C1q in PyMT transgenic mice affects mammary tumour growth, primary mammary tumours were processed, fixed in 4% paraformaldehyde and paraffin embedded. The primary mammary tumour tissues were sectioned and stained with haematoxylin and eosin. Clinical features of H&E-stained primary mammary tumour sections from PyMT\(^+/-\) and PyMT\(^+/-\) C1q\(^+/-\) mice were analysed by a veterinary pathologist (Dr. Lucy Woolford,
The University of Adelaide) blinded to genotype. The primary tumour of each mouse was assessed for the parameters of tumour grade, cytological atypia, tumour necrosis, and inflammation (Table 2.3).

The four stages of tumour progression (Fig. 5.6A-D), from hyperplasia to late carcinoma, are based on criteria stipulated in a previous paper on mouse mammary tumour pathology in the PyMT transgenic model (Lin et al. 2003).

The hyperplastic lesion (Fig. 5.6A) typically consists of multiple alveolar (acini) formed on the duct that connects to the main collecting duct. This hyperplastic acinus is occasionally filled with epithelial cells (arrows indicated) (Fig. 5.6A). Adenoma is the advanced stage of the premalignant tumour (Fig. 5.6B). This stage is often found in PyMT transgenic mice at 8-9 weeks of age (Lin et al. 2001), exhibiting extensive cell proliferation to expand the closely packed acini and ducts, and leading to a bigger primary tumour size. The key feature of this lesion is that epithelial proliferation is still confined to the basement membrane. The lesions are composed of solid relatively uniform cells with round to ovoid nuclei. Leukocytic infiltration is not a feature of these lesions (Fig. 5.6B).

Tumour tissue at early carcinoma stage is mainly enlarged but still well defined, however increased cytological atypia, particularly in the centre of tumours is observed (Fig. 5.6C). Tumour cells are pleomorphic, with moderate variation in nuclear morphology, size, and shape. Loss of outline of tumour acini and early stromal invasion (arrows indicated) are key features, and leukocytic infiltration can also be seen especially within the tumour surrounding acini (Fig. 5.6C). Late carcinoma is the term for the advanced carcinoma stage (Fig. 5.6D). Tumour tissue at this stage mainly exhibits solid sheets of neoplastic epithelial cells with very little or no remaining acinar or tubular structures. Malignant cells in the tumour have marked variation in cellular and nuclear size and shape with vesicular nuclei and prominent nucleoli (Fig. 5.6D). Carcinomas (early and late) exhibit stromal invasion and increased cytological atypia. The tumour cells show invasion of the stroma (arrows indicated), whilst the invasive tumour is retained within the mammary gland (Fig. 5.7A-B).

At 15 weeks of age, primary mammary tumours in only 1 out of 11 PyMT+/C1q+/- mice (9%) had developed to late carcinoma stage (Fig. 5.8A, Table 5.4A). In comparison, 7 out of 16 PyMT+/C1q+/-+ mice (44%) had tumours classified as late carcinoma (Fig. 5.8A, Table 5.4A). At 18 weeks of age, primary tumours collected from PyMT+/C1q+/-+ mice were mainly at a late carcinoma stage (21 out of 29, 72%), while late carcinomas were seen in only 29% of the PyMT+/C1q+/- mice (Fig. 5.8B, Table 5.4B). Overall, the study revealed a delay in tumour progression from hyperplasia to late carcinoma in PyMT+/C1q+/-+ primary tumours at both time points (15 weeks; p=0.05, n=11-16) (18
weeks, p=0.003, n=28-29) (Fig. 5.8A-B), suggesting a role for C1q in promoting advanced mammary tumour growth.

Cytological atypia is an indication of progression in malignancy. Classification of atypia ranges from none or minimal (Fig. 5.9A), mild (Fig. 5.9B), moderate (Fig. 5.9C), and marked (Fig. 5.9D), indicating degrees of variation in cell size (anisocytosis), variation in nuclear size (anisokaryosis), the nuclear to cytoplasmic ratio, and variation in cellular and nuclear shape (pleomorphism). Normal cells have zero atypia and occasionally they may show a slight increase in nuclear to cytoplasmic ratio (Fig. 5.9A). Highly malignant or transformed cells have a high level of atypia (i.e. they look less like their cell of origin, and are more pleomorphic in their appearance) and no longer form structures reminiscent of their tissue of origin, such as acini or tubules. Malignant cells have marked anisocytosis and anisokaryosis, marked pleomorphism with vesicular nuclei and prominent nucleoli. The highest level of atypia was seen in late carcinomas, associated with an increased mitotic index (as indicated by arrows) (Fig. 5.9D).

A significant proportion of PyMT+/C1q +/- primary mammary tumours developed to a more advanced stage of atypia compared to PyMT+/C1q +/- at 15 week time point (p=0.006, n=10-16) (Fig. 5.10A) (Table 5.4A). The mammary tumours from PyMT+/C1q +/- mice at 15 weeks showed approximately 25% of the total exhibited mild atypia, 68% were moderate and 6% were highly transformed cells with a high level of atypia (Table 5.4A). In comparison, about 20% of PyMT+/C1q +/- primary mammary tumours exhibited no or very minimal atypia, 60% were mild and 10% each for the moderate and marked (Fig. 5.10A, Table 5.4A). The effect of the null mutation in C1q on cytological atypia however, was not observed in 18 weeks of age PyMT+/C1q +/- primary mammary tumours (Fig. 5.10B).

Primary mammary tumour tissues of PyMT+/C1q +/- and PyMT+/C1q +/- mice were also assessed for presence of necrosis. Presence of tumour necrosis and degeneration and death of neoplastic cells within the tumour are key features of malignancy and rapid tumour growth. Necrotic cells are generally characterised by cytoplasmic dissolution and generation of nuclear karyolysis, karyorrhexis and pyknosis. Necrosis may affect a large area of tumour tissue (Fig. 5.11B) or can be scattered and affect individual or small clusters of cells (Fig. 5.11C). The majority of the tumours exhibited no necrosis (Fig. 5.11A) at either time point (15 weeks, Fig. 5.12A) (18 weeks, Fig. 5.12B) (Table 5.4) in both genotypes.
Overall, these findings suggest that although the C1q null mutation does not affect mammary hyperplasia in the PyMT tumour model, it does play a role in delaying mammary tumour development and progression.
Figure 5.4 Effect of C1q null mutation on tumour latency in PyMT transgenic mice

*PyMT*/C1q<sup>+</sup>/ and *PyMT*/C1q<sup>-/-</sup> female mice were monitored weekly from 6 weeks by palpation to determine tumour latency. There was a significant increase in mammary gland tumour free survival (A) and tumour latency (B) in *PyMT*/C1q<sup>-/-</sup> compared to *PyMT*/C1q<sup>+</sup>/ mice (14.3±0.3 weeks vs 13.5±0.3 weeks respectively; p=0.012, Kaplan-Meier test, n=43-45). Data shown as a mean±SEM. Asterisks (*) indicate statistical significance (p<0.05) between both genotypes.
Figure 5.5 Effect of C1q null mutation on mammary tumourigenesis in PyMT transgenic mice

PyMT+/C1q +/+ and PyMT+/C1q −/− female mice were sacrificed at 15 weeks or 18 weeks and the mammary tumours were dissected, weighed and processed for analysis. There was a significant reduction in the number of tumours in PyMT+/C1q −/− compared to PyMT+/C1q +/+ mice (p=0.028) at 15 weeks time point but this was not seen at 18 weeks time point (A). Primary tumour weight was defined as the first palpable mammary tumour with the highest tumour weight. There was no significant difference observed in the primary tumour weight of both genotypes at both time points (B). Total tumour burden was calculated as the sum of total measured mammary tumour weight per single animal. The total tumour burden was significantly less in PyMT+/C1q −/− compared to PyMT+/C1q +/+ mice (p=0.036) at 15 weeks time point but this was not observed at 18 weeks time point (C). Data shown as a mean±SEM, n=15-16 (15 weeks), n=28-29 (18 weeks) and were assessed using the Wilcoxon (Mann-Whitney) test. All tests were two-tailed and significance was assessed at the 5% alpha level. Asterisks (*) indicate statistical significance (p<0.05) between both genotypes.
Figure 5.6 Mammary tumour progression in PyMT transgenic mice

Representative images of H&E-stained primary mammary tumours indicative of a four-stage tumour classification that includes hyperplasia (A), adenoma (B), early carcinoma (C) and late carcinoma (D), taken at 40X magnification. The hyperplastic lesion is characterised by formation of multiple alveolar (acini) on the duct that connects to the main collecting duct, with hyperplastic acinus is occasionally filled with epithelial cells, identified by arrows. Cytological features of cells are bland, characterised by a mild increase in nuclear to cytoplasmic ratio only (A). Tumour progression to adenoma exhibits more extensive cell proliferation to expand the closely packed acini and ducts, leading to a bigger primary tumour size. At this stage, the epithelial proliferation is still confined to the basement membrane and the lesions are composed of solid relatively uniform cells with minimal nuclear atypia. No evidence of stromal invasion (B). Tumour cells at early carcinoma stage show increased atypia, illustrated by increased variation in nuclear morphology, size, and shape. In addition, tumours at this stage are also characterised by loss of outline of tumour acini and breaching of glandular basement membranes, leading to early stromal invasion (identified by arrows) (C). Tumour tissue at late carcinoma, the advanced carcinoma stage, exhibits solid sheets of neoplastic epithelial cells with reduced or no remaining acinar or tubular structures (D).
Figure 5.7 Stromal invasion by tumour cells into mammary fat pad

Higher grade primary mammary tumours collected demonstrated invasive tumour. Non-invasive tumours do not grown into or invade normal tissues within or beyond the breast. Invasive tumours grow into normal, healthy tissues (A). Representative image of H&E-stained primary mammary tumour indicative of stromal invasion by tumour cells identified by arrows, taken at 40X magnification (B). S, stroma; T, tumour.
Figure 5.8 Effect of C1q null mutation on mammary tumour development in PyMT transgenic mice

Primary mammary tumours were paraffin fixed, sectioned and stained with haematoxylin and eosin. The tumours were classified into four stages of tumour progression; hyperplasia, adenoma, early carcinoma and late carcinoma. Histopathological progression of primary mammary tumours to late carcinoma in PyMT+/C1q−/− was delayed compared to PyMT+/C1q+/+ female mice. Data shown as a percent distribution of PyMT+/C1q−/− and PyMT+/C1q+/+ mice in four histopathological tumour stages at 15 weeks (A) (n=11-16) and 18 weeks time point (B) (n=28-29). Data was assessed using Fisher’s Exact test. Asterisks (*) indicate statistical significance (p<0.05) between both genotypes.
Table 5.4 Analysis of 15 weeks and 18 weeks of age PyMT+/C1q+ and PyMT+/C1q− primary mammary tumours

Data shown as a frequency and percent distribution of each stage of tumour grade, cytological atypia, inflammation and presence of tumour necrosis, stratified by genotypes.

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Noor Din 170
Figure 5.9 Cytological atypia in PyMT transgenic mice

Representative images of H&E-stained primary mammary tumours indicative of mammary tumours without atypia (A) and with atypia present (B-D), taken at 40X magnification. Atypia, defined as abnormal nuclear morphology, is an indication of progression in tumour malignancy. Mild atypia is characterised by a mild increase in nuclear to cytoplasmic ratio (N:C), (B), moderate atypia is characterised by increased N:C and variation in nuclear size and shape (C). Marked atypia is characterised by high N:C, significant variation in nuclear size and shape, vesicular chromatin pattern and prominent, pleomorphic and sometimes presence of multiple nucleoli (mitotic cells identified by arrows) (D).
Primary mammary tumours were paraffin fixed, sectioned and stained with haematoxylin and eosin. The tumours were classified into four categories of cytological atypia; no presence or minimal atypia, mild, moderate and marked atypia. A significant proportion of PyMT+/C1q+/- female mice developed to a more advanced stage of cytological atypia compared to PyMT+/C1q-/- mice at 15 weeks time point (A) (p=0.006, n=10-16). There was no difference observed at 18 weeks time point (B) (n=28-29). Data shown as a percent distribution of PyMT+/C1q+/- and PyMT+/C1q-/- mice in four stages of cytological atypia at both time points. Data was assessed using Fisher’s Exact test. Asterisk (*) indicates statistical significance (p<0.05) between both genotypes.

**Figure 5.10 Effect of C1q null mutation on cytological atypia in PyMT transgenic mice**
Figure 5.11 Effect of C1q null mutation on tumour necrosis in PyMT transgenic mice

Representative images of H&E-stained primary mammary tumours indicative of mammary tumours without necrosis (A) and with necrosis present (B-C), taken at 40X magnification. Widespread necrosis of tumour cells identified by arrows, characterised by cytoplasmic dissolution and nuclear karyolysis, karyorrhexis and pyknosis (B). Individual necrotic cells sometimes observed scattered throughout the mass, characterised by shrunken hyperchromatic pyknotic nuclei and condensed hypereosinophilic cytoplasm, identified by arrows (C).
Primary mammary tumours were paraffin fixed, sectioned and stained with haematoxylin and eosin. The tumours were analysed for presence of tumour necrosis. Tumour necrosis was found as early as at 15 weeks in PyMT+/C1q+/+ but not in PyMT+/C1q−/− female mice (A) (n=10-16). Tumour necrosis at 18 weeks (B) (n=28-29). There was no difference in the presence of tumour necrosis in both genotypes at both time points. Data shown as a percent distribution of PyMT+/C1q−/− and PyMT+/C1q+/+ mice with or without tumour necrosis present at both time points. Data was assessed using Fisher’s Exact test.

Figure 5.12 Effect of C1q null mutation on tumour necrosis in PyMT transgenic mice
5.2.3 Effect of C1q null mutation on tumour-associated inflammation

Inflammation has been long associated with different stages of tumourigenesis including initiation, promotion and progression (Kundu and Surh 2008, Grivennikov et al. 2010). In this study, H&E-stained primary tumours were analysed for the presence of inflammation by veterinary pathologist, Dr. Lucy Woolford, who was blinded to mouse genotype. The grades of inflammation are no inflammation, mild, moderate, or marked, with the clinical features also described by distribution (focal – i.e. one area of tumour only, or multifocal – i.e. inflammation is associated with multiple areas of the tumour) (Fig. 5.13A-D and Table 2.3). Inflammation can be an indicator of early stromal invasion. Tissues without inflammation exhibit intact sheets of epithelial cells (Fig. 5.13A).

No significant difference in inflammation between PyMT+/C1q−/− and PyMT+/C1q+/+ mice was evident at 15 weeks of age when primary tumours were observed (Fig. 5.14A). The majority of primary mammary tumour tissues from PyMT+/C1q+/+ mice were graded as mild inflammation. Similarly, tumour tissues from PyMT+/C1q−/− mice were mostly mildly inflamed. There was no marked inflammation recorded for 15 weeks primary tumours in both genotypes (Fig. 5.14A, Table 5.4A). Unlike the mice from 15 weeks of age, the more advanced and progressive tumours from 18 week-old PyMT+/C1q+/+ and PyMT+/C1q−/− mice were associated with marked, diffuse and multifocal inflammation. However, there was no significant difference in the extent of inflammation in the primary mammary tumours of both genotypes at 18 week time point (Fig. 5.14B, Table 5.4B). This suggests that the delayed mammary tumour development and progression observed in the absence of C1q occurred independent of an inflammation-associated tumourigenic mechanism.
Figure 5.13 Effect of C1q null mutation on inflammation in PyMT transgenic mice

Representative images of H&E-stained primary mammary tumours indicative of primary tumour without inflammation (A) and with different degree of inflammation (B-D), taken at 40X magnification. Tissues without inflammation demonstrate intact sheets of epithelial cells (A). Mild inflammation exhibits scattered low numbers of inflammatory cells only (B). Moderate inflammation is characterised by moderate density infiltrates of inflammatory cells (C) and marked inflammation shows diffusion to florid and dense infiltration of inflammatory cells) (D). Tumour tissue is also shown at higher magnification in inset with examples of inflammatory leukocytic infiltration identified by arrows.
Primary mammary tumours were paraffin fixed, sectioned and stained with haematoxylin and eosin. The tumours were analysed for presence of inflammation. Classification of inflammation ranges from none to a progressive increase of severity and distribution. Mild, moderate and marked inflammation were defined by the number of inflammatory cells. Distribution of inflammation can be focal (one area of tumour only) or multifocal (inflammation associated with multiple areas of tumour). There was no significant difference in the extent of inflammation in PyMT+/C1q−/− and PyMT+/C1q+/- female mice at 15 weeks (A) (n=10-16) and 18 weeks (B) (n=28-29) time points. Data shown as a percent distribution of PyMT+/C1q− and PyMT+/C1q+/- mice in six categories of inflammation at both time points. Data was assessed using Fisher’s Exact test.
5.2.4 Effect of C1q null mutation on the spatial distribution of tumour-associated macrophages (TAMs) in primary mammary tumours

Although inflammation within PyMT tumours did not appear to be affected by C1q status, it was possible that the abundance of specific leukocyte types such as macrophages may be altered. To investigate the effect of C1q null mutation on tumour-associated macrophages (TAMs), paraffin embedded sections of PyMT+/C1q +/- and PyMT+/C1q -/- primary mammary tumours were stained with macrophage specific F4/80 antibody to identify macrophage abundance and location. Macrophages were located within the tumour area (Fig. 5.15B), mammary fat pad (Fig. 5.15C), stroma (Fig. 5.15D), and in the connective tissue (Fig. 5.15E). Quantification of TAMs within three selected tumour areas was done to investigate the effect of C1q null mutation on TAM abundance and location. The selected mammary tumours were at early and late carcinoma stages from 18 week mouse cohorts of both genotypes. There was no significant difference in TAM density between PyMT+/C1q +/- and PyMT+/C1q -/- mice (Fig. 5.15F), suggesting C1q deficiency does not affect TAM recruitment and infiltration into mammary tumour tissues.
Figure 5.15 Effect of C1q null mutation on TAM density in PyMT transgenic mice

Representative images of F4/80 antibody staining indicative of macrophage positive cells. Within the whole section of primary mammary tumour (A), macrophages were found located within tumour area (B), fat pad (C), stroma (D) and connective tissue (E), identified by arrows. Based on tumour classification, tumour grade early carcinoma and late carcinoma of 18 weeks time point were selected and analysed for macrophage density within tumour area (B) comparing PyMT+/C1q−/− and PyMT+/C1q+/+ female mice (F) (n=10-14). There was no difference observed in the macrophage number between both genotypes. Data shown as a mean±SEM of F4/80 positive cells/mm². Data was assessed using independent sample T test. TAM, tumour-associated macrophage.
5.2.5 Effect of C1q null mutation on pulmonary metastasis

Mammary tumours in the transgenic MMTV-PyMT model spontaneously progress to pulmonary metastatic disease (Lin et al. 2001). To assess whether pulmonary metastasis was affected by C1q deficiency, H&E-stained lung sections in 18 week mouse cohorts were manually analysed for the presence of tumour cells by veterinary pathologist, Dr. Lucy Woolford, who was blinded to mouse genotype. Metastasis of the mammary tumour in both genotypes was confirmed by histological analysis that showed small colonies of mammary tumour cells growing in the lung (Fig. 5.16B) as well as haemorrhagic pulmonary metastases (Fig. 5.16C-D). The majority of 18 weeks of age PyMT+/C1q +/- and PyMT+/C1q -/- mice showed no presence of metastatic lesions and the frequency of metastases was unaffected by genotype (Fig. 5.17A).

Further analysis using RT-PCR was conducted to quantify the expression of PyMT mRNA in the lungs, as the expression of this mRNA is a reliable biochemical marker for pulmonary metastasis (Guy et al. 1992). Expression of mRNA encoding PyMT is more sensitive than histology to detect micro metastases that are not widespread, and might not be visibly present in every H&E-stained section of lung. The abundance of PyMT mRNA was normalised to the expression of housekeeper gene Rpl19. Quantification of PyMT mRNA in the lungs of PyMT+/C1q +/- and PyMT+/C1q -/- mice at 15 weeks and 18 weeks of age found no significant difference (Fig. 5.17B-C). However, it was difficult to confirm whether the lung metastasis of the mammary tumours had already occurred earlier than 15 weeks of age. These findings suggest no effect of C1q deficiency on pulmonary metastasis of mammary tumours, however these time points may have been too early to properly assess metastasis as few metastatic lesions were identified histologically.
Figure 5.16 Effect of C1q null mutation on pulmonary metastasis in PyMT transgenic mice

Representative images of H&E-stained lung tissues indicative of normal lung tissue (A), taken at 10X magnification and lung tissue with mammary tumour metastasis present, identified by arrows (B), taken at 40X magnification. Example of haemorrhagic pulmonary metastasis (C), and inset shown tumour metastasis identified by arrows (D), taken at 40X magnification.
Lung tissues were paraffin fixed, sectioned and stained with haematoxylin and eosin to identify presence of mammary tumour metastasis. Percent distribution of pulmonary metastasis in $PyMT^+ / C1q^{-/-}$ was compared to $PyMT^+ / C1q^{+/+}$ female mice at 18 weeks time point (A) (n=29). Further analysis was done to investigate presence of PyMT mRNA in lung comparing $PyMT^+ / C1q^{+/+}$ and $PyMT^+ / C1q^{-/-}$ mice at 15 weeks (B) (n=11-16) and 18 weeks (C) (n=26-29) time points using qRT-PCR. The abundance of PyMT mRNA was normalised to the expression of housekeeper gene $Rpl19$. Data was assessed using independent sample T test.

Figure 5.17 Effect of C1q null mutation on pulmonary metastasis in PyMT transgenic mice
5.3 Discussion

It is well established that the effector activity of the complement system contributes to the recognition and destruction of tumour cells. The tumour immunosurveillance-associated complement system involves the recognition of non-self elements of the tumour cells, indirectly making these cells a target for elimination and destruction. Consistent with this activity, accumulated data from previous studies show complement activation to be part of tumour immunosurveillance in cancer patients (McConnell et al. 1978, Niculescu et al. 1992, Gasque et al. 1996, Lucas et al. 1996, Bu et al. 2007). However, complement roles in cancer are complex. Tumour cells can readily acquire genetic and epigenetic alterations during the cancer immunoediting process, allowing them to escape from the destructive effects of the activated complement system (Nicolson 1998, Compagni and Christofori 2000, Blackshear 2001, Stewart and Abrams 2008, Miyagi et al. 2012, Byler et al. 2014) (Fig. 1.2). Previous studies proposed that elements of the complement cascade can act as tumour promoters, and hence may facilitate tumourigenesis (Shearer et al. 1975, Markiewski and Lambris 2009, Markiewski and Lambris 2009, Rutkowski et al. 2010). Little is known about complement roles in mammary tumourigenesis. Using the PyMT transgenic cancer model, we demonstrate evidence that complement protein C1q promotes mammary tumour development and progression.

5.3.1 Deficiency in C1q affects antigen presentation by macrophages at early mammary tumourigenesis

Interaction between mammary tumours and the immune system elicits infiltration of innate and adaptive immune cells to the tumour site. However, whether these immune cells exert anti-tumourigenic or pro-tumourigenic activity is heavily dependent on the signals they receive from the tissue microenvironment. Our results indicate macrophage infiltration at the mammary tumour site both in the PyMT+/C1q +/- and PyMT+/C1q -/- mice and these may be tumour-associated macrophages (TAMs). Although macrophages are capable of eliminating tumour cells via different mechanisms including antibody-dependent phagocytosis and macrophage-dependent cytotoxicity (Evans and Alexander 1970, Mantovani et al. 1980, Jadus et al. 1996, Gul et al. 2014), TAMs have been shown to mediate tumour progression in established tumours, to sustain chronic inflammation through production of key mediators and to promote proliferation and survival of tumour cells (Mantovani et al. 1992, Mantovani et al. 2008, Noy and Pollard 2014). TAMs are also viewed as “M2” polarised macrophages (Mantovani et al. 2002), which is consistent with the pro-tumourigenic properties of “M2” macrophage phenotypes mentioned in the previous chapter.

There was no difference in the macrophage abundance in mice at 10 weeks of age, but the expression of MHC Class II on macrophages was reduced in PyMT+/C1q -/- mice. This could be
associated with reduced macrophage-mediated phagocytosis due to lack of C1q opsonisation, leading to impaired subsequent antigen presentation on antigen presenting cells (APCs). Presentation of MHC Class II molecules on APCs is one of the key events in initiating the specific CD4+ T cell response and activating intracellular cytokine signalling pathways to orchestrate an effective adaptive immune response (Holling et al. 2004, Roche and Furuta 2015). Reduction of MHC Class II expression on macrophages in the mammary gland could also be explained by migration of activated macrophages to the mammary gland draining lymph nodes to present the processed antigens to T lymphocytes, leading to adaptive immune responses that protect the mammary gland from tumourigenesis. However, the relationship of MHC Class II downregulation with tumour resistance in PyMT+/C1q−/− mice is not fully understood due to the complexity of macrophage functions within the tumour microenvironment. This finding could be strengthened by further analysis of the hyperplastic regions of the mammary gland by immunohistochemistry to quantify macrophage phenotypic markers.

Mammary hyperplasia can be detected in PyMT transgenic mice as early as 4 weeks of age (Guy et al. 1992, Lin et al. 2001). The morphology of tumour tissue at this age often started with a single focus stemming from the main collecting duct around the nipple. In older mice, hyperplastic lesions progressively developed multiple foci in the distant ducts (Lin et al. 2001, Lin et al. 2003). Consistently, our present results show similar morphological features in PyMT transgenic mice at 10 weeks of age, with multiple small nodules clustering within the nipple area and extending along more distal ducts. In the previous chapter, we reported normal mammary gland development in the C1q null mutant mice, therefore tumour development in PyMT+/C1q−/− mice is not affected by perturbed mammary gland development. The incidence of mammary hyperplasia was not affected in PyMT+/C1q−/− mice at 10 weeks of age, but the number of tumours and total tumour burden were reduced at 15 weeks of age. These findings suggest C1q does not affect epithelial cell proliferation, but there may be more subtle effects of C1q deficiency on mammary epithelial cells during the early onset phase of mammary tumourigenesis. Future studies may further investigate the cytological atypia histologically to define genome instability in the absence of C1q within these tissues.

5.3.2 Deficiency in C1q reduces mammary tumour susceptibility

The PyMT transgenic mouse model is widely used in cancer research to characterise early and late stages of mammary tumour development (Guy et al. 1994, Webster et al. 1998, Lin et al. 2001, Williams et al. 2004, Davie et al. 2007). PyMT is an oncogene, and when driven by the mammary gland specific MMTV promoter, mammary gland tumours occur with 100% penetrance with an average latency of 12 weeks in C57BL/6 mice (Lin et al. 2003). It was previously reported that the
PyMT model developed on a C57BL/6 background is more resistant to mammary tumourigenesis compared to the FVB strain (Lifsted et al. 1998). Our present results show a slight but significant increase in tumour latency and mammary tumour free survival in PyMT+/C1q−/− compared to PyMT+/C1q+/- mice. The difference in the tumour latency was very small but taking into consideration that the PyMT oncogene causes very aggressive tumour growth (Guy et al. 1992), a delay in tumour formation even by a week suggests the importance of C1q in promoting mammary tumour survival.

### 5.3.3 Deficiency in C1q delays mammary tumour development and progression

Cytological atypia is a staging tool used to measure the extent of cell abnormality and an indicator of progression in tumour malignancy. It is also a strong predictor of breast cancer incidence, as previous studies found an increased risk of breast cancer in women with abnormal cytology in breast fluid (Wrensch et al. 1993, Wrensch et al. 2001, Hartmann et al. 2005, Baltzell et al. 2008). Similarly, patients with atypical endometrial hyperplasia have a significant risk of endometrial cancer (Hunter et al. 1994). Our results show a reduction in the progression of cytological atypia in PyMT+/C1q−/− mice at 15 weeks of age, accompanied by a dramatic reduction in histological grade of primary mammary tumours both at 15 weeks and 18 weeks of age in PyMT+/C1q−/− mice. These findings are indicative of arrested or delayed disease progression in the absence of C1q, therefore supporting the proposed idea of tumour-promoting roles of complement during multistage tumourigenesis (Markiewski and Lambris 2009, Rutkowski et al. 2010, Pio et al. 2014). Consistently, previous studies reported complement element C5a enhances tumour growth by recruiting myeloid-derived suppressor cells (MDSCs) to tumours, followed by the activation of these cells, leading to suppression of anti-tumour CD8+ T cell-mediated responses. A subsequent study on C5a receptor inhibition also reported impairment in tumour growth (Markiewski et al. 2008, Markiewski and Lambris 2009). In addition, C3-deficient mice showed decreased ovarian tumour growth when compared to wildtype mice (Nunez-Cruz et al. 2012). These findings, hence, support the proposed notion of complement roles in promoting malignancy.

The presence of the C1q complement protein may also aid in tumour invasion and migration through extracellular matrix anchoring (Rutkowski et al. 2010). Complement proteins can degrade the extracellular matrix to allow neoplastic cells to invade and metastasise to distant sites from the origin (Rutkowski et al. 2010). Our present results show that the majority of 18 week old PyMT+/C1q−/− and PyMT+/C1q−/− mice showed no presence of metastatic lesions. The incidence of pulmonary metastasis was not affected in PyMT+/C1q−/− transgenic mice, suggesting pulmonary metastasis of mammary tumours is independent of C1q. However, the time point of 15 weeks and 18 weeks may
be too early to correctly dissect the effect of C1q on pulmonary metastasis. Given that pulmonary metastasis in PyMT transgenic mice on the C57BL/6 background is delayed compared to FVB mice, future studies are needed to investigate metastasis in the absence of C1q at time points beyond 18 weeks.

Taken together, these findings demonstrate impaired mammary tumourigenesis in C1q null mutant mice. Hence, it can be inferred that the presence of C1q in wildtype mice actually promotes tumourigenesis, potentially through promotion of an immune environment that favours tumour survival. However, the exact mechanism of this profound tumourigenesis phenotype seen in $PyMT^{+/-}C1q^{-/-}$ mice is yet to be understood due to our limited knowledge of the roles of C1q in mediating mammary gland development and tumourigenesis. One possible explanation of the delayed tumour development in $PyMT^{+/-}C1q^{-/-}$ mice could be attributed to oncogenic capabilities of the complement cascade during tumour-associated inflammation. It is well-established that inflammation can contribute to multiple hallmarks of cancer. Inflammation facilitates cancer growth by supplying the tumour microenvironment with various growth and survival factors, extracellular matrix-modifying enzymes that promote invasion and metastasis, inductive signals that activate epithelial-mesenchymal transition, and other hallmark-facilitating mechanisms (de Visser and Coussens 2006, Grivennikov and Karin 2010, Hanahan and Weinberg 2011). Activated complement proteins such as C1q, C3, C3a, C4, C5, and the membrane attack complex (MAC) are prominent features of a highly inflammatory tumour microenvironment (Niculescu et al. 1992, Bastrup et al. 1994, Yamakawa et al. 1994, Lucas et al. 1996, de Visser et al. 2004, Ytting et al. 2004, Bjorge et al. 2005). Previous studies have documented the association of these complement proteins with increased activity of mitogenic signalling pathways that are linked to neoplastic progression (Rutkowski et al. 2010). Inhibition of C1q during inflammation may impair the complement machinery, thus reducing the production of these complement split-products, protecting the tissue from tumour development. Although our present findings show no significant difference in the inflammation state and tumour necrosis in $PyMT^{+/-}C1q^{-/-}$ compared to $PyMT^{+/-}C1q^{++}$ mice, the more advanced primary mammary tumours of these mice are associated with moderate and marked inflammation. Stromal invasion is also evident in these tumours, indicating the capacity of tumour cells to break through the ductal basement membrane to invade the mammary fat pad, promoting malignancy.

Another possible explanation of the delayed tumour development in $PyMT^{+/-}C1q^{-/-}$ mice could be attributed to macrophage functions during mammary gland development. In the normal mammary gland, complement C1q opsonisation facilitates rapid removal of apoptotic bodies by macrophages,
which promotes self-tolerance and prevents autoimmunity. Self-tolerance is essential for normal mammary gland function, but may suppress anti-tumourigenic immune responses and result in an immune environment that is skewed in favour of tumourigenesis. Therefore, the tumour resistance effect seen in PyMT+/C1q−/− mice could also potentially be due to impairment of C1q-mediated phagocytosis, leading to an unfavourable environment for tumour promotion and survival, which then protects the mammary gland from tumourigenesis.

5.3.4 Limitations
It is important to note that there are several limitations to our study design. The mammary tumours that develop in the PyMT model almost exclusively metastasise to the lung, whilst in human breast cancers, the tumour frequently migrates to the nearest lymphatic vessels, liver and bone (Borowsky 2007). Further, the study end point of 18 weeks was too early to fully investigate the role of C1q in pulmonary metastasis. There were no calliper measurement of the mammary tumours done in this study. Hence, it is difficult to evaluate mammary tumour growth over time. However, despite these limitations, the advantage of this study is that the sample size, genetic homogeneity and ability to sample mice at timed ages allowed detection of key differences in tumour development and progression between PyMT+/C1q+/+ and PyMT+/C1q−/− genotypes.

5.4 Conclusion and future directions
The experiments in this chapter demonstrate an essential role of C1q in promoting mammary tumourigenesis. Absence of C1q increases the tumour latency and markedly delays tumour growth and progression. Future studies need to further address whether the macrophages in the mammary gland and mammary tumours are “M1” or “M2” polarised and to dissect the mechanism by which absence of C1q inhibits tumour development and progression. TUNEL staining could be conducted to identify apoptotic cells in the mammary tumours. Calliper measurement of mammary tumours could be informative in predicting tumour growth rate over time, hence future studies may include this method to improve the experimental design. The other mammary tumours collected need to be analysed for the degree of tumour progression, inflammation, cytological atypia, and tumour necrosis profiles to generate a better understanding of the impact of C1q in creating a favourable environment for tumour initiation and development. As the quantification of TAMs was done only in the higher grade of primary mammary tumours, a full analysis of TAM density in all primary mammary tumours collected and in hyperplastic lesions during early tumour development is warranted to more precisely dissect the C1q roles and possible C1q-macrophage crosstalk in driving cancer in the mammary gland.
Chapter 6 – General Discussion and Conclusions
6.1 Introduction

Growing evidence suggests macrophages play pivotal roles in ovarian-cycle associated mammary gland development and regression. Mammary gland regression occurs each ovarian cycle and is associated with macrophage-mediated phagocytosis of dying epithelial cells and tissue remodelling, reduced immune surveillance and increased susceptibility to mammary gland tumourigenesis. C1q is a complement protein that promotes rapid macrophage-mediated clearance of dying cells and tolerance to self-antigens. Given the extraordinary plasticity of macrophages, there may be roles for C1q signalling in regulating macrophage functions and phenotypes in the mammary gland. The dependence of the mammary gland on macrophage-mediated morphogenesis may affect the microenvironment of the tissue, in which it could potentially divert both macrophages and the immune system from appropriately responding to oncogenic changes in epithelial cells. However, the role of C1q in mammary gland development and tumourigenesis has not been elucidated.

The studies described in this thesis are the first to explore the effect of C1q null mutation in normal mammary gland development and breast cancer susceptibility and progression. Herein, we characterise the mammary gland developmental stages including puberty, proestrus phase of the ovarian cycle and pregnancy in the absence of C1q. Experiments utilising the hormone-induced regression model provide compelling evidence of the role of C1q in directing normal mammary gland regression and maintaining normal mammary gland morphogenesis, possibly through underlying C1q-macrophage crosstalk. In addition, we broadly define the induction of an immune system response to multiple doses of the chemical carcinogen 7, 12-dimethylbenz[a]anthracene (DMBA) in the absence of C1q, and compare the responses between the mammary gland, the mammary gland draining lymph nodes, cervical lymph nodes, para-aortic lymph nodes and the spleen. Flow cytometry analyses suggest the possibility of the CD4 T cell subset and the cytokine IFNG to play significant roles in protecting the mammary gland during the early stages of mammary gland tumourigenesis in the absence of C1q. We also demonstrate increased mammary tumour latency and delayed histopathological mammary tumour progression in PyMT+/C1q−/− mice, suggesting a role for C1q in promoting tumour growth. However, the underlying mechanism of these effects on mammary gland development and tumour susceptibility are yet to be understood. In this chapter, the possible crosstalk between C1q and macrophages in modulating macrophage phenotypes and functions in the mammary gland is discussed, which could significantly influence breast cancer susceptibility. The utilisation of different breast cancer models and the strengths and limitations of each model are elaborated. Further and directed approaches in generating a better understanding of the significance of C1q-macrophage regulatory networks in mammary gland development and
tumourigenesis are addressed. Opportunities for improved therapeutic interventions to reduce breast cancer risk in women are also highlighted.

6.2 C1q-macrophage crosstalk during mammary gland developmental stages

Historically acknowledged as one of the key effector arms of the innate immune system, recent discoveries demonstrate that various components of the complement cascade also participate in a variety of developmental, proliferative and regenerative processes (Rutkowski et al. 2010, Mastellos et al. 2013, Schraufstatter et al. 2015). However, the role of complement components, specifically C1q protein, in normal mammary gland development and function has never been elucidated. To the best of our knowledge, this is the first study to investigate the role of factor C1q in mammary gland development and breast cancer susceptibility in mouse models.

Mammary gland development occurs during specific growth stages; in the fetus, during puberty and during reproductive cycles which are governed by different hormones and growth factors (Macias and Hinck 2012). During pregnancy, the mammary gland ductal epithelial network develops and differentiates into alveolar epithelium capable of milk production, and then involutes back to a ductal network upon weaning. Over the course of the ovarian cycle, the mammary gland rotates through a sequence of development and regression phases similar to that which occurs during pregnancy, lactation and involution, although to a lesser extent. Herein, we demonstrate that deficiency in C1q does not affect terminal end bud numbers, epithelial cell proliferation or alveolar bud development, indicating a normal mammary gland during puberty and pregnancy. Therefore, the delayed mammary tumour latency and growth observed in the carcinogen DMBA and PyMT transgenic mouse models was not due to perturbed mammary gland development.

Our studies utilising a hormone-induced mammary gland regression mouse model reveal a developmental role for C1q during mammary gland regression. This model employs RU486 (mifepristone), which is a well-characterised anti-progestin that inhibits progesterone activity through antagonising the progesterone receptor (PR), leading to impairment of a wide range of PR-dependent cellular processes including mammary gland development (Baulieu 1991, Baulieu 1997, Humphreys et al. 1997, Han et al. 2007). Taking advantage of the progesterone antagonist effect of RU486 and endogenous treatment of estrogen and progesterone, this model provides a controlled environment of modulating hormone levels in ovariectomised mice, hence standardising analysis of the effects of C1q deficiency in a cycling mammary gland. We show an increase in the ductal branch points and TUNEL positive apoptotic cells within ductal epithelium and reduced macrophage
abundance at 24 h post-RU486 in ovariectomised mice deficient in C1q, suggesting a delayed mammary gland regression due to null mutation in C1q.

The underlying mechanism of how C1q delays mammary gland regression is yet to be elucidated. One possible explanation could be attributed to C1q-macrophage crosstalk which mediates normal mammary gland development. Macrophages play a key role in orchestrating epithelial proliferation and alveolar development at metestrus and diestrus and mammary gland regression at proestrus (Chua et al. 2010, Hodson et al. 2013), and it is possible that C1q affects macrophage functions during regression. During mammary gland regression, macrophages phagocytose dying epithelial cells and promote tissue remodelling to return the gland to its basic architecture. In this context, it is possible that macrophages are specifically positioned in the mammary gland to maximise removal of dying epithelial cells during mammary gland regression, hence indicating “specialised” tissue-resident macrophages that support mammary gland function over the course of the ovarian cycle. Non-complement functions of C1q may assist macrophage-mediated phagocytosis of dying epithelial cells through opsonisation. Apoptotic epithelial cells opsonised by C1q are rapidly taken up by macrophages, promoting self-tolerance, which is essential for normal mammary gland function. The increased abundance of ductal branching and TUNEL positive apoptotic cells within the ductal epithelium suggests that in the absence of C1q, the macrophage-mediated apoptotic machinery is impaired, causing delayed mammary gland regression. This effect of C1q null mutation was not observed in naturally cycling mice at proestrus, however the natural rise and fall of progesterone over the course of the ovarian cycle varies between mice. The process of mammary gland regression is very rapid and time-restricted. Utilisation of the hormone replacement model enables very specific investigation of progesterone withdrawal within defined time frames, with differences in the TUNEL positive cells and ductal remodelling observed at the 24 h time point but not the 48 h time point. Therefore, future studies to further dissect the role of C1q in mammary gland regression should be conducted within a tight timeframe of 24 hours post-RU486 administration.

Cells undergoing apoptosis display a number of “eat-me” markers to direct recognition, engulfment and elimination by professional and non-professional phagocytes (Jeannin et al. 2008, Tennant et al. 2013). Some of these markers include the exposure of phosphatidyserine (PtdSer) and expression of recognition sites on the membrane surface that can bind to soluble C1q molecules (Navratil et al. 2001, Paidassi et al. 2008, Paidassi et al. 2011). Although it is well documented that C1q can bind to immune complexes containing immunoglobulin G (IgG), particularly through CH2 domain of the Fc portion of the molecule (Burton et al. 1980, Duncan and Winter 1988, Idusogie et al. 2000), little is known about the exact C1q-binding sites on the apoptotic cell surface membrane,
largely due to the continuum of modifications taking place on the surface of cells undergoing apoptosis. Our study did not investigate further the exact location of C1q binding site(s), however, it is likely that C1q binds to surface blebs generated by apoptotic epithelial cells or PtdSer exposed on the apoptotic cell membrane, thereby sending cues for macrophage-mediated phagocytosis (Navratil et al. 2001, Elward et al. 2005, Paidassi et al. 2008, Paidassi et al. 2011). Therefore, lack of C1q leads to insufficient signals for phagocytosis, providing another possible explanation on the increased branch points and abundance of TUNEL positive apoptotic cells observed in C1q−/− mice. The reduced abundance of macrophages within ductal epithelium suggests there may be a role of C1q for macrophage migration. Hence, it would be of interest to further investigate macrophage trafficking to mammary gland draining lymph nodes by utilising flow cytometry, which in turn will generate a better understanding on C1q roles in macrophage migration and localisation within the mammary gland and other tissues.

6.3 Breast cancer models

Breast cancer is a heterogeneous disease, involving a complex, multistage process that exhibits specific molecular changes, distinct histopathological key features and diverse physiological and clinical outcomes. Therefore, it is unlikely that a single experimental model system will completely recapitulate all aspects of this disease. We utilised two different models in this study; the chemical carcinogen DMBA model and the MMTV-PyMT transgenic mouse model. Investigations using these two models enable us to address different aspects of mammary gland tumour initiation and progression in the absence of C1q.

DMBA metabolises to reactive intermediates that have carcinogenic effects (Baskaran et al. 2010), which lead to cellular changes within 24 hours of carcinogen DMBA exposure (Russo and Russo 1987). Hence, this model depicts the pathological process of tumour induction in the mammary gland and enables the evaluation of tumour susceptibility. The development of mammary tumours is generally hormone-dependent (Russo and Russo 1996), hence making it comparable to human disease. However, studies have demonstrated that multiple histologic and pathological features of DMBA-initiated mammary tumours developed depending on the carcinogen dose and upon the age of the host at the time of exposure (Nagasawa et al. 1976, Lindsey et al. 1981, Ratko and Beattie 1985, Ratko et al. 1988, Braun et al. 1989, Medina and Smith 1999, Ip and Asch 2000). Although this model has generated valuable knowledge on hormone-dependent breast cancer and the protective effect of pregnancy in breast cancer (Russo et al. 2006, Russo et al. 2007), it has two critical limitations – there is an enormous diversity of tumour subtypes and this model rarely metastasizes, which limits the study of tumour progression (Russo and Russo 2000).
In contrast, the MMTV-PyMT transgenic mouse model has been well characterised to accurately recapitulate the stepwise mammary tumour progression to malignancy and metastasis (Guy et al. 1992, Lin et al. 2003). Indeed, we show that the mammary tumours have metastasised to the lung, evident by the immunohistological and PyMT mRNA gene expression analysis in the lung tissues. MMTV-PyMT mice spontaneously initiate tumours in the mammary gland, which progress through different stages including hyperplasia, adenoma and early/late carcinoma (Lin et al. 2003), recapitulating the basic stages of human epithelial cell cancer development. Several aspects of this model may not correspond to human breast cancers. For example, most of the mammary tumours metastasise only to the lungs and the majority of the advanced tumours are hormone-independent as opposed to the human breast cancers (Cardiff et al. 2000). The MMTV promoters are selective but not specific to the mammary gland, as the transgene is expressed in other tissues including lungs, kidneys, salivary glands, seminal vesicles, testes and prostate (Choi et al. 1987, Henrard and Ross 1988). Thus, effect of MMTV transgene expression can be systemic, which may subsequently alter overall mammary gland development and tumourigenesis. Moreover, the level of oncogene PyMT expression driven by the MMTV promoters may not illustrate the expression level of oncogenes in human breast cancer. The MMTV promoters may target different cell lineages than the cellular origins of human breast cancer, hence establishing different molecular and histological tumour subtypes (Vargo-Gogola and Rosen 2007). Taken together, although these two models do not fully recapitulate the behaviours and properties of human breast cancer, it is important to acknowledge the utility of these models in gaining better understanding of the underlying mechanisms of tumour initiation, progression and metastasis.

6.4 C1q-macrophage crosstalk affects mammary cancer susceptibility

Complement has been traditionally viewed as part of the effector system in tumour immunosurveillance. However, recent studies show complement-deficient mice exhibit reduced tumour growth compared to wildtype mice (Markiewski et al. 2008, Corrales et al. 2012, Nunez-Cruz et al. 2012), hence challenging this conventional view and suggesting a role for C1q in tumour promotion and malignancy. In line with this hypothesis, our investigation in C1q null mice administered DMBA exhibit a reduced susceptibility to mammary tumourigenesis compared to wildtype mice (Hodson, unpubl). We show that PyMT+/C1q−/− mice exhibit delayed mammary tumour development and progression, supporting the finding from the carcinogen DMBA model. However, the underlying mechanism is still unclear. The findings generated from our studies utilising these two models suggest that the increased risk of mammary cancer observed in wildtype mice might be...
associated with C1q-macrophage interactions with the innate and adaptive arms of the immune system that results in adverse consequences, promoting cancer development.

It is now established that the immune system not only can provide protection against tumorigenesis, but it can also facilitate tumour progression through “cancer immunoediting” (Dunn et al. 2002, Dunn et al. 2004, Dunn et al. 2004, Swann and Smyth 2007, Schreiber et al. 2011, Pandya et al. 2016). The conflicting role of immune responses to be tumour suppressive or tumour promoting is highly dependent on the balance in immune system-tumour interactions that shape consequent effects. Our DMBA studies give insight into the likely interaction of C1q and immune cells, particularly macrophages, to dampen tumour immunosurveillance in the mammary gland, leading to an immune environment that favours tolerance and tumorigenesis. It appears that C1q-macrophage crosstalk during the early onset phase of mammary tumorigenesis alters the proportion and activation of immune cells within the mammary gland, particularly CD4+ T lymphocytes, MHC Class II antigen presentation on macrophages and IFNG production. C1q is a powerful attractant for “M2” macrophages (Vogel et al. 2014), and TAMs isolated from solid and metastatic tumours exhibit characteristics of the “M2”-like phenotype (Imtiyaz et al. 2010, Steidl et al. 2010, Edin et al. 2012). C1q-macrophage crosstalk may also assist in sculpting the mammary gland immune microenvironment to be skewed to tolerance and tumorigenesis by promoting an immunosuppressive microenvironment through the suppression of T cell-mediated anti-tumour response. TAMs, as well as T\textsubscript{reg} cells and myeloid-derived suppressor cells (MDSCs), are known as immunosuppressive cells which are involved in the induction of suppressive immune responses (Zou 2005). Confirmation of the identity of CD4+ T cells in our DMBA studies will be useful to elucidate a possible link of complement and immunosuppression. Indeed, T\textsubscript{reg} differentiation is correlated with the local C5a concentration within tumour microenvironment (Gunn et al. 2012). Complement C5a is also associated with the recruitment and activation of MDSCs in the tumour site (Markiewski et al. 2008). Co-engagement of CD3 and the complement regulator CD46 can mediate generation of inducible T\textsubscript{reg} cells (Kemper et al. 2003). Nonetheless, it should be highlighted that the host’s immune system-tumour interaction network is extremely complex. Polarisation of macrophages to exhibit a “M1” or “M2” phenotype and exert tumour suppressor or promoter functions are very much dependent on the signals these macrophages receive from the surrounding cells and environment, hence the immunological implications cannot be viewed too simplistically. The studies herein suggest that the pro-tumourigenic effects of C1q-macrophage crosstalk may be a secondary consequence of combined autocrine and paracrine signalling that directs T cell function and IFNG action within the mammary tumour microenvironment.
Hanahan and Weinberg have discussed in detail the hallmarks of cancer which include six original and two emerging biological capabilities acquired during the events of tumourigenesis; (1) sustenance of proliferative signalling, (2) evasion from growth suppression, (3) resistance to cell death, (4) replicative immortality, (5) induction of angiogenesis, (6) activation of invasion and metastasis, (7) reprogramming of energy metabolism and (8) evasion of immune destruction (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011). The contributions of C1q to these biological hallmarks are yet to be understood. Emerging data from a number of in vivo and in vitro studies show dual roles of complement within the tumour microenvironment (Nishioka et al. 1976, Maness and Orengo 1977, Ytting et al. 2004, Bjorge et al. 2005, Markiewski et al. 2008, Markiewski and Lambris 2009, Stover 2010, Corrales et al. 2012, Pio et al. 2014). For examples, although inhibition of complement C5aR function did not affect tumourigenesis in the murine TC-1 cervical cancer model (Markiewski et al. 2008), a C3-deficient mouse model of epithelial ovarian cancer showed impaired tumour vascularization (Nunez-Cruz et al. 2012). Hence, there is a potential role for C1q to promote tumour progression and invasion through induction of angiogenesis. Consistent with the pro-angiogenic capability of C1q in wound healing (Bossi et al. 2014) and murine melanoma cells (Bulla et al. 2016), complement C1q, therefore may assist angiogenesis during the development of cancer in the mammary gland.

The mammary gland undergoes substantial changes in both the stromal compartment and extracellular matrix (ECM) during mammary gland regression and tissue remodelling (Kass et al. 2007, Maller et al. 2010). Transformation of mammary tumour cells to become malignant is also associated with significant modifications of ECM organization coupled with a progressive stiffening of the stroma, enabling migration of tumour cells to surrounding stroma and subsequent extravasation into secondary sites (Kass et al. 2007, Chaudhuri et al. 2014). These, hence raised a question of potential C1q capability to promote tumour invasion or migration. Although our findings did not show a significant difference in the pulmonary metastasis of PyMT+/C1q+/- mice compared to PyMT+/C1q++ mice, previous studies have reported that complement proteins promote the degradation of tissue extracellular matrix (Rutkowski et al. 2010, Gonzalez et al. 2011, Bandyopadhyay and Rohrer 2012). Moreover, C3a has been reported to mediate the chemotaxis of human bone marrow–derived mesenchymal stem cells and reduce expression of E-cadherin, illustrating its role in invasion and metastasis (Schraufstatter et al. 2009, Tang et al. 2009). In line with our studies, C1q was recently reported to be expressed in the human malignant tumours and mice lacking C1q exhibit a prolonged survival with slower tumour growth (Bulla et al. 2016). Further
studies dissecting the role of C1q for tumour invasion and metastasis would be informative in unravelling the underlying mechanisms of C1q in promoting mammary cancer.

6.5 C1q-macrophage crosstalk defines cell death pathways and its immunological consequences

Cell death pathways are important determinants of the nature of immune responses, which can either be tolerogenic or immunogenic (Elliott and Ravichandran 2010, Garg et al. 2016). These eventual immunological consequences are governed by a number of factors (Zitvogel et al. 2010, Ravichandran 2011, Garg et al. 2016). We propose that during mammary gland regression, efficient and rapid removal of dying mammary epithelial cells by C1q-mediated macrophage phagocytosis promotes tolerance to self-antigens and prevents the generation of harmful adaptive immune responses which would scar the tissue and impair subsequent lactation. Induction of tolerogenic phagocytosis is accompanied by an active immunosuppression, characterised by downregulation of pro-inflammatory cytokines and polarisation of macrophages to “M2” type, which exhibit a pro-tumourigenic phenotype (Green et al. 2009, Barth et al. 2015, Vaught and Cook 2015, Garg et al. 2016). Hence, the role of C1q in macrophage-mediated regression may result in a pro-tumourigenic immune environment and ultimately increase breast cancer risk.

On the other hand, apoptotic cells undergoing inefficient macrophage-mediated apoptosis in the absence of C1q may succumb to another programmed cell death pathway called secondary necrosis, characterised by release of intracellular content, which induces an inflammatory, immunogenic immune response (Green et al. 2009, Galluzzi et al. 2015). Necrotic cells undergoing immunogenic cell death expose calreticulin on the cell membrane and release danger signals such as the damage-associated molecular pattern high mobility group box chromosomal protein 1 (HMGB1) to promote non-homeostatic phagocytosis by professional phagocytes such as “M1” macrophages (Apetoh et al. 2007, Green et al. 2009). Rather than promote tolerance, these events result in increased production of pro-inflammatory cytokines that synergistically generate immunostimulatory immune response, hence mediating anti-tumour immunity (Garg et al. 2016).

C1q contributes in promoting rapid apoptotic cell clearance by directly bridging apoptotic cells to phagocytes (Ogden et al. 2001, Green et al. 2009, Fraser et al. 2010), hence suggesting a role for C1q-macrophage crosstalk in dictating different cell death pathways and its inherent immunological consequences. Although we were not able to identify the macrophage phenotype as “M1” or “M2”, or the mode of cell death, our study has raised the possibility of increased breast cancer risk with each regression phase of the menstrual cycle.
6.6 Implications and future directions
The findings reported herein clearly demonstrated the fundamental relationship between C1q and macrophages in mammary gland development and cancer susceptibility in mouse models. Understanding and possibly manipulating the C1q-macrophage regulatory network can have important implications for the study of breast cancer risk, and development of immunotherapeutic interventions to prevent breast cancer (Figure 6.1).

The immunological outcomes of cell death pathways are not only attributed to the cell death mode, but several factors work in concert including cell type, characteristics of dying cells and the location of dying cells and phagocytes. Developmental-associated apoptosis and damage-induced apoptosis both have specific and distinctive markers, and there is tremendous heterogeneity in the mechanisms of cell death. Therefore, the preferential induction of tolerogenic and immunogenic responses in the mammary gland is a complex process and all these factors should be taken into account when predicting the impact of different cell death mechanisms on the host tissue and the immune system. Disruption to C1q-mediated macrophage phagocytosis in the mammary gland may induce apoptotic epithelial cells to progress into secondary necrosis, driving an immune response that better protects the tissue from tumourigenesis. However, it is still unclear whether this would create genome instability in the cell proliferation and cell death regulatory networks, hence this hypothesis remains to be tested.

Epithelial cell death during normal postpartum mammary gland involution requires macrophages and “M2”-polarised macrophages (O’Brien et al. 2012), hence roles of C1q-macrophage crosstalk could be explored in the involuting mammary gland. This could be done by doing a physiological readout of regression associated with involution, utilising a forced weaning model. If the mammary tissue is scarred by a deleterious immune response that occurs during forced weaning, it is expected that ability to feed the subsequent litter would be affected. This would be beneficial to fully characterise the role of C1q during different mammary gland developmental stages.

It would be of interest to explore potential roles of C1q in the immune response to mammary gland regression by utilising the antigen ovalbumin model (OVA), which is a common test antigen used to explore the induction of adaptive immune response. To address this, MMTV-OVA transgenic mice could be generated and bred onto a C1q null background. Mammary gland regression could be induced in MMTV-OVA/C1q−/− mice. Phagocytosis will be impaired in the absence of C1q and the OVA-expressing dying epithelial cells are expected to progress into secondary necrosis. T cells from OTI or OTII mice will be pulsed with a CFSE fluorescent tracer and injected at the time of induction.
of mammary gland regression. Fluorescence of the CFSE tracer allows tracking of the injected T cells, and also provides a measurement of proliferative index of the cells (Moldenhauer et al. 2009). Investigation of the impact of MHCI or MHCII-restricted presentation of OVA to T cells can determine the initiation and type of adaptive immune responses.

Although there is still much to explore and discover about novel roles of C1q during mammary gland regression and tumour promotion, our studies have generated fundamental information relevant to development of therapeutic interventions that target the complement C1q-regulated opsonic pathway in macrophage-mediated phagocytosis during mammary regression in mouse models of cancer to reduce breast cancer risk. SLE patients genetically deficient in C1q exhibit a significant reduced risk of breast cancer (Bernatsky et al. 2013), hence implying manipulation of the C1q regulatory networks during menstruation may redirect the immune response to protective tumour immunity and, thus may offer a promising option to reduce the impact of menstrual cycling on mammary tumourigenesis and ultimately reduce breast cancer incidence in women. There may be harmful effects of antagonistic blockade of C1q action, therefore, further exploration of C1q roles and improved animal models to identify mechanisms of manipulating C1q-macrophage crosstalk are essential to develop approaches for the treatment and prevention of breast cancer.

6.7 Conclusions
These studies have revealed the important role for C1q-macrophage crosstalk in mammary gland development and tumourigenesis. We have demonstrated that C1q is not essential during mammary gland developmental stages. Despite normal mammary gland development, we show delayed regression in a hormone replacement model, suggesting a role for C1q in regulating mammary gland regression. In addition, we have also revealed a role for C1q in promotion of tumourigenesis, demonstrating by increased tumour latency and mammary tumour-free survival and delayed histopathological progression of mammary tumours to carcinoma in C1q-/- mice. Collectively, the current work provides insight on C1q-macrophage crosstalk, and highlights the impact of C1q on the mammary gland and breast cancer susceptibility. These studies suggest there may be an opportunity to prevent breast cancer through inhibition of C1q action during mammary gland regression. Further studies are required to elucidate the underlying mechanism of C1q-macrophage signalling to regulate mammary gland morphogenesis and promotion of breast cancer. The knowledge generated will provide fundamental information that will assist in development of novel therapeutic interventions to cure and prevent breast cancer.
Figure 6.1 Suggested C1q and macrophage regulatory crosstalk in mammary gland regression and cancer susceptibility

The schematic illustration depicts the underlying mechanism of C1q-macrophage crosstalk in normal mammary gland development and tumour susceptibility. Our studies have demonstrated the role of C1q in rapid macrophage-mediated phagocytosis during regression to induce tolerogenic cell death that is essential for tissue homestasis but may increase tumour susceptibility. C1q can directly bridge dying epithelial cells through engagement with C1qR, or indirectly through antigen-antibody complex to macrophages to promote rapid clearance. The dying cells express multiple “eat-me” signals to initiate efferocytosis by macrophages. Activated macrophages can prime naive CD4+ T cells to differentiate into inducible regulatory T cells (T_{reg}), which inhibit proinflammatory immune responses. This elicits a “M2” macrophage phenotype and a pro-tumourigenic immune environment, resulting in increased breast cancer risk with each regression phase of the menstrual cycle. Inefficient macrophage-mediated phagocytosis of dying cells due to inhibition of C1q leads to secondary necrosis, which elicits a “M1” macrophage phenotype and induces inflammatory, immunogenic immune response that better protects the tissue from tumorigenesis. Ag, antigen, C1qR, C1q receptor, MHCII, major histocompatibility class II, TCR, T cell receptor.
Mammary gland regression

- Antibodies
- "Eat-me" signals
- Dying epithelial cell
- C1q
- C1qR
- Ag
- Rapid uptake by macrophages

Aptosis of epithelial cell
- "M2" macrophage phenotype
- Pro-tumour immune environment
- Breast cancer risk

C1q deficient

- Antibodies
- Dying epithelial cell
- C1qR
- Ag
- Delayed uptake

Secondary necrosis of epithelial cell
- "M1" macrophage phenotype
- Anti-tumour immune environment
- Breast cancer risk

- Processing and presentation of antigen from dying cell
- Secondary necrosis

- MHC II
- TCR
- Activation of adaptive immune response
- Draining lymph node
References


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