The assessment of T-cell subsets in response to treatment in patients with breast or oesophageal cancer

by

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I, Opope Oyaka Wedi, declare that this dissertation, which I hereby submit for the degree Masters of Science in medical immunology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

I am aware that, should the dissertation be accepted, I must submit the additional copies as required by the relevant regulations at least six weeks before the next graduation ceremony, and that the degree will not be conferred if this requirement is not fulfilled.

Signature………………………… Date…………………………
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</tr>
<tr>
<td>AFP</td>
<td>α-fetoprotein</td>
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<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
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<td>APC7</td>
<td>Anaphase promoting complex subunit 7</td>
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<td>CD25-APC</td>
<td>Anti-human CD25-Allophycocyanin</td>
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<td>APO-1</td>
<td>Apoptosis antigen 1</td>
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<td>BCA</td>
<td>Breast cancer</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<td>Conc.</td>
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<td>CTLA-4</td>
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<td>DN</td>
<td>Double negative</td>
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<td>EDTA</td>
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<td>FasL</td>
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<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<td>FoxP3</td>
<td>Foxhead box P3</td>
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<td>GITR</td>
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<td>HER-2</td>
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<td>ICAM-1</td>
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<td>INF</td>
<td>Interferon</td>
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<td>IPEX</td>
<td>Immune dysregulation polyendocrinopathy, enteropathy, X-linked syndrome</td>
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<td>Not applicable</td>
<td></td>
</tr>
<tr>
<td>NK1.1 receptor</td>
<td>Natural killer antigen 1.1</td>
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<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
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<tr>
<td>NKG2D ligand</td>
<td>Natural killer group 2 member D</td>
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<tr>
<td>NKT</td>
<td>Natural killer T-cell</td>
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<tr>
<td>PB</td>
<td>Pacific Blue</td>
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<tr>
<td>PC7</td>
<td>Phycoerythrin cyanin 7</td>
<td></td>
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<tr>
<td>PC5</td>
<td>Phycoerythrin cyanin 5</td>
<td></td>
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<tr>
<td>PD-L1</td>
<td>Programmed death-I ligand</td>
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<tr>
<td>PE</td>
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<td>PGE</td>
<td>Prostaglandin E</td>
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<td>PR</td>
<td>Progesterone receptor</td>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SS</td>
<td>Side light-scatter</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
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<tr>
<td>T&lt;sub&gt;FH&lt;/sub&gt;</td>
<td>Follicular helper T-cell</td>
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<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
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<tr>
<td>Th cell</td>
<td>T-helper cell</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TNM</td>
<td>Tumor, Nodes, Metastases</td>
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<tr>
<td>TNMG</td>
<td>Tumor, Nodes, Metastases, Grade</td>
</tr>
<tr>
<td>Treg cell</td>
<td>Regulatory T-cell</td>
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<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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Background: Cancer is a crippling disease affecting 32.6 million people globally. It is currently ranked as the leading cause of death worldwide, and is associated with significant morbidity and mortality. Despite advances made in the prevention, detection and treatment of malignancy, there remains a dearth of accurate and feasible prognostic and predictive factors for use in the management of patients suffering from cancer in developing countries. It is widely recognized that the immune system plays a fundamental role in regulating the development and progression of malignancy. T-lymphocytes in particular have been categorized into T-cell populations which either promote (T regulatory cells) or prevent (CD4$^+$ and CD8$^+$ T-lymphocyte) cancer development and progression.

Aim: The aim of this thesis was to evaluate whether the levels of the respective pro- (T regulatory) and anti- (CD4$^+$ and CD8$^+$ T-lymphocytes) cancer T-lymphocyte subsets before treatment, and alterations of these levels following conventional anticancer treatment (surgery, chemotherapy and radiotherapy) could be used as markers of prognosis in conjunction with other well known prognostic factors, or as predictors of short term (5-7 weeks post-treatment) survival.

Methods: Blood samples were collected from 25 breast cancer, and 10 oesophageal cancer patients before antitumor treatment, at day 1 post-treatment and at 5-7 weeks post-treatment. The circulating concentrations and percentages of CD4$^+$, CD8$^+$ and T regulatory cells were determined from whole blood by flow cytometry. These levels were used to determine the
CD4: CD8⁺ T-cell ratio, the CD4⁺ Treg: total CD4⁺ Treg ratio and the CD8⁺ Treg: total CD8⁺ Treg ratio.

**Results:** Early (stage 1 and 2) HER-2/neu negative breast cancer was associated with a higher CD4⁺ (558 versus 133 cells/µl; \(P=0.05\)) and CD8⁺ T-cell count (198 versus 58 cells/µl; \(P=0.05\)) as compared to early HER-2/neu positive breast cancer. Breast cancer patients showed a significant decline in CD8⁺CD25⁺CD127⁺ Treg cell subsets (\(P=0.012\)) following antitumor treatment. However, oesophageal cancer patients showed an increasing trend in CD8⁺CD127⁺FoxP3⁺ Treg cells at day 1 post-treatment (\(P=0.045\)) and at 5-7 weeks post-treatment (\(P=0.044\)). Patients who demised displayed a significantly lower CD4⁺: CD8⁺ T-cell ratio (1.2 versus 2.2; \(P=0.044\)) before treatment, as well as a lower CD4⁺ (111 versus 390 cells/µl; \(P=0.0046\)) and CD8⁺ T-cell count (88 versus 160 cells/µl; \(P=0.058\)) at day 1 post-treatment, as compared to those who survived. While patients who survived displayed a higher CD8⁺ Treg: total CD8⁺ T-cell ratio (1.48 versus 0.53 \(P=0.0126\)) before treatment, and a higher CD4⁺CD25⁺CD127⁺ Treg cell level at day 1 post-treatment (9 versus 2 cells/µl; \(P=0.047\)), as compared to those who demised.

**Conclusion**

Taken together the findings of this thesis suggest that the CD4⁺ and CD8⁺ T-cell count and CD4:CD8 T-cell ratio may serve as predictors of short-term survival in the management of breast and oesophageal cancer. Furthermore, HER-2/neu receptor status may reflect the underlying immune status and antitumor effector activity of a patient. These findings also suggest that T regulatory cell subsets are a heterogeneous group of cells with variable frequencies at presentation and in responses to antitumor treatment depending on the cancer
type, stage of disease and antitumor treatment employed. This further illustrates the need for more work aimed at better characterizing the different Treg cell subsets to better identify which subsets are associated with patient prognosis. In summary, this work presents evidence to suggest a role for the T-cell immune profile as a prognostic and predictive parameter in the management of cancer. It argues for early assessments of cancer patients’ immune status as part of the diagnostic work up and that the immune status is taken into consideration when decisions are made regarding treatment.
1 Introduction

Cancer is a crippling disease affecting an estimate 32.6 million people globally (IARC 2013). It is currently ranked as the leading cause of death worldwide, accounting for 13% of all deaths in 2008; and is associated with considerable morbidity and mortality (WHO 2013). During the past three decades, Africa has experienced an alarming rise in the prevalence of infectious diseases including human immunodeficiency virus (HIV), tuberculosis and malaria. These conditions account for seven times more deaths than cancer in sub-Saharan Africa and have been the main focus of international donors and health ministries in this region (Morhason-Bello et al. 2013). Although infectious diseases continue to plague the continent, recent reports suggest that the burden of disease attributable to cancer in sub-Saharan Africa is on the increase, with a projected 85% increase in the incidence of malignancy by 2030 (Bray et al. 2012). Furthermore, by the year 2050, 70% of the predicted 24 million people diagnosed with cancer will reside in low and middle-income countries (Parkin et al. 2001, Kingham et al. 2013).

The prognosis associated with malignancy in developing countries has been shown to be considerably worse than that observed in high-income settings (Ferlay et al. 2010). This has been attributed to a multitude of factors including; the shortage of healthcare professionals, poor infrastructure, advanced stage of disease at first presentation, limited treatment options, reliance on traditional medicine and poor treatment compliance (Kingham et al. 2013). Despite advances made in the prevention, detection and treatment of cancer on the African continent, this has not translated into improved prognosis (Kingham et al. 2013).

Over the past decades extensive research efforts have improved our understanding of the genetic, pathological and clinical components of malignancy. This information has been used
to direct clinical management and has facilitated the development of a wide variety of therapeutic interventions for patients suffering from cancer. However, despite the substantial progress made in cancer research, its immune-pathogenesis is still not completely understood. Furthermore, a large proportion of commonly used anticancer treatments are associated with considerable morbidity and even mortality due to their side effect profile (Esteva et al. 2004, Weigelt et al. 2005, Hayes et al. 2010). This is in addition to the fact that a number of these interventions fail to control the progression of the malignancy (Weigelt et al. 2005, Hayes et al. 2010). Irrespective of the clinical setting, the delicate and complex nature of cancer, as well as the broad spectrum of treatment options available makes the diagnosis and management of cancer extremely heterogeneous. This makes it imperative to have mechanisms that facilitate the selection of an appropriate therapeutic intervention for individual patients (Weigelt et al. 2005, Riley et al. 2009). These mechanisms should also facilitate the monitoring of treatment response, which would in turn enable clinicians to identify and respond to early signs of treatment failure.

Survival estimates and prognostic markers are essential in physicians’ decision making, in all phases of illness (Christakis et al. 2000, Glare et al. 2005, Riley et al. 2009). Prognostic markers, defined as clinical measures used to estimate an individual’s risk of future outcome (Riley et al. 2009) have been shown to enable the stratification of patients into different treatment groups and facilitate the counselling of oncology patients (Riley et al. 2009). Prognostic estimates in advanced cancer have also been shown to have increasing importance as a patient nears the end of life (Christakis et al. 2000). It is a time to formally re-evaluate the goals of treatment, which may change from life prolongation to palliation (Lamont et al. 2012).
Christakis and colleagues (2000) conducted a study which assessed the accuracy of doctors’ prognostic estimates in 504 terminally ill patients, and found that only 20% of prognoses were accurate (Christakis et al. 2000). Although physicians have tried to provide their patients with a reasonable estimate of response to treatment and duration of survival, studies have shown that physicians are typically too optimistic in their estimates of patients’ survival, with doctors overestimating survival by a factor of 5 (Christakis et al. 2000). These optimistic survival estimates have been associated with a delay in urgent hospice referrals and missed opportunities to devote more time to improving the quality of life of affected patients (Christakis et al. 2000). Furthermore, the physician’s inaccurate prognostication may contribute to the perpetuation of the patients’ misconception regarding the future and may result in patients making decisions which may have negative health outcomes (Christakis et al. 2000).

There is a growing body of evidence focusing on the identification of clinical (Glare et al. 2005), biochemical (Alcaraz et al. 2013) and histological (Nakamura et al. 2009) predictors of survival and tolerance to anticancer treatment, in addition to mechanisms whereby treatment response may be monitored (de Geus-Oei et al. 2009, Lilja et al. 2008). However, poor study design, lack of internal and external validity, inappropriate analysis and poor reporting have resulted in concerns regarding the validity of the results from numerous studies assessing prognostic markers (Altman et al. 1994, Altman et al. 1998, Kyzas et al. 2005). Commonly used modalities in our settings include patients’ clinical signs and symptoms, performance status, biochemistry (e.g. calcium, C-reactive protein and α-fetoprotein levels), lymph node stage, histological grade and type, vascular invasion, tumour markers (AFP), menopausal status, tumour receptor status (HER-2/neu) and hormone receptor status (oestrogen-ER, and progesterone-PR) (Lamont et al. 2001, Glare et al. 2005,
Ray-Coquard et al. 2009, Watanabe et al. 2010, Hayes et al. 2010, Hurria et al. 2012). Other authors suggest prognostication models which incorporate information regarding the tumour (e.g. primary site, size, grade, tumour markers, metastatic sites etc.) and patient-related factors (demographics, co-morbidities, performance status, biochemistry, socio-economic status etc.) to give an individual patient a specific estimate of survival (Glare et al. 2005). Despite all these modalities we are still unable to accurately determine a patient’s prognosis and monitor treatment response to anticancer treatment.

Recently there has been growing evidence that host defences against cancer play a significant role in predicting the clinical outcome of patients (Mafune et al. 2000). This has sparked a wave of interest in the role of the host’s immune response against cancer, and the possibility of identifying immunological markers which may be used to monitor treatment response and reliably determine prognosis in patients presenting with cancer.

1.1 The immune system

The immune system is a vast and complex structure which protects the body against infectious agents and other harmful substances e.g. toxins. It is divided into two main components, namely the innate immune system and the adaptive immune system, which work in conjunction to perform four main functions, including (Murphy 2012):

• **Immunological recognition**: this enables cells of the immune system to recognize sources of infection within the human body;

• **Containment and eradication of infection**: immunological cellular and soluble factors with effector functions neutralize infective agents identified within the body;

• **Immune regulation**: the self-regulation of the immune system to ensuring that the body does not damage itself;
• *Immunological memory*: Following exposure to a pathogen, the immune system is able to mount a stronger and faster response to the same pathogen on subsequent exposures, thus rapidly and effectively eradicating the pathogen.

### 1.1.1 The innate immune system

Innate immunity comprises of a non-specific, cellular and biochemical defence mechanism including (Abbas et al. 2007):

- Physical and chemical barriers e.g. epithelial surface;
- Phagocytic cells e.g. neutrophils, macrophages;
- Natural killer (NK) cells;
- Blood proteins e.g. complement system, mediators of inflammation, cytokines.

Innate immunity can distinguish between normal tissues belonging to the organism (self) and newly encounter ‘non-self’ proteins or living cells thus serving as the first line of defence against pathogens (Bremers et al. 2000, Abbas et al. 2007). All “non-self” structures including malignant cells, viruses, bacteria etc. are recognized in a non-specific way and are attacked by non-specific effector cells (Bremers et al. 2000, Abbas et al. 2007). This ability to discriminate between tumor cells and normal cells demonstrates the crucial role which the innate immune system plays in the process of mounting an immune response against malignant cells (Dunn et al. 2004).

### 1.1.2 The adaptive immune system

In contrast to the innate immune system, the adaptive immune system comprises of antigen specific lymphocytes that mount a response against a wide variety of intracellular and extracellular pathogens encountered (Abbas et al. 2007, Murphy 2012). Adaptive immunity is characterized by the ability to increase in magnitude and defensive capability with each
successive exposure, and has been identified as a critical weapon in the quest for the solution to cancer (Seremet et al. 2011).

There are two main types of lymphocytes namely, B-lymphocytes and T-lymphocytes (Murphy 2012). All lymphocytes arise from common lymphoid progenitor cells which are found within the bone marrow. Precursor B-lymphocytes complete their maturation within the bone marrow and are morphologically similar to T-lymphocytes despite differences in protein production and function (Abbas et al. 2007, Ruffell et al. 2010, Murphy 2012). Following exposure to a foreign antigen, B-lymphocytes differentiate into plasma cells. These cells produce antibodies which function as soluble forms of B-cell receptors and mediate humoral (antibody-mediated) immunity (Abbas et al. 2007, Murphy 2012). The antigen specificity of the secreted antibodies is identical to their origin and facilitates the identification of pathogens by effector cells including T-lymphocytes (Abbas et al. 2007). As the focus of this thesis is on the role of T-lymphocytes in cancer, B-lymphocyte function and role in anticancer immunity will not be elaborated on.

1.1.2.1 T-lymphocyte maturation

T-lymphocytes also originate in the bone marrow from hematopoietic stem cells; however, the precursor cells migrate to the thymus via the blood stream where the process of maturation occurs. Once in the thymus the Notch1 receptor, found on T-lymphocyte precursor cells, receives a signal from thymic stromal cells, which instructs the cells to commit to the T-cell lineage (Murphy 2012b). This results in the precursor cells differentiating for a period of one week and then progressing into a phase of proliferation where $5 \times 10^7$ new T-cells are produced daily (Murphy 2012b). These cells express CD2 receptors, but are regarded as double negative as they do not express any receptors which
Chapter 1: Introduction

1.1. The Immune System

define a mature T-lymphocyte, including, CD4\(^+\), CD8\(^+\) or CD3\(^+\)-T-cell receptor complex, they thus enter the double negative stage of differentiation (Lee et al. 2010, Murphy 2012b).

**Double negative thymocytes**

Within the thymus, 5% of all thymocytes are regarded as double negative T-cells. At the beginning of the maturation process, this pool of double negative thymocytes comprises of double negative cells committed to becoming mature CD4\(^+\) and CD8\(^+\) T-lymphocytes, in addition to a mature T-cell population which has already arranged its T-cell receptor genes to encode for the \(\gamma\delta\)-T-cell receptor and are regarded as \(\gamma\delta\)-T-lymphocytes; as well as the Invariant natural killer T-lymphocyte population, which express \(\alpha\beta\)-T-cell receptors of limited variability and NK1.1 receptors (Lee et al. 2010, Murphy 2012b). The “double-negative” stage brings about the rearrangement of the immature thymocytes genes to eventually generate the T-cell receptor (TCR) \(\alpha\) and \(\beta\) chains (Lee et al. 2010). The double negative stage is subdivided into 4 separate intervals including:

i. **Stage 1: Double Negative (DN) 1 thymocyte**

   This period is characterized by the expression of Kit and CD44\(^+\) on all double negative cells, with genes encoding for the \(\alpha\beta\)-chains of the TCR being in the germ line configuration (Murphy 2012b).

ii. **Stage 2: DN2 thymocyte**

   Thymocytes begin to express CD25 (IL-2 receptor) and the TCR-\(\beta\) chain commences the process of rearrangement (Murphy 2012b).

iii. **Stage 3 and 4: DN3 thymocyte and DN4**

   Thymocytes begin to lose the expression of Kit and CD44 (CD44\(^\text{low}\)CD25\(^+\)) and the rearrangement of the TCR-\(\beta\) chain continues. Once the \(\beta\)-chain gene has completed
the rearrangement, the thymocytes lose the expression of CD25$^+$ and progress to stage DN4. Cells which do not complete the rearrangement process remain at stage DN3 and soon die (Murphy 2012b). The expressed β chain combines with a pre-T-cell receptor-α chain to form a complete pre-T-cell receptor, which is expressed in conjunction with a CD3 molecule (CD3-pre-T-cell receptor complex) (Abbas et al. 2007B, Murphy 2012b). Once the CD3-pre-TCR molecule has been assembled, the β-chain gene rearrangement is discontinued and the proliferation and differentiation of thymocytes into double positive cells begins (CD3$^+$-pre-TCR$^+CD4^+CD8^+$) (Abbas et al. 2007b, Murphy 2012B). At this stage thymocytes have now lost the expression of CD44 and CD25 (Murphy 2012b).

**Double positive thymocytes**

Following this proliferative period the α-chain gene of the double-positive thymocyte undergoes rearrangement and completes the differentiation of the TCR-αβ receptor (CD4$^+$CD8$^+$CD3$^+$TCR-αβ) (Abbas et al. 2007B, Murphy 2012b). All lymphocytes contain a highly diverse TCR that enables the identification of almost any pathogen which enters the body (Abbas et al. 2007b, Murphy 2012b). The TCR-αβ facilitates the interaction between the T-lymphocytes and antigen presenting cells, thus initiating the process of inducing an antigen specific immune response (Abbas et al. 2007b).

**Positive selection of thymocytes**

The double positive thymocytes have not been exposed to any antigenic stimulation, and consequently encounter epithelial cells within the thymic cortex, which display a variety of self-peptides bound to class I and II Major-Histocompatibility-Complexes (MHC). If the TCR
on a double positive cell recognizes the self-peptide being presented and concurrently, the CD8$^+$ receptor interacts with the class I MHC molecule, the T-lymphocyte will receive a signal that simultaneously prevents cell death and elicits the loss of CD4$^+$ receptor expression (Abbas et al. 2007b). It subsequently matures into class I MHC-restricted CD8$^+$ T-lymphocyte (Abbas et al. 2007b, Lee et al. 2010). A similar process occurs for the CD4$^+$ T-lymphocyte, resulting in a class II restricted CD4$^+$ T-lymphocyte (Abbas et al. 2007b). Cells which are not positively selected or whose TCR gene fails to complete rearrangement die (Lee et al. 2010).

- **Negative selection of thymocytes**

The single positive cells (CD4$^+$ or CD8$^+$ T-lymphocyte) then undergo a process of negative selection, which involves the exposure of T-lymphocytes to self-antigens derived from widely expressed proteins within the body (Murphy 2012b). T-lymphocytes which have a high reactivity to self-antigens are signalled to undergo apoptosis (Abbas et al. 2007b). Thus self reactive T-cells which are potentially dangerous to the body are destroyed (Abbas et al. 2007B, Murphy 2012b). Only two percent of cells which begin as double positive thymocytes and undergo this process of differentiation, maturation and screening over a 3 week period will eventually exit the thymus to form the peripheral T-cell repertoire (Murphy 2012b). At the end of the maturation and differentiation process 60% of cells which began as immature double negative thymocytes would have differentiated into mature T-lymphocytes expressing either CD3$^+$TCR-αβCD4$^+$ or CD3$^+$TCR-αβCD8$^+$ receptors (Murphy 2012b).
1.1.2.2 Cell mediated Immunity

Cell mediated immunity is a process characterized by the identification and processing of a pathogen-derived-antigen by an antigen presenting cell (APC), followed by the presentation of this processed antigen to a lymphocyte, which subsequently results in an immune response to the pathogen (Abbas et al. 2007b). T-lymphocytes are one of the mediators of cellular immunity, with the two major T-lymphocyte subsets being, helper T-lymphocytes (CD4\textsuperscript{+} T-lymphocyte) and cytotoxic T-lymphocytes (CD8\textsuperscript{+} T-lymphocyte) (Martorelli et al. 2010, Murphy 2012b). These cells, in association with APCs, play a vital role in mounting an immune response against malignant cells.

- **CD4\textsuperscript{+} T-lymphocytes**

CD4\textsuperscript{+} T-lymphocytes are traditionally known as “helper cells” and have been shown to play a crucial role in the promotion of a variety of responses to different foreign antigens, in addition to the augmentation of antibody production by B-lymphocytes (Murphy 2012, Koretzky 2010). CD4\textsuperscript{+} T-lymphocytes have also been shown to play a substantial role in antitumor immunity by eliminating malignant cells irrespective of the presence of the MHC class II complex, and facilitating CD8\textsuperscript{+} T-lymphocyte antitumor response (Alcaraz et al. 2013). In the absence of an infection, T-lymphocytes are small featherless cells which circulate within the body with no functional activity and are termed “naïve T-lymphocytes” (Murphy 2012). Following exposure to an antigen, naïve CD4\textsuperscript{+} T-lymphocytes may differentiate into several functional classes of effector cells with specific functions (Ruffell et al. 2010, Murphy 2012c) (Figure 1.1 and Table 1.1).
Figure 1.1: Differentiation of naïve CD4⁺ T-lymphocytes (Martorelli et al. 2010)
Table 1.1: CD4$^+$ T-lymphocyte sub-populations

<table>
<thead>
<tr>
<th>CD4$^+$ T-helper cell</th>
<th>Functions</th>
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<tbody>
<tr>
<td><strong>Th1</strong></td>
<td>Regulates delayed hypersensitivity reaction and cell mediated immunity to intracellular pathogens and tumor cells (Mosmann et al. 1996, Goto et al. 1999).</td>
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<tr>
<td><strong>Th2</strong></td>
<td>Controls allergy and parasitic infection e.g. helminthes by coordinating responses mediated by eosinophils, mast cells and IgE antibody isotype. It also helps coordinates humoral immunity and can be reprogrammed to differentiate into Th9 cells (Mosmann et al. 1996, Goto et al. 1999, Murphy 2012, Kaplan 2013).</td>
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<tr>
<td><strong>Th3</strong></td>
<td>Found predominantly within the mucosal immune system and controls the activation and suppression of the immune response in the mucosae (Murphy 2012 c).</td>
</tr>
<tr>
<td><strong>Th9</strong></td>
<td>Present in very low frequencies in humans but have been shown to exert an effect on mast cells, eosinophils, T-lymphocytes and epithelial cells. May play a role in autoantibody production in systemic lupus erythematosus (SLE) (Wong et al. 2010)</td>
</tr>
<tr>
<td><strong>Th17</strong></td>
<td>Protects the body against extracellular bacteria and fungal infection by stimulating neutrophil response. Coordinates tissue inflammation and autoimmunity (Murphy 2012 c)</td>
</tr>
<tr>
<td><strong>Th22</strong></td>
<td>Found in human skin and plays a role in skin homeostasis (Duhen et al. 2009)</td>
</tr>
<tr>
<td><strong>T_{FHI}</strong></td>
<td>Migrates to B-cell follicles after activation and plays a role in B-cell activation and antibody production (Murphy 2012 c)</td>
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1.2. Antitumor immunity

i. CD4$^+$ T-helper cell

This group of cells consists of a large number of T-helper subsets which may be differentiated according to their cytokine secretion profiles and functional properties (Martorelli et al. 2010). The main effector subsets including CD4$^+$ T-helper cell Type 1 (Th1), Th2, Th17 and Follicular helper T-cell (T$_{FH}$) with additional cell types identified including Th3, Th9 and Th22 (Staudt et al. 2010, Murphy 2012c, Basu et al. 2012). Despite the multiple roles these cells play in coordinating and facilitating an immune response against infective and non-infective pathogens, only Th1 has been shown to have a significant role in antitumor activity (Martorelli et al. 2010). Th1 regulates cell-mediated immunity against tumor cells by producing large quantities of interferon-γ (INF-γ), tumor necrosis factor-α (TNF-α) and IL-2 (Martorelli et al. 2010). The Th17 lineage has been identified fairly recently with an increased frequency of IL-17 producing cells in patients with prostate, ovarian and gastric cancer, with its presence in hepatocellular carcinoma patients being associated with poor prognosis (Ruffell et al. 2010). Studies have suggested that disequilibrium in circulating T regulatory cells and T$_{H17}$ cells may also promote the development of autoimmune diseases (Wong et al. 2010).

ii. CD4$^+$ T regulatory cells

This group’s key function is to maintain homeostatic control and suppression of the immune response (Sakaguchi et al. 1995). It has also been implicated in the pathogenesis of autoimmune disease as well as malignancy, reviewed by Taams et al (2006). This cell population will be discussed in more detail shortly.
iii. CD4⁺ Type II natural killer T-cells
This cell also develops in the thymus and expresses the TCR-αβ receptor; however, they are characterized by a limited TCR repertoire and have the ability to recognize lipid epitopes presented by non-classical MHC molecules (CD1d) (Godfrey et al. 2007). These cells share characteristics of both NK cells and T-lymphocytes, and produce IFN-γ, TNF-α, IL-4 and IL-13, which direct a Th1 or Th2 polarized immune response (Ruffell et al. 2010). They are considered as part of the innate and adaptive immune system.

iv. Cytotoxic CD4⁺ T-cells
This cell population was identified within the last decade as CD4⁺ T-cells with granzyme B and perforin-containing granules (Martorelli et al. 2010). They normally represent a small proportion (2%) of the entire CD4⁺ T-cell population in healthy people; however, these numbers increase in the presence of chronic viral infection with up to 50% of CD4⁺ T-cells displaying cytotoxic potential in patients infected with HIV (Appay et al. 2002). These cells display immunophenotypic features consistent with antigen-experienced CD4⁺ T-cells and end-stage differentiated T-cells (Appay et al. 2002). While CD8⁺ T-lymphocytes have always been considered as the main cells capable of killing tumor cells, cytotoxic CD4⁺ T-cells may present a good supplementary alternative since there are many factors which make cancer cells inherently resistant to CD8⁺ T-cell antitumor activity including:

- The fact that malignant cells exhibit unpredictable antigen presenting capabilities and;
- The production of immunosuppressive substances by malignant cells (Martorelli et al. 2010).

Cytotoxic CD4⁺ T-lymphocytes can assist by eradicating the cancer cells directly via presentation of tumor antigens on a MHC class II molecule or indirectly by the recognition of the tumor cell via an APC (Mumberg et al. 1999, Heller et al. 2006).
CD8$^+$ T-lymphocytes

CD8$^+$ T-lymphocytes have always been regarded as the more efficient and effective cell with regards to the eradication of virus infected cells and antitumor immunity (Martorelli et al. 2010). CD8$^+$ T-cells identify peptide-MHC class I complexes expressed by tumor cells and eradicate these malignant cells directly whilst, NK cells have been shown to regulate and facilitate CD8$^+$ T-lymphocyte function through the release of INF-γ (Dunn et al. 2004, Ruffell et al. 2010). Similar to the CD4$^+$ T-cells, there are numerous subtypes in the CD8$^+$ T-cell family including: type 1, type 2, type 17 CD8$^+$ effector T-lymphocytes and CD8$^+$ regulatory T-lymphocytes (Croft et al. 1994, Lu et al. 2008, Hamada et al. 2009).

1.2 Antitumor immunity

The immune system has been implicated in the pathogenesis of numerous medical conditions including cancer (Gallimore et al. 2008). Cancer arises from the uncontrolled proliferation of cells with the dispersal of clones of malignant cells (Abbas et al. 2007 c). Cancer growth is broadly determined by the ability of the cancer cells to evade non-immunological and immunological surveillance, the proliferative capability of the malignant cells and the ability of these cells to invade adjacent tissues locally as well as distally (Abbas et al. 2007 c, Kim et al. 2007).

1.2.1 Carcinogenesis

As stated by Devi and colleagues (2004), in 1948 Berenblum and Schubik described a multi-step process that disrupts normal cell regulation and results in the uncontrollable proliferation of rogue cells, which develop into solid tumors or haematological malignancies (Devi 2004). The development of cancer has been described in 3 phases including initiation, promotion and progression:
1.2.1.1 Phase 1: Initiation

During this stage, stable changes occur within the genome of somatic cells, naturally or following exposure to a carcinogen (e.g. viral infections, tobacco and alcohol) (Devi 2004). A proto-oncogene is regarded as a normal DNA sequence which following a mutation undergoes conversion to active oncogenes (American Cancer Society 2011) (Figure 1.2). This renders the affected cells and its progeny susceptible to neoplastic transformation (Devi 2004). These oncogenes may affect cellular behaviour, interrupt biochemical pathways that regulate cell proliferation and disturb normal development and differentiation of cells (United Nations Scientific Committee On The Effects Of Atomic Radiation 2000). For the oncogene to be expressed phenotypically, subsequent activation of multiple oncogenes need to occur, in addition to possible changes in the surrounding cellular environment (Devi 2004).

Figure 1.2: Carcinogenesis (Broad Institute of MIT 2014)
1.2.1.2 Phase 2: Promotion

Following the initiation of the somatic cell, phenotypic expression of the oncogenic mutation occurs as a result of exposure to both intracellular (e.g. additional oncogenic mutations) and extracellular (e.g. cytokines) factors (Devi 2004). These factors promote cellular growth and may disrupt regulatory signals that coordinate cell development and maintenance (Devi 2004).

1.2.1.3 Phase 3: Progression

During this stage the premalignant cells commit to neoplastic transformation and undergo further proliferation (Devi 2004). Consecutive changes and proliferation of the malignant cells result in the development of addition mutations, growth of the tumor in size and creation of a heterogeneous cluster of malignant cells within the tumor (Devi 2004). Subsequently, tumor progression advances to a point where malignant cells detach from the primary tumor mass and invade the adjacent tissue including the haematological and lymphatic system (Devi 2004). They are transported to other organs where they form secondary tumors.

1.2.2 Non-immunological surveillance

Non-immunological surveillance has been described as a process whereby host cells monitor and regulate tumor growth. This occurs either by:

- The identification of DNA damage by intracellular mechanisms which results in the arrest of cell growth and the induction of DNA reparation e.g. p53 pathway or;
- The induction of cell apoptosis following the identification of irreparable DNA damage or the activation of oncogenes (Kim et al. 2007).

These mechanisms work in conjunction with immunological mechanisms of tumor surveillance to prevent the development of malignancy (Kim et al. 2007).
1.2.3 Immune surveillance

As quoted by Khazaie and colleagues (2006), Paul Ehrlich was the first to introduce the concept of immune surveillance in 1909 when he proposed that, “the incidence of cancer would be much greater were it not for the vigilance of our immune defence system in identifying and eliminating nascent tumor cells” (Khazaie et al. 2006). Thereafter, Sir Burnet (1957) postulated that alterations in self-antigens, as a consequence of oncogenesis, may induce an immune response against the accumulating malignant cells (Burnet 1957). This would in turn culminate in the regression of the tumor, rendering the tumor clinically undetectable (Burnet 1957); however, the limitations in immunological research at the time prevented the validation of this hypothesis (Dunn et al. 2004). In the mid-20th century and early 1990’s experimental evidence, including tumor transplantation models and knockout mouse models were used to validate the concept of immune surveillance against chemically (Old et al. 1962, Kaplan et al. 1998), virally (Klein et al. 1977) and spontaneously (Street et al. 2004, Smyth et al. 1999) induced tumors.

While experimental models have been extremely beneficial and have played a substantial role in the progress made in the field of tumor immunology, tumor development in immunocompromised mice have been difficult to interpret by virtue of the substantial defects imposed by each mutation (Smyth et al. 1999). This makes it difficult to translate this information into clinically relevant material, which can be used in a clinical setting for patient management. While it has long been recognised that patients suffering from congenital or acquired immune deficiencies are at an increased risk of developing malignancies (reviewed by Gatti et al. 1971), recent studies in the field of transplantation medicine has clearly demonstrated the importance of a functional immune system for anticancer activity. This comes following observations that immunocompromised organ transplant recipients
demonstrate an increased frequency of non-virally induced malignancies, which are not present in age-matched immunocompetent control patients (Sheil 1984, Birkeland et al. 1995, Hoshida et al. 1997, Jensen et al. 1999, Penn 2000, Adami et al. 2003, Dunn et al. 2004). These findings have further substantiated the concept of immune surveillance and suggest that, in the future, clinical modulation of the immune system could serve as an alternative form of therapeutic intervention to facilitate regression of an existing malignancy.

Originally, immune surveillance was described as a mechanism in which the adaptive immune system inhibited cellular transformation in the early stages of cancer (Dunn et al. 2004). However, studies have now demonstrated that host-protective mechanisms are instigated by both the innate and adaptive immune systems (Carey et al. 1976, Bauer et al. 1999, Shankaran et al. 2001), which in some instances protects the host from tumor development, while in other instances facilitates tumor progression and growth by selecting for malignancies with lower immunogenicity (Dunn et al. 2004). This led to the use of the phrase, "immunoediting" instead of "immune surveillance" to describe the immune systems dual roles of preventing the onset of cancer and moulding the already formed tumor (Dunn et al. 2002). Immunoediting is described in 3 stages: elimination, equilibrium and escape (Figure 1.3).
1.2. Antitumor immunity

1.2.1 Stage 1: Elimination

This stage refers to the original concept of immune surveillance and entails the eradication of developing malignant cells (Kim et al. 2007). This requires a coordinated response by both the innate and adaptive components of the immune system (Kim et al. 2007). Following neoplastic transformation, tumor cell proliferation commences and continues until the tumor mass reaches a measurement of 2-3mm. This is followed by the initiation of angiogenesis and

Figure 1.3: Schematic process illustrating immunoediting in cancer (Kim et al. 2007).
invasion of the local tissue for tumor growth to continue (Hanahan et al. 1996, Kim et al. 2007). The stromal remodelling promotes the release of proinflammatory molecules that alert the innate immune system to the presence of malignant cells (Dunn et al. 2002). Tumor cells have also been shown to release chemokines (e.g. CXCL-9, CXCL-10), which together with the proinflammatory molecules summon the cellular components of the innate immune system, including NK cells, NKT-cells, γδT-cells and macrophages to the site of malignancy (Dunn et al. 2002, Kim et al. 2007).

The effector cells of the innate immune system initiate the process of eliminating the malignant cells directly via the; Perforin, TNF-related apoptosis-inducing ligand (TRAIL) and FasL dependent mechanisms, production of tumoricidal products, and indirectly through the production of IL-12 and IFN-γ (Coughlin et al. 1998, Smyth et al. 2001, Takeda et al. 2001, Dunn et al. 2004). The production of IFN-γ contributes significantly to antitumor progression by promoting anti-proliferative (Bromberg et al. 1996), pro-apoptotic (Kumar et al. 1997) and angiostatic effects (Luster et al. 1993, Coughlin et al. 1998, Qin et al. 2000), in addition to facilitating the activation of tumor infiltrating macrophages and NK cells (Dunn et al. 2004). The release of IL-12 by macrophages stimulates the NK cells further, which in turn results in the release of larger quantities of INF-γ (Dunn et al. 2002).

The apoptosis of tumor cells results in the liberation of tumor-specific antigens (Dunn et al. 2004). NK cells promote the maturation of circulating dendritic cells, with additional activation occurring post exposure to proinflammatory molecules and cytokines released during the initial phase of the antitumor immune response (Gerosa et al. 2002). The liberated tumor antigens undergo phagocytosis by dendritic cells, where they are processed and transported to the draining lymph nodes and secondary lymphoid organs, where they are
presented to naive CD4$^+$ T-lymphocytes and CD8$^+$ T-lymphocytes (Kim et al. 2007), resulting in the maturation of these cells.

Tumor specific CD4$^+$ T-lymphocytes and CD8$^+$ T-lymphocytes migrate to the primary tumor site and attack and, eradicate immunogenic malignant cells (Kim et al. 2007). The production of IL-2, by CD4$^+$ T-lymphocytes, and IL-15 by host cells, facilitates the maintenance of CD8$^+$ cytotoxic T-lymphocyte function and viability (Dunn et al. 2004). Tumor specific CD8$^+$ T-cells play a crucial role in the eradication of the malignant cells by lysing tumor cells via death cell ligands e.g. TRAIL, granzyme/perforin pathways as well as producing large quantities of INF-$\gamma$, which in turn contributes to INF-$\gamma$ led tumorolysis (Dunn et al. 2004, Töpfer et al. 2011).

CD4$^+$ T-lymphocytes have also demonstrated additional non-redundant antitumor effector activity, independent of the CD8$^+$ T-lymphocytes (Pardoll et al. 1998, Töpfer et al. 2011). They play a central role in the initiation and maintenance of antitumor response with Th1 and Th2 effector pathways eliminating tumors through the activation and recruitment of effector cells including macrophages and eosinophils (Pardoll et al. 1998). Following the demonstration of the essential role of CD4$^+$ T-lymphocytes and CD8$^+$ T-lymphocytes in antitumor immunity, studies demonstrated that the peripheral blood CD4$^+$: CD8$^+$ T-cell ratio could be used as an indicator of patients’ immunity (Mafune et al. 2000). These studies demonstrated that a progressive decrease in both circulating CD4$^+$ and CD8$^+$ T-lymphocytes was associated with the spread of disease in patients with breast, malignant melanoma, lung and colorectal cancers (Blake-Mortimer et al. 2004).
1.2.3.2 Stage 2: Equilibrium

During the elimination period T-lymphocyte and INF-γ associated selection pressure results in the survival of variants of malignant cells with increased resistance to the antitumor immune response (Dunn, Old et al. 2004). A state of equilibrium is attained whereby effector cells of the immune system continually destroy immunogenic malignant cells, while new variants of genetically unstable tumor cells arise carrying mutations rendering them resistant to a competent immune system (Kim et al. 2007). This process continues for numerous years and these resistant variants ultimately progress to clinically detectable malignancies (Loeb et al. 2003).

1.2.3.3 Stage 3: Escape

In the escape phase, malignant cells which survived the elimination and equilibrium phase have accumulated genetic mutations which grant them resistance to attacks by the immune system, thus allowing the tumor to grow to clinically detectable volumes without immunological threat (Dunn et al. 2004). The tumor cells employ numerous evasive strategies including tumor related mechanisms and immunosuppressive cells (Figure 1.4) (Dunn et al. 2002, Kim et al. 2007, Morse et al. 2009, Töpfer et al. 2011).
Mechanisms which malignant cells use to evade antitumor immunity

Tumor related mechanisms

- Tumor-related mechanisms
  - Low immunogenicity
    - 1. Loss of antigen expression
    - 2. Loss of MHC component
    - 3. Loss of co-stimulatory molecules
    - 4. Shedding of NKG2D ligands
    - 5. Development of IFN-γ insensitivity
    - 6. Tumor induced privileged site
  - Resistance to effector cell-mediated killing
    - 1. Alteration of tumor apoptotic pathway
    - 2. Apoptosis of effector T-cells
  - Induction of effector cell anergy
    - 1. B7 family expression
    - 2. TCR signaling transduction alteration

Tumor derived soluble factors

- Tumor derived soluble factors
  - 1. TGF-β
  - 2. PGE2
  - 3. Galectin-1
  - 4. IDO
  - 5. GM-CSF
  - 6. IL10
  - 7. Mucin1
  - 8. MICA/B
  - 9. VEGF

Immunosuppressive immune cells

- Immunosuppressive immune cells
  - 1. Regulatory T cells
  - 2. Myeloid-derived suppressor cells
  - 3. Immunoregulatory antigen-presenting cells

Chapter 1: Introduction

1.2. Antitumor immunity

Tumor related mechanisms (Figure 1.4)

I. Tumor-derived barriers

i. Low immunogenicity

One of the mechanisms employed by malignant cells, to evade the immune response, is by the reduction of their immunogenicity (Murphy 2012d). The malignant cells may conceal themselves from antitumor effector cells by modulating the tumor within the tumor microenvironment through the creation of a physical barrier which prevents the interaction of immune cells and the tumor (Murphy 2012d). This process is mediated through the secretion of materials e.g. collagen, and surrounding non-tumor cells including fibroblasts, endothelial cells and extracellular matrix (Kim et al. 2007, Murphy 2012d). The fibroblasts and endothelial cells compete with dendritic cells for tumor antigens while the extracellular matrix bind tumor antigens (Murphy 2012d). This process is known as immunological ignorance. In conjunction to additional mechanisms, which renders the tumor undetectable, T-lymphocytes and NK cells, including the down-regulation or loss of MHC class 1 molecules, tumor-antigen expression, co-stimulatory molecules family, CD40, CD80, HLA-DR) and NKG2D ligands (Marincola et al. 2002, Groh et al. 2002, Rodriguez 2008, Murphy 2012d). Lastly, tumor cells induce the down-regulation or dysfunctional expression of adhesion molecules on blood vessels e.g. addressin, E-selectin and intracellular adhesion molecule 1), which results in impaired extravasation of T-lymphocytes at the tumor site (Clark et al. 2008).
ii. Resistance to effector cell-mediated killing

- **Apoptosis of effector cells:** This mechanism is mediated by 2 groups of receptors including Fas/Fas ligand and programmed death-1 ligand (PD-L1/PD-H1). Fas receptor, which is also known as apoptosis antigen 1 (APO-1) or CD95, is a member of the TNF receptor family and are highly expressed on effector T-lymphocytes following activation in the absence of co-stimulatory molecules (Morse et al. 2009, Töpfer et al. 2011). Fas ligands (FasL) are over-expressed in tumor cells, which initiate the apoptosis of effector T-cells following interaction with the Fas receptor on the T-cell (Webb et al. 2002). Programmed death ligands interact with Fas or PD-1 receptors on activated T-lymphocytes and NK cells (Morse et al. 2009).

- **Alteration of tumor apoptotic pathways:** One of the mechanisms which CD8+ T-cells, NK cells and NKT-cells use to eliminate malignancy is via the perforin-dependent cell death pathway (de Koning et al. 2011). However, tumor cells have been shown to evade this mechanism through the interference of perforin/granzyme signaling pathway via the expression of cell death inhibitors including caspase-inhibitors XIAP and FLIP, as well as granzyme B inhibiting serine protease inhibitor, SERPINB9 (de Koning et al. 2011, Töpfer et al. 2011). Tumor cells have also been shown to evade the immune response through the down-regulation or inactivation of death receptors at the transcriptional level, as well as the expression of decoy transmembrane or soluble receptors with truncated non-functional or missing death domains (Peli et al. 1999, Töpfer et al. 2011).
iii. Induction of effector cell anergy

- **B7 family expression**: Tumor cells have been shown to express B7 family receptors, which interact with inhibitory co-receptors Cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) and PD-1 (Zou et al. 2008). CTLA-4 interaction results in decreased IL-2 production, impaired TCR signaling and cell cycle arrest (Parry et al. 2005, Hodi 2007). PD-1 is upregulated by IFN-γ (Blank et al. 2004).

- **TCR signal transduction alteration**: TCR/CD3-ζ signal is one of 3 essential signals required for Th1 polarization, T-lymphocyte proliferation, tumor antigen expression and successful tumor response (Kim et al. 2007). Tumor induced myeloid derived suppressor cells (MDSC) have been shown to deplete the extracellular milieu of L-arginine, which leads to the inhibition CD3-ζ chain expression of the TCR-CD3 receptor complex and subsequently induces the arrest of T-cell proliferation (Rodríguez et al. 2008). The loss of CD3-ζ chain in tumor infiltrating lymphocytes is also associated with the down-regulation of INF-γ and an increase in TGF-β and IL-10 cytokine levels (Kim et al. 2007). This alteration has been noted in numerous tumors including, oral cancer (Reichert et al. 1998), pancreatic cancer (Schmielau et al. 2001) and ovarian cancer (Lockhart et al. 2001) with studies suggesting a possible associations to poor prognosis. Furthermore, TCR/CD3-ζ receptor is also considered as a caspase 3 substrate, which tumor cells may utilize to trigger apoptosis of effector T-lymphocyte (Gastman et al. 1999, Gastman et al. 2000).

II. Tumor-derived soluble factors

i. **Mucins**: Mucin1 (MUC 1), a membrane glycoprotein expressed by tumor cells become soluble when cleaved from the cell membrane and have been shown to down-regulate the immune response through cell-to-cell directed mechanism and cell-to-stroma
directed interaction (Morse et al. 2009). Mouse models have shown a correlation between MUC I expression and increased percentage of T regulatory cells (Morse et al. 2009).

ii. *TGF-β*: TGF-β is secreted by tumor cells and MDSC, and has been shown to inhibit the activation, proliferation and differentiation of CD4+ and CD8+ T-lymphocytes (Terabe et al. 2003, Li et al. 2008). High levels of intratumoral TGF-β have also been shown to facilitate the development of immunosuppressive cells including T regulatory cells (Shevach et al. 2008).

iii. *Prostaglandin E2 (PGE2)*: Tumor cells produce cyclooxygenase enzyme and prostaglandin E synthase that degrades arachidonic acid into PGE2 and Thromboxane (Morse et al. 2009). Increased intratumoral expression of PGE has been shown to stimulate tumor growth by shifting the immune response from a Th1-type response (TGF-α, IFN-γ and IL2), which promotes the proliferation of tumor-specific effector T-lymphocytes, to a Th2-type response (IL-4, IL-10 and IL-6), which promotes a humoral immune response and down-regulates the tumor-specific cell-mediated response (Huang et al. 1998, Harris et al. 2002, Morse et al. 2009, Töpfer et al. 2011). PGE2 has also been shown to impair dendritic cell maturation, T-lymphocyte proliferation, NK cell and macrophage effector function (Morse et al. 2009, Töpfer et al. 2011). In addition, PGE2 facilitates the induction of immunosuppressive FoxP3+ T regulatory cells (Baratelli et al. 2005).

iv. *Galectin-1*: This glycan-recognising protein, with an affinity for β-galactoside, has been shown to have an increased frequency of expression in numerous malignancies including melanoma (Mourad-Zeidan et al. 2008), breast cancer (Jung et al. 2007) and
ovarian cancer (van den Brûle et al. 2003). They have been shown to induce T-cell apoptosis and impair the assembly of the TCR complex (Perillo et al. 1995, Chung et al. 2000).

v. **Indoleamine 2,3-dioxygenase enzyme (IDO):** IDO is an intracellular tryptophan-degrading enzyme, which has been shown to induce immune suppression by the depletion of the essential amino acid, tryptophan (Mellor et al. 2004). IDO is expressed in antigen presenting cells and a variety of tumor cells, and promotes the development of T regulatory cells, and apoptosis of activated T-lymphocytes (Munn et al. 2007, Mellor et al. 2004, Morse et al. 2009, Bonanno et al. 2012).

vi. **VEGF:** Vascular endothelial growth factor (VEGF) is released by the tumor, and is involved in the recruitment of immature myeloid cells (macrophages and dendritic cells) from the bone marrow to the tumor microenvironment (Bellamy et al. 2001). It plays an essential role in the inhibition of dendritic cell maturation and proliferation, and facilitates the induction of T-cell and dendritic cell dysfunction through the promotion of intratumoral T-cell and dendritic activation by immature myeloid cells (Gabrilovich 2004).

vii. **MICA/B:** MHC class I chain-related proteins A and B molecules (MICA/B) are expressed on tumor cells and released as immunosuppressive soluble factors that binds to NKG2D receptors expressed on NK cells, CD8⁺ T-cells and γδT-lymphocytes (Bauer et al. 1999, Dunn et al. 2004). The interaction of MICA/B with the effector cells results in the down-regulation of T-cell and NK cell antitumor activity (Morse et al. 2009).
**Immunosuppressive immune cells** (Figure 1.4)

i. *Myeloid-derived suppressor cells (MDSC):* MDSC are a heterogeneous population of myeloid cells, including macrophages, dendritic cells and granulocytes, at different stages of development. They are generated in response to tumor-derived cytokines and granulocyte macrophage colony-stimulating factor (GM-CSF) (Bronte et al. 1999, Wislez et al. 2001, Nagaraj et al. 2007, Lechner et al. 2010). These cells are shown to be inversely correlated to intratumoral effector T-cells, and inhibit T-cell activity through the production of immunosuppressive substances (e.g. TGF-β) and amino acid metabolizing enzymes (e.g. arginase, nitric oxide synthetase and reactive oxygen species (ROS)) (Schmielau et al. 2001, Rodríguez et al. 2008, Töpfer et al. 2011).

ii. *Immunoregulatory antigen presenting cells:* Macrophages and dendritic cells have been shown to present immunoregulatory molecules including B7.1 and B7-H4 that initiate T-cell anergy and generate T regulatory cells (Dong et al. 1999, Dong et al. 2002, Kryczek et al. 2006). Furthermore, macrophages facilitate the inhibition of T-cell directed antitumor response through the secretion of immunosuppressive cytokines including IL-10, TGF-β and PGE₂ (Young et al. 1987).

iii. *T regulatory cells (Treg cells):* Tumor cells have been shown to increase the amount of circulating Treg cells within the tumor microenvironment or the peripheral blood of patients by activating naturally occurring Treg cells or converting non-Treg cells into suppressor Treg cells in the periphery (Orentas et al. 2006). Treg cells have been shown to inhibit antitumor activity by dendritic cells, CD4⁺ effector T-cells, CD8⁺ effector T-cells, NK cells and NKT-cells, through a number of mechanisms. This T-cell population will be discussed in more detail in section 1.3
1.3 T regulatory cell

T regulatory (Treg) cells were first described in 1970 as a specialised population of CD4\(^+\) T-lymphocytes in the healthy population (Gershon et al. 1970). These cells facilitate the maintenance of immune tolerance to self, and regulate the body’s immune response to commensal organisms, pathogens, and malignant cells (Gershon et al. 1970, Teng et al. 2011). These lymphocytes account for approximately 10-15% of resident CD4\(^+\) T-lymphocytes circulating within the peripheral system and are also found in the spleen, thymus and other lymphoid tissue (Sakaguchi et al. 1995, Setoguchi et al. 2005). Treg cells have been shown to have a lifespan of ± 5 months, with the ability to expand via lymphopenia-driven or antigen driven proliferation (Setoguchi et al. 2005, Anderson 2011).

1.3.1 Treg cell expression

Treg cells were initially defined by the expression of CD4\(^+\)CD25\(^+\) (α-chain of IL-2R receptor) surface markers (Sakaguchi et al. 1995), with additional surface and intracellular markers being identified over the past decade including: glucocorticoid-induced TNFR family-related receptor (GITR), CTLA-4, CD127 (α-chain of the IL-7 receptor), CD134 (OX40) (Ramsdell 2003), CD62L (L-selectin) and Foxhead box P3 (FoxP3) (Khattiri et al. 2003, Ramsdell 2003, Liu et al. 2006). However, markers identified as specific for this population of cells have been met with a substantial amount of controversy owing to the fact that CD25\(^+\), CD127\(^+\), GITR\(^+\) and CTLA-4\(^+\) are also expressed on non-suppressor effector T-cells (Linsley et al. 1992, Hernandez-Caselles et al. 1993, Ronchetti et al. 2004, Allan et al. 2007).

Recently CD127\(^+\) (IL-7Rα), which has high levels of expression in effector T-cells and memory T-cells, has been shown to be dimly expressed in Treg cells secondary to FoxP3
mediated down regulation of CD127 transcription (Liu et al. 2006). This results in the expression of CD127 being inversely correlated to intracellular FoxP3 expression, with effector T-cells being identified as FoxP3$^{−}$CD127$^{+}$ hi and suppressor T-cells being identified as FoxP3$^{+}$CD127$^{low/−}$ (Liu et al. 2006). Furthermore, suppressive activity by cells expressing CD4$^{+}$CD127$^{low/−}$CD25$^{−}$ surface markers suggested that CD127 could be used as a marker to identify different subtypes of peripheral Treg cells with variable CD25 or FoxP3 expression (Liu et al. 2006). CD127 is dimly expressed on the majority of CD4$^{+}$ Treg cells, and its expression in combination with CD25 makes it an excellent marker for identification of the majority of suppressor cells within the peripheral blood (Liu et al. 2006, Seddiki et al. 2006).

FoxP3 is an X-chromosome-linked transcription factor related to the forkhead/winged family, which is selectively expressed as an intracellular marker in naturally occurring, thymically derived Treg cells (Fontenot et al. 2003). It has been shown to be crucial for the development and function of naturally occurring Treg cells, and studies in humans have demonstrated that genetic mutations in this transcription factor leads to the development of a multi-organ autoimmune syndrome known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) (Bennett et al. 2001). FoxP3 was initially said to only be expressed in Treg cells which originated within the thymus (thymically derived/naturally occurring Treg cells); however, studies have demonstrated that CD4$^{+}$ T-cells could be induced to become suppressor T-cells once it had already left the thymus (peripherally derived Treg cells) with FoxP3 expression also being induced in the presence of TGF-β, IL-2 and retinoic acid (Hori et al. 2003, Chen et al. 2003, Coombes et al. 2007, Zheng et al. 2007).

Despite the high specificity that FoxP3 demonstrates in the identification of Treg cells, its presence alone does not correlate with the immunosuppressive capability of Treg cells. This
is in light of the fact that the suppressive activity of Treg cells is dependent on the levels of FoxP3 expression, and T-cells with low FoxP3$^+$ expression either display defective suppressive function or convert to Th-2 type effector T-lymphocytes (Wan et al. 2007). Furthermore, certain Treg cell populations do not express FoxP3 (Vieira et al. 2004). Lastly, Miyara and colleagues (2009) identified a CD4$^-$FoxP3$^+$ population which has no suppressive function but secretes the proinflammatory cytokine IL17.

Despite the continuous influx of new Treg markers, and the controversy associated with the more traditional markers, CD3$^+$CD4$^+$FoxP3$^+$CD127$^{low}$ is still considered as the definitive phenotypic expression of human peripheral blood Treg cells, with FoxP3 still being regarded as the most reliable marker of the Treg cell population (Seddiki et al. 2006, Sakaguchi et al. 2008).

### 1.3.2 Classification of Treg cells

Treg cells are classified into two brought groups according to their site of origin. The first group is regarded as naturally immunosuppressive cells, which originate in the thymus (nTreg) (Figure 1.5), and express FoxP3$^+$ as an intracellular marker (CD4$^+$FoxP3$^+$) (Sakaguchi et al. 2006). The second group of Treg cells (iTreg) (Figure 1.5) originate from naïve CD4$^+$ T-lymphocytes which are induced to become suppressive cells in the peripheral system following exposure to immunosuppressive cytokines or naturally occurring CD4$^+$FoxP3$^+$ Treg cells (Zheng et al. 2007).
Mechanisms which malignant cells use to evade antitumor immunity

Tumor related mechanisms

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5. Development of IFN-γ insensitivit
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Resistance to effector cell-mediated killing

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2. Apoptosis of effector T-cells

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iv. **Galectin-1:** This glycan-recognising protein, with an affinity for β-galactoside, has been shown to have an increased frequency of expression in numerous malignancies including melanoma (Mourad-Zeidan et al. 2008), breast cancer (Jung et al. 2007) and
ovarian cancer (van den Brûle et al. 2003). They have been shown to induce T-cell apoptosis and impair the assembly of the TCR complex (Perillo et al. 1995, Chung et al. 2000).

v. **Indoleamine 2,3-dioxygenase enzyme (IDO):** IDO is an intracellular tryptophan-degrading enzyme, which has been shown to induce immune suppression by the depletion of the essential amino acid, tryptophan (Mellor et al. 2004). IDO is expressed in antigen presenting cells and a variety of tumor cells, and promotes the development of T regulatory cells, and apoptosis of activated T-lymphocytes (Munn et al. 2007, Mellor et al. 2004, Morse et al. 2009, Bonanno et al. 2012).

vi. **VEGF:** Vascular endothelial growth factor (VEGF) is released by the tumor, and is involved in the recruitment of immature myeloid cells (macrophages and dendritic cells) from the bone marrow to the tumor microenvironment (Bellamy et al. 2001). It plays an essential role in the inhibition of dendritic cell maturation and proliferation, and facilitates the induction of T-cell and dendritic cell dysfunction through the promotion of intratumoral T-cell and dendritic activation by immature myeloid cells (Gabrilovich 2004).

vii. **MICA/B:** MHC class I chain-related proteins A and B molecules (MICA/B) are expressed on tumor cells and released as immunosuppressive soluble factors that binds to NKG2D receptors expressed on NK cells, CD8+ T-cells and γδT-lymphocytes (Bauer et al. 1999, Dunn et al. 2004). The interaction of MICA/B with the effector cells results in the down-regulation of T-cell and NK cell antitumor activity (Morse et al. 2009).
Immunosuppressive immune cells (Figure 1.4)

i. Myeloid–derived suppressor cells (MDSC): MDSC are a heterogeneous population of myeloid cells, including macrophages, dendritic cells and granulocytes, at different stages of development. They are generated in response to tumor-derived cytokines and granulocyte macrophage colony-stimulating factor (GM-CSF) (Bronte et al. 1999, Wislez et al. 2001, Nagaraj et al. 2007, Lechner et al. 2010). These cells are shown to be inversely correlated to intratumoral effector T-cells, and inhibit T-cell activity through the production of immunosuppressive substances (e.g. TGF-β) and amino acid metabolizing enzymes (e.g. arginase, nitric oxide synthetase and reactive oxygen species (ROS)) (Schmielau et al. 2001, Rodriguez et al. 2008, Töpfer et al. 2011).

ii. Immunoregulatory antigen presenting cells: Macrophages and dendritic cells have been shown to present immunoregulatory molecules including B7.1 and B7-H4 that initiate T-cell anergy and generate T regulatory cells (Dong et al. 1999, Dong et al. 2002, Kryczek et al. 2006). Furthermore, macrophages facilitate the inhibition of T-cell directed antitumor response through the secretion of immunosuppressive cytokines including IL-10, TGF-β and PGE₂ (Young et al. 1987).

iii. T regulatory cells (Treg cells): Tumor cells have been shown to increase the amount of circulating Treg cells within the tumor microenvironment or the peripheral blood of patients by activating naturally occurring Treg cells or converting non-Treg cells into suppressor Treg cells in the periphery (Orentas et al. 2006). Treg cells have been shown to inhibit antitumor activity by dendritic cells, CD4⁺ effector T-cells, CD8⁺ effector T-cells, NK cells and NKT-cells, through a number of mechanisms. This T-cell population will be discussed in more detail in section 1.3.
1.3 T regulatory cell

T regulatory (Treg) cells were first described in 1970 as a specialised population of CD4$^+$ T-lymphocytes in the healthy population (Gershon et al. 1970). These cells facilitate the maintenance of immune tolerance to self, and regulate the body’s immune response to commensal organisms, pathogens, and malignant cells (Gershon et al. 1970, Teng et al. 2011). These lymphocytes account for approximately 10-15% of resident CD4$^+$ T-lymphocytes circulating within the peripheral system and are also found in the spleen, thymus and other lymphoid tissue (Sakaguchi et al. 1995, Setoguchi et al. 2005). Treg cells have been shown to have a lifespan of ± 5 months, with the ability to expand via lymphopenia-driven or antigen driven proliferation (Setoguchi et al. 2005, Anderson 2011).

1.3.1 Treg cell expression

Treg cells were initially defined by the expression of CD4$^+$CD25$^+$ (α-chain of IL-2R receptor) surface markers (Sakaguchi et al. 1995), with additional surface and intracellular markers being identified over the past decade including: glucocorticoid-induced TNFR family-related receptor (GITR), CTLA-4, CD127 (α-chain of the IL-7 receptor), CD134 (OX40) (Ramsdell 2003), CD62L (L-selectin) and Foxhead box P3 (FoxP3) (Khattari et al. 2003, Ramsdell 2003, Liu et al. 2006). However, markers identified as specific for this population of cells have been met with a substantial amount of controversy owing to the fact that CD25$^+$, CD127$^+$, GITR$^+$ and CTLA-4$^+$ are also expressed on non-suppressor effector T-cells (Linsley et al. 1992, Hernandez-Caselles et al. 1993, Ronchetti et al. 2004, Allan et al. 2007).

Recently CD127$^+$ (IL-7Rα), which has high levels of expression in effector T-cells and memory T-cells, has been shown to be dimly expressed in Treg cells secondary to FoxP3
mediated down regulation of CD127 transcription (Liu et al. 2006). This results in the expression of CD127 being inversely correlated to intracellular FoxP3 expression, with effector T-cells being identified as FoxP3−CD127\(^{\text{high}}\), and suppressor T-cells being identified as FoxP3\(^{+}\)CD127\(^{\text{low/−}}\) (Liu et al. 2006). Furthermore, suppressive activity by cells expressing CD4\(^{+}\)CD127\(^{\text{low/−}}\)/CD25\(^{−}\) surface markers suggested that CD127 could be used as a marker to identify different subtypes of peripheral Treg cells with variable CD25 or FoxP3 expression (Liu et al. 2006). CD127 is dimly expressed on the majority of CD4\(^{+}\) Treg cells, and its expression in combination with CD25 makes it an excellent marker for identification of the majority of suppressor cells within the peripheral blood (Liu et al. 2006, Seddiki et al. 2006).

FoxP3 is an X-chromosome-linked transcription factor related to the forkhead/winged family, which is selectively expressed as an intracellular marker in naturally occurring, thymically derived Treg cells (Fontenot et al. 2003). It has been shown to be crucial for the development and function of naturally occurring Treg cells, and studies in humans have demonstrated that genetic mutations in this transcription factor leads to the development of a multi-organ autoimmune syndrome known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) (Bennett et al. 2001). FoxP3 was initially said to only be expressed in Treg cells which originated within the thymus (thymically derived/naturally occurring Treg cells); however, studies have demonstrated that CD4\(^{+}\) T-cells could be induced to become suppressor T-cells once it had already left the thymus (peripherally derived Treg cells) with FoxP3 expression also being induced in the presence of TGF-β, IL-2 and retinoic acid (Hori et al. 2003, Chen et al. 2003, Coombes et al. 2007, Zheng et al. 2007).

Despite the high specificity that FoxP3 demonstrates in the identification of Treg cells, its presence alone does not correlate with the immunosuppressive capability of Treg cells. This
is in light of the fact that the suppressive activity of Treg cells is dependent on the levels of FoxP3 expression, and T-cells with low FoxP3\(^+\) expression either display defective suppressive function or convert to Th-2 type effector T-lymphocytes (Wan et al. 2007).

Furthermore, certain Treg cell populations do not express FoxP3 (Vieira et al. 2004). Lastly, Miyara and colleagues (2009) identified a CD4\(^-\)FoxP3\(^+\) population which has no suppressive function but secretes the proinflammatory cytokine IL17.

Despite the continuous influx of new Treg markers, and the controversy associated with the more traditional markers, CD3\(^+\)CD4\(^+\)FoxP3\(^+\)CD127\(^{low}\) is still considered as the definitive phenotypic expression of human peripheral blood Treg cells, with FoxP3 still being regarded as the most reliable marker of the Treg cell population (Seddiki et al. 2006, Sakaguchi et al. 2008).

### 1.3.2 Classification of Treg cells

Treg cells are classified into two brought groups according to their site of origin. The first group is regarded as naturally immunosuppressive cells, which originate in the thymus (nTreg) (Figure 1.5), and express FoxP3\(^+\) as an intracellular marker (CD4\(^+\)FoxP3\(^+\)) (Sakaguchi et al. 2006). The second group of Treg cells (iTreg) (Figure 1.5) originate from naïve CD4\(^+\) T-lymphocytes which are induced to become suppressive cells in the peripheral system following exposure to immunosuppressive cytokines or naturally occurring CD4\(^+\)FoxP3\(^+\) Treg cells (Zheng et al. 2007).
1.3.2.1 CD4⁺ Treg cells

CD4⁺ Treg cells consists of numerous subsets including thymically derive CD4⁺CD25⁺FoxP3⁺ Treg cells (Fontenot et al. 2003), antigen-induce CD4⁺CD25⁺FoxP3⁺ Treg cells (Zheng et al. 2007) and CD4⁺FoxP3⁻ Trl cell (Miyara et al. 2009, Grazia et al. 2006).

Figure 1.6: Differentiation of naïve CD4⁺ T-cells into Treg cells and effect (Sakaguchi et al. 2008)
i. **Thymically derived Treg cell (CD4⁺CD25⁺FoxP3⁺):** These cells arise through a process of central tolerance and migrate to the periphery where they induce effector suppression by a cell-to-cell dependent contact mechanism without specific antigen stimulation (Sakaguchi et al. 2006).

ii. **Antigen-induced Treg cell (CD4⁻CD25⁺FoxP3⁺):** These cells arise following the exposure of CD4⁺CD25⁻ naïve T-cells and effector T-lymphocytes to a suppressive cytokine environment (Zheng et al. 2007) or following an encounter with naturally occurring CD4⁺CD25⁺FoxP3⁺ Treg cells (Qiao et al. 2007). These cells mediate immune suppression through a cell-to-cell contact mechanism, as well as through the secretion of soluble factors such as IL-10 and TGF-β (Shevach 2002).

iii. **Trl cells (CD4⁺FoxP3⁻):** These cells do not express FoxP3⁺ and are induced into becoming Treg cells through a process of MHC/peptide stimulation in the presence of a suppressive cytokine, namely IL-10 (Groux et al. 1997, Grazia et al. 2006).

A number of additional peripherally-derived Treg cells have been identified including, the Th3 (CD4⁺TGF-β⁺) Treg cell population which, like Trl cells, has been shown to suppress cells through the secretion of IL-10 and TGF-β1 (Weiner 2001). These cells have been shown to inhibit the development of autoimmune diseases such as autoimmune encephalitis (Bridoux et al. 1997).

Recently a new population of suppressive Th17FoxP3⁺ Treg cells have been identified in humans, with authors speculating that they may be essential in the regulation of
autoimmunity, inflammation and protection against bacterial pathogens (Voo et al. 2009). Th17FoxP3+ Treg cells are induced peripherally through the stimulation of the CD4++FoxP3++CCR6+ Treg cell’s T-cell receptor in the presence of human serum, IL-1β, IL-2, IL-21, and IL-23 (Voo et al. 2009). This results in the differentiation of these non-IL-17 secreting regulatory T-cells into IL-17 producing Treg cells (Voo et al. 2009). Numerous cytokines in combination with TGF-β have been shown to determine whether these cells become Treg suppressor cells, IL-17 producing T-helper cells or a combination of the two with both suppressor and effector function (Zhou et al. 2008).

1.3.2.2 \textit{CD8}^+ Treg cells

CD8\(^+\) Treg cells have also been identified following the demonstration of a population of Treg cells which did not conform to the CD4++CD25++FoxP3+ phenotype (Kiniwa et al. 2007). Like CD4\(^+\) Treg cells, these cells can also be thymically derived or peripherally induced (Kiniwa et al. 2007). In 2003, Cosmi and colleagues (2003) were the first to identify thymically derived CD8++CD25++GITR++FoxP3 in the thymic tissue of humans, which have similar localisation, phenotypic function and mechanism of action as CD4++CD25++FoxP3\(^+\) Treg cells (Cosmi et al. 2003). Following activation, these CD8\(^+\) Treg cells express TGF-β1 and CTLA-4 surface markers, which mediate the suppression of effector T-cell proliferation and activation through a cell-to-cell contact dependent mechanism (Cosmi et al. 2003). This interaction results in the suppression of IL-2Ra chain on effector T-cells, which renders them unresponsive to IL-2 (Cosmi et al. 2003). The absence of perforin or granzyme in CD8\(^+\) Treg cells excluded the possibility of suppression being induced via apoptosis of the target effector T-cell (Cosmi et al. 2003). It has been suggested that these cells
play a crucial role in the regulation of T-cell mediated response against virally infected cells and malignant cells (Cosmi et al. 2003). Thereafter, Jarvis and colleagues (2005) also identified a population of CD8⁺CD25⁺CTLA4⁺FoxP3⁺ Treg cells in the peripheral blood of patients suffering from autoimmune Ankylosing Spondylitis. These cells were very similar to those found by Cosmi and colleagues (2003), except that they were shown to produce IL-4, IL-5 and IL-13, which was not demonstrated in the CD8⁺ Treg population identified by Cosmi and colleagues (2003). These cells were also found to express CD69 continuously, which has been shown to induce the production of TGF-β1 by NK cells and T-lymphocytes (Jarvis et al. 2005). It is hypothesised that the CD8⁺ Treg cell population identified within the thymus by Cosmi and colleagues (2003), may be precursors to the CD8⁺ Treg cell population identified by Jarvis and colleagues in the peripheral blood circulation (Jarvis et al. 2005). This however is yet to be confirmed.

Numerous subpopulations of peripherally derived CD8⁺ Treg cells have been identified, including: CD8⁺CD122⁺ Treg cell, IL-10⁺CCR7⁺CD45RA⁺CD8⁺ Treg cell and CD8⁺LAG-3⁺CCL4⁺ Treg cell (Joosten et al. 2007, Kiniwa et al. 2007). These cells may be induced to become suppressive by immature dendritic cells, plasmacytoid dendritic cells, as well as malignant cells (Kiniwa et al. 2007). Following the finding of CD8⁺LAG-3⁺CCL4⁺ Treg cells in granulomatous lesions of Mycobacterium-infected lymph nodes, it has been suggested that they may play a role in the regulation and promotion of chronic infection (Joosten et al. 2007).

CD8⁺ Treg cells have been shown to inhibit the proliferation of antigen specific CD4⁺ and CD8⁺ effector T-cells through a cell-cell contact dependent mechanism, as well as
through the secretion of immunosuppressive soluble substances (Endharti et al. 2005, Kiniwa et al. 2007). Recently, the CD8\(^+\) Treg cell population specific for heme-oxygenase-1 (HO-1-CD8\(^+\)) was identified in the peripheral blood of cancer patients (Andersen et al 2009). These cells are said to inhibit proliferation, cytotoxicity and cytokine production of antitumor effector cells including CD4\(^+\) T-lymphocyte, CD8\(^+\) T-lymphocyte and possibly NK cells (Andersen et al. 2009). Despite the identification of a variety of CD8\(^+\) Treg subpopulations, there is still very little known about CD8\(^+\) Treg cells in comparison to CD4\(^+\) Treg cells which have been extensively researched. However, dysfunction of these cells have been implicated as major role players in the pathogenesis of cancer and autoimmune disease, thus opening up new avenues to be explored in the search for therapeutic interventions (Qiao et al. 2007, Andersen et al. 2009).

**1.3.3 T regulatory cell function**

Within healthy individuals, Treg cells have been shown to maintain immune tolerance by suppressing host immune response directed towards self-antigens, thus playing a crucial role in the prevention of the development of autoimmune diseases (Wilke et al. 2010). They contribute to the maintenance of tolerance to allogeneic tissues in transplantation medicine, with new studies demonstrating their ability to prevent both acute and chronic rejection of allografts (Pasquet et al. 2013). Furthermore, Treg cells limit tissue damage during inflammatory responses (D'Alessio et al. 2009), and play a significant role in the maintenance of fetomaternal tolerance during pregnancy (Habicht et al. 2007).
Treg cells have been shown to suppress host effector T-cell activity in numerous disease states and down-regulates T-cell function in antimicrobial (Lundgren et al. 2005), antiviral (Weiss et al. 2004) and antitumor response (Curiel et al. 2004, Taams et al. 2006). Over the last decade a substantial amount of attention has been given to the ability of Treg cells to down-regulate effector cells involved in mediating anticancer immunity, including NK cells (Trzonkowski et al. 2004), CD4\(^+\) T-lymphocytes (Itoh et al. 1999), CD8\(^+\) T-lymphocytes (Trzonkowski et al. 2004), NKT cells (Azuma et al. 2003), dendritic cells (DiPaolo et al. 2007) and B-lymphocytes (Lim et al. 2005).

Treg cells have been shown to mediate their immune suppressive mechanism in numerous overlapping ways, which may occur via a cell-to-cell directed mechanism or through the secretion of immunosuppressive substance.

These mechanism include:

i. Cytokine inhibition via release of IL-10, IL-35 and TGF-β (Grazia et al. 2006, Coombes et al. 2007, Collison et al. 2007);

ii. Direct cytolysis of effector T-cells by means of granzyme and perforin secretion (Gondek et al. 2005, Zhao et al. 2006);

iii. Metabolic disruption through the release of intracellular or extracellular adenosine nucleosides (Deaglio et al. 2007);

iv. Inhibition of dendritic cell function through binding of CTLA-4 to CD80/86 and induction of indoleamine 2,3-dioxygenase (IDO) (DiPaolo et al. 2007, Oderup et al. 2006);

v. Expression of negative costimulatory molecules (Joosten et al. 2007);

vi. Induction of anti-inflammatory biochemical pathways in effector T-cells and APCs (Andersen et al. 2009);

vii. Consumption of proinflammatory cytokines e.g. IL-2 (Pandiyan et al. 2007).
1.3.4 T regulatory cells and cancer

During the past two decades there has been a growing interest in the role that Treg cells play in the development and progression of cancer in vivo. This interest was triggered by the demonstration of increased levels of Treg cells in the peripheral blood of patients suffering from cancer, as compared to healthy individuals (Woo et al. 2001, Liyanage et al. 2002, Sasada et al. 2003, Liu et al. 2008, Jaberipour et al. 2010). Numerous studies have emerged which demonstrated an increase in the frequency of Treg cell activity in patients suffering from various cancers including solid tumors: lung (Petersen et al. 2006), pancreatic (Hiraoka et al. 2006), breast (Bates et al. 2006), liver (Ormandy et al. 2005), ovarian (Curiel et al. 2004), gastric (Kono, a et al. 2006), oesophageal (Kono et al. 2006), cervical (Nakamura et al. 2007), head and neck cancer (Strauss et al. 2007) and haematological malignancies (Karube et al. 2004). This increase in Treg cells was identified in either the peripheral blood, regional lymph nodes or within the tumor microenvironment itself, with the highest numbers of Treg cells identified in patients with untreated or refractory disease (Kono et al. 2006, Sellitto et al. 2011).

This was followed by the investigation of Treg cells as a marker of prognosis, and numerous studies began to emerge which demonstrated an increase in the expansion of Treg cell populations in the peripheral blood of patients with metastatic disease (Nakamura et al. 2009, Wang, et al. 2012). The presence of Treg cells within the tumor microenvironment as well as the peripheral blood has been associated with a poor outcome in numerous cancers including ovarian, breast, gastric, oesophageal and non-small cell lung cancer (Curiel et al. 2004, Kono et al. 2006, Ohara et al. 2009, Xu et al. 2009, Merlo et al. 2009). Merlo and colleagues (2009) even went so far as to
suggest that the expression of FoxP3 in Treg cells correlates with the metastatic capability of cancer, instead of tumor immune suppression.

The general consensus in the medical world was that an increase in Treg cells correlated with poor survival, which was an exciting discovery as it would mean that a reliable prognostic marker for malignancy had been discovered. However, studies emerged which contradicted these findings and suggested that the presence of Treg cells within the tumor micro-environment correlated with improved patient outcome, and could even be used as an independent prognostic marker for better regional control of the malignancy (Alvaro, et al. 2005, Salama et al. 2009, Haas et al. 2009, Correale et al. 2010).

In clinical medicine there has been increased interest in possible methods of manipulating Treg levels in oncology patients, with the aim of improving response to antitumor treatment. This interest developed following observations that Treg cells impaired the efficacy of immunotherapeutic agents, and that the disruption of Treg cell pathways could result in the augmentation of immune surveillance or the facilitation of an immunotherapeutic cure (Nagai et al. 2004, Dannull et al. 2005). Multiple attempts have been made to deplete or block Treg cells in patients presenting with cancer and has resulted in the improvement of effector CD8$^+$ T-cell mediated antitumor response (Onizuka et al. 1999, Nagai et al. 2004, Dannull et al. 2005). Ontak (Denileukin diftitox), a fusion protein of diphtheria-toxin and IL-2, has received FDA approval for use in the treatment of persistent or relapsed CD25$^+$ cutaneous T-cell lymphoma (Manoukian 2009). Following the administration of Ontak, patients presenting with ovarian or renal cancer display reduced numbers of
peripherally circulating Treg cells, and improved T-cell activation (Barnett et al. 2005, Dannull et al. 2005). Additional pharmacological interventions directed at Treg cells have been proposed, including the use of agents directed against CTLA-4 (anti-CTLA-4-antibodies) e.g. Ipilimumab, in patients presenting with malignant melanoma (Hodi et al. 2003, O'Day et al. 2010), prostate cancer (Small et al. 2007) and ovarian cancer (Hodi et al. 2003). Following the administration of the anti-CTLA-4 agent, there was objective clinical improvement and improved long term survival, however the induction of autoimmune diseases in these patients following immunotherapy still remains a problem (Hodi et al. 2008, O'Day et al. 2010). Combination therapy (anti-CD25 and anti-CTLA-4), as well as anti-GITR antibodies are also being investigated and have given promising results; however, additional investigations need to be performed before these agents can be used in the clinical management of patients (Sutmuller et al. 2001, Ko et al. 2005).

1.4 Antitumor treatment and immunity

Surgery, chemotherapy and radiotherapy are the most commonly used antitumor treatment modalities in our setting. Although these anticancer therapies are effective in eradicating malignancies in a large proportion of cases, they have been shown to have a multitude of complications, including immunosuppression (Mafune et al. 2000). As more studies continue to assess the role of the host immune defence against cancer and possible ways in which new immunotherapeutic agents can facilitate these processes, conflicting research has emerged which question numerous dogmas about the more traditional antitumor interventions currently in use.
1.4.1 Surgical oncology

In an effort to evade effector T-lymphocytes and NK cells, malignant cells induce the Th1/Th2 balance to shift towards a Th2 response, thus inhibiting the induction of cell-mediated immunity and promoting a B-lymphocyte and humoral led immune response (Goto et al. 1999). This shift from a Th1 to a Th2 response has also been demonstrated in patients as a response to surgical stress (Ishikawa et al. 2009). Hesler and colleagues (1997) conducted a study where they assessed the immunological changes following major surgery. The indication for surgery was malignancy-related in 83% of cases; however, contrary to previous findings they did not observe any malignancy-induced immune dysregulation pre-operatively (Hensler et al. 1997). They did however demonstrate that surgery impaired the activation, proliferation and cytokine production of T-lymphocytes in the early post-operative period (day 1-2) (Hensler et al. 1997). Although Th1 cytokines and Th2 cytokines were impaired, the secretion of IL-10, an anti-inflammatory cytokine, was not impaired and continued to rise even in the late post-operative period (day 3-5) (Hensler et al. 1997). The levels of IFN-γ and IL-2 were seen to return to normal pre-operative levels in the late post-operative period, (day 5) (Hensler et al. 1997).

Similar findings were demonstrated by Mafune and colleagues (2000) who observed a decrease in NK cell activity in the post-operative period, in conjunction with an increase in the CD4⁺:CD8⁺ T-cell ratio secondary to diminished CD8⁺ T-lymphocytes quantities. Prolonged immunosuppression following surgical intervention in cancer patients has been demonstrated in numerous studies (Yoshihara et al. 1986, Pollock et al. 1991, Hensler, et al. 1997, Ogawa et al. 2000), which may accelerate the development of pre-existing micrometastases and facilitate post-operative...

Despite the negative reports regarding surgical intervention in malignant conditions, proponents of surgical intervention claim that surgical excision of the tumor facilitates the immune response by decreasing the tumor cell burden, thus diminishing tumor associated mechanisms of antitumor immunity and decreasing the number of local Treg cells (González et al. 2007, Baumgartner et al. 2009). This results in the improvement of immune surveillance mechanism and possible impediment of tumor growth (González et al. 2007, Baumgartner et al. 2009). Studies have demonstrated improved survival following surgical excision of the primary tumor mass in patients presenting with renal cell carcinoma (Flanigan et al. 2001), colorectal (Rosen et al. 2000), breast (Gnerlich et al. 2007) and ovarian cancer (Griffiths et al. 1978). Leung and colleagues (2010) conducted similar studies in stage IV breast cancer patients and found that patients who received surgical treatment had a survival benefit over patients who received no surgical treatment. This finding corresponded with previous findings (Leung et al. 2010). However, following the standardisation of results for patients who received chemotherapy in addition to surgical treatment, they concluded that surgical intervention in itself does not provide a survival benefit but the addition of a chemotherapeutic regimen does (Leung et al. 2010).

1.4.2 Chemotherapy

Chemotherapy is the process whereby chemical agents with cytotoxic activity are administered systemically to patients with the aim of eradicating or controlling the cancer growth (Morgan 2003). The use of chemotherapeutic agents in clinical
medicine has spanned numerous decades, and has been shown to be beneficial in a multitude of conditions including autoimmune diseases, transplantation medicine and lymphoproliferative conditions (Rasmussen et al 1982). Chemotherapeutic agents however target rapidly replicating cells in a non-selective manner thus malignant cells as well as normal cells are targeted (Rasmussen et al 1982); consequently, the majority of oncology patients receiving chemotherapy experience chemotherapy-induced immune dysregulation (Lehrnbecher et al. 2008, Chu-Yuan et al. 2013).

Chemotherapy has been shown to affect multiple components of the immune system starting with the innate immune defence where chemotherapy-induced mucositis breaks down the external protective barrier (Ek et al. 2005, Fanning et al. 2006). NK cells are shown to decline significantly during treatment and although NK cell levels recover following treatment, studies have suggested that due to their crucial role in antitumor defence, such a significant decline may contribute to cancer relapse (Lehrnbecher et al. 2008). The functional capacity, chemotactic ability and frequency of neutrophils have also been shown to be affected with patients presenting with a clinical condition known as febrile neutropenia, which consists of a fever of >38°C and an total neutrophil count of <1.0 x 10^9 cells/l (Lehrnbecher et al. 1997, Mendonça et al. 2006, Naik et al. 2010). This condition renders the patient susceptible to life threatening bacterial infection (Naik et al. 2010).

Numerous studies have demonstrated the severe chemotherapy-induced lymphopenia associated with the majority of chemotherapeutic agents, with B-cell, CD4^+ and CD8^+ T-cell populations being affected (Sewell et al. 1993). The remaining circulating lymphocytes have also been shown to have impaired effector function thus rendering the patient susceptible to opportunistic infections (Lehrnbecher et al. 2008). Follow
up of these patients have also demonstrated that the frequency of circulating CD4\(^+\) T-lymphocytes remains low for up to six months following treatment; however, the frequency of CD8\(^+\) T-lymphocyte is said to recover one month after chemotherapy (Ek et al. 2005).

One of the benefits of the global lymphopenia induced by the chemotherapy is the fact that no T-lymphocyte population is spared, including Treg cells. Treg cells have been shown to be extremely sensitive to the chemotherapeutic agent cyclophosphamide (Ghiringhelli, Larmonier et al. 2004, Traverso, Fenoglio et al. 2012). Cyclophosphamide, a nitrogen mustard alkylating agent, is one of the most commonly used chemotherapeutic agents both in the treatment of cancer and autoimmune disease (Emadi et al. 2009, Brodsky et al. 2010). It has been shown to inhibit CD4\(^+\) and CD8\(^+\) Treg cell proliferation and suppressive activity, with Treg proponents suggesting that it should be regarded as a vital component of any antitumor treatment protocol (Lutsiak et al. 2005, Traverso et al. 2012).

Following this finding, it was assumed that alternative chemotherapeutic agents could also inhibit Treg cell proliferation and activity. However, Tohyama and colleagues (2013) conducted a study which assessed the influence of 4 widely used chemotherapeutic agents (methotrexate, arsenic trioxide, dacarbazine and 5-fluorouracil) on the cellular component of the immune system of healthy human participants. They found that not only did these agents induce a state of neutropenia, but they also decreased the quantity of effector T-cells and inhibited effector T-cell proliferation and function (Tohyama et al. 2013). Interestingly, they were shown to increase the relative numbers of Treg cells, possibly through the elimination of other...
T-cell subsets (Tohyama et al. 2013). Thus raising many questions regarding the possibility of Treg cells being chemo-resistant, as well as the possibility that certain chemotherapeutic agents attenuate the antitumor immune response, and tilt the balance towards a more immunosuppressive tumor-microenvironment, which augments tumor growth and progression.

1.4.3 Radiotherapy

The use of external beam radiotherapy in the treatment of cancer is also extremely common and can be used as a primary form of treatment (De-en, et al. 1988), or in combination with additional antitumor treatments, such as chemotherapy or surgery (Standish et al. 2008, Kang et al. 2009, Kachikwu et al. 2011). As already mentioned, surgery and chemotherapy suppress the patients immune response, and the use of radiotherapy as a supplementary antitumor intervention in these patients enhances the level of immune suppression even further (Standish et al. 2008, Standish et al. 2008b).

Standish and colleagues (2008b) conducted a study which assessed the influence of external beam radiotherapy on the innate and adaptive immunity of patients being treated for breast cancer. Following a standard course of external beam therapy, patients presented with a significant decline in the lymphocyte population which did not return to normal levels at 6 weeks after the termination of treatment (Standish et al. 2008b). This persistent lymphopenia was also observed in a study conducted by Bachtiary and colleagues (2005), where patients receiving radiation treatment for cervical cancer developed severe immunosuppression, which rendered them susceptible to opportunistic infections (Bachtiary et al. 2005). CD8\(^+\) T-lymphocytes are more radiosensitive than CD4\(^+\) T-lymphocytes, with studies showing an increase
in the CD4⁺: CD8⁺ T-cell ratio shortly after radiotherapy (Mafune et al. 2000). Radiotherapy has also been shown to reduce the frequency and effector activity of monocytes and NK cell, however neutrophils seem to be radio-resistant as they were not affected by the radiation and remained within normal range (Standish et al. 2008b).

Similar to neutrophils, studies have demonstrated that Treg cells do not decline following exposure to radiotherapy (Lissoni et al. 2009, Kachikwu et al. 2011). Some have even suggested that they may be the most radio-resistant T-cell subset following findings by Lissoni and colleagues (2009) who demonstrated a significant decline in the total lymphocyte count and CD4⁺ T-cell count, while no change was observed in the circulating Treg cell count in cancer patients who received a course of radiotherapy. Some studies have even demonstrated that Treg cells increase following exposure to radiotherapy (Horwitz et al. 2008, Kachikwu et al. 2011). This increase is said to be secondary to radiation induced secretion and activation of TGF-β, which has been shown to induce the peripheral conversion of CD4⁺CD25- T-cells into Treg cells, thus exacerbating the state of immune suppression and promoting tumor tolerance (Horwitz et al. 2008, Kachikwu et al. 2011).
1.5 **Breast cancer and immunology**

1.5.1 **Breast cancer epidemiology**

Breast cancer (BCA) is the most common malignancy affecting women in sub-Saharan Africa, as well as the most common cause of cancer-related deaths in this region (Ferlay et al. 2010, American Cancer Society 2011). In the last 2 decades, sub-Saharan Africa has seen a significant rise in the prevalence of BCA (American Cancer Society 2011). This has been attributed to an increase in economic development, which is associated with increased BCA risk factors including obesity, early menarche, late child bearing and having fewer children (American Cancer Society 2011). It may also be attributed to improvements in access to healthcare facilities, which would increase the identification of patients suffering from BCA, as well as improved screening methods, increased self examination and increased BCA community awareness programs which would allow for the identification of malignancies which are not yet clinically detectable (Kingham, et al. 2013).

According to the World Bank, South Africa is classified as a middle-income country and has a diverse population consisting of 52,982,000 people (Stats SA 2013, World Bank 2014). Despite the fact that the only available national cancer statistics for South Africa dates back to 2006, the statistics are still similar to what is currently being observed in the rest of sub-Saharan Africa, with BCA still being the most common cancer in women in South Africa (Figure 1.7). The current age standardised incidence rate of BCA in South Africa is 29.29/100,000, and a South African woman’s lifetime risk of developing BCA is 1 in 33 (National Health Laboratory Services 2014, CANSA 2014, African Cancer Registry Network 2014).
South Africa has been shown to have a higher BCA mortality: incidence ratio as compared to the world standard, which suggests that patients suffering from the condition in South Africa have a higher risk of death (Wadler et al. 2011). This is in agreement with reports stating that women in developing countries who are diagnosed with BCA have a poorer prognosis than those in developed countries (Ferlay et al. 2010). Despite all efforts to keep track of the evolution of BCA in South Africa through the National registry, there is a tendency to underestimate the true incidence of cancer as these statistics only represent pathology-based incidences rather than reporting a population-based incidence of BCA (Vorobiof et al. 2001, Wadler et al. 2011).
1.5.2 Pathology

BCA is regarded as an epithelial malignancy which affects the breast tissue and is classified according to the cytolytic features, biological behaviour and growth patterns of the malignancy. There are two main groups of BCA, including Ductal (intraductal) carcinoma insitu and lobular carcinoma insitu, which are further subdivided into several histological subtypes including: infiltrating ductal, invasive lobular, ductal/lobular, mucinous (colloid), tubular, medullary, papillary, metaplastic and invasive micropapillary breast cancer (Schnitt et al. 2004, Li et al. 2005). Infiltrating (invasive) ductal carcinoma insitu is the most commonly occurring histological type of breast cancer, accounting for 50-80% of diagnosed BCA (Table 1.2) (Weigelt et al. 2005).

1.5.3 Breast cancer prognosis

The search for a molecular marker that can be used as a prognostic and predictive marker in BCA is still on going with minimal success (Perez et al. 2007). Numerous potential markers have been identified (Table 1.3)(Weigelt et al 2005); however, their value in the treatment of BCA is continuously being questioned because of the lack of adequate quality control and the absence of the validation of these markers in an adequately powered clinical trial (Esteva et al. 2004). Despite the deficiencies of some of these markers, they are still being used during the assessment and management of BCA.
Table 1.2: Histopathological types of invasive breast cancer (Weigelt et al. 2005)

<table>
<thead>
<tr>
<th>Histopathological type of invasive breast carcinoma</th>
<th>Frequency</th>
<th>10-year survival rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive ductal carcinoma, not otherwise specified</td>
<td>50–80%</td>
<td>35–50%</td>
</tr>
<tr>
<td>Invasive lobular carcinoma</td>
<td>5–15%</td>
<td>35–50%</td>
</tr>
<tr>
<td>Mixed type, lobular and ductal features</td>
<td>4–5%</td>
<td>35–50%</td>
</tr>
<tr>
<td>Tubular/invasive cribriform carcinoma</td>
<td>1–6%</td>
<td>90–100%</td>
</tr>
<tr>
<td>Mucinous carcinoma</td>
<td>&lt;5%</td>
<td>80–100%</td>
</tr>
<tr>
<td>Medullary carcinoma</td>
<td>1–7%</td>
<td>50–90%</td>
</tr>
<tr>
<td>Invasive papillary carcinoma</td>
<td>&lt;1–2%</td>
<td>Unknown</td>
</tr>
<tr>
<td>Invasive micropapillary carcinoma</td>
<td>&lt;3%</td>
<td>Unknown</td>
</tr>
<tr>
<td>Metaplastic carcinoma</td>
<td>&lt;5%</td>
<td>Unknown</td>
</tr>
<tr>
<td>Adenoid cystic carcinoma</td>
<td>0.1%</td>
<td>Unknown</td>
</tr>
<tr>
<td>Invasive apocrine carcinoma</td>
<td>0.3–4%</td>
<td>Unknown</td>
</tr>
<tr>
<td>Neuroendocrine carcinoma</td>
<td>2–5%</td>
<td>Unknown</td>
</tr>
<tr>
<td>Secretory carcinoma</td>
<td>0.01–0.15%</td>
<td>Unknown</td>
</tr>
<tr>
<td>Lipid-rich carcinoma</td>
<td>&lt;1–6%</td>
<td>Unknown</td>
</tr>
<tr>
<td>Acinic-cell carcinoma</td>
<td>7 cases</td>
<td>Unknown</td>
</tr>
<tr>
<td>Glycogen-rich, clear-cell carcinoma</td>
<td>1–3%</td>
<td>Unknown</td>
</tr>
<tr>
<td>Sebaceaeous carcinoma</td>
<td>4 cases</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
The most commonly used markers of prognosis in our setting includes:

- **Tumor size and axillary node status:** Both tumor size and nodal status has been shown to have independent adverse effects on prognosis (Carter et al. 1989, Gupta et al. 2007). The use of tumor size to determine prognostic status has been used for more than 4 decades with studies demonstrating low risk of metastasis in patients presenting with tumor size <2cm and patients having a high risk of metastasis when presenting with tumor size >5cm (Carter et al. 1989, Weigelt et al. 2005). Lymph node involvement is also commonly assessed and is always considered in conjunction with the tumor size, with the effect of size on prognosis being greater in patients presenting with no nodal involvement (Carter et al. 1989). Irrespective of tumor size, patients presenting with the involvement of adjacent lymph nodes have a worse outcome then node negative patients, and if found to have tumor invasion of ≥4 axillary lymph nodes they have a significantly poorer outcome and the highest risk of metastasis (Carter et al. 1989, Weigelt et al. 2005).
### Table 1.3: Breast cancer metastatic determinants (Weigelt et al. 2005)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Use in clinic</th>
<th>Metastatic determinants</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour size</td>
<td>Established</td>
<td>Tumours under 2 cm in diameter have a low risk of metastasis; tumours of 2–5 cm have a high risk of metastasis; tumours over 5 cm have a very high risk of metastasis</td>
<td>Independent prognosis marker</td>
</tr>
<tr>
<td>Axillary lymph-node status</td>
<td>Established</td>
<td>If there are no lymph-node metastases, the risk of metastasis is low; if lymph-node metastases are present, the risk of metastasis is high; the presence of over 4 lymph-node metastases is associated with very high metastasis risk</td>
<td>Related to tumour size</td>
</tr>
<tr>
<td>Histological grade</td>
<td>Established</td>
<td>Grade 1 tumours have a low risk of metastasis; grade 2 tumours have an intermediate risk of metastasis; grade 3 tumours have a high risk of metastasis</td>
<td>Related to tumour size</td>
</tr>
<tr>
<td>Angioinvasion</td>
<td>Established in patients with lymph-node-negative tumours</td>
<td>The presence of tumour emboli in over 3 blood vessels is associated with metastasis</td>
<td>In patients with lymph-node-negative tumours</td>
</tr>
<tr>
<td>uPA/PAI1 protein level</td>
<td>Newly established marker</td>
<td>High protein levels of uPA and PAI1 are associated with high metastasis risk</td>
<td>Independent prognosis marker</td>
</tr>
<tr>
<td>Steroid-receptor expression</td>
<td>Established for adjuvant therapy decision</td>
<td>Low steroid-receptor levels are associated with metastasis</td>
<td>Short-term predictor of metastasis risk (5 years); related to histological grade</td>
</tr>
<tr>
<td>ERBB2 gene amplification and protein expression</td>
<td>Established for adjuvant therapy decision</td>
<td>ERBB2 amplification/overexpression is associated with metastasis</td>
<td>In patients with lymph-node-positive tumours</td>
</tr>
<tr>
<td>Gene-expression profiling</td>
<td>Currently being tested</td>
<td>A ‘good signature’ of 70 genes is associated with low metastasis risk; a ‘poor signature’ of 70 genes is associated with high metastasis risk</td>
<td>Tested in patients with lymph-node-negative tumours</td>
</tr>
</tbody>
</table>

PAI1, plasminogen activator inhibitor 1; uPA, urokinase-type plasminogen activator.
ii. **Histological grade**: In 1991 Elston and colleagues (1991) graded BCA according to 3 categories based on the architectural and cytological features, which include: well differentiated (grade 1), moderately differentiated (grade 2) and poorly differentiated (grade 3) BCA. They demonstrated that patients whose malignancy was classified as a grade 3 tumor (poorly differentiated) had a significantly worse prognosis then those presenting with grade 1 or 2 tumors (Elston et al. 1991).

iii. **Human Epidermal growth factor 2 receptors (HER-2/neu)**: This proto-oncogene, which is also known as c-erbB-2 or neu, is over-expressed in 60% of ductal carcinomas insitu and 20-30% of infiltrating breast carcinoma (Slamon et al. 1987, Esteva et al. 2004). It is the most commonly used prognostic and predictive marker in breast cancer and the assessment of HER-2/neu status is regarded as the standard of care in the management of BCA (Esteva et al. 2004, Weigelt et al. 2005). Over-expression of HER-2/neu receptors in patients with breast cancer, are associated with a poor prognosis and shortened disease free survival rate in patients with axillary node positive BCA (Allred et al. 1992). The presence of the HER-2/neu receptor has also been shown to dictate the treatment protocol, following an observed beneficial response to Trastuzumab (a monoclonal antibody directed against HER-2/neu), hormonal therapy and doxorubicin-based chemotherapy in patients with increased expression of the receptor (Paik et al. 1998, Slamon et al. 2001). However, over-expression of
HER-2/neu has also been shown to confer radio-resistance and is associated with ipsilateral breast cancer recurrence following radiotherapy (Akamatsu et al. 2003). Soluble forms of HER-2/neu receptor (HER-ECD) is currently being evaluated as a predictive marker of treatment response, however there are still a number of limitations which need to be addressed before it can be used in the clinical setting (Kostler et al. 2004, Kostler et al. 2004b, Perez et al. 2007).

iv. **Estrogen and progesterone receptor status:** The over-expression of estrogen receptors (ER-α) in BCA is a well-known positive prognostic marker, and facilitates the identification of patients who will benefit from hormonal treatment (Esteva et al. 2004). The incidence of BCA in African women, as compared to Caucasian women, is relatively low; however, only a small proportion of African women who develop BCA express ER positivity. Furthermore, a higher proportion of African women over-express the HER-2/neu receptor, which may contribute to the poorer outcomes observed in Africa women (Bird et al. 2008, Bogaert 2013). The contribution of progesterone receptor status in the prognostication of BCA is purely as a functional assay to ensure that the estrogen pathway is intact (Esteva et al. 2004). Although ER status is commonly used and facilitates treatment, the assays used to determine estrogen status are not standardized and the definition of positivity differs between laboratories (Esteva et al. 2004).

v. **Proliferation markers:** Ki-67 is a nuclear antigen expressed during the proliferative stages of the cell cycle and has been shown to be over-expressed in
breast cancer patients with increased risk of recurrent disease (Veronese et al. 1993).

Despite the large number of prognostic markers currently available in clinical use, the deficiencies associated with traditional prognostic markers result in the identification of only 30% of patients with either the best and worse prognosis (Weigelt et al. 2005).

1.5.4 Breast cancer and T regulatory cells

Following the discovery of Treg cells, their abundance within the tumor microenvironment of a variety of cancers, and their possible contribution to tumor progression, numerous studies began to investigate the role of Treg cells in BCA. The first study to demonstrate the presence of Treg cells in breast cancer was by Liyanage and colleagues (2002), who showed that the frequency of Treg cells within the peripheral blood and tumor microenvironment of patients with BCA was significantly higher than that in healthy controls. They also found no difference between the prevalence of Treg levels in healthy controls and patients who presented with benign disease (Liyanage et al. 2002). Liyanage and colleagues (2002) did not assess FoxP3 expression within the Treg cell population, as suggested by the literature, with only CD4, CD25, CTLA-4 and CD152 being used as markers of the suppressive Treg cell population; however, suppressive activity was confirmed with the use of functional assays. These findings were replicated in numerous studies thereafter (Bates et al. 2006, Ladoire et al. 2008, Ohara et al. 2009) and gave strong evidence to suggest that Treg cells have a crucial role in the development and progression of breast cancer.

Questions regarding the use of Treg cells as a prognostic factor began to arise and Bates and colleagues (2006) were the first to show that elevated intratumoral
FoxP3^+Treg cells correlated significantly with a more aggressive tumor phenotype, higher tumor grade and positive nodal status. They identified a novel group of high-risk patients whose tumors expressed ER-positivity, but had as poor a prognosis as patients with ER-negative tumors, with high intratumoral Treg levels being the only identifying feature (Bates et al. 2006). They observed that patients with elevated intratumoral FoxP3^+Treg cells had a shorter overall survival (Hazard Ratio (HR) 1.85 and relapse-free survival (HR 1.98 (Bates et al. 2006). The frequency of FoxP3^+Treg cells within the tumor was deemed as a marker of prognosis in invasive and non-invasive BCA and a marker for identification of patients with a risk of late relapse (Bates et al. 2006). These findings were also replicated in other studies (Ladoire et al. 2008, Aruga et al. 2009), including a study by Ladoire and colleagues (2008), who observed that elevated levels of intratumoral CD8^+ T-cells was also associated with poor prognosis.

The functional status of FoxP3 expression in breast cancer has also yielded interesting results, with increased expression of FoxP3 being associated with more invasive breast carcinomas, as compared to carcinoma insitu or normal tissue (Gupta et al. 2007). Gupta and colleagues (2007) demonstrated higher intratumoral FoxP3 expression in patients who presented with stage III BCA, as compared to patients presenting with stage II BCA. They also found a linear association between FoxP3 expression and tumor size, and suggest that FoxP3 expression is a marker of tumor progression and metastasis in breast carcinoma (Gupta et al. 2007).

The frequency of FoxP3^+Treg cells within the peripheral blood has also been shown to correlate with the stage of disease, with patients demonstrating significantly higher
levels of Treg cells in patients presenting with metastatic disease, as compared to those presenting with either stage I, II or III BCA (Rech, et al. 2010). The metastasis of BCA from the primary cancer site to other sites occurs via haematogenous, lymphatic and direct routes, with preferential sites including the lungs, brain, liver and bones (Tannock et al. 1998, Nguyen et al. 2007). Invasion into the adjacent axillary lymph nodes continues to be one of the most important determinants of cancer progression, especially when this involves the sentinel lymph nodes, which are located on the lymphatic drainage pathway of the breast (Watanabe et al. 2010). The accumulation of FoxP3⁺ Treg cells within these Sentinel lymph nodes have also been shown to be associated with undetected micrometastases, and is an independent predictor of prognosis in patients presenting with node negative cancer (Nakamura et al. 2009).

The Treg cell population has also been suggested as a predictive marker of treatment response following a study by Ladoire and colleagues (2008) who observed a significant decrease in Treg cell frequency in BCA following complete histological response to neo-adjuvant chemotherapy, independent of chemotherapeutic regimen, compared to non responders. However, cyclophosphamide was included as part of the chemotherapeutic regimen, which may be responsible for the observed responses, as previously discussed (Ladoire et al. 2008).

The presence and activity of Treg cells in BCA patients has also been shown to correlate with the more traditional prognostic and predictive markers (Perez et al. 2007). Perez and colleagues (2007) demonstrated a significantly higher level of peripherally circulating Treg cells in patients with HER⁺ breast cancer compared to
HER− breast cancer, and normal controls. Ohara and colleagues (2009) also observed elevated levels of circulating Treg cell in patients presenting with HER2+ breast cancer, as well as in patients presenting with progesterone receptor negative BCA. Although Horlock and colleagues (2009) also observed similar results, unlike Perez and colleagues, they found that patients presenting with HER+ and HER− breast cancer had similar levels of Treg cell.

Perez and colleagues (2007) observed a significant decrease in Treg cell frequency in patients who showed a good clinical response to Trastuzumab therapy. Furthermore, one month before the clinical confirmation of disease progression in patients with disease recurrence, Treg levels were shown to increase significantly (Perez et al. 2007). This was confirmed in a study by Horlock and colleagues (2009), who observed a significant decrease in patients presenting with metastatic BCA following treatment with Trastuzumab. Perez and colleagues (2007) proposed the use of circulating Treg levels as a surrogate marker of response to Trastuzumab therapy in patients with BCA.

Although it is logical to assume that Treg cell possibly plays a role as a prognostic marker in breast cancer, there are still numerous conflicting reports and unanswered questions. Further studies need to be conducted assessing the relationship between Treg cells in breast cancer and how it can be manipulated in the management of patients presenting with BCA.
1.6 Oesophageal cancer and immunology

1.6.1 Oesophageal cancer epidemiology

Oesophageal cancer is the 8th most common cancer in the world and is the only cancer which has shown a steady increase in incidence across the world (Tettey et al. 2013, World Cancer Research Fund International 2013). According to the 2008 International Agency for Research on Cancer (IARC) statistics, South Africa has the highest incidence of oesophageal cancer in the world, and is the 3rd most common cause of cancer both in men (incidence rate: 23.5/100000) and women (incidence rate 12.6/100000) (IARC 2013).

Oesophageal cancer is associated with a significant morbidity and mortality and despite advances in multimodal anticancer treatment, the five year survival rate of these patients’ remains below 10% (Pickens et al. 2003). This has been attributed to the late stage of presentation and consequent advanced stage of disease, which results in patients only being eligible for palliative treatment (Liakos et al. 2010, Tettey et al. 2013).

1.6.2 Pathology

Eighty percent of primary oesophageal cancers are malignant in nature with 98% of histopathological types being squamous cell carcinoma (SCC) and adenocarcinoma (AdenoCa) (Patel et al. 2003, Pickens et al. 2003). The remaining cancer types include sarcoma (1%), lymphoma (0.5%), cylindroma (0.2%) and primary melanoma
Despite reports demonstrating a decline in the incidence of SCC, it is still the most commonly occurring histological type of oesophageal cancer worldwide, and has been found to be 5 times more common in African patients as compared to Caucasian patients (Brown et al. 2001, Pickens et al. 2003, Tettey et al. 2013). The increased risk of Africans to develop SCC has been attributed to a possible genetic predisposition, as well as the exposure to certain risk factors including; low-income status, increased alcohol consumption, heavy smoking and low intake of raw fruit and vegetables (Brown et al. 2001, Pickens et al. 2003). The incidence of adenocarcinoma is showing an upward trend and is associated with a premalignant condition called Barret metaplasia, which develops following a prolonged duration and severity of gastro-oesophageal reflux disease (Patel et al. 2003).

1.6.3 Oesophageal cancer prognosis

The mortality rate associated with oesophagus cancer is almost 100% (Patel et al. 2003), and the prognosis of oesophageal cancer remains poor despite aggressive multimodal treatment (Liakos et al. 2010). Early detection of malignancies offers the best prognosis, however very few prognostic and predictive factors have been identified for clinical use in oesophageal cancer (Wang et al. 2011). There have been numerous studies looking at the possible role of Ki-67, p53, HER-1 and ProExC expression in detecting early oesophageal cancer and serving as a prognostic and predictive marker. Yang and colleagues (1999) observed that p53 decreases the responsiveness of the malignancy to chemoradiotherapy, however these observations were not found in other studies (Akamatsu et al. 2003). Ki-67 and ProExC are both markers of cell proliferation and have been shown to identify early stage oesophageal cancer.
cancer (Wen-Chuang et al 2011). As in breast cancer, the over expression of HER-1 or HER-2 receptors are associated with a significantly worse outcome (Vallböhmer et al. 2006). Furthermore, a study by Akamatu and colleagues (2003) found that HER-2 over-expression correlated with a poor prognosis, rendered the cells radio-resistant and induced local resistance to cisplatinum-based chemoradiotherapy (Akamatsu, Matsumoto et al. 2003).

Traditional prognostic factors have been assessed including tumor size, nodal status and metastatic spread, however they have been shown as insufficient for accurate prognostication of early recurrence in oesophageal cancer (Zhu et al. 2011). Zhu and colleagues (2011) identified additional prognostic factors; including grade of differentiation, depth of invasion, lymph node metastasis, number of lymph node metastases and margin status. These factors were shown to predict early death by recurrence of the primary tumor following the removal of the primary tumor (Zhu et al. 2011).

1.6.4 Oesophageal cancer and T regulatory cells

Oesophageal cancer patients are presumed to be in a poorer immunological state in comparison to other oncology patients due to the malnutrition associated with dysphagia (Mafune et al. 2000). Furthermore, aggressive multimodality treatment, such as major surgery with thoracotomy and laparotomy, combined radiotherapy and adjuvant chemotherapy further depress the immunological state (Mafune et al. 2000). Although the clinical and pathological features of oesophageal cancer at time of diagnosis have been established as prognostic factors, host defences against cancer are also thought to contribute to prognosis (Tsutsui et al. 1996). Despite increasing
clarity in the relationship between nutritional status, tumor staging and immunological parameters in patients with oesophageal cancer, the search for immunological markers of prognosis has not yielded any conclusive results (Mafune et al 2000). This is further complicated by the fact that there is controversy regarding whether the host immunological parameters can accurately be identified from the peripheral blood (Mafune et al. 2000). This has resulted in numerous studies focusing on the immunohistochemical analysis of post-oesophagectomy tumour specimens for prognostic and predictive parameters (Tsutsui et al. 1996, Schumacher et al. 2001, Xu et al. 2009, Xu et al. 2011).

Cho and colleagues (2003) investigated the prevalence of immune cells within oesophageal tumor samples and observed that patients presenting with high quantities of CD4\(^+\) and CD8\(^+\) T-lymphocytes within the stroma and cancer cell nest had a better prognosis compared to other groups. The balance between CD4\(^+\) and CD8\(^+\) T-lymphocytes has been suggested as a critical marker in the prognosis of patients with oesophageal cancer, furthermore CD4\(^+\): CD8\(^+\) T-cell status was suggested as a possible prognostic marker and index of post-surgical host immune reactivity (Cho et al. 2003). This substantiated the use of CD4\(^+\): CD8\(^+\) T-cell ratio as a prognostic marker in immunohistochemical analysis, however the use of these cells as markers of prognostication when sampled from the peripheral blood still remains unanswered.

Yoskioka and colleagues (2008) conducted a similar study; however, in addition to CD4\(^+\) and CD8\(^+\) T-cells, they also assessed the frequency of Treg cells within the tumor microenvironment. They found that high quantities of intratumoral FoxP3\(^+\) Treg cells correlated with improved prognosis in patients presenting with early and
advanced oesophageal cancer (Yoshioka et al. 2008). Furthermore, Yoshioka and colleagues (2008) also observed that Treg cell status did not correlate with the TNM classification, and had no influence on the progression of the malignancy. Treg cell populations were not shown to have any suppressive effect on effector CD4$^+$ and CD8$^+$ T-cells, and elevated levels of Treg cells were associated with an increased CD4$^+$: CD8$^+$ T-cell status (Yoshioka et al. 2008). This contradicted all studies that claimed that high Treg cell levels correlated with poor prognosis, and it was suggested that the presence of Treg cells within the microenvironment is compatible with a normal anticancer immune response (Yoshioka et al. 2008).

Studies then started assessing whether similar results would be found in the peripheral blood of patients with oesophageal cancer. A study by Xu and colleagues (2009) demonstrated elevated levels of Treg cells in the peripheral blood of patients presenting with oesophageal cancer. Furthermore, Treg: CD4$^+$ T-cell ratio and FoxP3$^+$ expression was significantly decreased following chemotherapeutic treatment (Xu et al. 2009).

In summary breast and oesophageal carcinomas are extremely prevalent conditions in our setting, which are associated with significant morbidities and mortalities. The immune system has now been shown to have a substantial influence on the patients’ health outcomes following the diagnosis of cancer. This has also led to the search for an immunological marker, which will serve as a prognostic, and predictive marker that facilitates the management of oncology patients. The discovery of Treg cells has rejuvenated the search for an explanation for immune escape; however, there are still numerous inconsistencies and conflicting findings regarding the T regulatory cell
population. Despite these discrepancies, if the Treg cell population proves to be a valid prognostic and predictive indicator in the management of cancer, it will guide treatment practices and facilitate the monitoring of patients receiving anticancer treatment. Furthermore, it will facilitate the identification of patients who are treatment resistance and enable the cessation of unnecessary therapy in patients with poor prognosis.
2 Aim and objectives

2.1 Aim

This study aimed to evaluate whether the levels of T-lymphocyte subsets, and alterations of these levels within the peripheral blood of HIV-negative patients presenting with either breast or oesophageal cancer at Steve Biko Academic hospital and Kalafong hospital, at the onset of treatment as well as following conventional anticancer treatment (surgery, chemotherapy and radiotherapy) could be used as markers of prognosis in conjunction with other well known prognostic factors, or as predictors of short term survival.

2.2 Objectives

2.2.1 Primary objectives

- To assess the relationship between levels of peripheral CD4\(^+\), CD8\(^+\) and Treg T-lymphocyte subsets and prognosis in patients presenting with breast cancer or oesophageal cancer.
- To assess the relationship between circulating levels of CD4\(^+\), CD8\(^+\) and Treg T-lymphocyte subsets and 7 weeks post-treatment survival of breast and oesophageal cancer patients.

2.2.2 Secondary objectives

- To assess the distribution of tumor stage at presentation in South African patients presenting with breast or oesophageal cancer.
• To quantify the T-lymphocyte immune profile in South African patients presenting with breast and oesophageal cancer.

• To assess the efficacy and agreement of the two different techniques used in the characterization and measurement of circulating Treg cell subsets.
3 Study cohort

3.1 Setting

Study participants were identified at the Surgery outpatient clinic at Kalafong Hospital and Steve Biko Academic Hospital, Pretoria, Gauteng, South Africa.

3.2 Eligibility criteria

Inclusion criteria:

1. Histological diagnosis of breast cancer or oesophageal cancer.

2. Human Immunodeficiency Virus negative status confirmed by serological test (ELISA testing). HIV-infection in the human population has been shown to promote T-cell activation and induce a progressive decline in CD4\(^+\) and CD8\(^+\) T-lymphocytes (Altfeld et al. 2000, Fiore et al. 2006). Furthermore, Treg cells have been shown to be elevated in HIV-positive individuals compared to healthy controls (Suchard et al. 2010). In light of the influence of HIV-infection on these variables of interest, the inclusion of HIV-infected participants into the study would serve as a substantial confounding factor, and would prevent the assertion of whether the observed effect was as a result of the antitumor treatment which the participant received or whether it was a result of the HIV-infection.

Exclusion criteria:

1. Refused to give consent to partake in the study.

2. Had received any antitumor or immunosuppressive treatment during the preceding 12 months as this would serve as a confounding factor.
3.3 Breast cancer cohort

All patients seen at the Family Medicine Clinic and Casualty Department with clinical symptoms suggestive of breast cancer were referred to the Surgery Outpatients Department, where a core needle biopsy of the breast mass was taken. Once a histological diagnosis of breast cancer was confirmed, participants were referred to the surgical-oncology team, consisting of surgeons, oncologists and radio-oncologists, for multi-modal treatment.

Staging of the malignancy was conducted following a number of investigations including: radiological examination, mammography, nuclear studies, electrolyte and haematological studies. Radical versus palliative treatment options were proposed for each patient after consideration of the following factors:

- TNM staging according to the American Joint Committee on Cancer (AJCC);
- Hormonal and HER-2/neu receptor status (ER, PR and HER);
- Comorbidities, especially those rendering the patient unable to tolerate anaesthetic;
- Performance status.

Depending on the factors specified above, participants received a combination of the following treatment interventions:

- Chemotherapy;
- Radiotherapy;
- Surgery (Mastectomy/ lumpectomy);
- Hormonal treatment (Tamoxifen).
3.4 Oesophageal cancer cohort

All patients seen at the Family Medicine and Casualty Department at Steve Biko Academic Hospital and Kalafong Hospital with signs and symptoms suggestive of oesophageal cancer were referred to the Surgery Outpatients Department. In this cohort it was noted that the majority of oesophageal cancer patients presented in a substantially poorer physical state compared to those suffering from breast cancer, with 7 out of the 10 participants presenting with symptoms of moderate to severe inability to swallow, dehydration, severe wasting and malnutrition.

Participants were admitted to the surgical wards for rehydration and oncology work-up which included a barium swallow for identification of the anatomical level at which the malignancy was situated, and a gastroscopy-guided biopsy and dilatation of the oesophageal mass. The surgical-oncology team proposed an individualised palliative or radical treatment plan depending on:

- TNM-staging of oesophageal carcinoma;
- Performance status;
- Length of the lesion and degree of narrowing;
- Associated comorbidities.

Treatment was then divided into:

- Radical treatment (chemotherapy and/or radiotherapy and, or surgery);
- Palliative treatment (Radiotherapy and/or surgical-bypass).


3.5 Recruitment

Between March 2012 to August 2012 participants were approached to volunteer for the current study following the clinical and histological confirmation of breast or oesophageal cancer. All potential participants were required to undergo an HIV (ELISA) test before admission into the study.

Each participant served as their own control and they were recruited into the study once they gave written informed consent. All research protocols were submitted to the University of Pretoria Ethics Committee, and the recruitment of participants began once final approval had been granted.

The breast cancer cohort comprised of 25 women (17 African and 8 Caucasian) with a mean age of 59, (range: 38-87). (Shown in Table 3.1) The oesophageal cancer cohort comprised of 10 participants (7 females and 3 males), with a mean age of 62 years (range: 41-85) amongst the females and 61 years amongst the males (range: 50-73). Eight of these participants were African while only 2 were Caucasian. The histological subtypes, steroid hormone receptor status (estrogen receptor and progesterone receptor) and HER-2/neu status was determined by the National Health Laboratory Services with the histologic types being defined according to the WHO classification.

Patients suffering from breast cancer were categorised into a surgical (modified radical mastectomy and lumpectomy), radiotherapy and chemotherapy/tamoxifen group, while the oesophageal cancer patients were categorised into surgical, radiotherapy or chemotherapy groups.
The majority of breast cancer patients received surgical intervention (19 patients) while the rest (6 patients) received neo-adjuvant chemotherapeutic treatment in combination with hormonal treatment (Tamoxifen). None of the breast cancer participants received radiotherapy as a first-line treatment.

Eight out of the 10 oesophageal cancer patients received palliative radiotherapy in conjunction with recurrent oesophageal dilatation or the insertion of an oesophageal stent (e.g. Procto-livingston tube), and the remaining 2 received major surgical intervention including a 3 stage oesophagectomy with gastric pull-up and a transthoracic-partial-oesophago-gastrectomy.

None of the patients had any history of autoimmune disease or recent infection. Information regarding the clinicopathological characteristics of all patients is summarised in Table 3.1.
### Table 3.1: Clinicopathological characteristics of the patients

<table>
<thead>
<tr>
<th></th>
<th>Breast cancer</th>
<th>Oesophageal cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total patients recruited (N)</strong></td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Females</td>
<td>25</td>
<td>7</td>
</tr>
<tr>
<td>2. Males</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td><strong>Mean age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Females</td>
<td>59</td>
<td>62</td>
</tr>
<tr>
<td>2. Males</td>
<td>0</td>
<td>61</td>
</tr>
<tr>
<td><strong>Race</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. African</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>2. Caucasian</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td><strong>Histological diagnosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Infiltrating ductal carcinoma</td>
<td>21</td>
<td>N/A</td>
</tr>
<tr>
<td>2. Infiltrating lobular carcinoma</td>
<td>3</td>
<td>N/A</td>
</tr>
<tr>
<td>3. Mucinous</td>
<td>1</td>
<td>N/A</td>
</tr>
<tr>
<td>3. Squamous cell carcinoma</td>
<td>N/A</td>
<td>9</td>
</tr>
<tr>
<td>4. Adenocarcinoma carcinoma</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td><strong>Stage of disease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 1</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>
## Chapter 3: Patient demographics

### 3.1. Study cohort

<table>
<thead>
<tr>
<th>Stage</th>
<th>Breast cancer</th>
<th>Oesophageal cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 2</td>
<td>5</td>
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</tr>
<tr>
<td>Stage 3</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Stage 4</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

**Treatment**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Breast cancer</th>
<th>Oesophageal cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Surgery only</td>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td>2. Chemotherapy and Tamoxifen</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>3. Radiotherapy only</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>

**Number of participants who died before 7 weeks post-treatment**

<table>
<thead>
<tr>
<th></th>
<th>Breast cancer</th>
<th>Oesophageal cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

**Number of patients who were still alive at 7 weeks post-treatment**

<table>
<thead>
<tr>
<th></th>
<th>Breast cancer</th>
<th>Oesophageal cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>19</td>
<td>0</td>
</tr>
</tbody>
</table>

**Number of patients who were lost to follow-up at 7 weeks post-treatment**

<table>
<thead>
<tr>
<th></th>
<th>Breast cancer</th>
<th>Oesophageal cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>
4 Materials and methods

4.1 Flow cytometric analysis

Whole blood (5ml) was drawn from each participant in an EDTA tube at specified time points including:

vi. Before treatment,

vii. Day 1 after treatment,

viii. 5-7 weeks post-treatment.

After sample collection the whole blood was stained using 2 staining techniques with fluorescent-tagged monoclonal antibodies including:

- **Intracellular**: FoxP3, CD25, CD4, CD8, CD127, CD45 and,

- **Extracellular**: CD25, CD4, CD8, CD127, CD45.

This is in light of the fact that the identification of Treg cell populations with intracellular staining techniques is a time consuming and technically challenging process, and sufficient identification of Treg cell populations (CD4⁺CD25⁻CD127<sub>low</sub>), in the absence of FoxP3⁺, has previously been demonstrated (Decker et al. 2012). T-lymphocyte subsets were identified using a Gallios flow cytometer (Beckman Coulter, Miami, FL), and each sample was run in duplicate to ensure the consistency of the results and then averaged to give a final result. Due to the limited frequency of Treg cells in the peripheral blood, a minimum of 100 000 events were recorded for each sample. Kaluza software (Beckman Coulter 2010, Miami, FL) was used for the digital compensation and analysis of results.
4.2 Monoclonal antibodies

4.2.1 Intracellular staining

The following monoclonal antibodies were used: CD4-FITC (Beckman Coulter), CD127-PC5 (Beckman Coulter), CD45-Pacific Blue (Beckman Coulter), Human Regulatory T-cell staining kit containing FoxP3-PE, CD4-FITC, CD25-APC (eBioscience, Hatfield, UK). Staining of samples was performed as specified by the manufacturer. Briefly, 100µl of whole blood was stained with surface antibodies (CD45 (10µl), CD4 (10µl), CD25 (10µl), CD127 (10µl), CD8 (5µl)) and incubated in the dark, on ice for 30 minutes. FoxP3 Fixation/Permeabilization buffer (1:4 conc) (1ml) was added to the sample, which was then incubated in the dark on ice for an additional 30 minutes. FoxP3 normal rat serum (2µl) was added to sample and incubated for 15 minutes at room temperature. FoxP3 (10µl) monoclonal antibody was added to the sample, which was again followed by a 30 minute incubation period in the dark. Flow count fluorosphere beads (Beckman Coulter) (100µl) were added to the sample immediately before acquisition.

4.2.2 Extracellular staining

The following monoclonal antibodies were used: CD8-PC7 (Beckman Coulter), CD4-FITC (Beckman Coulter), CD127-PC5 (Beckman Coulter), CD45-Pacific Blue (Beckman Coulter), and CD25-APC (Biolegend, San Diego, CA). Staining of samples was performed as specified by the manufacturer. Briefly, 100µl of whole blood, in the presence of monoclonal antibodies (CD45 (10µl), CD4 (10µl), CD25 (10µl), CD127 (10µl), CD8 (5µl)), was incubated in the dark, on ice for 30 minutes. The red blood cells were lysed and fixed using the automated TQ Prep (Beckman Coulter) sample processor. Flow count fluorosphere beads (Beckman Coulter) (100µl) were added to the sample and events were immediately acquired using the
flow cytometer. Prior to each patient acquisition, flow-check fluorescence (Beckman Coulter) was run to verify the optical alignment.

The spill-over coefficients of the 6 fluorochromes was determined by using strongly positive single-colour monoclonal antibodies in separate tubes for each of the fluorochromes: FITC, PE, APC, PC5, APC7 and PB (Carrock et al. 1997). Thereafter, these compensation standards for the lymphocyte population were used to derive a compensation matrix with Kaluza software (Beckman Coulter). CD4$^+$ and CD8$^+$ T-lymphocytes were quantified as a percentage of the total lymphocyte population while Treg T-lymphocytes populations were quantified as a percentage of total CD4$^+$CD45$^+$ and CD8$^+$CD45$^+$ T-lymphocyte subsets at each time point. Flow count fluorosphere beads (Beckman Coulter) were used to determine the concentration (cells/µl) for the counting of specific T-lymphocyte populations.

### 4.3 Gating strategy

#### 4.3.1 Step 1: Identification of lymphocyte population

The lymphocyte population was identified using the CD45 fluorescence (CD45) versus the side light-scatter (SS). This approach has been suggested as a more accurate method for lymphocyte identification as compared to the more conventional light scatter gating (forward scatter versus side-scatter-approach) as it reduces the likelihood of non-lymphocyte contamination within the gate (Nicholson et al. 1996, Storie 2005). Lymphocytes were characterised as CD45$^+$ bright, with low side scatter, (Figure 4.3 A).
4.3.2 Step 2: Identification of total CD4\(^{+}\) (CD4\(^{+}\)CD45\(^{+}\)) and total CD8\(^{+}\) (CD8\(^{+}\)CD45\(^{+}\)) T-lymphocyte populations

Sequential gating of the total CD45\(^{+}\) T-lymphocyte population (Figure 4.3 A: N) was used to identify the CD4\(^{+}\) and CD8\(^{+}\) T-lymphocytes subsets (Figure 4.3 B). These cell population represent the total CD4\(^{+}\) T-cell (CD4\(^{+}\)CD45\(^{+}\)) population and total CD8\(^{+}\) T-cells (CD8\(^{+}\)CD45\(^{+}\)).

4.3.3 Step 3: Identification of CD25\(^{+}\) expression by CD4\(^{+}\) T-lymphocyte and CD8\(^{+}\) T-lymphocyte

The frequency of CD25-expressing T-lymphocyte population was identified by sequentially gating on CD45\(^{+}\) lymphocyte region (N). CD4\(^{+}\) (Figure 4.3 C) and CD8\(^{+}\) T-cells were gated on region N (Figure 4.3 D). The CD4\(^{+}\) and CD25\(^{+}\) cells were gated on the CD45\(^{+}\) T-lymphocytes. This cell subset will be referred to as CD4\(^{+}\)CD25\(^{+/-}\) and CD8\(^{+}\)CD25\(^{+/-}\) in the remainder of the text.

4.3.4 Step 4: Identification of CD4\(^{+}\) and CD8\(^{+}\) regulatory T-lymphocytes

Treg cells were identified by three different gating strategies:

1. Firstly, Treg cells were characterised as CD4\(^{+}\) (Figure 4.3 E) or CD8\(^{+}\) T-lymphocytes (Figure 4.3 F) which co-expressed CD127\(^{+}\) and CD25\(^{+}\) receptors (Simonetta et al. 2010).

2. Secondly, Treg cells were characterized as CD4\(^{+}\) (Figure 4.3 G) or CD8\(^{+}\) T-lymphocytes (Figure 4.3 H), which co-expressed CD25\(^{+}\), surface marker and intracellular FoxP3\(^{+}\).

3. Lastly, Treg cells were characterized as CD4\(^{+}\) (Figure 4.3 I) or CD8\(^{+}\) T-lymphocyte (Figure 4.3 J) which co-expressed CD127\(^{+}\) surface marker and intracellular FoxP3\(^{+}\).
Chapter 4: Materials and methods

4.3 Gating strategy

Figure 4.3 A: Total lymphocyte population. Figure representative of $n$ samples.

Figure 4.3 B: Total CD4$^+$ T-lymphocyte and CD8$^+$ T-lymphocyte. Figure representative of $n$ samples.
Figure 4.3 C: CD25$^+$ expression on CD4$^+$ T-lymphocyte. Figure representative of $n$ samples.

Figure 4.3 D: CD25$^+$ expression on CD8$^+$ T-lymphocyte. Figure representative of $n$ samples.
Figure 4.3 E: CD4⁺CD25⁺CD127⁺ Treg cell population. Figure representative of n samples.

Figure 4.3 F: CD8⁺CD25⁺CD127⁺ Treg cell population. Figure representative of n samples.
4.3. Gating strategy

**Figure 4.3 G:** CD4⁺CD25⁺FoxP3⁺ Treg cell population. Figure representative of $n$ samples.

**Figure 4.3 H:** CD8⁺CD25⁺FoxP3⁺ Treg cell population. Figure representative of $n$ samples.
Chapter 4: Materials and Methods

4.3. Gating strategy

Figure 4.3 I: CD4⁺CD127⁺FoxP3⁺ Treg cell population. Figure representative of \( n \) samples.

Figure 4.3 J: CD8⁺CD127⁺FoxP3⁺ Treg cell population. Figure representative of \( n \) samples.
4.4 Statistical analysis

Statistical analysis was undertaken using STATA version 13 (Statacorp. 2 Station, TX: StataCorp LP). All statistical comparisons between the clinical profiles of patients were analysed using an unpaired Student t-test, Chi-squared test and Fisher's exact test, as appropriate, and results are reported as mean ± standard deviation (SD) or 95% confidence interval. The association between immunological levels and categorical variables were analysed with the non-parametric Wilcoxon rank-sum test and ANOVA test. The association between repeated immunological measurements from the sample groups were carried out using the Wilcoxon sign-rank (matched pairs) statistic, and results are reported as median ± interquartile range (IQR). A P-value of <0.05 was considered statistically significant.
5 T-lymphocyte subsets (CD4\(^+\), CD8\(^+\) and Treg cells) in breast and oesophageal cancer and association with stage of disease

5.1 Introduction

Within the South African healthcare setting, affliction with breast or oesophageal cancer is associated with significant morbidity and mortality. Numerous factors have been shown to contribute to the poor outcome of these patients, with advanced stage of disease at presentation playing a substantial role in a large proportion of these cancer-associated mortalities (American Cancer Society 2011, Kingham et al. 2013). Late presentation to healthcare facilities limits the treatment options available and consequently diminishes patient survival. This chapter focuses on the clinicopathological distribution and immune profile of 35 patients presenting with breast and oesophageal cancer at Steve Biko Academic Hospital and Kalafong Hospital between March 2012-August 2012.

5.2 Results

5.2.1 Determination of stage of disease at first presentation in patients suffering from breast or oesophageal cancer

The majority of participants diagnosed with cancer who were eligible for the study presented with stage 3 or stage 4 disease, with less than 20% of cancer patients presenting with stage 1 or stage 2 disease (Figure 5.1). This analysis was statistically significant (P=0.028). All patients suffering from oesophageal cancer presented at a late disease stage (stage 3 and 4) (as described in Chapter 3: Table 3.1). The analysis of patients presenting with breast cancer showed that a statistically significant number of African patients (88%) presented with stage 3 and 4 disease compared to Caucasian patients (12%), P=0.001 (Figure 5.2).
Squamous cell carcinoma was the most common histopathological subtype (90%) of oesophageal cancer noted in this setting (as described in Chapter 3: Table 3.1), which correlates with other previously described African (Tettey et al. 2013) and Asian studies (Zhu et al. 2011). Eighty four percent of participants presenting with breast cancer presented with a histopathological diagnosis of infiltrating ductal carcinoma (as described in Chapter 3: Table 3.1), with infiltrating lobular carcinoma and mucinous carcinoma contributing the remaining 16%. Despite the late presentation of the majority of patients presenting with breast cancer, mortality was significantly less (P=0.0004) than that seen in the oesophageal cancer cohort (60%), with 8% of breast cancer participants demising within 3 months of diagnosis.
Figure 5.1: The distribution of breast and oesophageal cancer patients and stage of disease at presentation. This Figure represents the frequency distribution of breast and oesophageal cancer patients in the different stages of disease, at presentation. Less than 20% of patients presented with stage 1 disease, with only breast cancer patients presenting in the early stages of disease (stage 1 or stage 2) (P=0.028.) All oesophageal cancer patients presented with stage 3 or stage 4 disease. (NS =not significant, *P<0.05, **P<0.01, ***P<0.001)
Figure 5.2: The distribution of breast cancer according to race and stage of disease at presentation. This Figure represents the frequency distribution of African and Caucasian patients at different stages of disease, at presentation. 88% of African patients presented with late stage (stage 3 or 4) disease, compared to 12% of Caucasian patients (P=0.001). (NS = not significant, *p<0.05, **p<0.01, ***p<0.001).
5.2.2 The prevalence of CD4<sup>+</sup>CD25<sup>+</sup> and CD8<sup>+</sup>CD25<sup>+</sup> T-lymphocyte cell populations in breast and oesophageal cancer patients

The included participants were evaluated for signs of immune suppression through the quantification of the circulating T-lymphocyte subsets. The circulating total CD4% in breast and oesophageal cancer patients was compared to a previously described reference ranges drawn from clinically healthy volunteers in the Gauteng region (Lawrie et al. 2009). This range was found to be between 27.7-58%. Normal levels of CD8% has been described as 13-42% with a CD4<sup>+</sup>/C8<sup>+</sup> T-cell ratio of 1.4-1.66 being considered as normal (Bofill et al. 1992, CDC 2004).

Cancer patients were found to have a median total CD4<sup>+</sup> T-lymphocyte percentage, defined as CD4<sup>+</sup>CD45<sup>+</sup>, of 48.4% (95% Confidence Interval (CI) 43.3%-52.2%) which falls within the normal range despite the late stage of cancer presentation (Figure. 5.3). However, the total CD4<sup>+</sup> concentration (CD4<sup>+</sup> count) was found to be lower in this cohort (246 cells/µl (95% CI 210-399 cells/µl)), as compared to levels described previously in healthy volunteers (500-2010 cells/µl) in the Gauteng region (Lawrie et al. 2009).

The median total CD8<sup>+</sup> T-lymphocyte percentage, defined as CD8<sup>+</sup>CD45<sup>+</sup>, was determined as 22.43% (95% CI 20.03-26.36), which fell within normal limits. However, the total CD8<sup>+</sup> T-cell concentration (CD8<sup>+</sup> count) was also found to be lower (141 cells/µl, (95% CI 111-190)) in this cohort as compared to that observed in healthy volunteers (209-924 cells/µl). The CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratio was calculated to be 2 (95% CI 1.46-2.5), which is >1 and is thus considered as normal. Interestingly, a large proportion of CD4<sup>+</sup> (89%; P=0.0017) and CD8<sup>+</sup> (57.2%; P=0.009) T-lymphocytes also expressed CD25<sup>+</sup> surface marker (Figure. 5.3).
5.2 Results

5.2.3 CD4+ and CD8+ Treg cell populations identified

The levels of circulating CD4+ and CD8+ Treg cell populations were quantified from the peripheral blood of the breast and oesophageal cancer patients. They were expressed as a percentage of the circulating CD4+ and CD8+ T-lymphocytes following findings which suggest that homeostatic changes in the systemic Treg pool makes the use of total count as a marker of change unreliable (Sasada et al. 2003, Rech et al. 2010).

CD4+ (CD4+CD25+CD127+, CD4+CD25+FoxP3+ and CD4+CD127+FoxP3+) and CD8+ (CD8+CD25+CD127+, CD8+CD25+FoxP3+ and CD8+CD127+FoxP3+) Treg cell populations were identified in this cohort (Figure 5.4). Individual Treg populations represented less than 2% of CD4+ T-lymphocyte populations; however, CD8+CD25+CD127+ Treg cells constituted 4.16% of CD8+ T-lymphocytes (Figure 5.4). Although reference ranges for normal CD8+ Treg levels have not been described, the levels of CD4+ Treg was substantially lower than the 3.72% (range 1.3-7.5%) of CD4+ T-cell population previously observed in a healthy South African control group (Suchard et al. 2010). CD4+CD25+CD127+ was found to be the most dominant CD4+ Treg cell subset, while CD8+CD25+CD127+ was the most dominant CD8+ Treg population (Figure 5.4).

Figure 5.3: General T-lymphocyte profile in breast and oesophageal cancer patients

![Graph showing T-lymphocyte subsets](image)

**Figure 5.3**: The CD4+ and CD8+ T-lymphocyte subsets of breast and oesophageal cancer patients at first presentation. The total CD4+, CD4+CD25+, CD4+CD25−, total CD8+, CD8+CD25+, CD8+CD25− T-lymphocytes percentage of patients presenting with breast and oesophageal cancer are shown as the median % ± IQR. The median total CD4+ and total CD8+ lymphocyte concentration and IQR at presentation are 246 cells/µl (IQR: 133-566) and 141 cells/µl (IQR:56-306). (NS =not significant, *P<0.05, **P<0.01, ***P<0.001)
Figure 5.4: The distribution of circulating CD4\(^+\) and CD8\(^+\) T regulatory cell subsets of breast and oesophageal cancer patients at first presentation. The CD4\(^+\) and CD8\(^+\) Treg cell subsets are presented as the median % ± IQR. (NS = not significant, *P<0.05, **P<0.001, ***P<0.0001)
5.2.4 Association between circulating levels of CD4\(^+\) and CD8\(^+\) T-lymphocytes and pathological stage of breast cancer and oesophageal cancer

To explore the relationship between the frequency of peripherally circulating CD4\(^+\) and CD8\(^+\) T-lymphocytes and pathological stage of disease in breast and oesophageal cancer, whole blood was drawn from participants before receipt of antitumor treatment and was analysed for the frequency of CD4\(^+\)CD25\(^-\), CD4\(^+\)CD25\(^+\), CD8\(^+\)CD25\(^-\) and CD8\(^+\)CD25\(^+\) T-lymphocytes subsets. Although previous studies have assessed Treg cell status as those co-expressing CD4\(^+\) and CD25\(^+\) surface markers, a large proportion of these cells have been shown to not display suppressive activity and are regarded as activated T-lymphocytes (Stanzani et al. 2004).

A statistically significant variation in the circulating levels of CD4\(^+\)CD25\(^+\) T-lymphocytes was observed in cancer patients at different stages of disease at presentation (Figure 5.5) (P=0.0572); however, pairwise comparison did not show a significant difference between the different stages of disease. The circulating levels of CD8\(^+\)CD25\(^+\) T-lymphocytes were also increased as compared to CD8\(^+\)CD25\(^-\) T-lymphocytes, however it did not reach statistical significance (Figure 5.5).
Breast and oesophageal cancer: CD25 distribution and association with pathological stage of disease

Figure 5.5: The distribution of CD25 in CD4$^+$ and CD8$^+$ T-lymphocyte populations in breast and oesophageal cancer patients according to stage of disease progression at presentation. The CD4$^+$ and CD8$^+$ T-lymphocyte subsets expressing CD25$^{+/−}$ are presented as the median $±$ IQR. (NS = not significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$)
5.2.5 Association between circulating levels of CD4$^+$ and CD8$^+$ T-lymphocytes and pathological stage of breast cancer

Patients presenting with breast cancer were staged according to the American Joint Committee on Cancer (AJCC) TNM staging and then further subdivided into 4 stages depending on their TNM status (stage I-IV). Breast cancer patients were found to have a substantially higher proportion of circulating CD25$^+$ expressing CD4$^+$, and CD8$^+$ T-lymphocytes (Figure 5.6), as compared to CD4$^-$CD25$^-$ and CD8$^-$CD25$^-$ T-lymphocytes. A significantly higher circulating level of CD25$^+$ expressing CD4$^+$ T-cells as compared to CD4$^-$CD25$^-$ T-lymphocytes observed in patients presenting with stage 3 disease (P=0.0039) (Figure 5.6). A significant variation was observed in the frequency of CD4$^+$CD25$^-$ T-lymphocytes (P=0.021) in breast cancer patients presenting at different stages of disease, however pairwise comparison did not yield a significant result (Figure 5.6). This may be due to the small sample size in each “disease stage” group.
Figure: 5.6. The distribution of CD25 in CD4\(^+\) and CD8\(^+\) T-lymphocyte populations in patients presenting with breast cancer, according to stage of disease progression at first presentation. The CD4\(^+\) and CD8\(^+\) T-lymphocyte subsets expressing CD25\(^+/−\) are presented as the median % ± IQR. (NS = not significant, \(*P<0.05\), \(**P<0.01\), ***P<0.001)
5.2.6 Association between circulating levels of CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes and pathological stage of oesophageal cancer

Patients presenting with oesophageal carcinoma were also staged according to the American Joint Committee on Cancer (AJCC) TNMG staging and then further subdivided into 4 groups depending on their TNMG status (American Cancer Society 2013). Elevated levels of CD4<sup>+</sup>CD25<sup>−</sup> and CD8<sup>+</sup>CD25<sup>−</sup> T-lymphocytes were observed in patients presenting with stage 3 disease as compared to stage 4 disease (Figure 5.7); however, this did not reach significance (P=0.3938 and P=0.6698). Patients presenting with stage 4 disease displayed significantly higher circulating percentage of CD4<sup>+</sup>CD25<sup>+</sup> T-lymphocytes (P=0.0550) and CD8<sup>+</sup>CD25<sup>+</sup> (P=0.0550), as compared to patients presenting with stage 3 disease (Figure 5.7).
Figure: 5.7 The distribution of CD25 in CD4$^+$ and CD8$^+$ T-cell subsets in patients presenting with oesophageal cancer, according to stage of disease progression at first presentation. The expression of CD25$^{+/−}$ by CD4$^+$ and CD8$^+$ T-lymphocytes are presented as the median% ± IQR.
5.2.7 **Intracellular versus extracellular staining**

In order to explore the benefits of characterising and measuring circulating Treg cell subsets, with an extracellular staining technique, as opposed to the conventional combination of both intracellular and extracellular staining technique, blood samples from breast and oesophageal cancer patients were stained using both techniques. The extracellular staining technique was substantially quicker, and yielded similar frequencies of CD4⁺CD127⁺CD25⁺ and CD4⁺CD127⁺CD25⁺ Treg cell subsets, as compared to the conventional intra- and extracellular Treg staining methods. However, patients in this cohort also exhibited FoxP3⁺ Treg cell subsets (Figure 5.4), which were missed when only the surface staining technique was employed.
5.3 Discussion

Despite efforts to improve cancer awareness in South Africa, patients suffering from breast and oesophageal cancer continue to present to healthcare centres with progressive disease. In this study, 88% of African women suffering from breast cancer were found to present to the healthcare centres with stage 3 and 4 disease, compared to only 12% of caucasian patients. This correlates well with findings by Vorobiof et al. (2001) who assessed the different stages of disease presentation in 8411 women suffering from breast cancer in South Africa, and found that 77.7% of African women suffering from breast cancer presented with stage 3 and 4 disease, compared to 30.7% of non-African patients.

All the oesophageal cancer patients presented with stage 3 and 4 disease, rendering the majority of these participants eligible only for palliative treatment. Consequently, 60% of these oesophageal cancer participants demised within 3 months of presentation and 40% were lost to follow-up. This finding demonstrates that despite an 11 year lapse between this study and that conducted by Vorobiof et al. (2010), racial disparities in health seeking behaviour has not improved and Black patients are still presenting with late stage disease. The delay by patients to seek conventional medical care has been attributed to a myriad of reasons including the patients’ indigenous beliefs, fear of the stigma associated with a cancer diagnosis and mistrust of conventional treatment modalities (Vorobiof et al. 2001, American Cancer Society 2011). This finding illustrates that there is an urgent need to raise awareness regarding the morbidity and mortality associated with these conditions, the availability of treatment and the benefits of early presentation.
Chapter 5

5.3 Discussion

The extensive role which CD4\(^+\) and CD8\(^+\) T-lymphocytes play in the induction, regulation and mediation of antitumor immunity has already been described (Akhmetzyanova et al. 2013), and a high prevalence of intratumoral effector CD4\(^+\) and CD8\(^+\) T-cells has been associated with a better prognosis. T-lymphocytes express certain activation markers when stimulated, including CD25\(^+\), CD26\(^+\), CD69\(^+\) CD71\(^+\), and HLA-DR\(^+\), depending on the stage of T-cell activation (Reddy et al. 2004, Starska et al. 2011). The early activation markers are regarded as CD69\(^+\) and CD71\(^+\), while CD25\(^{high}\), CD26\(^+\) and HLA-DR are regarded as markers of late activation (Starska et al. 2011). Thus the increased frequency of CD25\(^+\) expressing CD4\(^+\) and CD8\(^+\) T-lymphocytes observed in this cohort may represent the immune systems response to the malignancy. These cell subsets could represent activated effector CD4\(^-\)CD25\(^+\) and CD8\(^-\)CD25\(^+\) T-cells, which are being recruited to the tumor site.

Although CD25\(^+\) expression in the absence of FoxP3 and CD127\(^{high}\) is representative of an active T-cell phenotype (Stanzani et al. 2004), it may also be associated with abnormal clonal expansion of antigen-specific T-lymphocytes (Starska et al. 2011, Nowicka et al. 2012). In this cohort, a large expansion in CD25\(^+\) expressing CD4\(^+\) and CD8\(^+\) T-lymphocytes was observed, and although the small sample size prevented statistical analysis of some of the different groups, a statistically significant higher proportion of CD4\(^+\)CD25\(^+\) T-lymphocytes was identified in patients presenting with stage 3 breast cancer (P=0.0039). This correlates with findings by Sasada and colleagues (2003) who reported significantly higher rates of CD4\(^+\)CD25\(^+\) T-lymphocytes, ranging from 43.7- 86% of CD4\(^+\) T-lymphocytes, in the peripheral blood of patients presenting with a variety of cancers including gastric, colorectal, pancreatic, oesophageal and liver carcinomas (Sasada et al. 2003). Contrary to Sasada and colleagues, we found a difference in the expression of CD25\(^+\) T-lymphocytes in cancer patients at different stages of disease (Sasada et al. 2003). This could be because they only
included participants with gastrointestinal cancer, while the majority of this cohort consisted of breast cancer participants. This could suggest that CD25$^+$ expression may be tumor-specific; however, differences where observed in the frequency of CD25$^+$ expressing T-lymphocytes in both breast and oesophageal cancer participants in this cohort when stratified according to stage of disease (Figure 5.6 and Figure 5.7). Thus genetic and environmental factors would need to be investigated to identify which factors contribute to the differences observed in this cohort.

When stimulated in vitro CD25$^+$ expressing CD4$^+$ T-lymphocytes were shown to produce large quantities of Th2 cytokines, IL-4 and IL-10, with only small quantities of IL-2 and IFN-\(\gamma\). Furthermore, CD4$^+$CD25$^+$ cells were shown to inhibit IL-2 and IFN-\(\gamma\) production by CD4$^+$CD25$^-$ T-lymphocytes (Sasada et al. 2003). The diversity associated with Treg cells makes it most probable that this population of cells could represent a CD25$^+$CD127$^-$FoxP3$^-$ Treg cell population, which has expanded secondary to the loss of immune surveillance. Liyanage and colleagues (2002) also observed a significantly higher prevalence of CD4$^+$CD25$^+$ T-lymphocytes in patients presenting with breast (P<0.001) and pancreatic cancer (P<0.015), as compared to normal controls. Although they did not analyse these cells for the expression of FoxP3 or CD127, they did find that these cell populations secreted large amounts of IL-10 and TGF-\(\beta\), irrespective of whether they were isolated from cancer patients or normal controls (Liyanage et al. 2002). This could suggest that tumor-induced immune suppression may be mediated by the induction of CD25$^+$ expression and concurrent promotion of IL-10, IL-4 and TGF-\(\beta\) production by CD4$^+$ and CD8$^+$ T-lymphocyte. Further studies will need to be conducted to explore whether this population of CD25$^+$CD127$^-$FoxP3$^-$ cells express any additional markers of anergy or activation, as well as the functional suppressive activity and secreted cytokine profile of these cells.
Oesophageal cancer patients also showed an expansion in the CD4^+CD25^+ and CD8^+CD25^+ T-cell populations. Interestingly, patients presenting with stage 3 disease displayed higher frequencies of CD4^+CD25^- and CD8^+CD25^- T-lymphocytes as compared to patients presenting with stage 4 disease; however, significance was not achieved. Sasada and colleagues (2003) reported that CD4^+CD25^- cells secreted IL-2 and IFN-γ when stimulated *in vitro*, which suggested a Th-1 like phenotype. This suggests that this cell population could represent an effector T-cell subset. Patients suffering from stage 4 disease generally present with substantial protein calorie malnutrition secondary to tumor-induced partial or total occlusion of the oesophagus, in conjunction with the metastatic spread of the disease. Therefore, the decline observed in the frequency of CD4^+CD25^- effector T-lymphocytes in stage 4 patients as compared to stage 3 patients may be as a result of severe protein-energy malnutrition secondary to stage 4 oesophageal cancer, which has shown to induce immune paresis and decreases the frequency and functionality of T-lymphocyte subsets, with adverse consequences on the patient’s prognosis (Haffejee et al. 1979). Furthermore, the increase in tumor burden and progression to a more advanced stage of disease could be as a direct response to a decline in CD4^+CD25^- T-lymphocyte population.

Selective reduction in the number of CD4^+CD25^- T-lymphocytes may also be attributed to their sensitivity to clonal deletion and apoptosis following induction by tumor derived factors, or CD4^+CD25^- T-lymphocytes (Sasada et al. 2003).

Despite numerous studies reporting elevated levels of Treg cells in the peripheral blood of patients presenting with cancer (Liyanage et al. 2002, Wolf et al. 2003, Xu et al. 2009), this was not observed in our study (Wang et al. 2012). This may be as a consequence of our small samples size, or it could be due to insufficient and inadequate cellular markers which were
used in the identification of Treg cells in the majority of studies conducted previously (Wang et al. 2012). Breast and oesophageal cancer patients were shown to exhibit a variety of CD4$^+$ and CD8$^+$ Treg cell subpopulations, however CD4$^+$FoxP3$^+$ Treg cell levels were substantially lower than what was observed in a previously described South African control group (median 3.72% range 1.3%-7.5%) (Suchard et al. 2010). This correlates with findings by Szczepanik et al (2011), who also found lower rates of Treg cells in gastric cancer patients as compared to healthy control. This may suggest that conventional peripheral Treg cells are not elevated in the presence of cancer in this population or it may be that the current markers used in this study, including FoxP3, were not sufficient to identify Treg cells within the CD4$^+$ and CD8$^+$ T-cell populations.

Similar to Liu and colleagues (2006), we found that the extracellular staining technique of Treg cell subsets with CD127$^+$ and CD25$^+$ was efficient and substantially less time consuming, as compared to the more traditional intracellular (FoxP3) and extracellular Treg staining technique. However, this cohort was found to exhibit a wide variety of FoxP3$^+$ Treg cell subsets, including those which lacked CD127 expression. This suggests that the use of an extracellular staining technique would miss a substantial proportion of circulating Treg cells, which may have quantitative and qualitative implications in different disease types or different stages of disease (Liu et al. 2006).

Future work will include further characterisation of these T-cell subsets (CD4$^+$CD25$^+$, CD8$^+$CD25$^+$, CD4$^+$CD25$^-$ and CD8$^+$CD25$^-$ T-lymphocyte) and Treg cell subsets (CD4$^+$CD25$^+$FoxP3$^+$, CD4$^+$CD127$^+$FoxP3$^+$, CD4$^+$CD25$^+$CD127$^+$ and CD8$^+$CD127$^+$FoxP3$^+$, CD8$^+$CD25$^+$CD127$^+$ and CD8$^+$CD25$^+$FoxP3$^+$ Treg cell), the suppressive activity of each subset, and the role in immune response (e.g. cytokine production).
Chapter 6: Breast cancer receptor status and Immunity

6 The evaluation of the relationship between CD4$^+$, CD8$^+$ and Treg cell populations and markers of prognosis in breast cancer: HER-2/neu and estrogen receptor status

6.1 Introduction

The quantification of hormonal and HER-2/neu receptor status in clinical practice has been shown as valuable prognostic and predictive markers in the treatment of breast cancer (Esteva et al. 2004, Weigelt et al. 2005). Over-expression of hormonal receptors has been associated with good prognosis in patients presenting with breast cancer, and predicts treatment response to hormonal therapy, including Tamoxifen treatment (Esteva et al. 2004). However, over-expression of the HER-2/neu oncogene has been associated with the development of more aggressive cancer with a poorer prognosis, higher recurrence rate and shorter survival time (Ciocca et al. 2006, Mitri et al. 2012). Recently, studies have demonstrated the role of HER-2/neu receptor as a predictor of treatment response to anti-Her-2/neu immunotherapy, including Trastuzumab (Ciocca et al. 2006). Numerous studies have demonstrated that an increase in the frequency of circulating or tumor associated T regulatory cells in patients presenting with breast cancer is also associated with poor prognosis (Nakamura, et al. 2009, Decker et al. 2012); however, other authors have disputed this claim (West et al. 2013). This chapter focuses on the association between the immune profile of patients presenting with breast cancer and HER-2/neu and oestrogen receptor status. Furthermore, the role of circulating Treg cells as a marker of prognosis was investigated. It was hypothesised that patients presenting with a “poor” prognostic receptor status would display elevated levels of circulating Treg cells as compared to patients presenting with a “good” prognostic receptor status.
6.2 Results

6.2.1 HER-2/neu, estrogen receptor status and association with stage of disease

The immunohistochemical staining for the hormonal and HER-2/neu receptor status of patients presenting with breast cancer at Kalafong hospital and Steve Biko Academic hospital was conducted by the National Health Laboratory Services of South Africa. The results of all patients included in this study were retrieved and analysed. The relationship between the stage of disease at presentation, oestrogen receptor, progesterone receptor and HER-2/neu receptor expression was evaluated.

Despite the fact that 64% of breast cancer patients presented with progressive disease (stage 3 and 4), the majority of patients presented with ER-positive (74% versus. 26%; \( P<0.0001 \)), and HER-2/neu negative breast cancer (61% versus. 39%; \( P<0.0001 \)) (Figure 6.1). Only two patients were found to be both ER-negative and HER-2/neu-positive, which together indicates a much poorer prognosis than all other receptor combinations.

When patients were stratified according to the stage of disease at presentation, the majority of patients presenting with early breast cancer (stage 1 and 2) were found to express ER positivity (87%), compared to only 66% of patients presenting with progressive cancer (\( P=0.28 \)) (Figure 6.2). Interestingly, similar levels of HER-2/neu expression were found in patients presenting with early (37.5%) and progressive breast cancer (40%) (\( P=0.63 \)) (Figure 6.2).
As shown in figure 6.2, patients who presented with early disease (stage 1 and 2) were found to have a significantly higher prevalence of ER⁺/PR⁺/HER-2/neu⁻ breast cancer, than patients with progressive disease (stage 3 and 4). In comparison to patients presenting with progressive disease, a significant proportion (50% versus 6%, P=0.026) of patients presenting with early disease expressed (Figure 6.2). Only 12% of patients with early cancer as opposed to 20% of patients with progressive cancer presented with triple negative receptor status (ER⁻/PR⁻/HER-2/neu⁻) (P=0.026) (Figure 6.2). While triple positive receptor status was observed in 13% of patients with early cancer, this was not observed in any of the patients presenting with progressive cancer (Figure 6.2). However, this finding did not reach statistical significance (P=0.405).
Figure 6.1: The percentage distribution of ER, PR and HER-2/neu receptor status in patients presenting with early and progressive breast cancer. A significantly higher proportion (61%) of breast cancer patients presented with HER-2/neu negative breast cancer, compared to HER-2/neu positive breast cancer (39%) (P<0.0001). A higher percentage of HER-2/neu negative women expressed ER-positivity, however this did not reach significance (P=0.078). The majority of women (74%) presented with ER+ breast cancer (P<0.0001).
Figure 6.2: The relationship between receptor status and stage of disease, shown as a percentage distribution: 87% of patients presenting with stage 1 and 2 cancer expressed ER-positive receptor, compared to 66% of patients presenting with progressive cancer. Triple positive status (HER2/neu pos/ER+/PR+) was only found in early cancer. (NS = not significant, *P<0.05, **P<0.01, ***P<0.001)
6.2 Results and Immunity

6.2.2 Evaluation of the immune profile, HER-2/neu and ER-receptor status

To determine whether the immune profile of breast cancer patients was associated with HER-2/neu and ER-receptor status, we compared the frequencies of non-Treg and Treg T-cell subsets in patients according to their tumor expression of HER-2/neu and ER-receptor. Furthermore, participants were stratified according to their stage of disease progression.

6.2.2.1 HER-2/neu receptor status

- Non-Treg T-lymphocyte population

Patients presenting with HER-2/neu positive breast cancer displayed a higher median CD4$^+$ count (388 cells/µl versus 291 cells/µl) but lower median CD8$^+$ count (153 cells/µl versus 171 cells/µl) as compared to patients presenting with HER-2/neu negative breast cancer (Table 6.1). These differences were not statistically significant (Table 6.1). HER-2/neu positivity was also associated with a significantly higher prevalence of circulating CD8$^+$CD25$^-$ T-lymphocytes (P=0.01) (Table 6.1).

When patients were stratified according to stage of disease at presentation, patients presenting with early HER-2/neu negative breast cancer displayed a significantly higher circulating CD4$^+$ count as compared to their HER-2/neu positive counterparts (P=0.05). Furthermore, patients presenting with early HER-2/neu negative breast cancer displayed a significantly higher CD4$^+$ count as compared to all patients presenting with late stage disease, irrespective of HER-2/neu status (P=0.05) (Table 6.2).
Table 6.1: Association between the general immune profile and HER-2/neu receptor status.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>HER-2/neu positive breast cancer</th>
<th>HER-2/neu negative breast cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>IQR</td>
</tr>
<tr>
<td>CD4⁺ count (cells/µl)</td>
<td>388</td>
<td>134-554</td>
</tr>
<tr>
<td>CD8⁺ count (cells/µl)</td>
<td>153</td>
<td>56-263</td>
</tr>
<tr>
<td>Total CD4⁺%</td>
<td>50.05</td>
<td>37.95-52.24</td>
</tr>
<tr>
<td>Total CD8⁺%</td>
<td>22.43</td>
<td>15.97-26.09</td>
</tr>
<tr>
<td>CD4⁺CD25⁺%</td>
<td>45.85</td>
<td>35.36-48.93</td>
</tr>
<tr>
<td>CD4⁺CD25⁻%</td>
<td>4.15</td>
<td>2.30-6.75</td>
</tr>
<tr>
<td>CD8⁺CD25⁺%</td>
<td>17.62</td>
<td>9.98-19.47</td>
</tr>
<tr>
<td>CD8⁺CD25⁻%</td>
<td>4.76</td>
<td>1.35-8.40</td>
</tr>
</tbody>
</table>

(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

Patients presenting with early HER-2/neu negative breast cancer also displayed significantly higher CD8⁺ T-lymphocyte counts as compared to early HER-2/neu positive cancer patients (P=0.05) (Table 6.2). A significantly higher percentage of CD8⁺CD25⁻ T-lymphocytes was observed in patients presenting with late HER-2/neu positive breast cancer, as compared to late HER-2/neu negative (P=0.04) and early HER-2/neu negative breast cancer (P=0.01) (Table 6.2).

Although the median percentage of the total CD4⁺ and CD4⁺CD25⁺ T-lymphocytes observed in patients presenting with early HER-2/neu-negative breast cancer was increased as compared to patients presenting with HER-2/neu-positive early and progressive breast cancer; this was not statistically significant (Table 6.2).

No difference was observed in the remaining general immune T-cell populations.
Table 6.2: Association between the general immune profile and HER-2/neu receptor status, stratified according to the stage of disease.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>HER-2/neu positive breast cancer</th>
<th>HER-2/neu negative breast cancer</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4⁺ count (cells/µl)</td>
<td>133 56-412</td>
<td>558 172-905</td>
<td>=0.05*</td>
</tr>
<tr>
<td>CD8⁺ count (cells/µl)</td>
<td>56 30-187</td>
<td>198 61-257</td>
<td>=0.05*</td>
</tr>
<tr>
<td>Total CD4⁺ %</td>
<td>48.49 47.91-50.65</td>
<td>56.16 48.41-60.39</td>
<td>=0.10 (NS)</td>
</tr>
<tr>
<td>Total CD8⁺ %</td>
<td>22.95 20.41-26.36</td>
<td>19.9 13.76-25.13</td>
<td>=0.10 (NS)</td>
</tr>
<tr>
<td>CD4⁺CD25⁺ %</td>
<td>45.85 42.07-48.28</td>
<td>51.63 45.96-59.18</td>
<td>=0.10 (NS)</td>
</tr>
<tr>
<td>CD4⁺CD25⁻ %</td>
<td>2.4 2.36-6.79</td>
<td>2.06 0.66-11.23</td>
<td>=0.18 (NS)</td>
</tr>
<tr>
<td>CD8⁺CD25⁺ %</td>
<td>15.18 14.27-16.58</td>
<td>14.22 7.70-19.50</td>
<td>=0.46 (NS)</td>
</tr>
<tr>
<td>CD8⁺CD25⁻ %</td>
<td>4.01 0.56-8.63</td>
<td>0.69 0.17-3.42</td>
<td>=0.17 (NS)</td>
</tr>
<tr>
<td>CD4⁺ count (cells/µl)</td>
<td>393 156-1133</td>
<td>259 154-341</td>
<td>=0.19 (NS)</td>
</tr>
<tr>
<td>CD8⁺ count (cells/µl)</td>
<td>203 62-301</td>
<td>141 19-239</td>
<td>=0.44 (NS)</td>
</tr>
<tr>
<td>Total CD4⁺ %</td>
<td>51.03 33.7-60.05</td>
<td>43.6 35.13-57.42</td>
<td>=0.81 (NS)</td>
</tr>
<tr>
<td>Total CD8⁺ %</td>
<td>21.23 14.33-26.41</td>
<td>21.86 8.39-32.43</td>
<td>=0.47 (NS)</td>
</tr>
<tr>
<td>CD4⁺CD25⁺ %</td>
<td>44.63 33.53-56.80</td>
<td>37.42 26.27-47.05</td>
<td>=0.24 (NS)</td>
</tr>
<tr>
<td>CD4⁺CD25⁻ %</td>
<td>3.42 0.95-8.15</td>
<td>1.97 0.43-12.02</td>
<td>=0.28 (NS)</td>
</tr>
<tr>
<td>CD8⁺CD25⁺ %</td>
<td>14.34 9.01-16.77</td>
<td>11.89 4.72-28.85</td>
<td>=0.72 (NS)</td>
</tr>
<tr>
<td>CD8⁺CD25⁻ %</td>
<td>5.89 1.42-9.59</td>
<td>1.81 0.53-3.1</td>
<td>=0.04*</td>
</tr>
</tbody>
</table>

(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
• *Treg T-lymphocyte population*

No difference was observed in the circulating percentages of the different Treg cell subsets in patients presenting with early versus progressive breast cancer. When breast cancer patients were stratified according to their expression of HER-2/neu receptor status, patients presenting with early cancer displayed similar Treg profiles irrespective of HER-2/neu expression (Figure 6.3). However, patients presenting with progressive HER-2/neu negative cancer displayed a significantly higher frequency of CD4^+^CD127^−^FoxP3^+^ Treg cells (P=0.036) (Figure 6.4), as compared to patients with progressive HER-2/neu positive breast cancer. CD4^+^CD25^+^FoxP3^+^ (P=0.21) and CD8^+^CD25^+^FoxP3^+^ (P=0.25) Treg levels in patients presenting with HER-2/neu negative cancer were also elevated in comparison to patients presenting with HER-2/neu positive breast cancer; however, this did not reach significance (Figure 6.4).
5.2.7 **Intracellular versus extracellular staining**

In order to explore the benefits of characterising and measuring circulating Treg cell subsets, with an extracellular staining technique, as opposed to the conventional combination of both intracellular and extracellular staining technique, blood samples from breast and oesophageal cancer patients were stained using both techniques. The extracellular staining technique was substantially quicker, and yielded similar frequencies of CD4⁺CD127⁺CD25⁺ and CD4⁺CD127⁺CD25⁺ Treg cell subsets, as compared to the conventional intra- and extracellular Treg staining methods. However, patients in this cohort also exhibited FoxP3⁺ Treg cell subsets (Figure 5.4), which were missed when only the surface staining technique was employed.
5.3 Discussion

Despite efforts to improve cancer awareness in South Africa, patients suffering from breast and oesophageal cancer continue to present to healthcare centres with progressive disease. In this study, 88% of African women suffering from breast cancer were found to present to the healthcare centres with stage 3 and 4 disease, compared to only 12% of caucasian patients. This correlates well with findings by Vorobiof et al (2001) who assessed the different stages of disease presentation in 8411 women suffering from breast cancer in South Africa, and found that 77.7% of African women suffering from breast cancer presented with stage 3 and 4 disease, compared to 30.7% of non-African patients.

All the oesophageal cancer patients presented with stage 3 and 4 disease, rendering the majority of these participants eligible only for palliative treatment. Consequently, 60% of these oesophageal cancer participants demised within 3 months of presentation and 40% were lost to follow-up. This finding demonstrates that despite an 11 year lapse between this study and that conducted by Vorobiof et al (2010), racial disparities in health seeking behaviour has not improved and Black patients are still presenting with late stage disease. The delay by patients to seek conventional medical care has been attributed to a myriad of reasons including the patients’ indigenous beliefs, fear of the stigma associated with a cancer diagnosis and mistrust of conventional treatment modalities (Vorobiof et al. 2001, American Cancer Society 2011). This finding illustrates that there is an urgent need to raise awareness regarding the morbidity and mortality associated with these conditions, the availability of treatment and the benefits of early presentation.
The extensive role which CD4\(^+\) and CD8\(^+\) T-lymphocytes play in the induction, regulation and mediation of antitumor immunity has already been described (Akhmetzyanova et al. 2013), and a high prevalence of intratumoral effector CD4\(^+\) and CD8\(^+\) T-cells has been associated with a better prognosis. T-lymphocytes express certain activation markers when stimulated, including CD25\(^+\), CD26\(^+\), CD69\(^+\) CD71\(^+\), and HLA-DR\(^+\), depending on the stage of T-cell activation (Reddy et al. 2004, Starska et al. 2011). The early activation markers are regarded as CD69\(^+\) and CD71\(^+\), while CD25\(^{\text{high}}\), CD26\(^+\) and HLA-DR are regarded as markers of late activation (Starska et al. 2011). Thus the increased frequency of CD25\(^+\) expressing CD4\(^+\) and CD8\(^+\) T-lymphocytes observed in this cohort may represent the immune systems response to the malignancy. These cell subsets could represent activated effector CD4\(^-\)CD25\(^+\) and CD8\(^+\)CD25\(^+\) T-cells, which are being recruited to the tumor site.

Although CD25\(^+\) expression in the absence of FoxP3 and CD127\(^{\text{high}}\) is representative of an active T-cell phenotype (Stanzani et al. 2004), it may also be associated with abnormal clonal expansion of antigen-specific T-lymphocytes (Starska et al. 2011, Nowicka et al. 2012). In this cohort, a large expansion in CD25\(^+\) expressing CD4\(^+\) and CD8\(^+\) T-lymphocytes was observed, and although the small sample size prevented statistical analysis of some of the different groups, a statistically significant higher proportion of CD4\(^+\)CD25\(^+\) T-lymphocytes was identified in patients presenting with stage 3 breast cancer (P=0.0039). This correlates with findings by Sasada and colleagues (2003) who reported significantly higher rates of CD4\(^+\)CD25\(^+\) T-lymphocytes, ranging from 43.7- 86% of CD4\(^+\) T-lymphocytes, in the peripheral blood of patients presenting with a variety of cancers including gastric, colorectal, pancreatic, oesophageal and liver carcinomas (Sasada et al. 2003). Contrary to Sasada and colleagues, we found a difference in the expression of CD25\(^+\) T-lymphocytes in cancer patients at different stages of disease (Sasada et al. 2003). This could be because they only
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Despite numerous studies reporting elevated levels of Treg cells in the peripheral blood of patients presenting with cancer (Liyanage et al. 2002, Wolf et al. 2003, Xu et al. 2009), this was not observed in our study (Wang et al. 2012). This may be as a consequence of our small samples size, or it could be due to insufficient and inadequate cellular markers which were
used in the identification of Treg cells in the majority of studies conducted previously (Wang et al. 2012). Breast and oesophageal cancer patients were shown to exhibit a variety of CD4\(^+\) and CD8\(^+\) Treg cell subpopulations, however CD4\(^+\)FoxP3\(^+\) Treg cell levels were substantially lower than what was observed in a previously described South African control group (median 3.72% range 1.3%-7.5%) (Suchard et al. 2010). This correlates with findings by Szczepanik et al (2011), who also found lower rates of Treg cells in gastric cancer patients as compared to healthy control. This may suggest that conventional peripheral Treg cells are not elevated in the presence of cancer in this population or it may be that the current markers used in this study, including FoxP3, were not sufficient to identify Treg cells within the CD4\(^+\) and CD8\(^+\) T-cell populations.

Similar to Liu and colleagues (2006), we found that the extracellular staining technique of Treg cell subsets with CD127\(^+\) and CD25\(^+\) was efficient and substantially less time consuming, as compared to the more traditional intracellular (FoxP3) and extracellular Treg staining technique. However, this cohort was found to exhibit a wide variety of FoxP3\(^+\) Treg cell subsets, including those which lacked CD127 expression. This suggests that the use of an extracellular staining technique would miss a substantial proportion of circulating Treg cells, which may have quantitative and qualitative implications in different disease types or different stages of disease (Liu et al. 2006).

Future work will include further characterisation of these T-cell subsets (CD4\(^+\)CD25\(^+\), CD8\(^-\)CD25\(^+\), CD4\(^-\)CD25\(^-\) and CD8\(^-\)CD25\(^-\) T-lymphocyte) and Treg cell subsets (CD4\(^+\)CD25\(^+\)FoxP3\(^+\), CD4\(^+\)CD127\(^+\)FoxP3\(^+\), CD4\(^+\)CD25\(^-\)CD127\(^+\) and CD8\(^+\)CD127\(^+\)FoxP3\(^+\), CD8\(^-\)CD25\(^+\)CD127\(^+\) and CD8\(^-\)CD25\(^+\)FoxP3\(^+\) Treg cell), the suppressive activity of each subset, and the role in immune response (e.g. cytokine production).
6 The evaluation of the relationship between CD4$^+$, CD8$^+$ and Treg cell populations and markers of prognosis in breast cancer: HER-2/neu and estrogen receptor status

6.1 Introduction

The quantification of hormonal and HER-2/neu receptor status in clinical practice has been shown as valuable prognostic and predictive markers in the treatment of breast cancer (Esteva et al. 2004, Weigelt et al. 2005). Over-expression of hormonal receptors has been associated with good prognosis in patients presenting with breast cancer, and predicts treatment response to hormonal therapy, including Tamoxifen treatment (Esteva et al. 2004). However, over-expression of the HER-2/neu oncogene has been associated with the development of more aggressive cancer with a poorer prognosis, higher recurrence rate and shorter survival time (Ciocca et al. 2006, Mitri et al. 2012). Recently, studies have demonstrated the role of HER-2/neu receptor as a predictor of treatment response to anti-Her-2/neu immunotherapy, including Trastuzumab (Ciocca et al. 2006). Numerous studies have demonstrated that an increase in the frequency of circulating or tumor associated T regulatory cells in patients presenting with breast cancer is also associated with poor prognosis (Nakamura, et al. 2009, Decker et al. 2012); however, other authors have disputed this claim (West et al. 2013). This chapter focuses on the association between the immune profile of patients presenting with breast cancer and HER-2/neu and oestrogen receptor status. Furthermore, the role of circulating Treg cells as a marker of prognosis was investigated. It was hypothesised that patients presenting with a “poor” prognostic receptor status would display elevated levels of circulating Treg cells as compared to patients presenting with a “good” prognostic receptor status.
6.2 Results

6.2.1 HER-2/neu, estrogen receptor status and association with stage of disease

The immunohistochemical staining for the hormonal and HER-2/neu receptor status of patients presenting with breast cancer at Kalafong hospital and Steve Biko Academic hospital was conducted by the National Health Laboratory Services of South Africa. The results of all patients included in this study were retrieved and analysed. The relationship between the stage of disease at presentation, oestrogen receptor, progesterone receptor and HER-2/neu receptor expression was evaluated.

Despite the fact that 64% of breast cancer patients presented with progressive disease (stage 3 and 4), the majority of patients presented with ER-positive (74% versus 26%; P<0.0001), and HER-2/neu negative breast cancer (61% versus 39%; P< 0.0001) (Figure 6.1). Only two patients were found to be both ER-negative and HER-2/neu-positive, which together indicates a much poorer prognosis than all other receptor combinations.

When patients were stratified according to the stage of disease at presentation, the majority of patients presenting with early breast cancer (stage 1 and 2) were found to express ER positivity (87%), compared to only 66% of patients presenting with progressive cancer (P=0.28) (Figure 6.2). Interestingly, similar levels of HER-2/neu expression were found in patients presenting with early (37.5%) and progressive breast cancer (40%) (P=0.63) (Figure 6.2).
As shown in figure 6.2, patients who presented with early disease (stage 1 and 2) were found to have a significantly higher prevalence of ER⁺/PR⁺/HER-2/neu⁻ breast cancer, than patients with progressive disease (stage 3 and 4). In comparison to patients presenting with progressive disease, a significant proportion (50% versus 6%, P=0.026) of patients presenting with early disease expressed (Figure 6.2). Only 12% of patients with early cancer as opposed to 20% of patients with progressive cancer presented with triple negative receptor status (ER⁻/PR⁻/HER-2/neu⁻) (P=0.026) (Figure 6.2). While triple positive receptor status was observed in 13% of patients with early cancer, this was not observed in any of the patients presenting with progressive cancer (Figure 6.2). However, this finding did not reach statistical significance (P=0.405).
Figure 6.1: The percentage distribution of ER, PR and HER-2/neu receptor status in patients presenting with early and progressive breast cancer. A significantly higher proportion (61%) of breast cancer patients presented with HER-2/neu negative breast cancer, compared to HER-2/neu positive breast cancer (39%) (P<0.0001). A higher percentage of HER-2/neu negative women expressed ER-positivity, however this did not reach significance (P=0.078). The majority of women (74%) presented with ER+ breast cancer (P<0.0001).
Figure 6.2: The relationship between receptor status and stage of disease, shown as a percentage distribution: 87% of patients presenting with stage 1 and 2 cancer expressed ER-positive receptor, compared to 66% of patients presenting with progressive cancer. Triple positive status (HER2/neu pos/ER+/PR+) was only found in early cancer. (NS = not significant, *P<0.05, **P<0.01, ***P<0.001)
6.2.2 Evaluation of the immune profile, HER-2/neu and ER-receptor status

To determine whether the immune profile of breast cancer patients was associated with HER-2/neu and ER-receptor status, we compared the frequencies of non-Treg and Treg T-cell subsets in patients according to their tumor expression of HER-2/neu and ER-receptor. Furthermore, participants were stratified according to their stage of disease progression.

6.2.2.1 HER-2/neu receptor status

• Non-Treg T-lymphocyte population

Patients presenting with HER-2/neu positive breast cancer displayed a higher median CD4$^+$ count (388 cells/μl versus. 291 cells/μl) but lower median CD8$^+$ count (153 cells/μl versus. 171 cells/μl) as compared to patients presenting with HER-2/neu negative breast cancer (Table 6.1). These differences were not statistically significant (Table 6.1). HER-2/neu positivity was also associated with a significantly higher prevalence of circulating CD8$^+$CD25$^-$ T-lymphocytes (P=0.01) (Table 6.1).

When patients were stratified according to stage of disease at presentation, patients presenting with early HER-2/neu negative breast cancer displayed a significantly higher circulating CD4$^+$ count as compared to their HER-2/neu positive counterparts (P=0.05). Furthermore, patients presenting with early HER-2/neu negative breast cancer displayed a significantly higher CD4$^+$ count as compared to all patients presenting with late stage disease, irrespective of HER-2/neu status (P=0.05) (Table 6.2).
Table 6.1: Association between the general immune profile and HER-2/neu receptor status.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>HER-2/neu positive breast cancer</th>
<th>Median</th>
<th>IQR</th>
<th>HER-2/neu negative breast cancer</th>
<th>Median</th>
<th>IQR</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4⁺ count (cells/µl)</td>
<td>388</td>
<td>134 - 554</td>
<td>291</td>
<td>213-473</td>
<td>P=0.80 (NS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8⁺ count (cells/µl)</td>
<td>153</td>
<td>56 - 263</td>
<td>171</td>
<td>102-230</td>
<td>P=0.87 (NS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CD4⁺%</td>
<td>50.05</td>
<td>37.95 – 52.24</td>
<td>50.31</td>
<td>42.6-56.66</td>
<td>P=0.75 (NS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CD8⁺%</td>
<td>22.43</td>
<td>15.97-26.09</td>
<td>18.17</td>
<td>15.74-28.16</td>
<td>P=0.89 (NS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4⁺CD25⁺%</td>
<td>45.85</td>
<td>35.36-48.93</td>
<td>44.33</td>
<td>34.3-51.91</td>
<td>P=0.80 (NS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4⁺CD25%</td>
<td>4.15</td>
<td>2.30-6.75</td>
<td>2.00</td>
<td>0.73-4.44</td>
<td>P=0.10 (NS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8⁺CD25⁺%</td>
<td>17.62</td>
<td>9.98-19.47</td>
<td>17.07</td>
<td>9.85-18.29</td>
<td>P=0.48 (NS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8⁺CD25%</td>
<td>4.76</td>
<td>1.35-8.40</td>
<td>1.10</td>
<td>0.47-2.39</td>
<td>P=0.01**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

Patients presenting with early HER-2/neu negative breast cancer also displayed significantly higher CD8⁺ T-lymphocyte counts as compared to early HER-2/neu positive cancer patients (P=0.05) (Table 6.2). A significantly higher percentage of CD8⁺CD25⁺ T-lymphocytes was observed in patients presenting with late HER-2/neu positive breast cancer, as compared to late HER-2/neu negative (P=0.04) and early HER-2/neu negative breast cancer (P=0.01) (Table 6.2).

Although the median percentage of the total CD4⁺ and CD4⁺CD25⁺ T-lymphocytes observed in patients presenting with early HER-2/neu-negative breast cancer was increased as compared to patients presenting with HER-2/neu-positive early and progressive breast cancer; this was not statistically significant (Table 6.2).

No difference was observed in the remaining general immune T-cell populations.
Table 6.2: Association between the general immune profile and HER-2/neu receptor status, stratified according to the stage of disease.

<table>
<thead>
<tr>
<th></th>
<th>HER-2/neu positive breast cancer</th>
<th>HER-2/neu negative breast cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell type</td>
<td>Median (IQR)</td>
</tr>
<tr>
<td></td>
<td>CD4⁺ count (cells/µl)</td>
<td>133 (56-412)</td>
</tr>
<tr>
<td></td>
<td>CD8⁺ count (cells/µl)</td>
<td>56 (30-187)</td>
</tr>
<tr>
<td>Early cancer (Stage 1-2)</td>
<td>Total CD4⁺ %</td>
<td>48.49 (47.91-50.65)</td>
</tr>
<tr>
<td></td>
<td>Total CD8⁺ %</td>
<td>22.95 (20.41-26.36)</td>
</tr>
<tr>
<td></td>
<td>CD4⁺CD25⁺ %</td>
<td>45.85 (42.07-48.28)</td>
</tr>
<tr>
<td></td>
<td>CD4⁺CD25⁻ %</td>
<td>2.4 (2.36-6.79)</td>
</tr>
<tr>
<td></td>
<td>CD8⁺CD25⁺ %</td>
<td>15.18 (14.27-16.58)</td>
</tr>
<tr>
<td></td>
<td>CD8⁺CD25⁻ %</td>
<td>4.01 (0.56-8.63)</td>
</tr>
<tr>
<td>Late cancer (Stage 3-4)</td>
<td>CD4⁺ count (cells/µl)</td>
<td>393 (156-1133)</td>
</tr>
<tr>
<td></td>
<td>CD8⁺ count (cells/µl)</td>
<td>203 (62-301)</td>
</tr>
<tr>
<td></td>
<td>Total CD4⁺ %</td>
<td>51.03 (33.7-60.05)</td>
</tr>
<tr>
<td></td>
<td>Total CD8⁺ %</td>
<td>22.93 (14.33-26.41)</td>
</tr>
<tr>
<td></td>
<td>CD4⁺CD25⁺ %</td>
<td>44.63 (33.53-56.80)</td>
</tr>
<tr>
<td></td>
<td>CD4⁺CD25⁻ %</td>
<td>3.42 (0.95-8.15)</td>
</tr>
<tr>
<td></td>
<td>CD8⁺CD25⁺ %</td>
<td>14.34 (9.01-16.77)</td>
</tr>
<tr>
<td></td>
<td>CD8⁺CD25⁻ %</td>
<td>5.89 (1.42-9.59)</td>
</tr>
</tbody>
</table>

(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
• *Treg T-lymphocyte population*

No difference was observed in the circulating percentages of the different Treg cell subsets in patients presenting with early versus progressive breast cancer. When breast cancer patients were stratified according to their expression of HER-2/neu receptor status, patients presenting with early cancer displayed similar Treg profiles irrespective of HER-2/neu expression (Figure 6.3). However, patients presenting with progressive HER-2/neu negative cancer displayed a significantly higher frequency of CD4⁺CD127⁻FoxP3⁺ Treg cells (P=0.036) (Figure 6.4), as compared to patients with progressive HER-2/neu positive breast cancer. CD4⁺CD25⁺FoxP3⁺ (P=0.21) and CD8⁺CD25⁺FoxP3⁺ (P=0.25) Treg levels in patients presenting with HER-2/neu negative cancer were also elevated in comparison to patients presenting with HER-2/neu positive breast cancer; however, this did not reach significance (Figure 6.4).
Stage 1 and 2 breast cancer: HER-2/neu positive

Stage 1 and 2 breast cancer: HER-2/neu negative

Figure 6.3: The percentage distribution of CD4\(^+\) and CD8\(^+\) Treg cell subsets in patients presenting with stage 1 and 2 breast cancer, stratified according to HER-2/neu receptor status, shown as the median percentage ±IQR. No significant difference observed in the median lymphocyte percentage between patients presenting with HER-2/neu positive and HER-2/neu negative.

(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
Stage 3 and 4 breast cancer: HER-2/neu positive Treg cell population

Stage 3 and 4 breast cancer: HER-2/neu negative Treg cell population

Figure 6.4: The percentage distribution of CD4$^+$ and CD8$^+$ Treg cell subsets in patients presenting with stage 3 and 4 breast cancer, stratified according to HER-2/neu receptor status, shown as the median percentage ± IQR. Patients presenting with progressive HER-2/neu negative cancer displayed a significantly higher frequency of CD4$^+$CD127$^+$FoxP3$^+$ Treg cells (P=0.036) (Figure 6.4) as compared to patients presenting with HER-2/neu positive patients. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
6.2 Results

6.2.2 Oestrogen receptor status

- Non-Treg T-lymphocyte population

Patients presenting with ER-positive and ER-negative breast cancer were found to have similar immune profiles at presentation (Table 6.3). However, in comparison to patients presenting with ER-positive cancer, patients presenting with ER-negative cancer were found to have a significantly elevated circulating percentage of CD4⁺CD25⁺ T-lymphocytes (42.71% versus. 53.33%; P=0.01). ER-positivity was also associated with an increased percentage of circulating total CD4⁺ T-lymphocytes; however, this did not reach significance (56.33% versus. 48.49%; P=0.08) (Table 6.3). Surprisingly, although patients displaying ER-positivity are said to have a better prognosis, these patients demonstrated a lower CD4⁺ and CD8⁺ T-cell count as compared to patients presenting with ER-negative breast cancer (Table 6.3)

Table 6.3: Association between non-Treg immune profile and ER-receptor status

<table>
<thead>
<tr>
<th>Cell type</th>
<th>ER-positive breast cancer</th>
<th>ER-negative breast cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>IQR</td>
</tr>
<tr>
<td>CD4⁺ count (cells/µl)</td>
<td>303</td>
<td>172-425</td>
</tr>
<tr>
<td>CD8⁺ count (cells/µl)</td>
<td>153</td>
<td>108-227</td>
</tr>
<tr>
<td>Total CD4⁺ %</td>
<td>48.49</td>
<td>39.33-52.2</td>
</tr>
<tr>
<td>Total CD8⁺ %</td>
<td>22.43</td>
<td>20.03-26.35</td>
</tr>
<tr>
<td>CD4⁺CD25⁺ %</td>
<td>42.71</td>
<td>34.70-46.43</td>
</tr>
<tr>
<td>CD4⁺CD25⁻ %</td>
<td>2.36</td>
<td>1.73-6.79</td>
</tr>
<tr>
<td>CD8⁺CD25⁺ %</td>
<td>14.27</td>
<td>11.07-15.64</td>
</tr>
<tr>
<td>CD8⁺CD25⁻ %</td>
<td>1.81</td>
<td>0.69-4.00</td>
</tr>
</tbody>
</table>

(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
When patients were stratified according to the stage of disease, ER-negativity, in comparison to ER-positivity, was still significantly associated with increased circulating levels of CD4⁺CD25⁺ T-lymphocytes (34.75 versus. 48.98; P=0.007) in patients presenting with progressive breast cancer. Furthermore, progressive ER-negative breast cancer was associated with increased circulating levels of total CD4⁺ T-lymphocytes, however this also did not reach statistical significance (41.30-52.26; P=0.066). Only one patient presented with early ER-negative breast cancer, thus statistical analysis could not be performed.

**Table 6.4:** Association between non-Treg immune profile and ER-receptor status, stratified according to stage of disease

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Early cancer (Stage 1-2)</th>
<th></th>
<th>Late cancer (Stage 3-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ER positive breast cancer</td>
<td>ER negative breast cancer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>95% Confidence interval</td>
<td>Median</td>
</tr>
<tr>
<td>CD4⁺ count (cells/µl)</td>
<td>412</td>
<td>80-795</td>
<td>741</td>
</tr>
<tr>
<td>CD8⁺ count (cells/µl)</td>
<td>187</td>
<td>38-242</td>
<td>198</td>
</tr>
<tr>
<td>Total CD4⁺ %</td>
<td>50.65</td>
<td>48.07-56.26</td>
<td>60.39</td>
</tr>
<tr>
<td>Total CD8⁺ %</td>
<td>20.41</td>
<td>15.70-25.97</td>
<td>16.14</td>
</tr>
<tr>
<td>CD4⁺CD25⁺ %</td>
<td>46.69</td>
<td>43.26-52.81</td>
<td>59.18</td>
</tr>
<tr>
<td>CD4⁺CD25⁻ %</td>
<td>2.36</td>
<td>1.16-9.83</td>
<td>0.66</td>
</tr>
<tr>
<td>CD8⁺CD25⁺ %</td>
<td>15.18</td>
<td>8.76-18.58</td>
<td>14.22</td>
</tr>
<tr>
<td>CD8⁺CD25⁻ %</td>
<td>1.45</td>
<td>0.29-7.18</td>
<td>0.25</td>
</tr>
<tr>
<td>CD4⁺ count (cells/µl)</td>
<td>281</td>
<td>173-438</td>
<td>280</td>
</tr>
<tr>
<td>CD8⁺ count (cells/µl)</td>
<td>153</td>
<td>108-260</td>
<td>135</td>
</tr>
<tr>
<td>Total CD4⁺ %</td>
<td>41.30</td>
<td>33.78-55.03</td>
<td>52.26</td>
</tr>
<tr>
<td>Total CD8⁺ %</td>
<td>22.56</td>
<td>20.47-28.23</td>
<td>15.63</td>
</tr>
<tr>
<td>CD4⁺CD25⁺ %</td>
<td>34.75</td>
<td>27.96-42.85</td>
<td>48.98</td>
</tr>
<tr>
<td>CD4⁺CD25⁻ %</td>
<td>2.69</td>
<td>0.99-11.34</td>
<td>2.30</td>
</tr>
<tr>
<td>CD8⁺CD25⁺ %</td>
<td>13.82</td>
<td>10.44-16.34</td>
<td>9.64</td>
</tr>
<tr>
<td>CD8⁺CD25⁻ %</td>
<td>2.54</td>
<td>0.59-8.62</td>
<td>2.22</td>
</tr>
</tbody>
</table>

(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
In addition to stratification according to ER-receptor status and stage of disease at presentation, patients were further stratified according to their HER-2/neu receptor status.

- **Early (Stage 1 and 2) ER-positive: HER-2/neu positive versus. HER-2/neu negative**

Patients presenting with early ER-receptor positive, HER-2/neu negative receptor status displayed a higher median CD4$^+$ count (492 cells/µl, 95% CI 172-905) as compared to patients presenting with early ER-receptor positive, HER-2/neu positive receptor status, (133 cells/µl, 95% CI= 56-412). However, this did not reach significance (P=0.077).

Similarly, patients with early ER-positive, HER-2/neu negative receptor status were also found to have higher median CD8$^+$ T-lymphocyte count (61 cells/µl, 95%CI 61-257) as compared to patients presenting with early ER-positive, HER-2/neu positive breast cancer (56 cells/µl, 95%CI 30-187). However, this difference did not reach statistical significance (P=0.077), difference was observed in the remaining non-Treg immune markers (Figure 6.5).

- **Late (stage 3 and 4) HER-2/neu positive: ER-positive versus. ER-negative**

In comparison to patients presenting with progressive HER-2/neu positive, ER-positive breast cancer, patients presenting with progressive HER-2/neu positive, ER-negative breast cancer displayed an elevated median percentage of total CD4$^+$ T-lymphocytes (P=0.064) and CD4$^+$CD25$^+$ T-lymphocytes (P=0.064) (Figure 6.6). However, patients presenting with ER-positive, HER-2/neu positive breast cancer were found to have elevated levels of circulating total CD8$^+$ T-lymphocytes (P=0.064) and CD8$^+$CD25$^+$ T-lymphocytes (P=0.064) (Figure 6.6). These findings were not statistically significant.
• **Late (stage 3 and 4) HER-2/neu negative: ER-positive versus. ER-negative**

Patients presenting with late HER-2/neu negative, ER-negative breast cancer were found to have a significantly higher prevalence of circulating CD4^+CD25^+ T-lymphocytes, as compared to patients with ER-positive breast cancer (P=0.039) (Figure 6.7). Interestingly, patients who presented with ER-positive breast cancer displayed substantially higher levels of CD4^+CD25^- Treg cells as compared to patients presenting with ER-negative breast cancer (Figure 6.7), however this did not reach significance.
6.2 Results

Figure 6.5: The CD4$^+$ and CD8$^+$ T-lymphocyte subsets in patients presenting with stage 1 and 2 breast cancer, stratified according to ER-receptor status, stage of disease and HER-2/neu receptor status, shown as the median % ±IQR. No difference was observed in the median non-Treg T-lymphocyte subsets in patients presenting with HER-2/neu positive and HER-2/neu negative patients. (NS= Not statistically significant, *= statistically significant: *P<0.05, **P<0.01, ***P<0.001)
Figure 6.6: The CD4$^+$ and CD8$^+$ T-lymphocyte subsets in HER-2/neu positive breast cancer patients presenting with stage 3 and 4 disease, stratified according to ER-receptor status, and stage of disease and, shown as the median % ± IQR. The median CD4$^+$CD45$^+$ and CD4$^+$CD25$^+$ T-lymphocytes were elevated in patients presenting with ER-negative cancer compared to ER-positive cancer. The median CD8$^+$CD45$^+$ and CD8$^+$CD25$^+$ T-lymphocytes were elevated in patients presenting with ER-positive cancer, compared to ER-negative cancer, however, this did not reach significance. (NS= Not statistically significant, *= statistically significant: *P<0.05, **P<0.01, ***P<0.001)
Figure 6.7: The CD4\(^+\) and CD8\(^+\) T-lymphocyte subsets in HER-2/neu negative breast cancer patients presenting with stage 3 and 4 disease, stratified according to ER-receptor status and stage of disease, shown as the median % ± IQR. The median CD4\(^+\)CD25\(^+\) T-lymphocyte was found to be significantly elevated in patients presenting with ER-negative cancer as oppose to patients with ER-positive cancer (P=0.039) (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
Chapter 6: Breast cancer receptor status and Immunity

6.2 Results

- *Estrogen receptor status: Treg cell population*

A similar Treg cell profile was observed in patients presenting with early ER-positive, HER-2/neu positive breast cancer and early ER-positive, HER-2/neu negative breast cancer (Figure 6.8). Furthermore, no difference was observed in the Treg immune profile of patients presenting with progressive HER-2/neu positive breast cancer (ER-positive versus ER-negative) (Figure 6.9) or HER-2/neu negative breast cancer (ER-positive versus ER-negative) (Figure 6.10).

The results of the association between the immune profile and the breast cancer tumor receptor status are summarised in table 6.5.
Chapter 6: Breast cancer receptor status

6.2 Results and Immunity

Figure 6.8: The CD4$^{+}$ and CD8$^{+}$ Treg subsets in patients presenting with ER-positive stage 1 and 2 breast cancer, stratified according to HER-2/neu receptor status, shown as the median % ±IQR. The median CD4$^{+}$CD25$^{+}$CD127$^{-}$ (P=0.4) and CD8$^{+}$CD25$^{+}$FoxP3$^{+}$ (P=0.6) T-lymphocytes were higher in patients presenting with HER-2/neu negative patients compared to HER-2/neu positive patients. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
Figure 6.9: The CD4⁺ and CD8⁺ Treg subsets in patients presenting with HER-2/neu-positive stage 3 and 4 breast cancer, stratified according to ER-receptor status, shown as the median % ±IQR. No difference was observed in the median Treg cell subsets in ER-positive and ER-negative women.

(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
Chapter 6: Breast cancer receptor status

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Figure 6.10: The CD4$^+$ and CD8$^+$ Treg subsets in patients presenting with HER-2/neu-negative stage 3 and 4 breast cancer, stratified according to ER-receptor status, shown as the median % ±IQR. The median CD8$^+$CD25$^+$FoxP3$^+$ and CD4$^+$CD25$^+$FoxP3$^+$Treg cell subsets were higher in patients presenting with ER-negative cancer compared to ER-positive cancer. The median CD8$^+$CD25$^+$CD127$^+$ Treg cells were higher in patients presenting with ER-positive, compared to ER-negative cancer.

(NS= Not statistically significant, *= statistically significant: *P<0.05, **P<0.01, ***P<0.001)
### Summary table 6.5: Association between immune profile and breast cancer tumor receptor status

<table>
<thead>
<tr>
<th>HER-2/neu receptor status</th>
<th>Non Treg T-cell population</th>
<th>Treg T-cell population</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HER-2/neu positive</strong></td>
<td>All breast cancers</td>
<td>High CD4⁺ count (NS)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low CD8⁺ count (NS)</td>
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<tr>
<td></td>
<td></td>
<td>High CD8⁺CD25⁻ ** (£)</td>
</tr>
<tr>
<td></td>
<td>Early breast cancer</td>
<td>Low CD4⁺ count* (£)</td>
</tr>
<tr>
<td></td>
<td>(Stage 1 and 2)</td>
<td>Low CD8⁺ count* (£)</td>
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<tr>
<td></td>
<td></td>
<td>Low total CD4% (NS)</td>
</tr>
<tr>
<td></td>
<td>Late breast cancer</td>
<td>Low CD4⁺ count* (£)</td>
</tr>
<tr>
<td></td>
<td>(Stage 3 and 4)</td>
<td>High CD8⁺CD25⁻ ** (%)</td>
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<tr>
<td></td>
<td></td>
<td>Low CD4⁺CD25⁻ (NS)</td>
</tr>
<tr>
<td><strong>HER-2/neu negative</strong></td>
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</tr>
<tr>
<td></td>
<td>High CD8⁺ count (NS)</td>
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<td>Early breast cancer</td>
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<td>Late breast cancer</td>
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<td></td>
<td>(Stage 3 and 4)</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Low CD4⁺CD25⁻ (NS)</td>
</tr>
<tr>
<td><strong>ER-receptor status</strong></td>
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<td>Low CD4⁺ count (NS)</td>
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<tr>
<td></td>
<td>Low CD8⁺ count (NS)</td>
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<tr>
<td></td>
<td>Early breast cancer</td>
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</tr>
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<td>the ER negative group.</td>
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<td>the ER negative group.</td>
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<td></td>
<td>No difference observed</td>
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<td></td>
<td></td>
<td>No difference observed</td>
</tr>
<tr>
<td></td>
<td>(Stage 3 and 4)</td>
<td>Low total CD4(^+)% (NS)</td>
</tr>
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<td>--------------------</td>
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<td>-----------------------------</td>
</tr>
<tr>
<td><strong>ER-negative</strong></td>
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<td></td>
</tr>
<tr>
<td>All breast cancer</td>
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<td>High CD4(^-)CD25(^+) ** (¶)</td>
</tr>
<tr>
<td></td>
<td>High CD4(^-)% (NS)</td>
<td>Low total CD4(^+)% (NS)</td>
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<td>High CD4(^-) count (NS)</td>
<td>Low total CD4(^+)% (NS)</td>
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<tr>
<td></td>
<td>High CD8(^-) count (NS)</td>
<td>No difference observed</td>
</tr>
<tr>
<td>Early breast cancer (Stage 1 and 2)</td>
<td>Unable to do statistics due to only 1 patient in the group</td>
<td>Unable to do statistics due to only 1 patient in the group</td>
</tr>
<tr>
<td>Progressive cancer (Stage 3 and 4)</td>
<td>High CD4(^-)CD25(^+) ** (ἔ)</td>
<td>High total CD4(^+)% (NS)</td>
</tr>
<tr>
<td></td>
<td>No difference observed</td>
<td></td>
</tr>
</tbody>
</table>

| **Early ER-positive** | HER-2/neu positive | Low CD4\(^+\) count (NS) |
|                       | Low CD8 count (NS) | No difference observed |
| HER-2/neu negative    | High CD4\(^+\) count (NS) |
|                       | High CD8\(^+\) count (NS) | No difference observed |

| **Late HER-2/neu positive** | ER-positive | Low total CD4\(^+\)% (NS) |
|                            |             | Low CD4\(^-\)CD25\(^+\)% (NS) |
|                            |             | High total CD8\(^-\)% (NS) |
|                            |             | High CD8\(^-\)CD25\(^+\) (NS) |
|                            | ER-negative | High total CD4\(^-\)% (NS) |
|                            |             | High CD4\(^-\)CD25\(^+\)% (NS) |
|                            |             | Low total CD8\(^-\)% (NS) |
|                            |             | Low CD8\(^-\)CD25\(^+\) (NS) |
|                            | No difference observed |                             |

| **Late HER-2/neu negative** | ER-positive | Low CD4\(^-\)CD25\(^+\) * (อยากให้) |
|                            | High CD4\(^-\)CD25\(^+\) (NS) | No difference observed |
|                            | ER-negative | High CD4\(^-\)CD25\(^+\) * (ạt) |
|                            | Low CD4\(^-\)CD25\(^+\) (NS) | No difference observed |

NS = Not statistically significant, *= statistically significant: *P=0.05, **P=0.01, ***P>0.001
¶ = Statistically significance difference observed between patients presenting with HER-2/neu negative and HER-2/ neu positive breast cancer.
§ = Statistically significance difference observed between patients presenting with early HER-2/neu negative and early HER-2/ neu positive breast cancer.
€= Statistically significance difference observed between patients presenting with early HER-2/neu negative and late disease irrespective of HER-2/ neu receptor status.
¥= Statistically significance difference observed between patients presenting with early HER-2/neu negative and early HER-2/ neu positive breast cancer
%= Statistically significance difference observed between patients presenting with late HER-2/neu positive and both early and HER-2/ neu negative breast cancer
$=$ Statistically significant difference observed between patients presenting with late HER-2/neu negative and HER-2/neu positive breast cancer.
¶$=$ Statistically significant difference observed between patients presenting with ER-negative and ER-positive breast cancer.
⌘$=$ Statistically significant difference observed between patients presenting with late ER-negative and ER-positive breast cancer.
万余元$=$ Statistically significant difference observed between patients presenting with late HER-2/neu negative ER-negative and HER-2/neu negative ER-positive breast cancer.
6.3 Discussion

This chapter aimed to investigate the association between non-Treg and Treg immune profile, hormonal and HER-2/neu receptor status in patients presenting with early and progressive breast cancer.

The majority (74%) of patients in this cohort presented with ER-positive breast tumors, which is said to be associated with decreased rates of local invasion and distant metastases to other organs (Gago et al. 1998). This incidence is higher than that reported by other sub-Saharan African settings including, 43.2% by Yarney and colleagues (2008), in Ghana, and 24% by Bird and colleagues (2008) in Kenya (Yarney et al. 2008, Bird et al. 2008). Positive hormone receptor status has also been associated with improved overall survival and breast cancer-specific survival irrespective of additional clinically relevant factors (Zell et al. 2009). ER-negativity was only found in 12% of patients presenting with early disease, and 33% of patients presenting with progressive disease (P=0.28), and is a better determinant of poor survival rather than HER-2/neu status (Zell et al. 2009). In this cohort no mortalities occurred in patients presenting with ER-negative receptor status. However, of the patients who presented with ER-positive receptor status, 1 patient who presented with progressive HER-2/neu positive receptor status demised.

The over-expression of HER-2/neu receptor was found in 39% of breast cancer patients in this cohort, which was also higher than the 19% reported by Yarney and colleagues (2008) in a Ghanaian cohort, and 26% reported by Bogaert and colleagues (2013) in a rural South African cohort. HER-2/neu receptor expression is found in 25-30% of breast cancer, and develops during the late stages of carcinogenesis (Slamon et al. 1987, Gusterson et al. 1992, Ciocca et al. 2006). HER-2/neu positivity is said to give rise to aggressive tumors, which are
associated with shorter disease free periods and overall survival (Gusterson et al. 1992). HER-2/neu expression is said to be inversely correlated to ER-receptor status (Ciocca et al. 2006). However, despite our limited sample size, 31% of breast cancer patients over-expressed both HER-2/neu and oestrogen receptor, which is said to be uncommon, and has been associated with a poor overall survival (Ciocca et al. 2006). Co-expression is also associated with an up-regulation of HER-2/neu expression, which induces resistance to hormonal therapy e.g. Tamoxifen (Konecny et al. 2003). This data suggests that co-expression may not be as infrequent as previously suggested, and raises questions regarding the treatment options for this group of patients. Further studies need to be conducted on a larger sample size to evaluate the co-expression of these receptors in the South African population, and the impact that this status has on treatment outcome.

In contrast to findings by Bird and colleagues (2008), this cohort had lower levels of ER\(^+\)/PR\(^-\)/HER-2/neu\(^+\) (17.6% versus 8.7%), ER\(^+\)/PR\(^+\)/HER-2/neu\(^+\) (8.8% versus 4.3%) and triple negative (ER\(^-\)/PR\(^-\)/HER-2/neu\(^-\)) (44.1% versus 17.4%) breast cancer (Bird et al. 2008). On the other hand, we found a similar incidence of triple negative breast cancer to that reported by Cubasch and colleague (2013) in a different South African setting (19.1% versus 17.4%) (Cubasch et al. 2013). This may suggest that genetic or even environmental factors may play a role in the differences observed in receptor status in patients in the West African cohort, versus those found in this setting. However, irrespective of the location, this subgroup is a cause for concern considering the fact that triple negative status has been associated with more advanced histological grade, higher recurrence rate, a decreased overall survival and substantial hormonal insensitivity (Rakha et al. 2007). This was substantiated by this cohort, where early disease was significantly associated with ER\(^+\)/PR\(^+\)/HER-2/neu negative status.
(P=0.026), while progressive disease was significantly associated with triple negative status (ER\(^{-}/PR^{-}/HER-2/neu\)) (P=0.026).

Patients presenting with early HER-2/neu negative cancer were found to have a higher circulating CD4\(^{+}\) and CD8\(^{+}\) count, as compared to patients presenting with early HER-2/neu positive disease or late disease. This may suggest that a positive HER-2/neu receptor status may be associated with a poorer immune profile, and possibly poorer antitumor effector activity. This could potentially contribute to the poor survival outcome which has been attributed to this marker.

Patients presenting with early HER-2/neu positive breast cancer displayed a higher frequency of circulating CD8\(^{+}\)CD25\(^{-}\) T-lymphocytes (P=0.01) as compared to patients presenting with HER-2/neu negative early (P=0.01) and late breast cancer (P=0.04). As previously discussed (Chapter 5), CD8\(^{+}\)CD25\(^{-}\) T-lymphocyte is not clearly characterised in the literature but could represent an effector T-lymphocyte population.

ER-receptor status alone did not seem to have an impact on the immune profile; however, when assessed in conjunction with the stage of disease at presentation, patients presenting with progressive ER-negative cancer displayed a significantly higher prevalence of CD4\(^{+}\)CD25\(^{+}\) T-lymphocytes (P=0.007), as compared to patients presenting with progressive ER-positive cancer. As mentioned previously (chapter 5), this T-cell population could potentially represent a suppressive T-cell subset. As the remaining non-Treg cell subsets were similar between the groups, this suggests that ER-negativity may be associated with an up-regulation of suppressive T-cell activity, and suggests that these cells may be involved in
mediating a suppressive and tolerant immune milieu that facilitates tumor progression. This suggests that ER-receptor status may serve as an indicator of the patients’ immune status, with ER-negativity possibly being associated with a poorer immune status.

Early ER-positive, HER-2/neu-negative breast cancer were found to have a higher CD4$^+$ count (492 cells/µl-133 cells/µl, $P=0.07$), and CD8$^+$ count (61 cells/µl-56 cells/µl; $P=0.07$), (Figure 6.5) as compared to patients presenting with early ER-receptor positive, HER-2/neu positive receptor status and all patients with progressive breast cancer. Although this was not statistically significant, within the clinical setting it may suggest that CD4$^+$ and CD8$^+$ T-cell immunity in this group of patients is still intact, compared to patients with more progressive disease. This suggests that the role of CD4$^+$ and CD8$^+$ T-lymphocytes in the induction and maintenance of antitumor immunity is sustained in this group of patients. Furthermore, this suggests that HER-2/neu status may also reflect the immune status of a patient, with HER-2/neu over-expression possibly being associated with a poorer immune status.

Interestingly, a variety of CD4$^+$ and CD8$^+$ Treg cell populations were also identified in this cohort, with a trend towards higher frequencies of CD8$^+$CD25$^+$CD127$^+$, CD8$^+$CD25$^+$FoxP3$^+$ and CD4$^+$CD25$^+$CD127$^+$ Treg cells being detected in patients diagnosed with early cancer compared to late cancer, though significance was not reached. However, we did not observe an increased prevalence of Treg cells in the peripheral blood of patients presenting with HER-2/neu positive cancers as was reported by Perez & colleagues (2007) and Rech and colleagues (2010). In fact, the majority of Treg cell subsets were identified in patients presenting with progressive HER-2/neu negative cancer (Figure 6.10). Furthermore, patients presenting with late HER-2/neu positive receptor status (Figure 6.9) demonstrated similar
Treg immune profiles to patients presenting with early ER-positive receptor status (Figure 8). This suggests that an increase in Treg cell subset is not associated with the clinical stage of disease or the ER-receptor status. It may suggest that within our setting the presence of Treg cell subsets is associated with good HER-2/neu receptor status (HER-2/neu negative).

Furthermore, as previously mentioned, early HER-2/neu negative receptor status was also associated with an increase in non-Treg immune profile as compared to early HER-2/neu positive receptor status, which may suggest that HER-2/neu receptor status may serve as a fair representation of a patients’ immune status and vice-versa. Further studies with a larger sample size will need to be conducted to confirm or refute this finding.

To the best of my knowledge, this is the first study to compare biological immune profiles with hormonal and HER-2/neu receptor status in African patients presenting with breast cancer. Furthermore, this is the first study to demonstrate circulating CD4+ and CD8 Treg cell populations in African patients presenting with breast cancer. Although I have found a number of contradictory findings as compared to what has been previously reported, this could be as a result of the small sample size or due to the fact that no other study has assessed the prevalence of Treg cell populations in the presence of breast cancer within an African population. Further studies need to be conducted with a larger sample size to investigate this further.
Chapter 7: Effect of antitumor treatment

7 Effect of antitumor treatment on T-lymphocyte distribution

7.1 Introduction

Research efforts focused on the augmentation of antitumor immunity through the manipulation of cellular and soluble components of the immune system has been ongoing for more than 100 years (Agarwala et al. 2010). These efforts have been intensified following the discovery of Treg cells, with numerous studies attempting to facilitate immune surveillance through the depletion of circulating and intratumoral Treg cell populations in cancer patients (Tuve et al. 2007). This mode of treatment would be used as an adjunct to conventional antitumor treatment (surgery, chemotherapy and radiotherapy) and would result in the amplification of the host's antitumor immunity while simultaneously attenuating the profound immunosuppressive effects associated with orthodox antitumor treatment (Lennard et al. 1985, Baumgartner et al. 2009).

Surgery, chemotherapy and radiotherapy have all been shown to induce defects in T-lymphocyte proliferation, function and cytokine secretion, with a consequent reduction in the circulating T-lymphocyte population (Campbell et al. 1973, Rasmussen et al. 1982, Lennard et al. 1985, De-en et al. 1988, Hensler et al. 1997, González et al. 2007). This creates an environment that is conducive to tumor dissemination, and is associated with an increased risk of relapse following treatment (De-en et al. 1988, Kachikwu et al. 2011, Kang et al. 2009). However, conventional antitumor treatment has also been shown to facilitate antitumor immunity through the depletion of Treg cells. This occurs either following the surgical removal of the tumor mass, which contains a large proportion of the Treg cells, or via apoptosis of Treg cells following exposure to chemotherapeutic agents or radiation (Liu et al. 2012, Schmidt et al. 2012, Wang et al. 2013, Baumgartner et al. 2008). Therefore, in
theory conventional treatment should diminish the Treg mediated immunosuppressive effect and promote antitumor immunity.

Despite numerous reports on the effect which cancer treatment has on cells involved in antitumor immunity (CD4\(^+\) and CD8\(^+\) T-cells) (De-en et al. 1998, Ishikawa et al. 2009, Kang et al. 2009) and Treg cell populations (Ladoire et al. 2008, Kachiwu et al. 2011, Schmidt et al. 2012), little is known about the overall effect of antitumor treatment on the relationship between T-cells which promote tumor progression (Treg cells) and those which promote tumor regression (non-Treg CD4\(^+\) and CD8\(^+\) T-cells). We aimed to investigate whether the overall effect of antitumor treatment creates an environment which is more conducive to tumor growth (increased Treg and decreased non-Treg cells) or tumor eradication (decreased Treg and increased non-Treg cells).

In order to evaluate the effect of conventional antitumor treatment, including surgery, chemotherapy and radiotherapy, on T-lymphocyte subsets in patients presenting with breast and oesophageal cancer, peripheral blood samples were collected from the treatment naïve patients, to establish a baseline T-lymphocyte profile, and at day 1 post antitumor treatment. An additional blood sample was collected at 5-7 weeks post-treatment to determine whether any change observed at day 1 post-treatment had returned to baseline levels. The 5-7 week time frame was chosen because most patients who received surgical intervention were started on adjuvant chemotherapy no later than 5-7 weeks post-surgery.
Twenty-five breast cancer and 10 oesophageal cancer patients supplied blood samples for examination of baseline T-lymphocyte profile. At day 1 post-treatment only 24 breast and oesophageal cancer patients in total were able to supply blood samples for post-treatment examination. At the 5-7 week follow-up only 13 cancer patients from the original 35 were able to supply blood samples for examination. The reasons for patients not supplying samples included death, early start on 2nd line treatment and loss to follow-up.

7.2 Results

7.2.1 Breast and oesophageal cancer: All antitumor treatment

7.2.1.1 Detection of variation in circulating non-Treg CD4+ and CD8+ T-lymphocyte percentage at day 1 post-treatment and at 5-7 weeks post-treatment, in comparison to baseline levels

In order to assess the effect of antitumor treatment on T-lymphocyte immune profile, blood samples were taken at baseline (before treatment), day 1 post-treatment and at 5-7 weeks post-treatment. All CD4+ T-cell populations showed a non-significant decline in the median T-cell percentages at 5-7 weeks post-treatment as compared to baseline levels (Figure 7.1). An analysis of variance (Kruskal-Wallis) revealed a non-significant variation in the median percentage of total CD4+, CD4+CD25− and CD4+CD25+ T-lymphocytes at day 1 post-treatment and 5-7 weeks post-treatment, as compared to baseline T-lymphocyte levels (Figure 7.1). However, the median percentage of CD4+CD25− T-lymphocyte was significantly higher than the median percentage of CD4+CD25+ T-lymphocytes at day 1 post-treatment (P<0.0001) and at 5-7 weeks post-treatment (P=0.0005) (Figure 7.1). In comparison to baseline levels (246 cells/µl), an increasing trend in the CD4+ count was observed at day 1
post-treatment (256 cells/µl), and at 5-7 weeks post-treatment (388 cells/µl), however this did not reach significance (P=0.32) (Table 7.1).

CD8$^+$ T-lymphocyte populations also showed a declining progression from baseline to 5-7 weeks post-treatment, with an increase in frequency only being noted in the CD8$^+$CD25$^+$ T-cell subset at day 1 post-treatment. The analysis of variance revealed a non-significant variation in the frequency of circulating total CD8$^+$, CD8$^+$CD25$^+$ and CD8$^+$CD25$^-$ T-lymphocyte during the follow-up period. The prevalence of circulating CD25$^+$ expressing CD8$^+$ T-lymphocytes was also significantly higher than CD8$^+$CD25$^-$ at day 1 post-treatment (P=0.0001) and at 5-7 weeks post-treatment (P=0.0049) (Figure 7.1). In comparison to the baseline total CD8$^+$ T-cell concentration (149 cells/µl) levels, patients at day 1 post-treatment showed a decline in the total CD8$^+$ T-cell concentration (119 cells/µl); however, this was followed by an increase in frequency at 5-7 weeks post-treatment (132 cells/µl), although not to the same level as that observed at baseline (Table 7.1). This variation was not statistically significant (P= 0.59) (Table 7.1).
Breast and oesophageal cancer T-lymphocyte immune profile in response to antitumor treatment

CD4\(^+\) and CD8\(^+\) T-cell population

Figure 7.1: The frequency distribution of CD4\(^+\) and CD8\(^+\) T-lymphocyte subsets in breast and oesophageal cancer patients before treatment (red bar), at day 1 after treatment (yellow bar) and 5-7 weeks post-treatment (blue bars). This Figure represents the median % ± IQR of T-lymphocyte subsets detected at different time points. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
Table 7.1: The CD4<sup>+</sup> and CD8<sup>+</sup> T-cell counts (cells/µl) in breast and oesophageal cancer patients before treatment, at day 1 post-treatment and 5-7 weeks post-treatment

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Median ±IQR</th>
<th>Statistical significance</th>
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<tr>
<td></td>
<td>Before treatment</td>
<td>Day 1 post-treatment</td>
</tr>
<tr>
<td>Cells/µl</td>
<td>Time point 1</td>
<td>Time point 2</td>
</tr>
<tr>
<td>Total CD4&lt;sup&gt;+&lt;/sup&gt; count</td>
<td>246 (172-425)</td>
<td>256 (154-447)</td>
</tr>
<tr>
<td>Total CD8&lt;sup&gt;+&lt;/sup&gt; count</td>
<td>149 (57-306)</td>
<td>119 (82-197)</td>
</tr>
<tr>
<td>CD4:CD8 ratio</td>
<td>2.0 (1.3-2.6)</td>
<td>2.2 (1.0-3.3)</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD25&lt;sup&gt;+&lt;/sup&gt;CD127&lt;sup&gt;+&lt;/sup&gt;</td>
<td>10 (4-21)</td>
<td>5 (1-11)§</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD127&lt;sup&gt;+&lt;/sup&gt;FoxP3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0 (0-0.1)</td>
<td>0 (0)</td>
</tr>
<tr>
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<tr>
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<td>24 (12-38)</td>
</tr>
<tr>
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<td>0 (0-4)</td>
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<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;CD127&lt;sup&gt;+&lt;/sup&gt;FoxP3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0 (0-0.1)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Statistically significant difference between time point 1 and 2 =§ (P=0.05)
Statistically significant difference between time point 1 and 3=¶ (P=0.045)
Statistically significant difference in variation (P>0.05) between time point 1, 2 and 3*
(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
7.2.1.2 Detection of circulating CD4+ and CD8+ Treg cell subsets before treatment, at day 1 post-treatment and at 5-7 weeks post-treatment

A downward trend was observed in the frequency of CD4+CD25+FoxP3+, CD4+CD25+CD127−, CD8+CD127+Fox3+ and CD8+CD25+CD127+ Treg cell percentage at day 1 post-treatment and 5-7 weeks post-treatment (Figure 7.2). However, these variations in frequency did not reach significance. Interestingly, a similar trend was observed in the circulating CD4+CD25+CD127− (P=0.037) and CD8+CD25+CD127+ (P=0.045) Treg cell concentrations (cells/µl) (Table 7.1), with these subsets achieving significance.

An upward trend was observed in the median percentage of CD8+CD25+FoxP3+ and CD4+CD127+FoxP3+ Treg cells from baseline to day 1 post-treatment, however this was followed by a decline to baseline levels at 5-7 weeks post-treatment (Figure 7.2). These variations were not statistically significant.

Interestingly, the median percentage and cell concentration (cells/µl) of circulating CD4+CD25+CD127− Treg cells were significantly higher than CD4+CD25+FoxP3+ Treg cells at day 1 post-treatment (P<0.0001) and at 5-7 weeks post-treatment (P=0.002) (Figure 7.2 and Table 7.1). Furthermore, the prevalence of CD4+CD25+CD127− Treg cell was also significantly higher than CD4+CD127+FoxP3+ Treg cells at day 1 post-treatment (P<0.0001) and 5-7 weeks post-treatment (P=0.0020) (Figure 7.2 and Table 7.1).

The median percentage and cell concentration of CD8+CD25+CD127+ Treg cells were also found to be significantly higher than CD8+CD25+FoxP3+ Treg cell at baseline (P=0.0001), at day 1 post-treatment (P<0.0001) and at 5-7 weeks post-treatment (P=0.0039) (Figure 7.2 and Table 7.1). Moreover, the median percentage and cell concentration of CD8+CD25+CD127+ Treg cell subset was also found to be significantly higher than CD8+CD127+FoxP3+ at
Breast and oesophageal cancer Treg immune profile

in response to antitumor treatment

CD4⁺ and CD8⁺ Treg cell population

Figure 7.2: The frequency distribution of CD4⁺ and CD8⁺ Treg subsets in breast and oesophageal cancer patients before treatment (brown bar), at day 1 after treatment (green bar) and 5-7 weeks post-treatment (blue bars). This Figure represents the median % ± IQR of Treg subsets detected at different time points. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
baseline (P< 0.0001), at day 1 post-treatment (P<0.0001) and at 5-7 weeks post-treatment (P=0.0039) (Figure 7.2 and Table 7.1). A large interquartile range was observed in the CD8⁺CD25⁺FoxP3⁺ Treg cell subset at baseline and day one after treatment (Figure 7.2), which was driven by the presence of high frequency of CD8⁺CD25⁺FoxP3⁺ Treg cell in patients with advanced disease. By 5-7 week follow-up these participants had demised or were lost to follow-up (Figure 7.2). Due to the small sample size we were unable to further investigate the effect of the stage of disease on the Treg subset.

7.2.1.3 Effect of antitumor treatment on breast and oesophageal cancer CD4⁺:CD8⁺ T-lymphocyte, CD4⁺ Treg: total CD4⁺ and CD8⁺ Treg: total CD8⁺ ratio

In order to assess the overall effect of antitumor treatment on immunity, this study investigated the variation in the ratio between the total CD4⁺ or CD8⁺ Treg cells percentage and the total CD4⁺ (CD4⁺CD45⁺) and total CD8⁺ (CD8⁺CD45⁺) T-lymphocyte percentages. Furthermore the variation in the total CD4⁺: total CD8⁺ T-cell concentration ratio across the different time points under investigation was assessed.

The total CD4⁺ Treg cell percentage at each time point was calculated by tallying the percentage CD4⁺CD25⁺FoxP3⁺, CD4⁺CD25⁺CD127⁺ and CD4⁺CD127⁺FoxP3⁺ Treg cell subsets observed at the respective time points. While the total CD8⁺ Treg cell percentage at each time point was calculated by tallying the percentage CD8⁺CD25⁺FoxP3⁺, CD8⁺CD25⁺CD127⁺ and CD4⁺CD127⁺FoxP3⁺ Treg cell subsets.

A non-significant declining trend was observed in the CD4⁺ Treg: total CD4⁺ T-cell ratio at day 1 post-treatment and at 5-7 weeks post-treatment (P=0.499), in comparison to the baseline (Figure 7.3). A similar trend was observed in the CD8⁺ Treg: total CD8⁺ T-cell ratio, however it also did not reach significance (P=0.40) (Figure 7.3). Interestingly, The CD8⁺
Treg: total CD8⁺ T-cell ratio was higher than the CD4⁺ Treg: total CD4⁺ T-cell ratio at all time points (Figure 7.3).

Unlike the downward trend observed in the CD4⁺ Treg: total CD4⁺ T-cell ratio and CD8⁺ Treg: total CD8⁺ T-cell ratio, the total CD4⁺: total CD8⁺ T-cell concentration ratio was seen to have an upward trend over the follow-up period, moving from a ratio of 2.0 (IQR: 1.3-2.6) at baseline and peaking at a ratio of 3.14 (IQR: 2.4-4.3) at 5-7 weeks post-treatment (Table 7.1). However, this trend was not statistically significant (P=0.71).
Breast and oesophageal cancer

Overall effect of antitumor treatment

CD4\(^+\) Treg (%): total CD4\(^+\) (%)

CD8\(^+\) Treg (%): total CD8\(^+\) (%)

Figure 7.3: The frequency distribution of CD4\(^+\) Treg (%): total CD4\(^+\)(%) and CD8\(^+\) Treg (%): total CD8\(^+\)(%) ratio in breast and oesophageal cancer patients before treatment, at day 1 after treatment and 5-7 weeks post-treatment. This Figure represents the median % ± IQR of CD4\(^+\) Treg/total CD4\(^+\) or CD8\(^+\) Treg/total CD8\(^+\) ratio detected at different time points. (NS= Not statistically significant, *= statistically significant: *P<0.05, **P<0.01, ***P<0.001).
7.2.2 Breast cancer patients: Surgical versus chemotherapeutic intervention

In order to assess the effect of surgical and neo-adjuvant chemotherapeutic intervention on the circulating T-lymphocyte subsets in patients with breast cancer, the prevalence of T-lymphocytes was quantified before surgery or chemotherapy, at day 1 post-surgery or chemotherapy and at 5-7 weeks post-surgical or chemotherapeutic intervention.

Breast cancer patients who were eligible for neo-adjuvant chemotherapy presented with a more progressive stage of disease as compared to those eligible for surgical intervention. All chemotherapy patients received combination therapy as determined by the treating oncologist, which consisted of cyclophosphamide in combination with adriamycin 5FU or methotrexate. Of the patients who were eligible for surgical intervention, 94% received a modified radical mastectomy (MRM) with or without axillary clearance, while only 5.3% of patients received a lumpectomy. This was generally followed by a course of adjuvant chemotherapy, which was commenced no later than 7 weeks post surgery.

7.2.2.1 Detection of Non-Treg T-lymphocyte population in breast cancer patients receiving surgical or chemotherapeutic intervention, before treatment, at day 1 post-treatment and at 5-7 weeks post-treatment

Patients who received surgical treatment showed a significant variation in the circulating levels of total CD4+ (P=0.037) and CD4+CD25+ (P=0.009) T-lymphocyte percentages between the different time-points (Figure 7.4). The total CD4+ T-lymphocyte percentage observed before treatment 48.49% (IQR: 39.29-56.16) remained stable at day 1 post surgery (48.91%; IQR 38.87-54.97), however it declined to 47.17% (IQR: 40-56.47%) at 5-7 weeks post-treatment (Figure 7.4). Similarly, the median CD4+CD25+ T-lymphocyte percentage
showed a downward trend from 45.85% (IQR: 37.42-51.63%) before treatment to 41.49% (IQR: 38.8-52.12) at 5-7 weeks post-treatment. A post-hoc pairwise comparison did not show a significant difference between the time points. Interestingly, the total CD4⁺ T-cell concentration showed an increasing trend during this period from 260 cells/µl before treatment to 440 cells/µl at 5-7 weeks post-treatment (P=0.62) (Table 7.2).

When the total CD4⁺ percentage in surgical patients was stratified according to the CD25⁺ expression at different time points, a significantly higher median percentage of CD4⁺CD25⁺ T-lymphocytes were observed before treatment (P≤0.001), at day 1 after treatment (P≤0.001) and at 5-7 weeks post-treatment (P=0.0039), as compared to CD4⁺CD25⁻ T-lymphocytes (Figure 7.4).

A downward trend in the median percentage of total CD8⁺ T-lymphocyte was observed at 5-7 weeks post surgery (16.81; IQR 14.4-22.82) as compared to baseline levels (22.43%; IQR 16.14-26.83). However, although a decline was also observed in the total CD4⁺ T-cell population, the decline observed in the total CD8⁺ T-cell subset was more acute and severe, as compared to that observed in the total CD4⁺ T-cell subset. This acute decline is best observed at day 1 post-treatment, where the median total CD8⁺ T-lymphocyte percentage declined from 22.43% to 19.66% (15.75%-25.67%), as apposed to the total CD4⁺ T-cell population which remained stable at day 1 post-treatment (48.49% before treatment versus 48.91% at day 1 post-treatment) (Figure 7.4).

The decline observed in the median total CD8⁺ T-lymphocyte percentage was also associated with a decline in the total CD8⁺ T-cell concentration from 187 cells/µl at baseline to 160 cells/µl at day 1 post-treatment (Table 7.2). At 5-7 weeks post-treatment, the CD8⁺ count
remained similar to levels observed at day 1 post-treatment (160 cells/µl). This variation was not statistically significant (P=0.48).

A significantly higher proportion of CD8⁺ T-lymphocytes expressed CD25⁺ receptor before treatment (P≤0.002), after treatment (P≤0.001) and at 5-7 weeks post-treatment (P=0.0117), as compared to CD8⁺CD25⁻ T-lymphocytes (Figure 7.4). Although a decline was observed in the frequency of circulating CD8⁺CD25⁻% and CD8⁺CD25⁺% at 5-7 weeks post-treatment, in comparison to baseline levels, this was not statistically significant (Figure 7.4).
Breast cancer
Chemotherapeutic and Surgical intervention
CD4\(^+\) and CD8\(^+\) T-cell population

Figure 7.4: The frequency distribution of CD4\(^+\) and CD8\(^+\) T-lymphocyte subsets in breast cancer patients receiving chemotherapy and surgical intervention, before treatment (red bar), at day 1 after treatment (yellow bar) and 5-7 weeks post-treatment (blue bars). This Figure represents the median % ± IQR of T-lymphocyte subsets detected at different time points. (NS = Not statistically significant, * = statistically significant: \*P<0.05, **P<0.01, ***P<0.001).
Patients receiving chemotherapy demonstrated a similar median total CD4\(^+\) T-lymphocyte percentage at day 1 post-treatment as compared to baseline levels, while the remaining non-Treg T-lymphocyte subsets increased in frequency at day 1 post-treatment (Figure 7.4). This was followed by a substantial decline in the median percentage of all non-Treg T-cell subsets at 5-7 weeks post-treatment, far below levels observed at baseline (Figure 7.4). Due to the small sample size in the chemotherapy cohort, statistical analysis could not be conducted.

The circulating total CD4\(^+\) T-lymphocyte concentration in patients receiving chemotherapy was shown to decline from 292 cells/µl (IQR: 222-345 cells/µl) at baseline, to 234 cells/µl (154-281 cells/µl) at day 1 post-treatment (Table 7.2). This was followed by a substantial decline in the CD4\(^+\) count (39 cells/µl) at 5-7 weeks post-treatment (Table 7.2). Similarly, the CD8\(^+\) T-lymphocyte concentration observed at baseline (151 cells/µl IQR: 135-153) declined to 109 cells/µl (IQR: 98-232) at day 1 post-treatment with a further decline observed at 5-7 weeks post-treatment (55 cells/µl) (Table 7.2).

Patients receiving chemotherapy displayed a higher median percentage of CD25\(^+\) expressing CD4\(^+\) T-lymphocytes before treatment, at day 1 post-chemotherapy and 5-7 weeks post-chemotherapy as compared to CD4\(^+\)CD25\(^-\) T-lymphocyte (Figure 7.4). Similarly, a higher proportion of CD8\(^-\)CD25\(^+\) T-lymphocytes (%) was observed before chemotherapy and at day 1 post-chemotherapy as compared to CD8\(^-\)CD25\(^-\) T-lymphocyte (%). Due to the small sample size, statistical analysis could not be conducted (Figure 7.4).

In comparison to chemotherapy patients, surgical patients displayed a significantly higher median CD8\(^-\)CD25\(^-\) (P=0.05) T-lymphocyte percentage at baseline, however the remaining
non-Treg T-lymphocyte (%) profiles were similar (Figure 7.4). Interestingly, at day 1 post-treatment chemotherapy patients displayed a higher median percentage of almost all the non-Treg cell subsets, with surgical patients only displaying a higher total CD4⁺ T-lymphocyte percentage (Figure 7.4). At 5-7 weeks post-treatment the T-lymphocyte levels in surgical patients were substantially higher than that observed in chemotherapy patients; however, the small sample size in the chemotherapy group prevented further statistical analysis (Figure 7.4).

When the T-lymphocyte cell concentrations were compared, chemotherapy patients were found to have a higher CD4⁺ T-lymphocyte concentration (292 cells/µl, IQR: 222-345) as compared to surgical patients (260 cells/µl, IQR: 150-559) (Table 7.2) at baseline. However, surgical patients displayed a substantially higher CD4⁺ T-lymphocyte count at day 1 post-treatment (396 cells/µl versus 234 cells/µl) and at 5-7 weeks post-treatment (440 cells/µl versus 39 cells/µl), as compared to chemotherapy patients. Furthermore, in comparison to chemotherapy patients, surgical patients were found to have a substantially higher level of CD8⁺ T-lymphocytes at baseline (187 cells/µl versus 151 cells/µl), at day 1 post-treatment (161 cells/µl versus 109 cells/µl) and at 5-7 weeks post-treatment (160 cells/µl versus 55 cells/µl).
Table 7.2: The CD4$^+$ and CD8$^+$ T-cell counts (cells/µl) in breast cancer patients receiving surgical or chemotherapeutic intervention; before treatment, at day 1 post-treatment and 5-7 weeks post-treatment

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<td>post-treatment</td>
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Chemotherapy patients

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<tr>
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<td>B</td>
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<td>5-7 weeks</td>
<td></td>
</tr>
<tr>
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<td></td>
<td>treatment</td>
<td>post-treatment</td>
<td>post-treatment</td>
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</tr>
<tr>
<td>Total CD4$^+$ count</td>
<td>292 (222-345)</td>
<td>234 (154-281)</td>
<td>39 (39-39)</td>
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<td>Total CD8$^+$ count</td>
<td>151 (135-153)</td>
<td>109 (98-232)</td>
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Statistically significant difference between time point 1 and 2 =§ (P=0.05)
Statistically significant difference between time point 1 and 3=¶ (P=0.045)
Statistically significant difference in variation (P>0.05) between time point 1, 2 and 3*
(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
7.2.2.2 Detection of Treg CD4\(^+\) and CD8\(^+\) Treg cell subsets in breast cancer patients receiving chemotherapy and surgical treatment intervention; before treatment, at day 1 after treatment and at 5-7 weeks post-treatment intervention

A variety of CD4\(^+\) and CD8\(^+\) Treg cell populations were observed in both treatment groups at baseline (Figure 7.5). Surgical patients displayed a significantly higher median percentage and circulating concentration (cells/µl) of CD4\(^+\)CD25\(^+\)CD127\(^+\) Treg cells as compared to both CD4\(^+\)CD25\(^+\)FoxP3\(^+\) Treg cells (P<0.0001) and CD4\(^+\)CD127\(^+\)FoxP3\(^+\) (P< 0.0001) Treg cell subsets before surgery (Figure 7.5 and Table 7.2). The median CD4\(^+\)CD25\(^+\)CD127\(^+\) Treg cell percentage and circulating concentration (cells/µl) remained significantly elevated in comparison to CD4\(^+\)CD25\(^+\)FoxP3\(^+\) at day 1 post-treatment (P=0.0005) and at 5-7 weeks post-treatment (P=0.0059) (Figure 7.5 and Table 7.2). Similarly, the median CD4\(^+\)CD25\(^+\)CD127\(^+\) Treg cell percentage and circulating concentration (cells/µl) was also significantly elevated in comparison to CD4\(^+\)CD127\(^+\)FoxP3\(^+\) at day 1 post-treatment (P=0.001) and 5-7 weeks post-treatment (P=0.0107) (Figure 7.5 and Table 7.2). The median percentage of CD4\(^+\)CD25\(^+\)FoxP3\(^+\) Treg was significantly higher than CD4\(^+\)CD127\(^+\)FoxP3\(^+\) at day 1 post-treatment (P=0.0010) and at 5-7 week post-treatment (P=0.0107) (Figure 7.5).

The CD8\(^+\)CD25\(^+\)CD127\(^+\) Treg cell prevalence and circulating concentration in surgical patients was also found to be significantly higher than CD8\(^+\)CD25\(^+\)FoxP3\(^+\) Treg cells (P<0.0001) and CD8\(^+\)CD127\(^+\)FoxP3\(^+\) (P<0.0001) Treg cells before surgery, at day 1 post-surgery [CD8\(^+\)CD25\(^+\)FoxP3\(^+\) Treg cells (P=0.0001), CD8\(^+\)CD127\(^+\)FoxP3\(^+\) Treg cells (P=0.0001)] and at 5-7 weeks post-surgery [CD8\(^+\)CD25\(^+\)FoxP3\(^+\) Treg cells (P=0.0051), CD8\(^+\)CD127\(^+\)FoxP3\(^+\) Treg cells (P=0.0051)] (Figure 7.5 and Table 7.2). The median prevalence of CD8\(^+\)CD25\(^+\)FoxP3\(^+\) Treg cells was significantly higher than
Chapter 7: Effect of antitumor treatment

7.2 Results

CD8\(^+\)CD127\(^+\)FoxP3\(^+\) Treg cells at baseline (P=0.025), at day 1 post-treatment (P=0.05) and 5-7 weeks post-treatment (P=0.02) (Figure 7.5).

Breast cancer patients who were eligible for chemotherapy displayed a higher median percentage of CD4\(^+\)CD25\(^+\)CD127\(^+\) Treg cells (3.49, IQR: 0.8-7.37) as compared to CD4\(^+\)CD127\(^+\)FoxP3\(^+\) (0.06, IQR: 0-0.11) and CD4\(^+\)CD25\(^+\)FoxP3\(^+\) (0.205, IQR: 0.13-0.31), at baseline, at day 1 post-treatment [(CD4\(^+\)CD25\(^+\)CD127\(^+\) Treg cells: 2.68 IQR 2.18-5.92), (CD4\(^+\)CD127\(^+\)FoxP3\(^+\); 0.01 IQR 0-0.02), (CD4\(^+\)CD25\(^+\)FoxP3; 0.49 IQR 0.05-1.76)] and at 5-7 weeks post-treatment [(CD4\(^+\)CD25\(^+\)CD127\(^+\) Treg cells: 0.5), (CD4\(^+\)CD127\(^+\)FoxP3\(^+\)): 0), (CD4\(^+\)CD25\(^+\)FoxP3: 0.01)] (Figure 7.5).

Similarly, the median percentage of CD8\(^+\)CD25\(^+\)CD127\(^+\) Treg cells was higher than CD8\(^+\)CD25\(^+\)FoxP3\(^+\) Treg cells and CD8\(^+\)CD127\(^+\)FoxP3\(^+\) Treg cells and CD8\(^+\)CD25\(^+\)FoxP3\(^+\) Treg cells at baseline, at day 1 post-treatment and 5-7 weeks post-treatment (Figure 7.5). Statistical analysis could not be performed due to the small sample size.

Surgical patients demonstrated a statistically significant decline in the median prevalence of CD8\(^+\)CD25\(^+\)CD127\(^+\) Treg cell from baseline levels to 5-7 weeks post-treatment (P=0.012), while chemotherapy patients demonstrated a downward trend in the circulating prevalence of CD4\(^+\)CD25\(^+\)CD127\(^+\) Treg cell at 5-7 weeks post-treatment in comparison to baseline levels; however, this did not reach significance (P=0.097) (Figure 7.5). Although the remaining Treg cell subsets showed a declining trend from baseline to 5-7 weeks post-treatment, this also did not reach significance (Figure 7.5).
Chemotherapy patients were shown to have a higher prevalence of circulating CD8⁺CD25⁺FoxP3⁺ (0.054) and CD4⁺CD25⁺FoxP3⁺ (P=0.0157) Treg cells at baseline, in comparison to surgical patients (Figure 7.5). Although, chemotherapy patients appeared to display higher median CD4⁺ and CD8⁺ Treg levels at day 1 post-treatment, and lower CD4⁺ and CD8⁺ Treg levels at 5-7 weeks post-treatment, this could not be confirmed statistically due to the small sample size.

Interestingly, despite differences observed in the median percentages of CD4⁺ and CD8⁺ Treg cell population in surgical and chemotherapy patients, these two groups were found to have similar circulating concentrations of Treg cell counts at the different time points, irrespective of the treatment prescribed.
Figure 7.5: The frequency distribution of CD4$^+$ and CD8$^+$ Treg cell subsets in breast cancer patients receiving chemotherapeutic and surgical intervention, before treatment (brown bar), at day 1 after treatment (green bar) and 5-7 weeks post-treatment (blue bars). This Figure represents the median % ± IQR of T-lymphocyte subsets detected at different time points. CD4$^+$CD25$^+$CD127$^+$ and CD8$^+$CD25$^+$CD127$^+$ Treg cells were the most abundant Treg populations at all time intervals, in both surgical and chemotherapy patients. (NS= Not statistically significant, *= statistically significant: *P<0.05, **P<0.01, ***P<0.001)
7.2.2.3 Effect of surgical and chemotherapeutic treatment on the CD4⁺ Treg: total CD4⁺ T-cell ratio and the CD8⁺ Treg: total CD8⁺ T-cell ratio in patients presenting with breast cancer

- **CD4⁺Treg: total CD4⁺ T-cell ratio**

Patients who received chemotherapeutic treatment showed an upward trend in the CD4⁺Treg: total CD4⁺T-cell ratio with a higher ratio found at the 5-7 weeks follow-up as compared to baseline levels (Figure 7.6). Interestingly, surgical patients demonstrated the opposite effect with a decreasing trend in the CD4⁺(%) : total CD4⁺ T-cell ratio over time, and a lower CD4⁺(%) : total CD4⁺ T-cell ratio being found at 5-7 weeks follow-up as compared to that observed at baseline (Figure 7.6). These findings however did not reach statistical significance. Although, in comparison to surgical patients, patients receiving chemotherapy displayed higher CD4⁺(%) : total CD4⁺ T-cell ratios, this did not reach significance (before treatment: P=0.89, day 1 post-treatment =0.31; 5-7 week post-treatment).

- **CD8⁺Treg: total CD8⁺ T-cell ratio**

Chemotherapy patients showed a non-significant variation (P=0.47) in the CD8⁺ Treg: total CD8⁺ T-cell ratio during the follow-up period, with a decline in CD8⁺ Treg: total CD8⁺ T-cell ratio observed at day 1 post-treatment, which was followed by an increase in CD8⁺ Treg: total CD8⁺ T-cell ratio at 5-7 weeks post-treatment (Figure 7.7).
Surgical patients also displayed a non-significant variation in the CD8\(^+\) Treg: total CD8\(^+\) T-cell ratio during the follow-up period. A decline was observed in the CD8\(^+\) Treg: total CD8\(^+\) T-cell ratio at day 1 post-treatment (P=0.27) and at 5-7 weeks post-treatment (P=0.14).

In comparison to surgical patients, chemotherapy patients were found to have an increased CD8\(^+\) Treg: total CD8\(^+\) T-cell ratio before treatment (P=0.94) and at 5-7 weeks post-treatment (P=0.75) (Figure 7.7). However, surgical patients were found to have a higher CD8\(^+\) Treg: total CD8\(^+\) T-cell ratio at day 1 post-treatment (P=0.57), as compared to patients receiving chemotherapy (Figure 7.7). This was not statistically significant.

- **CD4\(^+\): CD8\(^+\) T-cell ratio**

Surgical patients were found to have an upward trend in the CD4\(^+\): CD8\(^+\) T-cell ratio over the follow-up period with patients having a higher CD4\(^+\): CD8\(^+\) T-cell ratio at day 1 post-treatment (2; IQR: 1.5-3.4) and at 5-7 weeks post-treatment (3.2, IQR: 1.7-3.5), in comparison to baseline levels (2.2, IQR: 1.4-3.5). However, this trend was not significant (Table 7.2)

However, chemotherapy patients showed a decreasing trend on the CD4\(^+\): CD8\(^+\) T-cell ratio over the follow-up period with a lower, CD4\(^+\): CD8\(^+\) T-cell ratio at 5-7 weeks post-treatment (0.7) as compared to day 1 post-treatment (2, IQR: 1-3) and 5-7 weeks post-treatment (2, IQR: 1.4-2.1) (Table 7.2).
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Figure: 7.6: The frequency distribution of CD4⁺ Treg (%): total CD4⁺ (%) T-cell ratio in breast cancer patients receiving chemotherapeutic (green bars) and surgical (blue bars) intervention, before treatment, at day 1 after treatment and 5-7 weeks post-treatment. This Figure represents the median % ± IQR of T-lymphocyte subsets detected at different time points. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
Figure 7.7: The frequency distribution of CD8⁺ Treg (%): total CD8⁺ (%) ratio in breast cancer patients receiving chemotherapy (blue bars) and surgical (red bars) intervention, before treatment, at day 1 after treatment and 5-7 weeks post-treatment. This Figure represents the median ± IQR of T-lymphocyte subsets detected at different time points. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
7.2.3 Oesophageal cancer patients

In order to evaluate the effect of antitumor treatment on the T-lymphocyte profile in patients presenting with oesophageal cancer, the frequency of circulating T-lymphocyte subsets before treatment, at day 1 post-treatment and at 5-7 weeks post-treatment were quantified. Due to the small sample size and high number of mortalities and loss to follow-up in this group, all results were analysed together irrespective of the type of treatment received.

7.2.3.1 Detection of CD4$^+$ and CD8$^+$ non-Treg T-lymphocyte population in oesophageal cancer patient before treatment, at day 1 post-treatment and at 5-7 weeks post-treatment

In comparison to baseline levels, a decline was observed in the median percentage of the majority of CD4$^+$ and CD8$^+$ non-Treg cell populations (Figure 7.8), with CD8$^+$CD25$^+$ T-lymphocyte being the only T-cell population to show an increase at day 1 post-treatment (Figure 7.8). A significant variation in the median percentage of circulating CD4$^+$CD25$^-$ (P=0.0072) and CD8$^+$CD25$^-$ (P=0.031) T-lymphocytes were observed between baseline, day 1 post-treatment and 5-7 weeks post-treatment. Pairwise comparison revealed a significant decline in the median CD4$^+$CD25$^-$ (P=0.011), and CD8$^+$CD25$^-$ (P=0.04) at day 1 post-treatment, in comparison to levels observed before treatment (Figure 7.8). Substantial variation was also observed in the median percentage of CD8$^+$CD25$^+$ (P=0.062), but this did not reach significance. Only 1 patient was present for follow-up at 5-7 weeks post-treatment.
The CD4⁺CD25⁺ T-lymphocytes were significantly higher than the median CD4⁺CD25⁻ T-lymphocyte percentage before treatment (P=0.005); however, due to the small sample size at day 1 post-treatment (0.067) and 5-7 weeks post-treatment (P=0.31), a statistically significant difference was not observed (Figure 7.8).

Similarly, in comparison to the median percentage of CD8⁺CD25⁻ T-lymphocytes, CD8⁺CD25⁺ T-lymphocytes were significantly higher before treatment (P=0.046); however, significance was lost at day 1 post-treatment (P=0.067) and 5-7 weeks post-treatment (P=0.31) (Figure 7.8).

The decline in the CD4⁺ T-lymphocytes were also associated with a decline in the total CD4⁺ T-lymphocyte concentration from 241 cells/µl (IQR: 210-416) to 112 cells/µl (IQR: 54-153) at day 1 post-treatment and 106 cells/µl at 5-7 weeks post-treatment (Table 7.3). Furthermore, the CD8⁺ T-lymphocyte concentration also declined from 125 cells/µl to 65 cells/µl at day one post-treatment, however a mild recovery was observed at 5-7 weeks post-treatment (86 cells/µl) (Table 7.3).
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Figure 7.8: The frequency distribution of CD4$^+$ and CD8$^+$ T-lymphocyte subsets in oesophageal cancer patients before treatment (red bar), at day 1 after treatment (yellow bar) and 5-7 weeks post-treatment (blue bars). This figure represents the median % ± IQR of T-lymphocyte subsets detected at different time points. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)

Oesophageal cancer: immune profile in response to treatment
CD4$^+$ and CD8$^+$ T-cell population

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Table 7.3: The CD4⁺ and CD8⁺ T-cell counts (cells/µl) in oesophageal cancer patients before treatment, at day 1 post-treatment and 5-7 weeks post-treatment

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Median ±IQR</th>
<th>Time point 1</th>
<th>Time point 2</th>
<th>Time point 3</th>
<th>Statistical significance (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells/µl</td>
<td></td>
<td>Before treatment</td>
<td>Day 1 post-treatment</td>
<td>5-7 weeks post-treatment</td>
<td></td>
</tr>
<tr>
<td>Total CD4⁺ count</td>
<td>241 (210-416)</td>
<td>112 (54-153)</td>
<td>106 (106)</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>Total CD8⁺ count</td>
<td>125 (74-180)</td>
<td>65 (38-96)</td>
<td>86 (86)</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>CD4:CD8 ratio</td>
<td>1.8 (1.2-2.5)</td>
<td>1.2 (0.5-2.4)</td>
<td>1.2 (1.2)</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>CD4⁺CD25⁺CD127⁺</td>
<td>11 (7-22)</td>
<td>2 (0-4)</td>
<td>29 (29)</td>
<td>0.301</td>
<td></td>
</tr>
<tr>
<td>CD4⁺CD127⁺FoxP3⁺</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.705</td>
<td></td>
</tr>
<tr>
<td>CD4⁺CD25⁺FoxP3⁺</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>CD8⁺CD25⁺CD127⁺</td>
<td>26 (18-35)</td>
<td>12 (6-22)</td>
<td>72 (72)</td>
<td>0.071</td>
<td></td>
</tr>
<tr>
<td>CD8⁺CD25⁺FoxP3⁺</td>
<td>0 (0)</td>
<td>0 (0-5)</td>
<td>9 (9)</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>CD8⁺CD127⁺FoxP3⁺</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.26</td>
<td></td>
</tr>
</tbody>
</table>

Statistically significant difference between time point 1 and 2 =§ (P=0.05)
Statistically significant difference between time point 1 and 3=¶ (P=0.045)
Statistically significant difference in variation (P>0.05) between time point 1, 2 and 3*
(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
7.2.3.2 Detection of CD4$^+$ and CD8$^+$ Treg T-lymphocyte population in oesophageal cancer patients before treatment, at day 1 post-treatment and at 5-7 weeks post-treatment

Oesophageal cancer patients presented with a variety of CD4$^+$ and CD8$^+$ Treg cell subsets at the respective time points (Figure 7.9). The majority of CD4$^+$ and CD8$^+$ Treg cells showed an increasing trend from baseline to 5-7 week follow-up, with a significant variation in the trend observed in the CD8$^+$CD25$^+$CD127$^+$ (P=0.0026), CD8$^+$CD127$^+$FoxP3$^+$ (P=0.0139) and CD4$^+$CD25$^+$CD127$^+$ (P=0.025) Treg cell populations (Figure 7.9). Pairwise comparison revealed a significant increase in the median percentage of CD8$^+$CD127$^+$FoxP3$^+$ Treg at 5-7 weeks after treatment, in comparison to baseline levels (P=0.0443) and day 1 post-treatment (P=0.0455) (Figure 7.9).

A decrease in the CD4$^+$CD25$^+$CD127$^+$ Treg cell concentration (cells/µl) and CD8$^+$CD25$^+$CD127$^+$ was also observed at day 1 post-treatment (Table 7.3), however this was followed by a substantial increase in both cell subsets at 5-7 weeks post-treatment (Table 7.3). Furthermore, although CD8$^+$CD25$^+$FoxP3$^+$ Treg cells were undetectable before treatment and at day 1 post-treatment, an increase was observed at 5-7 weeks post-treatment (9 cells/µl) (Table 7.3).
Figure 7.9: The frequency distribution of CD4$^+$ and CD8$^+$ Treg subsets in oesophageal cancer patients before treatment (brown bar), at day 1 after treatment (green bar) and 5-7 weeks post-treatment (blue bars). This Figure represents the median % ± IQR of Treg subsets detected at different time points.

(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
The median percentage and cell concentration (cells/µl) of CD8^+CD25^+CD127^+ Treg cell subset was significantly elevated as compared to the median percentage of CD8^+CD25^+FoxP3^+ Treg cells before treatment (0.0051), however significance was lost at day 1 post-treatment (P=0.067), and at 5-7 weeks post-treatment (P=0.31) (Figure 7.9 and Table 7.3). Similarly, the median CD8^+CD25^+CD127^+ Treg percentage and circulating concentration (cells/µl) was significantly higher than CD8^+CD127^+FoxP3^+ Treg percentage before treatment, but non-significantly higher at day 1 post-treatment (P=0.069) and at 5-7 weeks post-treatment (P=0.31) (Figure 7.9 and Table 7.3). The median percentage of CD8^+CD25^+FoxP3^+ Treg cell was significantly higher than CD8^+CD127^+FoxP3^+ Treg cell (0.027) before treatment, with a non-significant difference observed at day 1 post-treatment (P=0.0679) and at 5-7 weeks post-treatment (P=0.317).

The median percentage and cell concentration of CD4^+CD25^+CD127^+ Treg cells was significantly higher than the median percentage of CD4^+CD25^+FoxP3^+ Treg cells (P=0.0069) and CD4^+CD127^+FoxP3^+ Treg cells (P=0.0059) before treatment. Furthermore, the median percentage of CD4^+CD25^+FoxP3^+ Treg cells was significantly higher than the median percentage of CD4^+CD127^+FoxP3^+ Treg cells (P=0.0076) before treatment, however statistical significance did not persist at day 1 post-treatment (P=0.0947) or at 5-7 weeks post-treatment (P=0.317).
7.2.3.3 Effect of antitumor treatment on the $CD4^+\text{Treg}$: total $CD4^+$ T-cell ratio and the $CD8^+\text{Treg}$: total $CD8^+$ T-cell ratio in patients presenting with oesophageal cancer

- **$CD4^+\text{Treg}$: total $CD4^+$ T-cell ratio**

Oesophageal cancer patients displayed a statistically significant variation in the CD4$^+$Treg:total CD4$^+$ T-cell ratio between the time points under investigation ($P=0.049$). Due to the small sample size, pairwise analysis was not significant. The CD4$^+$Treg:total CD4$^+$ T-cell ratio at 5-7 weeks post-treatment was substantially higher than at baseline and at day 1 after treatment.

- **$CD8^+\text{Treg}$: total $CD8^+$ T-cell ratio**

The analysis of variance between the different time points suggested that the variation in the CD8$^+$Treg:total CD8$^+$ T-cell ratio was significant ($P=0.0003$). Pairwise analysis was not significant, however the CD8$^+$Treg:total CD8$^+$ T-cell ratio was substantially higher at 5-7 weeks post-treatment as compared to day 1 post-treatment and before treatment.

- **CD4$^+$: CD8$^+$ T-cell ratio**

Oesophageal cancer patients demonstrated a declining CD4$^+$: CD8$^+$ T-cell ratio from baseline levels (1.8; IQR, 1.2-2.5) to day 1 post treatment (1.2; IQR 0.5-2.4) (Table 7.3). This ratio was maintained at 5-7 weeks post-treatment (Table 7.3).
Figure: 7.10: The frequency distribution of $\text{CD}^4^+$ Treg (%): total $\text{CD}^4^+$ (%) ratio and $\text{CD}^8^+$ Treg (%): total $\text{CD}^8^+$ (%) ratio in oesophageal cancer patients receiving intervention, before treatment, at day 1 after treatment and 5-7 weeks post-treatment. This Figure represents the median % ± IQR of T-lymphocyte subsets detected at different time points. A significant variation was observed in the frequency of $\text{CD}^4^+$ Treg (%): total $\text{CD}^4^+$ (%) ratio ($P=0.049$), and $\text{CD}^8^+$ Treg (%): total $\text{CD}^8^+$ (%) ratio ($P=0.0003$) during the follow-up period. Pairwise comparison did not yield a significant result. (NS= Not statistically significant, * = statistically significant: *$P<0.05$, **$P<0.01$, ***$P<0.001$)
7.3 Discussion

CD4$^+$ and CD8$^+$ T-lymphocyte populations have been shown to play a major role in the instigation, maintenance and regulation of antitumor immunity, with alterations in the distribution and ratios of these T-cell subsets serving as an indicator of immune impairment and disease progression. Surgery, chemotherapy and radiotherapy are the most common antitumor treatment modalities currently in use in this setting, and this study aimed to investigate the effect which antitumor treatment has on the T-lymphocyte immune profile. Furthermore, this study set out to determine whether antitumor treatment induces a shift in the T-cell immune profile towards one which promotes tumor progression and attenuates antitumor immunity (increased Treg cells: decreased non-Treg cells), or one which augments antitumor immunity and facilitates tumor eradication (decreased Treg cells: increased non-Treg cells).

Previous studies have demonstrated the effect of antitumor treatments on circulating T-lymphocyte subsets (Campbell et al. 1973, Colachio et al. 1994, De-en et al. 1998, Goldfarb et al. 2006, Gottschalk et al. 2012, Chu-yuan et al. 2013), however, to the best of my knowledge, this is the first report which assesses the influence of antitumor treatment on the circulating T-lymphocyte and T regulatory cell profile in South African patients presenting with breast and oesophageal cancer. Furthermore, this analysis was conducted on whole blood, as compared to a large proportion of previous studies where Ficoll-derived peripheral blood mononuclear cells were used (Verma et al 2013.) This ensures a better reflection of the circulating Treg and non-Treg immune status, as minimal manipulation of the participants’ blood did not occur prior to analysis (Sellito et al. 2011).
When assessed together, anticancer treatment (surgery, chemotherapy and radiotherapy) was found to induce a non-significant decline in the CD4$^+$ and CD8$^+$ non-Treg and Treg cell profile (%) in breast and oesophageal cancer patients. This was associated with an increasing trend in the total CD4$^+$ T-cell concentration and a decreasing trend in the total CD8$^+$ T-cell concentration over the follow-up period. Consequently, breast and oesophageal cancer patients demonstrated an upward trend in the CD4$^+$:CD8$^+$ T-cell ratio during the follow-up period, which suggests that total CD4$^+$ T-cells may be more resistant to the effects of antitumor treatment, in comparison to the total CD8$^+$ T-cells.

Dederichsen and colleagues (2003) reported a significant inverse relationship between the intratumoral CD4$^+$:CD8$^+$ T-cell ratio and survival in patients presenting with colorectal cancer. The implications of this ratio in the peripheral blood has not yet been determined; however, a higher CD4$^+$:CD8$^+$ T-cell ratio in the peripheral blood suggests a weaker CD8$^+$ effector arm of the antitumor immune system, which should in turn be associated with a poorer antitumor immunity. CD8$^+$ T-lymphocytes are directly involved in cancer eradication (Akhmetzyanova et al. 2013). Furthermore, CD4$^+$ T-cells have not been shown to fully compensate for the effector functions of CD8$^+$ T-cells in the absence of CD8$^+$ T-lymphocytes, thus a decline in the CD8$^+$ T-cell subset as a consequence of antitumor treatment may result in a compromised antitumor immunity, and prevent tumor eradication (Akhmetzyanova et al. 2013).

The overall treatment effect showed a declining trend in both the CD4$^+$Treg:total CD4$^+$ T-cell ratio and the CD8$^+$Treg:total CD8$^+$ T-cell ratio, which suggests that despite the fact that antitumor treatment induces a decline in both the Treg and non-Treg components of the immune system, Treg cells are more sensitive to the effects of anticancer treatment, resulting
in a higher relative decline during the follow up period as compared to non-Treg cells. This finding confirms reports by Schmidt and colleagues (2000), who found that the percentage of CD4^+ Treg cells in colorectal and breast cancer patients declined distinctly following radiochemotherapy, while the percentage of CD4^+ T-lymphocytes remained relatively constant. This finding suggests that the overall effect of anticancer treatment results in an improved antitumor immune profile.

Campbell and colleagues (1973) suggested that all antitumor treatment induces a state of lymphopenia, however in the cohort of patients, described in this study it was demonstrated that the T-cell response to anticancer treatment is heterogeneous and varies depending on the type of malignancy, the clinical stage of disease and the type of antitumor treatment that the patient receives.

Breast cancer patients who received surgical intervention demonstrated a significant variation in the total CD4^+ and CD4^+CD25^+ T-lymphocyte percentages over the follow-up period. The total CD8^+ T-lymphocyte percentage was seen to decline more sharply (-5.62%) in comparison to total CD4^+ T-lymphocyte (-1.32%). This was associated with a decline in the total CD8^+ T-cell count, while the total CD4^+ T-cell count increased almost 2 fold over the follow-up period. This finding contradicts those observed by Lissoni and colleagues (2009), who found a significant decline in the total CD4^+ T-cell count in the immediate post-operative period. This difference could be linked to the fact that malignancies under investigation by Lissoni and colleagues were gastrointestinal cancers, as opposed to breast cancer. Furthermore, this difference could also be because immune analysis by Lissoni and colleagues was performed at day 5 post-treatment, while analysis in our cohort occurred at day 1 post-treatment.
Interestingly, a decline in the total CD8$^+$ T-lymphocyte percentage and concentration was observed during the follow-up period with a resultant increase trend in the CD4$^+$:CD8$^+$ T-cell ratio. This suggests that CD8$^+$ T-lymphocytes may be more vulnerable to the effects of surgical treatment than CD4$^+$ T-lymphocytes. This finding motivates for previous reports, which suggested that surgical patients are vulnerable to metastatic implantation of disseminated tumors and enhanced tumor growth during the immediate post operative period (Colachio et al. 1994).

Chemotherapy was initially associated with an increase in the majority of non-Treg cell subsets (%) at day 1 post-treatment, even exceeding levels observed in the surgery group. This was then followed by a substantial decline in both the percentage and count of non-Treg and Treg cell subsets at 5-7 weeks post-treatment, far below levels observed in surgical patients. Similar to findings by Rech and colleagues, breast cancer patients displayed a declining trend in the total CD4$^+$ count, with a CD4$^+$ T-cell count less than 200 cells/µl observed at 5-7 weeks post chemotherapy treatment. This translated to a substantial decline in the CD4$^+$:CD8$^+$ T-cell ratio from 2 to 0.7 at 5-7 weeks post-treatment, suggesting a substantially compromised antitumor immune profile. This finding illustrates the severe lymphopenia induced by chemotherapy, which has been identified as an independent prognostic factor in patients presenting with colorectal cancer (Chu-yuan et al. 2013). Furthermore, this finding re-emphasizes the importance of assessing an oncology patients’ immune profile before the commencement of treatment (Chu-yuan et al. 2013).
As surgery and chemotherapy are generally used in conjunction during the management of breast cancer, it can be assumed that the dual effect of these anticancer modalities on the T-cell immune profile may compromise the patients’ immune status to a point that renders them susceptible to opportunistic infections, as seen in patients presenting with Acquired Immune Deficiency Syndrome (AIDS). Therefore, mechanisms need to be employed to optimise oncology patients before treatment and eliminate alternative causes of lymphopenia e.g malnutrition, prior to commencement of treatment (Fock et al. 2010). Secondly, prophylactic treatment against opportunistic infection, similar to those observed in other immune compromised states, should be considered when patients are receiving both surgical and chemotherapeutic treatment for cancer.

Surgical patients demonstrated a substantial decline in the circulating percentage and cell concentration of all Treg cells following exposure to surgical treatment, with the decline in the circulating CD8+CD25+CD127+ and CD4+CD25+FoxP3+ Treg cells subsets reaching significance. This confirms findings by Tokuno and colleagues (2009), Kono and colleagues (2006), Sellito and colleagues (2011), who found that surgical resection of the tumor results in a significant decline in Treg cells. Some studies have even reported a decline to levels observed in healthy volunteers if all macroscopic tumor was removed (Tokuno et al. 2009, Sellito et al. 2011). This decline could be due to redistribution of Treg cells from the peripheral blood to the site of surgical inflammation (Saito et al. 2013), or it could be that the tumor contains a large induced and expanded Treg pool, which is removed during surgical resection (Tokuno et al. 2009).

All patients who were recipients of chemotherapeutic treatment received a cyclophosphamide-base treatment. This has been shown to inhibit the generation and function
of CD4\(^+\) and CD8\(^+\) Treg cells, and induce apoptosis in circulating Treg cells (Traverso et al. 2012). Similar to previous reports (Traverso et al. 2012, Rech et al. 2010), the use of cyclophosphamide-based chemotherapy in this cohort was associated with a substantial decline in the circulating CD4\(^+\) and CD8\(^+\) Treg cell subsets at day 1 post-treatment and at 5-7 weeks post-treatment. Furthermore, although patients received cyclophosphamide in combination with adriamycin 5FU and methotrexate, the latter two treatments have been shown to have a limited effect on the circulating levels of Treg cells (Calasan et al. 2012). In comparison to surgical treatment, chemotherapy induced a more substantial decline in all Treg cell subsets, with surgical patients demonstrating higher Treg levels (%) at 5-7 weeks post-treatment. This could be because chemotherapy has been shown to not only induce apoptosis of Treg cells, but also inhibits FoxP3\(^+\) expression at transcriptional level (Xu et al. 2011). As FoxP3\(^+\) has been shown as a key regulator in the function of Treg cells, the decrease expression of mRNA inhibits the proliferation of Treg cells in the peripheral blood with a consequent decline in the circulating Treg cell population in the blood (Xu et al. 2011).

Interestingly, despite differences observed in the circulating Treg cell percentages, the Treg cell concentrations was similar in patients who received surgical or chemotherapeutic treatment (Table 7.2). This verifies the findings by Rech and colleagues who reported no difference in the CD4\(^+\)FoxP3\(^+\) Treg count in early or progressive breast cancer patients who had received surgical or chemotherapeutic anticancer treatment (Rech et al. 2000). Furthermore, they found that the patients presenting with breast cancer displayed similar levels of circulating Treg concentrations to healthy controls (Rech et al. 2000). This may be the result of homeostatic control mechanisms governing the concentration of Treg cell subsets within the peripheral compartment irrespective of the stage of disease or the treatment status (Rech et al. 2010, Parker et al. 2012).
Interestingly, the CD4+ Treg: total CD4+ T-cell ratio in surgical patients decreased during the follow-up period while an upward trend was observed in chemotherapy patients. Furthermore, the CD8+ Treg: total CD8+ T-cell ratio was seen to decline at day 1 post-treatment in both surgical and chemotherapy patients. This trend was maintained in surgical patients with a further decline at 5-7 weeks follow-up; however, chemotherapy patients showed an increasing trend from day 1 post-treatment to 5-7 weeks post-treatment. This suggests that CD4+ and CD8+ Treg cells are more susceptible to the effects of surgical intervention, as compared to non-Treg cells. Furthermore, it also suggests that although chemotherapy induces a severe lymphopenia in all T-cell subsets, total CD4+ T-cells are more susceptible to this effect as compared to CD4+ Treg cells, thus resulting in an overall increase in the CD4+ Treg: total CD4+ T-cell ratio. This finding contradicts reports by Lutsiak and colleagues (2005) who found that CD4+ Treg cells are more susceptible to apoptosis following antitumor treatment with chemotherapy, as compared to non-Treg cell subsets. This difference may be because Lutsiak and colleagues (2005) reported findings from in vitro and in vivo experiments using mouse models. This finding suggests that the surgical removal of the tumor burden is associated with an increase in the relative proportion of antitumor effector CD4+ and CD8+ T-cells, which would translate to a decline in malignancy and Treg mediated immune suppression and improved antitumor response.

The decline observed in the CD8+ Treg: total CD8+ T-cell ratio at day 1 post chemotherapy, and subsequent increase in this ratio at 5-7 weeks post-treatment suggests that CD8+ Treg cells are transiently susceptible to the effects of chemotherapy treatment and their recovery rate post-treatment occurs earlier and at a faster pace than in the total CD8+ T-cell population. This confirms findings by Lutsiak and colleagues (2005) who demonstrate that
cyclophosphamide induces a temporary inhibitory effect on Treg cell populations in the immediate post-treatment period, however Treg cells regain their suppressive function at 10 days post-treatment.

This however does not explain why a similar pattern is not observed in the CD4$^+$ Treg population, suggesting that CD4$^+$ and CD8$^+$ Treg cell populations may have intrinsic differences which results in different responses to the same antitumor treatment.

The increase in the CD4$^+$ Treg: total CD4$^+$ T-cell ratio and CD8$^+$Treg: total CD8$^+$ T-cell ratio following chemotherapy treatment, may direct the immune system towards a more immunosuppressive milieu, which would inhibit active antitumor effector cells as well as, facilitate the conversion of naïve T-lymphocytes to Treg cells thus resulting in the further loss of immune surveillance, tumor progression and even cancer relapse following treatment (Kono et al. 2006).

Due to the late stage of presentation, the majority of patients presenting with oesophageal cancer received palliative radiotherapy with only 20% of patients receiving surgical intervention. A decline was observed in the percentage and count of all CD4$^+$ non-Treg cell subsets; however, despite substantial fluctuation in the different CD8$^+$ T-cells subsets during the follow-up period, a decline was observed in in CD8$^+$ non-Treg T-cell percentage and concentration during the follow-up period. As this downward trend was observed in this cohort despite the majority receiving palliative treatment, it serves as a testament to the aggressive nature of oesophageal cancer and the consequent immune suppression associated with this condition.

Oesophageal cancer patients demonstrated a decline at day 1 post-treatment, which was then followed by an increase in Treg cells at 5-7 weeks post-treatment. Kono and colleagues
(2006) reported that, following the surgically induced decline in Treg cells, a rebound increase in the Treg cell level was observed in patients who presented with disease relapse (Kono et al. 2006). Furthermore, Sellito and colleagues (2011) found that patients who had received palliative surgical excision, and still had remnant cancer or metastatic spread still displayed higher Treg levels than those observed in healthy control patients. (Sellito et al. 2011) This again suggests an association between the tumor burden and circulating Treg levels, with the tumor cells possibly contributing to the underlying mechanism involved in the maintenance and regulation of Treg cells (Tokuno et al. 2009, Sellito et al. 2011). This suggests that Treg cells may potentially be useful as a marker of treatment-response and disease relapse.

Interestingly, an increase was observed in the median percentage of all CD4$^+$ and CD8$^+$ Treg cells over the follow-up period, peaking at 5-7 weeks post-treatment. Curiel and colleagues (2004) demonstrated that CD4$^+$CD25$^+$FoxP3$^+$ Treg cells accumulated during tumor progression in patients presented with ovarian cancer, and that these Treg cells suppressed antitumor immunity, and promoted tumor growth (Curiel et al. 2004). In addition to increasing CD4$^+$CD25$^+$FoxP3$^+$ Treg cells, oesophageal cancer patients in our cohort demonstrated increasing levels of CD8$^+$CD25$^+$FoxP3$^+$ Treg cells, CD4$^+$CD127$^+$FoxP3$^+$ Treg, CD8$^+$CD127$^+$FoxP3$^+$ Treg, CD4$^+$CD25$^+$CD127$^+$ Treg and CD8$^+$CD25$^+$CD127$^+$ Treg cells. Breast cancer patients who received chemotherapy or surgical intervention, with curative intent, displayed a decrease in Treg cells over time, while oesophageal cancer patients, the majority (80%) of which received palliative radiotherapy, showed an increasing trend in the Treg profile. This may suggest that either Treg cells may not be susceptible to radiotherapeutic intervention, or the low dose employed during palliative treatment, rather than curative radiotherapy, is insufficient to reduce the circulating levels of Treg cells. However,
the increasing Treg levels in oesophageal cancer patients could also be interpreted as marker of tumor progression, as well as a concomitant predictive marker of response to antitumor treatment.

This increase in Treg cells juxtaposed to the decreasing non-Treg cell profile translated to an increase in the CD4⁺ Treg: total CD4⁺ T-cell ratio and CD8⁺ Treg: total CD8⁺ T-cell ratio during the follow-up period which suggests a declining antitumor immune response and loss of immune surveillance.

Interestingly, breast cancer patients receiving chemotherapy and oesophageal cancer patients displayed substantially higher levels of FoxP3⁺ Treg cells (%) in comparison to breast cancer patients who received surgical intervention. Oesophageal cancer patients displayed higher levels of CD8⁺CD25⁺FoxP3⁺ Treg cells (%), as compared to all breast cancer patients at all time points assessed, while chemotherapy patients displayed the highest percentage of CD4⁺CD25⁺FoxP3⁺ Treg cells at all times assessed. Surgical patients expressed very little FoxP3⁺ Treg cells. As patients with oesophageal cancer and those requiring chemotherapy treatment presented at a more advanced stage of disease as compared to patients who received surgical intervention, these findings suggest that the frequency of FoxP3⁺ expressing Treg cells may be associated with the clinical stage of disease at presentation. This corroborates with findings by Sellito and colleagues (2011) who found that higher peripheral Treg levels were associated with advanced stage of colorectal cancer and the presence of distant metastases. Furthermore, Tokuna and colleagues (2009) also reported a significant increase in the frequency of CD4⁺FoxP3⁺ Treg cells in patients with stage 4 gastrointestinal cancer, including oesophageal cancer, as compared to patients with stage I, II and III gastrointestinal cancer or healthy controls. This may suggest that although, as previously
reported, an increased frequency of Treg cells is observed in patients presenting with malignancy, late stage of disease may be associated with an increased frequency of Treg cells expressing FoxP3 receptor.

Although this study demonstrated the alterations which occur in the peripheral system in response to anticancer treatment, this may not be a true reflection of the physiology of non-Treg and Treg cells at the tumor microenvironment. Thus future studies would aim to analyse the T-lymphocyte levels in both the peripheral blood and tumor microenvironment, thus revealing the true
8 Treatment outcome and association with T-lymphocyte immune profile

8.1 Introduction

Predictive and prognostic markers in cancer play an important role in the decision making process during the management of patients. Despite the identification and use of a number of prognostic and predictive factors in breast and oesophageal cancer, the search for a feasible and more reliable marker is still ongoing. Following studies that demonstrated the crucial contribution that the host immune system makes towards tumor eradication, research efforts have been directed towards the identification of immunological markers which can be used in clinical practice. This marker would facilitate the identification of patients who would benefit from treatment and those at high risk of mortality, as well as to monitor the progress of patients receiving treatment.

Numerous studies have evaluated the relationship between CD4\(^+\), CD8\(^+\) and Treg T-lymphocyte distribution and patient outcome, however this has yielded conflicting results (Alvaro et al. 2005, Bates et al. 2006, Ladoire et al. 2008, Aruga et al. 2009, Salama et al. 2009, Haas et al. 2009, Correale et al. 2010). This study aimed to determine the relationship between T-lymphocyte subsets before and after treatment compared to patient outcome at 5-7 week follow-up. The outcomes of interest included survival or demise before the 5-7 week follow-up period. Statistical analysis was conducted using the unpaired non-parametric Mann-Whitney statistic with a two-sided P-value. A P-value of <0.05 was considered as statistically significant.
8.2 Results

8.2.1 Detection of CD4\(^+\) and CD8\(^+\) T-lymphocyte populations in patients who survived compared to those who demised during the 5-7 week follow-up period

Patients who demised were found to have substantially lower CD4\(^+\) T-lymphocyte percentage (Figure 8.1) and cell concentration (cells/µl) (Table 8.1) before treatment and at day 1 post-treatment, as compared to patients who survived; with a significant difference observed in the total CD4\(^+\) T-cell percentage (P=0.034) before treatment, as well as the CD4\(^+\)CD25\(^-\) T-cell percentage (P=0.038) and total CD4\(^+\) T-lymphocyte concentration (P=0.0046) at day 1 post-treatment (Figure 8.1 and Table 8.1). Furthermore, in comparison to patients who survived, patients who demised where also found to have a higher circulating percentage of CD8\(^+\) non-Treg T-lymphocyte subsets, with a significant difference observed in the median percentage of CD8\(^-\)CD45\(^+\) T-lymphocyte (P=0.01) and CD8\(^-\)CD25\(^-\) T-lymphocytes (P=0.013) (Figure 8.1). This however did not translate to a higher CD8\(^+\) T-cell concentration, with patients who demised found to have a lower CD8\(^+\) T-lymphocyte concentration before receiving anti-tumor treatment (190 cells/µl versus. 140 cells/µl; P=0.79) and at day 1 post-treatment (160 cells/µl versus. 88 cells/µl; P=0.058) (Table 8.1). This translated to a CD4\(^+\): CD8\(^+\) T-cell ratio of 1.2 in patients who demised compared to 2.2 in patients who survived, this was statistically significant (P=0.044) (Table 8.1). The CD4\(^+\): CD8\(^+\) T-cell ratio was seen to improve in patients who survived at day1 after treatment (CD4\(^+\): CD8\(^+\) T-cell ratio = 2.5), however this ratio remained stable in patients who demised (CD4\(^+\): CD8\(^+\) T-cell ratio = 1.2) (Table 8.1).
Table 8.1: The total CD4\(^+\) T-lymphocyte count, total CD8\(^+\) T-lymphocyte count, and CD4\(^+\):CD8\(^+\) T-cell ratio in breast and oesophageal cancer patients who survived and demised following antitumor treatment.

<table>
<thead>
<tr>
<th></th>
<th>Survived</th>
<th>Demised</th>
<th>Statistical significance (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total CD4(^+) count (cells/µl)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>345 (IQR: 172-558)</td>
<td>205 (IQR: 147-316)</td>
<td>0.137</td>
</tr>
<tr>
<td>After treatment</td>
<td>390 (IQR: 230-472)</td>
<td>111 (IQR: 101-153)</td>
<td>0.0046**</td>
</tr>
<tr>
<td><strong>Total CD8(^+) count (cells/µl)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>190 (IQR: 61-253)</td>
<td>140 (IQR: 79-252)</td>
<td>0.790</td>
</tr>
<tr>
<td>After treatment</td>
<td>160 (IQR: 91-214)</td>
<td>88 (IQR: 64-112)</td>
<td>0.058*</td>
</tr>
<tr>
<td><strong>CD4(^+):CD8(^+) T-cell ratio</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>2.2 (IQR: 1.5-3.5)</td>
<td>1.2 (IQR: 1.1-1.9)</td>
<td>0.044*</td>
</tr>
<tr>
<td>Day 1 after treatment</td>
<td>2.5 (IQR: 1.6-3.4)</td>
<td>1.2 (IQR: 0.9-2.4)</td>
<td>0.186</td>
</tr>
</tbody>
</table>

(NS= Not statistically significant, * = statistically significant; \(*P<0.05, **P<0.01, ***P<0.001\)
Breast and oesophageal cancer  
Association of immune profile to patient outcome

Figure 8.1: The frequency distribution of CD4$^+$ and CD8$^+$ T-lymphocyte subsets of breast and oesophageal cancer in patients who survived (red bar) and demised (red bar), before treatment and at day 1 post-treatment. This is shown as the median percentage (%) ±IQR. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
8.2.2 Detection of CD4$^+$ and CD8$^+$ Treg cell populations in patients who survived compared to those who demised during the 5-7 week follow-up period

Patients who survived displayed a similar Treg percentage and count as compared to patients who demised. However, a significantly higher prevalence of CD8$^-$CD25$^+$CD127$^+$ Treg cells (%) was observed before treatment in patients who survived, as opposed to patients who demised (P=0.019) (Figure 8.2). Furthermore, the CD8$^-$CD25$^+$CD127$^+$ Treg concentration in patients who survived was higher as compared to those who demised before treatment and at day 1 post-treatment, however this did not reach significance (Table 8.2). This translated to a significantly higher CD8$^+$ Treg: total CD8$^+$ T-cell ratio (P=0.0126), before treatment, in patients who survived compared to patients who demised (Table 8.2 and Table 8.3).
**Table 8.2:** The CD4$^+$ and CD8$^+$ Treg cell count (cells/µl) in breast and oesophageal cancer patients who survived and demised, before receiving antitumor treatment and at day 1 post-treatment.

<table>
<thead>
<tr>
<th></th>
<th>Survived</th>
<th>Demised</th>
<th>Statistical significance (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD4$^+$ Treg cells (cells/µl)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4$^+$CD25$^-$FoxP3$^+$</td>
<td>0 (IQR: 0-0)</td>
<td>0 (IQR: 0-0)</td>
<td>0.89</td>
</tr>
<tr>
<td>CD4$^+$CD127$^+$FoxP3$^+$</td>
<td>0 (IQR: 0-0)</td>
<td>0 (IQR: 0-0)</td>
<td>0.311</td>
</tr>
<tr>
<td>CD4$^+$CD25$^+$CD127$^+$</td>
<td>10 (IQR: 2.8-21.9)</td>
<td>9 (IQR: 4-11)</td>
<td>0.473</td>
</tr>
<tr>
<td><strong>CD8$^+$ Treg cells (cells/µl)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8$^+$CD25$^-$FoxP3$^+$</td>
<td>0 (IQR: 0-0.3)</td>
<td>0 (IQR: 0-0)</td>
<td>0.6</td>
</tr>
<tr>
<td>CD8$^+$CD127$^+$FoxP3$^+$</td>
<td>0 (IQR: 0-0)</td>
<td>0 (IQR: 0-3)</td>
<td>0.362</td>
</tr>
<tr>
<td>CD8$^+$CD25$^+$CD127$^+$</td>
<td>46 (IQR: 18-72)</td>
<td>21 (IQR: 11-38)</td>
<td>0.137</td>
</tr>
<tr>
<td><strong>Day 1 after treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD4$^+$ Treg cells (cells/µl)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4$^+$CD25$^-$FoxP3$^+$</td>
<td>0 (IQR: 0-1)</td>
<td>0 (IQR: 0-0)</td>
<td>0.395</td>
</tr>
<tr>
<td>CD4$^+$CD127$^+$FoxP3$^+$</td>
<td>0 (IQR: 0-0)</td>
<td>0 (IQR: 0-0)</td>
<td>0.629</td>
</tr>
<tr>
<td>CD4$^+$CD25$^+$CD127$^+$</td>
<td>9 (IQR: 4-17)</td>
<td>2 (IQR: 2-4)</td>
<td>0.047*</td>
</tr>
<tr>
<td><strong>CD8$^+$ Treg cells (cells/µl)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8$^+$CD25$^-$FoxP3$^+$</td>
<td>0 (IQR: 0-0)</td>
<td>0 (IQR: 0-4)</td>
<td>0.113</td>
</tr>
<tr>
<td>CD8$^+$CD127$^+$FoxP3$^+$</td>
<td>0 (IQR: 0-0)</td>
<td>0 (IQR: 0-0)</td>
<td>0.942</td>
</tr>
<tr>
<td>CD8$^+$CD25$^+$CD127$^+$</td>
<td>27 (IQR: 16-42)</td>
<td>15 (IQR: 12-22)</td>
<td>0.237</td>
</tr>
</tbody>
</table>

(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
Table 8.3: The CD4$^+$ Treg (%): total CD4$^+$ (%) and CD8$^+$ Treg (%) total CD8$^+$ (%) T-cell ratios in breast and oesophageal cancer patients who survived and demised, before treatment and at day 1 after treatment.

<table>
<thead>
<tr>
<th>CD4$^+$ Treg: total CD4$^+$ T-cell ratio and CD8$^+$ Treg: total CD8$^+$ T-cell ratio in breast and oesophageal cancer patients</th>
<th>Survived</th>
<th>Demised</th>
<th>Statistical significance (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4$^+$ Treg: total CD4$^+$ T-cell ratio</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>0.06 (IQR: 0.02-0.11)</td>
<td>0.08 (IQR: 0.03-0.13)</td>
<td>0.7101</td>
</tr>
<tr>
<td>Day 1 after treatment</td>
<td>0.04 (IQR: 0.03-0.11)</td>
<td>0.06 (IQR: 0.04-0.73)</td>
<td>0.924</td>
</tr>
<tr>
<td><strong>CD8$^+$ Treg: total CD8$^+$ T-cell ratio</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>1.48 (0.69-1.98)</td>
<td>0.53 (0.26-0.91)</td>
<td>0.012**</td>
</tr>
<tr>
<td>Day 1 after treatment</td>
<td>0.84 (IQR: 0.53-1.33)</td>
<td>0.87 (IQR: 0.58-1.00)</td>
<td>0.7768</td>
</tr>
</tbody>
</table>

(NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
Figure 8.2: The frequency distribution of CD4$^+$ and CD8$^+$ Treg cell subsets in breast and oesophageal cancer patients who survived (red bar) and demised (blue bar), before treatment and at day 1 post-treatment. This is shown as the median percentage (%) $\pm$IQR. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
8.2.3 Detection of CD4$^+$ and CD8$^+$ Treg cell populations in patients who survived compared to those who demised during the 5-7 week follow-up period, stratified according to tumor type

8.2.3.1 CD4$^+$ Treg cell population

Patients presenting with oesophageal cancer displayed a higher frequency of FoxP3-expressing Treg cells as compared to patients presenting with breast cancer (Figure 8.3). Due to the small sample size in each cancer type, statistical analysis could not be conducted.

8.2.3.2 CD8$^+$ Treg cell population

Patients presenting with breast and oesophageal cancer displayed similar levels of CD8$^+$ FoxP3-expressing and non-FoxP3 Treg cells (Figure 8.4). Due to the small sample size in each cancer type, statistical analysis could not be conducted.
Figure 8.3: The frequency distribution of CD4\(^+\) Treg cell subsets in breast and oesophageal cancer patients who survived (red bar) and demised (blue bar), before treatment and at day 1 post-treatment. This is shown as the median percentage (%) ±IQR. (NS= Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
8.2: Discussion

Breast cancer patients

CD8^+ Treg cell

Oesophageal cancer patients

CD8^+ Treg cell

Figure 8.4: The frequency distribution of CD8^+ Treg cell subsets in breast and oesophageal cancer patients who survived (red bar) and demised (blue bar), before treatment and at day 1 post-treatment. This is shown as the median percentage (%) ±IQR. (NS = Not statistically significant, * = statistically significant: *P<0.05, **P<0.01, ***P<0.001)
8.3 Discussion

CD4⁺, CD8⁺ and Treg T-lymphocytes have all been implicated as possible predictors of prognosis, and for monitoring of treatment response. Numerous studies have reported that the increased levels of tumor infiltrating and circulating CD4⁺ and CD8⁺ T-lymphocytes correlate with a good prognosis in the context of cancer (Ayoub et al 1998, Zingg et al. 2010), while increased levels of Treg cells in the peripheral blood and tumor microenvironment has been associated with a poor prognosis. (Curiel et al. 2004, Bates et al. 2006, Wolf et al. 2007).

In this cohort, it was demonstrated that patients who demised presented with a lower prevalence of CD4⁺ T-lymphocyte percentage and concentration, which supports the findings by Ray-Coquad and colleagues (2009) who reported that CD4⁺ lymphopenia is associated with poor outcome. Furthermore, patients who survived displayed a significantly higher CD8⁺ T-lymphocyte count at all time points, as compared to patients who demised. This confirms findings by Blake-more and colleagues (2010), who reported that a higher CD8⁺ T-lymphocyte count was associated with longer survival in patients presenting with breast cancer, independent of the effects of medical treatment (Blake-more et al. 2010).

A CD4⁺: CD8⁺ T-cell ratio of 2.1 was found in patients who survived, compared to a ratio of 1.2 in those who demised. This verifies findings by Diederichsen and colleagues (2003) who demonstrated that patients suffering from colorectal cancer, with a good clinical outcome and higher 5-year survival displayed a CD4⁺:CD8⁺ T-lymphocyte ratio of > 2. However, they
assessed this ratio in tumor infiltrating lymphocytes, while this study assessed this ratio in the peripheral blood.

Despite numerous reports suggesting that a higher Treg cell level in the peripheral blood is associated with a poor prognosis (Kono et al. 2006), this was not observed in this cohort. On the contrary, patients who survived displayed higher levels of CD8<sup>+</sup>CD25<sup>−</sup>CD127<sup>+</sup> Treg cell percentage and cell concentration before treatment and at day 1 post-treatment, which translated to a significantly higher CD8<sup>+</sup>Treg: total CD8<sup>+</sup> T-cell ratio, as compared to patients who demised. Furthermore, the CD4<sup>+</sup>CD25<sup>−</sup>CD127<sup>+</sup> Treg cell count in patients who survived was significantly higher than that observed in patients who demised. This suggests that FoxP3-negative Treg cells may be associated with improved outcome, which contradicts findings by Liu and colleagues (2008) who reported a poor prognosis in cancer patients who displayed a high percentage of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells.

Interestingly, patients who survived displayed an absence of FoxP3-positive Treg cells, while patients who demised, most notably patients presenting with oesophageal cancer, displayed a higher prevalence of FoxP3<sup>+</sup> Treg cell. This correlates with findings by Yamamato and colleagues (2012), who reported that pancreatic cancer patients presenting with a low circulating percentage of FoxP3<sup>+</sup> Treg cells displayed a longer period of survival as compared to patients with higher levels of FoxP3<sup>+</sup> Treg cells. However, FoxP3-negative T-lymphocytes were not evaluated by Yamamato and colleagues (2012).

When patients were stratified according to the type of cancer, oesophageal cancer patients displayed higher levels of circulating FoxP3<sup>+</sup> Treg cells, as compared to breast cancer patients who survived and demised. This suggests that the increase in FoxP3<sup>+</sup> expression may be tumor specific, which contradicts other studies which have identified FoxP3<sup>+</sup> Treg cells in
peripheral circulatory system and within the tumor microenvironment in patients presenting with breast cancer (Ladoire et al. 2008, Perez et al. 2007). The expression of FoxP3+ could also be as a result of the aggressive nature of the malignancy, the late stage of presentation of all oesophageal cancer patients, the severe immune deficiency as a consequence of both the nutritional depletion and the progressive malignancy. The absence of oesophageal patients who survived until 5-7 weeks follow-up prevents further investigation of this point. Future studies, with larger sample sizes would enable us to investigate which factors in oesophageal cancer patients contribute to the induction of FoxP3+ expression. The clear differences observed between FoxP3+ and FoxP3− Treg cells in this cohort, implies that therapeutic treatments aimed at the eradication of all Treg cells in oncology patients may have beneficial effects, but may also result in the depletion of T-cell subsets which are involved either directly or indirectly in the improvement of patient outcome. This is a contentious issue considering reports that the effect which Treg cells confers their effect in the host is heterogeneous and may be organ or tumor specific (Zingg et al. 2010). More efforts need to be employed to further discern the role of these cells in malignancy, the clinical implications of Fox-P3 expression versus non-FoxP3 Treg cells and how best to manipulate these cells so as to promote improved patient outcome.
9 Conclusion

Within the context of cancer, the immune system has been shown to have two distinct arms which play opposing roles. These include immune cells which facilitate and augment tumor growth and progression, while the other component attenuates tumor progression and eradicates existing malignant cells. The conundrum in the management of malignancy lies in the question of how best to up-regulate the antitumor arm of the immune system and simultaneously attenuate the pro-tumor component. The pivotal regulatory role that the immune system plays in the development and progression of cancer suggests that elements of this complex system may vary depending on the type, size, location and aggressiveness of a tumor. This implies that changes to the tumor as a consequence of antitumor treatment, would also result in a change in the patients’ immune status. Taken together this suggests that the patients’ immune status may be the predictive and prognostic marker which the world has been searching for. Despite the limitations of this study, there is strong evidence to suggest that the immune profile of a cancer patient should be taken into consideration when deciding on the mode and aggressiveness of treatment, as well as when estimating patient survival.
10 References


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