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THE MOLECULAR BASIS OF HUMAN DISEASE

Master Thesis

Definition and Clinical Implication of Human FoxP3$^+$ CD4$^+$ Treg cell subsets in Non-Small Cell Lung Cancer Patients

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Περίληψη

Τα Τ ρυθμιστικά λεμφοκύτταρα (Tregs) παίζουν ένα σημαντικό ρόλο στην τροποποίηση της ανοσιακής απάντησης, σε ασθενείς με καρκίνο. Ο πληθυσμός των Τ ρυθμιστικών λεμφοκυττάρων παρουσιάζει υψηλό βαθμό ετερογένειας. Ως εκ τούτου, πολλοί βιοδείκτες επιτρέπουν τον προσδιορισμό φαινοτυπικά διακριτών υποσυνόλων του πληθυσμού των Tregs, που παρουσιάζουν διαφορετικά λειτουργικά χαρακτηριστικά. Στόχος αυτής της μελέτης ήταν ο προσδιορισμός των φαινοτυπικών και λειτουργικών χαρακτηριστικών των διαφορετικών υποτύπων των Tregs στην κυκλοφορία του αίματος, σε ασθενείς με μη μικροκυτταρικό καρκίνο του πνεύμονα (ΜΜΚΠ), και η μελέτη της επίδρασης της χημειοθεραπείας στη συχνότητά τους.

Φλεβικό περιφερικό αίμα συλλέχθηκε από 145 ασθενείς με ΜΜΚΠ, πριν την έναρξη της χημειοθεραπείας πρώτης γραμμής, καθώς επίσης και μετά τον 3ο και τον 6ο κύκλο. Στην μελέτη εντάχθηκαν, επίσης, 31 φυσιολογικοί αιμοδότες ίδιας ηλικίας. Η συχνότητα των υποτύπων των κυκλοφορούντων Tregs στο ολικό αίμα εξετάστηκε με κυτταρομετρία ροής. Η κατασταλτική ικανότητα αυτών των κυττάρων ελέγχθηκε μετά από την απομόνωσή τους από το σύνολο των μονοπύρηνων κυττάρων του περιφερικού αίματος, με χρήση μαγνητικών σφαιριδίων.

Η συχνότητα εμφάνισης των $CD3^+CD4^+CD25^+$ Τ ρυθμιστικών λεμφοκυττάρων στο αίμα ασθενών με ΜΜΚΠ ήταν στατιστικά υψηλότερη, σε σχέση με αυτή που παρατηρήθηκε σε υγιείς δότες, και τα κύτταρα αυτά παρουσίασαν ισχυρή κατασταλτική δράση. Το ποσοστό των Terminal Memory Effector Tregs ($CCR7^-CD25^{high}CD127^-CD152^+FOXP3^+CD45RO^+$) ήταν, επίσης, σημαντικά υψηλότερο σε ασθενείς με ΜΜΚΠ, στην έναρξη της μελέτης, σε σύγκριση με τους υγιείς δότες, ενώ υψηλό ποσοστό τους συσχετίστηκε με βελτιωμένη προγνωστική αξία. Επιπροσθέτως, η χημειοθεραπεία είχε ως αποτέλεσμα μια στατιστικά σημαντική αύξηση στα επίπεδα των Terminal effector ($CD25^{high}CD127^-CD152^+FOXP3^+CD45RO^+$) και Terminal Memory Effector Tregs ($CCR7^-CD25^{+high}CD127^-CD152^+FOXP3^+CD45RO^+$), κάτι το οποίο συσχετίστηκε με καλύτερη ανταπόκριση στη θεραπεία. Αντίθετα, το ποσοστό των Naïve Tregs ($CCR7^+CD25^{high}CD127^{-/low}CD152^-FOXP3^{+low}CD45RO^-$) ήταν σημαντικά αυξημένο στους ασθενείς με ΜΜΚΠ, σε σύγκριση με τους υγιείς δότες, γεγονός που συσχετίστηκε με κακή προγνωστική αξία. Επιπλέον, τα επίπεδα των Naïve Tregs αυξήθηκαν ως συνέπεια της επίδρασης της χημειοθεραπείας, ενώ η αύξηση αυτή συσχετίστηκε με κακή κλινική έκβαση.

Εν κατακλείδι, η παρούσα εργασία κατέδειξε σαφώς, για πρώτη φορά, τις δύο αντίθετες όψεις των $CD4^+$ Τ ρυθμιστικών λεμφοκυττάρων όσων αφορά την κλινική έκβαση, ανάλογα με την κατάσταση ωρίμανσης τους. Η μείωση ή ο αποκλεισμός της μετανάστευσης τους στις καρκινικές περιοχές μπορεί να αποτελέσει μια αποτελεσματική στρατηγική.

Abstract

Regulatory T cells (Tregs) play an important role in the modification of immune responses in malignancy. Treg population presents a high degree of heterogeneity, therefore several markers allow the identification of phenotypically distinct Treg subsets which present different functional characteristics. However, little is known the different subtypes of the regulatory T cells (Tregs) in the peripheral blood of non-small cell lung cancer (NSCLC) patients. The aims of this study were to determine the phenotypic and functional characteristics of different circulating Treg subtypes in the peripheral blood in NSCLC patients and the significant effect of therapy on the frequency of the circulating Tregs subset was also investigated.

Peripheral blood was collected from 145 treatment-naïve NSCLC patients and 31 aged-matched heathy donors, as well as the 3rd and the 6th cycle of treatment. The presence of circulating Treg subsets was examined by studying the presence of intra- and extracellular markers in whole blood using flow cytometry. The functionality of these cells was tested after their isolation from PBMCs, using magnetic separation.

The results showed that the frequencies of $CD3^{+}CD4^{+}CD25^{+high}$ Tregs in NSCLC patients were significantly higher than in normal controls exhibiting a highly suppressive activity. The percentage of the Terminal Memory Effector Treg cells ($CCR7^{-}$ $CD25^{+high}CD127^{-}CD152^{+}FOXP3^{+}CD45RO^{+}$) was significantly higher in baseline, in NSCLC patients, compared to normal control. Furthermore, higher percentage of Terminal Memory Effector Treg cells was associated with improved prognostic value. Moreover, the levels of Terminal effector ($CD25^{+high}CD127^{-}CD152^{+}FOXP3^{+}CD45RO^{+}$) and Terminal Memory Effector Treg cells ($CCR7^{-}CD25^{+high}CD127^{-}CD152^{+}FOXP3^{+}CD45RO^{+}$) increased after the chemotherapy treatment which was associated with an improved prognosis. In contrast, percentage of the Naïve Treg cells ($CCR7^{+}$ $CD25^{+high}CD127^{-/low}CD152^{-}FOXP3^{+low}CD45RO^{-}$) was significantly higher in baseline, in NSCLC patients, compared to normal control, which was associated with poor prognostic value. Moreover, the levels of Naïve Treg cells increased after the chemotherapy treatment and were correlated with poor clinical outcome.

In conclusion, this thesis has clearly demonstrated, for the first time, two opposite faces of $CD4^{+}$ Treg cells in respect to clinical outcome, depending on their maturation status. The use of depletion or blocking their migration in the tumour sites may be an effective strategy.

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List of Abbreviations

AP	**:**	Alkaline Phosphatase
APCs	**:**	Antigen-Presenting Cells
ATP	**:**	Adenosine Triphosphate
BCR	**:**	B-cell Receptor
BSA	**:**	Bovine Serum Albumin
CCL21	**:**	CC Chemokine Ligand
CCR7CC	**:**	Chemokine Receptor 7
CDA	**:**	Cytidine Deaminase
CDKIs	**:**	Cyclin-Dependent Kinases Inhibitors
CDKs	**:**	Cyclin-Dependent Kinases
CI	**:**	Confidence Intervals
CIS	**:**	Carcinoma in Situ
COX-2	**:**	Cyclooxygenase-2
CSF-1	**:**	Colony-Stimulating Factor 1
CTL	**:**	Cytotoxic T Lymphocyte
CTLA-4	**:**	Cytotoxic T Lymphocyte–Associated Antigen 4
dCK	**:**	Deoxycytidine Kinase
DCs	**:**	Dendritic Cells
dFdCMP	**:**	dFdC-5'-Monophosphate
EGF	**:**	Epidermal Growth Factor
EIA	**:**	Enzyme Immunoassay
ELISA	**:**	Enzyme-Linked Immunosorbent Assay
EMT	**:**	Epithelial to Mesenchymal Transition
E-NTPDase	**:**	Ecto-Nucleoside Triphosphate Diphosphohy-Drolase
Fc R	**:**	Fc Receptors
FCS	**:**	Fetal Calf Serum
FGF1	**:**	Fibroblast Growth Factors
FSC	**:**	Forward Scatter
G-CSF	**:**	Granulocyte Colony-Stimulating Factor
GM-CSF	**:**	Granulocyte-Macrophage Colony-Stimulating Factor
hCNT-1	**:**	Human Concentrative Nucleoside Transporter-1

hCNT-3	**:**	Human Concentrative Nucleoside Transporter-3
hENT1	**:**	Human Equilibrative Nucleoside Transporter-1
HLA	**:**	Human Leukocyte Antigen
HR	**:**	Hazard Ratios
HRP	**:**	Horseradish Peroxidase
IFN	**:**	Interferon
IFN-γ	**:**	Interferon-γ
IL-10	**:**	Interleukin-10
IL-2R	**:**	Receptor of Interleukin-2
IL-8	**:**	Interleukin-8
iNOS	**:**	Onducible Nitric Oxide Synthase
iTregs	**:**	Induced Tregs
LTi	**:**	Lymphoid Tissue Inducer
mAb	**:**	Monoclonal Antibody
MDSCs	**:**	Myeloid-Derived Suppressor Cells
MRP	**:**	Myeloid-Related Protein
NF-AT	**:**	Nuclear Factor of Activated T Cells
NF-κB	**:**	Nuclear Factor-κB
NK	**:**	Natural Killer cells
NLR	**:**	Neutrophil to Lymphocyte Ratio
NSCLC	**:**	Non- Small Cell Lung Cancer
nTreg	**:**	Naïve Treg
PB	**:**	Peripheral Blood
PBMCs	**:**	Peripheral Blood Mononuclear Cells
PBS	**:**	Phosphate Buffered Saline
PD	**:**	Progressive Disease
PGE2	**:**	Prostaglandin E2
PR	**:**	Partial Resonse
RBC	**:**	Red Blood Cell
RR	**:**	Ribonucleotide Reductase
SCLC	**:**	Small Cell Lung Cancer
SD	**:**	Stable Disease
SSC	**:**	Sideward Scatter

TAM	**:**	Tumour-Associated Macrophages
TAN	**:**	Tumour-Associated Neutrophils
Tc	**:**	Cytotoxic
TCR	**:**	T-cell Receptor
TF	**:**	Transcription Factor
TGFα	**:**	Tumour Growth Factor α)
TGFβ	**:**	Transforming Growth Factor beta
Th	**:**	T Helper
TIL	**:**	Tumour Infiltrating Lymphocytes
TIM-3	**:**	T cell Immunoglobulin-3
TKIs	**:**	Tyrosine Kinase Inhibitors
TNF-a	**:**	Tumour Necrosis Factor
TNM	**:**	Tumour Node Metastasis
Tregs	**:**	Regulatory T Cells
Ts	**:**	T Suppressor
VEGF	**:**	Vascular Endothelial Growth Factor
VEGFRs	**:**	VEGF Receptors

Chapter 1

Introduction

1.1 CANCER

Cancer is a generic term for a large group of diseases that develop across time and can affect any part of the body. Cancer is usually viewed as an evolutionary process that results from the accumulation of somatic mutations in the progeny of a normal cell, leading to a selective growth advantage in the mutated cells and ultimately to uncontrolled proliferation (Greaves & Maley , 2012; Merlo et al., 2006). Although cancer can develop in virtually any of the body's tissues, and each type of cancer has its unique features, the basic processes that produce cancer are quite similar in all forms of the disease. The most frequent human cancers arise in epithelial tissues such as the skin, colon, breast, prostate or lung, and collectively lead to several million deaths per year (Ferlay et al., 2010).

Cancer is a multifactorial disease. There are many factors involved and, as a result, there is no single cause for any one type of cancer. Cancer incidence rates increase with age and with exposure to carcinogens (Falandry et al., 2014; Vineis & Wild , 2008). The genetic makeup also increases the frequency of cancer presence. Familial cancers are caused by variants in multiple genes. Each genetic variant causes a slight increase in risk (Weber et al., 2001). The overall risk of developing cancer depends on the number of cancer risk genetic variants that a person inherits and the environmental factors that interact with those genes. Hereditary cancers are cancers that are associated with a change in a single cancer susceptibility gene (like BRCA1 or BRCA2) (Fostira et al., 2007). Moreover, the presence of a chronic inflammation or immunosuppression is also contributes to the development of cancer. Many cancer cases are also linked to lifestyle factors such as smoking, alcohol, diet, or being overweight (Vineis & Wild , 2008). Viruses can also cause genetic changes that increase the risk of cancer development (HPV infection) (White et al., 2014).

Cancer is an abnormal growth of cells caused by multiple changes in gene expression leading to deregulated balance of cell proliferation and cell death and evolving into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity and death of the host (Ruddon , 2007). It begins when a single cell with an altered DNA sequence (mutation) evade from the normal restraints on cell division and begins an uncontrolled proliferation (Merlo et al., 2006). All of the cells produced by division of this first ancestral cell also display inappropriate proliferation. Uncontrolled proliferation of these abnormal cells is

followed by a second mutation leading to the mildly aberrant stage (Greaves & Maley , 2012). Successive rounds of mutation and selective expansion of these cells results in the formation of a tumour mass, which remain within the tissue in which it originated or begin to invade nearby tissues (Ruddon , 2007).

Tumorigenesis in humans is a multistep process and these steps reflect genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives. The essential alterations in cell physiology that dictate malignant growth are: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (figure 1.1) (Hanahan & Weinberg , 2000).

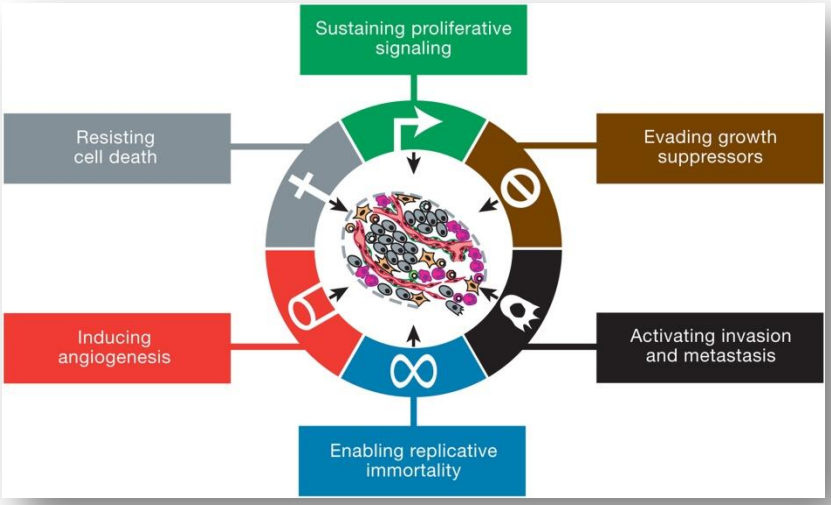

Figure 1.1 The Hallmarks of Cancer. This illustration encompasses the six essential alterations proposed in cell physiology that dictate malignant growth (Hanahan & Weinberg , 2000)

Underlying these characteristics are genome instability and inflammation, which fosters multiple hallmark functions (figure 1.2) (Hanahan & Weinberg , 2011). New studies added two new hallmarks to this list—reprogramming of energy metabolism and evading immune destruction. All these hallmarks contribute to the creation of the tumour microenvironment (Hanahan & Weinberg , 2011).

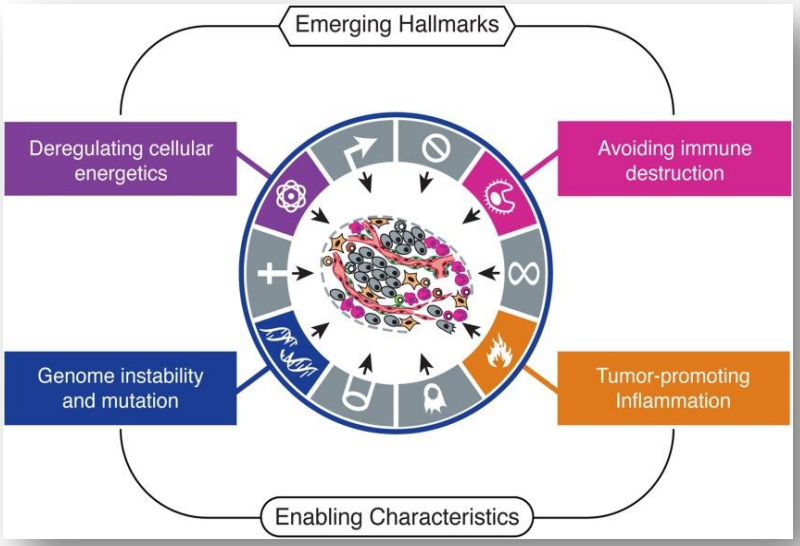

Figure 1.2: Emerging Hallmarks and Enabling Characteristics. Four additional hallmarks of cancer were proposed to be involved in the pathogenesis of cancers. The first involves the capability to modify, or reprogram, cellular metabolism in order to most effectively support neoplastic proliferation. The second allows cancer cells to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells. These two hallmarks are characterized as Emerging Hallmarks. Additionally, there were proposed two Enabling Characteristics. Genomic instability and thus mutability endow cancer cells with genetic alterations that drive tumour progression. Inflammation designed to fight infections can result in tumour-promoting consequences of inflammatory responses (Hanahan & Weinberg , 2011).

Mitogenic growth signals are required in order a normal cell to move into an active proliferative state. Some of the proto-oncogenes act as mimics of normal growth signals (Hanahan & Weinberg , 2011). As a result, tumour cells generate many of their own growth signals, thereby reducing their dependence on stimulation from their normal tissue microenvironment (Amit et al., 2007; Fedi et al., 1999; Witsch , 2010). This event disrupts the homeostatic mechanism that normally operates to ensure a proper behaviour of the various cell types within a tissue (Witsch , 2010). The production of TGFα (tumour growth factor α) by sarcomas is an example of growth signal that produced autologous of the cancer cells (Fedi et al., 1999).

Within a normal tissue, cellular quiescence and tissue homeostasis is maintained by the operation of multiple anti-proliferative signals. Soluble growth inhibitors and immobilized inhibitors both embedded in the extracellular matrix and on the surfaces of nearby cells

(Hanahan & Weinberg , 2011). An example is TGFβ which suppresses expression of the c-myc gene, which regulates the G1 cell cycle machinery (Moses et al., 1990). Lose of TGFβ responsiveness through downregulation of Transforming growth factor beta (TGFβ) receptors or by downstream alterations that disable the tumour-suppressive arm of this pathway in cancer cells results in the production of autocrine mitogens or the releasing of pro-metastatic cytokines (Fynan & Reiss , 1993).

Except from the rate of cell proliferation, the rate of cell attrition also contributes to the ability of tumour cell populations to expand in number. Apoptosis, the programmed cell death, represents a major source of this attrition (Alberts et al., 2002). Acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer. Two classes of components, sensors and effectors, take part in the apoptotic machinery (Ding & Liu , 2007). The sensors are responsible for monitoring the extracellular and intracellular environment for conditions of normality or abnormality that influence whether a cell should live or die (Ding & Liu , 2007). These signals regulate the second class of components, which function as effectors of apoptotic death (Ding & Liu , 2007). Resistance to apoptosis can be acquired by cancer cells through a variety of strategies. Surely, the most commonly occurring loss of a pro-apoptotic regulator through mutation involves the p53 tumour suppressor gene (Norbury & Zhivotovsky , 2004). The resulting functional inactivation of its product is seen in greater than 50% of human cancers and results in the removal of a key component of the DNA damage sensor that can induce the apoptotic effector cascade (Norbury & Zhivotovsky , 2004; Sherr & McCormick , 2002; Sigal & Rotter , 2000).

Normal cells have a finite replicative potential. After a certain number of doublings, normal cells stop growing, a process termed senescence (Kastan & Bartek , 2004). The senescence reflects a protective mechanism that can be activated by shortened telomeres or conflicting growth signals that forces aberrant cells irreversibly into a G_0-like state, thereby rendering them incapable of further proliferation (Lim & Campisi , 2001). Circumvention of senescence, due to lose of telomerase activity, may represent an essential step in tumour progression that is required for the subsequent approach to and breaching of the crisis barrier (Blagosklonny , 2001; Kastan & Bartek , 2004).

The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival (Hoeben et al., 2004). Neoplasias develop angiogenic ability in order to progress in a larger size. Positive and negative signals promote or block angiogenesis (Adams & Alitalo , 2007). One class of these signals is conveyed by soluble factors and their receptors (Adams & Alitalo , 2007). The angiogenesis-initiating signals are exemplified by vascular endothelial growth factor (VEGF) (Byrne et al., 2005) and acidic and basic fibroblast growth factors (FGF1/2) (Compagni et al., 2000). Each binds to transmembrane tyrosine kinase receptors displayed by endothelial cells (Compagni et al., 2000; Neufeld et al., 1999). During tumour development, the ability to induce and sustain angiogenesis is acquired via an "angiogenic switch" from vascular quiescence (Hanahan & Weinberg , 2000). Tumours appear to activate the angiogenic switch by changing the balance of angiogenesis inducers and countervailing inhibitors (Hanahan & Folkman , 1996). One common strategy for shifting the balance involves altered gene transcription. Many tumours evidence increased expression of VEGF and/or FGFs compared to their normal tissue counterparts.

The capability for invasion and metastasis enables cancer cells to escape the primary tumour mass and colonize new terrain in the body where nutrients and space are not limiting (Coghlin & Murray , 2010). The newly formed metastases arise as amalgams of cancer cells and normal supporting cells conscripted from the host tissue (Fidler , 2003). The activation of extracellular proteases and the altered binding specificities of cadherins, CAMs, and integrins are central to the acquisition of invasiveness and metastatic ability (Coghlin & Murray , 2010; Fidler , 2003).

Defects in genome maintenance and repair are selectively advantageous and therefore instrumental for tumour progression because they accelerate the rate at which evolving premalignant cells can accumulate favourable genotypes (Negrini et al., 2010). Advances in the molecular-genetic analysis of cancer cell genomes have provided demonstrations of function-altering mutations and of genomic instability during tumour progression (Campbell et al., 2010; Chin et al., 2004). Altered energy metabolism is also a hallmark of cancer cells (Hanahan & Weinberg , 2011). Cancer cells can reprogram their glucose metabolism, and thus their energy production, by limiting their energy metabolism largely to glycolysis, leading to a state that has been termed aerobic glycolysis (Jones & Thompson , 2009). Aerobic glycolysis is programmed by proliferation-inducing oncogenes (Dang , 2012).

Moreover, tumour promotes the inflammation. Tumour-associated inflammatory response helps incipient neoplasias to acquire hallmark capabilities (Hanahan & Weinberg , 2011). Inflammation can contribute to multiple hallmark capabilities by supplying bioactive molecules to the tumour microenvironment, including growth factors that sustain proliferative signalling, survival factors that limit cell death, pro-angiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis (Landskron et al., 2014). In addition, cancer cells may evade immune destruction by disabling components of the immune system that have been dispatched to eliminate them (Gajewski et al., 2013). For example, secretion of TGF-β or other immunosuppressive factors by cancer cells paralyze the infiltrated CTLs and NK cells (Wilson et al., 2011). The recruitment of inflammatory cells that are immunosuppressive, including regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) also take part in the suppression of the cytotoxic lymphocytes.

1.2 CANCER IMMUNOLOGY

It has been proposed that a natural function of the immune system is to confront and destroy malignantly transforming cells continuously produced in the body, as a result of the plethora of spontaneous mutations taking place during one's lifetime. However, in cases where tumours have emerged, the immune system has apparently failed due to immune tolerance or escape.

Tumour cells produce danger signals and express tumour antigens that are recognized as foreign, resulting in the induction of immune responses (Kim et al., 2007). It has been reported that the levels of tumour antigen-specific immune responses, such as FRα, HER-2/neu, CEA, and NY-ESO-1, are elevated in cancer patients compared to normal healthy individuals (Beckhove et al., 2004; Disis et al., 2000; Li et al., 1996; Rentzsch et al., 2003; Sabharanjak & Mayor , 2004). Tumour antigen specific IgG responses have also been observed in cancer patients and in some cases are associated with improved survival (Goodell et al., 2006). The findings of both humoral and T cell immunity to tumour antigens might indicate that the immune system is just as capable of eliciting a coordinated immune response to tumours as it is to a foreign antigen.

Moreover, immune cells are attracted by tumours very early the course of disease. In many cases, T cell infiltration correlates with disease outcomes. Patients with high levels of T cells (CD4 or CD8) infiltrating into the tumours have an improved outcome relative to those with

lower levels or without detectable infiltrating T cells (Pages et al., 2005; Sato et al., 2005; Zhang et al., 2003). As a result, infiltrating T cells are associated with improved survival in cancer patients (Pages et al., 2005). Moreover, immune effectors may protect against disease progression, since the lack of immune infiltration is associated with the development of metastases (Pages et al., 2005).

The relationship between cancer and the immune system is explained by the term "cancer immunoediting" (figure 1.3). In its most complex form, cancer immunoediting occurs in three sequential phases: elimination, equilibrium, and escape (Dunn et al., 2004; Schreiber et al., 2011). Elimination, the initial step, is a modern view of the older notion of cancer immunosurveillance, in which innate and adaptive immunity work together to detect and destroy transformed cells long before they become clinically apparent (Dunn et al., 2004; Schreiber et al., 2011; Vesely & Schreiber , 2013). However, sometimes, tumour cell variants may not be completely eliminated but instead enter into an equilibrium phase in which the immune system controls net tumour cell outgrowth. During this step, the cancer cells that begin to prevail are those that have developed the ability to deviate from immune attack (equilibrium) (Dunn et al., 2004; Vesely & Schreiber , 2013). Obviously, the surviving cancer cells develop an immunogenic phenotype mirroring the immunoreactive mechanisms of the host. Finally, the functional dormancy of the tumour cell population may be broken, leading to progression of the cells into the escape phase, in which edited tumours of reduced immunogenicity begin to grow progressively in an immunologically unrestrained manner, establish an immunosuppressive tumour microenvironment, and eventually become clinically apparent (Dunn et al., 2004).

In this theory, the immune system on one hand protects the body from cancer and on the other hand, it shapes the immunogenicity of these cancers. As a result, the differences in the immunogenicity of each cancer reflect the differences of the immune functions between the hosts. Moreover, the tumour escape mechanisms do not develop during the clinically evident period of the disease, but rather they represent the final outcome of a long-lasting interrelation between rapidly transforming cells and the immune system, during the second step of the immunoediting process.

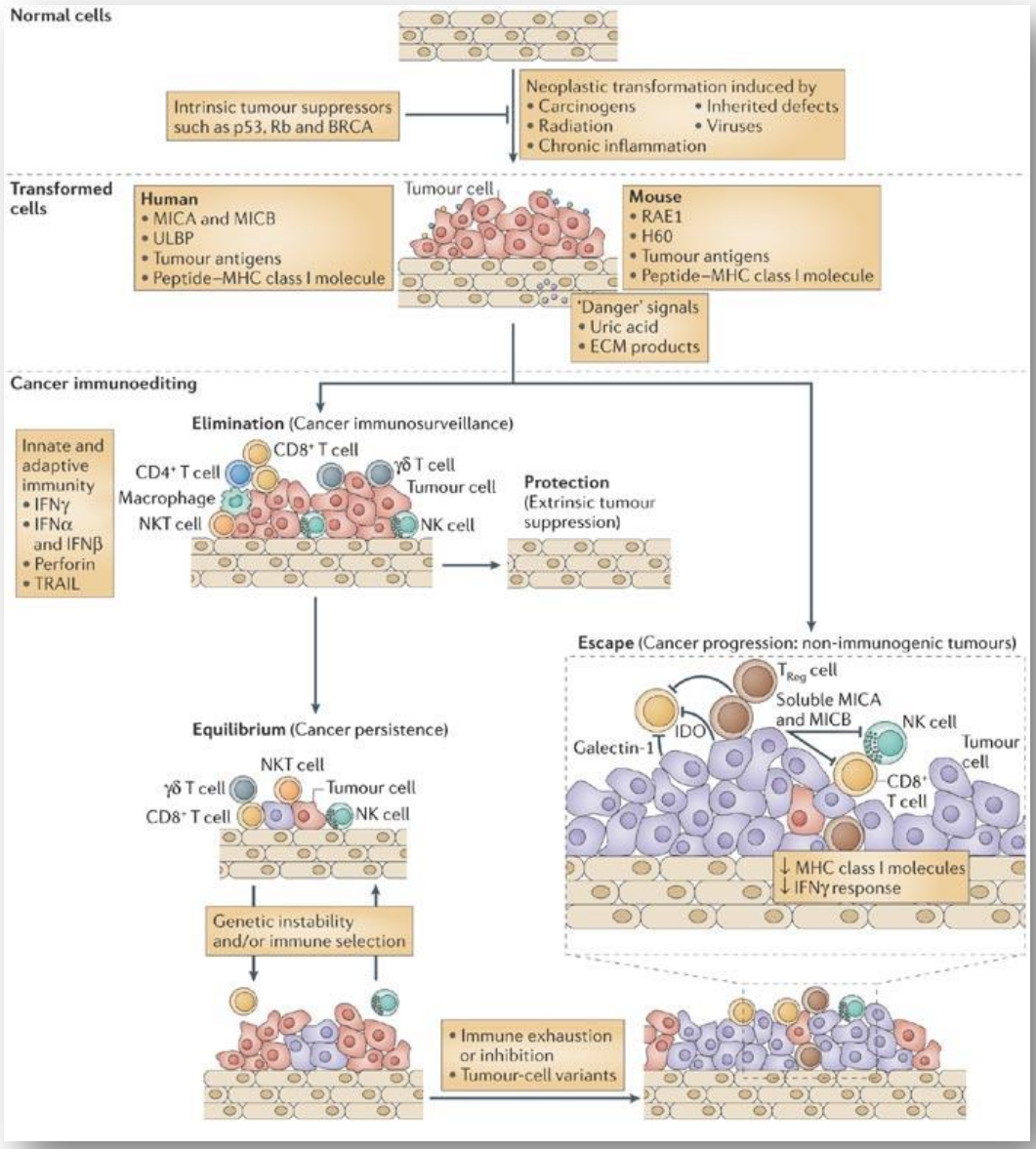

Figure 1.3: The three phases of immunoediting. Elimination-the process of detecting and destroying transformed cells; Equilibrium- where the proliferation and destruction of the tumour cells is at a balance; Escape- where the edited tumour of reduced immunogenicity begin to grow progressively in an immunologically unrestrained manner. NK: natural killer cells; NKT: variants expressing an extremely limited T cell repertoire; $CD4^+$, $CD8^+$, $CD4^+CD25^+$ Treg, and γδ cells: types of T cell (Dunn et al., 2006).

It has been suggested that immune functions during oncogenesis result in the selection of tumour variants, capable of surviving within an immunologically intact environment, very much like various pathogens (Germenis & Karanikas , 2006). Immune-induced tumour

alterations include those that would not otherwise be associated with immune escape such as transformation and increased aggressiveness (i.e. inflammation-induced tumour promotion) (Bui & Schreiber , 2007). The genetic instability of cancer cells allows the induction of several alterations with the final consequences being the bypassing of immune effector mechanisms and the shaping of tumour immunogenicity (Germenis & Karanikas , 2006). Immunosculpting encompass all immune-mediated changes in the tumour and includes the most minimal changes such as amino acid substitutions in key antigenic proteins (i.e. mutations) to major reprogramming strategies such as epithelial to mesenchymal transition (EMT). The genes those are often lost from the final consequences code for various tumour antigens, elements that participate in the processing and presentation of antigens to T cells of the host or factors that intervene with the interferon (IFN)-g receptor signal transduction. Further, immunosculpting includes both permanent (e.g. mutations) and non-permanent (e.g. reversible ligand induced cytokine production) events. Immunoediting might be thought as the component of immunosculpting, which imparts immune escape properties. As a result, interactions between the tumour and the immune system may result in sculpting of the tumour for increasingly aggressive growth and further resistance to immune destruction.

The immune system is linked to tumour development and progression as the tumour is promoted by the induction of inflammation. Chronic inflammation is linked with the presence of cancer and is thought as a major contributor to cancer pathogenesis. Inflammatory bowel diseases are associated with an increased risk of cancer. Moreover, chronic exposure to irritants that cause inflammation, such as cigarette smoke, are associated with an elevated risk of cancers and many cancers are linked with chronic pathogen exposure. Moreover, the inflammatory mediators produced by inflammation drive further mutation and selection resulting in immunosculpting. So, there is a complex and often paradoxical role of the immune system in cancer in which immune cells are cast in a protagonist versus antagonist role. On one hand, immunity may protect against cancer and on the other, it appears to be pathologic.

Immune cells, both adaptive and innate effectors, as well as their products (e.g. cytokines) have a role in immunosculpting. Phagocytic cells are capable of sculpting the phenotype of dysplastic and neoplastic cells. Macrophages are among the first cells recruited into aberrant tissues. A variation of chemokines and cytokines are released by neoplastic lesions and result in the influx and differentiation of macrophages, including GM-CSF, TGF-β and CCL3. On the other hand,

M2-macrophages modulate the tumour microenvironment by producing survival/growth factors (e.g. vascular endothelial growth factor) and are associated with a poor prognosis. Multiple intracellular signalling pathways are used by tumour cells in response to macrophage exposure, including both the NF-κB and JNK pathways. Moreover, macrophages express Epidermal Growth Factor (EGF) that acts directly on tumours to increase both invasiveness and increased tumour cell production of colony stimulating factor 1 (CSF-1). Neutrophils may also immunosculpt tumours for increased aggressiveness. Neutrophils are attracted into lesions by IL-8 tumour production and once in the tumour microenvironment, neutrophils produce many factors that sculpt the tumour. Tumour cells also produce a factor that induces HGF production by the neutrophils. HGF is well known for its abilities to induce scattering and migration of a variety of cell types, for example by upregulating chemokine receptors such as CXCR4. In addition, other studies showed that tumour cells can also induce N2-neutrophils to produce oncostatin M, an IL-6-related cytokine. Oncostatin M also augments tumour cell invasiveness. Tumours therefore appear to interact with multiple phagocytic cells using several different pathways, a complex network that is likely to pose a significant challenge to target with exogenous agents aimed at preventing such interactions. In addition, regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) also take part in the suppression of the cytotoxic lymphocytes.

T cells and effector molecules also have a role in increasing cancer aggressiveness. For example, TNF-α, a T cell and macrophage-produced pleiotropic cytokine, can result in one of at least two potential outcomes depending on its prevalence. The first outcome is the induction of inflammation and cell survival in which TNF-α causes the release of variety of growth factors and upregulates negative regulators of apoptosis, such as c-FLIPL and Bcl-2. At higher doses, however, the effects are the opposite and include activation of apoptosis and lead to haemorrhagic necrosis. Moreover, TNF-α has widely been implicated in both early and late events in initiation, promotion, and progression of cancers. Progression of cancer is associated with the acquisition of the tumour to produce its own TNF- α, which then act in an autocrine and paracrine fashion. TNF-α can also increases its own activity by promoting the release of other soluble mediators. In addition, TNF-α triggers activation of the NF-kB signalling pathway, which results in the upregulation of pro-survival pathways.

The nuclear factor-κB (NF-κB) pathways also play an important role in mediating development and progression of cancer. In addition to its anti- apoptotic activity, NF-κB contributes to tumour development by stimulating cell proliferation, because it activates the expression of growth factor genes, proto-oncogene c-Myc, and cell cycle regulator cyclin D1. NF-κB may also play an essential role in late-stage cancer development. NF-κB is activated by inflammatory stimuli and its constitutive activation is found in cancer; as a result, it has long been suspected to be a critical promoter facilitating the development from inflammation into cancer.

1.3 PHYSIOLOGY OF THE IMMUNE SYSTEM

The immune system is a versatile defence system that has evolved to protect animals from invading pathogenic microorganisms and cancer. Its function is based on its ability to generate an enormous variety of cells and molecules, which are able to specifically recognize and eliminate a limitless variety of foreign invaders (Janaway et al., 2001). These cells and molecules act together in a dynamic complex network. Functionally, an immune response can be divided into two related activities; recognition and response. Immune recognition is remarkable for its specificity. The immune system is able to recognize subtle chemical differences that distinguish one foreign pathogen from another (Rabson et al., 2005). Furthermore, the immune system is able to discriminate between foreign molecules and the body's own cells and proteins.

Immunity is divided into two types determined by the speed and specificity of the reaction. Innate (natural) immunity, which is the first line of defence, includes physical, chemical and microbiological barriers and occurs to the same extent however many times the infectious agent is encountered (Goldsby et al., 2003). Moreover, the cells that participate in the innate immunity are phagocytic cells (neutrophils, monocytes, and macrophages), cells that release inflammatory mediators (basophils, mast cells, and eosinophils) and natural killer cells which provide immediate host defence (Rabson et al., 2005). Complement, acute-phase proteins and cytokines such as the interferons (IFN) are the molecular components that are included in the innate immunity (Goldsby et al., 2003). The innate responses are highly conserved, which is a proof of its importance in survival.

The hallmark of the immune system of higher animals is the adaptive immunity. Adaptive immunity involves antigen-specific reactions through T lymphocytes and B lymphocytes, which

occurs when the surface receptors of these cells bind to antigen (Delves & Roitt , 2000). Antigen-presenting cells display the antigen via the MHC complex to lymphocytes and collaborate with them in the response to the antigen. B cells secrete immunoglobulins, the antibodies, which are antigen-specific and responsible for eliminating extracellular microorganisms (Delves & Roitt , 2000). On the other hand, T cells eradicate intracellular pathogens by activating macrophages and by killing virally infected cells, while they stimulate the B cells to turn on antibody production (Delves & Roitt , 2000). Whereas, the innate response is rapid but sometimes damages normal tissues through lack of specificity, the adaptive response is precise, but takes several days or weeks to develop. In addition, adaptive immunity has memory and improves on repeated exposure to a given infection (Delves & Roitt , 2000). Innate and adaptive responses work together to eliminate pathogens.

The cells of innate and adaptive immunity develop from pluripotent stem cells in the fetal liver and in bone marrow and then circulate in the blood. The maturation of the B cells occurs in the bone marrow, while T cells complete their development in the thymus (Rabson et al. 2005). Lymphocytes are activated in the secondary lymphoid tissues, which include the lymph nodes, the spleen, and the mucosa-associated lymphoid tissue (figure 1.4). The activation of lymphocytes by antigen occurs in distinctive B- and T-cell compartments of lymphoid tissue. In the lymph nodes, B lymphocytes are localized in follicles, with T cells more diffusely distributed in surrounding paracortical areas, also referred to as T-cell zones (Janaway et al., 2001). Some of the B-cell follicles include germinal centres, where B cells are undergoing intense proliferation after encountering their specific antigen and their cooperating T cells (Janaway et al., 2001). The gut-associated lymphoid tissues (GALT), which include the tonsils, adenoids, and appendix, and specialized structures called Peyer's patches in the small intestine, collect antigen from the epithelial surfaces of the gastrointestinal tract (Janaway et al., 2001).

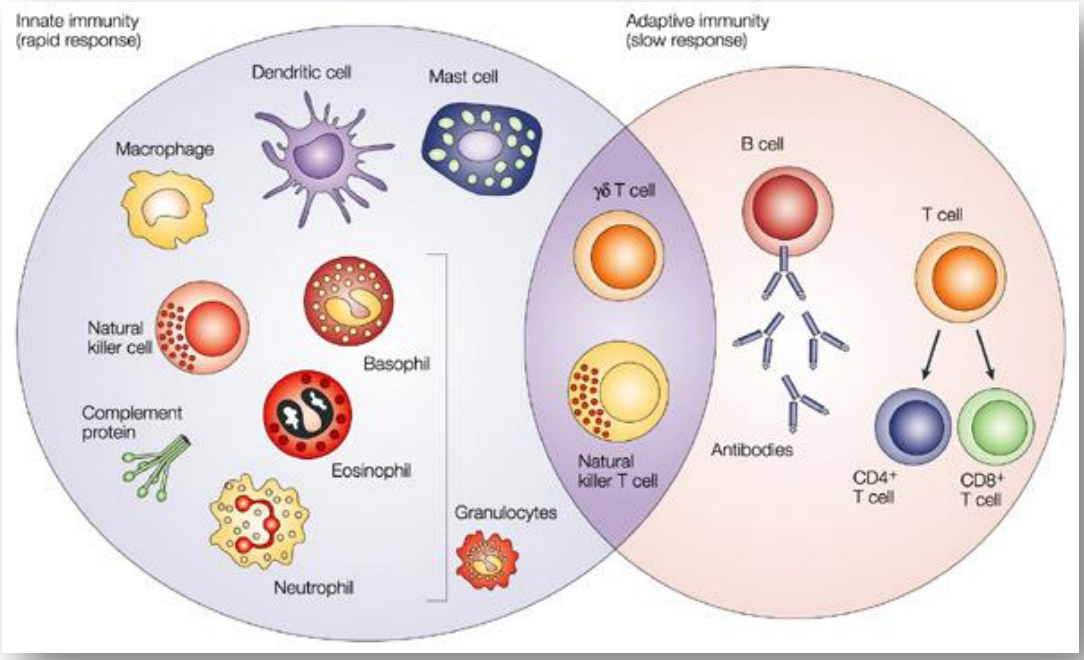

Figure 1.4: Physiology of the immune system. Innate (natural) immunity, which is the first line of defence, includes physical, chemical and microbiological barriers. It consists of soluble factors, such as complement proteins, and diverse cellular components including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells. The adaptive response takes several days or weeks to develop, but manifests as increased antigenic specificity and memory. It involves antigen-specific reactions through T lymphocytes and B lymphocytes, which occurs when the surface receptors of these cells bind to antigen. Natural killer T cells and γδT cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity. (Dranoff 2004)

1.3.1 Innate Immune

Innate immunity can be seen to comprise four types of defensive barriers: anatomic, physiologic, phagocytic, and inflammatory. The innate immune responses lack immunologic memory and remain unchanged, however often the antigen is encountered. These types of responses are evolutionarily primitive and defects in them can be fatal (Goldsby et al., 2003).

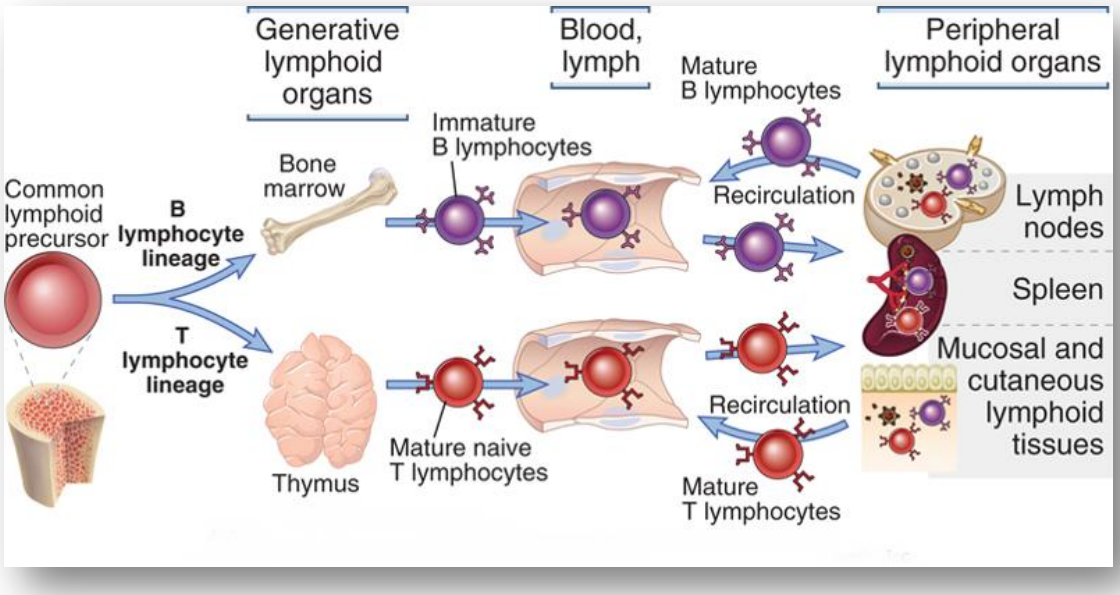

Figure 1.5. Maturation of lymphocytes. (Abbas & Lichtman , 2001)

1.3.1.1 The first line of defence

The first defence against infection is the physical and anatomic barriers that tend to prevent the entry of pathogens. The skin and the surface of mucous membranes are included in this category, as they are effective barriers to the entry of most microorganisms (Kawamura et al., 2014). The skin consists of two distinct layers: a thinner outer layer—the epidermis—and a thicker layer—the dermis. Breaks in the skin resulting from scratches, wounds, or abrasion are routes of infection (Visscher & Narendran , 2014). The body surfaces are defended by epithelia, which provide a physical barrier between the internal milieu and the external world that contains pathogens(Aderem & Underhill , 1999). Epithelial cells are held together by tight junctions, which effectively form a seal against the external environment. The internal epithelia are known as mucosal epithelia because they secrete a viscous fluid called mucus, which contains many glycoproteins called mucins (Ryu et al., 2010). Surface epithelia also produce chemical substances that are microbicidal or inhibit microbial growth.

The physiologic barriers that contribute to immunity include temperature, pH, and various soluble and cell associated molecules. The acid pH of the stomach and the digestive enzymes of the upper gastrointestinal tract create a substantial chemical barrier to infection (Janaway et al., 2001). To establish an infection, the pathogen must first overcome these surface barriers that

either are directly antimicrobial or inhibit attachment of the microbe. Any organism that breaks through this first barrier encounters the two further levels of defence, the innate and acquired immune responses.

1.3.1.2 Cellular Components of the Innate Immune

Macrophages play a central role in host defence in collaboration with antibody, complement, and T lymphocytes (Ezekowitz , 1989; Gordon et al., 1988; Papadimitriou & Ashman , 1989). The macrophages function as effector cells and contribute to the induction of the immune response. Tissue macrophages form a lattice beneath epithelial surface in the lung, gut, kidney, and skin and play a role in first line host defence (Gordon et al., 1988; Papadimitriou & Ashman , 1989). Macrophages have the capacity to internalize a variety of particulate and soluble components via specific cell surface receptors (Ezekowitz & Stahl , 1988; Gordon et al., 1988; Ravetch & Kinet , 1991). Macrophages express receptors for carbohydrates that are not normally exposed on the cells of vertebrates (Fraser et al., 1998), and therefore can discriminate between "foreign" and "self" molecules. In addition, macrophages possess receptors for antibodies and complement, so the phagocytosis of microorganisms coated with antibodies, complement, or both is enhanced (Aderem & Underhill , 1999). A wide range of toxic intracellular molecules (including superoxide anion, hydroxyl radicals, hypochlorous acid, nitric oxide, antimicrobial cationic proteins and peptides, and lysozyme) are secreted by macrophages and take part in the lysis of the phagocytized cells. In addition, apoptotic cells express molecules on their cell surface, such as phosphatidyl serine, and are recognized and phagocytized by macrophages (Savill , 1997).

One other feature of the innate immunity is the recruitment and the activation of neutrophils at the site of infection to eradicate pathogens (Witko-Sarsat et al., 2000). Activated macrophages release cytokines during the early stages of infection or tissue damage. Granulocyte (G-CSF) and granulocyte-macrophage colony (GM-CSF) stimulating factors stimulate the division of myeloid precursors in the bone marrow, which has as a result the release of cells into the circulation. Neutrophils are not static within a particular compartment, but they normally flow in the blood or roll along the vascular endothelium. At the site of infection, neutrophils use a multistep process involving proinflammatory mediators, adhesion molecules, chemoattractants, and chemokines (von Andrian & Mackay , 2000). Neutrophils phagocytose organisms by making pseudopodia, which form a membrane-bound vesicle (phagosome) around the particle. Phagosome fuses with cytoplasmic granules to form the phagolysosome (Garred et al., 1995).

The organism in the phagolysosome is killed by a combination of two mechanisms. The first mechanism is the oxygen-dependent response, which involves the sequential reduction of oxygen by an NADPH oxidase leading to production of toxic oxygen metabolites, such as hydrogen peroxide, hydroxyl radicals, and singlet oxygen (Garred et al., 1995). The other mechanism is the oxygen-independent response, which uses the highly toxic cationic proteins and enzymes (eg, myeloperoxidase and lysozyme) contained within the neutrophil cytoplasmic granules. The opsonisation of the phagocytized particle with specific antibody or complement upgrades the effect of ingestion and killing of the organism. These molecules bind to neutrophil receptors, increasing adhesion between particle and phagocyte and priming the cell for activation. Some organisms are not susceptible to neutrophil phagocytosis unless first coated with antibody.

The main physiological role of eosinophils is in the elimination of parasites too large to be engulfed. Such infections induce antigen-specific IgE production, the antibodies coating the organism (Rabson et al., 2005). Eosinophils bear Fc receptors specific for the constant region of IgG, as well as high-affinity receptors for IgE. Aggregation of these receptors triggers the release of toxic substances, such as eosinophilic cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin, onto the surface of the parasite (Wardlaw et al., 1995). These proteins are highly cytotoxic when released onto the surface of organisms. They also secrete leukotrienes, prostaglandins, and various cytokines (Wardlaw et al., 1995).

Basophils and mast cells have similar functional characteristics (Abraham & Arock , 1998), but there is little evidence that blood basophils develop into tissue mast cells (Siraganjan , 1998). Although basophils and mast cells are relatively few in number compared with the other white blood cells, they are involved in some of the most severe immunological reactions. Mast cells are large cells containing distinctive cytoplasmic granules that contain a mixture of chemical mediators, including histamine, that act rapidly to make local blood vessels more permeable. There are at least two populations of mast cells, based on the enzymes they contain and their tissue location. T mast cells (mucosal mast cells) contain only trypsin, whereas connective tissue mast cells contain both trypsin and chymotrypsin (Benoist & Mathis , 2002). Basophils are morphologically similar to mast cells found in the blood. Mast cells and basophils possess high-affinity receptors for IgE (FcγRI) (CD23) (Kinet , 1999) and IgG (FcγRIII) which rapidly absorb any local IgE and can be activated to release their granules, and to secrete lipid inflammatory

mediators and cytokines, via antibody bound to Fc receptors (Janaway et al., 2001). Crosslinking of these receptors by the binding of antigen to IgE leads to degranulation and release of inflammatory mediators such as histamine, prostaglandins, and leukotrienes.

Natural killer cells (NK cells) develop in the bone marrow from the common lymphoid progenitor cell and circulate in the blood. They have the morphology of lymphocytes but do not bear a specific antigen receptor (Biron et al., 1999). They are larger than T and B lymphocytes, have distinctive cytoplasmic granules, and are functionally identified by their ability to kill certain lymphoid tumour cell lines in vitro without the need for prior immunization or activation. NK cell-killing is triggered by invariant receptors, and their known function in host defence is in the early phases of infection with several intracellular pathogens. They recognize abnormal cells in two ways. Like many other cells, they possess Fc receptors that bind IgG (Fc R). These receptors link natural killer cells to IgG-coated target cells, which they kill by a process called antibody-dependent cellular cytotoxicity (Moretta et al., 1997). Secondly, they have receptors on their surface for MHC class I. If this receptor is not bound during its interaction with a cell, the natural killer cell is programmed to lyse the target (Lanier , 1998). The mechanism of NK cell killing is the same as that used by the cytotoxic T cells generated in an adaptive immune response; cytotoxic granules (perforin and granzyme B) are released onto the surface of the bound target cell, and the effector proteins (IFNγ) they contain penetrate the cell membrane and induce programmed cell death (Biron et al., 1999). NK cells are activated in response to interferons or macrophage-derived cytokines. Tumour cells and viruses often cause downregulation of class I. Although, this may offer some advantage to the pathogen impairing recognition by cytotoxic T cells, it does leave them open to natural killer cell attack.

The erythrocytes have complement receptors and they play an important part in the clearance of immune complexes consisting of antigen, antibody, and components of the complement system.

1.3.1.3 Soluble Factors in Innate Defence

A variety of soluble factors contribute to innate immunity, among them the soluble proteins lysozyme, interferon, and complement. Lysozyme, a hydrolytic enzyme found in mucous secretions and in tears, is able to cleave the peptidoglycan layer of the bacterial cell wall (Glynn AA & Milne , 1965). Interferon comprises a group of proteins produced by virus-infected cells

(Bloom , 1980). Among the many functions of the interferons is the ability to bind to nearby cells and induce a generalized antiviral state (Bloom , 1980).

Another important component of the innate immune is the complement which is a system of distinct plasma proteins that activates a cascade of proteolytic reactions on microbial surfaces but not on host cells (Abbas et al., 2012). Activation of a single molecule leads to generation of a plethora of molecules. A number of complement proteins are proteases that are themselves activated by proteolytic cleavage. There are three distinct pathways through which complement can be activated on pathogen surfaces (Law & Reid , 1995). These pathways depend on different molecules for their initiation, but they converge to generate the same set of effector molecules (figure 1.6). There are three ways in which the complement system protects against infection (Abbas et al., 2012). First, it generates large numbers of activated complement proteins that bind covalently to pathogens, opsonizing them for engulfment by phagocytes bearing receptors for complement. Second, the small fragments of some complement proteins act as chemoattractants to recruit more phagocytes to the site of complement activation, and also to activate these phagocytes. Third, the terminal complement components damage certain bacteria by creating pores in the bacterial membrane (Law & Reid , 1995). The activation of compliment can me be driven by three pathways, the classical by antigen-antibody reactions, the alternative by polysaccharides from yeasts and gram negative bacteria and the mannan binding lectin pathway (Wallis & Drickamer , 1999). Many of the components of the classical and alternative pathway are homologous, suggesting the pathways were initially derived from the same sequence (Abbas et al., 2012). The classical pathway can be initiated by the binding of C1q, the first protein in the complement cascade, directly to the pathogen surface. The alternative pathway can be initiated when a spontaneously activated complement component binds to the surface of a pathogen (Abbas et al., 2012; Law & Reid , 1995). The mannan binding lectin pathway is the more recently identified and is initiated by binding of the mannan-binding lectin, a serum protein, to mannose-containing carbohydrates on bacteria or viruses. The common component of the three pathways is the activated central C3 (Dodds & Law , 1988). The proteolytic cleavage fragment of complement component C3, the C3b molecule, becomes deposited on the surface of microorganisms (Abbas et al., 2012). This event enhances phagocytosis of the microbe, because phagocytic cells have cell surface receptors for C3b. The complement fragments C3a, C4a, and C5a cause the release of inflammatory mediators from mast cells (Dodds & Law , 1988). C3 leads to a final common pathway with the forming of C5–C9 transmembrane pore in the cell

surface and death by osmotic lysis (Abbas et al., 2012). The cells of the host bear the complement receptor type 1 and decay accelerating factor, which inhibit C3 convertase and prevent progression of complement activation. Cells that lack these molecules are susceptible to complement. Moreover, complement has other anti-infective functions, such as the release of soluble C3a and C5a, which are anaphylatoxins, and its activation helps to target complement-receptor bearing antigen-presenting cells (Law & Reid , 1995).

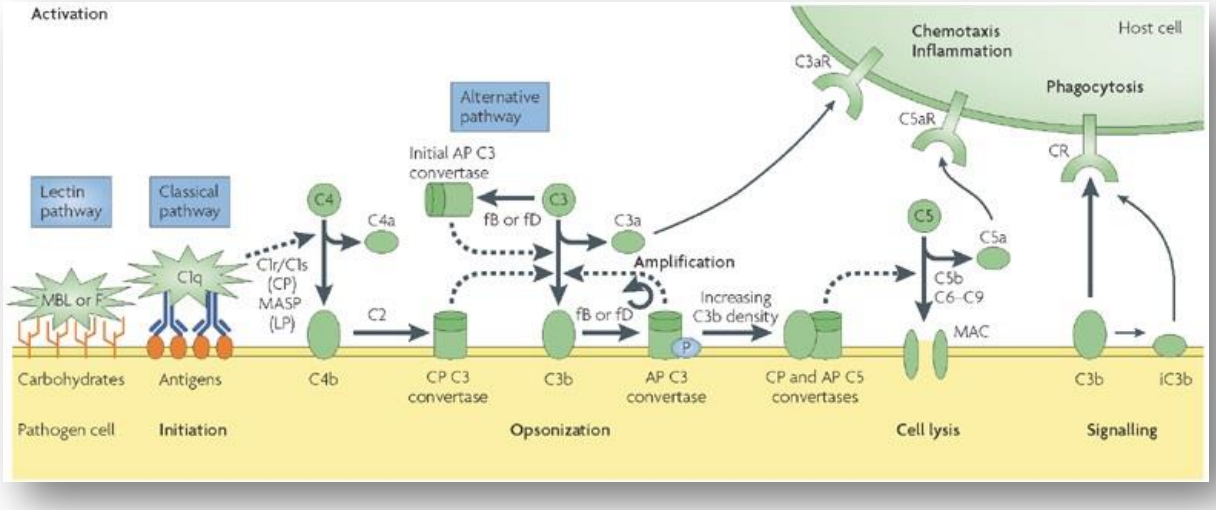

Figure 1.6: Complement activation pathways. The classical, lectin, and alternative pathways converge into a final common pathway when C3 convertase (C3 con) cleaves C3 into C3a and C3b. Ab = antibody; Ag = antigen; C1-INH = C1 inhibitor; MAC = membrane attack complex; MASP = MBL-associated serine protease; MBL = mannose-binding lectin. Overbar indicates activation (Lambris et al., 2008).

Acute phase proteins enhance resistance to infection and promote the repair of damaged tissue (Gabay & Kushner , 1999). Infection, inflammation and tissue injury change the levels of these proteins. Moreover, the acute-phase proteins include C-reactive protein, serum amyloid A protein, proteinase inhibitors, and coagulation proteins (Pepys & Hirschfield , 2003). Another group of soluble mediators of immune are the cytokines. Cytokines act as messengers within the immune system and between the immune system and other systems of the body (Mire-Sluis & Thorpe , 1998). The cells bear specific cytokine receptors by which they sense the presence of

a cytokine (Heaney & Golde , 1998; Merlos-Suarez et al., 1998). In addition, some cytokines have a direct role in defence.

1.3.2 Adaptive Immune

Adaptive immunity is capable of recognizing and selectively eliminating specific foreign microorganisms and molecules (i.e., foreign antigens). Unlike innate immune responses, adaptive immune responses are not the same in all members of a species but are reactions to specific antigenic challenges. Adaptive immunity displays four characteristic attributes: antigenic specificity, diversity, immunologic memory and self/non-self recognition (Alberts et al., 2002).

The initial stages of lymphocyte development do not require the presence of an antigen, but once these cells express a mature antigen receptor, their survival and further differentiation become antigen-dependent. First, the antigen is presented to and recognized by the antigen specific T or B cell leading to cell priming, activation, and differentiation, which usually occurs within the specialized environment of lymphoid tissue (Janaway et al., 2001). Second, the effector response takes place, either due to the activated T cells leaving the lymphoid tissue and homing to the disease site or due to the release of antibody from activated B cells (plasma cells) into blood and tissue fluids, and thence to the infective focus (Janaway et al., 2001).

Once the immune system has recognized and responded to an antigen, it exhibits immunologic memory; that is, a second encounter with the same antigen induces a heightened state of immune reactivity (Goldsby et al., 2003). Because of this attribute, the immune system can confer life-long immunity to many infectious agents after an initial encounter.

An effective immune response involves two major groups of cells: T lymphocytes and antigen-presenting cells. The development of lymphocytes from stem cells is guided by interactions with stromal cells and by cytokines (Koning & Mebius , 2012). Lymphocytes are one of many types of white blood cells develop from progenitor cells within the bone marrow by the process of haematopoiesis (Akashi et al., 1999). B cells remain within the marrow for the duration of their development, but T cells migrate to the thymus at an early stage as thymocytes (Janaway et al., 2001).

The antigenic specificity of the immune system permits it to distinguish subtle differences among antigens. Antibodies can distinguish between two protein molecules that differ in only a single amino acid. The immune system is capable of generating tremendous diversity in its recognition molecules, allowing it to recognize billions of unique structures on foreign antigens (Abbas & Lichtman , 2001). The production of antigen-specific receptors in both cell types is the result of an unusual process of random rearrangement and splicing together of multiple DNA segments that code for the antigen-binding areas of the receptors (Abbas & Lichtman , 2001). Gene rearrangement occurs before the exposure to antigen. There are four segments of gene involved in receptor formation called the variable (V), diversity (D), joining (J), and constant (C) regions (Alberts et al., 2002). These are found on different chromosomes within the developing cell. The segments are cut out by nucleases and spliced together using ligases (Parkin & Cohen , 2001).

Finally, the immune system normally responds only to foreign antigens, indicating that it is capable of self/non-self recognition (Gonzalez et al., 2011). The ability of the immune system to distinguish self from non-self and respond only to non-self molecules is essential, for, as described below, the outcome of an inappropriate response to self-molecules can be fatal (Gonzalez et al., 2011).

1.3.2.1 B lymphocytes

B lymphocytes mature within the bone marrow; when they leave it, each expresses a unique antigen-binding receptor on its membrane. This antigen-binding or B-cell receptor (BCR) is a membrane-bound antibody molecule. This serves to neutralize toxins, prevents organisms adhering to mucosal surfaces, activates complement, opsonizes bacteria for phagocytosis, and sensitizes tumour and infected cells for antibody dependent cytotoxic attack by killer cells (figure 1.7) (Parkin & Cohen , 2001).

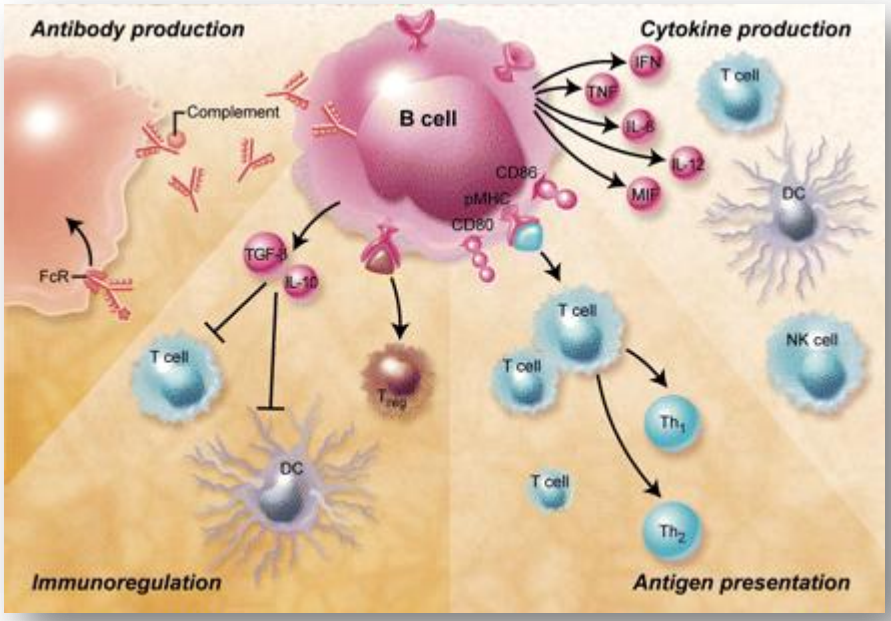

Figure 1.7: Overview of the B-cell functions. B cells contribute to immune responses by antibody-mediated and antibody-independent mechanisms. B cells produce antibodies that lead to activation of complement, antibody-dependent cell-mediated cytotoxicity, and Fc-receptor antigen uptake and phagocytosis. Moreover, B cells secrete a large number of proinflammatory cytokines, such as IL-2, TNF-α, IL-6, IL-12, MIF and interferon-γ, which activate T cells (including Th17 cells), macrophages, and natural killer (NK) cells. Activated B cells have up-regulated major histocompatibility complex and co-stimulatory molecules such as CD80 and CD86. These cells take part in antigen presentation, which leads to $CD4^+$ and $CD8^+$ T-cell activation and differentiation. Furthermore, some B lymphocytes can also act as immunoregulatory cells that induce peripheral $CD4^+$ and $CD8^+$ T-cell tolerance, inhibit dendritic cells, and induce and expand regulatory T cells.(Shimabukuro-Vornhagen et al., 2009)

Antibodies are glycoproteins that consist of two identical heavy polypeptide chains and two identical light polypeptide chains that are held together by disulfide bonds (Parham , 2009). The N terminal of each chain possesses a variable domain that binds antigen through three hypervariable complementarity-determining regions (Alberts et al., 2002). The C terminal domains of the heavy and light chains form the constant regions, which define the class and subclass of the antibody and govern whether the light chain is of the κ or λ type (Alberts et al., 2002). The amino acid sequence of the constant region of the heavy chains specifies five classes of immunoglobulins (IgG, IgA, IgM, IgD, and IgE), four subclasses of IgG, and two subclasses of IgA (Alberts et al., 2002). These classes and subclasses have different functions. Each type of

antibody can be produced as a circulating molecule or as a stationary molecule. Although ultimately antibody is the secreted product of activated B cells with the functions listed, early in B-cell development it is a membrane bound molecule that acts as the B-cell receptor. In this role it internalizes antigen and processes it to act as an antigen-presenting cell for T-cell responses.

When a naive B cell (one that has not previously encountered antigen) first encounters the antigen that matches its membrane bound antibody, the binding of the antigen to the antibody causes the cell to divide rapidly; its progeny differentiate into memory B cells and effector B cells called plasma cells (Goldsby et al., 2003). Memory B cells have a longer life span than naive cells, and they express the same membrane-bound antibody as their parent B cell (McHeyzer-Williams & McHeyzer-Williams , 2004). Plasma cells produce the antibody in a form that can be secreted and have little or no membrane-bound antibody (McHeyzer-Williams & McHeyzer-Williams , 2004). Although plasma cells live for only a few days, they secrete enormous amounts of antibody during this time. Secreted antibodies are the major effector molecules of humoral immunity (McHeyzer-Williams & McHeyzer-Williams , 2004).

Antigen recognized by the surface IgM of the B cell, is internalized, processed, and re-expressed on the MHC class II molecule of the B cell (Janaway et al., 2001). This can then present the antigen to a primed specific T cell (which recognizes a different part of the same antigen). The T cell in turn produces cytokines (B-cell growth factors) leading to B cell division and maturation to antibody secreting cells (McHeyzer-Williams & McHeyzer-Williams , 2004). Further T-cell interactions, in particular the binding of CD40 on B cells with the CD40 ligand on T cells induces isotype switching from the initial IgM response (Banchereau et al., 1994; Quezada et al., 2004). Once the switch from IgM to another isotype has occurred, some of the activated cells become long-lived memory cells.

B cells can also respond to some antigens in a T-cell independent reaction (Mond et al., 1995). The antigens, that can induce this, have numerous repeating epitopes (mainly polysaccharides) that bind multiple B-cell receptors and activate the B cell directly to secrete IgM antibody. However, as there is no germinal centre formation, no affinity maturation takes place, and there is no class switching or generation of memory. Therefore T-cell independent responses are IgM limited, of poor specificity, and short-lived (Mond et al., 1995).

1.3.2.2. T lymphocytes

T lymphocytes also arise in the bone marrow. Stem cells continuously migrate from the bone marrow to the thymus, where they develop into T cells (figure 1.8) (Kruisbeek , 1999) and continue to develop throughout life (Jamieson et al.,1999). During its maturation within the thymus, the T cell comes to express a unique antigen-binding molecule, called the T-cell receptor (TCR), on its membrane (Alberts et al., 2002). Once receptor rearrangement has occurred, T cells are able to respond to their antigen and induce an immune response. However, cell activation is tightly regulated to ensure that only damaging antigens elicit a reaction (Parkin & Cohen , 2001).

The T-cell receptor recognizes short peptides that result from the intracellular processing of protein antigens, which are presented to the T-cell receptor by MHC molecules on the cell surface (Alberts et al., 2002; Janaway et al., 2001; Parkin & Cohen , 2001). MHCs are polymorphic (genetically diverse) glycoproteins found on cell membranes (Janaway et al., 2001). There are two major types of MHC molecules. Class I MHC molecules, which are expressed by nearly all nucleated cells of vertebrate species, consist of a heavy chain linked to a small invariant protein called β2-microglobulin (Alberts et al., 2002). Class II MHC molecules consist of an alpha and a beta glycoprotein chain and are expressed only by antigen-presenting cells (Alberts et al., 2002). Since MHC molecules are highly polymorphic, the desirable immature T cells in each person in an outbred population are those that can recognize self MHC molecules but that are not autoreactive. This objective is achieved by thymic education, a process that involves both positive and negative selection (Fink & Bevan , 1995; Kruisbeek & Amsen , 1996; Rathmell & Thompson , 1999). Cells are positively selected if they express a T-cell receptor capable of interacting with the MHC complexes on the person's own epithelial cells in the thymic cortex. Positive selection switches off the signal for spontaneous apoptosis that is otherwise triggered naturally in developing T cells. More than 95% of T cells are not selected at this stage and therefore die in the thymus (Abbas et al., 2012). In contrast, negative selection involves the induction of apoptosis in any lymphocyte that expresses a T-cell receptor with a high affinity for the complex of a self-peptide plus a self MHC molecule on dendritic cells and macrophages in the thymic medulla (Starr et al., 2003).

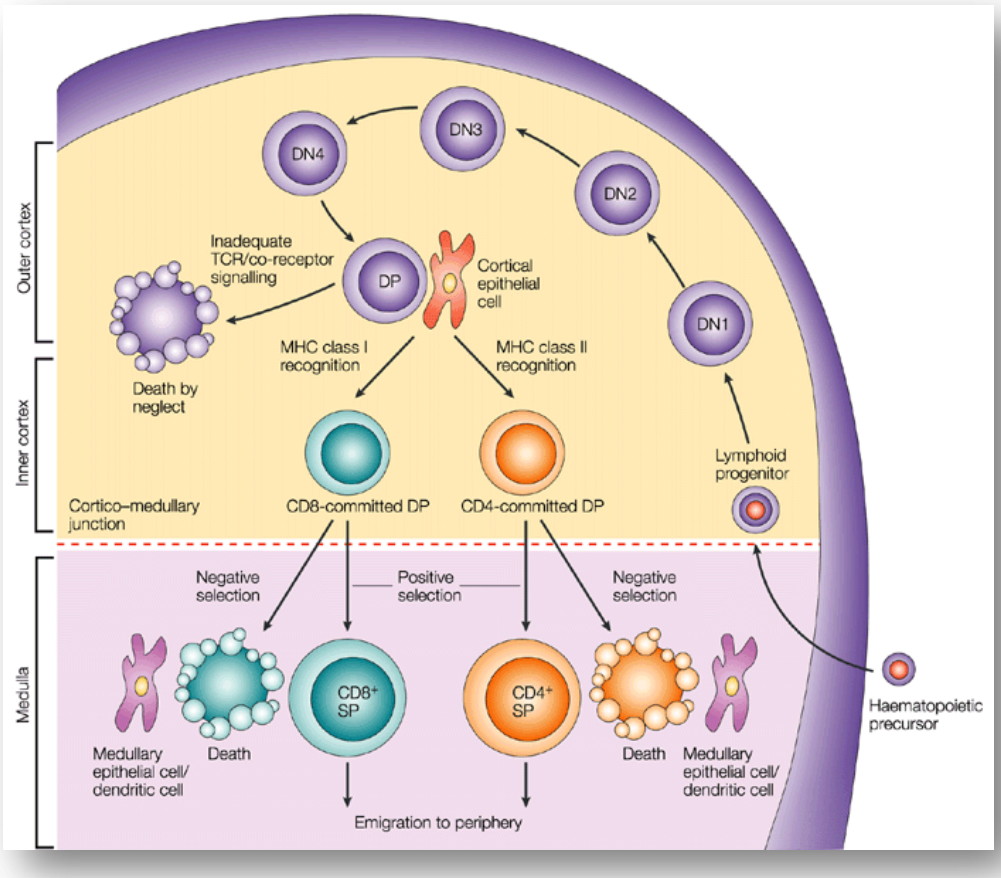

Figure 1.8: T-cell development and the CD4-CD8 lineage decision. Lymphoid progenitors arise in the bone marrow and migrate to the thymus. Early T cells lack expression of T-cell receptor (TCR) and are characterized as double-negative thymocytes (DN; no CD4 or CD8). During differentiation, DN thymocytes can be subdivided into four stages (DN1, $CD44^{+}CD25^{-}$; DN2, $CD44^{+}CD25^{+}$; DN3, $CD44^{-}CD25^{+}$; and DN4, $CD44^{-}CD25^{-}$). Pre-TCR expression leads to cell transition to double positive (DP) stage. The $CD4^{+}CD8^{+}$ (DP) thymocytes then interact with cortical epithelial cells that express a high density of MHC class I and class II molecules associated with self-peptides.. Too little signalling results in delayed apoptosis while too much signalling can promote acute apoptosis (negative selection). The appropriate, intermediate level of TCR signalling initiates effective maturation (positive selection). Thymocytes that bind self-peptide–MHC-class-I complexes become $CD8^{+}$ T cells, whereas those that bind self-peptide–MHC-class-II ligands become $CD4^{+}$ T cells. (Germain , 2002)

Naive T cells bear receptors (peripheral node addressins; PNAds) that bind to adhesion molecules on the high endothelial venules of lymph nodes, enter the nodes, and pass through binding transiently to the multiple antigen-presenting cells (Janaway et al., 2001). Although

about 95% of T lymphocytes are sequestered within the lymphoid tissue, they are not static but move continuously from one lymphoid tissue to another, via the blood or lymph, travelling around the whole body in 1–2 days (Cose , 2007). When the T cell meets an antigen-presenting cell bearing its antigen, activation occurs over the next 2–3 days (Grakoui et al., 1999). The antigen is brought to the lymphoid tissue directly in the lymphatics, or within dendritic (or other antigen presenting cells) cells that have endocytosed the antigen locally (Parham , 2009). The antigens may be further processed by antigen-presenting cells (macrophages, interdigitating dendritic cells, and B cells) ready to attract antigen-specific T cells and induce an immune response.

There are two ways in which antigen loading onto MHC can occur. The antigen may have been produced endogenously within the cell (such as viral or tumour proteins) and is complexed with MHC class I through intracellular processing pathways (Vyas et al., 2008). Alternatively, specialised professional antigen-presenting cells might have taken up exogenous antigen by endocytosis. Antigen presenting cells include dendritic cells, B cells, and macrophages (Abbas et al., 2012). Exogenous antigen is processed via a different pathway to endogenous, and re-expressed with MHC class II molecules (Robinson & Delvig , 2002). MHC class II has restricted expression, in normal circumstances being expressed only on these specialised cells.

When a naive T cell encounters antigen combined with a MHC molecule on a cell, the T cell proliferates and differentiates into memory T cells and various effector T cells (Janaway et al., 2001). There are two well-defined subpopulations of T cells: T helper (Th) and T cytotoxic (Tc) cells. Although a third type of T cell, called a T suppressor (Ts) cell, has been postulated, recent evidence suggests that it may not be distinct from Th and Tc subpopulations (Goldsby et al., 2003). T helper and T cytotoxic cells can be distinguished from one another by the presence of CD4 or CD8 membrane glycoproteins on their surfaces, respectively. T cells displaying CD4 generally function as Th cells (Zhu , 2008), whereas those displaying CD8 generally function as Tc cells (Fung-Leung et al., 1991). After a Th cell recognizes and interacts with an antigen–MHC class II molecule complex, the cell is activated—it becomes an effector cell that secretes various growth factors known as cytokines (Janaway et al., 2001). The secreted cytokines play an important role in activating B cells, Tc cells, macrophages, and various other cells that participate in the immune response. Differences in the pattern of cytokines produced by activated Th cells result in different types of immune response (Janaway et al., 2001).

Under the influence of Th-derived cytokines, a Tc cell that recognizes an antigen–MHC class I molecule complex proliferates and differentiates into an effector cell called a cytotoxic T lymphocyte (CTL) (Alberts et al., 2002). In contrast to the Th cell, the CTL generally does not secrete many cytokines and instead exhibits cell-killing or cytotoxic activity (Goldsby et al., 2003). The CTL has a vital function in monitoring the cells of the body and eliminating any that display antigen, such as virus-infected cells, tumour cells, and cells of a foreign tissue graft. Cells that display foreign antigen complexed with a class I MHC molecule are called altered self-cells; these are targets of CTLs (Alberts et al., 2002).

1.4 REGULATORY T CELLS (Tregs)

The regulatory T cells play an important role in the modification of immune response in malignancy. Recent studies confirmed that regulatory T lymphocytes (Tregs) inhibit the functionality of $CD4^+$ and $CD8^+$ T cells, natural killer (NK, NKT) cells and dendritic cells (DCs) (Nishikawa & Sakaguchi , 2010; Vasievich & Huang , 2011) and play an important role in the immune surveillance and tolerance (Baecher-Allan et al., 2004; Nielsen et al., 2004). Tregs have been found in peripheral blood (PB) [in low proportion (2-5% of lymphocytes)], lymph nodes and ascites. They also infiltrate the tumour microenvironment in a variety of solid cancers (figure 1.9) (Kawaida et al., 2005; Liyanage et al., 2002; Miller et al., 2006; Viguier et al., 2004).

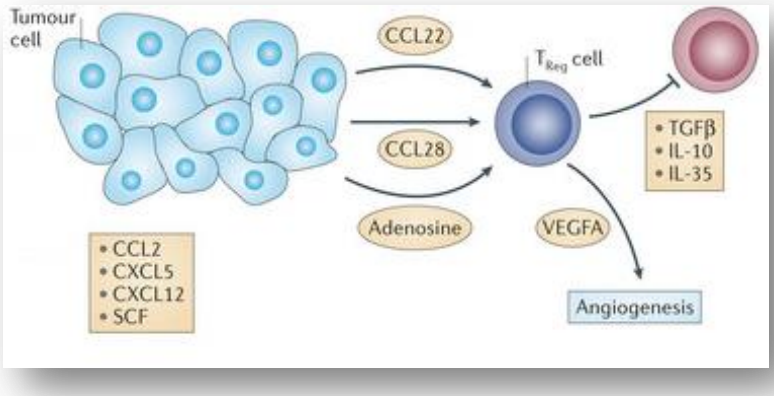

Figure 1.9: Treg cells and inflammatory signalling. Tumours recruit T regulatory (Treg) cells and manipulate their function. CC-chemokine ligand 22 (CCL22) and CCL28 attract Treg cells that express the respective receptors CC-chemokine receptor 4 (CCR4) and CCR10 to the site of tumour growth. Treg cells suppress effector T cells by the secretion of soluble mediators and induce an angiogenesis programme. CXCL, CXC-chemokine ligand; SCF, stem cell factor. (Elinav et al., 2013)

Multiple Treg subpopulations have been reported [Tr1 ($CD4^+$ Tregs induced by IL-10), Th2, Th3 (acting by TGF-β), $CD8^+$ Tregs, NKT ($CD3^+$, $CD56^+$)] (Garra et al., 2004), while naturally occurring Tregs are the most widely studied and well characterized subpopulation (Shevach , 2001). Natural Tregs (nTregs) express surface markers such as CD4, CD25, low CD127 (interleukin-7 receptor-α), CD152 (CTLA-4, cytotoxic T lymphocyte–associated antigen 4) and the transcription factor FoxP3 (Takahashi et al., 2000). However, one recent study observed high CD127 expression in the activated state of Tregs (Simonetta et al., 2010). Natural Tregs are derived from thymus, while induced Tregs (iTregs) are differentiated in periphery by cytokines released by $CD4^+$ cells (cell-to-cell contact) (Orentas et al., 2006).

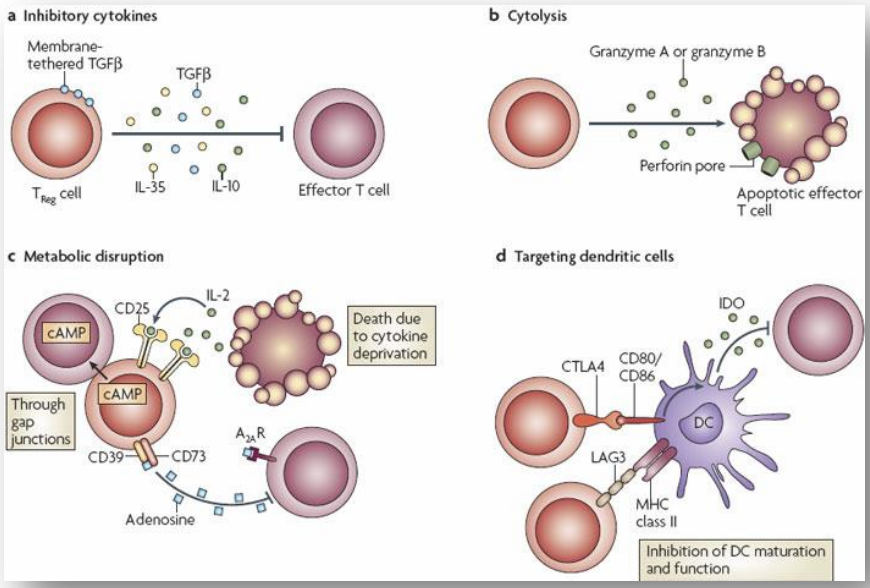

Figure 1.10: The functions of regulatory T cells. The mechanisms of regulatory T (Treg)-cell follow four basic modes of action. (**a**) Inhibitory cytokines include interleukin-10 (IL-10), IL-35 and transforming growth factor-beta (TGFβ). (**b**) Cytolysis includes granzyme-A- and granzyme-B-dependent and perforin-dependent killing mechanisms. (**c**) Metabolic disruption includes high-affinity CD25 (also known as IL-2 receptor alpha)-dependent cytokine-deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition, and CD39- and/or CD73-generated, adenosine receptor 2A (A2AR)-mediated immunosuppression. (**d**) Targeting dendritic cells (DCs) includes mechanisms that modulate DC maturation and/or function such as lymphocyte-activation gene 3 (LAG3)–MHC-class-II-mediated suppression of DC maturation, and cytotoxic T-lymphocyte antigen-4 (CTLA4)–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule made by DCs.(Vignali et al., 2008)

CD25 is the α-chain of the high-affinity receptor of interleukin-2 (IL-2R). Although nTregs do not produce IL-2, they are vitally dependent on IL-2 production by their environment (Allan et al., 2005). The constitutive expression of IL-2R on nTregs may reflect this dependence on external IL-2. $CD25^{+}$ T cells are not a homogenous population and could be split into subpopulations, based on their level of CD25 expression. $CD25^{high}$ T cells exhibit immune suppression function and their proportion in the PB correlated with the pathological stage of NSCLC (Baecher-Allan et al., 2001; Ju et al., 2009).

The FoxP3 gene encodes the transcription factor (TF) FoxP3 of the forkhead-box/winged-helix family. Extensive studies in mice and humans revealed the critical importance of FoxP3 transcription factor as a master regulator of nTreg development and function (Tai et al., 2005; Zhou et al., 2009). Concordant to CD25 expression-based characterization of Tregs, the majority of $CD4^{+}$ $FoxP3^{+}$ T cells are found to be $CD25^{high}$ (Baecher-Allan et al., 2004; Roncador et al., 2005). FoxP3 dimerizes with the nuclear factor of activated T cells (NF-AT) leading to suppression of IL-2, IL-4, and interferon-γ (IFN-γ) expression, while inducing expression of CD25 and cytotoxic T lymphocyte antigen 4 (CTLA-4) (Lopes et al., 2007; Wu et al., 2006).

The CTLA-4 molecule, a costimulator for Tregs, is an important factor in tumour progression. CTLA4 is constitutively expressed on Tregs and plays a role in regulating T cell tolerance (Chambers et al., 2001; Egen et al., 2002). CTLA-4 deficiency in Treg cells alone is sufficient to cause fatal disease, and maintenance of its expression in activated effector T cells is insufficient to prevent this outcome.

CD39, a cell surface-located prototypic member of the ecto-nucleoside triphosphate diphosphohy-drolase (E-NTPDase) family (Mizumoto et al., 2002), hydrolyzes ATP or ADP to AMP. CD39 is expressed by B cells, DCs, and subsets of T cells and has immune suppressive activity (Thiel et al., 2003). CD39 was initially described as an activation marker of lymphoid cells (Maliszewski et al., 1994) and is expressed by regulatory $FoxP3^{+}$ T (Treg) cells. CD39 is expressed on all $Foxp3^{+}$ T cells in mice, but in humans only a subset of nTreg cells coexpress Foxp3 and CD39 (Borsellino et al., 2007). $Foxp3^{+}CD39^{+}$ Treg cells play an important role in constraining pathogenic Th17 cells and in preventing autoimmune diseases (Borsellino et al., 2007).

Treg population presents a high degree of heterogeneity. Several markers allow the identification of phenotypically distinct Treg subsets which present different functional characteristics (figure 1.11). As described for conventional T cells, CD45RO expression has been notably considered to identify naïve and effector Tregs. On the other hand, loss of the CD127 marker expression was demonstrated to characterize the Terminal Effector from Effector/Memory subset. Moreover, Tregs are differentiated for the expression of $CCR7^{+}$ marker.

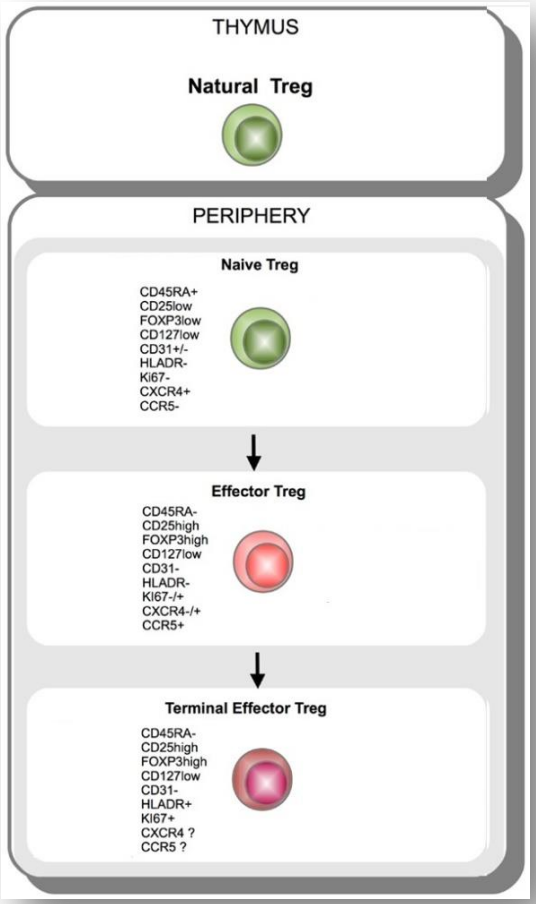

Figure 1.11: Schematic representation of Treg subtypes. CD4 T cells originate in the thymus as Natural $FOXP3^{+}$ Treg. Natural $CD45RA^{+}$ $FOXP3^{+/low}$ naïve Treg cells further differentiate into effector $CD45RA^{-}FOXP3^{high}$ and terminal effector $CD45RA^{-}FOXP3^{high}$ Tregs (Simonetta & Bourgeois , 2013).

Most $CD4^{+}CD25^{high}FOXP3^{+}$ regulatory T cells from adult peripheral blood express high levels of CD45RO (Fritzsching et al., 2006). However, a Treg subpopulation, which is consistently

apoptosis-resistant, was characterized by the absence of CD45RO marker. This subset has been detected in the peripheral blood and lymphoid organs and represents the precursor cells of conventional activated Treg cells (Fritzsching et al., 2006; Valmori et al., 2005). Naïve Tregs are Treg cells that released from the thymus and have not yet experienced antigen contact. Moreover, these cells are more specific for self rather than foreign antigens (Valmori et al., 2005). In addition, this subset express FOXP3 and are equally suppressive as their memory counterparts (Beyer & Schultze , 2007).

Effector Treg cells ($CD45RO^{+}CD127^{+/low}$) represent a short-lived differentiated population (Miyara et al., 2009). There are two types of effector Tregs, the "natural" or thymus-derived and the "induced" that are generated in the periphery in response to antigens. Effector Treg cells preferentially localize within nonlymphoid and inflamed tissues (Moser & Loetscher , 2001). Moreover, Effector Treg cells are found in higher frequency in tumour microenvironment compared to peripheral blood (Sugiyama et al., 2013). Terminal effector Treg population represents about 20–30% of human circulating Treg cells (Baecher-Allan et al., 2001; Baecher-Allan et al., 2006). Terminal Effector Treg cells are part of the effector Treg compartment of which they seem to constitute a terminally differentiated subset (Miyara et al., 2009). This subtype is differentiated by the absence of CD127 expression. It has been demonstrated that Treg function is inversely correlated with CD127 expression in human (Liu et al., 2006b). As a result, Terminal effector subpopulation was characterized as the most suppressive subset of Tregs (Baecher-Allan et al., 2006).

CC chemokine receptor 7 (CCR7) mediates the migration of naive $CD4^{+}$ T cells into the secondary lymphoid organs and is a prerequisite for their encounter with mature dendritic cells (Sallusto et al., 1999). CCR7 is expressed by naive and in resting memory Treg cells (Capitini et al., 2009; Sallusto et al., 1999). After T cell activation and differentiation, expression of CCR7 is lost on the memory T cell subset (Sallusto et al., 1999). $CCR7^{+}$ naïve Treg subset exhibits most naive Treg cell characteristics, such as high proliferative potential and low sensitivity to apoptotic cell death (Fritzsching et al., 2006). Similar to other naïve Treg subset, $CCR7^{+}$ naïve Treg cells proliferate vigorously in response to auto-antigens. Cells of the $CCR7^{+}$ naïve Tregs population are anergic and exert their suppressor activity through a mechanism that requires cell-to-cell contact. Moreover, $CCR7^{+}$ naïve Tregs express FoxP3 and have the ability to suppress the T cell function (Beyer & Schultze , 2007). The chemokine receptor CCR7 interacts

with the lymphoid chemokine CC chemokine ligand (CCL)21 and this interaction is sufficient for attracting naive T cells into secondary lymphoid tissues (Weninger et al., 2003).

As this subset is characterized by the expression of CCR7, which allows its recirculation through lymphoid tissues, Memory Tregs comprise two different subpopulations that differ in the expression of the lymphoid chemokine receptor CCR7; $CCR7^{+}$-Central Memory and $CCR7^{-}$-Terminal effector/memory Treg cells (Tosello et al., 2008). Central memory Treg cells home efficiently into lymphoid tissue, whereas Effector memory T cells have the potential to migrate into peripheral sites of inflammation. Both of them are differentiated from naïve on the basis of CD45RO expression. Central memory cells represent a non-polarized Ag-experienced cell population that lacks immediate effector cell functions (Miyara et al., 2009; Unsoeld et al., 2002). Previous studies demonstrated that the majority of peripheral Tregs, in healthy individuals, display a central memory phenotype (Mailloux & Epling-Burnette , 2013). On the other hand, effector memory cells are capable of producing cytokines after Ag recognition (Unsoeld et al., 2002). Terminal effector memory Treg cells are specialized to control ongoing immune responses directly within the inflammatory environment. Moreover, functionally, Terminal Effector Memory Treg cells are more suppressive than Central Memory Treg cells in vitro (Mailloux & Epling-Burnette , 2013).

1.5 NON SMALL CELL LUNG CANCER

Lung cancer has become the leading cause of cancer-related mortality worldwide and the overall 5-year survival rate is a dismal 15% (Jemal et al., 2002). The 2 main histopathological groups of lung cancer are small cell lung cancer (SCLC) (Schiller , 2001) and non-SCLC (NSCLC) (Zandwijk et al., 1995). Approximately 80% of lung cancers are diagnosed as non-small cell lung cancer (NSCLC), which is further classified, according to World Health Organization, into three main histologies: adenocarcinoma, which includes bronchiolar alveolar carcinoma, squamous cell carcinoma or epidermoid and large cell carcinoma (Travis , 2002). The tumour phenotype is dictated by the genetic alterations and the cell type in which the mutations occur (Jackson et al., 2001). The different tumour subclasses arise from distinct cells of origin localised within a defined regional compartment/microenvironment.

Adenocarcinomas are histologically heterogeneous peripheral masses that metastasize early and often occur in patients with underlying lung disease (Travis et al., 1995). Adenocarcinomas are the most common type of lung cancer and can be further divided into subgroups that differ not only in gene expression patterns, but also in clinical and pathological properties (Garber et al., 2001). One of these is bronchioloalveolar carcinoma, which is thought to arise from the epithelial lining of the alveolar spaces (Hammar , 1994). Moreover, adenocarcinomas have been implicated especially with K-ras and EGFR mutations (Husgafvel-Pursiainen et al., 1995).

Squamous cell carcinoma is thought to arise from a progressive dysplasia of metaplastic squamous epithelium (Hammar , 1994). Bronchial carcinogenesis is a multistep process involving transformation of the normal bronchial mucosa through a continuous spectrum of lesions, including basal cell hyperplasia, squamous metaplasia, dysplasia, and carcinoma in situ (CIS) (Lantuejoul et al., 2009; Travis & Brambilla , 2006). Morphologic changes are associated with a series of molecular events that accumulate as the squamous lesions progress through increasing dysplasia to invasive squamous cell carcinoma. Such changes include amplification of chromosome segment 3q26.33 (Bass et al., 2009). The transcription factor Sox2, which plays a key role in the proliferation and differentiation of basal progenitor cells, resides within 3q26.33, and was shown to be the relevant gene for the amplification (Bass et al., 2009)

The morphologic features that suggest squamous differentiation include intercellular bridging, squamous pearl formation, and individual cell keratinization. In well differentiated tumours these features are readily apparent; however, in poorly differentiated tumours they are difficult to find (Carlile & Edwards , 1986). Squamous cell carcinoma arises most often in segmental bronchi and involvement of lobar and mainstem bronchus occurs by extension. Squamous cell carcinoma can have papillary, clear cell, small cell and basaloid subtypes. Squamous cell carcinomas typically are centrally located endobronchial masses that may present with hemoptysis, postobstructive pneumonia, or lobar collapse (Patz , 2000). Squamous cell carcinoma tends to remain confined to the thorax, even when locally advanced, whereas adenocarcinoma and large cell carcinoma, which are not separable clinically, spread with great frequency, especially to the brain (Cox , 1986). Differences in serious haemorrhagic complications with vascular endothelial growth factor (VEGF) inhibitors in squamous carcinoma (Johnson et al., 2004) affect therapeutic choices. Squamous cell carcinomas are linked more strongly with smoking than other forms of non-small cell lung cancers, and are more

common in men than in women (Freedman et al., 2008). Large cell carcinoma a poorly differentiated subtype usually diagnosed by exclusion of the other three types of lung cancer (Garber et al., 2001). These tumours are large peripheral masses associated with early metastases (Travis et al., 1995).

Cancer of lung is staged using international TNM (Tumour, Node, Metastasis) classification in 4 stages. The 45% of the patients presenting with non-small cell lung cancer can be resected and are under Stage I (14%), Stage II (18.5%), Stage III (12.5%) and Stage IIIa (partial resectable). The other 55% of patients (Stage IIIb and IV) are non-respectable. Because 70% of lung cancers present in advanced stages, most patients are unresectable and the diagnosis is based on small biopsies and cytology. The choice of therapies is dependent on histology. For example, patients with adenocarcinomas and NSCLC not otherwise specified (NSCLC-NOS) are eligible for EGFR tyrosine kinase inhibitors (TKIs) if an EGFR mutation is present (Farina et al., 2011; Maemondo et al., 2010; Mitsudomi et al., 2010; Mok et al., 2009). Moreover, they are eligible for either pemetrexed-based (Ciuleanu et al., 2009; Scagliotti et al., 2009; Scagliotti et al., 2011; Scagliotti et al., 2008) or bevacizumab-based regimens (Johnson et al., 2004; Travis , 2002). In contrast, if the diagnosis is squamous cell carcinoma, patients are not eligible for these therapies.

1.5.1 Chemotherapy in Non–Small Cell Lung Cancer

Chemotherapy is a drug or combination of drugs that travels throughout the body to kill cancer cells. Effective treatment strategies are needed in order to reduce lung cancer mortality and recurrence rate. For advanced non–small-cell lung cancer, chemotherapy is often considered ineffective or excessively toxic. However, it was demonstrated that this strategy results in a small improvement in survival in patients with advanced non–small-cell lung cancer (Anelli et al., 2001). In addition, it was shown that chemotherapy reduces symptoms and improves the quality of life (Cullen et al., 1999).

A large variety of agents are available for the treatment of metastatic non–small-cell lung cancer, including the taxanes, gemcitabine, and vinorelbine. Combination of these agents with platinum compounds, results in high response rates and prolonged survival (Crino et al., 1997; Fossella et al., 2003; Sandler et al., 2000). The drugs that are included in the treatment depend on

the histological type of non-small cell lung cancer— adenocarcinoma, squamous cell carcinoma, or large cell lung cancer.

1.5.1.1 Chemotherapy drugs

Pemetrexed is an antifolate antimetabolite that targets multiple folate-dependent enzymatic pathways and inhibits multiple enzymes involved in purine and pyrimidine synthesis (Adjei , 2004). Pemetrexed enters cells via the reduced folate carrier and binds to folate receptor-α with a very high affinity (Zhao et al., 2000). Intracellularly, pemetrexed is polyglutamated to the active pentaglutamide by a reaction catalyzed by folylpolyglutamate synthase. Previous studies demonstrated that pemetrexed has antitumor activity in a variety of solid tumour cell lines. Pemetrexed can be combined with other cytotoxic agents, including cisplatin and has clinical activity in non-small-cell lung cancer (NSCLC) patients (Dubey & Schiller , 2005).

Gemcitabine (Gemzar) (difluorodeoxycytidine) is a potent and specific pyrimidine nucleoside antimetabolite which is structurally analogous to deoxycytidine (Hertel et al., 1988). Due to its hydrophilic nature, the induction of the drug into the cells requires to be active through highly specialized carriers, including the human equilibrative nucleoside transporter-1 (hENT1) and the human concentrative nucleoside transporter-1 and -3 (hCNT-1, hCNT-3) (Mini et al., 2006). Gemcitabine can be either deaminated to an inactive form (2', 2'-difluorodeoxyuridine, dFdU) by cytidine deaminase (CDA), or phosphorylated by deoxycytidine kinase (dCK) to dFdC-5'-monophosphate (dFdCMP) and to the active metabolites diphosphate and triphosphate (Peters et al., 2000). The dFdC-5'-diphosphate can inhibit the ribonucleotide reductase (RR), inhibits ribonucleotide reductase, an enzyme that produces deoxynucleotides that are required for DNA synthesis in S phase of dividing cells in S phase of dividing cells (Hertel et al., 1990). On the other hand, dFdC-5'-triphosphate results in early termination of DNA synthesis (Peters et al., 2000). Gemcitabine has emerged as an ideal partner for platinum compounds, because of its theoretical ability of interfering with the inhibition of repair of platinum-induced DNA damage.

Erlotinib (TARCEVA) and gefitinib (IRESSA) are two small molecular agents that target the tyrosine kinase domain of the EGFR (Sridhar et al., 2003). EGFR signalling plays a key role in promoting the growth and survival of various types of solid tumours, including non-small cell lung cancer (NSCLC). Both Erlotinib and Gefitinib have an inhibitory effect both on the autophosphorylation and downstream signalling, competing reversibly with the adenosine

triphosphate (ATP) for the catalytic domain of EGFR. Erlotinib has shown a significant improvement in median survival, quality of life, and related symptoms in NSCLC patients (Gridelli et al., 2007). On the other hand, the better quality of life and more favourable toxicity profile supported the use of gefitinib as a first-line therapy in patients with activating EGFR mutations (Costanzo et al., 2014).

Bortezomib (Velcade), a proteasome inhibitor, interferes with the cytosolic protein degradation machinery, namely the ubiquitin-proteasome complex, causing breakdown of cell-cycle regulators and cell-cycle arrest. The ubiquitin-proteasome complex is responsible for the degradation of cellular. Ubiquitin is the marker for the protein substrates that need to be degraded. Ubiquitin-marked proteins are presented to the 26S proteasome complex. The ubiquitin-proteasome pathway is responsible for the proteolysis of cellular proteins, including those involved in cell-cycle regulation, such as the cyclin-dependent kinases (CDKs) and their inhibitors (CDKIs) (King et al., 1996). Bortezomib is a proteasome inhibitor. By inhibiting proteasomes, bortezomib increases the levels of the CDKI p21 and causes G2-M cell-cycle arrest and, subsequently, apoptosis of tumour cells (Adams et al., 1999). Moreover, bortezomib inhibits vascular endothelial growth factor (VEGF) secretion in the bone marrow, inhibits VEGF-mediated caveolin phosphorylation, and decreases caveolin expression (Podar et al., 2004). Studies of bortezomib in combination with other antitumor agents in vitro and in vivo demonstrate that these combination regimens can offer additive/synergistic effects compared with the single agents (Schenkein , 2005).

Paclitaxel, a tubulin-binding agent, is widely used for the treatment of non-small cell lung cancer (NSCLC). The combination of paclitaxel and a platinum compound is an approved regimen for the treatment of advanced NSCLC. Paclitaxel and Docetaxel (Taxotere or Docecad) are members of the taxane family. Paclitaxel is the active ingredient in a crude extract from the bark of the Pacific yew tree (*Taxus Brevifolia*) which was found to have significant preclinical activity against a number of tumours (Wani et al., 1971). On the other hand, Docetaxel is a semi-synthetic combination formed by the esterification of a noncytotoxic precursor (Cortes & Pazdur , 1995). Both these agents promote microtubule assembly and polymerization. B y stabilizing the growing microtubule, theses drug disrupt the equilibrium between tubulin formation and breakdown, ultimately leading to the impairment of mitosis and then cell cycle arrest (Verweij et al., 1994). Docetaxel also has anti-angiogenic effect (Herbst & Khuri , 2003) and is able to induce

proinflammatory genes and proteins (Fitzpatrick & Wheeler , 2003). Docetaxel shown efficacy in the second-line treatment of non-small cell lung cancer (NSCLC), and has become a mainstay of NSCLC therapy.

Vinorelbine (Navelbine, NVB) as a single agent and in combination with cisplatin have demonstrated antitumor activity in patients with advanced non-small cell lung cancer (NSCLC). Vinorelbine (5′nor-anhydro-vinblastine) is a semi-synthetic vinca-alkaloid (Toso & Lindley , 2014). Vinorelbine induces cytotoxicity by inhibiting the polymerization of tubulin dimers into microtubules, which in turn disrupts mitotic spindle formation and prevents cell division. This promotes apoptosis of cancer cells (Bunn & Kelly , 1998). On the other hand, Zoledronic Acid (Zometa) is a nitrogen-containing bisphosphonate that inhibit the proliferation and induce apoptosis in a broad range of human cancer cells (Green , 2003). A number of studies suggest that zoledronic acid (ZOL) may provide clinically meaningful anticancer benefits in patients with advanced NSCLC (Zarogoulidis et al., 2009).

Cisplatin, *cis*-Diamminedichloro-platinum(II), is one of the most commonly used chemotherapeutic agents in the treatment of cancer, in particular non-small cell lung cancer (NSCLC) (Prestayko et al., 1979). Cisplatin interacts with DNA which has a result in the formation of DNA adducts which activate several signal transduction pathways and culminate in the activation of apoptosis (Kelland , 2000). Carboplatin is a derivative of cisplatin that has far less non-hematologic toxicity, although myelosuppression may be slightly greater than that observed with cisplatin (Brahmer & Ettinger , 1998).

1.5.1.2 Anti-VEGF therapy

Although adjuvant chemotherapy has made some progress in the treatment of locally advanced and advanced NSCLC, treatment outcomes are still disappointing (Bria et al., 2007). Better understanding of tumour biology and mechanisms of oncogenesis have allowed the addition of monoclonal antibodies direct against several molecular targets for NSCLC treatment. One of the targeted approaches, most widely studied in the treatment of NSCLC, is the inhibition of angiogenesis (Gridelli et al., 2006), which has been characterized as a promising strategy for treatment of cancer (Ferrara & Kerbel , 2005). Among angiogenesis inhibitors, the anti–vascular endothelial growth factor (VEGF) monoclonal antibody (mAb) bevacizumab (Avastin) represents the most successful targeted therapy. Bevacizumab (Avastin) is the leading anti-

angiogenic agent used currently in NSCLC and its effectiveness has also been investigated in multiple other solid tumours. Moreover, previous studies evaluated the contribution of bevacizumab to adjuvant chemotherapy (Soria et al., 2013). In chemotherapy-naive advanced NSCLC patients with non-squamous histology, the combination of bevacizumab with chemotherapy has demonstrated better efficacy outcomes than with chemotherapy alone (Sandler et al., 2006; Scagliotti et al., 2008).

Vascular supply is essential in order tumours to grow beyond 2 mm (Folkman , 1990). Neovascularization permits the growth of primary tumour and migrating tumour cells to gain access to the systemic circulation and to establish distant metastases. Loss of the angiogenic ability results in a dormant state of tumour, which is unable to metastasize (Folkman , 1971). In normal conditions, angiogenesis is well controlled. However, solid tumours secrete a range of factors that favour angiogenesis. Among these pro-angiogenic factors, VEGF is the most potent and specific of the endothelial cell mitogens (Herbst et al., 2005). Angiogenesis has an essential role in lung cancers. High microvessel density, in NSCLC patients, was demonstrated to be a prognostic factor; predictive for metastasis and poor survival (Meert et al., 2002). Moreover, high vascularity at the tumour periphery has been correlated with tumour progression (Ushijima et al., 2001).

Neovascularization is controlled by stimulatory (VEGF, basic fibroblast growth factor, platelet-derived growth factor, transforming growth factor-α, GM-CSF) and inhibitory (angiostatin, endostatin) factors, whose balance determines the degree of angiogenesis. VEGF is one of the most commonly upregulated angiogenic factor in tumours (Keshet & Ben-Sasson , 1999), and together with the VEGF receptors (VEGFRs), plays a pivotal role in normal and pathologic angiogenesis. Activation of the VEGF–VEGFR axis triggers multiple signalling networks that result in endothelial cell survival, mitogenesis, migration, and differentiation. Moreover, this activation results in the vascular permeability and the mobilization of endothelial progenitor cells from the bone marrow into the peripheral circulation. VEGF-A isoform, commonly referred to as VEGF, is a 45-kDa homo-dimeric glycoprotein with a diverse range of angiogenic activities. Bevacizumab, a recombinant humanized monoclonal antibody, blocks the binding of all VEGF-A isoforms to the receptors, inhibiting the biologic activities of VEGF.

Vascular endothelial growth factor (VEGF), detected in the tumour microenviroment, act as an obstacle against antitumor immunity (Zitvogel et al., 2006; Zou , 2006). It was demonstrated that VEGF is a suppressing factor in anti-tumour immunity.(Linderholm et al., 2000). VEGF induces development of Foxp3$^+$ Tregs through a TGF-β-dependent and/or independent pathway (Huang et al., 2006; Serafini et al., 2008; Yang et al., 2006). Moreover, it was shown that VEGF-A, could also directly induce Treg proliferation patients in a VEGFR2-dependent manner (Terme et al., 2013). Analysis of VEGFR expression on Tregs shows that Tregs express VEGFR2 only in tumour-bearing hosts (Terme et al., 2013). As a result, antiangiogenic molecules, especially those targeting VEGF-A signalling, can modulate Tregs. Treatment with Bevacizumab resulted in a drop in Treg numbers in many types of cancer, but this drop was observed only in responders (Passalacqua et al., 2008).

1.5.2 Immune Markers in Non–Small Cell Lung Cancer

Immune responses within the tumour microenvironment are implicated as a determining factor in tumour progression and aggressiveness. Immune markers are used to stratify prognosis of colorectal and ovarian cancer patients (Pages et al., 2005; Zhang et al., 2003). High levels of effector memory cells, within the tumour microenvironment, in colorectal cancer and CD3$^+$ T cells in ovarian cancer are associated with prolonged survival. The most published studies, which investigate the immune markers as prognostic markers in non–small cell lung cancer (NSCLC), are heterogeneous in histology and stage.

1.5.2.1 Tumour-infiltrating lymphocytes

The type, the density and the location of the immune cells in the tumour microenvironment influence the interactions of the tumour with immune cells (figure 1.12). Among T lymphocytes, which comprise 80% of tumour infiltrating lymphocytes (TIL) in NSCLC (Kataki et al., 2002), CD8$^+$ cytotoxic lymphocytes confer effector function in the adaptive immunity and are thought to have protective roles against tumours. However, no correlation between CD8$^+$ TIL infiltration and survival in NSCLC had been shown in recent published studies, while CD8$^+$ T-cell predominance was associated with smoking-related chronic obstructive pulmonary disease in NSCLC patients (Cosio et al., 2002) and it is thought to drive the progression of emphysema (Grumelli et al., 2004). This finding was explained in mouse models, as the presence of cigarette smoke impacted the activation of CD8$^+$ T cells (Kalra et al., 2000; Robbins et al., 2006). Other

studies indicate that B cells found in tumour-bearing mice are able to significantly reduce the T-cell proliferative response (Kiessling et al., 1999; Kusmartsev et al., 2000). The results about the correlation of $CD8^+$ TILs and prognosis are controversial (Ruffini et al.,; Wakabayashi et al., 2003). In addition, other study suggests that the $CD8^+$ T cells are able to infiltrate the tumour but they are not able to compute an antitumor response in the tumour nest (Trojan et al.,).

On the other hand, stromal $CD4^+$ (Al-Shibli et al., 2008; Ruffini et al.,) and the presence of stromal $CD8^+$ and $CD4^+$ cells in the tumour microenvironment (Hiraoka et al., 2006) are associated with better survival. The colocalization of those effector immune cells is thought to take part in the activation of an antitumor immune response (Hiraoka et al., 2006).

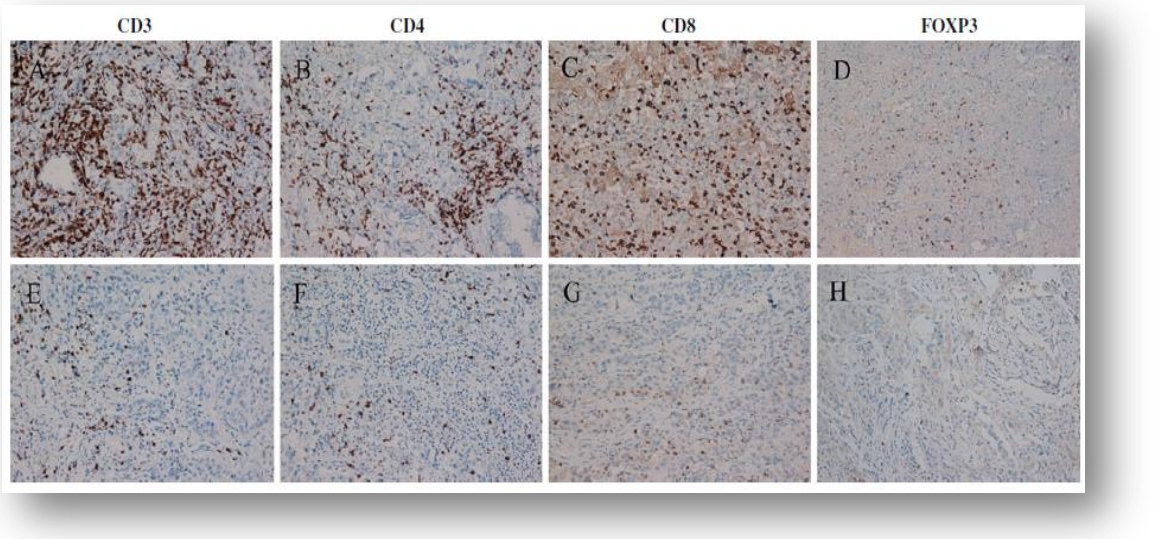

Figure 1.12: Immunohistochemical staining of intratumoural lymphocytes. Representative pictures of intratumoural lymphocyte infiltration in non-small cell lung cancer by immunohistochemical staining (x200). High density of $CD3^+$ TILs (a); $CD4^+$ TILs (b); $CD8^+$ TILs (c);and $FOXP3^+$ TILs (d). Low density of $CD3^+$ TILs (e); $CD4^+$ TILs(f); $CD8^+$ TILs (g); and $FOXP3^+$ TILs (h) (Liu et al., 2012).

1.5.2.2 Regulatory T cells

Preclinical and clinical studies have implicated regulatory T cells (Tregs) as one immunosuppressive entity (deLeeuw et al., 2012; Menetrier-Caux et al., 2012). The abundance and the immunosuppressive activity of Tregs can play a pivotal role in the inhibition of the immune response (figure 1.13). Previous studies provided direct evidence that patients with epithelial malignancies, in particular ovarian and non-small-cell lung cancer (NSCLC) displayed

increased levels of Tregs in the circulation and within the tumour infiltrating lymphocytes (TILs) (Woo et al., 2001). This increase may be chemokine receptors mediated. Treg frequency changes with treatment and response. Modulation of Tregs may have therapeutic implication in the management of advanced NSCLC. In previous studies, it was demonstrated that Tregs infiltrated in the tumour microenvironment were more proliferative than those found in the periphery, whereas peripheral Tregs were more immunosuppressive than tumour-infiltrating ones (Lutsiak et al., 2008). Because of the difficulty in obtaining biopsy material from metastatic carcinoma lesions, there is a trend in analysis of Treg immunosuppressive functions by the Tregs found in the periphery.

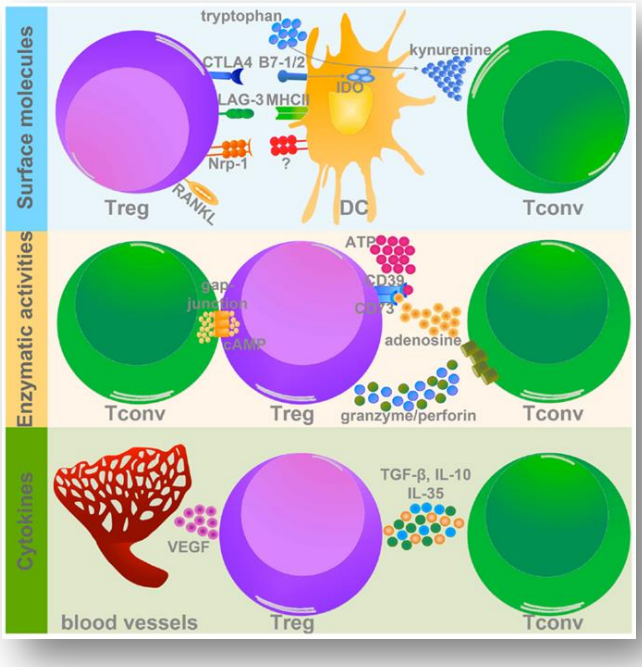

Figure 1.13: Treg suppressive mechanisms in tumour microenvironment. Treg cells use different strategies to inhibit target cells within the tumour mass. Three types of Treg-related molecules can mediate these suppressive mechanisms; surface molecules, enzymes and cytokines. Surface molecules expressed by Treg cells take part in the promotion of tumour progression, mainly modulating DC activation and function. CTLA-4 and LAG-3, binding to CD80/CD86 (B7-1/2) and MHCII respectively, impair DC capacity to activate T cells. In addition CTLA-4 promotes IDO expression and the production of the pro-apoptotic metabolite kynurenine. Nrp-1 stabilizes Treg-DC contact, allowing Treg cells to adequately suppress DC. The ecto-enzymes CD39 and CD73 generate adenosine, which is endowed with tolerogenic effects. Granzyme and perforin induce the apoptosis of target cells by cytolysis. Moreover, Treg cells secrete several immune-modulatory cytokines, which directly modulate T cell functions (TGF-β,

IL-10, and IL-35), or indirectly promote the establishment of pro-tumorigenic microenvironment (VEGF) (Burrochi et al., 2013).

Regulatory T cells (Tregs) are enriched between tumour infiltrated lymphocytes (TILs) in NSCLC patients. These Treg cells suppress the immune response possibly through the secretion of TGF-β or directly by contact inhibition (Woo et al., 2001; Woo et al., 2002). In addition, a number of studies have suggested a critical role for Tregs in dampening NK cell effector functions (Ghiringhelli et al., 2006). In addition to the down-regulation of the adaptive effector phase of immunity, Tregs would also modulate early and innate immune reactivity. NK cells are dynamic sentinels of immune surveillance, which resist to airborne intracellular infections and transformed cells (Curtis , 2005), and thus contribute to the first line of defence in lungs. The majority of the infiltrated Treg cells in NSCLC were characterized as TIM-3$^+$ (Gao et al., 2012). T cell immunoglobulin-3 (TIM-3) is a negative regulatory molecule and plays a critical role in immune tolerance (Gao et al., 2012). TIM-3 expression on Tregs was correlated with poor clinical outcome, such as nodal metastasis and advanced cancer stages, in NSCLC patients.
Some tumour cells secrete the chemokine CCL (Lee et al., 2004), which attracts Tregs to the tumour site (Zou , 2006). As a result, the activity of effector T cells is suppressed (Zou , 2006). Tumour-infiltrating FoxP3$^+$ Tregs were, also, correlated with cyclooxygenase-2 (COX-2) expression, as tumour-derived COX-2 and its product prostaglandin E2 (PGE2) induces in vitro lymphocyte expression of FoxP3 (Sharma et al., 2005). In previous studies, it was shown that, in NSCLC, COX-2/PGE2 is overexpressed and is correlated with a poor outcome in patients with early-stage disease (Laga et al., 2005). In addition, the production of cytokines such as TGF-β and interleukin-10 by infiltrated regulatory T cells was associated with tumour progress. The percentage of the Tregs within the TIL population is correlated with the prognosis of the NSCLC patients. Moreover, it was demonstrated that, in squamous NSCLC, the risk of tumour recurrence was correlated with higher proportion of Tregs between TILs (Lutsiak et al., 2008).

Little is known about the regulatory T cells (Tregs) in the peripheral blood of non-small cell lung cancer (NSCLC) patients. The Treg percentage in advanced stage patients (III-IV) was found to be significantly higher, compared the values shown by controls (Chen et al., 2014). As a consequence, it was indicated that the percentage of Tregs correlates with the pathological stage in NSCLC (Chen et al., 2014). Another phenotype of highly suppressive cells

($CD13^{+}CD4^{+}CD25^{high}$ Tregs) was defined (Ju et al., 2009). The proportion of this phenotype in the peripheral blood correlated with the pathological stage of NSCLC. Proteins with immunosuppressive function are highly expressed in this subset (CTLA-4, TGFβ and PD-L1) (Ju et al., 2009). The chemokine receptor CCR7 is another crucial lymphoid homing molecule for immune cells (Britschgi et al., 2008). Human peripheral blood Treg cells express CCR7 to some degree and this marker is crucial for their characterization as inactivated cells. Moreover, this marker is essential for their migration in response to CCL21, the ligand for CCR7 (Forster et al., 1999). Upon activation, the chemokine receptor expression on Treg cells is switched from CCR7 to CXCR5. This change in chemokine receptor expression is consistent with reduced migration toward CCL21 and increased migration toward CXCL13, a chemokine expressed in the B-cell zone (Britschgi et al., 2008). This suggests that upon activation, Treg-cell migration toward the T-cell zone changes to trafficking toward B-cell follicles.

The metastatic growth of tumours has been shown to be associated with a loss of MHC I expression and of other proteins involved in the processing of antigenic peptides (i.e., TAP1), thereby down-regulating the presentation of T cell activating epitopes (Kaklamanis et al., 1994). Previous studies demonstrated the negative impact of Tregs on the generation of tumour-specific CTLs as well as on effector mechanisms mediated by the innate immune response (Onizuka et al., 1999; Shimizu et al., 1999).

The levels of Tregs are elevated in the peripheral blood of NSCLC patients compared with normal healthy volunteers (Li et al., 2009; Okita et al., 2005), while the levels of TGF-b and IL-10, both known promoters of Treg development, are increased in serum and plasma of NSCLC patients compared with healthy controls (Baratelli et al., 2010; De Vita et al., 2000). However, the predictive value of these markers has not been investigated in NSCLC patients.

1.5.2.3Tumour-associated macrophages

Tumour-associated macrophages (TAM) show antitumor effects in the tumour microenvironment (Mantovani & Sica , 2010). The most common phenotype of TAMs in the tumour microenvironment in NSCLC is the M1, whereas protumour TAMs of the M2 phenotype accumulate in the stroma and express interleukin-8 (IL-8) and interleukin-10 (IL-10). IL-8 is an angiogenic factor and the higher density of protumour TAMs in the tumour microenvironment was correlated with higher counts of microvessels in NSCLC (Chen et al., 2003). The angiogenic role of protumour TAMs through IL-8 production was also correlated with poor patient

outcome. Moreover, IL-10 is an immunosuppressive cytokine, and its expression by protumour TAMs has been correlated with decreased OS (Zeni et al., 2007).

On the other hand, M1 TAMs are associated with increased survival (Kim et al., 2008). M1 TAMs are characterized by the expression of human leukocyte antigen (HLA)-DR, inducible nitric oxide synthase (iNOS), myeloid-related protein (MRP) 8/14, and tumour necrosis factor (TNF)-a (Ohri et al., 2009).

1.5.2.4 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSC) represent a heterogeneous population of myeloid cells, which include immature macrophages, granulocytes, and DCs at early stages of differentiation (figure 1.14). MDSCs are marked by $CD11b^{+}CD14^{-}CD15^{+}CD15^{+}$ and they exert their protumour effect by inhibiting T-cell proliferation and activation. They are mobilized by tumour-derived factors from bone marrow into the peripheral blood and accumulate in the tumour microenvironment (Kusmartsev et al., 2005). Increased levels of MDSCs have been observed in the peripheral blood of advanced stage NSCLC patients (Liu et al., 2010) and are associated with decreased levels of $CD8^{+}$ T cells.

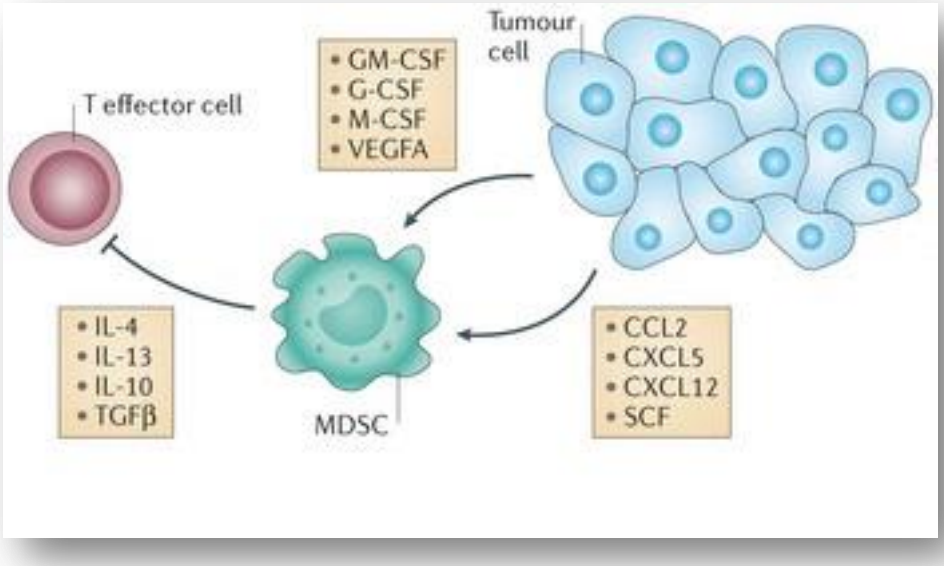

Figure 1.14: MDSC cells and inflammatory signalling. Tumours also promote the generation of myeloid-derived suppressor cells (MDSCs) via the secretion of growth factors and chemo attractants. MDSCs suppress antitumor T cell responses by the secretion of cytokines such as interleukin-4 (IL-4), IL-13, IL-10 and transforming growth factor-β (TGFβ). CXCL, CXC-chemokine ligand; G-CSF, granulocyte

colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; SCF, stem cell factor; VEGFA, vascular endothelial growth factor A. (Elinav et al., 2013)

1.5.2.5 Tumour-associated neutrophils

In mouse models of lung cancer, TGF-b induces a population of Tumour-associated neutrophils (TAN), which has a protumour function (N2), whereas TGF-b blockade results in antitumor neutrophils (N1) (Fridlender et al., 2009). Depletion of these N1 neutrophils results in increased tumour growth.

1.5.2.6 Peripheral blood lymphocytes

The use of peripheral blood lymphocytes as a prognostic marker is investigated in cancer, due to their availability. In NSCLC, increased levels of total lymphocyte counts are associated with lower hazard ratios (HR) for death (Sarraf et al., 2009). Moreover, the neutrophil to lymphocyte ratio (NLR) is correlated with survival (Sarraf et al., 2009).

1.6 AIM OF THE STUDY

Several types of immunosuppressive mechanisms in cancer patients have been reported to date. Regulatory T cells (Tregs), which express the transcription factor forkhead box P3 (FoxP3), are considered to play a major role in hampering antitumor immune response. T regulatory cells (Tregs) have been shown to increase significantly in hosts with advanced malignancies. An increase in Treg numbers enables cancer progression by dampening the immune system and allowing tumour cells to evade immune detection and destruction. However, the clinical significance of Tregs in patients with NSCLC cancer has not been fully elucidated.

The aims of this study were to determine the phenotypic and functional characteristics of circulating Treg subtypes in the peripheral blood in NSCLC patients. Moreover, we evaluated the percentage of different Treg subtypes, which were characterized in other cancer types, in the blood of NSCLC patients compared to normal control. In the next step, we determined the effect of chemotherapy in the percentage of Treg subpopulations, as compared with the levels at baseline, and the differences in the changes that are mediated from conventional treatment and bevacizumab-based therapy. Furthermore, the purpose of this study was to investigate the

relationship between the frequency of the circulating Treg subsets and the clinical outcome of patients with stage III and IV NSCLC. Specifically, peripheral blood in EDTA was obtained from treatment-naïve NSCLC patients and aged-matched heathy donors. The presence of circulating Treg subsets was examined by studying the presence of intra- and extracellular markers in mononuclear cells of blood using flow cytometry. The functionality of these cells was tested after their isolation from PBMCs, using magnetic separation. Finally, in order to determine the association between the percentages of Treg cells with the clinical outcome, patients who enrolled in the study were evaluated as clinically documented stable (SD) or progressive disease (PD) after three and six cycles of treatment.

Chapter 2

Materials & Methods

2.1 PATIENTS AND HEALTHY DONORS

Peripheral blood in EDTA was obtained from 145 treatment-naïve NSCLC patients at the time of diagnosis and before administration of any systemic or local treatment. All patients were older than 18 years old. Patients that received any immunosuppressive drugs or G-CSF injections prior to immune testing were excluded from the study. Patients enrolled were diagnosed with inoperable, locally advanced (Stage III with pleural effusion or severe respiratory failure) or metastatic (Stage IV) NSCLC.

One hundred seventeen patients with advanced NSCLC were treated with 4-6 cycles of platinum-based chemotherapeutic regimen (24. 8% and 75.2% with or without bevacizumab, respectively). Only 73 patients were evaluated for assessment of total clinical outcome, while the rest of them received only one cycle of treatment because of early death or received immunosuppressive drugs or G-CSF.

Thirty one (31) age- and sex-matched healthy volunteers (23 males and 8 females; age 64 years) were enrolled in the study. Blood was collected prior chemotherapy, at the 4th cycle and after the 6th cycle of treatment. The protocol was approved by the Ethics and Scientific Committees of the University Hospital of Heraklion. All patients and controls signed a written informed consent in order to take part to the study.

2.2 PATIENTS SAMPLES FOR IMMUNOPHENOTYPIC ANALYSIS

Peripheral blood (5 ml) was collected by venipuncture into tubes containing EDTA anticoagulant (BD Biosciences, Europe) and after centrifugation, the plasma was removed and stored at -80^{0}C.

2.3 CELL ISOLATION

2.3.1 Experimental procedure

Each peripheral blood sample (5 ml EDTA-treated whole blood) was incubated with 45ml 1X Red Blood Cell (RBC) lysing buffer at room temperature for 20 min. RBC Lysis Buffer is supplied as a 10X solution containing ammonium chloride, potassium carbonate, and EDTA, and is diluted in deionized water prior to use. The white blood cells were collected by centrifugation at 500xg for 5 minutes at room temperature. The supernatant was discarded and the white blood cell pellet was re-suspended in 1ml of FACs buffer (1% FCS, 0.01% NaN3 in PBS; Sigma, USA). 15 ml of flow buffer were added twice in the tube in order to wash the cells. Cells were then resuspended at 100µl of a FACs buffer (final concentration of $1x10^7$/ml) for immunophenotypic analysis.

2.4 IMMUNOPHENOTYPIC ANALYSIS OF CELLS

2.4.1 Principles of method

Flow cytometry provides rapid analysis of multiple characteristics of single cells. The information obtained is both qualitative and quantitative. Flow cytometry measures optical and fluorescence characteristics of single cells (or any other particle, including nuclei, microorganisms, chromosome preparations, and latex beads). Physical properties, such as size (represented by forward angle light scatter) and internal complexity (represented by right-angle scatter) can resolve certain cell populations (Baumgarth & Roederer , 2000). Antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells. When labelled cells are passed by a light source, the fluorescent molecules are excited to a higher energy state. Upon returning to their resting states, the fluorochromes emit light energy at higher wavelengths. The use of multiple fluorochromes, each with similar excitation wavelengths and different emission wavelengths (or "colours"), allows several cell properties to be measured simultaneously (McLaughlin et al., 2008). Commonly used dyes include propidium iodide, phycoerythrin, and fluorescein, although many other dyes are available. Tandem dyes with internal fluorescence resonance energy transfer can create even longer wavelengths and more colours (Baumgarth & Roederer , 2000).

Inside a flow cytometer, cells in suspension are drawn into a stream created by a surrounding sheath of isotonic fluid that creates laminar flow, allowing the cells to pass individually through an interrogation point. At the interrogation point, a beam of monochromatic light, usually from

a laser, intersects the cells (Perfetto et al., 2004). Emitted light is given off in all directions and is collected via optics that direct the light to a series of filters and dichroic mirrors that isolate particular wavelength bands. The light signals are detected by photomultiplier tubes and digitized for computer analysis. The resulting information usually is displayed in histogram or two-dimensional dot-plot formats (Baumgarth & Roederer , 2000) (figure 2.1).

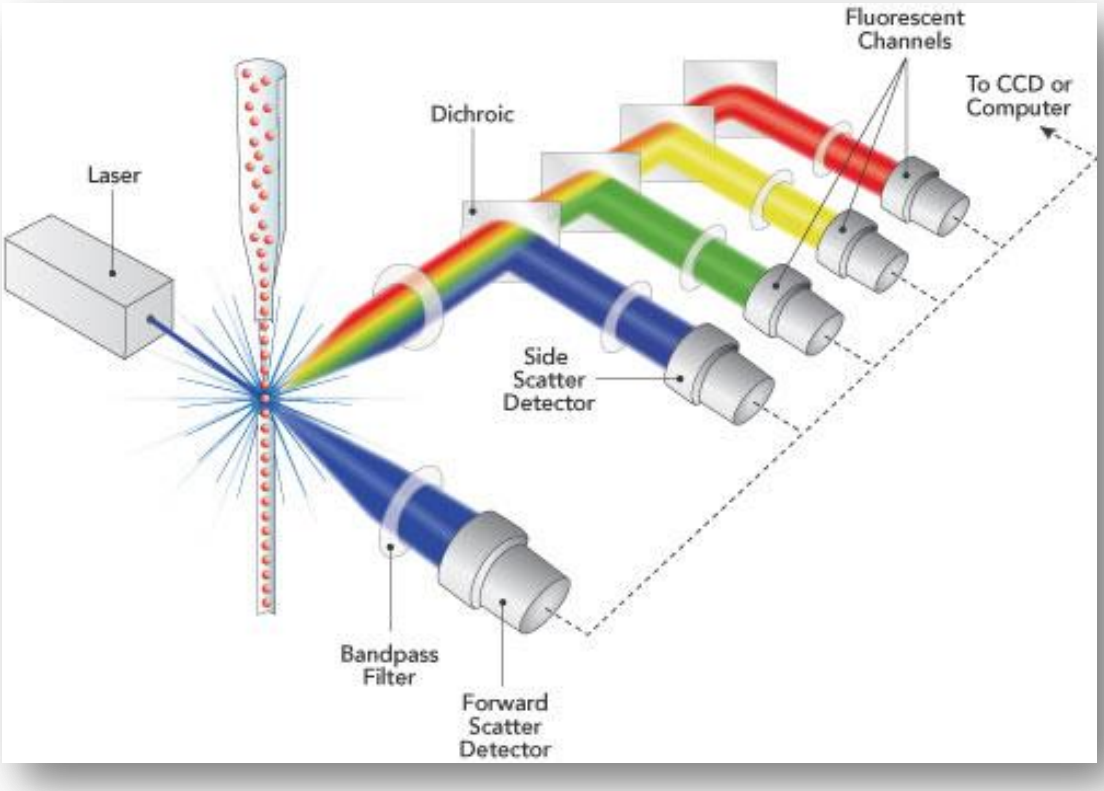

Figure 2.1: The flow cytometer. Scattered and emitted light signals are converted to electronic pulses that can be processed by the computer.

2.4.2 Experimental procedure

To determine the regulatory T cell phenotype, eleven-colour flow cytometry of whole blood was performed. The cell surface markers in the white blood cells were detected after labelling by human monoclonal antibodies conjugated with a fluorochrome. The specificity provided by monoclonal antibodies makes them ideal for use as diagnostic reagents, and therefore the ability to conjugate these proteins with a variety of fluorochromes adds to their flexibility and utility in flow cytometric applications. The antibodies used in staining are the following: anti-CD4-V500; anti- CD3-PE-CF594; anti-CD25-PE-Cy7; anti-CD127-V450; anti-CD8-APC-Cy7; anti-CD45RO-

Alexa700; anti- CD197 (CCR7)-PE; anti-CD13-PerCP-CY5.5 and anti-CD39-APC (all purchased from BD Biosciences, Biolegent and R&D). The white cells were incubated with the appropriate amounts of fluorochrome-labelled Abs for surface staining in the dark at 4^0C for 30 min followed by two washes.

Then, cells were fixed and permeabilized using FoxP3 Buffer set (BD Pharmygen). The FoxP3 Buffer A (10X concentrate) was diluted 1:10 with room temperature (20°C to 25°C) deionized water. The cells were fixed using 2 mL of 1X working solution Human FoxP3 Buffer A in each tube (incubation for 10 minutes at room temperature, protected from light) followed by two washes. To make a working solution of Buffer C, FoxP3 Buffer B (50X) was diluted into 1X FoxP3 Buffer A at a ratio of 1:50 (Buffer B: Buffer A). In order the cells to be permeabilized, 0.5 ml of 1X working solution Human FoxP3 Buffer C were added in each tube and the mix was incubated for 30 minutes, protected from light, followed by two washes. The cells were then stained with anti-FoxP3-FITC and anti-CTLA-4 (CTLA-4)-PE-Cy5 (all purchased from BD Biosciences) for 1 hour at 4^0C and washed twice. After washing, cells were resuspended in 0.5ml flow buffer and a multicolour analysis was performed on a BD LSR II Flow Cytometer (BD Biosciences).

Analysis of FACS data was done using FACS Diva Software (BD Biosciences). BD FACS Diva software is a data acquisition and analysis package specifically designed for digital-based flow cytometers. For T-cell subset the acquisition and analysis gates were restricted to the lymphocyte population. $CD3^+$ $CD4^+$ cells were calculated as a percentage of $CD3^+$ lymphocytes. Each measurement contained 100,000 events.

2.5 PATIENTS SAMPLES FOR Treg ISOLATION ASSAY

Peripheral blood (50 ml) from five treatment-naïve Non-Small Cell Lung Cancer (NSCLC) patients was collected by venipuncture into tubes containing EDTA anticoagulant (BD Biosciences, Europe). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Sigma, UK) density centrifugation and the Treg population was collected by magnetic separation.

2.6 PERIPHERAL BLOOD MONONUCLEAR CELLS ISOLATION

2.6.1 Principles of method

Ficoll-Hypaque density gradient centrifugation is a method of purifying peripheral blood mononuclear cells (PBMC). Ficoll is a neutral, highly branched, high-mass, hydrophilic polysaccharide which dissolves readily in aqueous solutions. It is prepared by co-polymerization of the polysaccharide with epichlorohydrin. The basis for this cell separation assay is the differential migration of cells during centrifugation according to their buoyant density, which results in the separation of different cell types into distinct layers. Mononuclear cells and platelets collect on top of the Ficoll-Hypaque layer because they have a lower density. In contrast, red blood cells (RBC) and granulocytes have a higher density than Ficoll-Hypaque and collect at the bottom of the Ficoll-Hypaque layer (figure 2.2).

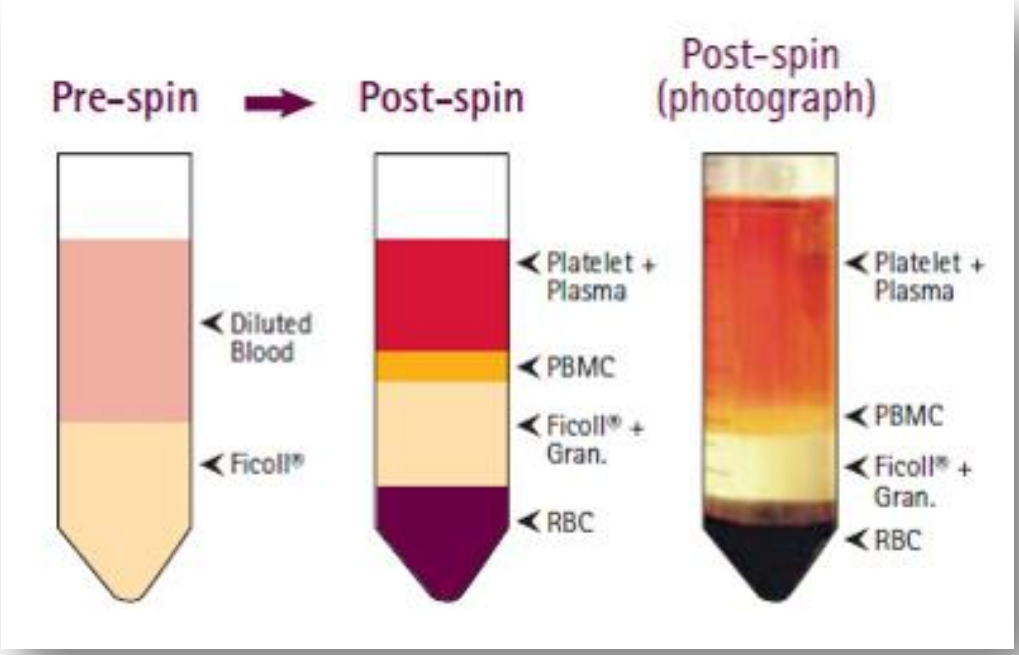

Figure 2.2: Schematic of Ficoll separation of human peripheral blood. Differential migration following centrifugation results in the formation of several cell layers. Mononuclear cells (lymphocytes and monocytes) and platelets are contained in the banded plasma-LSM interphase due to their density, and the pellet that is formed contains mostly erythrocytes and granulocytes, which have migrated through the gradient to the bottom of the tube. Lymphocytes are recovered by aspirating the plasma

layer and then removing the cells. Excess platelets, Ficoll and plasma can then be removed by cell washing with isotonic phosphate buffered saline.

2.6.2 Experimental procedure

Peripheral blood sample (50 ml EDTA-treated whole blood) was diluted with an equal volume of washing medium [RPMI 1640 (Gibco) supplemented with antibiotics P/G/S (40U/ml penicillin, 2Mm glutamine, 40U/ml streptomycin)], for a total volume of 100ml, in two sterile 50ml falcon tubes. Ficoll-Hypaque 1077 (Sigma) (12.5ml) was pipetted into four separate sterile 50ml falcon tubes. The diluted blood sample (25ml in each tube) was layered slowly onto the Ficoll-Hypaque 1077 (Sigma) (1:2), without disturbing the Ficoll layer. The four tubes were centrifuged at 1,200 rpm for 30 min without brake. Using a clean Pasteur pipette, the lymphocyte layer (middle milky ring) was transferred to two clean sterile 50ml falcon tubes. The lymphocytes were washed, using washing medium, and then centrifuged at 2,000 rpm for 15min, with brake. Then, the supernatant was discarded and the pellet was re-suspended in 1ml of washing medium. The two pellets were combined in one tube and then centrifuged at 2,000 rpm for 15min, with brake. The supernatant was discarded and the pellet was resuspended in 10 ml of complete medium [RPMI 1640 (Gibco) supplemented with 10% Fetal Calf Serum (FCS) and antibiotics P/G/S (40U/ml penicillin, 2Mm glutamine, 40U/ml streptomycin)]. Ten (10) μl of cells were diluted in Trypan Blue (1:10) and then counted in the cell counter. Trypan Blue is a dyazo dye that is derived from toluidine. The Trypan Blue stain is most commonly used to distinguish viable from nonviable cells. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, Eosin, or propidium, while nonviable cells absorb the dye and appear blue. Moreover Trypan Blue has a higher affinity for serum protein than for cellular proteins, so suspending cells in medium containing serum will generate a dark background. This method is also referred as dye exclusion test. Moreover, 200μl of the cell suspension were used for immunophenotypic analysis, as described above.

2.7 $CD4^+$ $CD25^+CD127^{-/dim}$ REGULATORY T CELL ISOLATION

2.7.1 Principles of method

Magnetic separation is an emerging technology using magnetism, sometimes in combination with conventional separation or identification methods, to purify cells, cell organelles and biologically active compounds (nucleic acids, proteins, xenobiotics) directly from crude samples. Cells of interest have to be tagged by a magnetic label to achieve the required contrast in magnetic susceptibility between the labelled and unlabelled cells. The attachment of magnetic labels is usually attained by the use of affinity ligands of various types, which can interact with target structures on the cell surface. Usually antibodies against specific cells surface epitopes are used, but other specific ligands can also be employed. The formed complexes have magnetic properties and can be manipulated using an appropriate magnetic separator.

There are two basic strategies for separating specific cell populations: positive selection and depletion. During positive selection, the target cells are magnetically labelled and collected as the positive fraction. During depletion, the unwanted cells are labelled and depleted from the target cells. The target cells are collected as the negative fraction. Sequential sorting allows the performance of two consecutive separations. The autoMACS Pro Separator provides a selection of twelve pre-set separation programs.

The autoMACS Pro Separator is a bench top instrument for high-speed magnetic cell sorting of multiple samples, which is designed for cell isolation in a fully automated, walk-away fashion. AutoMACS Columns contain a matrix composed of ferromagnetic spheres covered with a cell-friendly coating. When placed on a magnetic separator, the spheres amplify the magnetic field by 10,000-fold, thus inducing a high gradient within the column. This is crucial for isolation of cells which are only minimally labelled, leaving enough epitopes free for concurrent antibody staining.

A cocktail of biotin-conjugated monoclonal anti-human antibodies against CD8, CD19, CD123, and CD127 was used. Moreover, Anti-Biotin and $CD25^+$ MACS MicroBeads were used. MACS MicroBeads are 50-nm super paramagnetic particles that are conjugated to highly specific antibodies against a particular antigen on the cell surface. Due to the small size, they do not

activate cells and they do not saturate cell surface epitopes. Moreover, this type of beads does not have to be removed for any downstream application.

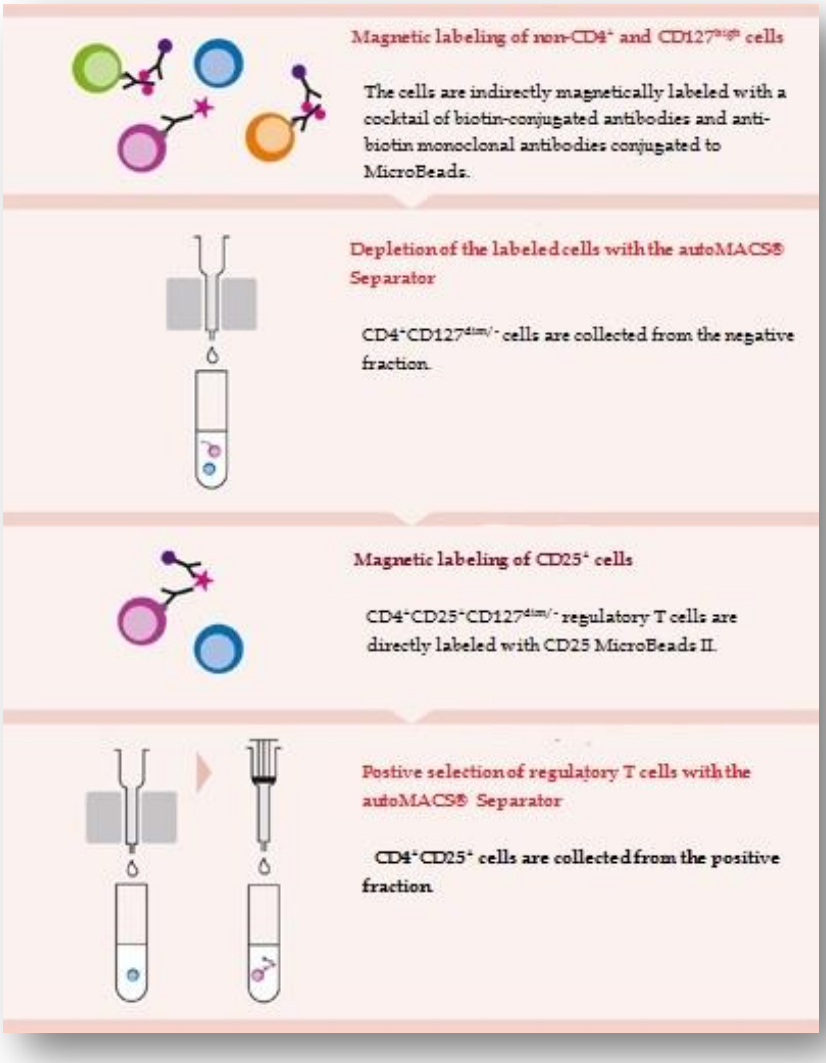

Figure 2.3: Magnetic isolation of CD4$^+$ CD25$^+$ CD127$^{dim/-}$ Regulatory T cell Isolation. The isolation of CD4$^+$ CD25$^+$ CD127$^{dim/-}$ regulatory T cells is performed in a two-step procedure. First, the non- CD4$^+$ and CD127^{high} cells are indirectly magnetically labelled with a cocktail of biotin-conjugated antibodies, as primary labelling reagent, and anti-biotin monoclonal antibodies conjugated to MicroBeads, as secondary labelling reagent. The labelled cells are subsequently depleted by separation with an autoMACS® Pro Separator. In the second step, the CD4$^+$ CD25$^+$ CD127$^{dim/-}$ regulatory T cells are directly labelled with CD25 MicroBeads II and isolated by positive selection from the pre-enriched CD4$^+$ T cell fraction by separation over an autoMACS Pro Separator.

2.7.2 Experimental procedure

The cell suspension of isolated PBMCs was centrifuged at 300xg for 10 minutes and the supernatant was aspired completely. The cell pellet was resuspended in 40μl of cold (2-8^{0}C) buffer (phosphate-buffered saline, ph 7.2, 0.5% bovine serum albumin (BSA) and 2mM EDTA) per 107 total cells. The isolation of $CD4^+$ $CD25^+CD127^{-/dim}$ Regulatory T cells was performed in a two-step procedure. First, the non-$CD4^+$ and $CD127^{high}$ cells were indirectly magnetically labelled with a cocktail of biotin-conjugated antibodies, as primary labelling reagent, and anti-biotin monoclonal antibodies conjugated to MicroBeads, as secondary labelling reagent. 10 μl of $CD4^+$ $CD25^+CD127^{-/dim}$ T Cell Biotin-Antibody Cocktail II per 10^7 cells were added in the cell suspension. The cell suspension was mixed well and was incubated for 10 minutes in the refrigerator (2-8^{0}C). 30μl of buffer and 20μl of Anti-Biotin Microbeads per 10^7 total cells were added and the cell suspension was mixed well and was incubated for 30 minutes in the refrigerator (2-8^{0}C). The cells were washed by adding 1-2ml of buffer per 10^7 total cells and centrifuged at 300xg for 10 minutes. The supernatant was aspirated completely and the pellet was resuspended in 500μl of buffer for up to 10^8 total cells.

Two autoMACS Columns were placed in the autoMACS Pro Separator. The instrument was prepared and primed and the sample tube was placed in the row A of the tube rack. The fraction collection tubes were also placed in rows B and C. The program Depletion: "Depl025" was chosen and the negative fraction was collected in row B of the rack tube. 200μl of the cell suspension were used for immunophenotypic analysis, as described above. The antibodies used in staining are the following: anti-CD4-V500; anti- CD3-PE-CF594; anti-CD25-PE-Cy7; anti-CD127-V450; anti-CD8-APC-Cy7 and anti-CD19-FITC. In addition, 10μl were diluted in Trypan Blue (1:10) and then counted in the cell counter.

In the second step, the $CD4^+$ $CD25^+CD127^{-/dim}$ regulatory T cells were directly labeled with CD25 MicroBeads II and isolated by positive selection from the pre-enriched $CD4^+$T cell fraction. The cell suspension was centrifuged at 300xg for 10 minutes and the supernatant was aspirated completely. The pellet was resuspended in 90μl of buffer and 10μl of CD25 Microbeats II were added, per 10^7 total cells. The mix was incubated for 15 minutes in the refrigerator (2-8^{0}C). The cells were washed by adding 1-2 ml of buffer and centrifuged at 300xg for 10 minutes. Then, the supernatant was aspirated completely and the pellet was resuspended in 500μl of buffer for up

to 10^8 total cells. The instrument was prepared and primed and the sample tube was placed in the row A of the tube rack. The fraction collection tubes were also placed in rows B and C. The program Positive Selection: "Posseld2" was chosen and the positive fraction was collected in row C of the rack tube, which was the enriched $CD4^+$ $CD25^+CD127^{-/dim}$ T cell fraction. 200μl of the cell suspension were used for immunophenotypic analysis, as described above. The antibodies used in staining are the following: anti-CD4-V500; anti- CD3-PE-CF594; anti-CD25-PE-Cy7; anti-CD127-V450; anti-CD8-APC-Cy7 and anti-CD19-FITC. Moreover, 10μl were diluted in Trypan Blue (1:10) and then counted in the cell counter.

2.8 RESPONDER CELLS' ACTIVATION ($CD4^+$ $CD25^-$)

2.8.1 Principles of method

Activation of $CD4^+$ $CD25^-$ cell was performed by the Dynabeads ® Human T-Activator CD3/CD28 (Gibco). Dynabeads® Human T-Activator CD3/CD28 offer a simple method for activation and expansion of T cells that does not require feeder cells (antigen-presenting cells) or antigen. This product contains 4×10^7 beads/mL in phosphate buffered saline (PBS), pH 7.4, with 0.1% human serum albumin (HSA). Dynabeads® Human T-Activator CD3/CD28 are uniform 4.5 μm, super paramagnetic polymer beads coated with an optimized mixture of monoclonal antibodies against the CD3 and CD28 cell surface molecules of human T cells. The CD3 antibody is specific for the epsilon chain of human CD3, which is a subunit of the TCR complex. The CD28 antibody is specific for the human CD28 co-stimulatory molecule, which is the receptor for CD80 (B7-1) and CD86 (B7-2). Both antibodies are mouse anti-human IgGs coupled to the same bead, mimicking in vivo stimulation by antigen-presenting cells (APCs). Both the bead size and the covalent antibody coupling technology are critical parameters to allow the simultaneous presentation of optimal stimulatory signals to the T cells in culture, thus allowing their full activation and expansion. Expansion of the T cell population can be stimulated using recombinant IL-2.

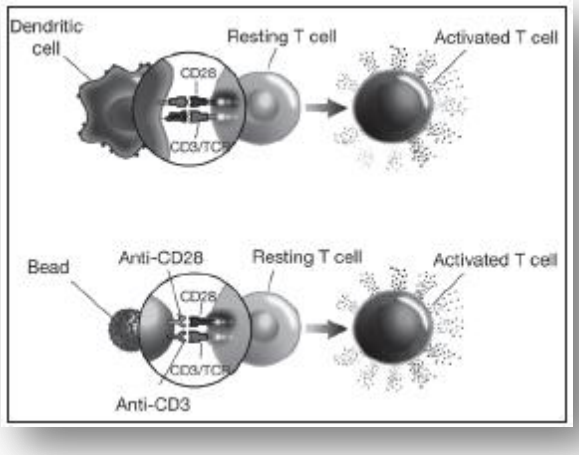

Figure 2.4: Activation of human T cells. The Dynabeads® Human T-Activator CD3/CD28 (Gibco) product mimics in vivo T cell activation from antigen-presenting cells (above) by utilizing the two activation signals CD3 and CD28, bound to a three-dimensional bead similar in size to the antigen-presenting cells (below).

2.8.2 Experimental procedure

The cells were activated with the Dynabeads Human T-Activator CD3/CD28 (Gibco). 2μl of pre-washed and resuspended Dynabeads were used for activation per $8x10^4$ total cells. In this experiment we activated the cells of total 11 wells, while each well contained $1.5x10^5$ cells. The total number of activated cells was $16,5x10^5$. As a result, we needed 42μl of Dynabeads. In order to be washed, the Dynabeads were resuspended in the vial and the desired volume was transferred to a tube. 1ml of buffer was added and the mix was vortexed for 5 sec. The tube was placed on a magnet for 1 minute and the supernatant was discarded. The tube was then removed from the magnet and the washed Dynabeads were resuspended in the same volume of culture medium as the initial volume of Dynabeads taken from the vial.

2.9 CELL CULTURE

2.9.1 Principles of method

Cell culture refers to the dispersal of cells in an artificial environment, generally outside of their natural environment. The cells may be removed from the tissue directly and disaggregated by

enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been established. Culture conditions vary widely for each cell type, but the artificial environment in which the cells are cultured invariably consists of a suitable vessel containing a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, and gases (O_2, CO_2), and regulates the physico-chemical environment (pH, osmotic pressure, temperature). Most cells are anchorage- dependent and must be cultured while attached to a solid or semi-solid substrate (adherent or monolayer culture), while others can be grown floating in the culture medium (suspension culture). In such a system, a researcher can precisely measure the response of the cell's alterations in culture alterations, prospective drugs, the presence or absence of different kinds of cells, carcinogenic agents, and viruses.

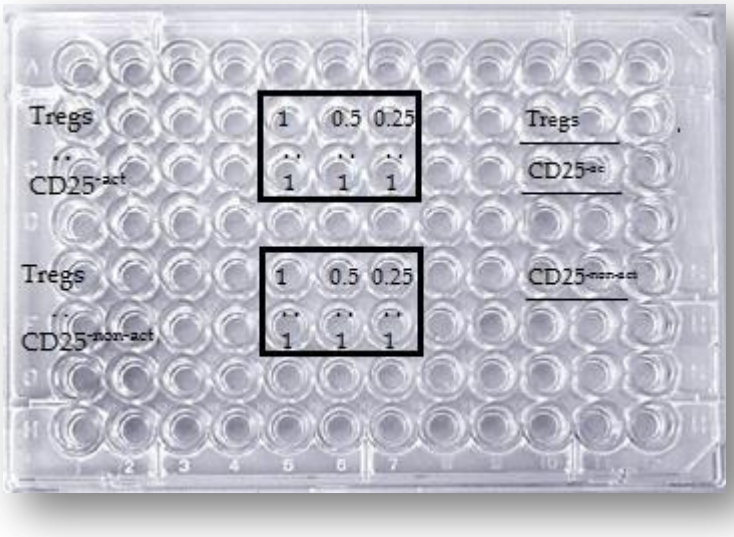

Figure 2.5: The design of the culture plate. Treg cells were co-cultured with CD25- activated or non-activated cells in concentration 1:1, 1:2 and 1:4. Tregs, CD25- activated and CD25- non-activated cells were also cultured.

2.9.2 Experimental procedure

100µl of responder and effector cells were pipetted in a round bottom 96-well tissue culture plate (figure 2.5). The wells that contained only one type of cells, 100µl of AIM-V-media containing Interleukin 2 (IL-2), 10% FCS, L-glutamine and Pen/Strep were added for a final volume of 200µl. The plate was incubated for two days in the incubator in order to make

optimization of the protocol (at 37^0C and 5%CO_2). After two days of incubation, the plate was centrifuged at 500xg for 5minutes. The cell culture supernatant was collected and stored for the Elisa assay.

2.10 ELISA ASSAY

2.10.1 Principles of method

The enzyme-linked immunosorbent assay (ELISA) is a common laboratory technique which is used to detect and quantify substances such as peptides, proteins, antibodies and hormones. Other names, such as enzyme immunoassay (EIA), are also used to describe the same technology. The basic ELISA is distinguished from other antibody-based assays because separation of specific and non-specific interactions occurs via serial binding to a solid surface, usually a polystyrene multiwell plate, and because quantitative results can be achieved. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction.

ELISAs begin with a coating step, where the first layer - either an antigen or an antibody - is adsorbed to a polystyrene 96 well plate. The antibodies used in an ELISA can be either monoclonal (derived from unique antibody producing cells called hybridomas and capable of specific binding to a single unique epitope) or polyclonal (a pool of antibodies purified from animal sera that are capable of binding to multiple epitopes). Coating is followed by blocking and detection steps. Since the assay uses surface binding for separation, several washes are repeated between each ELISA step to remove unbound materials. During this process it is essential that excess liquid is removed in order to prevent the dilution of the solutions added in the next stage. For greatest consistency specialized plate washers are used.

There are four basic ELISA formats. The ELISA provides a wealth of information in its simplest formats, but it can also be used in more complex versions to provide enhanced signal, more precise results, or if certain reagents are not available. In a direct ELISA test (figure 2.6a) the antigen is adsorbed to a multiwell plate, then an excess of another protein (normally bovine serum albumin) is added to block all the other binding sites. While an enzyme is linked to an

antibody in a separate reaction, the enzyme-antibody complex is applied to adsorb to the antigen. After excess enzyme-antibody complex is washed off, enzyme-antibody bound to antigen is left. By adding in the enzyme's substrate, the enzyme is detected illustrating the signal of the antigen. Indirect ELISA (figure 2.6b) is a two-step ELISA which involves two binding process of primary antibody and labelled secondary antibody. The unlabelled primary antibody, which is specific for the antigen, is incubated with the antigen followed by the incubation with an enzyme-labelled secondary antibody. The secondary antibody is usually an anti-species antibody and is often polyclonal. However, these reactions may lead to nonspecific signals because of cross-reaction that the secondary antibody may bring about.

Sandwich ELISAs (figure 2.6c) quantify antigens between two layers of antibodies (i.e. capture and detection antibody). The antigen to be measured must contain at least two antigenic epitope capable of binding to antibody, since at least two antibodies act in the sandwich. Either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in Sandwich ELISA systems. Monoclonal antibodies recognize a single epitope that allows fine detection and quantification of small differences in antigen. A polyclonal is often used as the capture antibody to pull down as much of the antigen as possible. The first antibody, termed the capture antibody, is coated to the polystyrene plate. Next, the analyte or sample solution is added to the well. A second antibody layer, the detection antibody, follows this step in order to measure the concentration of the analyte. Polyclonals can also be used for capture and/or detection in a sandwich ELISA provided that variability is present in the polyclonal to allow for both capture and detection of the analyte through different epitopes. If the detection antibody is conjugated to an enzyme, then the assay is called a direct sandwich ELISA. If the detection antibody is unlabelled, then a second detection antibody will be needed resulting in an indirect sandwich ELISA.

Competition or Inhibition ELISA is the most complex ELISA, and is used to measure the concentration of an antigen (or antibody) in a sample by observing interference in an expected signal output. Hence, it is also referred to as an inhibition ELISA. It can be based upon any of the above ELISA formats, direct, indirect, or sandwich, and as a result it offers maximum flexibility in set up. It is most often used when only one antibody is available to the antigen of interest or when the analyte is small and cannot be bound by two different antibodies. In this case samples are added to an ELISA plate containing a known bound antigen. After coating,

blocking, and washing steps, unknown samples are added the plate. Detection then follows pretty much as with other ELISA formats. If the antigen in the sample is identical to the plate-adsorbed antigen, then there will be competition for the detection antibody between the bound and free antigen. If there is a high concentration of antigen in the sample, then there will be a significant reduction in signal output of the assay. Conversely, if there is little antigen in the sample, there will be minimal reduction in signal. Therefore, with a competition ELISA, one is actually measuring antigen concentration by noting the extent of the signal reduction. If the detection antibody is labelled, then this would be a direct competition ELISA and if unlabelled, then this would be an indirect competition ELISA.

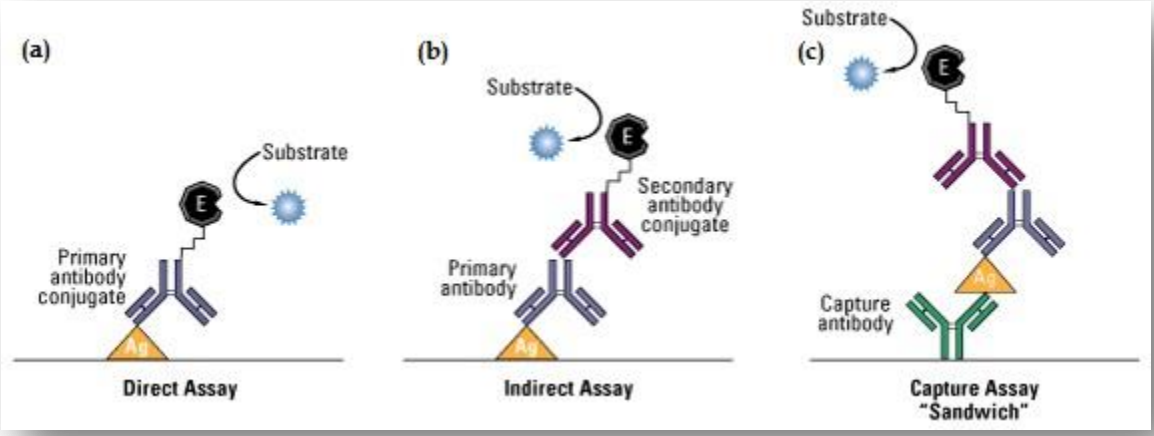

Figure 2.6: The common Elisa formats. In the assay, the antigen of interest is immobilized by direct adsorption to the assay plate or by first attaching a capture antibody to the plate surface. Detection of the antigen can then be performed using an enzyme-conjugated primary antibody (direct detection) (a) or a matched set of unlabelled primary and conjugated secondary antibodies (indirect detection) (b). In Sandwich ELISA, plate is coated with a capture antibody; sample is added, and any antigen present binds to capture antibody; detecting antibody is added, and binds to antigen; enzyme-linked secondary antibody is added, and binds to detecting antibody and substrate is added, and is converted by enzyme to detectable form (c).

A detection enzyme or other tag can be linked directly to the primary antibody or introduced through a secondary antibody that recognizes the primary antibody. It also can be linked to a protein such as streptavidin if the primary antibody is biotin labelled. The most commonly used enzyme labels are horseradish peroxidase (HRP) and alkaline phosphatase (AP). Other enzymes

have been used as well, but they have not gained widespread acceptance because of limited substrate options. These include β-galactosidase, acetylcholinesterase and catalase. A large selection of substrates is available for performing the ELISA with an HRP or AP conjugate. The choice of substrate depends upon the required assay sensitivity and the instrumentation available for signal-detection (spectrophotometer, fluorometer or luminometer). The final assay signal is measured with a spectrophotometric or fluorescent plate reader (depending upon the substrate chosen).

ELISA data is typically graphed with optical density vs log concentration to produce a sigmoidal curve as shown. Known concentrations of antigen are used to produce a standard curve and then this data is used to measure the concentration of unknown samples by comparison to the linear portion of the standard curve (figure 2.7). This can be done directly on the graph or with curve fitting software which is typically found on ELISA plate readers.

ELISAs are one of the most sensitive immunoassays available. The typical detection range for an ELISA is 0.1 to 1 fmol or 0.01 ng to 0.1 ng, with sensitivity dependent upon the particular characteristics of the antibody –antigen interaction. In addition, some substrates such as those yielding enhanced chemiluminescent or fluorescent signal, can be used to improve results.

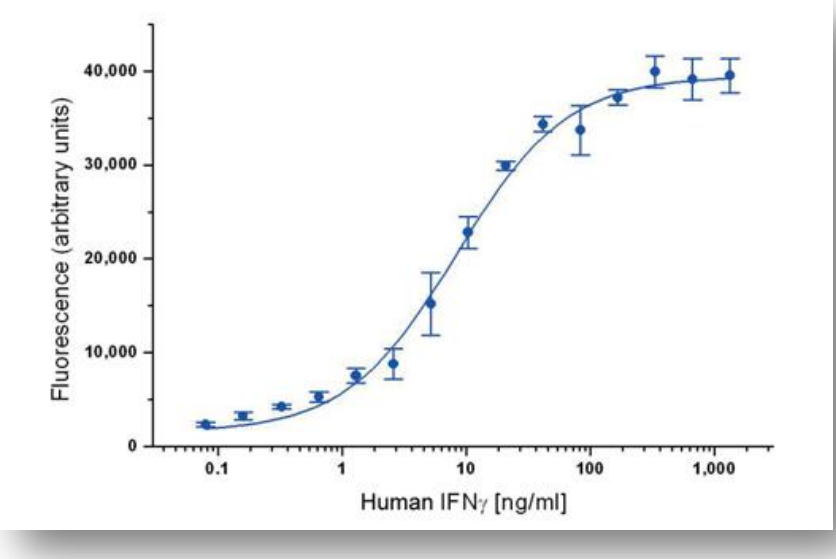

Figure 2.7: A typical ELISA standard curve.

2.10.2 Experimental procedure

In this experiment, the Duo Set ELISA Development kit was used. This kit contains the basic components required for the development of sandwich ELISAs to measure natural and recombinant human Interferon gamma (IFN-γ).

In this assay, a 96-well microplate was coated with 100µl Capture Antibody (mouse anti-human IFN-γ), diluted to a working concentration of 4µg/ml in PBS. The plate was sealed and incubated overnight at room temperature. After the incubation, each well was aspirated and washed three times with Wash Buffer (0.05% Tween 20 in PBS, pH 7.2-7.4, R&D). The plates were then blocked by adding 300µl of Block Buffer (1% BSA in PBS with 0.05% NaN_3) to each well. The plate was incubated at room temperature for minimum 1 hour, followed by 3 washes.

The sample (cell culture supernatant) or the standards in Reagent Diluent (0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline (20mM Trizma base, 150mM NaCl) pH 7.2-7.4, 0.2mM filtered) were added (100µl) in wells. The plate was covered with an adhesive strip and incubated overnight in the incubator (at 37oC and 5%CO2), followed by 3 washes. Detection antibody (biotinylated goat anti-human IFN-γ), diluted in working concentration of 50µg/ml in Reagent Diluent, was added (100µl) to each well. The plate was covered with an adhesive strip and incubated for 2 hours at room temperature, followed by 3 washes. Strepavidin-HRP (streptavidin conjugated to horseradish-peroxidase), diluted to the working concentration of 50µg/ml in Reagent Diluent. The plate was incubated for 20 minutes at room temperature, avoiding direct light, followed by three washes. Substrate solution [100µl of 1:1 mix of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine) into each well] was added and the plate was incubated for 20 minutes at room temperature, avoiding direct light. Stopping solution (2 N H_2SO_4) (R&D Systems) was added (50µl into each well) and the optical density of each well was determined, using a microplate reader set to 450nm. The wavelength correction was set to 450nm.

2.11 STATISTICAL ANALYSIS

Data were presented as mean ± SE. In the suppression assay statistical analysis was performed by Friedman test. In unpaired datasets, significance was determined with the Unpaired t-test

with Welch's correction when Gaussian distribution was occurred and with the Mann–Whitney test when data set did not exhibit Gaussian distribution. In paired datasets, significance was determined with the Paired t-test when sample followed Gaussian distribution and with the Mann–Whitney test when data set did not exhibit Gaussian distribution. In order to find the effect of chemotherapy, One-Way ANOVA test was used when Gaussian distribution was occurred, whereas when not we used The Nonparametric T-test. In Before-After Analysis the differences and the significance were evaluated by the Wilcoxon matched-pairs signed rank test, as no dataset exhibited Gaussian distribution. When compared two different groups, statistical analyses were performed with Multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

Overall survival (OS) is the length of time from either the date of diagnosis or the start of treatment for a disease, such as cancer, that patients diagnosed with the disease are still alive. In this study, it was estimated from the date of the start of treatment for the disease to the last contact or death. Progression-free survival (PFS) is the length of time during and after the treatment of a disease, such as cancer, that a patient lives with the disease but it does not get worse. Cumulative survival (progression-free survival, PFS; overall survival, OS) time was calculated by the Kaplan-Meier method and analysed by the log-rank test. The value of upper 95% confidence intervals (CI) of control was chosen as division standard for subgroups.

All statistical analyses were performed with the Software GraphPad Prism 6. Differences were considered statistically significant with $p \leq 0.05$ and highly significant with $p \leq 0.01$.

Chapter 3

Results

3.1 PATIENTS DEMOGRAPHICS

The mean age of 145 NSCLC patients who enrolled in the study was 64 years, while 82.1% of patients were male. The following histopathological tumour types were included (Table 3.1): adenocarcinoma (n=85); squamous cell carcinoma (n=44); and other types pf carcinoma (n=16). At study entry 117 patients had metastatic lung cancer in the most advanced stage (Stage IV) and during the study 34.5% of patients presented disease progression (PD). Seventy three patients were evaluated for assessment of clinical outcome, the rest of them (n= 72) either received only one chemotherapy cycle because of early death or refused systemic anticancer treatment and received only supportive care. Moreover 29.6% of patients completed the 6th cycle of chemotherapy while 14.9% presented stable or partial response at the end of the treatment.

Table 3.1: Patients' demographics.

Patients		Nº	%
Number of Patients	145		
Sex	**Male**	119	82.1
	Female	26	17.9
Age (Median)	**All**	64	
	Male	66.5	
	Female	61.5	
Histopathological Type of Lung Cancer	**Adenocarcinoma**	85	58.6
	Squamous Cell Carcinoma	44	30.4
	Other Types of Carcinoma	16	11
Disease Stage (at study entry)	**IIIA/IIIB**	28	19.3
	IV	117	80.7
Treatment	**Platinum-based Therapy**	109	75.2
	Avastin	36	24.8
Response Rate	**Stable Disease/Partial Resonse (SD/PR)**	50	34.48
	Progressive Disease (PD)	23	16.7
	Non Evaluated (NE)	72	48.8

3.2 CHARACTERIZATION OF CD4$^+$CD25$^+$ T CELLS IN NSCLC PATIENTS

Whole blood from NSCLC patients was stained and viable lymphocytes were gated for CD3 expression. In the next step, CD3$^+$ T cells were gated for CD4 and CD25 expression. The double negative (CD4$^-$CD25$^-$) population was used to define negative staining for CD4 and CD25 expression. The cells expressing CD25 above the 90% contour level were defined as CD25^{high}; remaining CD25$^+$ cells were defined as CD25^{low}. Comparing the percentage of CD4$^+$ CD25$^-$, CD25^{low} and CD25^{high} cells that expressed FoxP3, we observed that FoxP3 expression was most commonly seen among CD25^{high} (figure 3.1).

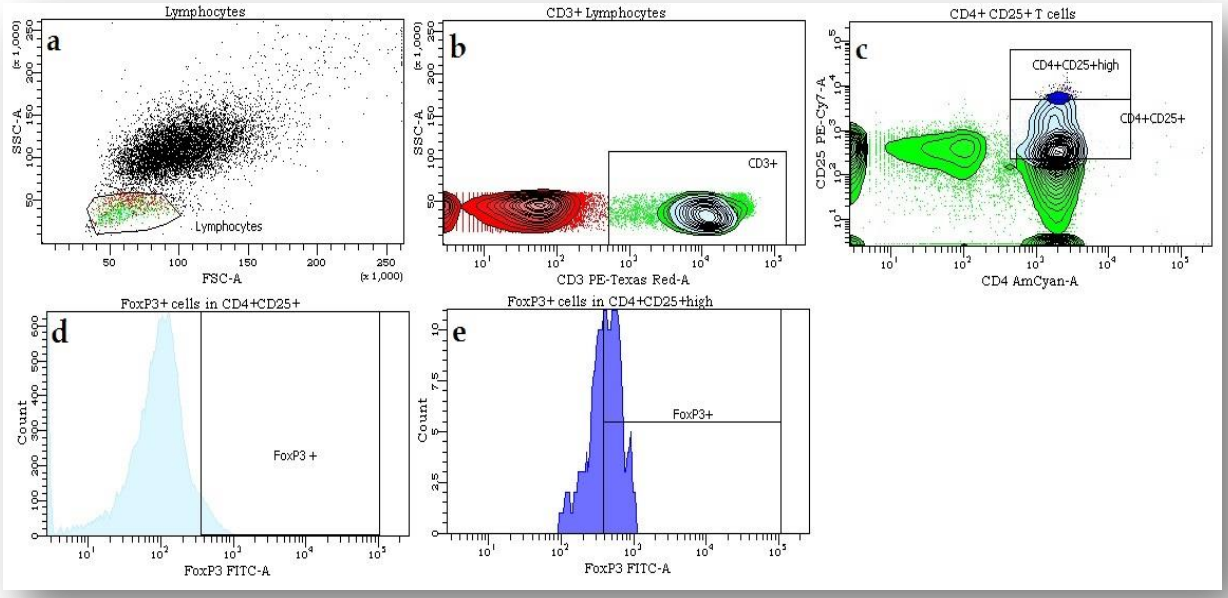

Figure 3.1: Representative plots showing the subpopulation of FoxP3$^+$ T cells from a NSCLC patient. (a) Lymphocytes were identified based on their characteristic properties shown in the forward scatter (FSC) and sideward scatter (SSC). (b) A representative gating was set for CD3$^+$ T cells from blood lymphocytes. (c) A representative dot plot shows expression of CD4$^+$CD25$^+$ Tregs in blood CD3$^+$ T cells. (d, e) Representative overlay histograms showing expression of FoxP3$^+$ in blood CD25$^+$ and CD25^{high} T cells, respectively.

Moreover, lymphocytes were gated for CD127 and CD152. The majority of $CD4^+$ $CD25^{high}$ cells were characterized as $CD127^{-/low}CD152^+$. Expression of FoxP3 was determined for $CD4^+CD25^{high}$ cells that were $CD127^+$ and $CD152^{-/low}$ (CTLA4). Moreover, in this population, we found cells positive for CD13 and double positive for CD13 and CD39 (figure 3.2).

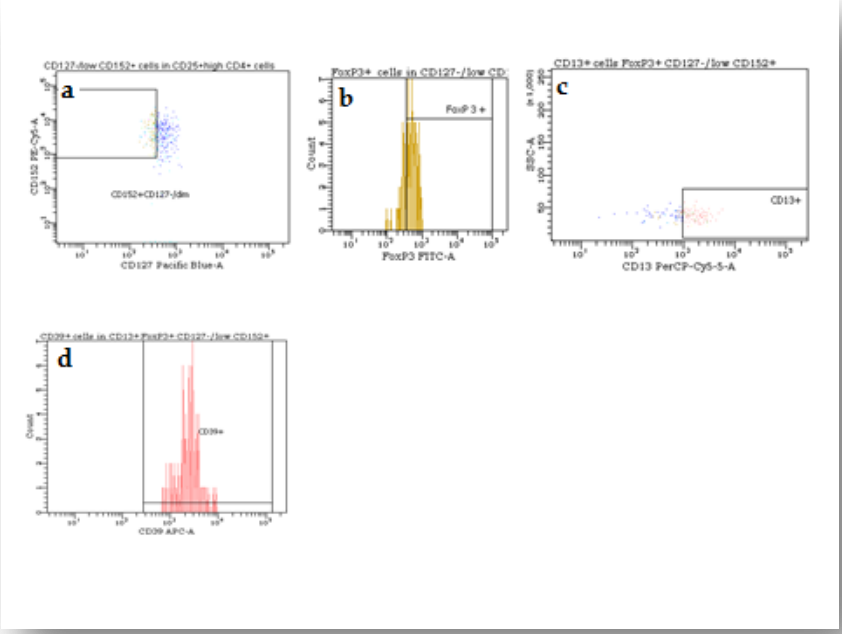

Figure 3.2: Representative plots showing the subpopulation of $CD152^+CD127^{-/low}FoxP3^+$ T cells from a NSCLC patient. (a) Representative dot-plot showing $CD127^{-/low}CD152^+$ cells. (b) A representative histogram shows expression of $FoxP3^+$ in blood $CD127^{-/low}CD152^+$ T cells. (c) Representative dot plots showing expression of $CD13^+$ in blood $FoxP3^+$ $CD127^{-/low}CD152^+$ T cells (d) Representative dot plot showing expression of $CD39^+$ in blood $CD13^+FoxP3^+$ $CD127^{-/low}CD152^+$ T cells.

In the $CD4^+CD25^{high}$ cells, we observed two different populations, based in the expression of CD45RO (figure 3.3). $CD45RO^+$ cells are antigen-experienced cells while CD45RO– represent a population of naïve Treg (nTreg) that have recently emigrated from the thymus and have not yet encountered self-ligands in an inflammatory context (Miyara et al., 2009; Valmori et al., 2005). $CD4^+CD25^{high}CD127^{-/low}FOXP3^{+low}CD45RO^-$ cells did not express CD152 and are characterized as naïve Tregs (Valmori et al., 2005). This population also expressed CCR7, a ligand that controls the migration of naive T cells to inflamed tissues. This population is characterized by bibliography as $CCR7^+$ Naïve Tregs (figure 3.3h) (Britschgi et al., 2008).

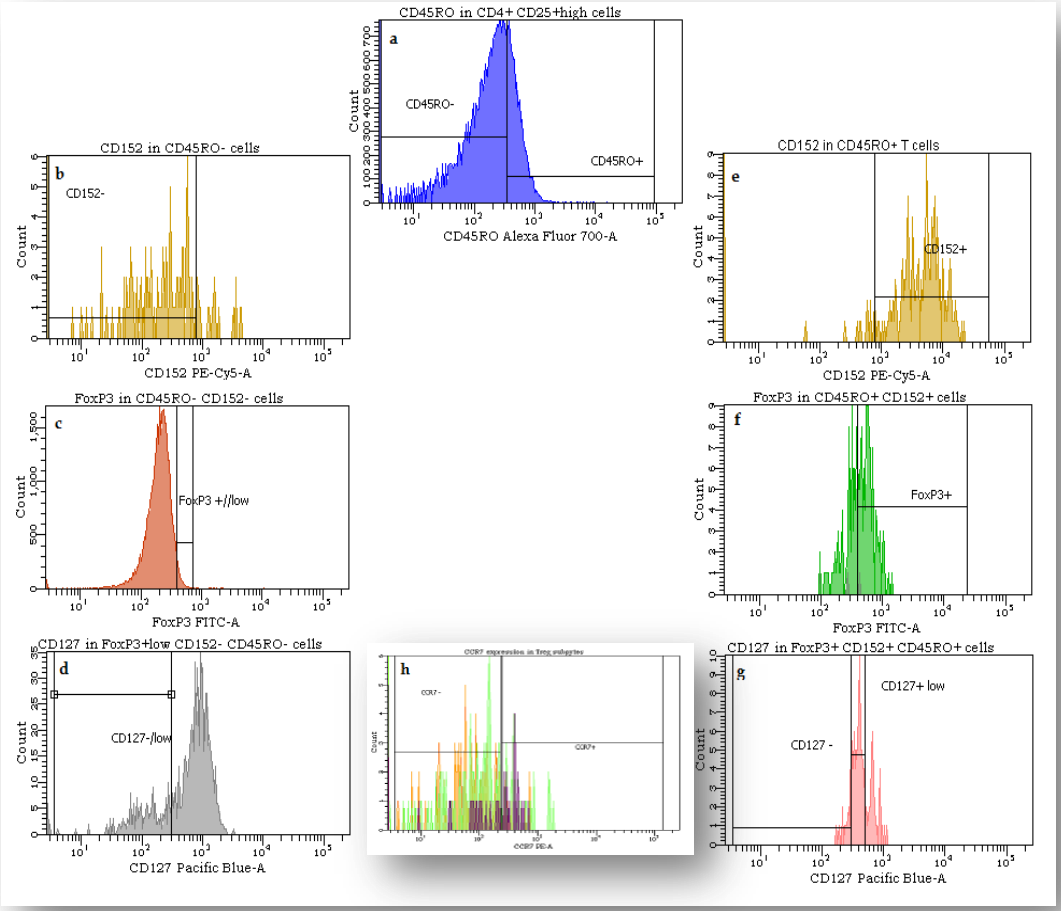

Figure 3.3: Representative plots showing the Treg subtypes from a NSCLC patient. (a) Representative histogram showing expression of CD45RO in blood $CD25^+$ T cells. $CD45RO^-$ cells were gated for (b) CD152 and the negative cells were gated for (c) FoxP3 expression. (d) $CD127^{-/low}$ cells inside the $FoxP3^{+/low}$ $CD152^-$ $CD45RO^-$ $CD25^+$ population were identified by histograms and are characterized as Naïve $CD4^+$ Tregs. On the other hand, $CD45RO^+$ cells were gated for (e) CD152 expression. (f) FoxP3 expression was identified by histogram. (g) Histogram showed the expression of CD127 in blood $FoxP3^+$ $CD152^+$ CD45RO+ $CD25^+$. $CD127^{+low}$ cells are characterized as Effector $CD4^+$ Tregs while $CD127^-$ cells are characterized as Terminal Effector $CD4^+$ Tregs. (h) Representative histogram showing CCR7 expression in Treg subtypes from a NSCLC patient. Naïve, Effector and Terminal Effector Tregs are represented with green, purple and yellow colour, respectively.

On the other hand, $CD45RO^+$ cells expressed CTLA4 more than CD45RO– (naıve) cells. The majority of this population also expressed FoxP3. The $CD4^+$ $CD25^{high}$ $FoxP3^+$ $CD152^+$ $CD45RO^+$ cells that expressed low levels of CD127 were characterized as Effector Tregs (figure 3.3). These

cells were further divided in central-memory Tregs ($CCR7^+$) which migrate to the draining lymph nodes (figure 3.4) (Sallusto et al., 2004; Tosello et al., 2008). However, $CD4^+CD25^{high}FoxP3^+CD152^+CD45RO^+$ that were negative for CD127 expression were characterized as Terminal Effector Tregs (figure 3.3), while cells inside this population that were negative for CCR7 expression are referred as Terminal Memory Effector Tregs ($CCR7^-$) and travel to inflamed tissues (figure 3.3h) (Sallusto et al., 2004; Tosello et al., 2008).

3.3 THE FUCNTIONALITY OF THE Tregs IN NSCLC PATIENTS

Interferon-gamma (IFN-γ) is produced by activated T lymphocytes and plays a regulatory role in immune responses. In order to evaluate the functionality of Tregs in NSCSC, they were isolated with magnetic separation and then were co-cultured with activated T cells in different concentrations. Detection of IFN-γ levels in the supernatant of the co-culture was measured by ELISA. As shown in figure 3.4, the presence of Tregs inhibited the production of IFN-γ in activated T cells in a concentration-dependent manner. The production of IFN-γ by activated $CD4^+$ cells co-cultured with Tregs in concentration 1:1 detected in similar levels with this of inactivated $CD4^+$ cells. Lower concentration of Tregs in the co-culture resulted in significant increased production of IFN-γ by activated $CD4^+$ cells. As a result, the presence of Tregs resulted in down regulation of T cell proliferation.

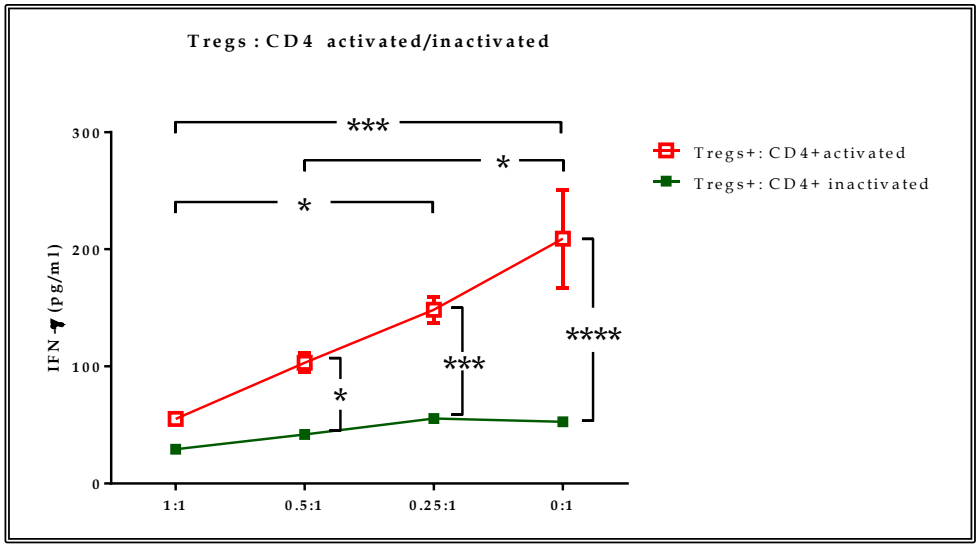

Figure 3.4: Suppression of CD4⁺ T cell proliferation by Tregs. ELISA based analysis of IFN-γ in the supernatant collected from co-cultures of CD4⁺ activated or inactivated T cells with Tregs isolated from NSCLC patients. Tregs in concentrations 1x, 0.5x, 0.25x were co-cultured with CD4⁺ activated and inactivated T cells. The IFN-γ levels detected in the co-culture of Tregs with CD4⁺ activated T cells (55.12±4.313) were similar to them of inactivated T cells. Lower concentration of Tregs (0.25:1) resulted in significant higher expression of IFN-γ (148.3 ±10.95) (P=0.0439). Moreover, in absence of Tregs, the expression of IFN-γ was significantly higher (209±41.65) (P=0.0003). Statistical analysis was performed by Friedman test. Points represent the mean percentage ± SEM of IFN-γ production from activated CD4⁺ T cells (red) and inactivated CD4⁺ T cells (green).

3.4 QUANTIFICATION OF RELATIVE CD4⁺CD25⁺ T CELL NUMBERS

Peripheral blood lymphocytes in patients with Non-Small Cell Lung Cancer (N=145) and in healthy donors (n=31) were examined for the prevalence of CD4⁺ T cells. The population of CD4⁺ cells as a percentage of total CD3⁺ cells was evaluated by flow cytometric analysis. There was no significant difference in the levels of CD3⁺CD4⁺T cells in the patients with Non-Small Cell Lung Cancer in comparison with those in healthy donors. Moreover, the percentage of CD3⁺CD4⁺ cells did not collate with neither the stage, nor the type of carcinoma (figure 3.5).

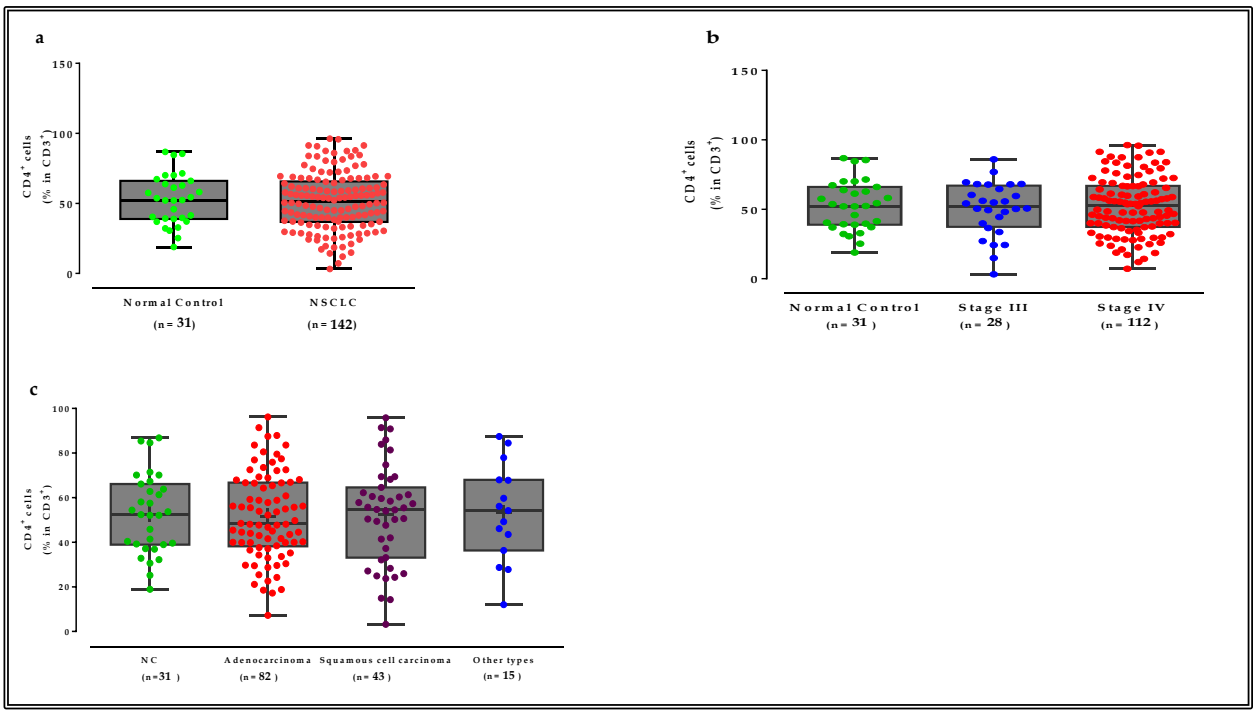

Figure 3.5: Unchanged levels of $CD3^+CD4^+$ T cells in peripheral blood of patients with Non-Small Cell Lung Cancer. The population of $CD4^+$ cells as a percentage of total $CD3^+$ cells in the PBMCs was evaluated by flow cytometric analysis. (a) Summarized data from all individuals showed that there was no significant difference in the levels of $CD3^+CD4^+$ T cells in patients with NSCLC in comparison with that in healthy donors (Unpaired t test with Welch's correction). Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented. The percentage of $CD3^+CD4^+$ did not collate with neither the stage (b) nor the type (c) of carcinoma.

Previous studies provided evidence of an increased pool of $CD4^+CD25^+$ regulatory T cells in the peripheral blood of cancer patients with potent immunosuppressive features. In the current study, in healthy controls (n = 31), the mean percentage of $CD4^+CD25^+$ cells in the peripheral blood was 14.64 ± 1.517% of all $CD4^+$ T cells. In contrast, peripheral blood of cancer patients (n = 140) exhibited a statistically significant 1.7-fold increase in $CD4^+CD25^+$ T cells (24.98 ± 1.044% of the $CD4^+$ population) ($P < 0.001$) compared to healthy controls (figure 3.6a). The number of $CD4^+CD25^+$ T cells did not associate with either tumour stage or tumour type (figure 3.6b, c).

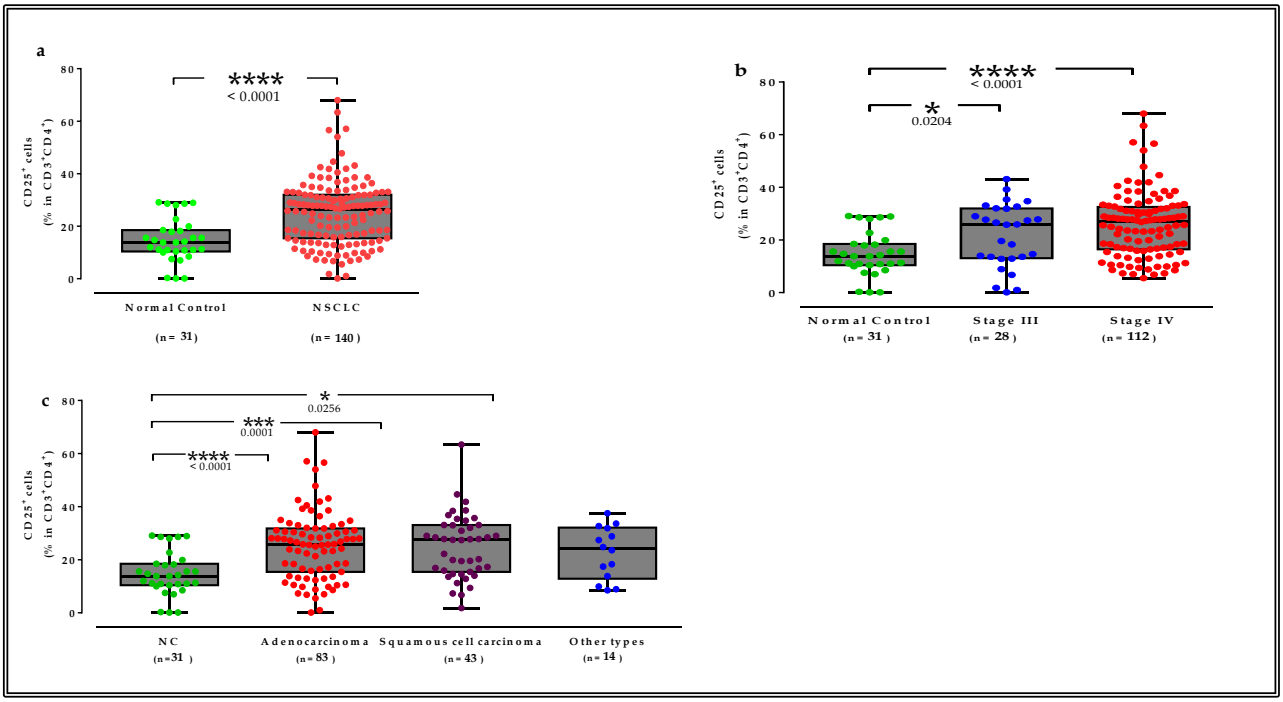

Figure 3.6: NSCLC patients show a significant increase in $CD4^+CD25^+$ T cells. The percentage of $CD4^+CD25^+$ T cells in the peripheral blood of 140 NSCLC patients was determined by flow cytometry and compared with 31 healthy controls (Unpaired t test with Welch's correction). (a)The mean percentage of $CD4^+CD25^+$ cells in the peripheral blood was significantly higher ($P<0.0001$) in NSCLC patients compared to normal control. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented. The number of $CD4^+CD25^+$ T cells did not correlate with either tumour stage (b) or tumour type (c).

Natural Treg cells possess high levels of CD25 ($CD25^{high}$) present on the surface of T cells and the expression of FOXP3 required for the generation and maintenance of their suppressive activity (Bluestone & Abbas , 2003; Fontenot & Rudensky , 2005; Sakaguchi , 2004). The

population of CD4⁺CD25^{high} T cells as a percentage of total CD3⁺ T cells was identified by flow cytometry after cell surface labeling for expression of CD4 and CD25 molecules. The CD4⁺ T cells with the highest level of CD25 (CD4⁺CD25^{high}) appear as a tail over the major population containing both CD4⁺CD25^{low} and CD4⁺CD25⁻ cells (figure 3.7a). The CD25^{high} cells represent 1–2% of the total CD4⁺ T cell population. As shown, the frequency of CD4⁺CD25^{high} T cells in NSCLC patients was lower in NSCLC patients (0.7850± 0.03675%) than in healthy donors (1.229 ± 0.1294%) (P<0.0001). The percentages of CD4⁺CD25^{high} T cells in NSCLC patients did not change depending on the stage or the type of the carcinoma.

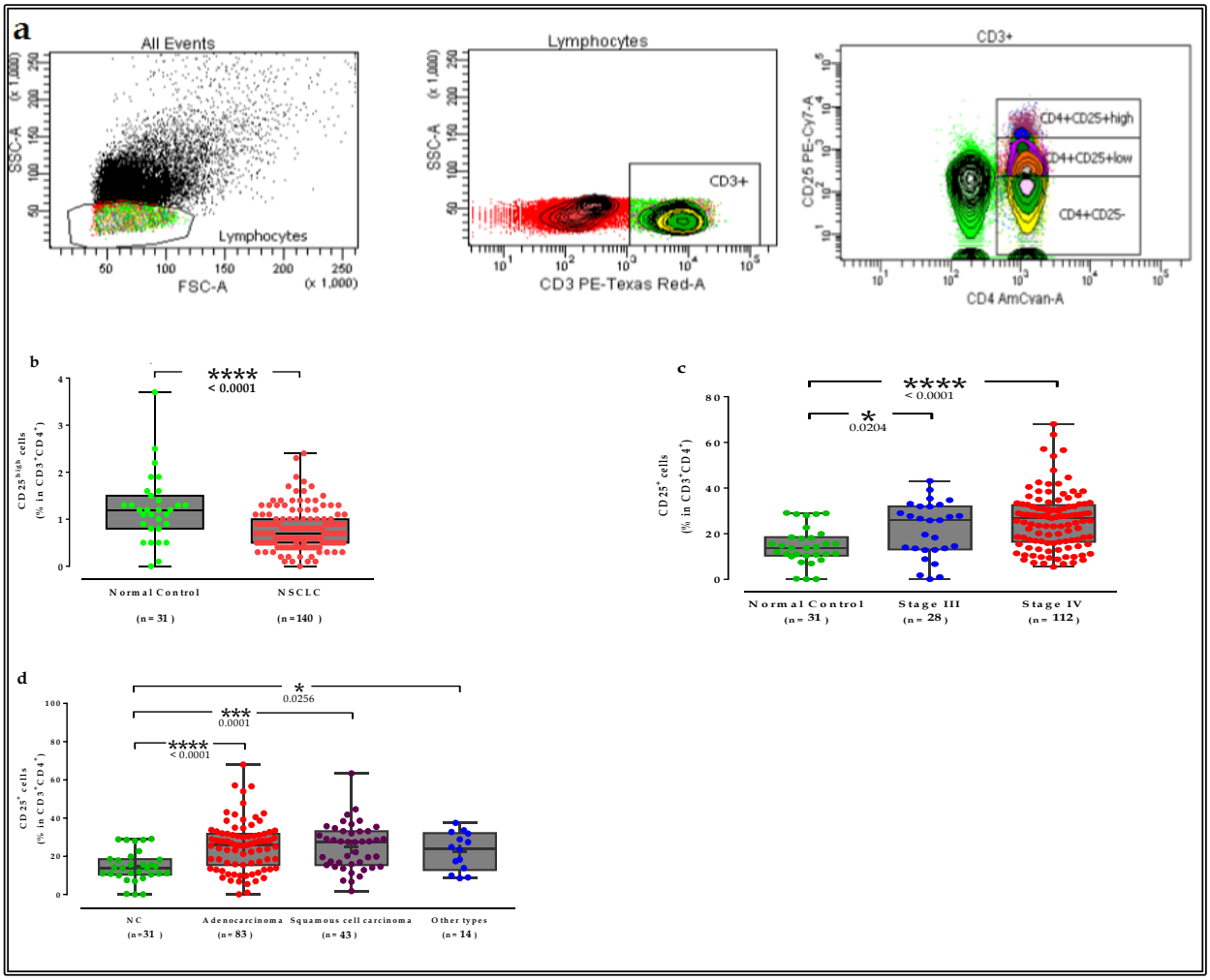

Figure 3.7: NSCLC patients show a significant decrease in CD4⁺CD25^{high} T cells. (a) Representative dot plots showing the subpopulation of CD4⁺CD25^{high} T cells from a NSCLC patient. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented. Groups were compared by Mann-Whitney test. (b) Percentage of CD4⁺CD25^{high} cells in the peripheral blood of healthy donors and NSCLC patients.

$CD4^+CD25^{high}$ cells are presented as a percentage of total $CD3^+$ cells. Prevalence of regulatory T cells in NSCLC patients was significantly lower than in healthy controls ($P < 0.0001$). (c , d) No correlation was found between the percentage of $CD4^+CD25^{high}$ T cells and the stage or type of carcinoma.

3.5 EXPRESSION OF FoxP3 IN $CD4^+CD25^+$ T CELLS

It has been well documented that $CD4^+CD25^+FoxP3^+$ Treg contributes to cancer-induced immunosuppression (Larmonier et al., 2007). We assessed FOXP3 expression on single cells by intracellular flow cytometry. However, in this study, no statistically significant difference was observed between $CD4^+CD25^+FoxP3^+$ Treg percent in PMBCs of NSCLC patients and healthy donors (31.27±2.995 vs 35.46±6.232, P=0.2) (figure 3.8a). Furthermore, one way ANOVA test showed no significant differences between subgroups according to stage and type of carcinoma (figure 3.8b, c).

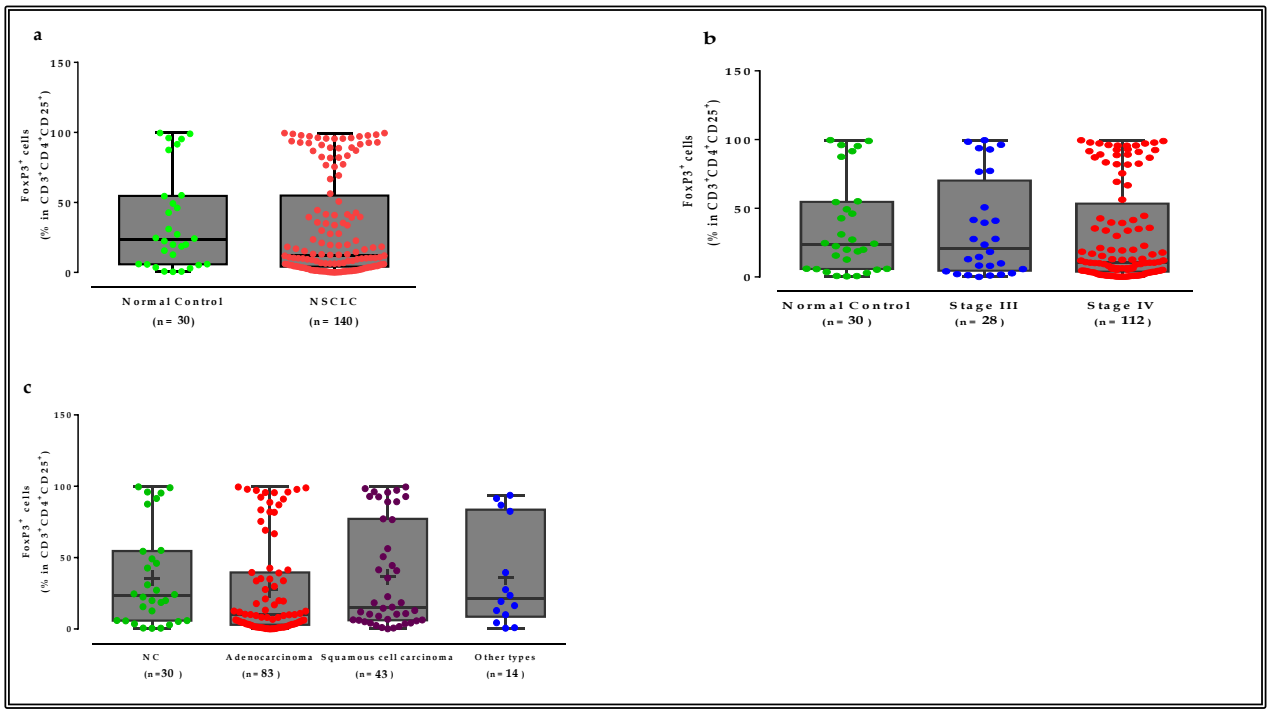

Figure 3.8: No statistical difference in $CD4^+CD25^+FoxP3^+$ T cells between NSCLC patients and healthy donors. The percentage of $CD4^+CD25^+FoxP3^+$ T cells was analyzed using flow cytometry. (a)The study group did not show significantly different percentage of $CD4^+CD25^+FoxP3^+$ Tregs than control. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented. Groups were compared by Mann-

Whitney test. One way ANOVA test showed no significant differences between each of the two subgroups of (b) stage and (c) type of carcinoma.

Previous studies have demonstrated that $CD4^+CD25^{high}FoxP3^+$ Treg cells are expanded in patients with solid tumours (Gray et al., 2003; Tokuno et al., 2009; Woo et al., 2001; Woo et al., 2002). However, in this study there were no statistically significant differences in the $CD4^+CD25^{high}FoxP3^+$ Treg populations among the NSCLC patients and the control group and among different stages of carcinoma (figure 3.9 a, b). Although, the population of $CD4^+CD25^{high}FoxP3^+$ cells were significantly higher in the squamous cell carcinoma group (59.71±5.057) than in the adenocarcinoma group (43.51±3.914) (P=0.0185) (figure 3.9 c), the percentages of these cells did not differ compared to the normal controls.

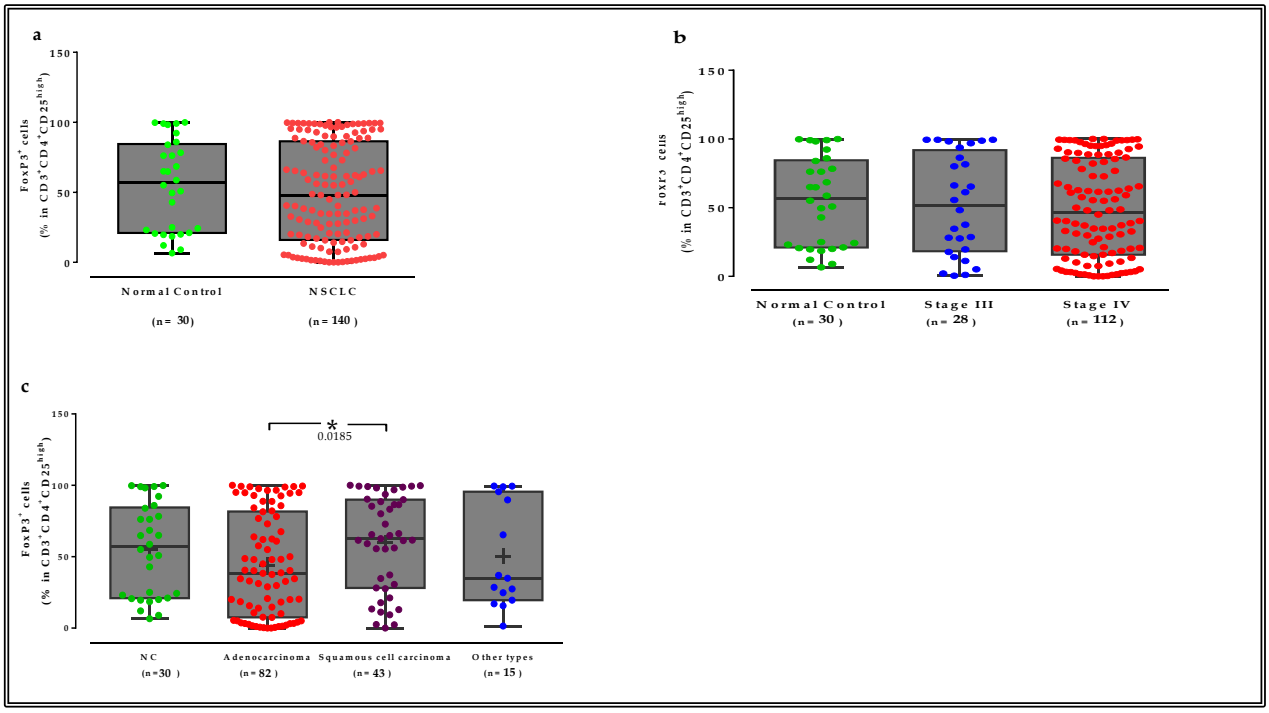

Figure 3.9: The population of $CD4^+CD25^{high}FoxP3^+$ T cells in NSCLC patients and healthy donors. The percentage of $CD4^+CD25^{high}FoxP3^+$ T cells was analyzed using flow cytometry. (a) No statistically significant difference was found between the study and control groups. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented. Groups were compared by Mann-Whitney test. (b) One way ANOVA test showed no significant differences between each of the two subgroups of stage. (c)

However, the mean percentage of the $CD4^+CD25^{high}FoxP3^+$ cells in the peripheral blood was significantly higher (P=0.0185) in the squamous cell carcinoma group compared to the adenocarcinoma group.

3.6 IDENTIFICATION OF $CD3^+CD4^+CD25^{high}$ T CELLS WITH REQULATORY PROPERTIES

Recent studies have shown that a lack or low expression of CD127 (the α chain of the IL-7 receptor) is linked with T_{reg} identification similar to $CD4^+CD25^+FoxP3^+$ T cells. Moreover, the CTLA-4 ($CD152^+$) is one of the main regulators of T cell activation. Surface expression of CTLA-4 increases after T cell activation and naturally occurring regulatory T cells (T regs) constitutively express this molecule (Sakaguchi , 2004). The population of $CD127^{-/low}CD152^+FoxP3^+$ cells as a percentage of total $CD3^+CD4^+CD25^{high}$ cells in the peripheral blood did not differ between the NSCLC patients and heathy donors and did not correlate with the stage of the carcinoma (figure 3.10 a, b). However, the mean percentage of $CD127^{-/low}CD152^+FoxP3^+$ cells was significantly higher in patients with squamous cell carcinoma than in those with adenocarcinoma and in healthy subjects (figure 3.10 c); the mean percentages were 30.58 ± 3.378% vs. 19.46 ± 2.346% (P=0.0064) and 19.31 ± 3.941% (P=0.0192) respectively.

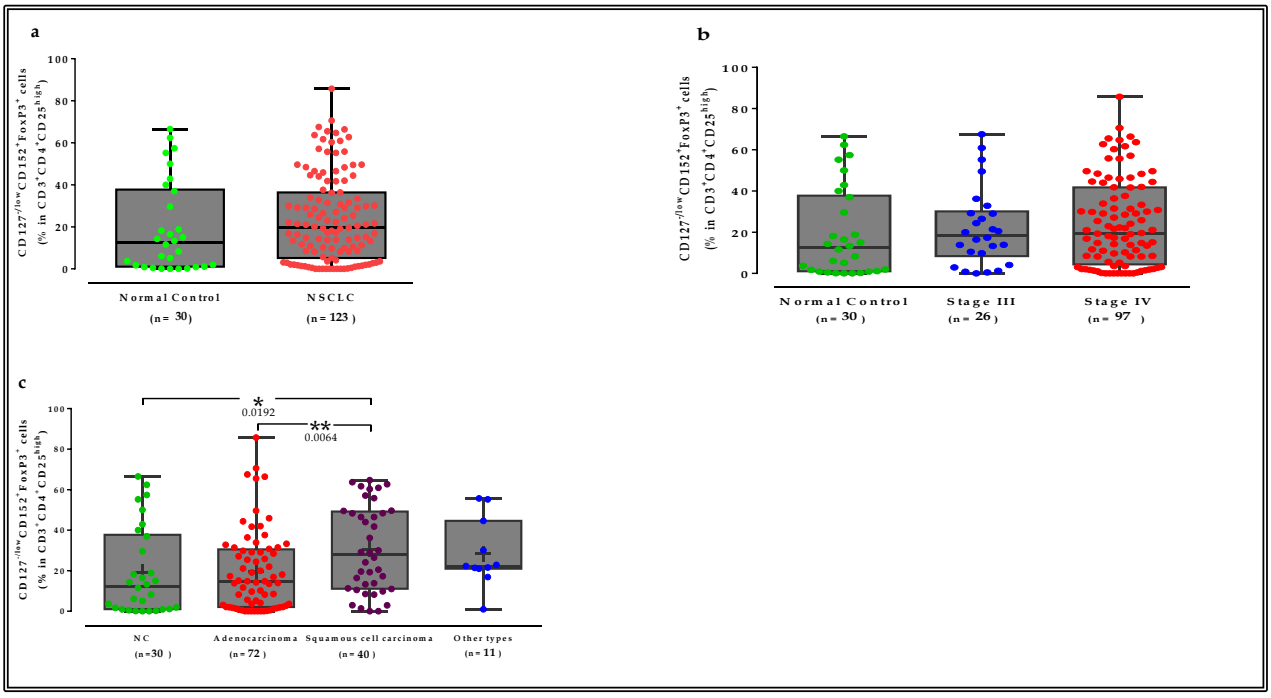

Figure 3.10: The population of CD127$^{-/low}$CD152^{+}FoxP3^{+} cells as a percentage of total CD3^{+}CD4^{+}CD25^{high} cells in peripheral blood of patients with Non-Small Cell Lung Cancer. The population of CD127$^{-/low}$CD152^{+}FoxP3^{+} cells as a percentage of total CD3^{+}CD4^{+}CD25^{high} cells in the PBMCs was evaluated by flow cytometric analysis. (a) No significant difference was shown in the levels of CD127$^{-/low}$CD152^{+}FoxP3^{+} T cells in patients with NSCLC in comparison with that in healthy donors (Unpaired t test with Welch's correction). Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented. Groups were compared by Mann-Whitney test. The percentage of CD3^{+}CD4^{+} did not collate with the (b) stage but (c) was significantly higher in patients with squamous cell carcinoma than in those with adenocarcinoma and in healthy subjects (One way ANOVA test).

CD13, a membrane-bound cell surface glycoprotein, was originally recognized as a marker for subsets of normal and malignant myeloid cells, but later was detected on other types of cells (Shipp , 1993). Lymphocytes in peripheral blood do not express CD13, but its expression can be induced on PMA-activated T lymphocytes when they are adhered directly to human gingival fibroblasts (Saho et al., 2003). CD13 is a Zn-dependent metalloprotease, which cleaves N-terminal neutral amino acids from proteins. In NSCLC patients, the surface expression of CD13^{+} did not differ in FoxP3^{+}CD127$^{-/low}$CD152^{+}CD3^{+}CD4^{+}CD25^{high} cells in NSCLC patients and healthy subjects and did not correlate with stage (figure 3.11 a, b). Although, the mean

expression was higher in patients with squamous cell carcinoma (8.845 ± 1.54%) than in patients with adenocarcinoma (6.021 ± 1.034%) (P=0.0097) (figure 3.11 c), there was not a difference compared to healthy controls.

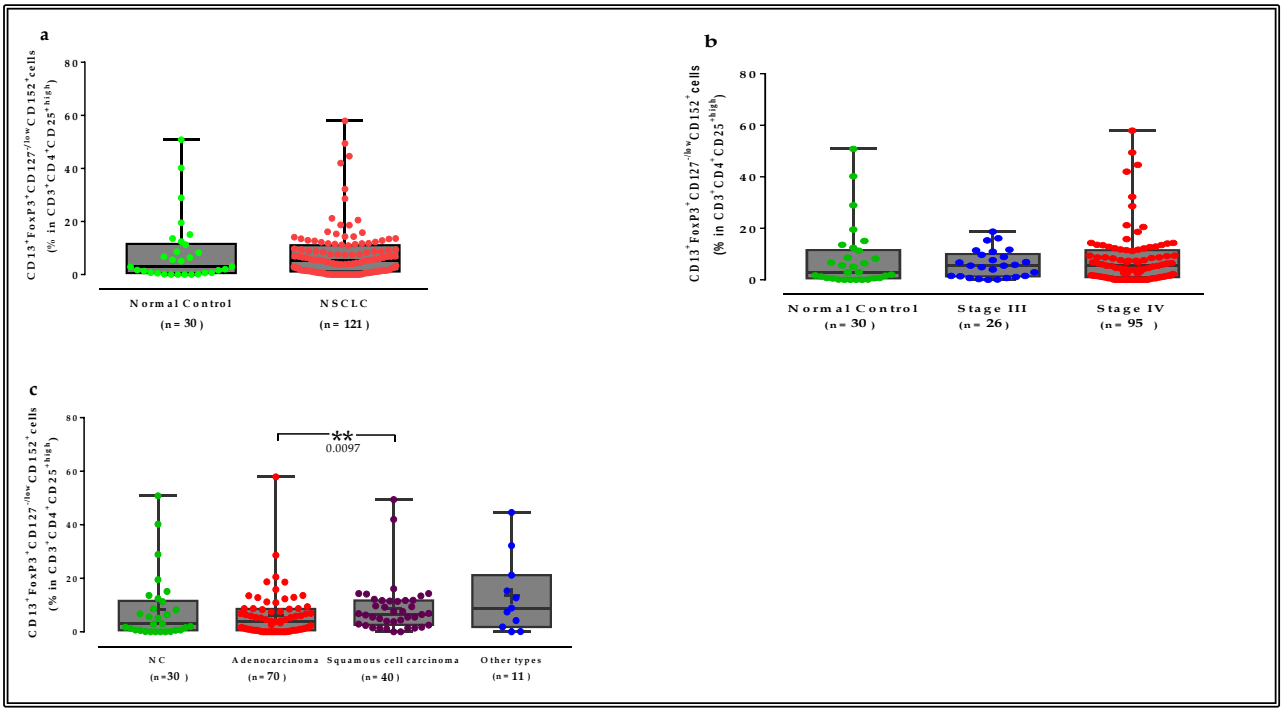

Figure 3.11: The expression of CD13⁺ in FoxP3⁺ CD127$^{-/low}$ CD152⁺ CD3⁺ CD4⁺ CD25^{high} cells in NSCLC patients and healthy donors. The percentage of CD13 in FoxP3⁺CD127$^{-/low}$CD152⁺CD3⁺CD4⁺CD25^{high} cells was analyzed using flow cytometry. (a) No statistically significant difference was found between the study and control groups and (b) between each of the two subgroups of stage. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented. However, the mean percentage of the CD13⁺ cells was significantly higher (P=0.0097) in the squamous cell carcinoma group compared to the adenocarcinoma group (c).

Moreover, recently CD39 has been shown to be an accurate marker for T_{reg} detection. The expression of CD39 by human Treg is restricted to a subset of T regulatory memory effector cells (Borsellino et al., 2007) capable of suppressing IL-17 production (Fletcher et al., 2009). The surface expression of CD39⁺ in Treg cells did not correlate with the presence of disease or with the stage of the carcinoma (figure 3.12 a, b). However, the expression was higher in patients with squamous cell carcinoma than in patients with adenocarcinoma; the mean was 7.640 ± 1.086% vs 5.341 ± 0.9162% (P=0.0048) (figure 3.12 c).

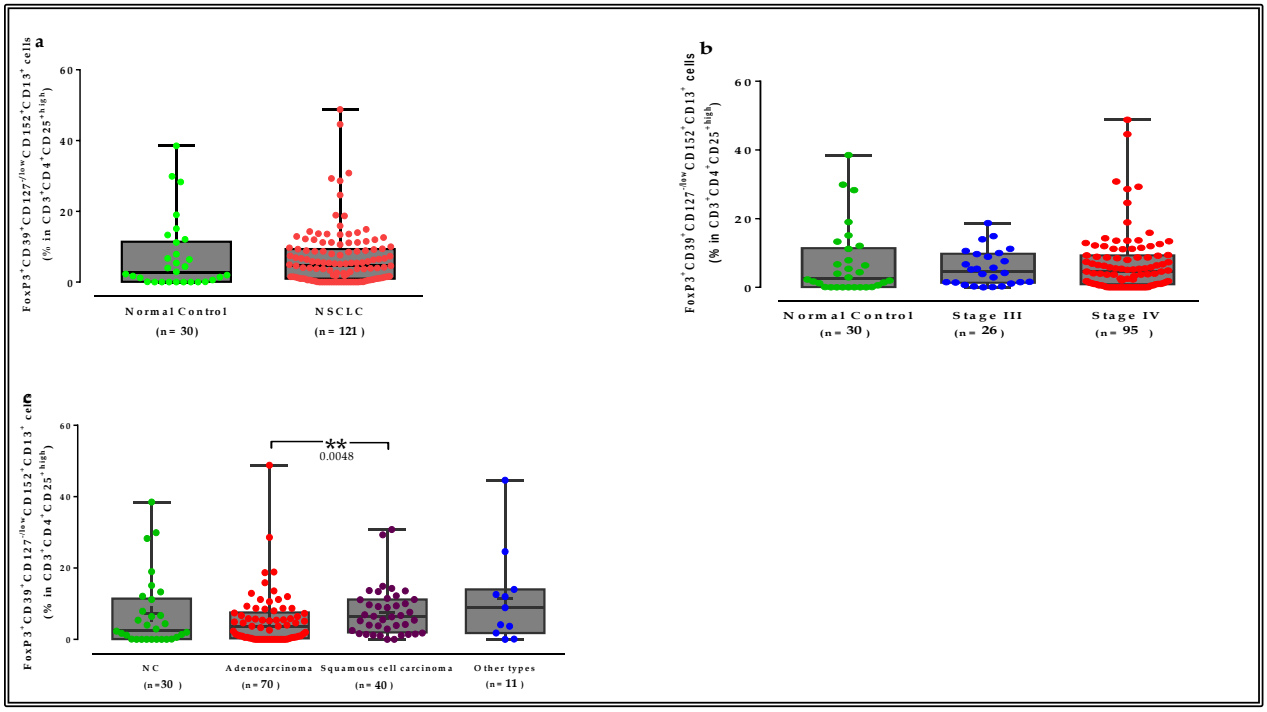

Figure 3.12: The expression of CD39⁺ in Treg cells in NSCLC patients and healthy donors. The expression of CD39⁺ in Treg cells did not correlate with the presence of (a) disease or with (b) the stage of the carcinoma. (c) However, the mean percentage of the CD39⁺ Treg cells in the peripheral blood was significantly higher (P=0.0018) in the squamous cell carcinoma group compared to the adenocarcinoma group. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented.

3.7 Treg SUBTYPES

Several distinct subsets of CD4+ T cells with regulatory activity have been described in humans. CD45RO expression has been notably considered to identify naïve and effector Tregs. Analysis of CD25 expression in human circulating CD4+ T lymphocytes identifies a distinct subset of $CD25^{+high}CD127^{-/low}$CTLA-4-$FOXP3^{+/low}$ cells contained in the $CD45RO^{-}$ naive fraction. This subset is named Naïve Tregs (nTregs). Analysis of Naïve Treg cells revealed no difference between the presence or the stage of the disease (figure 3.13 a, b). However, the mean percentage of the Naïve Tregs presents a significant reduction in patients with adenocarcinoma (1.362± 0.2863%) than in patients with squamous cell carcinoma (2.890 ± 0.6268%) (P=0.0418) (figure 3.13 c).

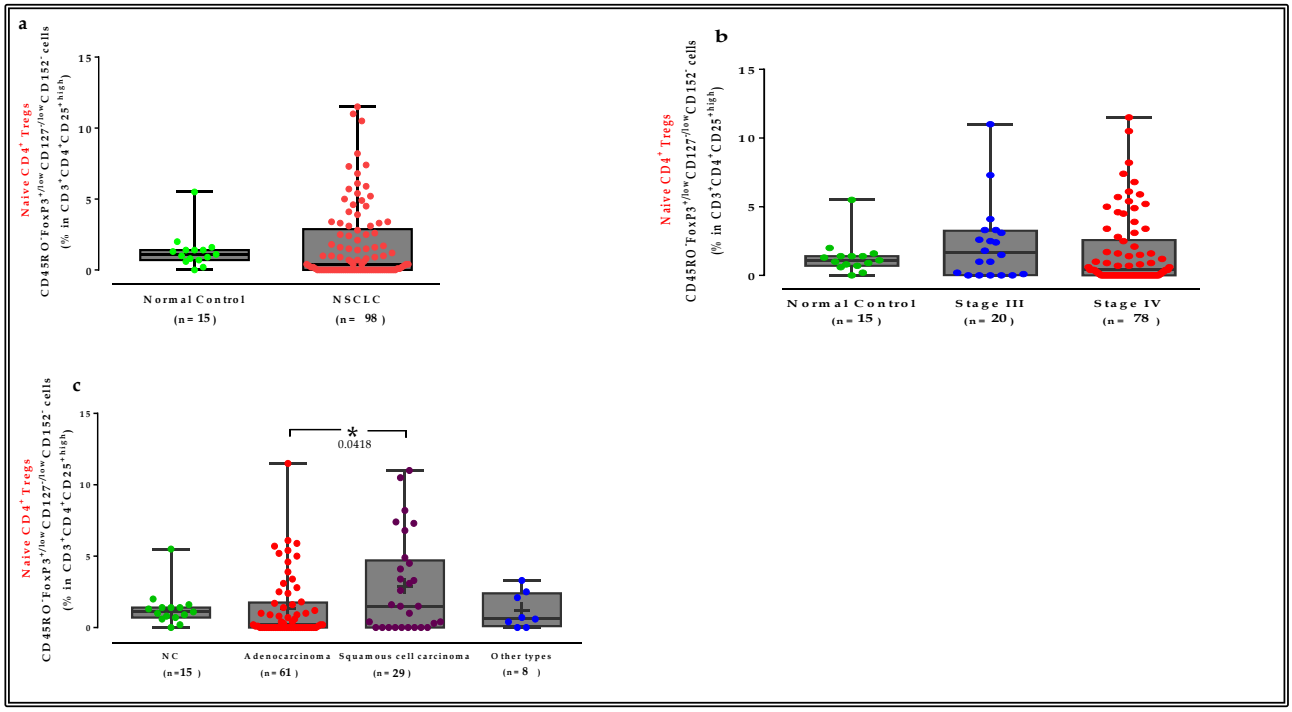

Figure 3.13: The percentage of Naïve Treg cells in NSCLC patients and healthy donors. (a) The percentage of Naïve Treg cells revealed no difference between the presence of disease and (b) the stage of the carcinoma. (c) Although, the mean percentage of the Naïve Tregs present a significant reduction in patients with adenocarcinoma than in patients with squamous cell carcinoma. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented.

No differences were reported in proportions of $CD25^{+high}CD127^{+/low}CD152^{+}FOXP3^{+}CD45RO^{+}$ Effector Treg cells during NSCLC disease and between the different types of the carcinoma (figure 3.14 a, c). In contrast, in stage IV patients presented a significant increase in effector Treg cell counts when compared with stage III (figure 3.14 b).

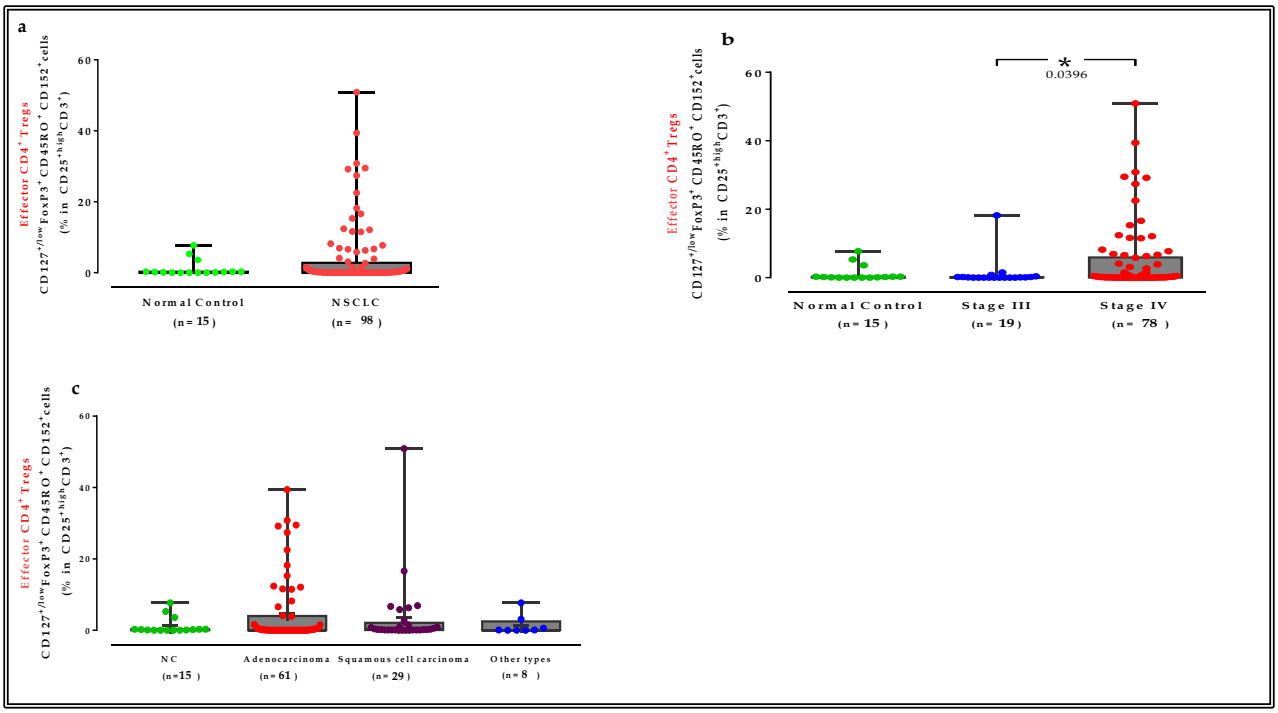

Figure 3.14: The percentage of effector Treg cells in NSCLC patients and healthy donors. (a) No difference was observed in the percentage of effector Treg cells between the presence of disease and (c) the type of the carcinoma. (b) Although, in stage IV patients present a significant increase in effector Treg cell counts when compared with stage III. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented.

There was no significant difference in the levels of the populations of $CD25^{+high}CD127^{-}CD152^{+}FOXP3^{+}CD45RO^{+}$ Terminal Effector Treg cells in the patients with Non-Small Cell Lung Cancer in comparison with those in healthy donors and between the stages of the carcinoma. However, we confirmed a significant increase in the percentages of Terminal Effector Treg cells in individuals with squamous cell carcinoma (15.74± 2.343%) and other types of carcinoma (15.26± 3.922%), as compared to individuals with adenocarcinoma (7.798± 1.385%) (P= 0.0011 and 0.0208 respectively) (figure 3.15).

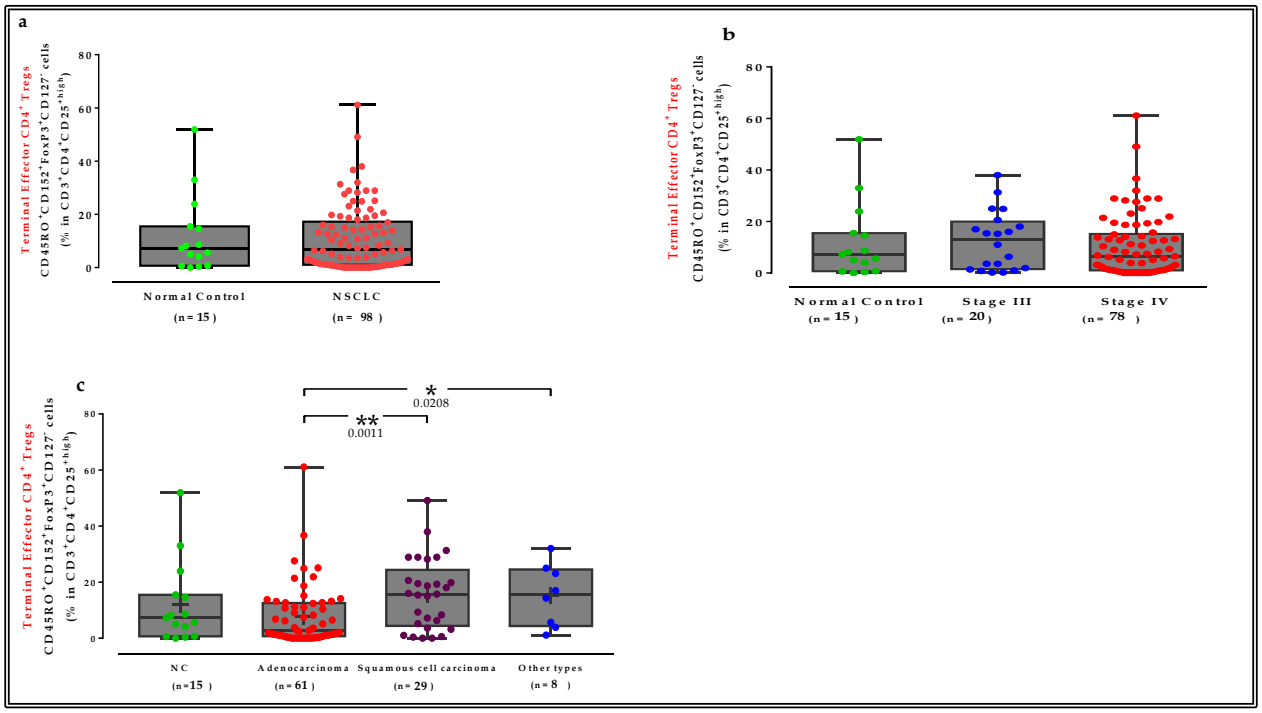

Figure 3.15: The levels of Terminal Effector Treg cells in NSCLC patients. There was no significant difference in the levels of the populations of terminal effector Treg cells (a) in the patients with Non-Small Cell Lung Cancer in comparison with those in healthy donors and (b) between the stages of the carcinoma. (c) Although, percentages of Terminal Effector Treg cells were significantly higher in both squamous cell carcinoma and other types of carcinoma patients, as compared to adenocarcinoma patients. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented.

CCR7 plays an important role in the initiation of adaptive immune responses. CCR7 mediates the migration of $CD25^{+high}CD127^{-/low}$CTLA-4-$FOXP3^{+/low}CD45RO^{-}$ Naïve $CD4^{+}$ T cells into the secondary lymphoid organs and is a prerequisite for their encounter with mature dendritic cells, the productive presentation of cognate antigen, and consequent T cell proliferation and effector differentiation (Sallusto et al., 1999). As a result, CCR7 expression on Treg differentially controls in vivo function of the naive- and effector/memory-like subsets (Menning et al., 2007).

In this study, the mean percentage of $CCR7^{+}$ $CD25^{+high}CD127^{-/low}$CTLA-4-$FOXP3^{+/low}CD45RO^{-}$ Naïve $CD4^{+}$ Tregs was significantly enhanced in the NSCLC patients (0.2684± 0.0805%) compared with healthy donors (0± 0%) (P=0.0089) (figure 3.16 a). Moreover, the percentages of $CCR7^{+}$ Naïve $CD4^{+}$ Tregs did not differ between the stage or the type of the carcinoma (figure 3.16 b, c).

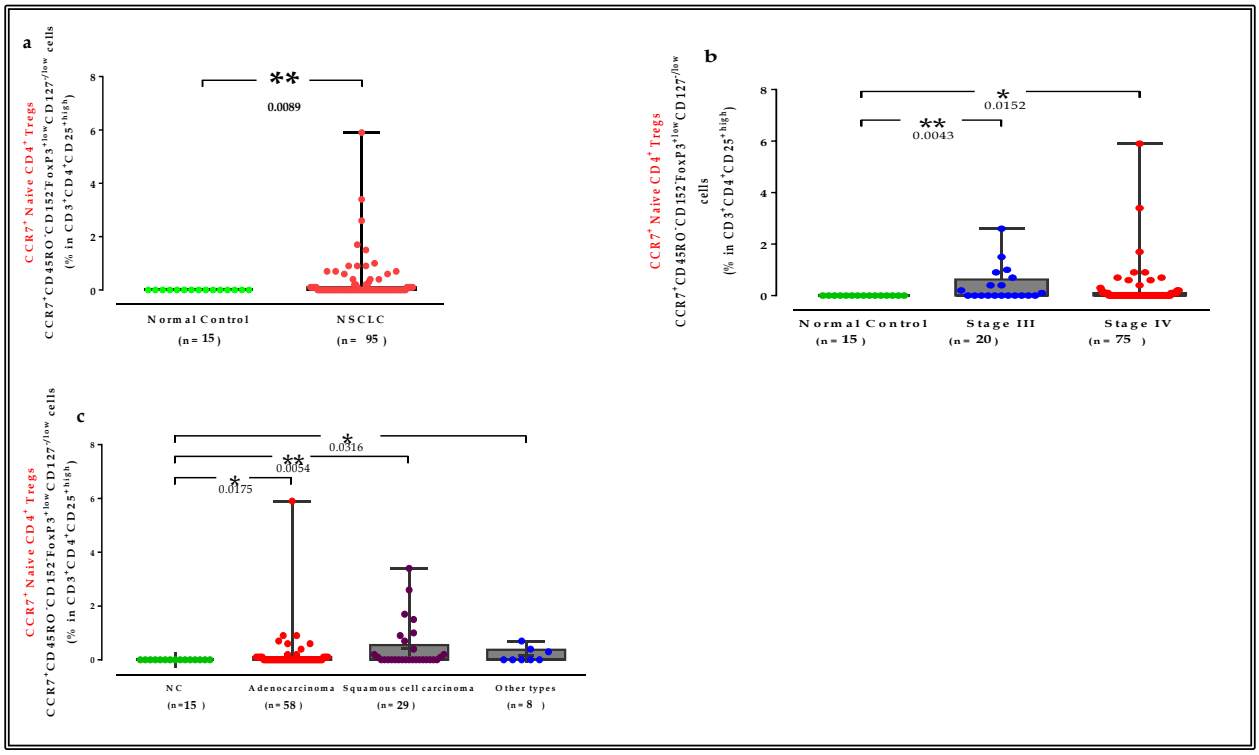

Figure 3.16: NSCLC patients show a significant increase in $CCR7^{+}$ Naïve $CD4^{+}$ Treg cells. The percentage of $CCR7^{+}$ Naïve $CD4^{+}$ Treg cells in the peripheral blood of NSCLC patients was determined by flow cytometry and compared with healthy controls (Unpaired t test with Welch's correction). (a)The mean percentage of $CCR7^{+}$ Naïve $CD4^{+}$ Treg cells in the peripheral blood was significantly higher (P=0.0089) in NSCLC patients compared to normal control. The number of $CCR7^{+}$ Naïve $CD4^{+}$ Treg cells did not correlate with either (b) tumour stage or (c) tumour type. Each point corresponds to an individual

patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented.

Central memory Tregs are characterized by the expression of CCR7 and CD45RO. Previous studies demonstrated that the majority of peripheral Tregs in healthy individuals display a central memory phenotype (Mailloux & Epling-Burnette , 2013). Central memory Treg cells may represent an inactive, long-term memory population. In this study, no significant difference was observed for the mean percentage of the $CCR7^{+}$ $CD25^{+high}CD127^{+/low}CD152^{+}FOXP3^{+}CD45RO^{+}$ Central memory Tregs in NSCLC patients and healthy individuals (figure 3.17).

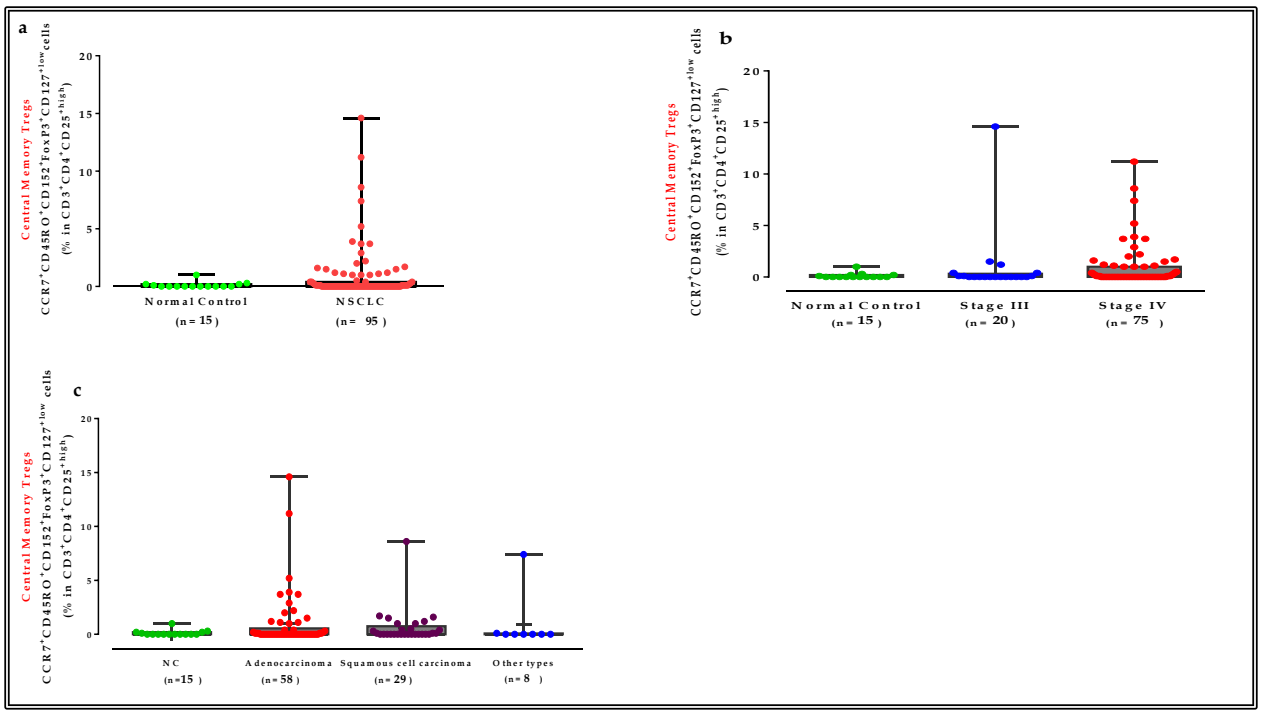

Figure 3.17: The levels of Central memory Treg cells in the presence of disease. The population of Central memory Treg cells was evaluated by flow cytometric analysis. No significant difference was found in the levels of Central memory Treg cells in patients with NSCLC in comparison with that in healthy donors (Unpaired t test with Welch's correction) (a). Moreover, the percentage of Central memory Tregs did not collate with neither the stage (b) nor the type (c) of carcinoma. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented.

Terminal Memory Effector Treg cells are more suppressive than CCR7^{+} CD25^{+high}CD127$^{+/low}$CD152^{+}FOXP3^{+}CD45RO^{+} Central Memory Treg cells in vitro (Mailloux & Epling-Burnette , 2013). The highly suppressive CCR7^{-} CD25^{+high} CD127^{-}CD152^{+} FOXP3^{+} CD45RO^{+} Terminal Memory Effector Treg cells are independent from other Treg subtypes and the analysis of their phenotype is suggested as a simple tool to predict an early immune escape (Mailloux et al., 2012). A significantly higher percentage of CCR7^{-} CD25^{+high}CD127^{-} CD152^{+}FOXP3^{+}CD45RO^{+} Terminal Memory Effector Tregs within the CD4^{+} T-cell compartment was observed in NSCLC patients (1.733± 0.3239%) compared to controls (9.196± 1.105%) (P=0.025) (figure 3.18 a). In addition, there was no statistical difference in the levels of Terminal Memory Effector Tregs between NSCLC patients with different stage or type of carcinoma (figure 3.18 b, c).

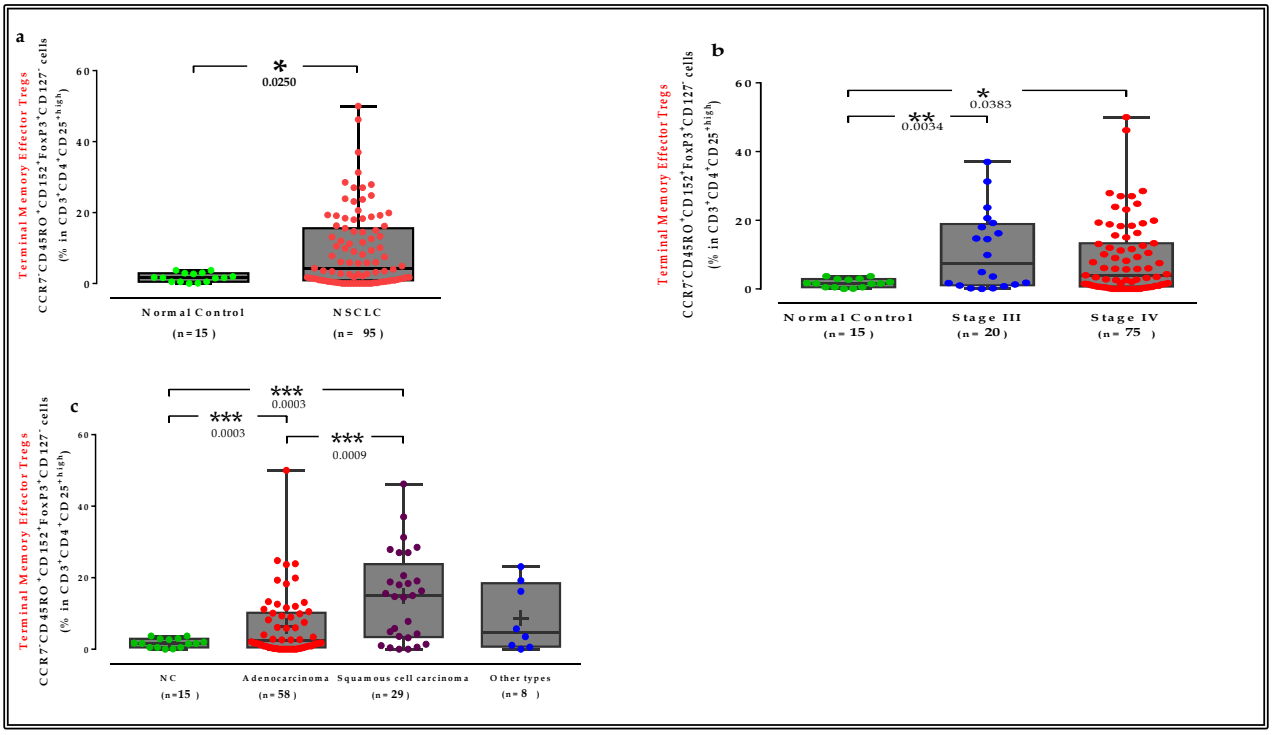

Figure 3.18: The percentage of naive Treg cells in NSCLC patients and healthy donors. A significantly higher percentage of Terminal Memory Effector Tregs was observed in NSCLC patients compared to controls (P=0.025) (a) Although, there was no statistical difference in the mean percentage of the Terminal Memory Effector Tregs between NSCLC patients with different (b) stage or (c) type of carcinoma. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented.

3.8 THE OVERAL EFFECT OF CHEMOTHERAPY ON THE POPULATIONS OF CD4+ Treg CELLS

To test the impact of chemotherapy in the immunosuppressive cells, the percentages of CD4+ T cells were evaluated by flow cytometric analysis after the 3rd and the 6th cycle of treatment. Figure 3.19 showed that there were no significant differences between the levels of the populations of CD4+T cells in patients who completed all the cycles of chemotherapy (figure 3.19 a). A significant decrease of CD25+ T cells was observed 6 months after the first cycle of chemotherapy (24.98± 1.044% to 20.16± 1.843%) (P=0.0245) (figure 3.19 b). Although, the mean percentage of CD25+high T cells significantly increased after the 3rd cycle of chemotherapy (0.8610± 0.09249% to 1.594± 0.5002%) (P=0.0069), it returned to the basal levels at the end of the treatment (non-significant decrease) (figure 3.19 c).

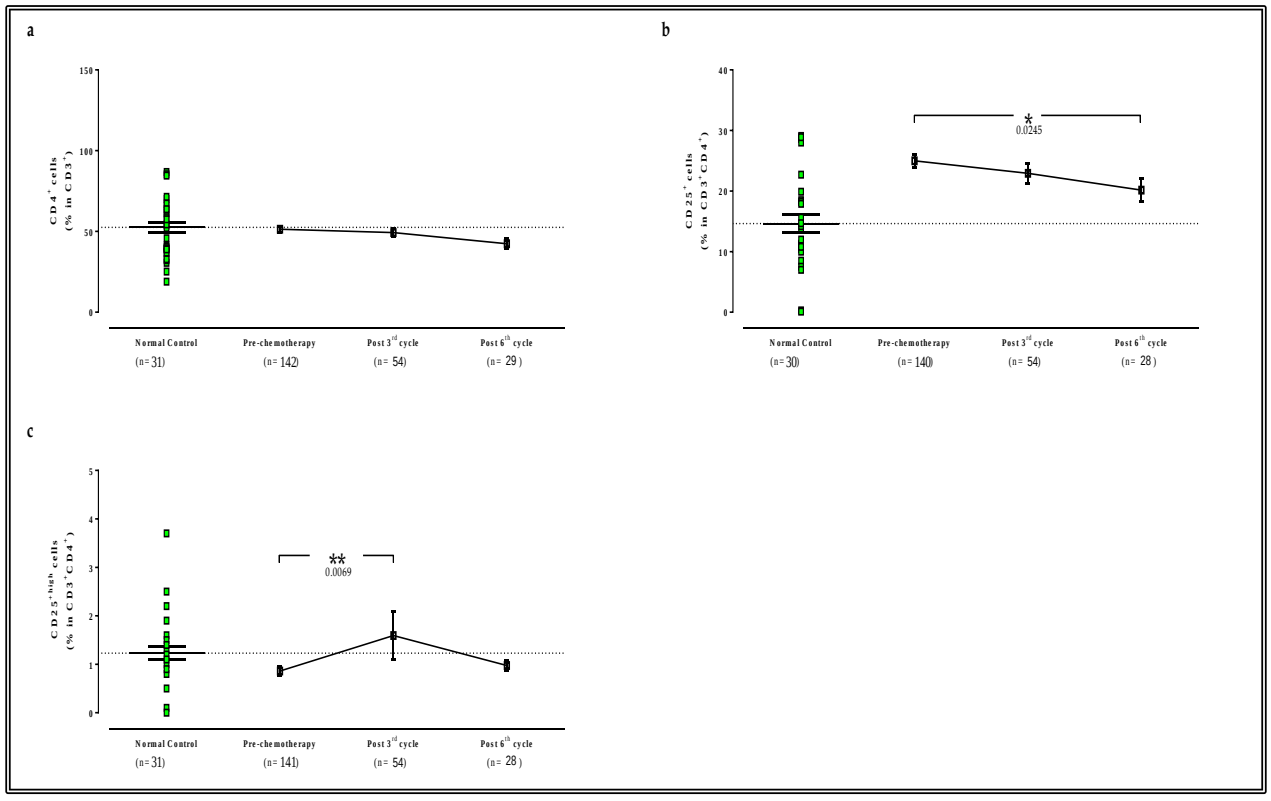

Figure 3.19: The effect of chemotherapy on CD4+ Treg cells of NSCLC patients. (a) No significant difference was observed in the mean percentage of CD4+T cells during the 6 cycles of treatment. (b) A significantly lower percentage of CD25+ T cells was observed after the 6th cycle of chemotherapy compared to non-treated patients (P=0.0245). (c) Although, the mean percentage of CD25+high T cells significantly increased after the 3rd cycle of chemotherapy (P=0.0069), but it returned to

the first levels at the end of the treatment (non-significant decrease). Dashed line represents the levels of corresponding cells in normal controls. Y axis shows the cells tested. Percentages indicated in the plots represent the percentages of phenotypic marker expression in the parental population, which are presented inside the brackets. The data are represented as the mean ± SEM and the p values are determined by Mann-Whitney test.

No statistically significant difference was observed in the mean percentage of $FoxP3^+$ T cell populations ($CD25^+$, $CD25^{+high}$) during the treatment. In contrast, the levels of $CD127^{-/low}CD152^+FoxP3^+$ $CD25^{high}$ cells statistically increased in response to chemotherapy in NSCLC patients (23.88± 1.865% to 37.15± 3.187%) at the end of treatment, as compared to the basal levels (P=0.0435) (figure 3.20 c). The same change did not observed in the levels of $CD13^+$ and $CD39^+$ $CD13^+$ Treg populations, which remained unchanged in all cycles of chemotherapy.

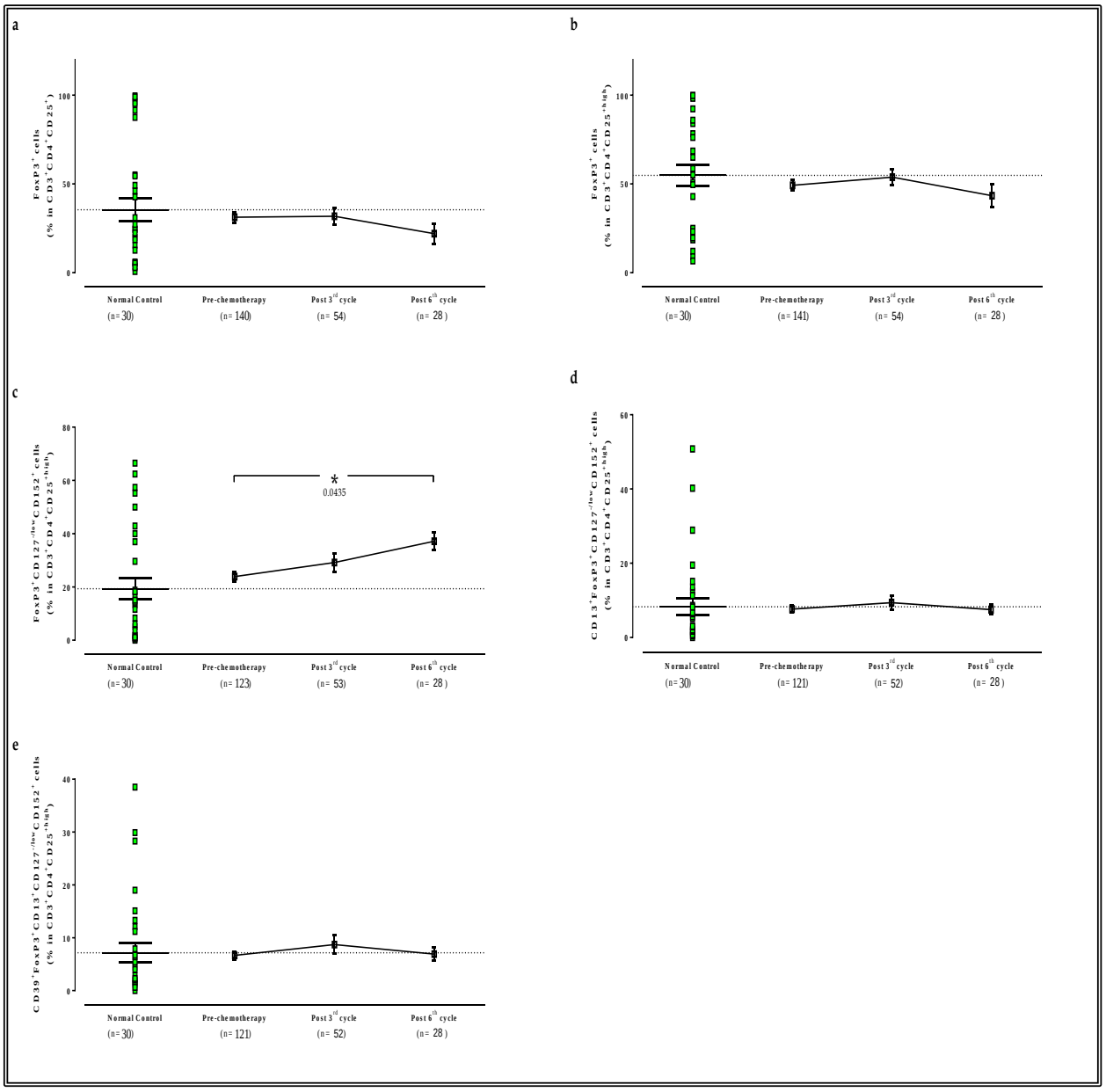

Figure 3.20: The effect of chemotherapy on FoxP3⁺ T cells of NSCLC patients. (a, b) No significant difference was observed in the mean percentage of FoxP3⁺CD25⁺ and FoxP3⁺CD25^{+high} T cells after gating for CD4⁺CD3⁺ during the 6 cycles of treatment. (c) A significantly higher percentage of Treg cells (FoxP3⁺CD127$^{-/low}$CD152⁺ cells in CD3⁺CD4⁺CD25^{+high})was observed at the end of the treatment compared to non-treated patients (P=0.0435). (d, e) The mean percentage of CD13⁺ and CD39⁺CD13⁺ Treg cells remained unchanged during the treatment. Dashed line represents the levels of corresponding cells in normal controls. Y axis shows the cells tested. Percentages indicated in the plots represent the percentages of phenotypic marker expression in the parental population, which are presented inside the brackets. The data are represented as the mean ± SEM and the p values are determined by Mann-Whitney test.

NSCLC patients exhibited a statistically significant increase in peripheral $CD25^{+high}CD127^{-/low}$CTLA-4-$FOXP3^{+/low}CD45RO^{-}$ Naïve $CD4^{+}$ Tregs, after the 3rd (1.801± 0.2672% to 3.095± 0.8453%) (P=0.00462) and the 6th (1.801± 0.2672% to 3.319± 0.8612%) (P=0.0012) cycle of chemotherapy (figure 3.21 a). An opposite picture was seen by determining the percentage of $CD25^{+high}CD127^{+/low}CD152^{+}FOXP3^{+}CD45RO^{+}$ Effector $CD4^{+}$ Tregs. We observed that those percentages were significantly lower at the end of treatment compared to that pre-treatment (4.140± 0.9287% to 1.419± 0.719%) (P=0.046) (figure 3.21 b). Moreover, a higher percentage of $CD25^{+high}CD127^{-}CD152^{+}FOXP3^{+}CD45RO^{+}$ Terminal Effector Tregs was noticed in the group of patients treated with 6 cycles of chemotherapy compared to that estimated in pre-chemotherapy NSCLC cancer patients (10.76± 1.203% to 20.69± 4.277%) (P=0.0296) (figure 3.21 c).

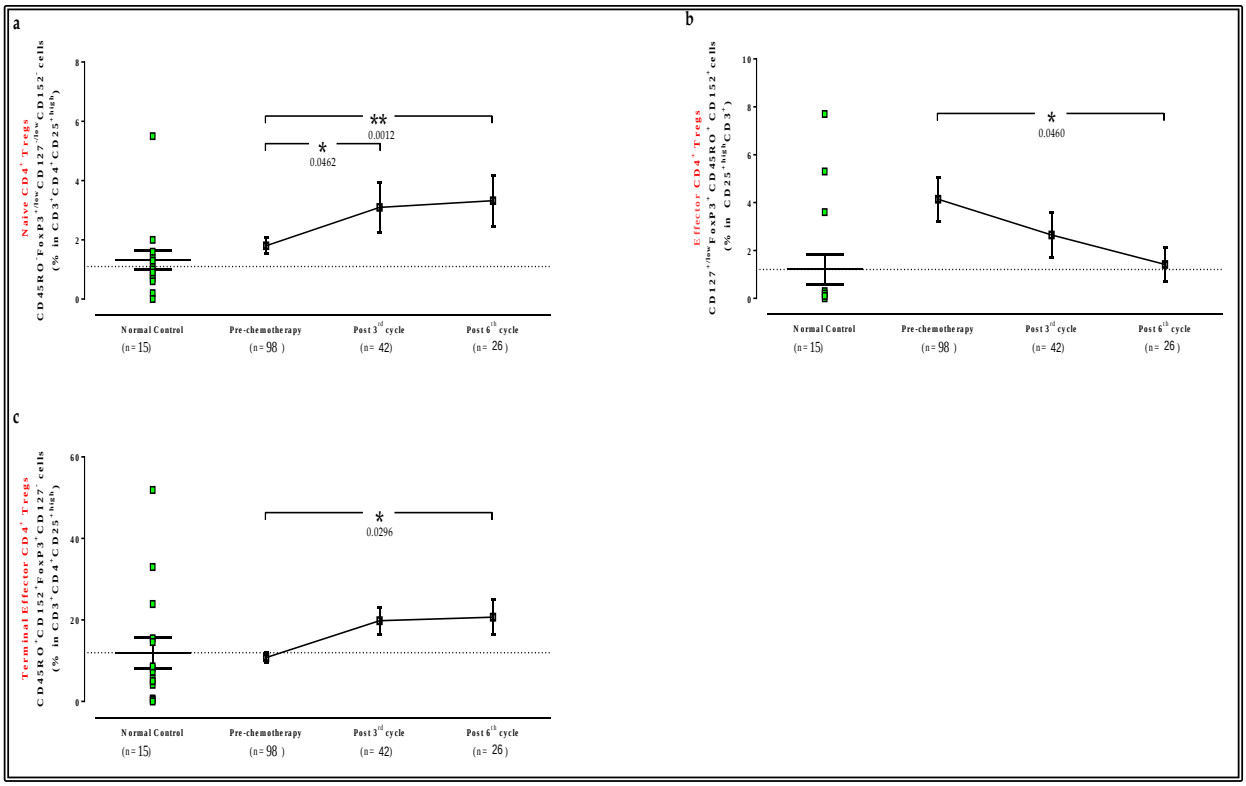

Figure 3.21: The effect of chemotherapy on Treg subtypes of NSCLC patients. (a) A significantly higher percentage of Naïve $CD4^{+}$ Treg cells was observed after the 3rd (P=0.00462) and the 6th (P=0.0012) cycle of chemotherapy. (b) The mean percentage of Effector $CD4^{+}$ Tregs was significantly reduced at the end of treatment compared to the pre-treatment levels (P=0.046). (c) Significantly higher percentage of Terminal Effector $CD4^{+}$ Tregs were noticed at the end of treatment compared to the pre-treatment levels (P=0.0296). Dashed line represents the levels of corresponding cells in normal controls. Y axis shows the cells tested. Percentages indicated in the plots represent the percentages of phenotypic

marker expression in the parental population, which are presented inside the brackets. The data are represented as the mean ± SEM and the p values are determined by Mann-Whitney test.

The mean percentage of CCR7+ Naive Tregs was elevated in the peripheral blood of the NSCLC patients undergoing chemotherapy (0.2684± 0.0805% to 0.7955± 0.233%) (P=0.0077) (figure 3.22 a). However, the percentage of CCR7+ CD25+highCD127+/lowCD152+FOXP3+CD45RO+ Central Memory Treg cells decreased after chemotherapy from a mean value of 0.8768% (± 0.2368) before chemotherapy to a mean value of 0.5091% (± 0.3241) after 3 cycles of treatment (P=0.0469) (figure 3.22 b). The proportion of CCR7- CD25+highCD127-CD152+FOXP3+CD45RO+ Terminal Memory Effector increased significantly in NSCLC patients treated with chemotherapy compared with those non-treated (9.196 ± 1.105% to 20.02± 4.229%) (P=0.0033) (figure 3.22 c).

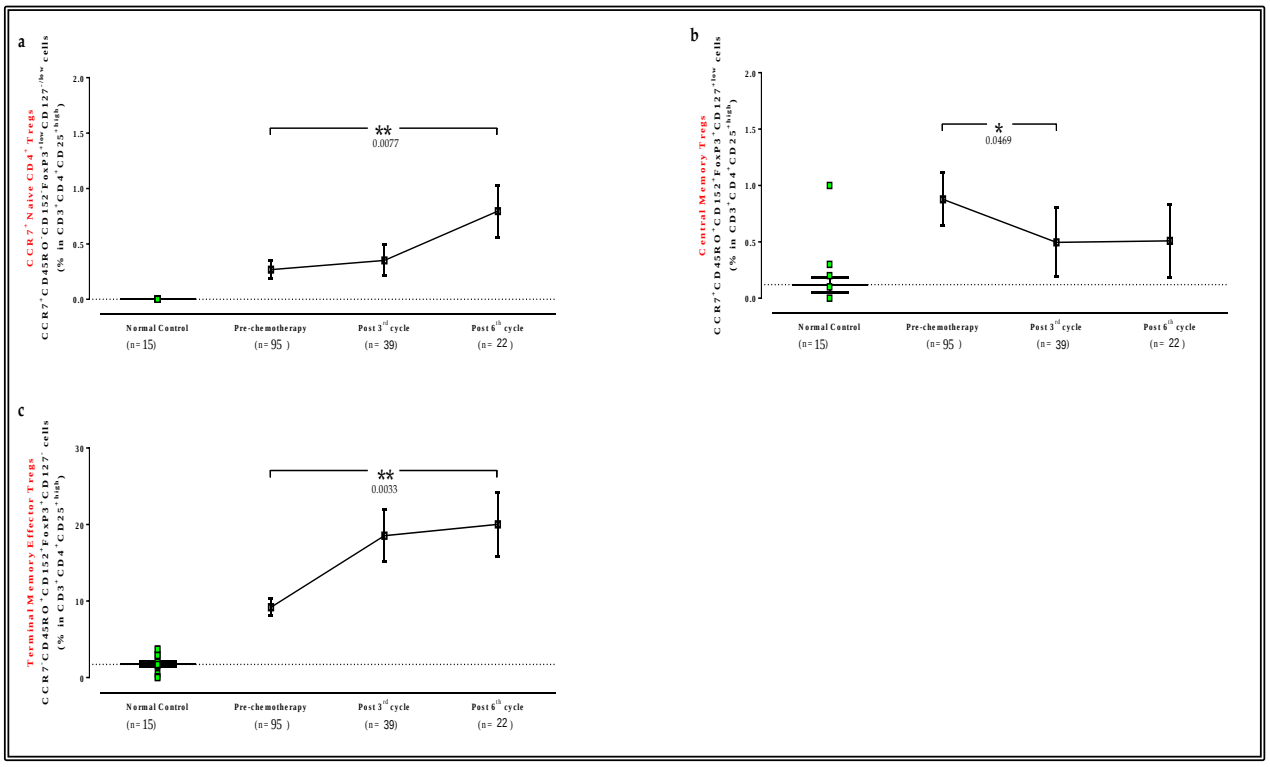

Figure 3.22: The effect of chemotherapy on CCR7+ Treg subtypes of NSCLC patients. (a) A significantly higher percentage of CCR7+ Naïve CD4+ Treg cells was observed in the peripheral blood of the NSCLC patients undergoing chemotherapy (P=0.077). (b) The mean percentage of Central Memory Tregs was significantly reduced after the 3rd cycle of chemotherapy (P=0.0469). (c) Significantly higher proportion of Terminal Memory Effector were noticed at the end of chemotherapy compared to this pre-treatment (P=0.0033). Dashed line represents the levels of corresponding cells in normal controls. Y axis shows the cells tested. Percentages indicated in the plots represent the percentages of phenotypic marker

expression in the parental population, which are presented inside the brackets. The data are represented as the mean ± SEM and the p values are determined by Mann-Whitney test.

3.9 THE EFFECT OF CHEMOTHERAPY IN EACH PATIENT

Before-after statistical analysis revealed the changes of each CD4+ Treg cell populations in each patient. The patients were divided in groups according to the change of each CD4+ Treg population after 3 or 6 cycles of chemotherapy (figure 3.23). The levels of FoxP3+CD127-/lowCD152+ Treg cells as a percentage of total CD3+CD4+CD25+high cells increased after the third cycle of chemotherapy in 25 patients, whereas in 21 patients were decreased. However, in 5 patients the mean percentage of FoxP3+CD127-/lowCD152+ Treg cells remained unchanged (figure 3.23 a, b). Similarly, the percentage of these cells after 6 cycles of treatment increased in 17, decreased in 8 and remained unchanged in one patient (figure 3.23 c, d).

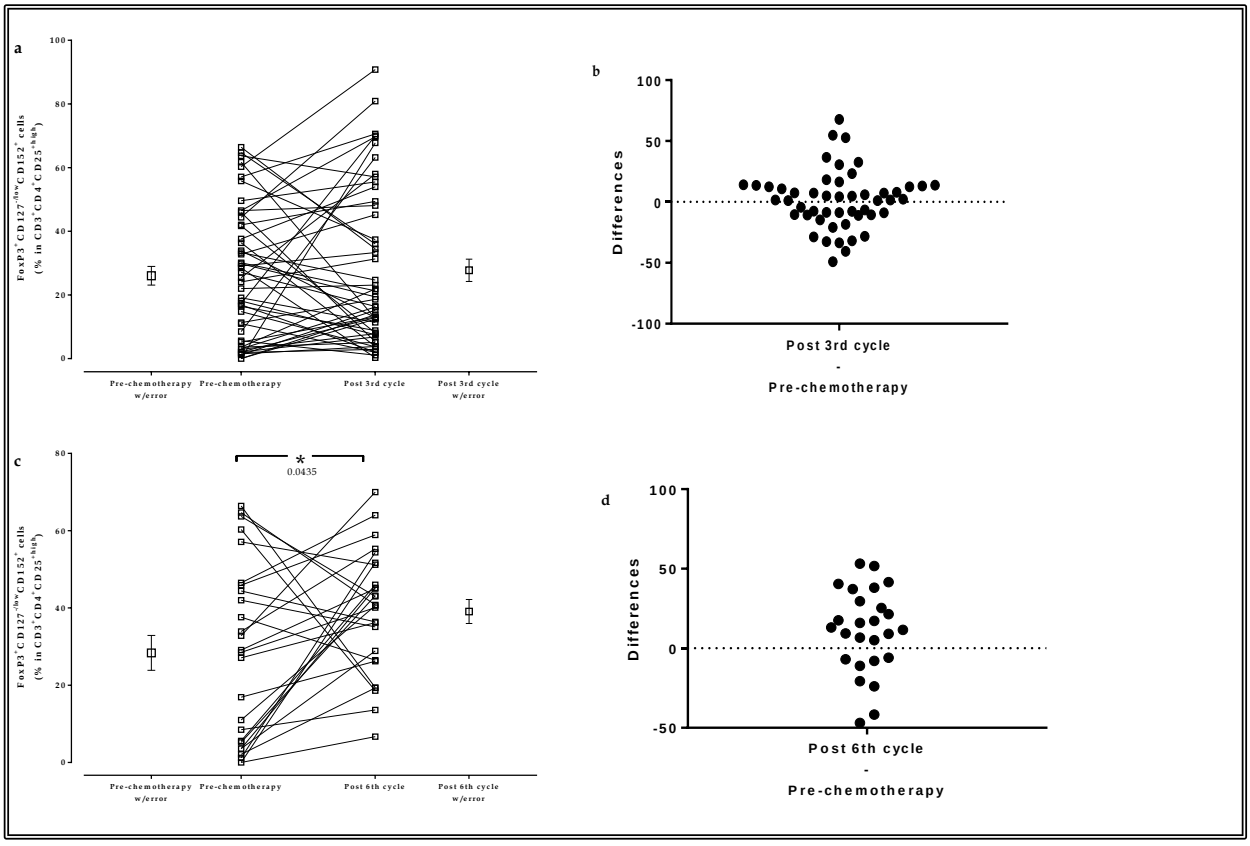

Figure 3.23: The changes of Treg population in NSCLC patients during the chemotherapy. The before after plot represents the changes in the percentage of FoxP3+CD127-/lowCD152+ Treg cells after

the (a) 3rd and the (c) 6th cycle of chemotherapy in each patient. The differences plot shows the subtraction of the percentage of FoxP3+CD127-/lowCD152+ Treg cells in the (b) 3rd or the (c) 6th cycle minus the percentage before the treatment. Each line corresponds to an individual patient. Each plot corresponds to the difference of the value at 4th or 6th cycle minus the basal value of an individual patient.

The percentage of CD25+highCD127-/lowCTLA-4-FOXP3+/lowCD45RO- Naïve CD4+ cells was higher in 16 patients after the 3rd cycle of chemotherapy, compared to the percentage before the treatment, while in 6 patients it was lower (figure 3.24 a, b). In the rest 17 patients, the percentage remained unchanged. However, after the 6th cycle of treatment the percentage of Naïve CD4+ cells increased in the half of the 20 patients, while in the rest 10 patients in remained unchanged (figure 3.24 c, d).

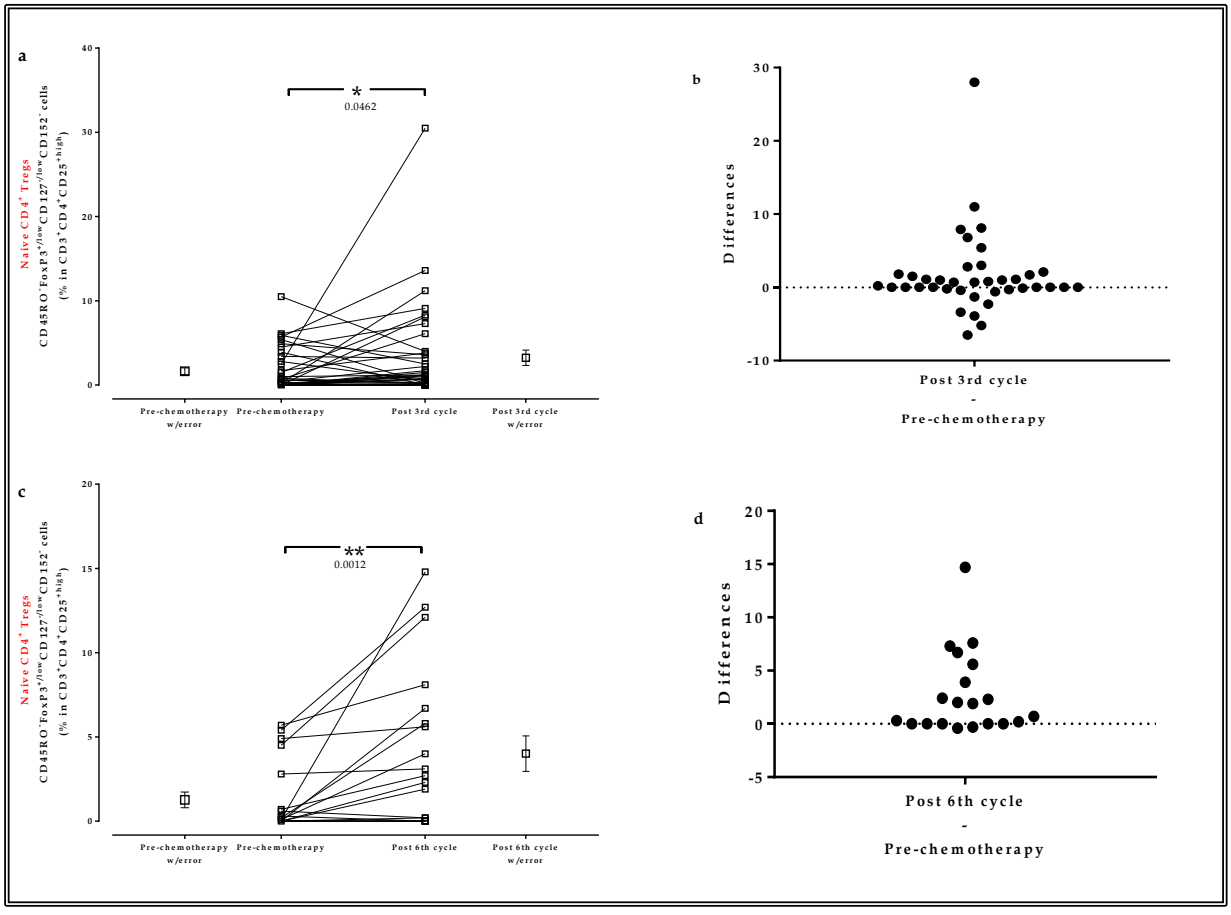

Figure 3.24: The changes of Naive Treg population in NSCLC patients during the chemotherapy. The changes in the percentage of Naive Treg cells in each patient during the first (a) three and (c) six cycles of chemotherapy are shown at the before-after plot. At the differences plot, it is shown that the subtractions of the percentage of Naïve Tregs minus the percentage before the treatment were equally distributed in the (b) 3rd cycle, whereas were mainly positive in the (c) 6th cycle. Each line corresponds to an individual

patient. Each plot corresponds to the difference of the value at 4th or 6th cycle minus the basal value of an individual patient.

In addition, the percentage of $CD25^{+high}CD127^{+/low}CD152^{+}FOXP3^{+}CD45RO^{+}$ Effector $CD4^{+}$ cells decreased in 10 patients remained at the same level at 23 patients and increased at 6 patients after the 3rd cycle of chemotherapy (figure 3.25 a, b). At the same pattern, after the 6th cycle of treatment the percentage of Effector $CD4^{+}$ cells was lower at 8 patients, higher at 1 patient and show no difference at 11 patients compared to the pre-treatment levels (figure 3.25 c, d).

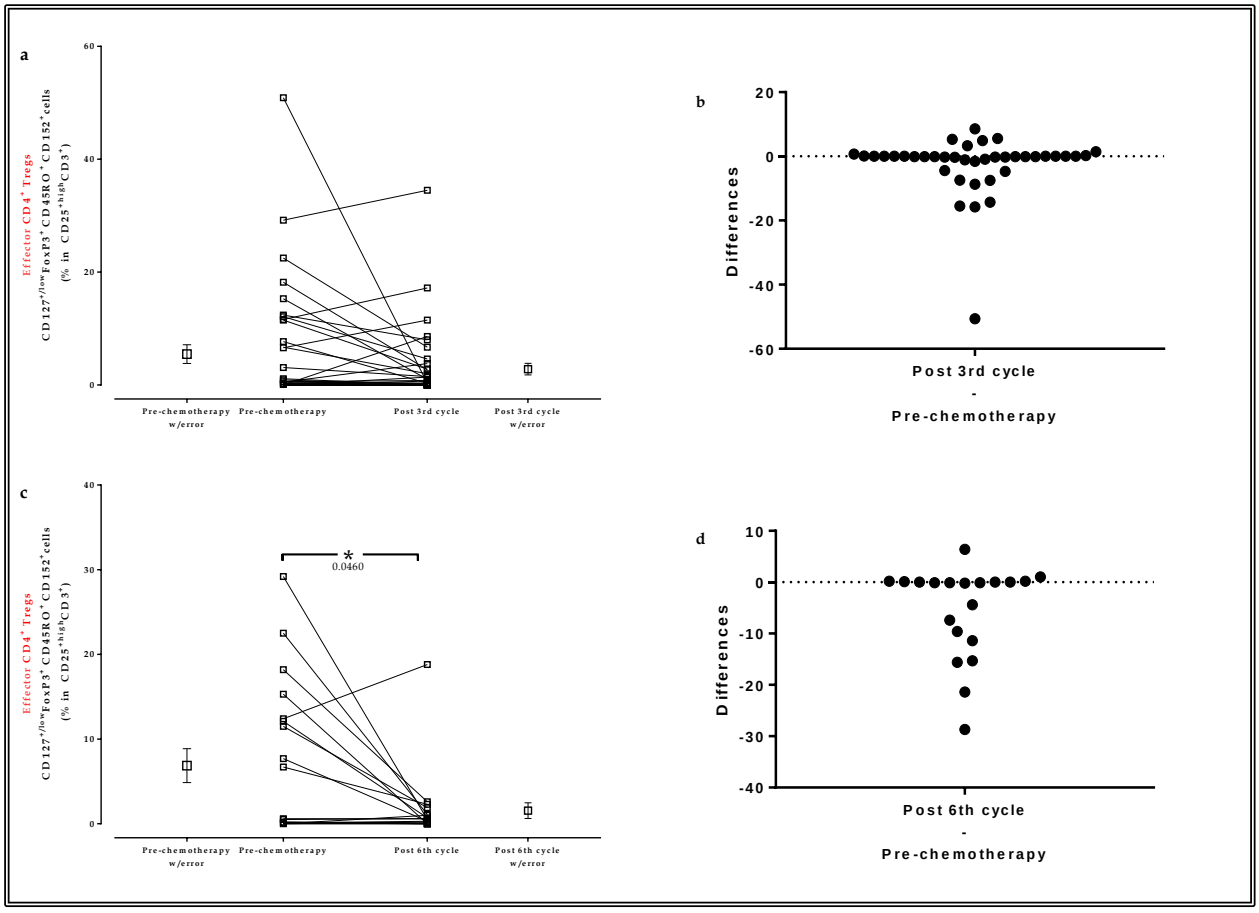

Figure 3.25: The changes of Effector Treg population in NSCLC patients during the chemotherapy. The changes in the percentage of Effector Treg cells in each patient during the first (a) three and (c) six cycles of chemotherapy are shown at the before-after plot. At the differences plot, it is shown that the subtractions of the percentage of Naïve Tregs minus the percentage before the treatment were equally distributed in the (b) 3rd cycle, whereas were mainly positive in the (d) 6th cycle. Each line corresponds to an individual patient. Each plot corresponds to the difference of the value at 4th or 6th cycle minus the basal value of an individual patient.

The level of $CD25^{+high}CD127^{-}CD152^{+}FOXP3^{+}CD45RO^{+}$ Terminal Effector Treg cells increased in 15 patients decreased in 12 patients and did not differ in 12 patients after three cycles of chemotherapy (figure 3.26 a, b). The majority of the differences between the percentage of Terminal Effector T cells in the 6th cycle and before the treatment were positive (12 patients), while 3 of them were negative (5 were stable) (figure 3.26 c, d).

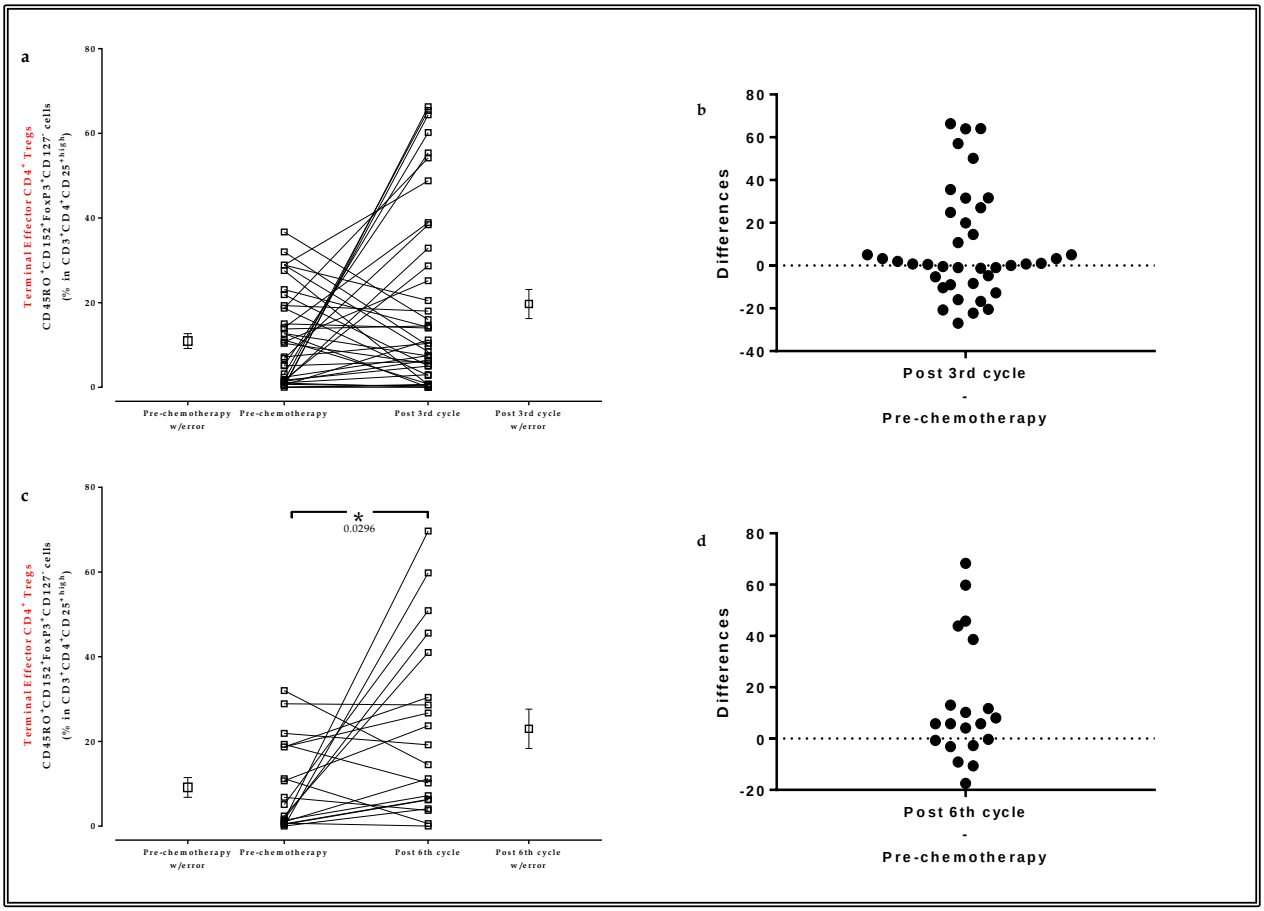

Figure 3.26: The changes of Terminal Effector Treg population in NSCLC patients during the chemotherapy. The variation of Terminal Effector Treg cell percentage in each patient during the first (a) three and (c) six cycles of chemotherapy is represented at the before-after plot. The differences plot shows the subtraction of the percentage of Terminal effector Treg cells in the (b) 3rd or the (d) 6th cycle minus the percentage before the treatment. Each line corresponds to an individual patient. Each plot corresponds to the difference of the value at 4th or 6th cycle minus the basal value of an individual patient.

The CCR7+ Naïve Treg cell population remained in the same levels during the first 3 cycles of chemotherapy in the majority of the patients (29) (increased at five patients and decreased in three) (figure 3.27 a, b). However, at the end of the treatment the majority of patients had higher percentage of CCR7+ Naïve Treg cells (12 of 18) (in two cases it decreased and in four remained stable) (figure 3.27 c, d).

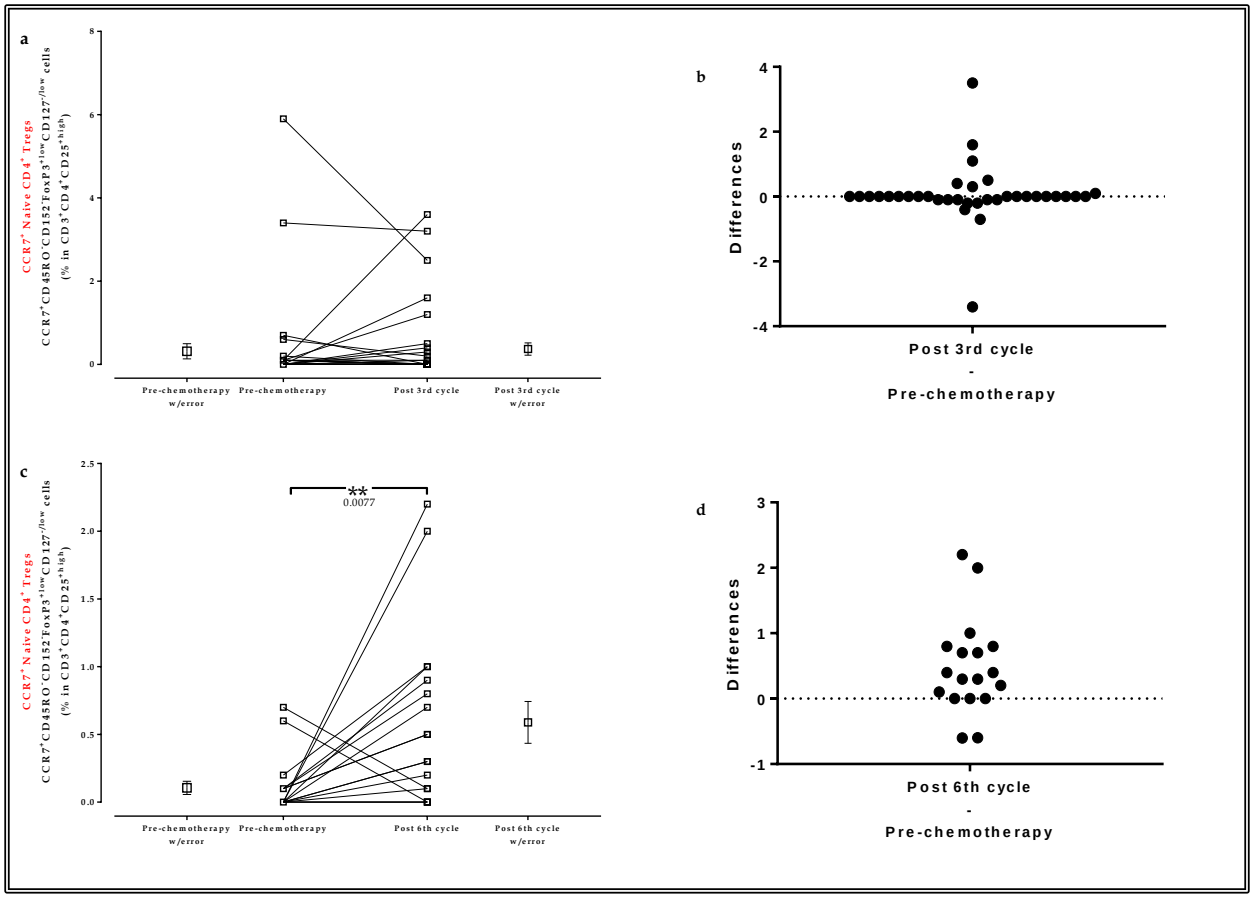

Figure 3.27: The variation of CCR7+ Naive Treg population in NSCLC patients during the chemotherapy. The changes in the percentage CCR7+ Naïve Treg cells in each patient during the first (a) three and (c) six cycles of chemotherapy are shown at the before-after plot. The differences plot shows the subtraction of the percentage of CCR7+ Naïve Treg cells in the (b) 3rd or the (c) 6th cycle minus the percentage before the treatment. Each line corresponds to an individual patient. Each plot corresponds to the difference of the value at 4th or 6th cycle minus the basal value of an individual patient.

The CCR7+ CD25+highCD127+/lowCD152+FOXP3+CD45RO+ Central Memory Treg population followed the same pattern with levels stable in the most of the patients during the 3 first cycles of chemotherapy (n=24 of total 37) (figure 3.28 a, b). In nine patients the levels were decreased, while in four were higher in the 3rd cycle, compared to the levels before the treatment. In addition, the percentage of the Central Memory Treg cells was stable in eleven patients, decreased in five patients and increased in 2 patients during all the cycles of the treatment (figure 3.28 c, d).

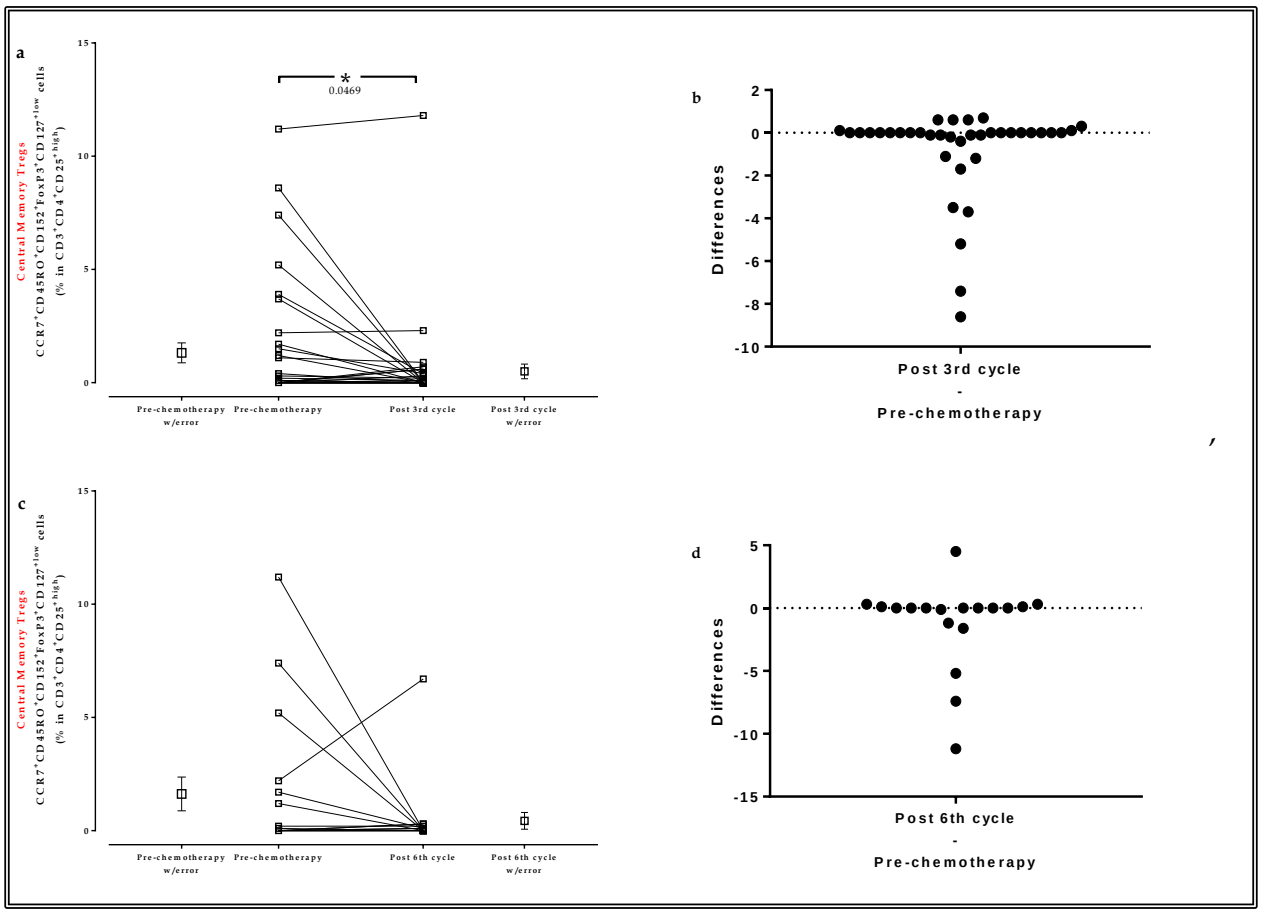

Figure 3.28: The changes of Central Memory Treg population in NSCLC patients during the chemotherapy. The changes in the percentage of Central Memory Treg cells in each patient during the first (a) three and (c) six cycles of chemotherapy are shown at the before-after plot. At the differences plot, it is shown that the subtractions of the percentage of Naïve Tregs minus the percentage before the treatment were mainly negative or zero in the (b) 3rd and in the (c) 6th cycle. Each line corresponds to an individual patient. Each plot corresponds to the difference of the value at 4th or 6th cycle minus the basal value of an individual patient.

Finally, the percentages of the CCR7- CD25+highCD127-CD152+FOXP3+CD45RO+ Terminal Memory Effector Treg cells were increased or stable after the 6th cycle of chemotherapy, compared to the levels before the treatment, in the majority of patients (figure 3.29 c, d). In contrast, there was no statistical significant difference after the 3rd cycles, as the level of Terminal Memory Effector Treg cells increased in 17, decreased in 11 and was unchanged in 9 patients (figure 3.29 a, b).

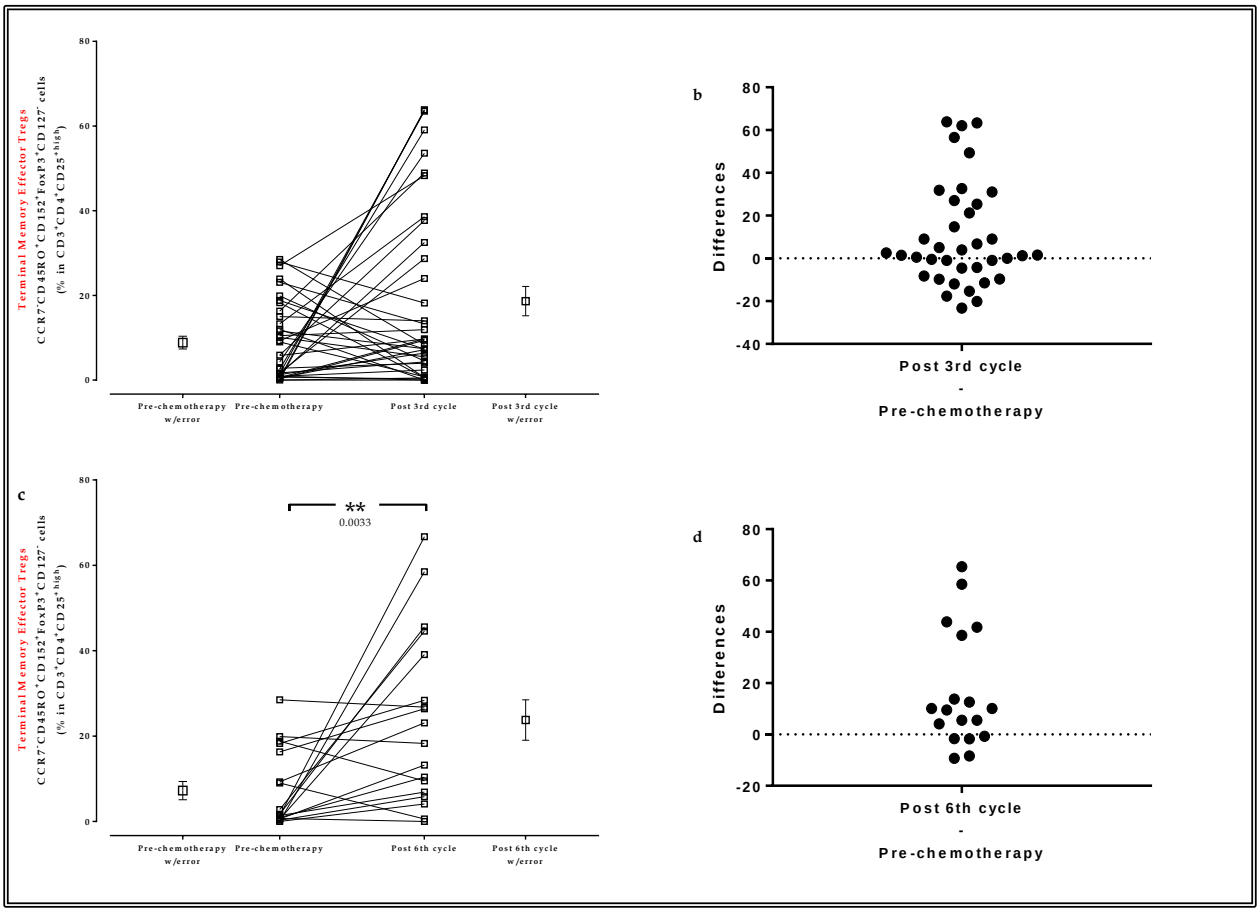

Figure 3.29: The changes of Terminal Memory Effector Treg population in NSCLC patients during the chemotherapy. The variation of Terminal Memory Effector Treg cell percentage in each patient during the first (a) three and (c) six cycles of chemotherapy is represented at the before-after plot. The differences plot shows the subtraction of the percentage of Terminal effector Treg cells in the (b) 3rd or the (c) 6th cycle minus the percentage before the treatment. Each line corresponds to an individual patient. Each plot corresponds to the difference of the value at 4th or 6th cycle minus the basal value of an individual patient.

3.10 THE EFFECT OF BEVACIZUMAB TREATMENT IN CD4+ Treg SUBPOPULATIONS

In order to evaluate the effect of bevacizumab treatment in the different CD4+ Treg subpopulations, NSCLC patients were divided in two groups. The change of the mean value was assessed after three and six cycles of treatment. The results were analyzed by multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

At baseline, the mean percentage of $CD3^+CD4^+CD25^{+high}CD127^{-/low}CD152^+Foxp3^+$ Tregs was equal in the two groups. However, the Avastin-based treatment had as a result the reduction of the mean percentage of $CD3^+CD4^+CD25^{+high}CD127^{-/low}CD152^+Foxp3^+$ Tregs, while the mean value was increased in the Non Avastin-based group. As a result, the mean percentage of $CD3^+CD4^+CD25^{+high}CD127^{-/low}CD152^+Foxp3^+$ Tregs was significantly higher in chemotherapy treated patients as compared with bevacizumab treated patients (figure 3.30).

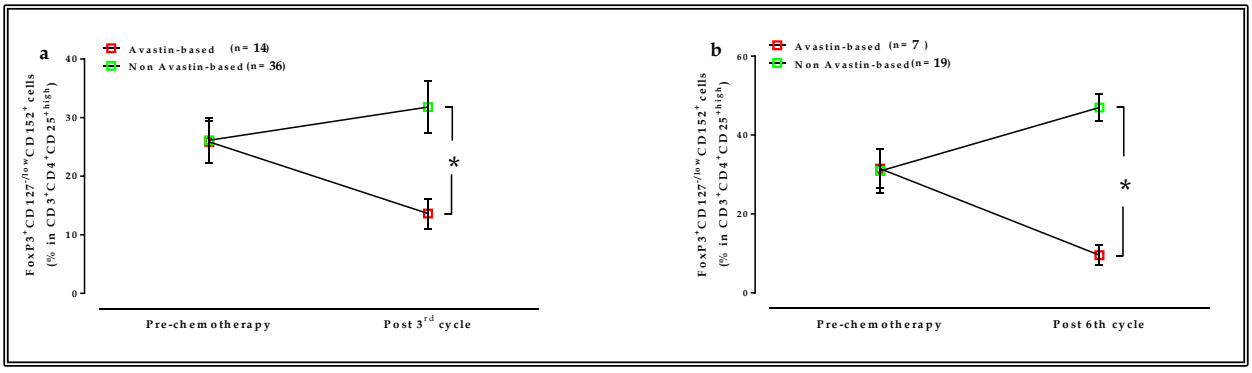

Figure 3.30: Comparison of the changes of $FoxP3^+CD127^{-/low}CD152^+$ Treg population in Avastin and non-Avastin treated NSCLC patients. (a) The mean percentage of $FoxP3^+CD127^{-/low}CD152^+$ Treg cells was significantly higher in patients treated with non-Avastin based chemotherapy (n = 36) (31.8 ± 4.464) after 3 cycles of treatment, compared to Avastin-based treatment group (n = 14) (13.269 ± 2.559) (P=0.017). (b) The mean percentage of $FoxP3^+CD127^{-/low}CD152^+$ Treg cells significantly decreased in Avastin-based treatment group (n = 7) (31.486 ± 4.962 to 9.571 ± 2.581) after six cycles of chemotherapy In contrast, the mean percentage in Non Avastin-based treatment group increased (n = 19) (30.911 ± 5.695 to 46.989 ± 3.383) (P<0.0001). The signal with row represents the mean value before and after the treatment. Statistical analyses were performed with Multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

In the Avastin-based treatment group, $CD25^{+high}CD127^{-/low}$CTLA-4-$FOXP3^{+/low}CD45RO^{-}$ Naïve $CD4^{+}$ Treg numbers were unchanged three and six months after treatment. On the other hand, in the Non Avastin-based treatment group, Naïve $CD4^{+}$ Treg numbers increased significantly after chemotherapy. The mean percentage of Naïve $CD4^{+}$ Treg was significantly higher at the post 3rd (P=0.0142) and post 6th (P=0.02) cycle time point in Non Avastin-based treatment group (6.038 ± 1.27 and 8.593 ± 1.901, respectively), compared to Avastin-based treatment group (1.246 ± 0.639 and 1.017 ± 0.642, respectively) (figure 3.31).

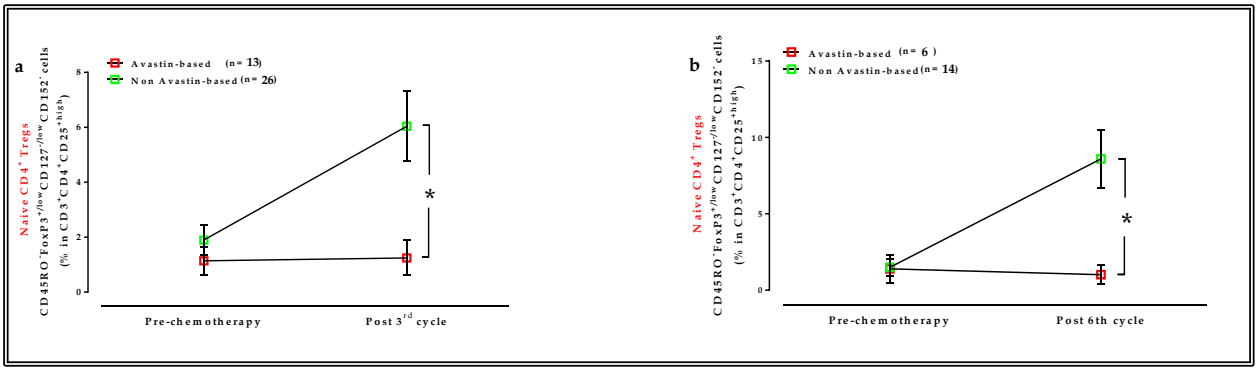

Figure 3.31: Comparison of the changes of Naïve $CD4^{+}$ Treg population in Avastin and non-Avastin treated NSCLC patients. (a) After three cycles of treatment, the percentage of Naïve $CD4^{+}$ Treg cells significantly increased in Non Avastin-based treatment group (n = 26) (1.896 ± 0.538 to 6.038 ± 1.27), while, in the Avastin-based treatment group, Naïve $CD4^{+}$ Treg numbers were unchanged. (b) The same impact was observed and after six cycles of treatment, where the percentage of Naïve $CD4^{+}$ Treg cells increased significantly in Non Avastin-based treatment group (n = 14) (1.493 ± 0.55 to 8.593 ± 1.901). The signal with row represents the mean value before and after the treatment. Statistical analyses were performed with Multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

At baseline, the mean percentage of $CD25^{+high}CD127^{+/low}CD152^{+}FOXP3^{+}CD45RO^{+}$ Effector $CD4^{+}$ Treg cells was higher in the Non Avastin based treatment group, compared to Avastin based treatment group, but the difference was not statistical significant (figure 3.32).

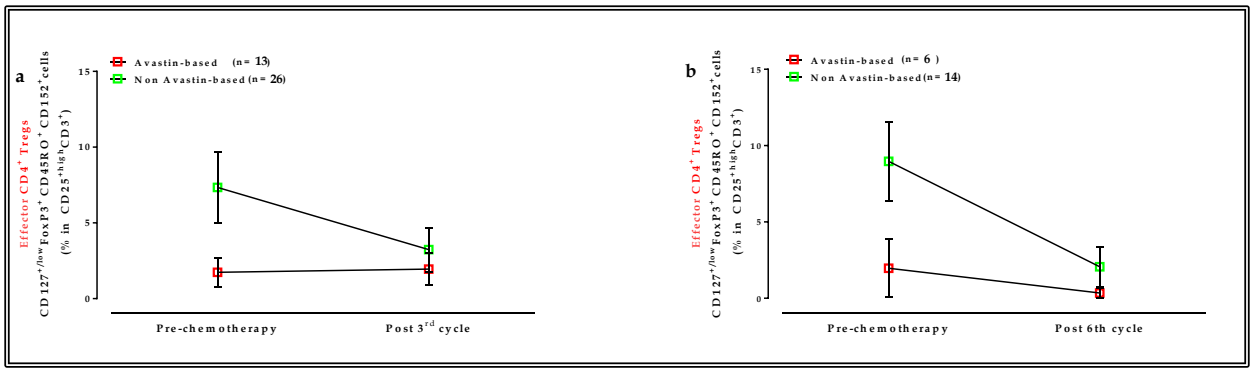

Figure 3.32: Comparison of the changes of Effector $CD4^{+}$ Treg population in Avastin and non-Avastin treated NSCLC patients. (a) Comparison of the change of Effector Treg cells between Non Avastin-based treatment group (n = 26) and Avastin-based treatment group (n = 13) after 3 cycles of chemotherapy. (b) Comparison of the change of Effector Treg cells between Non Avastin-based treatment group (n = 11) and responders Non Avastin-based treatment group (n = 21) after 6 cycles of chemotherapy. The signal with row represents the mean value before and after the treatment. Statistical analyses were performed with Multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

Before the treatment entry, the mean percentage of $CD25^{+high}CD127^{-}CD152^{+}FOXP3^{+}CD45RO^{+}$ Terminal Effector $CD4^{+}$ Treg cells was the same in the two groups. However, the effect of the Avastin-based chemotherapy was the reduction of the mean level of Terminal Effector $CD4^{+}$ Treg cells (non-significant), while Non Avastin-based chemotherapy had as a result the increase of the mean percent of Terminal Effector $CD4^{+}$ Treg subpopulation (P=0.0009). At the end of the 3rd cycle, the mean percentage of Terminal Effector $CD4^{+}$ Treg cells was significantly higher (P=0.0142) in the Non Avastin-based treatment group (23.873 ± 4.699), compared to Avastin-based treatment group (5.923 ± 1.602) (P=0.011) (figure 3.33 a). The same results were observed after the 6th cycle of treatment, where the mean percentage in the Non Avastin-based treatment group was 26.107 ± 5.872 and in the Avastin-based treatment group was 3.3 ± 1.075 (P=0.22) (figure 3.33 b).

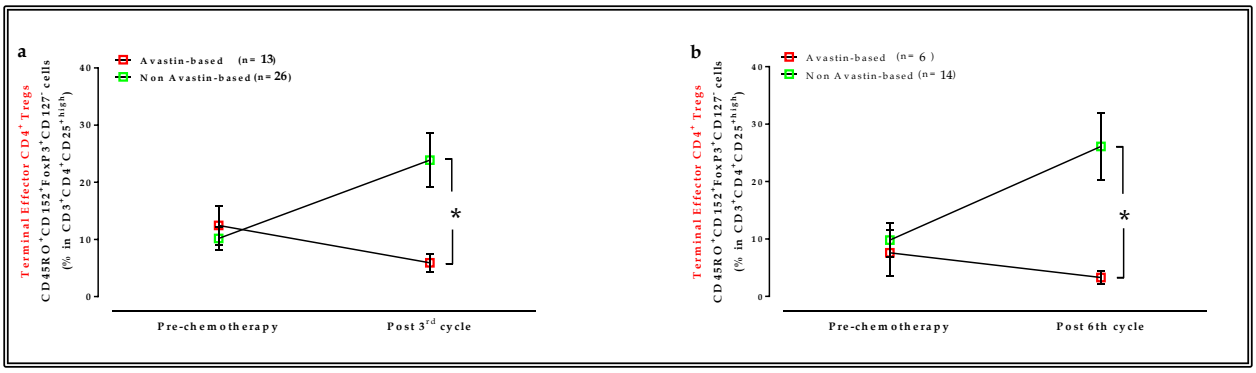

Figure 3.33: Comparison of the changes of Terminal Effector CD4⁺ Treg population in Avastin and non-Avastin treated NSCLC patients. In the baseline, the mean percentage of Terminal Effector CD4⁺ Treg cells was the same in the two groups. (a) However after the 3rd cycle of chemotherapy, it was significantly lower in Avastin-based treatment group (n = 13) (5.923 ± 1.602) compared to Non Avastin-based treatment group (n = 26) (23.873 ± 4.699) (P=0.011). (b) Moreover, after six cycles of chemotherapy, the mean level of Terminal Effector CD4⁺ Treg subset was significantly lower in Avastin-based treatment group (n = 6) (3.3 ± 1.075) compared to Non Avastin-based treatment group (n = 14) (26.107 ± 5.872) (P=0.022). The signal with row represents the mean value before and after the treatment. Statistical analyses were performed with Multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

In addition, we looked at the mean percentages of CCR7⁺ Naive CD4⁺ Treg subset but did not find any significant differences between Avastin-based treatment and Non Avastin-based treatment group (figure 3.34).

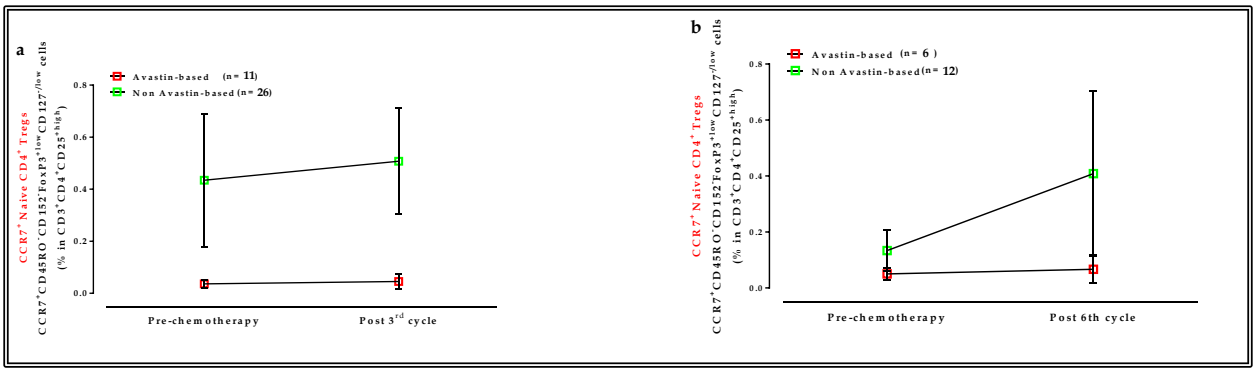

Figure 3.34: Comparison of the changes of CCR7⁺ Naive CD4⁺ Treg subset in Avastin and non-Avastin treated NSCLC patients. (a) Comparison of the change of CCR7⁺ Naive CD4⁺ Treg subset between Non Avastin-based treatment group (n = 26) and Avastin-based treatment group (n = 11) after 3 cycles of chemotherapy revealed no statistical difference. (b) No significant difference in the mean level of

CCR7+ Naive CD4+ Treg subset was observed in baseline before and after treatment between the two groups after 6 cycles of chemotherapy. The signal with row represents the mean value before and after the treatment. Statistical analyses were performed with Multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

Moreover, even if the mean percentages of CCR7+ CD25+highCD127+/lowCD152+FOXP3+CD45RO+ Central Memory CD4+ Treg in Non Avastin-based treatment group were higher in the baseline and decreased, the differences were not statistically significant (figure 3.35).

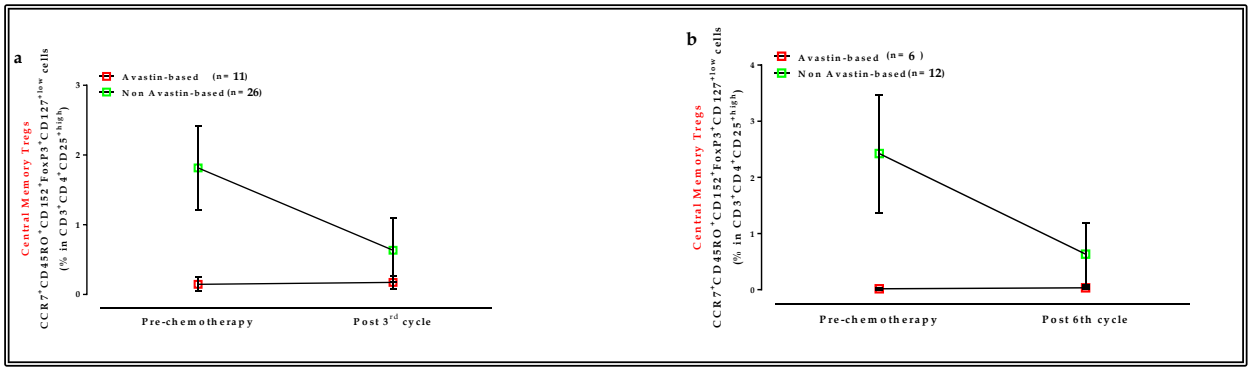

Figure 3.35: Comparison of the changes of Central Memory CD4+ Treg subset in Avastin and non-Avastin treated NSCLC patients. (a) Patients treated with Non-Avastin Based therapy (n = 26) had a non-significantly higher percentage in Central Memory Treg cells, compared to patients treated with Avastin Based therapy (n = 11) at baseline before therapy. (b) Similarly, no significant difference in the mean level of Central Memory Treg cells was observed in baseline before and after 6 cycles of treatment in the two groups. The signal with row represents the mean value before and after the treatment. Statistical analyses were performed with Multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

Finally, the Avastin-based treatment did not cause any statistically significant effect at the mean percentages of CCR7^{-} CD25^{+high}CD127^{-}CD152^{+}FOXP3^{+}CD45RO^{+} Terminal Memory Effector CD4^{+} Treg subpopulation, compared to Non Avastin-based treatment (figure 3.36).

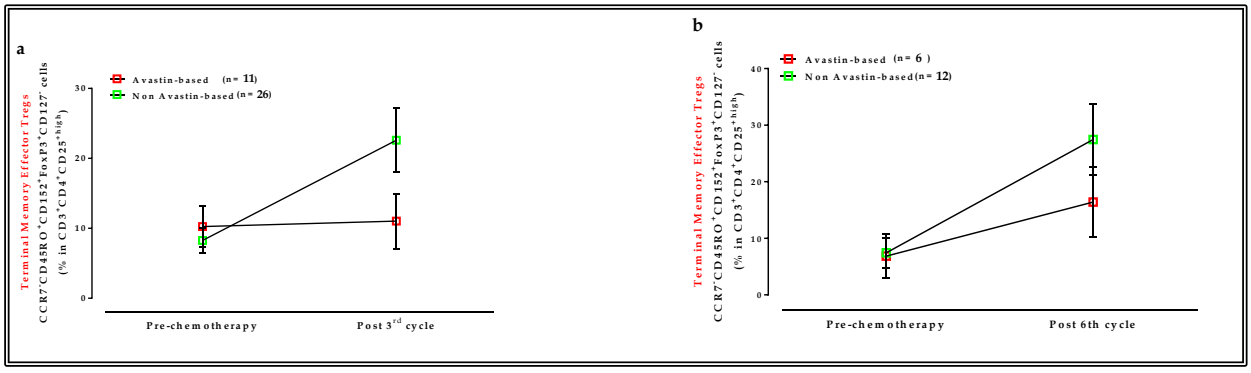

Figure 3.36: Comparison of the changes of Terminal Memory Effector CD4^{+} Treg subset in Avastin and non-Avastin treated NSCLC patients. (a) Comparison of the change of Terminal Memory Effector CD4^{+} Treg subset between Avastin-based treatment group (n = 11) and Non Avastin-based treatment group (n = 26) after 3 cycles of chemotherapy revealed no statistical difference. (b) No statistical difference was observed in the comparison of the change of Terminal Memory Effector Treg subset in baseline before and after six cycles of treatment in the two groups. The signal with row represents the mean value before and after the treatment. Statistical analyses were performed with Multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

3.11 THE CORRELATION OF THE BASELINE PERCENTAGES OF CD4^{+} Treg SUBPOPULATIONS WITH RESPONSE TO TREATMENT

Seventy-three, stage IV patients, treated with systemic chemotherapy, were evaluable for assessment of clinical outcome after three and six cycles of treatment. We found a significant positive correlation between increased percentage of CD3^{+}CD4^{+}CD25^{+high}CD127$^{-/low}$CD152^{+}Foxp3^{+} Tregs and response to treatment (figure 3.37). At baseline, responders had a significant higher percentage of CD3^{+}CD4^{+}CD25^{+high}CD127$^{-/low}$CD152^{+}Foxp3^{+} Tregs compared those with progressive disease at the 3rd or the 6th cycle.

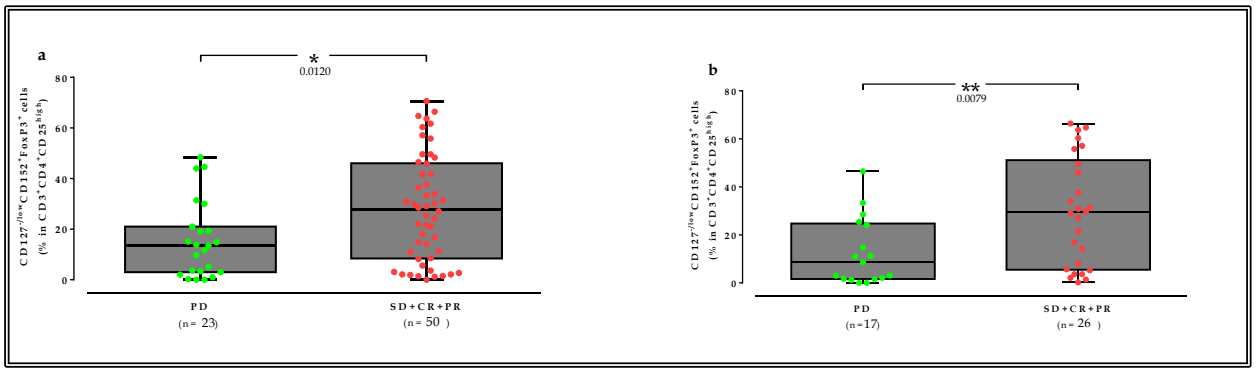

Figure 3.37: The percentage of CD3⁺CD4⁺CD25⁺^{high}CD127$^{-/low}$CD152⁺Foxp3⁺ Tregs was correlated with clinical outcome. Higher percentage of CD3⁺CD4⁺CD25⁺^{high}CD127$^{-/low}$CD152⁺Foxp3⁺ Tregs was correlated with poor clinical outcome after the (a) 3rd (P=0.012) and the (b) 6th (P=0.0079) cycle of treatment. Significance was evaluated by unpaired T-test. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented.

In contrast, the CD25⁺^{high}CD127$^{-/low}$CTLA-4-FOXP3$^{+/low}$CD45RO⁻ Naïve CD4⁺ Tregs correlated with the response to treatment. Patients with disease control (partial response/ stable disease [PR/CR]) showed a significantly lower percentage of Naïve CD4⁺ Tregs compared to those who developed progressive disease the post 3rd and 6th cycle (figure 3.38). In baseline, the mean percentage of Naïve CD4⁺ Tregs was 1.541± 0.347 in the patients that were evaluated as SD or PR after the 3rd cycle of treatment, while in patients with progressive disease it was 3.472 ± 0.8683 (P=0.012). In addition, in baseline, the percentage of Naïve CD4⁺ Tregs was significantly higher in non-responders of the 6th cycle (2.754± 0.709), compared to responders (0.805± 0.325).

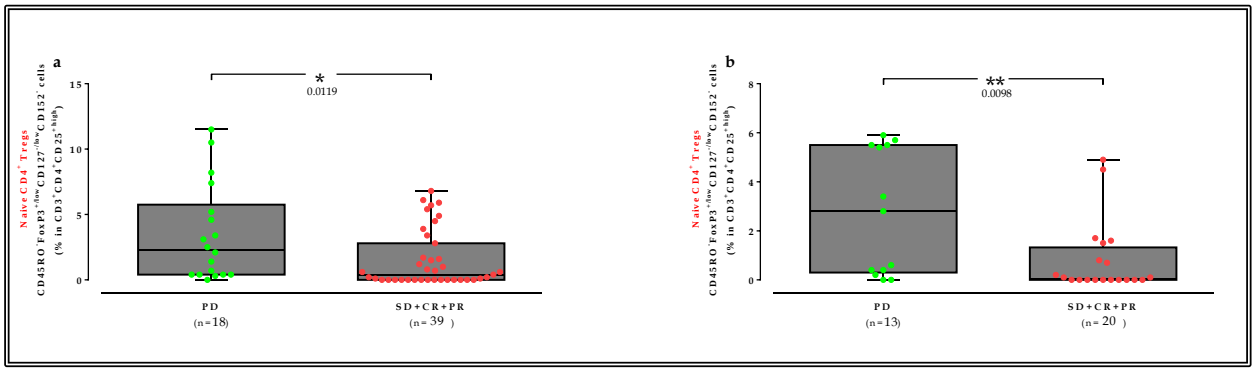

Figure 3.38: The percentage of Naïve CD4⁺ Tregs was correlated with clinical outcome. Patients presented progressive disease had significantly higher percentage of Naïve CD4⁺ Tregs in baseline, compared to responders. (a) The mean percentage of Naïve CD4⁺ Tregs was lower in the patients that

were evaluated as SD or PR after the 3rd cycle of treatment, compared to patients with progressive disease (P=0.012). (b) Similarly, higher levels of Naïve CD4$^+$ Tregs were observed in patients presented progressive disease after the 6th cycle of treatment, compared to responders (P=0.0098). Significance was evaluated by unpaired T-test. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented.

As far as the basal levels of CD25^{+high}CD127$^{+/low}$CD152$^+$FOXP3$^+$CD45RO$^+$ Effector CD4$^+$Tregs are concerned, there was no correlation with the response to treatment as no significant difference was observed between NSCLC patients whose disease progress after the 3rd or 6th cycle and those with disease control (figure 3.39).

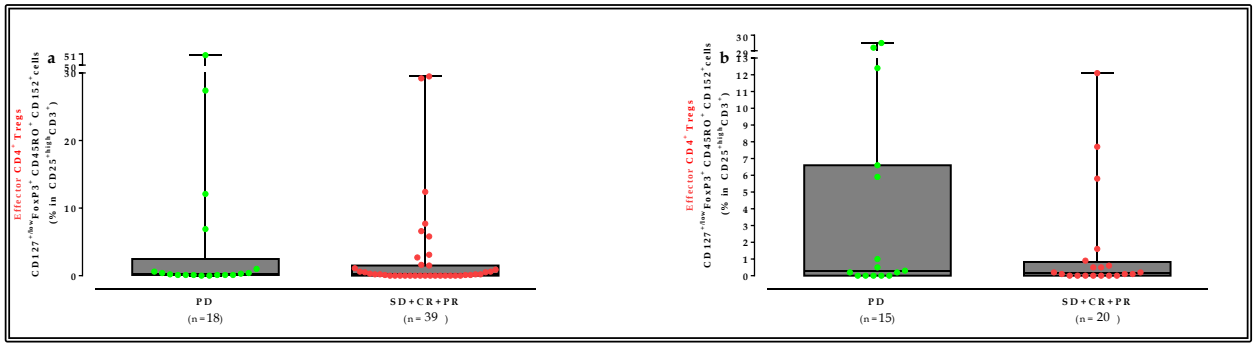

Figure 3.39: No correlation was observed between the percentage of Effector CD4$^+$ Tregs and clinical outcome. No statistical difference was observed in the percentage of Effector CD4$^+$ Tregs between responders and patients presented progressive disease after the (a) 3rd or (b) 6th cycle of treatment. Significance was evaluated by unpaired T-test. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented.

In addition, no correlation was found between the mean percentage of the CD25^{+high}CD127^{-}CD152^{+}FOXP3^{+}CD45RO^{+} Terminal Effector CD4^{+} Tregs and the respond after the 3rd cycle of chemotherapy (figure 3.40 a). However, high levels of Terminal Effector CD4^{+} Tregs in the baseline were correlated with improved clinical outcome after the 6th cycle of treatment (figure 3.40 b). The mean percentage of the Terminal Effector CD4^{+} Tregs in baseline was significantly higher in responders (partial response/ stable disease [PR/CR]) evaluated in after the 6th cycle of chemotherapy (14.08 ± 2.742), compared to patients with progressive disease (5.985± 1.603) (P=0.0164).

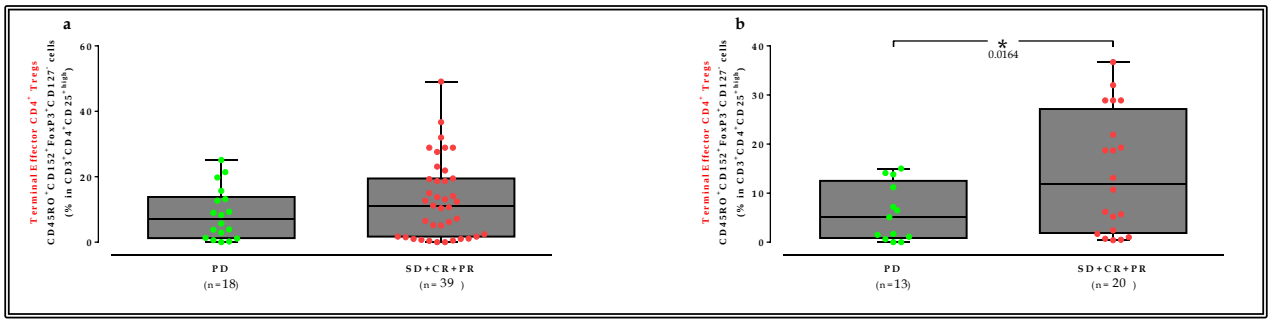

Figure 3.40: The percentage of Terminal Effector CD4^{+} Tregs was correlated with the clinical outcome of the post 6th cycle of chemotherapy. (a) No statistical difference was observed in the percentage of Terminal Effector CD4^{+} Tregs between responders and patients presented progressive disease after the 3rd cycle of treatment. (b) However, low levels of Terminal Effector CD4^{+} Tregs were correlated with progression of disease after the 6th cycle of treatment (P=0.0164). Significance was evaluated by unpaired T-test. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented.

Moreover, the high levels of CCR7^{+} CD25^{+high}CD127$^{-/low}$CTLA-4^{-}FOXP3$^{+/low}$CD45RO^{-} Naïve CD4^{+} Tregs were correlated with poor clinical outcome (figure 3.41). The mean percentage of CCR7^{+} Naïve CD4^{+} Tregs in baseline was higher in patients whose disease progress after the 3rd (0.6 ± 0.3472) or 6th (0.4589±0.2767) cycle of treatment, compared to responders (partial response/ stable disease [PR/CR]) (0.1579±0.0913 and 0.03158±0.01336, respectively) (P= 0.0209 and 0.0069).

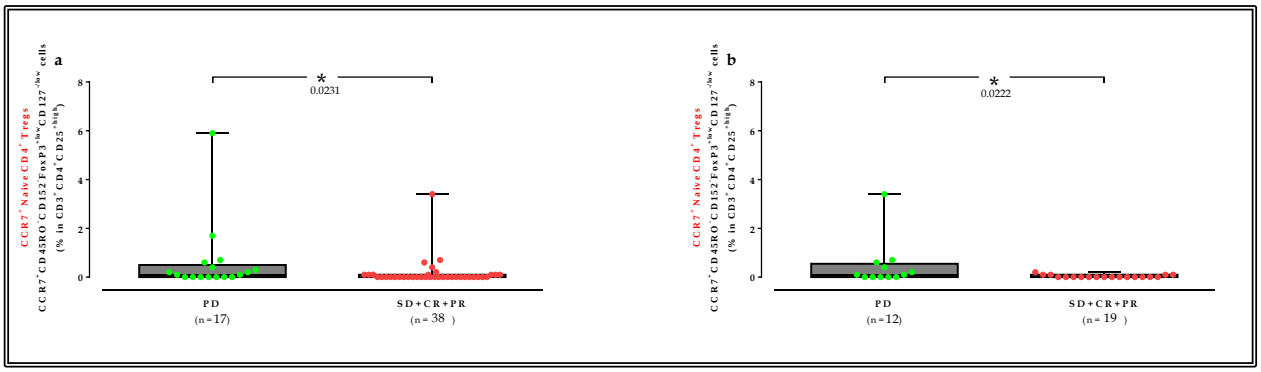

Figure 3.41: Higher percentage of CCR7^{+} Naïve CD4^{+} Tregs was correlated with poor clinical outcome. The mean percentage of CCR7^{+} Naïve CD4^{+} Tregs in baseline was increased in patients whose disease progress after the (a) 3rd (P= 0.0209) or (b) 6th (P= 0.0069) cycle of treatment, compared to responders (partial response/ stable disease [PR/CR]). Significance was evaluated by unpaired T-test. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented.

A significant correlation between high percentage of CCR7^{+} CD25^{+high} CD127$^{+/low}$ CD152^{+} FOXP3^{+}CD45RO^{+} Central Memory Tregs in baseline and poor clinical outcome was observed after the 6th cycle of treatment. Patients with high frequency of Central Memory Tregs in baseline (2.846 ± 1.083) presented with progressive disease after the 6th cycle of chemotherapy, compared to responders (0.1368 ± 0.082) (P=0.0068) (figure 3.42).

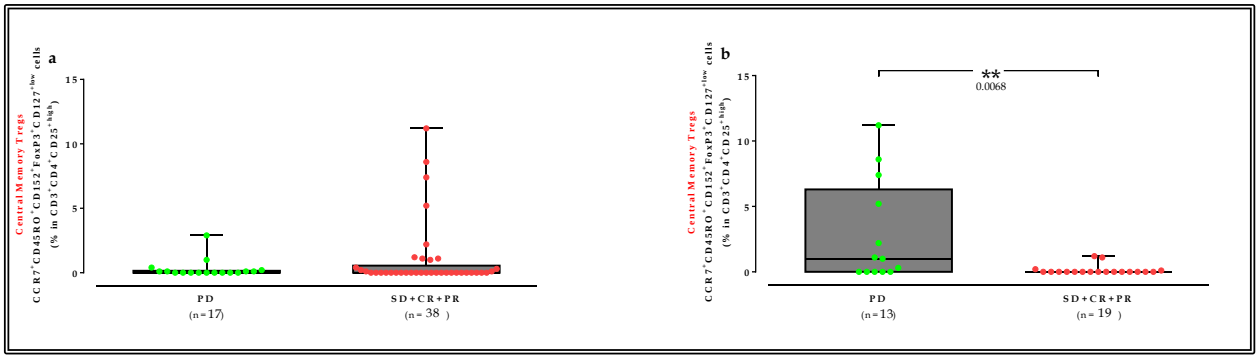

Figure 3.42: The percentage of Central Memory CD4^{+} Tregs was correlated with the clinical outcome of the post 6th cycle of chemotherapy. (a) No statistical difference was observed in the percentage of Central Memory CD4^{+} Tregs between responders and patients presented progressive disease after the 3rd cycle of treatment. (b) However, high levels of Central Memory CD4^{+} Tregs were correlated with progression of disease after the 6th cycle of treatment (P=0.0048). Significance was evaluated by unpaired T-test. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented.

In contrast, high levels of CCR7^{-} CD25^{+high}CD127^{-}CD152^{+}FOXP3^{+}CD45RO^{+} Terminal Memory Effector Tregs were correlated with improved clinical outcome. In baseline, the mean percentage of Terminal Memory Effector Tregs was significantly higher 11.22 (± 1.419) in the patients that were evaluated as SD or PR after the 3rd cycle of treatment, while in patients presented progressive disease it was 5.306 (± 1.745) (P=0.0209) (figure 3.43 a). In addition, in baseline, the percentage of Terminal Memory Effector Tregs was significantly higher in responders of the 6th cycle (11.41± 2.256), compared to non-responders (3.738± 1.345) (P=0.0069) (figure 3.43 b).

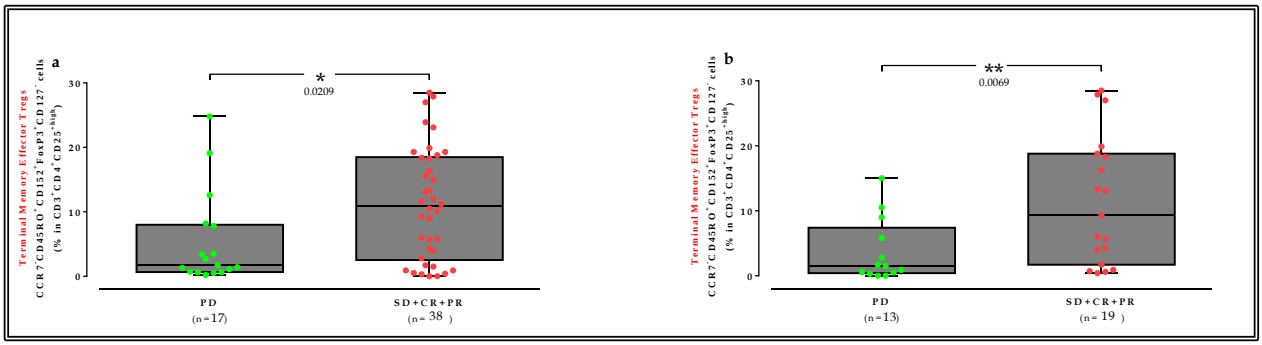

Figure 3.43: Low percentage of Terminal Memory Effector CD4^{+} Tregs was correlated with poor clinical outcome. (a) In baseline, the mean percentage of Terminal Memory Effector Tregs was significantly higher in the patients that were evaluated as SD or PR after the 3rd cycle of treatment, compared to patients presented progressive disease (P=0.0209). (b) In addition, in baseline, the percentage of Terminal Memory Effector Tregs was significantly higher in responders of the 6th cycle, compared to non-responders (P=0.0069). Significance was evaluated by unpaired T-test. Each point corresponds to an individual patient (red) or healthy controls (green). The medians, 75 percentile (box) and max and min (whiskers) are represented.

3.12 THE CORRELATION OF THE CHANGES OF CD4^{+} Treg SUBPOPULATIONS DURING CHEMOTHERAPY WITH THE RESPONSE TO TREATMENT.

The changes in the Treg subtypes during the treatment prompted us to determine whether changes in the frequency of these cell types correlated with the type of clinical outcome, in stage IV patients. Clinical responses were determined as part of the normal clinical evaluation process. Firstly, we looked at the level of CD4^{+}CD25^{+high}Foxp3^{+}CD127$^{-/low}$CD152^{+} (Treg) subset but did not find any significant differences between responders (partial response/ stable disease

[PR/CR]) and non-responders (progressive disease [PD]) (figure 3.44). Moreover, although the Treg subsets increased within the $CD4^+$ subset, the change was not correlated to clinical response.

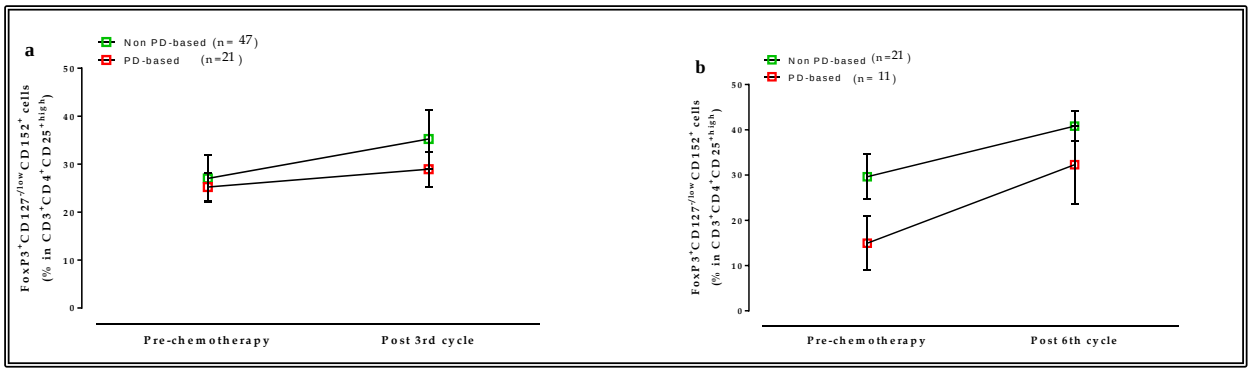

Figure 3.44: Comparison of the changes of Treg population in NSCLC patients during the chemotherapy. (a) Comparison of the change of Treg cells between nonresponders (progressive disease [PD]) (n = 21) and responders (partial response/ stable disease [PR/CR]) (n = 47) after 3 cycles of chemotherapy. (b) Comparison of the change of Treg cells between nonresponders (progressive disease [PD]) (n = 11) and responders (partial response/ stable disease [PR/CR]) (n = 21) after 6 cycles of chemotherapy. The signal with row represents the mean value before and after the treatment; red colour: non-responders, green colour: responders. Statistical analyses were performed with Multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

There was a trend towards an inverse association between the change in $CD25^{+high}CD127^{-/low}$CTLA-4-$FOXP3^{+/low}CD45RO^{-}$ Naïve $CD4^{+}$ Treg in the peripheral blood and clinical-benefit. An increase in the percentage of the Naïve $CD4^{+}$ Treg cells was associated with progression of the disease. The percentage of Naïve $CD4^{+}$ Treg cells tended to be significantly higher after the 3rd cycle of chemotherapy (9.2 ± 2.791) and after the 6th cycle of chemotherapy (6.6 ± 1.467) in the patients who developed progressive disease (PD) while the opposite was observed in the clinical-benefit group (2.589 ± 0.58 and 2.941 ± 0.621, respectively) (figure 3.45).

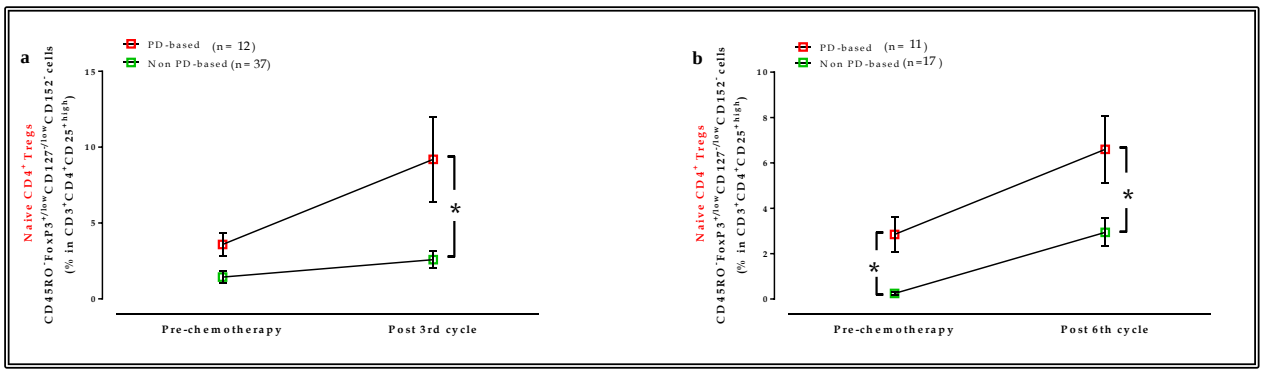

Figure 3.45: Comparison of the changes of Naïve $CD4^{+}$ Treg population in NSCLC patients during the chemotherapy. (a) The increase of Naïve $CD4^{+}$ Treg cells was significantly higher in nonresponders (progressive disease [PD]) (n = 12) (2.967 ± 0.839 to 9.2 ± 2.791) after 3 cycles of chemotherapy, compared to responders (partial response/ stable disease [PR/CR]) (n = 37) (1.657 ± 0.415 to 2.589 ± 0.580) (P<0.001). (b) The percentage of Naïve $CD4^{+}$ Treg cells increased significantly more in nonresponders (progressive disease [PD]) (n = 11) (0.964 ± 0.519 to 6.6 ± 1.467) after six cycles of chemotherapy, compared to responders (partial response/ stable disease [PR/CR]) (n = 17) (1.453 ± 0.537 to 2.94 ± 0.621) (P=0.014). The signal with row represents the mean value before and after the treatment; red colour: non-responders, green colour: responders. Statistical analyses were performed with Multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

Patients with significantly higher percentage of $CD25^{+high}CD127^{+/low}CD152^{+}FOXP3^{+}CD45RO^{+}$ Effector $CD4^{+}$ Treg cells before the entry of treatment tended to have a partial response (PR) or stable disease (SD) after three or six cycles of chemotherapy. However, the percentage of the Effector $CD4^{+}$ Treg cells approached the levels of the progressive disease (PD) group after the treatment (figure 3.46).

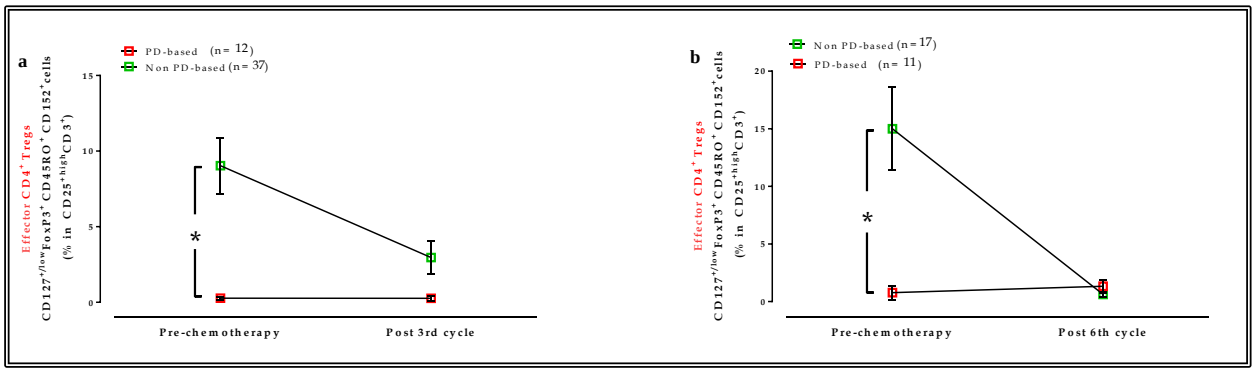

Figure 3.46: Comparison of the changes of Effector $CD4^{+}$ Treg population in NSCLC patients during the chemotherapy. (a) The percentage of Effector $CD4^{+}$ Treg cells was significantly lower in nonresponders (progressive disease [PD]) (n = 12) (0.275 ± 0.1) compared to responders (partial response/ stable disease [PR/CR]) (n = 37) (9.038 ± 1.844) (P=0.009) at baseline before treatment, for patients that were evaluated after the 3rd cycle of chemotherapy. (b) The mean level of Effector $CD4^{+}$ Treg subset was significantly lower in nonresponders (progressive disease [PD]) (n = 11) (0.773 ± 0.59) compared to responders (partial response/ stable disease [PR/CR]) (n = 17) (15 ± 3.612) (P=0.004) before entry treatment, for patients that were evaluated after six cycles of chemotherapy. The percentage of Effector $CD4^{+}$ Treg cells decreased significantly more in PD group, compared to clinical-benefit group. The signal with row represents the mean value before and after the treatment; red colour: non-responders, green colour: responders. Statistical analyses were performed with Multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

In contrast, the initial percentage of $CD25^{+high}CD127^{-}CD152^{+}FOXP3^{+}CD45RO^{+}$ Terminal Effector Treg cells and the change of their population were not correlated to clinical response, as no statistically significant differences in the percentage of Terminal Effector Treg cells at baseline before and after therapy were found between responders and nonresponders (figure 3.47).

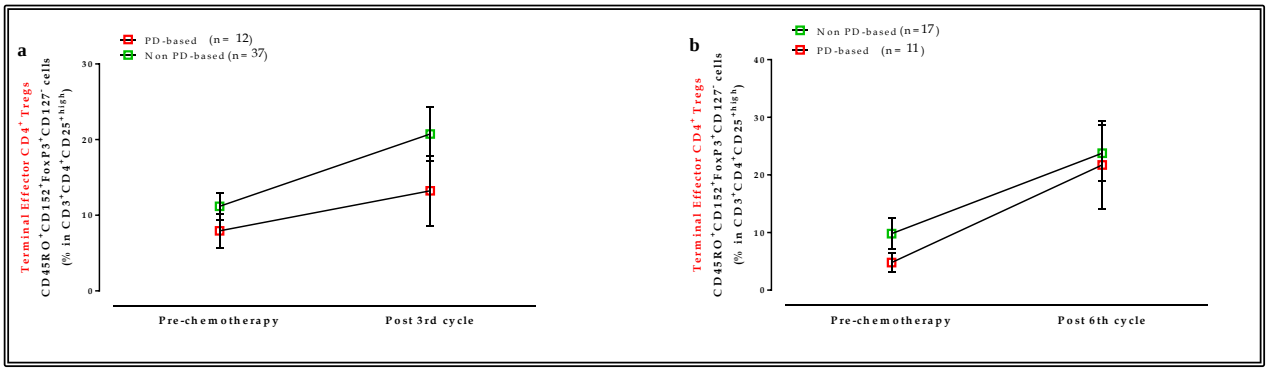

Figure 3.47: Comparison of the changes of Effector $CD4^{+}$ Treg subset in NSCLC patients during the chemotherapy. (a) Comparison of the change of Effector $CD4^{+}$ Treg subset between nonresponders (progressive disease [PD]) (n = 12) and responders (partial response/ stable disease [PR/CR]) (n = 37) after 3 cycles of chemotherapy revealed no statistical difference. (b) No difference in the mean level of Effector $CD4^{+}$ Treg subset was observed in baseline before and after treatment between nonresponders (progressive disease [PD]) (n = 11) and responders (partial response/ stable disease [PR/CR]) (n = 17) after 6 cycles of chemotherapy. The signal with row represents the mean value before and after the treatment; red colour: non-responders, green colour: responders. Statistical analyses were performed with Multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

A significantly higher percentage of CCR7+ CD25+highCD127-/lowCTLA-4-FOXP3+/lowCD45RO- Naïve Tregs was observed after the 3rd cycle of treatment in the progressive disease (PD) group (2.4± 1.545) compared to the clinical-benefit group (0.281± 0.12) (P=0.009), but not after the 6th cycle of chemotherapy. The CCR7+ Naïve Treg subset did not have a significantly higher increase in non-responders after cycle 6 of therapy (figure 3.48).

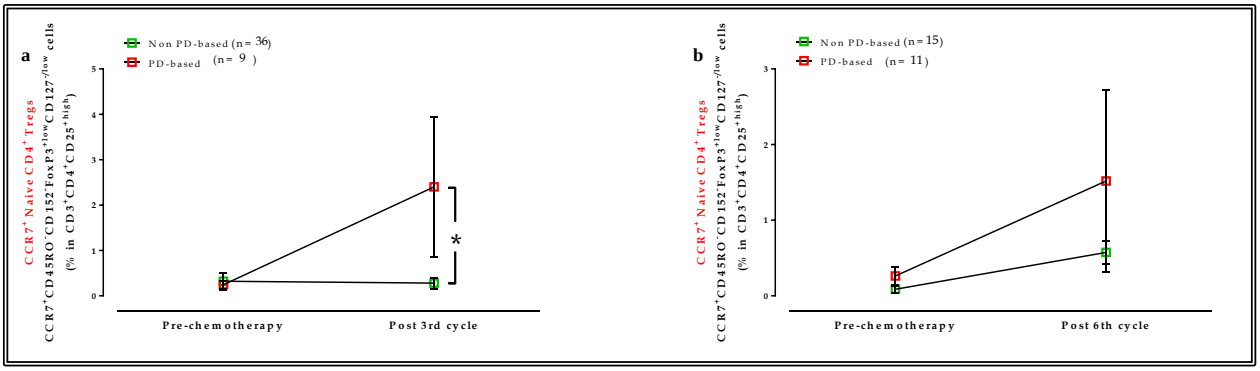

Figure 3.48: Comparison of the changes of CCR7+ Naive CD4+ Treg subset in NSCLC patients during the chemotherapy. (a) The change of CCR7+ Naïve CD4+ Treg cells after 3 cycles of chemotherapy was significantly higher in nonresponders (progressive disease [PD]) (n = 9) (0.244 ± 0.09 to 2.4 ± 1.545), compared to responders (partial response/ stable disease [PR/CR]) (n = 36) (0.322 ± 0.186 to 0.281 ± 0.12) (P=0.009). (b) No statistical difference was revealed in the comparison of the change of CCR7+ Naïve CD4+ Treg subset in baseline before treatment in patients evaluated after six cycles of chemotherapy. The mean level of the CCR7+ Naïve CD4+ Treg subset increased more in PD-group (n = 11) compared to PR/SD-group (n = 15), but this was not statistically significant. The signal with row represents the mean value before and after the treatment; red colour: non-responders, green colour: responders. Statistical analyses were performed with Multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

Although responding patients had a higher percentage in CCR7+ CD25+highCD127+/low CD152+FOXP3+CD45RO+ Central Memory Treg cells over non-responders at baseline before therapy, this was not statistically significant (figure 3.49).

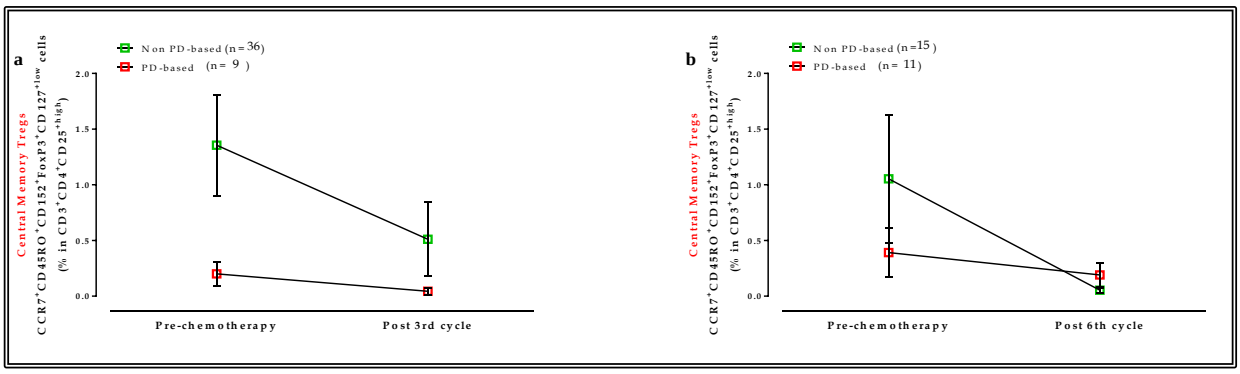

Figure 3.49: Comparison of the changes of Central Memory CD4+ Treg subset in NSCLC patients during the chemotherapy. (a) Responding patients (partial response/ stable disease [PR/CR]) (n = 36) had a non-significantly higher percentage in Central Memory Treg cells, compared to non-responders (progressive disease [PD]) (n = 9) at baseline before therapy. (b) No difference in the mean level of Central Memory Treg cells was observed in baseline before and after treatment between nonresponders (progressive disease [PD]) (n = 11) and responders (partial response/ stable disease [PR/CR]) (n = 15) after 6 cycles of chemotherapy. The signal with row represents the mean value before and after the treatment; red colour: non-responders, green colour: responders. Statistical analyses were performed with Multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

Finally, we looked at changes in the percentage of CCR7- CD25+highCD127- CD152+FOXP3+CD45RO+ Terminal Memory Effector Tregs and did not find significant differences between responders and nonresponders before and after chemotherapy (figure 3.50).

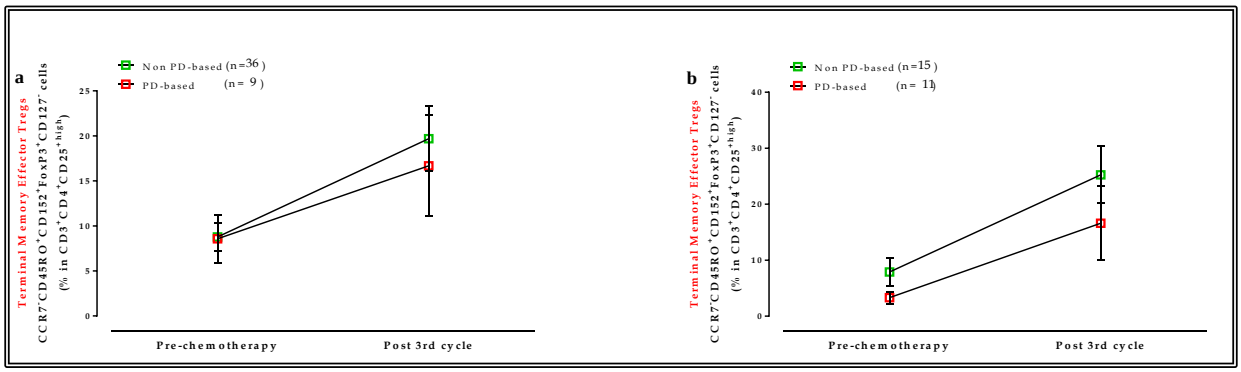

Figure 3.50: Comparison of the changes of Terminal Memory Effector CD4+ Treg subset in NSCLC patients during the chemotherapy. (a) Comparison of the change of Terminal Memory Effector CD4+ Treg subset between nonresponders (progressive disease [PD]) (n = 9) and responders (partial response/ stable disease [PR/CR]) (n = 36) after 3 cycles of chemotherapy revealed no statistical difference. (b) No statistical difference was revealed in the comparison of the change of Terminal Memory effector Treg subset in baseline before treatment in patients evaluated after six cycles of chemotherapy. The signal with row represents the mean value before and after the treatment; red colour: non-responders, green colour: responders. Statistical analyses were performed with Multiple t-test with correct for multiple comparisons using the Holm-Sidak method.

3.13 PROGNOSTIC VALUE OF PERCENTAGE OF NAÏVE CD4+ Treg SUBPOPULATIONS IN UNTREATED NSCLC PATIENTS.

Assuming increased levels of CD4+ Tregs subpopulations those that were over the 95% percentile of the controls (outliers excluded), stage IV patients were dichotomized into those (outliers excluded), patients were dichotomized into those with above normal range of CD4+ Treg subpopulation percentages (high expression > as indicated in each cell subtype) and those with in normal range (≤ as indicated in each cell subtype). The evaluation period was defined from the date that patients were included in the study till August 2014. Cumulative survival (progression-free survival, PFS; overall survival, OS) time was calculated by the Kaplan-Meier method and analyzed by the log-rank test.

Interestingly, patients with high percentage of $CD3^+CD4^+CD25^{+high}CD152^+CD127^{-/low}FoxP3^+$ Treg cells (above 27.57) exhibited a longer progression-free survival (PFS), while high Treg frequency group did not reached median value (P=0.01) (figure 3.51 a). Moreover, patients who had high percentages (above 27.57) of $CD3^+CD4^+CD25^{+high}CD152^+CD127^{-/low}FoxP3^+$ Treg had longer overall survival compared with those with lower Treg frequencies (equal or below 27.57) (P=0.0064) (figure 3.52 b). The median of survival of the high Treg frequency group was 24.07 months, while this of the low Treg frequency group was 12.63; however, this was not statistically significant.

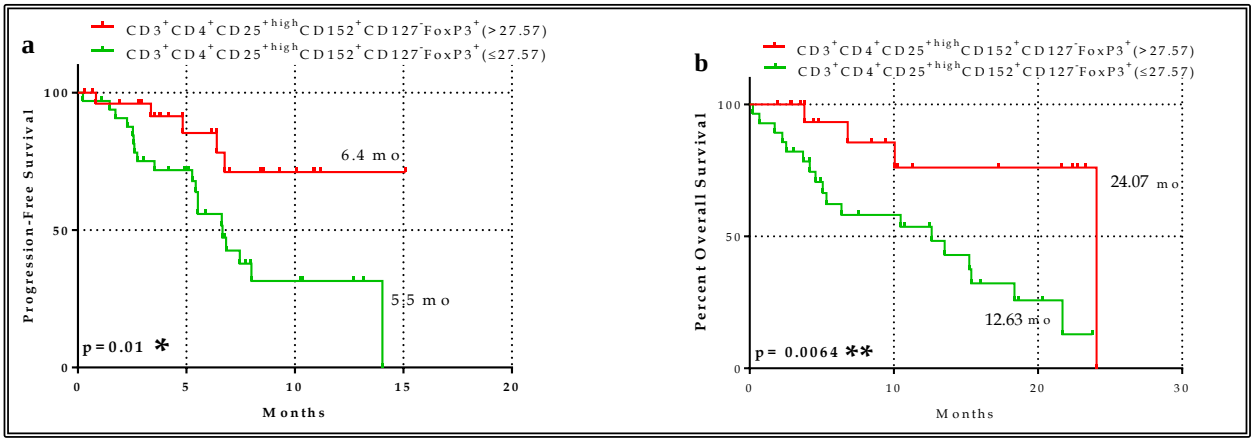

Figure 3.51: Kaplan-Meier survival analysis of patients divided according the frequency of Treg. (a) Curve shows overall survival time for patients who had lower or higher percentage than 27.57. Patients of Treg high group had higher progression-free survival (6.4 months), as compared with those with lower Treg frequencies (5.5 months) (0.01). (b) Moreover, the Treg high group was associated with longer OS

(24.07) (P=0.064), than low Treg group (12.63); green line: below 95% CI, red line: above 95% CI of normal control.

The detection of $CD25^{+high}CD127^{-/low}CTLA\text{-}4^{-}FOXP3^{+/low}CD45RO^{-}$ Naïve $CD4^{+}$ Tregs within the normal levels at baseline was associated with longer PFS and OS compared to those with high levels. The median progression-free and overall survival for high Naïve $CD4^{+}$ Tregs group was 6.4 and 10.07 months, respectively, and was significantly reduced compared to low group (11.2 and 18.67 months) (P=0.038 and 0.02, respectively) (figure 3.52).

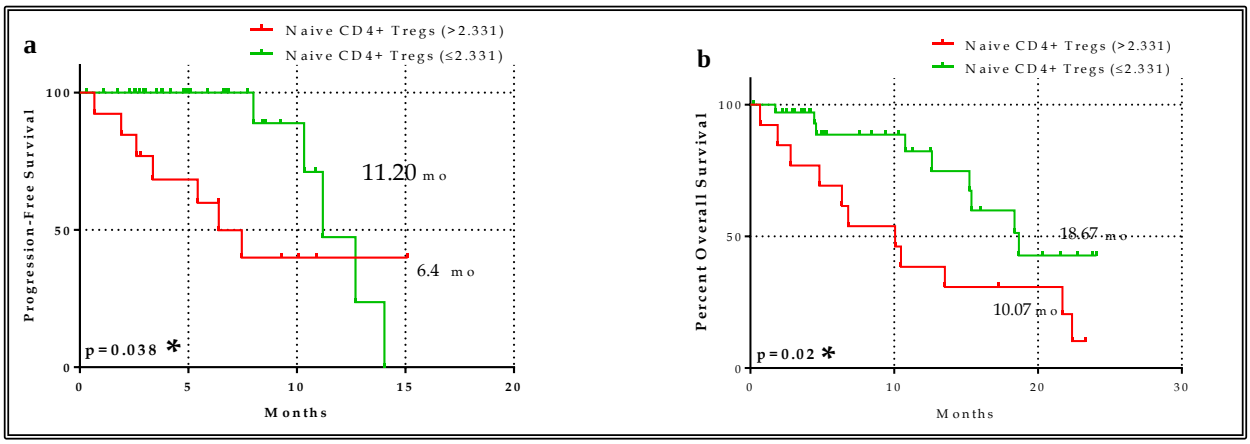

Figure 3.52: Prognostic significance of Naïve $CD4^{+}$ Tregs in NSCLC patients. Kaplan-Meier survival analysis of patients divided according the frequency of Naïve $CD4^{+}$ Tregs. Cumulative survival time was calculated by the Kaplan–Meier method and analyzed by the log-rank test. Low frequency of Naïve $CD4^{+}$ Treg was associated with improved (a) progression-free survival (P=0.038) and (b) overall survival (P=0.02); green line: below 95% CI, red line: above 95% CI of normal control.

Moreover, $CD25^{+high}CD127^{+/low}CD152^{+}FOXP3^{+}CD45RO^{+}$ Effector $CD4^{+}$ Tregs were negatively associated with survival. The subgroup of patients with high percentage of Effector $CD4^{+}$ Tregs demonstrated poor progression-free (median 14.03) and overall survival (median 18.37), compared to high group (median 6.633 and 5.333) (P= 0.0308 and 0.033, respectively) (figure 3.53).

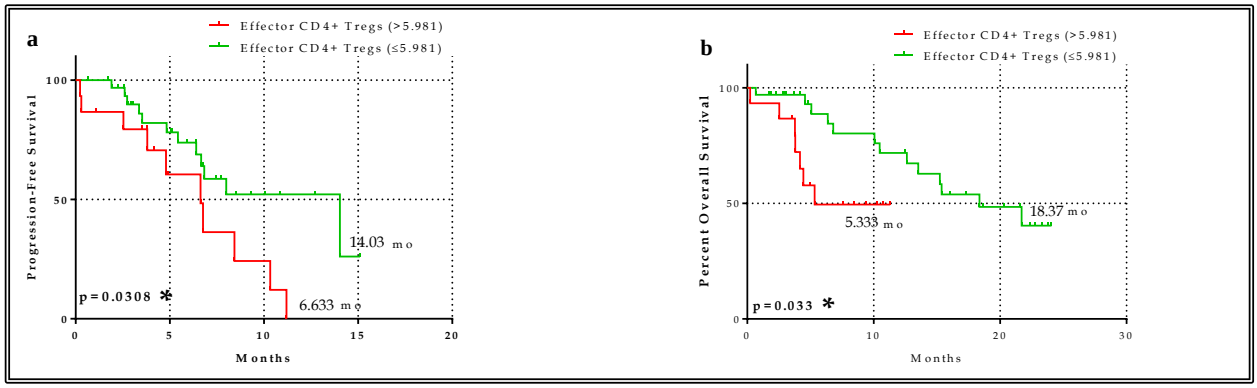

Figure 3.53: The frequency of Effector CD4⁺ Tregs modulates the survival of NSCLC patients. Kaplan–Meier curves displays the PFS and OS of groups of NSCLC patients divided according the frequency of Tregs. (a) In cumulative analysis, the high percentage of Effector CD4⁺ Tregs significantly decreased the progression free survival (P=0.0308), while NSCLC patients of Effector CD4⁺ Treg high group lived significantly lower as compared with those with lower Effector CD4⁺ Treg frequencies (P=0.033); green line: below 95% CI, red line: above 95% CI of normal control.

In contrast, our data show that higher levels of CD25⁺^{high}CD127⁻CD152⁺FOXP3⁺CD45RO⁺ Terminal Effector Tregs lead to a significant survival advantage. The progression free survival of high (above 13.15) Terminal Effector frequency group was significantly longer (14.03 months), as compared to low (equal or below 13.15) Terminal Effector frequency group (6.767 months) (P=0.0341) (figure 3.54). Patients who have high percentages of Terminal Effector Treg lived significantly longer as compared with those with lower Terminal Effector Treg frequencies (P=0.0492). The median of survival of the low Terminal Effector frequency group was 10.07 months while high Terminal Effector frequency group did not reached median value so the median of survival was undefined.

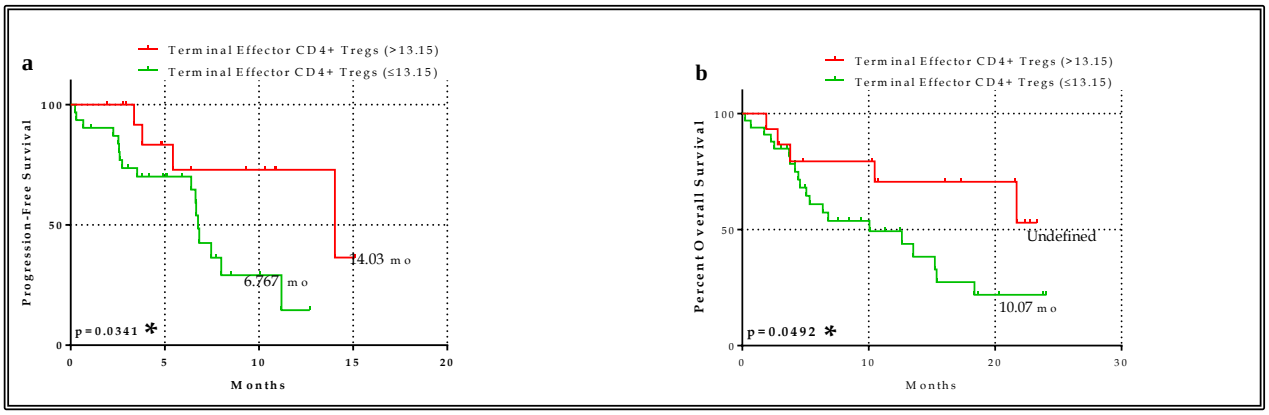

Figure 3.54: Prognostic significance of Terminal Effector CD4⁺ Tregs in NSCLC patients. Kaplan-Meier curves are shown for progression-free survival (PFS) and overall survival (OS) was stratified by the

percentage of Terminal Effector CD4+ Tregs to divide the patients into two groups. High percentage of Terminal Effector CD4+ Tregs was associated with both (a) longer PFS (P=0.0341) and (b) OS (P=0.0492); green line: below 95% CI, red line: above 95% CI of normal control.

A higher percentage of CCR7+ Naive Tregs (>0.4283) correlated with shorter progression-free survival (PFS) (2.6 months) (P=0.0063), and worse overall survival (OS) (6.367 months) (P=0.0022), as compared to low (≤13.15) CCR7+ Naive Treg frequency group (8.0 and 15.97 months. respectively) (figure 3.55).

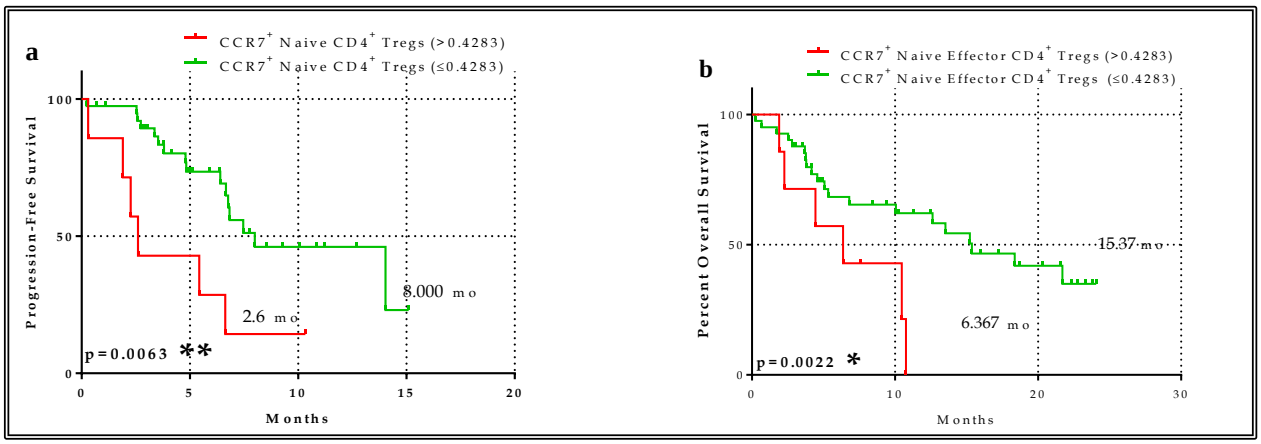

Figure 3.55: Prognostic significance of CCR7+ Naïve CD4+ Tregs in NSCLC patients. Kaplan-Meier survival analysis of patients divided according the frequency of CCR7+ Naïve CD4+ Tregs. High frequency of CCR7+ Naïve CD4+ Treg was associated with (a) poor progression-free survival (2.6 vs 8.00 months) (P=0.0063) and (b) overall survival (6.367 vs 15.97 months) (P=0.0022) ; green line: below 95% CI, red line: above 95% CI of normal control.

Central Memory (CCR7+ CD25+highCD127+/lowCD152+FOXP3+CD45RO+) CD4+ Tregs did not correlated with the clinical outcome of the patients. There was no significant difference in the progression-free and overall survival comparing patients with low and high percentage of Central Memory CD4+ Treg cells (>1.347 or ≤1.347) (p = 0.377 and 0.413, respectively, Log-Rank test) (figure 3.56).

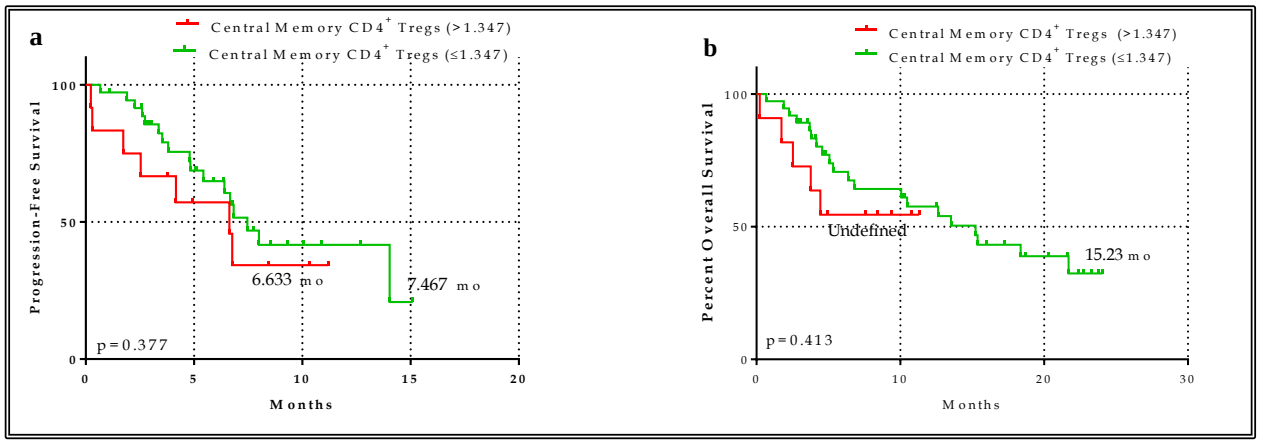

Figure 3.56: Prognostic significance of Central Memory CD4⁺ Treg cells in NSCLC cancer patients. (a) The percentage of Central Memory CD4⁺ Treg cells was not associated with improved progression-free survival using (mean cut-off: 1.347), (p = 0.377, Log-Rank test). (b) No significant difference in the overall survival comparing patients with low and high percentage of Central Memory CD4⁺ Treg cells (mean cut-off: 1.347), (p = 0.413, Log-Rank test); green line: below 95% CI, red line: above 95% CI of normal control.

The mean progression-free survival time was 10.9 months for patients with high percentage of CCR7⁻ CD25^{+high}CD127⁻CD152⁺FOXP3⁺CD45RO⁺ Terminal Memory Effector CD4⁺ Tregs and 6.667 months low percentage (P=0.0314). Furthermore, patients with high counts of Terminal Memory Effector CD4⁺ Treg cells showed a significant increase in survival (means did not reach median value so the median of survival was undefined) compared with patients with low Terminal Memory Effector CD4⁺ Tregs counts (12.5) (P=0.0354) (figure 3.57).

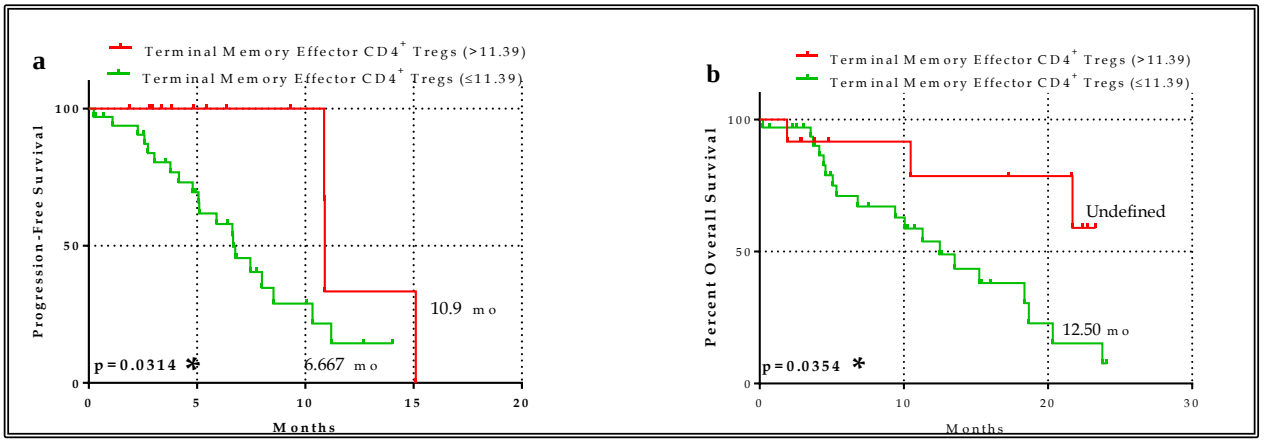

Figure 3.57: Prognostic significance of Terminal Memory Effector CD4⁺ Treg cells in NSCLC cancer patients. Kaplan-Meier survival analysis of patients divided according the frequency of Terminal Memory Effector CD4⁺ Tregs (a) Patients of Terminal Memory Effector CD4⁺ Treg high group had higher progression-free survival (10.9 months), as compared with those with lower Treg frequencies (6.667

months) (0.0314 months). (b) Moreover, the Treg high group was associated with longer OS (Undefined) (P=0.0354), than low Treg group (12.50 months); green line: below 95% CI, red line: above 95% CI of normal control.

3.14 THE CORRELATION OF THE CHANGES OF CD4$^+$ Treg SUBPOPULATIONS DURING TREATMENT WITH THE CLINICAL OUTCOME.

In order to evaluate the correlation of changes in CD4$^+$ Treg subpopulations with clinical outcome, stage IV patients were divided in three groups according to difference of the post 3rd or post 6th cycle value minus the value evaluated pre-chemotherapy. The patients whose difference value was negative were admitted into decrease group. In contrast, the patients whose difference value was positive were admitted into high percentage value group. Moreover, patients whose difference value was zero (± Standard Error of mean) were admitted into stable group. The evaluation period was defined from the date that patients were included in the study till August 2014. Cumulative survival (progression-free survival, PFS; overall survival, OS) time was calculated by the Kaplan-Meier method and analyzed by the log-rank test.

No correlation was observed between the changes of the percentage of CD3$^+$CD4$^+$CD25^{+high}CD152$^+$CD127$^{-/low}$FoxP3$^+$ Treg cells, during chemotherapy, with progression free survival and overall survival. The progressive free and the overall survival was longer in patients with decreased percentages of CD3$^+$CD4$^+$CD25^{+high}CD152$^+$CD127$^{-/low}$FoxP3$^+$ Treg cells in the post 3rd (n=25) and post 6th (n=8) cycle of treatment, but it was not statistically significant (figure 3.58).

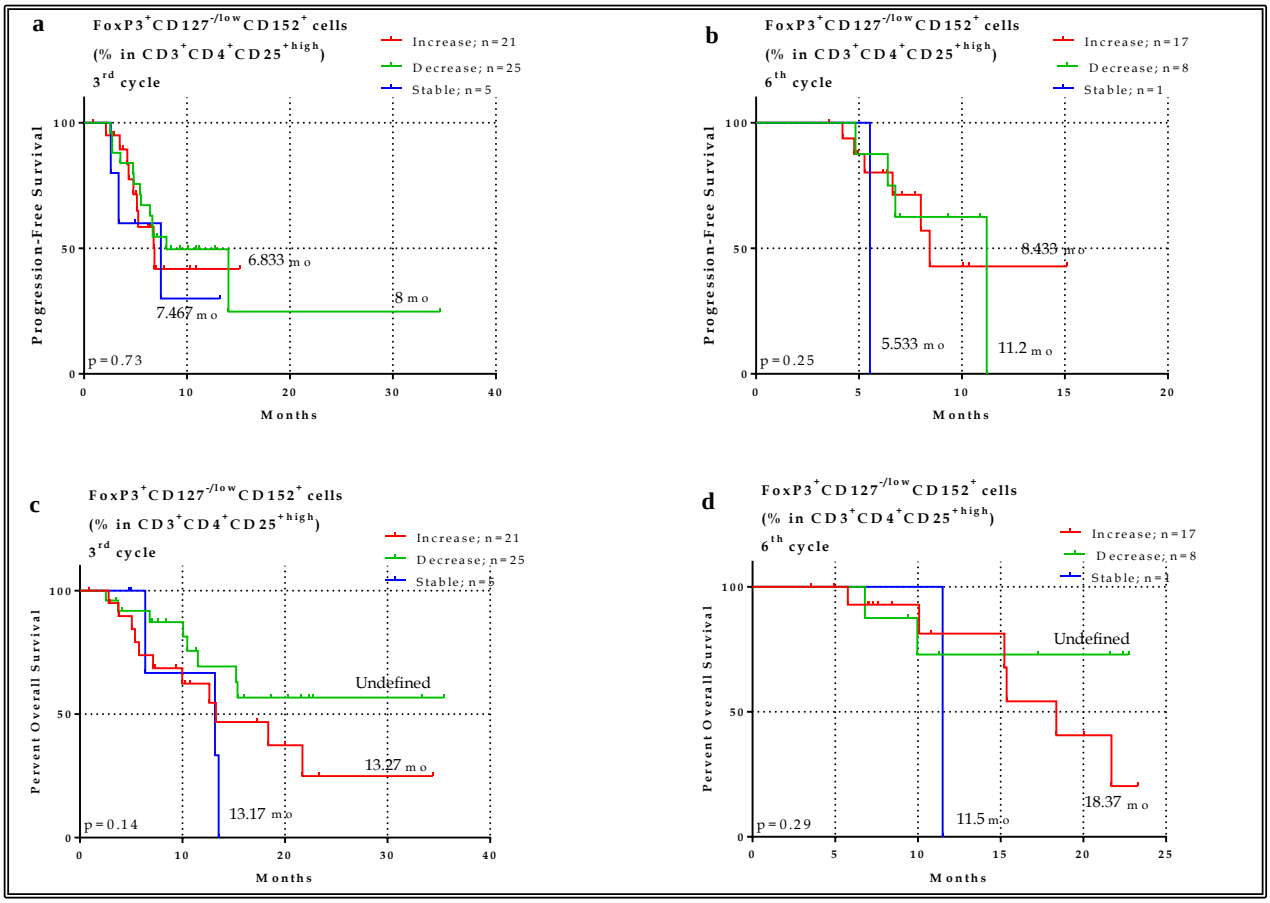

Figure 3.58: Prognostic significance of changes in the percentage of CD3⁺ CD4⁺ CD25⁺high CD152⁺ CD127-/low FoxP3⁺ Treg cells in NSCLC cancer during treatment. The changes of percentage of $CD3^{+}CD4^{+}CD25^{+high}CD152^{+}CD127^{-/low}FoxP3^{+}$ Treg cells were examined in relation to prognosis by Kaplan–Meier analysis. (a, c) A non-significant lower progression free (PFS) overall survival (OS) was observed in patients with decreased percentage of $CD3^{+}CD4^{+}CD25^{+high}CD152^{+}CD127^{-/low}FoxP3^{+}$ Treg cells after the 3rd cycle of chemotherapy. (b, d) The same results were observed and for the patients with decreased percentage of $CD3^{+}CD4^{+}CD25^{+high}CD152^{+}CD127^{-/low}FoxP3^{+}$ Treg cells after the 6th cycle of treatment. P value was determined by log-rank test; green line: decreased percentage, red line: decreased percentage, blue line: stable percentage as the effect of treatment.

The changes in the percentages of $CD25^{+high}CD127^{-/low}$CTLA-4-$FOXP3^{+/low}$$CD45RO^{-}$ Naïve $CD4^{+}$ Tregs followed therapy showed no significant prognostic values. The clinical outcome of the decrease group in the post 3rd cycle was improved in the decrease group, no significance was observed in the statistical evaluation of the results. Moreover, the number of patients with decreased percentage of Naïve $CD4^{+}$ Tregs was zero, so no evaluation could be performed (figure 3.59).

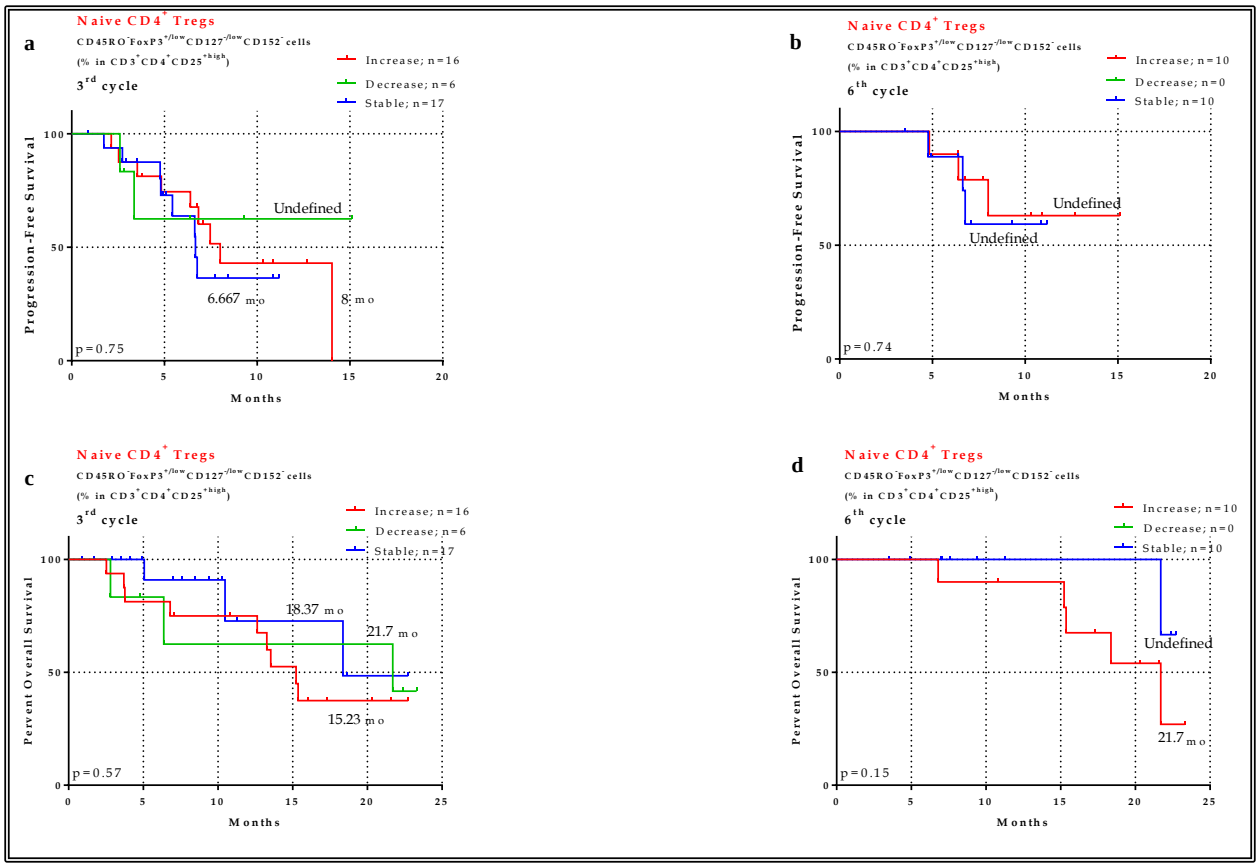

Figure 3.59: Prognostic significance of changes in the percentage of Naïve CD4⁺ Treg cells in NSCLC cancer during treatment. The changes of percentage of Naïve CD4⁺ Treg cells were examined in relation to prognosis by Kaplan–Meier analysis. (a, c) A non-significant poor clinical outcome was observed in patients with decreased percentage of Naïve CD4⁺ Treg cells after the 3rd cycle of chemotherapy. (b, d) No assessment could be performed for the patients evaluated after the 6th cycle of treatment, due to zero number in the decrease group. P value was determined by log-rank test; green line: decreased percentage, red line: decreased percentage, blue line: stable percentage as the effect of treatment.

Moreover, increase of CD25⁺^{high}CD127⁺$^{/low}$CD152⁺FOXP3⁺CD45RO⁺ Effector CD4⁺ Tregs in the post 3rd cycle of treatment was significantly correlated with sorter progression free survival (P=0.0012), but showed no association with overall survival. However, the assessment of the clinical outcome of the post 6th cycle of treatment groups was not possible due to the low number of patients in the increase group (n=1) (figure 3.60).

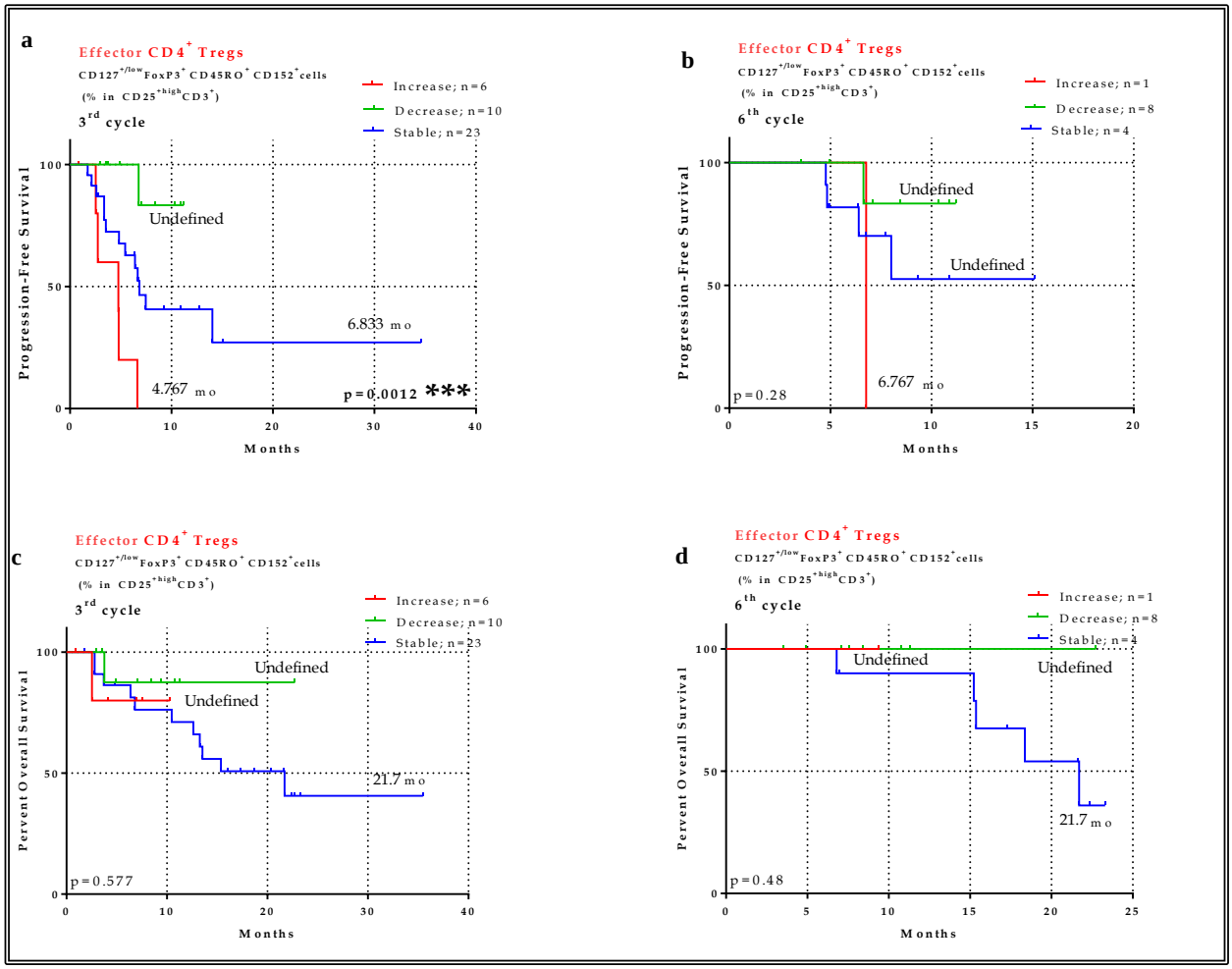

Figure 3.60: Prognostic significance of changes in the percentage of Effector CD4⁺ Treg cells in NSCLC cancer during treatment. The changes of percentage of Effector CD4⁺ Treg cells were examined in relation to prognosis by Kaplan–Meier analysis (a). A significant lower progression free survival was observed in patients with decreased percentage of Effector CD4⁺ Treg cells after the 3rd cycle of chemotherapy (4.767), compared to increase group (P=0.0012). (c) This result was not confirmed for the overall survival. (b, d) No assessment could be performed for the patients evaluated after the 6th cycle of treatment, due to low number in the increase group (n=1). P value was determined by log-rank test; green line: decreased percentage, red line: decreased percentage, blue line: stable percentage as the effect of treatment.

In twelve patients, a decrease in CD25⁺^{high}CD127⁻CD152⁺FOXP3⁺CD45RO⁺ Terminal Effector CD4⁺ Tregs was observed after 3 cycles of chemotherapy. The progression free and overall survival was significantly longer in these patients, compared to increase and stable group (P=0.0089 and 0.042, respectively). This finding was not confirmed in patients evaluated after

the 6th cycle of treatment, but can be associated with the low number of patients in the decrease group (n=3) (figure 3.61).

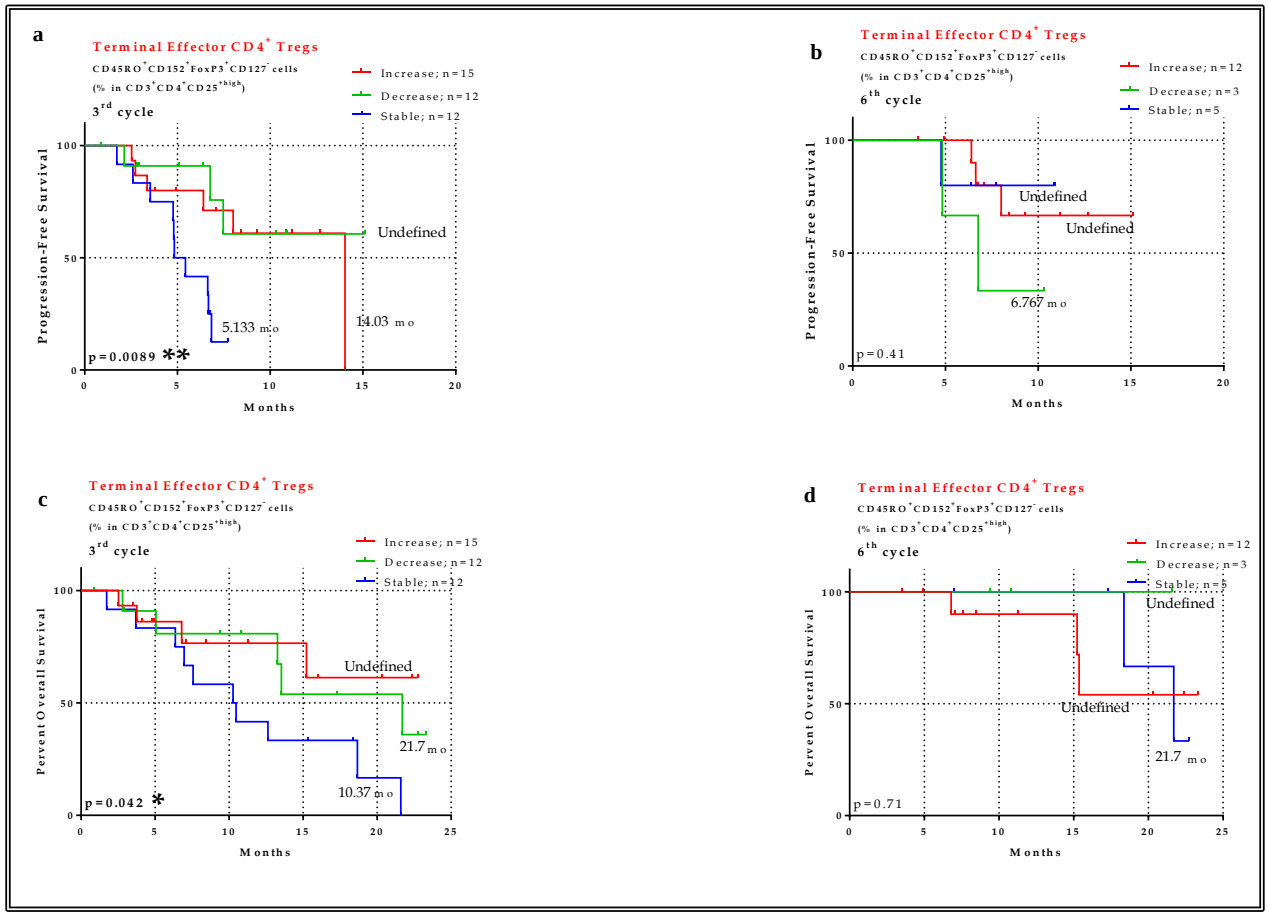

Figure 3.61: Prognostic significance of changes in the percentage of Terminal Effector CD4⁺ Treg cells in NSCLC cancer during treatment. The changes of percentage of Terminal Effector CD4⁺ Treg cells were examined in relation to prognosis by Kaplan–Meier analysis (a, c). A decreased Naïve CD4⁺ Treg level contributed to both improved (a) progression-free (PFS) and (b) overall survival (OS). PFS and OS were significantly shorter in the increase group, for the patients evaluated after the 3rd cycle of treatment (P= 0.0089 and 0.042, respectively). (b, d) This finding was not confirmed in patients evaluated after the 6th cycle of treatment, due to low number of patients in the decrease group (n=3). P value was determined by log-rank test; green line: decreased percentage, red line: decreased percentage, blue line: stable percentage as the effect of treatment.

Changes in the CCR7⁺ Naïve CD4⁺ Treg were not determined as a prognostic factor for patient survival. The difference in survival time between the increase (n=5) and decrease (n=3) groups of the post 3rd cycle was not significant, as the CCR7⁺ Naïve CD4⁺ Treg levels were stable in the

majority of patients. Moreover, no significant difference in the patient survival was observed between the groups of the post 6th cycle, due to the fact that the number of patients in the decrease group was too low (n=2) (figure 3.62).

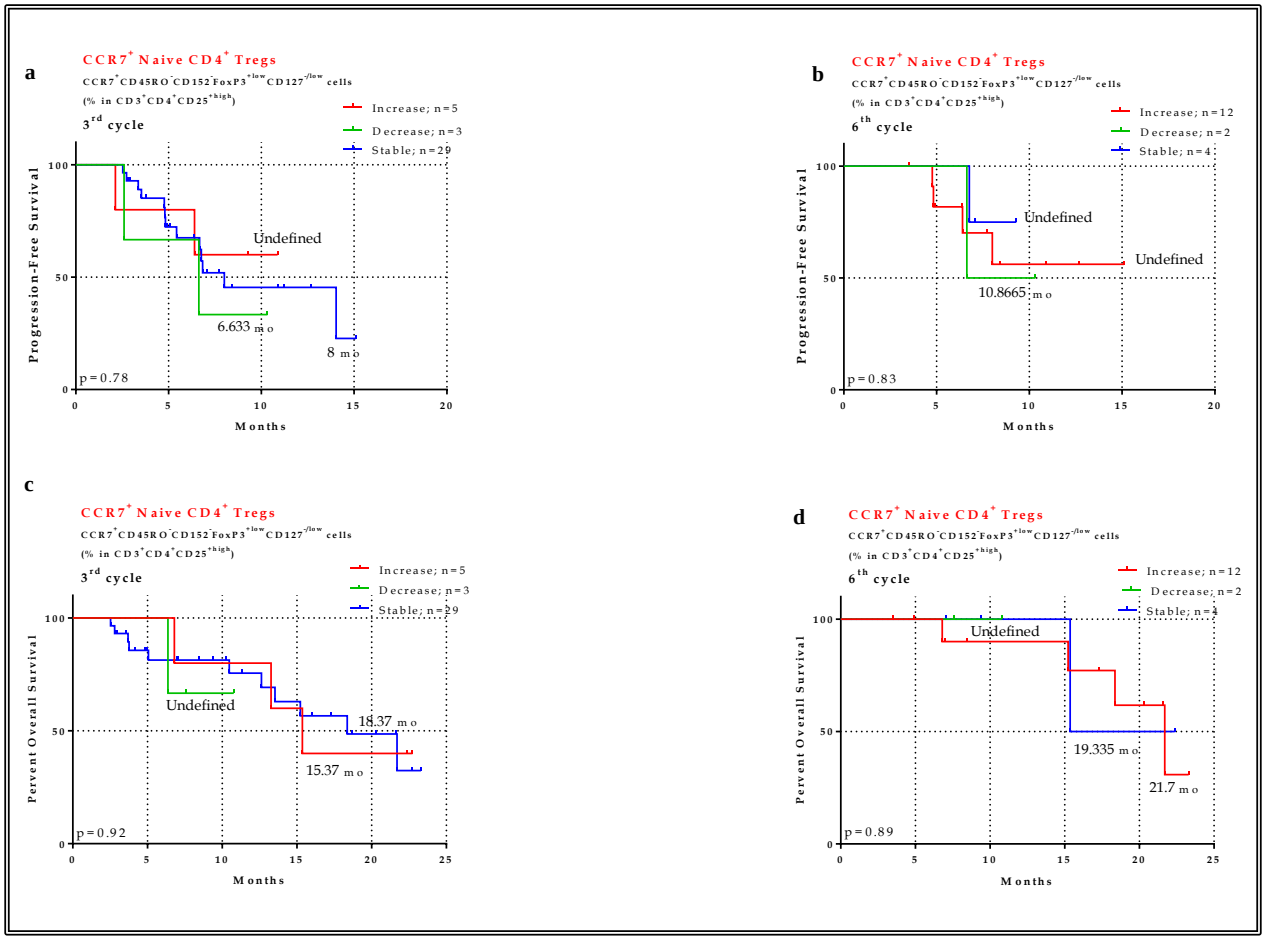

Figure 3.62: Prognostic significance of changes in the percentage of CCR7+ Naive CD4+ Treg cells in NSCLC cancer during treatment. The changes of percentage of CCR7+ Naïve CD4+ Treg cells were examined in relation to prognosis by Kaplan–Meier analysis (a, c). No correlation was observed between CCR7+ Naïve CD4+ Treg level with (a) progression-free (PFS) and (b) overall survival (OS) in patients evaluated after the 3rd cycle of treatment. (b, d) Similarly, no correlation was observed for the patients evaluated after the 6th cycle of treatment. P value was determined by log-rank test; green line: decreased percentage, red line: decreased percentage, blue line: stable percentage as the effect of treatment.

Changes in the percentage of CCR7+ CD25+highCD127+/lowCD152+FOXP3+CD45RO+ Central Memory Tregs were strongly correlated with progression free survival. Patients who had increased percentages of Central Memory Tregs after the 3rd cycle of chemotherapy (n=4) lived

without progression of disease significantly shorter as compared with those with decreased Central Memory Treg frequencies (n=9) (*P*=0.0461). This finding was also confirmed in the groups of the difference of the post 6th cycle. However, no correlation was observed between the changes in the percentage of Central Memory Tregs and overall survival (figure 3.63).

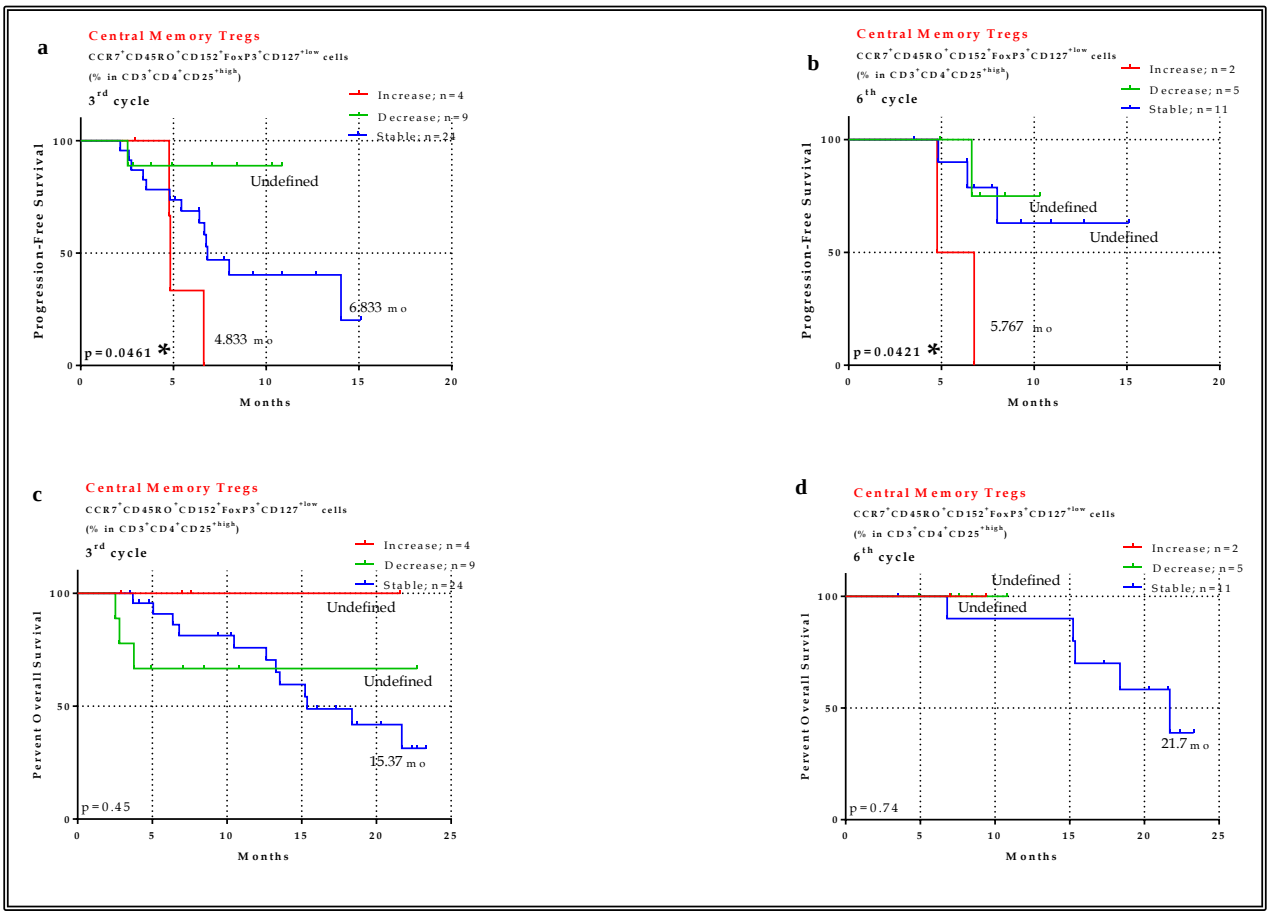

Figure 3.63: Prognostic significance of changes in the percentage of Central Memory CD4⁺ Treg cells in NSCLC cancer during treatment. The changes of percentage of Central Memory CD4⁺ Treg cells were examined in relation to prognosis by Kaplan–Meier analysis (a-b). Changes in the percentage of Central Memory Tregs were strongly correlated with progression free survival. A decreased Central Memory Treg level after the 3rd (a) and 6th (b) cycle of chemotherapy (n=4) lived significantly longer without progression of disease. The same result was not observed in the overall survival (no significance difference). P value was determined by log-rank test; green line: decreased percentage, red line: decreased percentage, blue line: stable percentage as the effect of treatment.

No significant difference in survival was observed between patients with increased or decreased percentage of CCR7- CD25+highCD127-CD152+FOXP3+CD45RO+ Terminal Memory Effector Tregs (figure 3.64). The overall and progression free survival showed controversial results, as decreased group showed improved progression free survival for values evaluated after the 3rd cycle of chemotherapy(n=11) and poor for values evaluated after the 6th cycle (n=2) of treatment. Moreover, these differences were not statistically significant. Finally, decrease group showed poor overall survival compared to increase group, without significance.

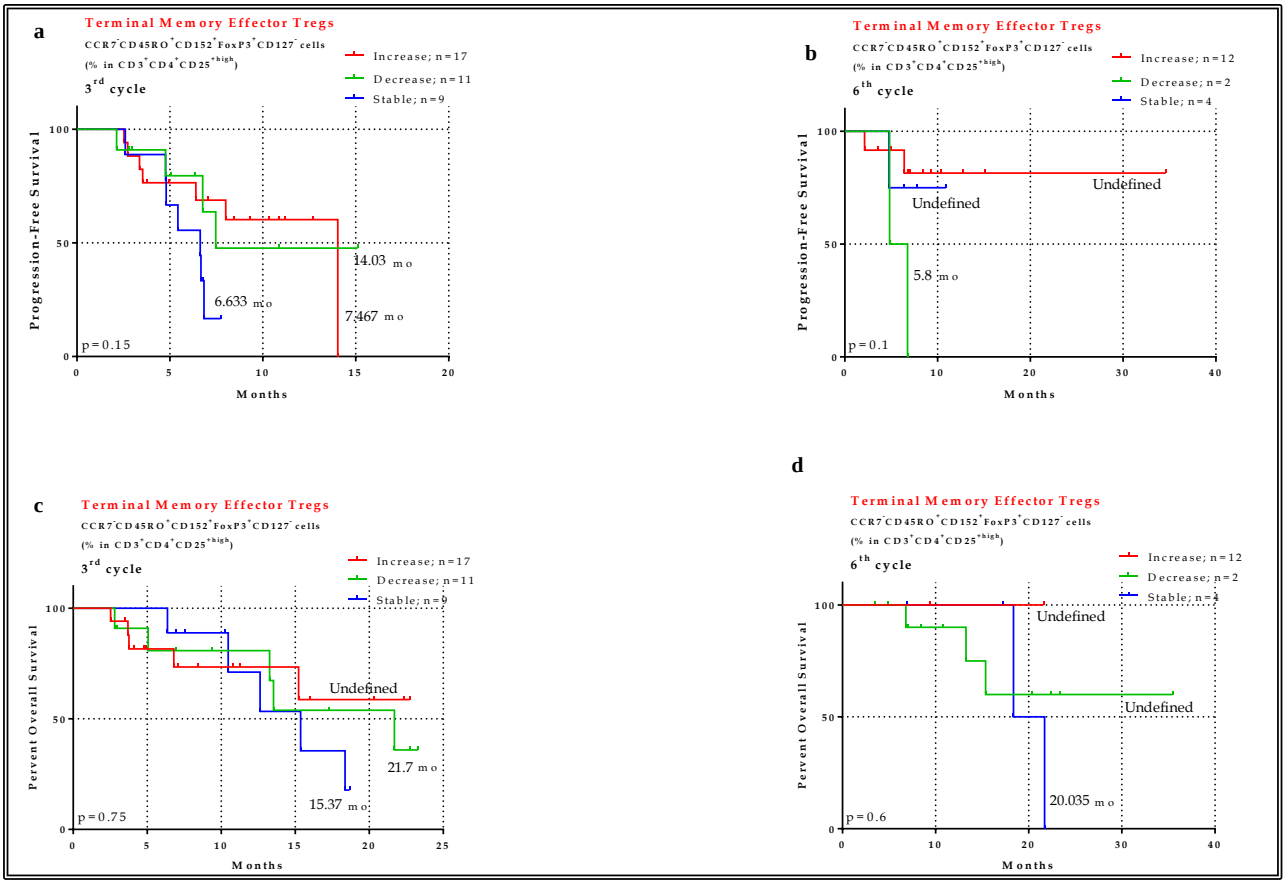

Figure 3.64: Prognostic significance of changes in the percentage of Terminal Memory Effector CD4+ Treg cells in NSCLC cancer during treatment. The changes of percentage of Terminal Memory Effector CD4+ Treg cells were examined in relation to prognosis by Kaplan–Meier analysis (a, c). No correlation was observed between Terminal Memory Effector CD4+ level with progression-free (PFS) (a) and overall survival (OS) (b) in patients evaluated after the 3rd cycle of treatment. (b, d) Similarly, no correlation was observed for the patients evaluated after the 6th cycle of treatment. P value was determined by log-rank test; green line: decreased percentage, red line: decreased percentage, blue line: stable percentage as the effect of treatment.

Chapter 4

Discussion

4.1 DISCUSSION

The immune system plays a dual role in cancer. On the one hand, immune responses suppress tumour growth by destroying cancer cells or inhibiting their outgrowth. On the other hand, immune system promote tumour progression either by establishing conditions within the tumour microenvironment that facilitate tumour outgrowth or by selecting tumour cells able to survive. The process by which immune cells protect the host against cancer formation is called cancer immunoediting. Malfunction of the immune system drives in the establishment and progressive growth of tumours (Koebel et al., 2007).

Oncogenic process generates multiple factors that neutralize the immune system, making it unable to hamper an efficient immune response, and as a result enable tumour to escape from the host immune system (Dunn et al., 2002). Regulatory cells have the ability to suppress the response of cytotoxic T cells against tumours and play a central (negative) role in tumour immune surveillance (Sakaguchi et al., 2008). Increased levels of T regulatory cells have been observed in the circulation and within the tumour infiltrating lymphocytes (TILs) in patients with epithelial malignancies, such as ovarian and non-small-cell lung cancer (NSCLC) (Woo et al., 2001). T regulatory cells are characterized by the expression of CTLA-4 and the lack of the CD127 marker extracellular. In addition, T regulatory cells exhibit suppressive effects by inhibiting the proliferation of conventional T cells and IFN-γ production.

In order to evaluate the functionality of T regulatory cells circulating in the blood of NSCLC patients, $CD4^+$ $CD25^+$ $CD127^{dim/-}$ Regulatory T cells were isolated using magnetic separation. After co-culture with activated $CD4^+$ cells, we observed that the isolated Tregs were able to inhibit the functionality of the activated T cells. The production of IFN-γ was significantly reduced by the presence of T regulatory cells in the wells (55.12±4.313), compared to wells that $CD4^+$ activated cells were cultured alone (209±41.65) (P=.0003) (figure 3.4). Moreover, the concentration of T regulatory cells in the well also affected the IFN-γ production. The differences in IFN-γ production reflect the effect of the isolated T regulatory cells on the cytotoxic activity of T cells ex vivo. This result demonstrates that the T regulatory cells circulating in NSCLC patients are functional and able to suppress immune responses.

In order to identify the mechanisms of immune dysfunction in NSCLC, T cells derived from patients with NSCLC were studied. In the present study, there was no significant difference in the levels of the populations of $CD4^+$T cells after gating for $CD3^+$ cells in the patients with Non-Small Cell Lung Cancer in comparison with those in healthy donors. Moreover, the percentages of $CD3^+CD4^+$ cells were not influenced by the stage or the type of carcinoma (figure 3.5). Previous reports have demonstrated a reduction of $CD3^+CD4^+$T lymphocytes in cancer patients. These studies included post-treated cancer patients who had undergone chemo/radiotherapy which could result in lymphodepletion (Laurent et al., 2010; Zitvogel et al., 2008). However, in our study, treatment naïve patients were recruited which may be the reason of this difference.

Recent studies have demonstrated that lymphocytes with the phenotype of $CD4^+CD25^+$ regulatory T cells (Tregs) contribute to immune dysfunction in cancer patients, and a relative increase in $CD4^+CD25^+$ regulatory T cells is related to immunosuppression and tumour progression in patients with some malignancies. In this study, we reported that NSCLC patients exhibited a significantly higher level of $CD4^+CD25^+$ T cells compared to healthy controls. This is in agreement with previous studies done by several other investigators (Okita et al., 2005). Peripheral blood of cancer patients (n = 140) exhibited a 1.7-fold increase in $CD4^+CD25^+$ T cells (24.98 ± 1.044% of the $CD4^+$ population), which is highly significant ($P < 0.001$), as compared with healthy controls (14.64 ± 1.517%) (figure 3.6). However, the increased $CD4^+CD25^+$ T cells found in NSCLC patients did not express high levels of Foxp3 transcription factor. Moreover, chemotherapy had as a result the significant decrease of $CD25^+$ T cells (24.98± 1.044% to 20.16± 1.843%) ($P=0.0245$). These result suggest that chemotherapy was capable of inducing severe depletion, as it was demonstrated from other studies (Mackall et al., 1994).

$CD4^+CD25^{high}$ cells inhibit the proliferation and the cytokine secretion by activated $CD4^+CD25^-$ responder T cells in a contact-dependent manner (Baecher-Allan et al., 2001). In this study, the frequency of $CD4^+CD25^{high}$ T cells in NSCLC patients was lower in NSCLC patients (0.7850± 0.03675%) than in healthy donors (1.229 ± 0.1294%) ($P<0.0001$). Previous studies demonstrated that the percentages of $CD4^+CD25^{high}$ cells were elevated in NSCLC patient group and other types of cancer (Kono et al., 2006; Liu et al., 2006a). The effect of chemotherapy on $CD4^+CD25^{high}$ cells had as a result a significant increase after the 3rd cycle of chemotherapy (0.8610± 0.09249% to 1.594± 0.5002%) ($P=0.0069$), but this effect was reversed at the end of the treatment (non-significant).

Previous studies shown that $CD4^{+}CD25^{high}$ Treg cells specifically express a transcription factor, Foxp3, which plays a major role in their development and function (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003; Ramsdell , 2003). Foxp3 is currently considered to be the most accurate marker to identify Treg (Ramsdell , 2003). Moreover, it have been demonstrated that $CD4^{+}CD25^{high}FoxP3^{+}$ Treg cells are expanded in patients with solid tumours (Gray et al., 2003; Tokuno et al., 2009; Woo et al., 2001; Woo et al., 2002). Although in this study there were no statistically significant differences in the $CD4^{+}CD25^{high}FoxP3^{+}$ Treg populations among the NSCLC patients and the control group and among different stages of carcinoma, the population of $CD4^{+}CD25^{high}FoxP3^{+}$ cells were significantly larger in the squamous cell carcinoma group (59.71±5.057) than in the adenocarcinoma group (43.51±3.914) (P=0.0185) (figure 3.7). No statistically significant difference was observed in the mean percentage of $FoxP3^{+}$ T cell populations ($CD25^{+high}$) during the treatment (figure 3.9).

$CD127^{low}$ was proposed as a marker of Treg by Liu and colleagues (Liu et al., 2006b). Moreover, CD127 is not only a Treg marker but as is the IL-7 receptor alpha chain plays a vital role in T cell survival and memory phenotype (Mazzucchelli & Durum , 2007). As a result, CD127 is not used as a Treg marker by itself, but in combination with CD25 and, Foxp3 (Dunham et al., 2008; Ndhlovu et al., 2008). Moreover, the expression of CD152 antigen, also called cytotoxic T lymphocyte associated antigen-4 (CTLA-4) (Vasu et al., 2004), is fundamental for the immunosuppressive activity of Treg cells (Takahashi et al., 2000). In addition, it was found in NSCLC that the CTLA4 proportion was higher intracellular than on the surface of circulating $CD4^{+}$ cells (Erfani et al., 2013). Therefore, the addition of a these markers may allow to define a more homogeneous cell population provided by a regulatory activity with respect to the simple $CD4^{+}CD25^{+high}$ expression (Dieckmann et al., 2002). This population was not characterized in the blood of NSCLC patients, but was increased in oral cancer patients (Lim et al., 2014), in laryngeal squamous cell carcinoma (Erfani et al., 2012) and in head and neck squamous cell carcinoma (Sun et al., 2014). In this study, the population of $CD127^{-/low}CD152^{+}FoxP3^{+}$ cells as a percentage of total $CD3^{+}CD4^{+}CD25^{high}$ cells in the peripheral blood did not differ between the NSCLC patients and heathy donors and did not correlate with the stage of the carcinoma (figure 3.10).

However, the mean percentage of $CD127^{-/low}CD152^{+}FoxP3^{+}CD4^{+}CD25^{high}$ cells was significantly higher in patients with squamous cell carcinoma than in those with adenocarcinoma and in

healthy subjects. The same result was observed and in $CD4^{+}CD25^{high}FoxP3^{+}$ Treg population (figure 3.10). Previous studies demonstrate that Tregs are present in all lung tissues, but with significant enrichment in adenocarcinoma (Black et al., 2013). However, squamous cell carcinoma mostly develops in smokers(Kawase et al., 2012). Smoking is one of the parameters that induce inflammation and as a result the presence of chronic inflammation may explain the higher expression of FoxP3 between the $CD4^{+}CD25^{high}$ cells.

Significantly higher mean percentage of $CD127^{-/low}CD152^{+}FoxP3^{+}$ cells was noticed at the end of the treatment, compared with this estimated before the entry of chemotherapy (23.88± 1.865% to 37.15± 3.187%) (P=0.0435) (figure 3.20). Previous studies have shown that chemotherapeutic agents such as fludarabine, paclitaxel and cyclophosphamide down-regulate the number and function of Tregs in cancer patients (Beyer et al., 2005; Ghiringhelli et al., 2007; Zhang et al., 2008). Fludarabine, and paclitaxel have been found to selectively deplete Tregs, while low-dose of cyclophosphamide induces a reduction in circulating Tregs, associated with a suppression of their inhibitory functions (Beyer et al., 2005). Moreover, after high-dose of CTX injection, the percentage of circulating memory and other $CD4^{+}$ T-cell subtypes increased, which resulted in a decrease of the Treg population (Hong et al., 2010).

In particular, in this study, the levels of $FoxP3^{+}CD127^{-/low}CD152^{+}$ Treg cells increased after the third cycle of chemotherapy in 25 patients, whereas in 21 patients were decreased. Similarly, the percentage of $CD127^{-/low}CD152^{+}FoxP3^{+}$ Treg cells increased in 17, decreased in 8 and remained unchanged in one patient at the end of treatment (figure 3.23). Most of the patients with decreased percentage of $FoxP3^{+}CD127^{-/low}CD152^{+}$ Treg cells had received avastin (bevacizumab) -based treatment. Bevacizumab is a monoclonal antibody targeting specifically vascular endothelial growth factor A (VEGF-A) (figure 3.30). This result is in agreement with previous studies, in which treatment with bevacizumab suppressed the percentage of Tregs in peripheral blood of cancer patients (Wada et al., 2009). VEGF-A induced by the tumour is suggested to enhance Treg proliferation, due to a direct action of VEGF-A on Tregs via the vascular endothelial growth factor receptor 2 (VEGFR-2). VEGF-A/VEGFR-2 blockade (avastin) also reduced Treg proportion and proliferation in metastatic colorectal cancer patients (Terme et al., 2013).

Previous studies demonstrated that increased Treg numbers enable cancer progression by dampening the immune system and allowing tumour cells to evade immune detection and destruction. Treg cells have a potential role in cancer development and progression because they have been shown to suppress antitumour immunity (Lizee et al., 2006; Sakaguchi , 2004). As a result, Treg cells play a pivotal role in tumour immunology, thereby having an important impact on the outcome of cancer patients (Beyer & Schultze , 2006; Dougan & Dranoff , 2009; Zou , 2006). However, in this study we found a significant positive correlation between increased percentage of $CD3^{+}CD4^{+}CD25^{+high}CD127^{-/low}CD152^{+}Foxp3^{+}$ Tregs and response to treatment (figure 3.37). At baseline, responders had a significant higher percentage of $CD3^{+}CD4^{+}CD25^{+high}CD127^{-/low}CD152^{+}Foxp3^{+}$ Tregs compared to patient developed progressive disease at the end of treatment (figure 3.44).

High levels of peripheral blood Tregs prior to therapy were associated with decreased progression-free survival in follicular lymphoma patients (Voo et al., 2014). In this study, patients with high percentage of $CD3^{+}CD4^{+}CD25^{+high}CD152^{+}CD127^{-/low}FoxP3^{+}$ Treg cells exhibited a longer progression-free survival (PFS; P=0.01) and lived significantly longer (OS; P=0.0064) (figure 3.51). A previous study demonstrated an opposite effect, showing that lower percentage of Tregs had as a result longer survival (Wada et al., 2008). However, in this study, researchers made an assumption that most of $CD4^{+}CD25^{high}$ T-cells expressed FOXP3, therefore they defined Tregs based only on CD25 high expression, without using any other phenotypic markers specific for Tregs.

These results are in contrast with previous studies. As the immune system can also promote cancer by provoking chronic inflammation and elaborating factors that drive tumour growth, survival, and angiogenesis, higher number of circulating Tregs at baseline may resulted in restriction of the inflammation related to better clinical outcome. So, in cancers that have an inflammatory component, Tregs can inhibit cancer progression by dampening inflammation (Banerjee et al., 2013).

Moreover, number of publications showed that progressive tumour growth evokes generation of immune-suppressor cells and suppressor T cells (Fujimoto et al., 1976) and that the percentage of Treg cells in the peripheral blood increases in parallel with tumour progression (Xu et al., 2009). However, in this study no correlation between tumour growth and changes in

the percentage of Treg cells was observed. Moreover, no correlation was observed between the changes of the percentage of $CD3^+CD4^+CD25^{+high}CD152^+CD127^{-/low}FoxP3^+$ Treg cells, during chemotherapy, with progression free survival and overall survival (figure 3.58).

In this study, it is the first time showing the presence and clinical association of different $CD4^+$ Treg subsets in the NSCLC, as previous studies, by others, referred to Tregs as $CD3^+CD4^+CD25^+$ population. With the dissection of the $CD3^+CD4^+CD25^{+high}FoxP3^+$ Tregs into subpopulations, it was shown the dynamics of Treg cell differentiation in Non-Small Cell lung Cancer patients. The subset of naive $CD4^+CD25^+CD45RO^-$ regulatory T cells has been detected in the peripheral blood and lymphoid organs and represents the precursor cells of conventional memory T cells (Fritzsching et al., 2006; Valmori et al., 2005). Naive Treg cells are less sensitive to apoptotic cell death and are in an earlier state of differentiation (Fritzsching et al., 2006). They have been recently released from the thymus and have not yet experienced Ag contact. These naïve Treg cells proliferated vigorously in response to auto-antigens suggesting that particularly this subpopulation was specific for self rather than foreign antigens (Valmori et al., 2005). The cells of this subset express high levels of FOXP3 and are equally suppressive as their memory counterparts and this pool of naïve Treg cells is relatively small in peripheral blood of healthy individuals (Beyer & Schultze , 2007). However, in this study, analysis of naïve Treg cells in NSCLC patients revealed no difference between the presence or the stage of the disease (figure 3.13). However, the mean percentage of the naïve Tregs presents a significant reduction in patients with adenocarcinoma than in patients with squamous cell carcinoma.

Chemotherapy had as a result a statistically significant increase in peripheral Naïve $CD4^+$ Tregs of NSCLC patients, after the 3rd ($P=0.00462$) and the 6th ($P=0.0012$) cycle of treatment (figure 3.21). In particular, the percentage of Naïve $CD4^+$ Treg cells was higher in 16 patients after the 3^{rd} cycle of chemotherapy, compared to the percentage before the treatment, while in 6 patients it was lower. In the rest 17 patients the percentage remained unchanged (figure 3.24). However, after the 6^{th} cycle of treatment, the percentage of Naïve $CD4^+$ Treg cells increased in half of the 20 patients, while in the rest 10 patients in remained unchanged. This increase was mostly observed in non-avastin based treated patients. In the Avastin-based treatment group, Naïve $CD4^+$ Treg numbers were unchanged three and six months after treatment. On the other hand, in the Non Avastin-based treatment group, Naïve $CD4^+$ Treg numbers increased significantly after chemotherapy (figure 3.31). The mean percentage of Naïve $CD4^+$ Treg was significantly

higher at the post 3rd and post 6th cycle time point in Non Avastin-based treatment group, compared to Avastin-based treatment group.

The effect of the naïve $CD4^+$ Treg subset in the disease progression of NSCLC patients has not been described previously. In this study, we observed, for the first time, that the high percentage of Naïve $CD4^+$ Tregs at baseline was correlated with progressive disease [PD] (figure 3.38). Responders (partial response/ stable disease [PR/CR]) showed a significantly lower percentage of Naïve $CD4^+$ Tregs compared to non-responders of the post 3rd and 6th cycle (progressive disease [PD]). Moreover, naïve $CD4^+$ Tregs were found to be prognostic for worse survival. The median progression-free and overall survival for high Naïve $CD4^+$ Tregs group was significantly reduced compared to low group (figure 3.52). As it was demonstrated, Naïve Treg cells are less sensitive to apoptotic cell death and are equally suppressive as their memory counterparts (Beyer & Schultze , 2007; Fritzsching et al., 2006). As a result, the cells of this subset are more resistant and contribute strongly in the down-regulation of the immune response against tumour antigens.

Moreover, we observed a trend towards an inverse association between the change at Naïve $CD4^+$ Treg levels in the peripheral blood followed treatment and the clinical-benefit. An increase in the percentage of the Naïve $CD4^+$ Treg cells was associated with progression of the disease. The percentage of Naïve $CD4^+$ Treg cells tended to be significantly higher after the 3^{rd} cycle and after the 6^{th} cycle of chemotherapy in the progressive disease (PD) group while the opposite was observed in the clinical-benefit group (figure 3.45). However, the change of Naïve $CD4^+$ Tregs showed no significant predictive value. Even if the clinical outcome was improved in the decrease group after the post 3^{rd} cycle, the statistical evaluation revealed no significance. Moreover, the number of patients with decreased percentage of Naïve $CD4^+$ Tregs was zero at time point of 6^{th} cycle, so no evaluation could be performed (figure 3.59).

Effector Treg cells represent a short-lived terminally differentiated population, which divides rapidly and disappears (Miyara et al., 2009). The two types of effector Tregs are "natural" or thymus-derived, and "induced" that are generated in the periphery in response to antigens under the influence of specific cytokines, for example TGFβ. Effector cells preferentially localize within non-lymphoid and inflamed tissues, which correlate with increased levels of a variety of adhesion molecules and chemokine receptors (Moser & Loetscher , 2001). Moreover, Effector

Treg cells are predominant among tumour-infiltrating FOXP3$^+$ T cells and much higher in frequency compared with those in peripheral blood (Sugiyama et al., 2013). This finding was confirmed in our study, as no difference was reported in proportions of Effector Treg cells during NSCLC disease and between the different types of the carcinoma (figure 3.14). In contrast, in stage IV patients present a significant increase in effector Treg cell counts when compared with stage III. The same result was observed in gastrointestinal cancer patients, as the prevalence of Effector Treg cells in the peripheral blood of stage IV was significantly higher than that in stage III patients (Tokuno et al., 2009).

Chemotherapy had as a result a significantly decrease of the percentage of Effector CD4$^+$ Tregs (figure 3.21). In particular, after the 6th cycle of treatment the percentage of Effector CD4$^+$ cells was lower at 8 patients, higher at 1 patient and show no difference at 11 patients compared to the pre-treatment levels (figure 3.25). The decrease was focused in the non-avastin based treatment group, as the percentage of the Effector Tregs in avastin treated patients was lower in the baseline (figure 3.32). Chemotherapy, also affects proliferating peripheral blood lymphocytes (Stahnke et al., 2001), and in particular can target for depletion unwanted Tregs (Zou , 2006). Paclitaxel has been shown to significantly reduce Tregs in both naive and tumour bearing mice (Vicari et al., 2009) and also in patients with non-small cell lung cancer (Zhang et al., 2003). In vitro studies demonstrate that chemotherapeutic drugs not only reduce Treg levels, but also affect their function as incubation of CD4$^+$CD25$^+$ T cells with paclitaxel reduces their FoxP3 expression and suppressive ability (Zhang et al., 2003). Therefore, chemotherapy has immuno-modulatory effects that can regulate the immune response.

No significant difference was observed in the mean percentage of the Effector CD4$^+$ Tregs in baseline between NSCLC patients whose disease progress after the 3rd or 6th cycle and responders (figure 3.39). However, higher percentage of Effector CD4$^+$ Tregs at baseline had a negative impact on clinical outcome, as the high percentage group demonstrated poor progression-free and overall survival, compared to low group (figure 3.53). Effector Tregs are directly suppressive and predominant among tumour-infiltrating FOXP3$^+$ T cells (Sugiyama et al., 2013). The impact of Effector Treg subset on clinical outcome suggests a functional suppression of immunosurveillance against the tumour. Moreover, increase of Effector CD4$^+$ Tregs during treatment was significantly correlated with sorter progression free survival, but showed no association with overall survival (figure 3.60). Effector Treg cells efficiently suppress

conventional effector T-cell responses in vitro which possibly reflect their effect in clinical outcome (Bour-Jordan et al., 2011).

Terminal effector Treg population represents about 20–30% of human circulating Treg cells and was identified by MHC-II expression (Baecher-Allan et al., 2001; Baecher-Allan et al., 2006). Several studies have reported that Treg function was inversely correlated with CD127 expression in human (Liu et al., 2006b). Terminal effector Tregs are $CD127^-$ and suppress responder T-cell proliferation and cytokine secretion more efficiently and more rapidly than other Treg subsets (Baecher-Allan et al., 2006). Terminal Effector Treg cells are part of the effector Treg compartment of which they seem to constitute a terminally differentiated subset (Miyara et al., 2009).

No significant difference was observed in the percentage of terminal effector Treg cells between patients and controls (figure 3.15). However, we confirmed a significant increase in the percentages of Terminal Effector Treg cells in individuals with squamous cell carcinoma and other types of carcinoma, as compared to individuals with adenocarcinoma. Tissue sample studies have reported a less extensive infiltration of Tregs in squamous tumours compared with non-squamous tumours (Kim et al., 2009; Yoshida et al., 2006). This could explain the increased percentage of Tregs in the circulation.

Terminal effector Treg cells are differentiated from the effector Tregs by the absence of CD127. A strong inverse relationship have been observed between CD127 expression and chemotherapy (Capitini et al., 2009). Chemotherapy results in down-regulation of the CD127 expression. As a result, the percentage of the $CD127^-$ Treg population (Terminal Effector Tregs) significantly increased as a result of chemotherapy treatment (figure 3.21). The level of Terminal Effector Treg cells increased in the majority of patient (12 patients) and decreased in 3 patients at the end of treatment (figure 3.26). Moreover, before the treatment entry, the mean percentage of Terminal Effector $CD4^+$ Treg cells was the same in the two treatment groups. The effect of the Avastin-based chemotherapy was the reduction of the mean level of Terminal Effector $CD4^+$ Treg cells (non-significant), while Non Avastin-based chemotherapy had as a result the increase of the mean percent of Terminal Effector $CD4^+$ Treg subpopulation (figure 3.33).

Previous studies have given apparently conflicting prognostic data in cancer. Given the immunosuppressive capacity of Tregs, it seems paradoxical that increased FoxP3 expressing cells have been repeatedly associated with an improved prognosis in follicular lymphoma (Carreras et al., 2006), ovarian cancer (Leffers et al., 2009) and gastric cancer (Haas et al., 2009). In this study, we observed a correlation between increased suppressive cells percentage in blood and improved clinical outcome (figure 3.40). High levels of Terminal Effector $CD4^+$ Tregs in the baseline were correlated with improved clinical outcome. Chronic inflammation is considered as one of the major risk factors for the development of lung cancer. Thus, the presence of large numbers of circulating Treg may help to dampen these local inflammatory responses. A possible mechanism may be that the effect of Treg is mediated via a down-regulation of harmful proinflammatory cytokines known to promote malignancy (Balkwill & Coussens , 2004). One other explanation is that the increased levels of circulating Terminal Effector Tregs may reflect a decreased number of tumour-infiltrating Terminal Effector Tregs. As these cells inhibit tumour immune reactions by direct cell-to-cell contact, the increased circulating Terminal Effector Treg population in the blood may not influence the immune response. As a result, high levels of Terminal Effector Tregs in the blood stream may lead to a significant survival advantage (longer progression free survival and overall survival) (figure 3.54).

In contrast, the initial percentage of Terminal Effector Treg cells and their increase following treatment were not correlated to clinical response (figure 3.61), as no statistically significant differences in the percentage of Terminal Effector Treg cells at baseline before and after therapy were found between responders and nonresponders (figure 3.47). However, the decrease in circulating Terminal Effector $CD4^+$ Tregs was associated with significantly shorter progression free and overall survival in these patients. This correlation strongly supports the immunosuppressive nature of these cells.

A novel subset of Tregs in adult peripheral blood was recently identified. The newly described population, which is defined as $CCR7^+$ naïve Tregs, exhibits most naive T cell characteristics, including a relatively high proliferative potential and low sensitivity to apoptotic cell death (Fritzsching et al., 2006). Similar to other naïve Treg subset, $CCR7^+$ naïve Treg cells proliferate vigorously in response to auto-antigens. As a result, this subpopulation is specific for self rather than foreign antigens (Valmori et al., 2005). Tumour-derived antigens able to stimulate Tregs are self-antigens (Darrasse-Jeze & Podsypanina , 2013). $CCR7^+$ naïve Tregs are clearly identified

as composed by naive cells based on their phenotypic characteristics ($CD45RO^-$, $CCR7^{+)}$. $CCR7^+$ naïve Tregs are distinguishable from other naive $CD4^+$ T cells based on their expression of CTLA-4 and FOXP3, which are also expressed by antigen-experienced Tregs, but not by other subsets of circulating $CD4^+$ T cells ex vivo.

CC chemokine receptor 7 (CCR7) is expressed by naive cells and some of the memory T cells and mediate lymphocyte homing to secondary lymphoid organs in search of antigen presented by dendritic cells (Capitini et al., 2009; Sallusto et al., 1999). After T cell activation and differentiation, expression of CCR7 is lost on a subset of memory T cells (Sallusto et al., 1999). Lack of CCR7 expression led to a strongly hampered migration of Tregs into lymph nodes accompanied by a severely reduced capacity to suppress antigen-induced naive T cell proliferation (Forster et al., 2008). Thus, CCR7 critically determines the Treg in vivo function underlining the importance of appropriate Treg localization for their in vivo suppressive capacity (Menning et al., 2007). Cells of the $CCR7^+$ naïve Tregs population are anergic and exert their suppressor activity through a mechanism that requires cell-to-cell contact. Moreover, $CCR7^+$ naïve Tregs express FoxP3 and have the ability to suppress the T cell function (Beyer & Schultze , 2007).

In this study, the mean percentage of $CCR7^+$ Naïve $CD4^+$ Tregs was significantly enhanced in the NSCLC patients compared with healthy donors (P=3.22) (figure 3.16). Previous studies suggested, also, an increased frequency of $CCR7^+$ Naïve Treg cells chronic lymphatic leukemia (CLL) and multiple myeloma (Beyer et al., 2006). However, the frequency of $CCR7^+$ Naïve $CD4^+$ Tregs in NSCLC patients was not evaluated by other studies. As expected, in our study, the frequency of these $CCR7^+$ naive Treg cells was relatively low in healthy individuals (Beyer & Schultze , 2007).

The mean percentage of $CCR7^+$ Naive Tregs was elevated at the end of the treatment in the peripheral blood of the NSCLC patients undergoing chemotherapy (figure 3.22). Chemotherapeutic drugs cause DNA damage and kill cancer cells mainly by apoptosis. p53 mediates apoptosis after DNA damage. p53-dependent apoptosis after DNA damage is mediated by the CD95 (APO-1/Fas) receptor/ligand system. However, Naïve $CD45RA^+$ Treg cells in adult life were characterized by resistance to CD95L-induced cell death (Fridlender et al., 2009; Fritzsching et al., 2006). As a result, chemotherapy is not able to induce apoptotic pathway

in this cells. Moreover, this subset is characterized a high proliferative potential. These characteristics could explain the elevation of the percentage of the CCR7+ Naive Tregs in majority of patients (12 of 18) (in two cases it decreased and in four remained stable), as a result of chemotherapy treatment (figure 3.27).

Moreover, high levels of CCR7+ Naïve CD4+ Tregs were correlated with poor clinical outcome. The mean percentage of CCR7+ Naïve CD4+ Tregs in baseline was increased in patients whose disease progress after the 3rd or 6th cycles of treatment, compared to responders (partial response/ stable disease [PR/CR]) (figure 3.41). The overexpression of the CCL21 chemokine has been documented in the tertiary lymphoid structures of lung and breast carcinoma patients (de Chaisemartin et al., 2011; Gu-Trantien et al., 2013). Moreover, invasive tumour cells, that are present in NSCLC, secrete CCL21 (Shields et al.,2007). CCL21 in tumour site is a main driver of lymphoid tissue formation (Drayton et al., 2006; Randall et al., 2008), as it attracts CCR7+ lymphoid tissue inducer (LTi) cells that drive the maturation of lymphoid stroma (Eberl & Littman , 2003). As a result, CCR7+ Naïve CD4+ Tregs are also attracted from the tumour cells. On the other hand, CCR7+ naïve Tregs are less sensitive to apoptotic cell death, express FoxP3 and are equally suppressive as other Treg subsets (Beyer & Schultze , 2007). Moreover, CCR7+ naïve Tregs taken from adult peripheral blood were shown to constitutively express FoxP3 even after the cell lines were maintained for several weeks, while other Treg subtypes (memory Tregs) cells lost their ability to express FoxP3 (Beyer et al., 2006). These characteristics indicate that the CCR7+ Naïve CD4+ Treg subset has the ability to migrate at the tumour tissue and enhance the immune response more efficient than other Treg subsets. Moreover, CCR7 is also expressed on mature dendritic cells (Forster et al., 1999). Mature dendritic cells are attracted to the tumour tissue, due to the CCL21 chemokine, and give rise to the differentiation and expansion of naive Tregs into memory/effector Tregs. CCR7+ Naïve CD4+ Treg cells are stimulated by the presentation of pMHCII by dendritic cells and then form effector or memory Treg cells under the influence of cytokines from the tumour. Our result was also confirmed by the clinical outcome evaluation, as a significantly higher percentage of CCR7+ Naïve Tregs circulating in blood at baseline correlated with shorter progression-free survival (PFS), and worse overall survival (OS). In order to confirm this demonstration, CCL21 expression in tumour site or plasma need to be evaluated (figure 3.55).

In addition, a higher percentage of CCR7^{+} Naïve Tregs was observed after the 3rd cycle of treatment in the progressive disease (PD) group compared to the clinical-benefit group, but not after the 6th cycle of chemotherapy (figure 3.48). However, changes in the CCR7^{+} Naïve CD4^{+} Treg were not determined as a prognostic factor for patient survival (figure 3.62).

Memory Tregs contain high proportions of inflammatory chemokine-expressing cells and comprise two populations that differ in the expression of the lymphoid chemokine receptor CCR7 and represent the counterparts of CCR7^{+} central memory and CCR7^{-} Terminal memory effector Treg cells (Tosello et al., 2008). Central memory Treg cells are CCR7-positive and home efficiently into lymphoid tissue whereas CCR7-negative memory effector T cells have the potential to migrate into peripheral sites of inflammation. Functionally Memory Treg subpopulations are differentiated from Naïve Tregs on the basis of CD45RO expression.

Central memory cells express CCR7 and represent a nonpolarized Ag-experienced cell population that lacks immediate effector cell functions and is more prone to apoptosis (Miyara et al., 2009; Unsoeld et al., 2002). Previous studies demonstrated that the majority of peripheral Tregs in healthy individuals display a central memory phenotype (Mailloux & Epling-Burnette , 2013). Moreover, Central Memory Tregs were significantly expanded in the blood of patients with multiple myeloma. However, in this study, no significant difference was observed between mean percentage of the Central memory Tregs in NSCLC patients and healthy individuals (figure 3.17).

The percentage of Central Memory Treg cells decreased after 3 cycles of treatment (figure 3.22). This finding could be associated with the increase of CCR7^{-} Terminal Memory Effector. CCR7^{+} Central Memory Treg cells can be converted to functional CCR7^{-} CD25^{+high}CD127^{-} CD152^{+}FOXP3^{+}CD45RO^{+} Terminal Memory Effector Tregs by antigen upon entry into non-lymphoid tissue (Marzo et al., 2007). Moreover, Central Memory Treg cells apparently cannot undergo self-renewal (Vukmanovic-Stejic et al., 2006). In particular, Central Memory Treg population decreased in nine, increased in four and was stable in the most of the patients during the 3 first cycles of chemotherapy (figure 3.28). In addition, the percentage was stable in eleven patients, decreased in five patients and increased in 2 patients at the end of the treatment. No significant difference was observed between the Non Avastin and Avastin-based treatment group (figure 3.35).

Central Memory Tregs expressing CCR7 are believed to have only minimal cytokine-secreting capacity and to migrate preferentially to the T cell areas of lymphoid organs and tumour expressing CCL21, where they can be restimulated by antigen. Moreover, this subset expresses FoxP3 and has suppressive activity. A significant correlation between high percentage of Central Memory Tregs in baseline and poor clinical outcome was observed (figure 3.42). Patients with high frequency of Central Memory Tregs at baseline presented with progressive disease after the 6th cycle of chemotherapy, compared to responders (3.42). However, Central Memory CD4^{+} Tregs were not prognostic for survival (3.56). On the other hand, changes in the percentage of Central Memory Tregs were strongly irreversibly correlated with progression-free survival (figure 3.49). Patients who had increased percentages of Central Memory Tregs after the 3rd cycle of chemotherapy lived without progression of disease significantly shorter as compared with those with decreased Central Memory Treg frequencies. However, no correlation was observed between the changes in the percentage of Central Memory Tregs and overall survival (figure 3.63).

In contrast to central memory, memory effector Treg cells have down-regulated CCR7 and are capable of immediately producing cytokines after Ag recognition (Unsoeld et al., 2002). Whereas naive and central memory Treg express molecules such as CC chemokine receptor 7 (CCR7), allowing recirculation through lymphoid tissues, Terminal memory effector Treg display a high chemokine and homing receptor versatility and efficiently migrate into peripheral tissues and inflamed sites (Eksteen et al., 2006; Huehn et al., 2004; Iellem et al., 2001; Kleinewietfeld et al., 2004; Szanya et al., 2002). So, terminal memory effector Treg cells are specialized to control ongoing immune responses directly within the inflammatory environment. Moreover, expression of trafficking receptors such as selectin ligands and chemokine receptors on Terminal memory effector Treg subsets critically influences their suppressive capacity in inflammation (Curiel et al., 2004; Lee et al., 2005; Siegmund et al., 2005), suggesting that appropriate localization is indispensable for Treg function (Huehn & Hamann ,2005). Moreover, functionally, Terminal Memory Effector Treg cells are more suppressive than Central Memory Treg cells in vitro (Mailloux & Epling-Burnette , 2013). In this study, a significantly higher percentage of Terminal Memory Effector Tregs within the CD4^{+} T-cell compartment was observed in NSCLC patients compared to controls (figure 3.18). No previous study has characterized the proportion of this subset in the blood of NSCLC or other types of cancer patients.

The proportion of Terminal Memory Effector increased significantly in NSCLC patients treated with chemotherapy compared with those non-treated (3.21). In particular, the percentages of the Terminal Memory Effector Treg cells were increased or stable after the 6th cycle of chemotherapy, compared to the levels before the treatment, in the majority of patients (figure 3.28). Moreover, the Avastin-based treatment did not cause any statistically significant different effect at the mean percentage of Terminal Memory Effector $CD4^+$ Treg subpopulation, compared to Non Avastin-based treatment (figure 3.36).

Because of their highly suppressive ability, Terminal Memory Effector Treg cells are independent from other Treg subtypes. Moreover, analysis of this subset has been proposed as a tool to predict an early immune escape (Mailloux et al., 2012). In contrast to their suppressive activity, in our study, high levels of Terminal Memory Effector Tregs were correlated with improved clinical outcome (figure 3.43). In baseline, the mean percentage of Terminal Memory Effector Tregs was significantly higher in the patients that were evaluated as SD or PR after the 3^{rd} or 6^{th} cycle of treatment, compared to them in patients presented progressive disease. In addition, the mean progression-free and overall survival time was longer in patients with high percentage of Terminal Memory Effector $CD4^+$ Tregs (figure 3.55).

Finally, we looked at changes in the percentage of Terminal Memory Effector Tregs and did not find significant differences between responders and non-responders before and after chemotherapy and no correlation of the changes with clinical outcome (figure 3.48, 3.62).

4.2 CONCLUDING REMARKS & FUTURE WORK

Novel evidence was presented in this thesis demonstrating that the increased levels of $CD3^+CD4^+CD25^{high}FoxP3^+CD127^{-/low}CD152^+$ (Terminal effector) and $CD3^+CD4^+CD25^{high}$ $FoxP3^+CD127^{-/low}CD152^+CCR7^-$ (Terminal memory effector) Treg cells following treatment were associated with improved prognosis, in contrast to high levels of $CCR7^+CD25^{high}CD127^{-/low}$CTLA-4-$FOXP3^{+/low}CD45RO^-$ (Naïve) Tregs, which was identified as a prognostic factor for worse PFS and OS. Previous studies demonstrated that chemotherapy decreases intratumoural Foxp3 Tregs infiltration (Hu et al., 2014) and this result was correlated with better clinical outcome (Pircher et al., 2014). Our hypothesis is that chemotherapy has as a result reduced production of chemokines by tumour cells, which play a potential role in the infiltration of Tregs

in the tumour tissue. Chemotherapy could reduce the production of chemokines, such as CCL21, and this could reflect the accumulation of effector- Tregs in the blood. Taking into account that the suppressive function of Treg cells requires cell to cell contact, Tregs in the peripheral may have reduced suppressive activity. On the other hand, it was shown that CCL21 is highly expressed in advanced-stage tumour site (Shields et al.,2007). CCL21 in tumour site drives lymphoid tissue formation (Drayton et al., 2006; Randall et al., 2008), as it attracts $CCR7^+$ lymphoid tissue inducer (LTi) cells. $CCR7^+$ Naïve $CD4^+$ Tregs are also attracted from the tumour cells. Beside of these cells, mature dendritic cells are attracted to the tumour tissue, due to the CCL21 chemokine, and give rise to the differentiation and expansion of naive Tregs into memory/effector Tregs in situ. Moreover, the high proliferative activity and less sensitive to apoptotic cell death of Naïve $CD4^+$ may explain their increased percentage in circulation. Thus, future work should be concentrated in the evaluation of the levels of different chemokines in plasma and tumour tissue of NSCLC patients and in the effect of chemotherapy in their production by cancer cells.

In conclusion, this thesis has clearly demonstrated, for the first time, the negative role of increased $CCR7^+$ Naïve Tregs in the peripheral blood of NSCLC patients. The effect of this population was not characterized in any type of cancer and as a result this finding is novel. Moreover, here we describe the effect of the percentage of activated ($FoxP3^+CD127^{-/low}CD152^+$, Terminal effector and Terminal memory effector) Treg cells in peripheral blood of NSCLC patients which is inversed compare to that of infiltrated Treg cells. This sheds further light in the role of suppressive cells in cancer immunology and of the tumour microenvironment in recruitment and expansion of CD4+ Tregs subpopulations in the tumour and periphery.

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