Role and prognostic importance of regulatory T cells in lung cancer patients, according to the presence of tertiary lymphoid structures

Priyanka Devi

To cite this version:

Priyanka Devi. Role and prognostic importance of regulatory T cells in lung cancer patients, according to the presence of tertiary lymphoid structures. Immunology. Université Pierre et Marie Curie - Paris VI, 2015. English. <NNT: 2015PA066345>. <tel-01298428>

HAL Id: tel-01298428
https://tel.archives-ouvertes.fr/tel-01298428

Submitted on 6 Apr 2016

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Université Pierre et Marie Curie

Ecole doctorale ED394 : Physiologie et Physiopathologie et thérapeutique

UMRS1138

Thèse de doctorat d’immunologie

Présentée par :

Priyanka DEVI

Pour obtenir le titre de Docteur de l’université Pierre et Marie Curie

Role and prognostic importance of regulatory T cells in lung cancer patients according to the presence of tertiary lymphoid structures

Présentée et soutenue publiquement le 5 Octobre 2015

Devant un jury composé de :

Prof. François Lemoine : Président du Jury
Prof. Eric Tartour : Rapporteur
Dr. Bruno Lucas : Rapporteur
Dr. Christine Caux : Examinatrice
Prof. François Ghiringhelli : Examinateur
Prof. Catherine Sautés-Fridman : Invitée
Dr. Marie-Caroline Dieu-Nosjean : Directrice de thèse
Acknowledgement

First, I would like to thank Prof. Herve Fridman and Current director Mr. Pascal Ferre, for accepting me to the Centre de recherche de Cordeliers.

I would like to thank you, Prof. Herve Fridman, for his scientific advices and valuable discussions during the lab-meetings which helped me to get insights of tumor immunology and helped this work to improve qualitatively.

I thank Prof. Catherine Sautes-Fridman for giving me an opportunity to join this laboratory as PhD student through the Erasmus Mundus scholarship program. I thank her for the kindness and scientific discussions during the lab meetings. I thank her for giving me an opportunity to talk in the departmental day. Since, my first day in the lab, I am so pleased with your and Herve’s love about Indian food and culture. I thank their affection about India and I hope they will continue loving India.

I thank Prof. François Lemoine for accepting to be president of the jury. I thank Prof. Eric Tartour and Dr. Bruno Lucas for reviewing the thesis manuscript. I thank Dr. Christine Caux and Prof. François Ghiringhelli for accepting to be examiners of the thesis.

I would like to sincerely acknowledge Dr. Marie-Caroline Dieu-Nosjean, who accompanied me throughout this journey. I thank her, for her patience and the confidence she showed in me. Her scientific parenthood helped me to understand the tumor immunology, which was quite a foreign field for me on my arrival in lab. I thank her for giving me an opportunity to learn so many new techniques and helping me to be an independent in performing the experiments and understanding the results. I cannot forget the long meetings with the designing experiments or discussing the results (especially writings on the white board!). This thesis could not have been better without her motivation and immense knowledge which widen my research from various aspects.

My sincere thanks also go to Dr. Jean-Luc Teillaud and Prof. Isabelle Cremer, who integrated me in this laboratory and allowed me to access the laboratory and research facilities. I also thank them for the kind follow up my work during these four years and their scientific contributions which helped this work to improve. I especially
thank Jean-Luc for sharing not only scientific interests, but also the political, geographic, historic and artistic views with me. I would like to thank Dr. Sophie Siberil for her contributions in the lab meeting and her kindness.

I thank Dr. Audrey Lupo, Dr. Diane Damotte, Dr. Marco Alifano and the team of clinicians and pathologists at Hotel Dieu and Cochin Hospital; also to the Dr. Pierre Validire and the team at the Institute Mutualiste Montsouris hospital for their valuable help for the surgical specimen and the clinic-pathological datas of the cancer patients. Without their help this project could not have been moved ahead.

I would like to thank CICC team for their grand technical support. My thanks goes to Hélène and Estelle for their great help for the flow cytometry and I thank Christophe for his help in the imaging. I wish Estelle a very good luck for end of her thesis.

I thank my dear friends (“100% women’s team Marie Caroline”) i.e. Claire, Samantha, Helene, Claudia and Myriam for their love and support throughout this thesis. I would like to thank a lot of my dear post-doc Claire, for her incredible help during the experiments and her kindness. Thanks for sharing the Calopix pain with me in all these years! Your hard work and perfectionism have always been inspiring for me! I would like thank Sam for her kindness and motherhood during the molecular biology experiments. Thank you for motivating me with your kind words “ca vas aller” during stressful times! I would like to thank my dear friend Helene for her happy and humorous nature. Thank you for making me laughs all the time! I wish you a very good luck for your thesis. I would like to thank you Claudia, my English speaking companion in the team for your help and support. Thank you for teaching me the Ingenuity software and it was nice to discuss with you a lot about gene expression data. Gracias mi amiga peruana! I would like to thank Myriam for her help in the last few months of my thesis and I hope you will continue liking the Indian food. I would like to thank my former DC-Lamp companion, “Mr. DOC GOC” for his help and support during the initial phase of my thesis. I hope he will be successful in his adventures in New-York.

I would also like to thank other post-docs Sarah, Jerome, Pauline in the lab. Your presence in the lab was helpful for me! I thank you Sarah for introducing the nanostring technology and most importantly for giving pleasure to hear some American
English in the lab! I thank you Jerome for always helping me with antibodies and also thank you for your curiosity with the Tregs project. I thank you Pauline for your kindness and help. I wish a good luck to new postdocs Mihaela and Angella for their projects in the lab.

I would like to thank the IHC “super girls” Ben Ben, Tick Tick, Laetitia, Hanane, Estephania and Marion, for their “super energy” to organize parties and fun times. I thank them for keeping the IHC lab always in a full energized mode. I thank you a tennis girl Tessa for your kindness and your help in the molecular biology experiments. Thanks for sharing and teaching me how to break correctly the “Noix”! I hope you will visit the Himalayas (Mt. Everst) and of course me:P one day! I also thank the Lucie, Nathalie Josseaume, Tania, Kris and Melanie for their help. I would like to thank Gabriela and Bernhard for their advice during the bioinformatics data analyses.

I would like to thank my dear Indian friend Saradiya for accompanying me in the initial years of the thesis. I was always lucky, to have you before and later Shambhu, to share Indian food and to speak in Hindi while learning French! I wish you very good luck Shambhu, to end of your thesis! Also, I would like to thank other PhD students in the lab who were sailing in the same boat with me! I would like to thank Nicolas, Etienne, Nicolas Merle, Claire Deligne, Mikael, and Benoit for their friendship throughout my thesis.

I would like to thank Dr. Lubka Roumenina, Dr. Véronique Fremeaux-Bacchi and Marie-Agnes Dragon-Durey for their scientific discussions in lab meetings.

I would like to thank the past and present M2 students in lab for keeping the lab young and fresh! Thanks to the past members of the lab Jeremy Cosette, Romain Remark, Caroline, Claire Galand and Duy for their help.

Je vous remercie de Nathalie et Eliane pour être mère à nous tous! La manière vous soignez pour moi a toujours été incroyable! Je remercie Johanna et Lamia pour toute l’aide administrative que vous avez fait pendant toutes ces années! Je remercie Jasmina pour amener l’humour pendant le déjeuner et en général aussi! Sans vous tout le laboratoire ne peut pas fonctionner sans problèmes!

I would like to thank our collaborators in this project Sadrine Katsahian and Sylvain Leveugle from team 22 CRC for their help in the statistical analyses. I thank
David Gentien and Benoit Albaud for their hospitality at the Curie institute. I also thank Emmanuel Donadieu and Houcine Bougerara for their help and hospitality in Cochin institute.

I would like to thank my professor Dr. Kanchanganga Gandhe for her enormous support and love during all this phase of my work. Her kind words have always motivated me all these years. I thank my dearest friend Rhucha for her incredible love and support. I would like to thank my friends in India Tejashree, Revatee, Sharvari, Kalyani, Mihir for their priceless love, friendship and their continuous support, although we were far from each other in all these thesis years.

I would like to thank my parents, parent’s in-laws and my younger brother for their precious love, their kindness and their continuous support throughout this phase of my life. Words feel shorter to thank them!

Last but not least, I thank deep from my heart to love of my life, my husband, Swanand who made this journey pleasant for me. I was lucky to have you with me in this beautiful and romantic city, Paris. There comes a time in PhD when you have to constantly remind yourself that not everyone is running the same race, and that you're only competing with who you were yesterday. In all those times, I thank you for being with me, for showering your relentless love and tremendous support.
“Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something and that this thing, at whatever cost, must be attained”

-Marie Curie
To my parents,
For their relentless love for me...
# Table of contents

Acknowledgement ................................................................................................... 2

Index of figures ...................................................................................................... 11

Index of tables ........................................................................................................ 12

Abstract 13

Abbreviations ......................................................................................................... 14

A. Introduction .................................................................................................... 17

1. Immune system and tumors: a complex discourse ....................................... 17

1.1. Origin of the concept of tumor microenvironment.............................. 17

1.2. Origin and concept of immune surveillance ............................................. 19

1.2.1. Imunoediting: 3 'E' concept................................................................. 20

1.3. Tumor microenvironment: a complex interactome................................ 22

1.3.1. Characteristics of contexture ................................................................. 24

1.3.2. Cancer associated TLS ........................................................................ 25

1.3.2.1. General characteristics of TLS ......................................................... 25

1.3.2.2. Formation of TLS ............................................................................. 26

1.3.3. TLS in anti-tumor immune response..................................................... 28

1.4. Infiltration of immune cells in solid tumor: a strong prognostic marker .... 31

1.4.1. Prognostic importance of the TLS in cancer ....................................... 32

2. Tregs: Key Regulators of anti-tumor immune response............................... 35

2.1. Discovery and features of regulatory T cells .............................................. 35

2.2. Regulatory T cell subsets.......................................................................... 38

2.3. Regulatory mechanisms exerted by Tregs ................................................. 41

2.3.1. Inhibitory cytokines ............................................................................ 42

2.3.2. Suppression by cytolysis ..................................................................... 43

2.3.3. Suppression by metabolic disruption ................................................. 44
2.3.4. Cell to cell contact dependent suppression: Involvement of co-stimulatory and co-inhibitory signals........................................................................................................................................45

2.4. How many mechanisms do Tregs need? Treg plasticity.....................................51

2.5. Infiltration, differentiation and activation of Tregs in tumor microenvironment 53

2.5.1. Infiltration of Tregs in tumor microenvironment .............................................53

2.5.2. Expansion and activation of Tregs .................................................................55

2.5.3. Antigen specificity of Tregs in cancer............................................................55

2.6. Tregs in cancer: ambiguity in prognostic importance ........................................56

3. Tregs and immunotherapy: a blessing in disguise?.............................................60

4. Lung cancer: a study model.................................................................................66

4.1. Etiology and histology of the lung cancer..........................................................67

4.2. TNM classification and survival of patients.......................................................68

4.3. Treatment of lung cancer patients.....................................................................71

4.4. Era of combined therapies: promising for NSCLC............................................72

B. Hypothesis and objectives ...................................................................................75

C. Results 80

References 106

Tregs in advanced stage chemotherapy treated lung cancer patients ..............141

D. Discussion.............................................................................................................146

TLS in lung tumors: centers of the protective immune responses.................146

Infiltration, activation of Tregs in cancer microenvironment...........................147

Anti-inflammatory role of Tregs in lung cancer...............................................150

Tregs in TLS: Proponents or opponents?..............................................................152

Expansion of the specific subsets of Tregs in TLS..........................................156

Modulation of the Treg phenotype in the neoadjuvant chemotherapy treated lung cancer .................................................................................................................157

Prognostic outcome of Tregs in lung cancer patients .......................................158
E. Limitations of this study ............................................................................... 161
F. Conclusion and perspectives ..................................................................... 162
G. References .............................................................................................. 165
Publication bibliography ................................................................................ 165
H. Annex 199
Index of figures

Figure 1: The hallmarks of cancer ------------------------------------------18
Figure 2: The cancer immunoediting concept-----------------------------22
Figure 3: Components of the tumor microenvironment (core of the tumor, invasive margin and the TLS) -----------------------------------24
Figure 4: Proposed model describing the principle events of the TLS neogenesis ----28
Figure 5: Association of immune cell infiltrates with prognosis in various types of cancer ----------------------------------------34
Figure 6: Basic immunoregulatory mechanisms used by Tregs. ---------------41
Figure 7: Immune checkpoints in regulating T cell responses ----------------48
Figure 8: Molecules expressed by the Tregs used for the immunotherapy trials in cancer patients -------------------------------62
Figure 9: Age-standardized lung cancer incidence rates by sex and world area. Source: GLOBOCAN 2008. -------------------------------66
Figure 10: Histological types of lung cancer. -----------------------------68
Figure 11: Characterization of Ti-BALT in NSCLC. -------------------------76
Figure 12: Evaluation of DC-Lamp as a marker of Ti-BALT, and its prognostic value. -----------------------------------------------77
Figure 13: Hypothesis about the role of Tregs in lung Tumor microenvironment. ----79
Figure 14: comparison of the Tregs in NAC treated and NAC untreated patients --- 143
Figure 15: Activation and immunosuppression profile of the Tumor Tregs in the NAC treated and untreated patients -------------------------- 144
Figure 16: Activation and immunosuppression profile of the Blood circulating Tregs in the NAC treated and untreated patients ------------------ 145
Index of tables

Table 1: The comparison among the general characteristics of SLO and TLS--------26
Table 2: Prognostic value of TLS-associated biomarkers in naïve and vaccinated cancer patients (Dubois et al., submitted) -----------------------------------------------30
Table 3: The regulatory T cell subsets and their suppressive mechanisms (Mougiakakos et al. 2010) ------------------------------------------------------------------------38
Table 4: Main differences between nTreg and iTreg cells ---------------------------------------------------------40
Table 5: Study of prognostic importance of Tregs in types of cancers ---------------------------------60
Table 6: Co-stimulatory and co-inhibitory molecules, activating or blocking antibodies in anti-cancer treatment -------------------------------------------------65
Table 7: TNM classification in human lung cancer (7th edition) ------------------------------------------69
Table 8: TNM classification and characteristics of each stage -------------------------------------------70
Abstract

Tumor comprise complex niche of the immune and non-immune components. The complex interaction between the tumor cells with its environment turns into either eradication or the growth and metastasis of the tumors. In our team we have previously demonstrated the role of TLS (tertiary lymphoid structures) in lung tumors, in the generation of the protective anti-tumor responses. These TLS act as foci of the generation of the humoral, Th1 and CD8 cytotoxic T cell responses. High densities of the mature DC, B cells in TLS and CD8+ T cells are associated with the good clinical outcome in the lung cancer patients. Despite of the anti-tumor responses generated by the immune system tumors do develop via exploiting the regulatory mechanisms. And one of these mechanisms includes the infiltration of the Tregs (regulatory T cells) in the tumor microenvironment.

The aim of thesis was to study the putative role of Tregs in regulating the immune responses in lung cancer. This study strongly demonstrates the presence of FoxP3+ Tregs in the TLS as well as non-TLS areas of the lung tumors. Ti-Tregs (tumor infiltrating Tregs) mainly exhibit central memory and effector memory phenotype expressing vast repertoire of the activation and ICP (immune checkpoint) molecules. The gene expression and flow cytometry experiments showed that Tregs express the co-stimulatory and co-inhibitory markers constituting the ICP which are known to be involved in the immune suppression. Tregs expressed peculiar set of genes in comparison to the CD4+ conventional T cells. At the end I showed that high density of the Ti-Tregs either in TLS or in nonTLS areas is associated with the poor survival of the NSCLC patients. When combined with the density of TLS mature DC or TLS B cells or conventional T cells or CD8+ T cells, it was observed that a group of patients with the low DC, B cells and CD8+ T cells but high Tregs densities, had the worst clinical outcome. Combination of the TLS mature DC or TLS B cells or conventional T cells or CD8+ T cells densities with Tregs allowed to identify the NSCLC patients with highest risk of death.

Thus, it be concluded that the Tregs create the immunosuppressive environment in the lung tumors by acting in both TLS and nonTLS areas of the tumors and thus could be possible reason for the reduced survival of the lung cancer patients.
Abbreviations

APC  Antigen presenting cell
BALT  Bronchus-associated lymphoid tissue
Bcl-2  B-cell lymphoma 2
BMDC  Bone marrow-derived cells
CTLA-4  Cytotoxic T-lymphocyte-associated protein 4
DC  Dendritic cell
ER  Estrogen receptor
GITR  Glucocorticoid-induced TNFR family related gene
GC  Germinal center
iBALT  Induced BALT
IDO  Indoleamine 2,3-dioxygenase
iTreg  Induced regulatory T cell
LAG-3  Lymphocyte activation gene 3
Lti  Lymphoid tissue inducer cell
mAB  Monoclonal antibody
MALT  Mucosa-associated lymphoid tissue
MDSC  Myeloid-derived suppressor cell
MHC  Major histocompatibility complex
NK  Natural killer cell
NKT  Natural killer T cell
NOTCH  Neurogenic locus notch homolog protein
NSCLC  Non-small-cell lung cancer
nTreg  Naïve regulatory T cell
PD-1  Programmed death 1
SCLC  Small-cell lung cancer
SLO  Secondary lymphoid organ
TAA  Tumor-associated antigen
T_{CM}  Central memory T cell
TCR  T cell receptor
T_{EM}  Effector memory T cell
T_{FH}  Follicular helper T cell
Th1  T helper type 1 cell
Th17  T helper type 17 cell
Th2  T helper type 2 cell
Th22  T helper type 22 cell
Th9  T helper type 9 cell
Ti-BALT  Tumor-induced BALT
TIGIT  T cell immunoreceptor with Ig and ITIM domains
Tim-3  T cell immunoglobulin domain and mucin domain 3
TLS  Tertiary lymphoid structure
TNF  Tumor necrosis factor
TNFR1  TNF receptor 1
TRAIL  TNF-related apoptosis-inducing ligand
Treg  Regulatory T cell
TSA  Tumor specific antigen
CD137/4-1BB  Tumor necrosis factor receptor super family member 9 (TNFRSF9)
mAB  Monoclonal antibody
GrA/GrB  Granzyme A/B
TIL  Tumor-infiltrating lymphocytes
Introduction
A. Introduction

Large amount of research is going on, to know, how the normal cells from body get transformed into the cancerous cell. Not only the oncologists but tumor immunologists are also participating to understand the microenvironment shaped around these tumor cells. It has been understood now that tumors modulate this microenvironment and escape the counteracting immune responses. Immune regulating factors (includes regulatory cells) also serve as a tool for this escape. In this study, we tried to decipher the immune regulation of the lung cancer microenvironment by regulatory T cells and especially their role in the tumor-induced TLS. Study of the phenotype of Tregs and their association with the prognostic importance in the lung may help to search new biomarkers for immunotherapy modulating Tregs in cancer patients.

1. Immune system and tumors: a complex discourse

1.1. Origin of the concept of tumor microenvironment

It all started with the discovery of tumor development as abnormal proliferation of cells which was summarized by reductionist’s as is a simple aggregate of malignant cell proliferation. This long prevailed concept later changed in year 2000 and it is now clearly established that tumor growth is accompanied by the formation of a complex niche that plays an important role in the progression of the malignancy (Hanahan, Weinberg 2000). With rapid advances in research about cancer revealed it as a disease with dynamic genomic changes. Complexities about this disease described in laboratories and clinical studies transformed into an underlying principle. These basic principles are termed as “hallmarks of cancer”.

In 2000, Hanahan and Weinberg suggested that malignant growth is accomplished through the different cancer cell genotypes and six essential alterations in cell physiology. These six <<Pillars>> of cancer growth are illustrated in the Fig. 1.

Each of these capabilities are acquired by the tumors to escape the recognition by the host cells (Hanahan, Weinberg 2000). Approximately all tumors may show in
common these six hallmarks. After 10 years of the first theory of “Hallmarks of cancer”, the two new pillars were added to this list that is <<deregulating cellular energetic and avoiding immune destruction>>(Hanahan, Weinberg 2011). The later one is particularly found to be important in tumor development because along with the transformed cells tumors also contain the stromal cells, matrix fibers, blood and lymph vessels and most importantly immune cells and interaction of these components leads to the development of the tumors.

**Figure 1: The hallmarks of cancer**
A. The primary six pillars of the cancer proposed in year 2000 (Hanahan, Weinberg 2000).
B. The addition of the two emerging hallmarks and two enabling characteristics of cancer growth in year 2011 (Hanahan, Weinberg 2011).
1.2. Origin and concept of immune surveillance

The concept of relation between the inflammation and cancer is ancient. This was noted first time by Rudolf Virchow in 1863. He first proposed that the leucocytes in the neoplastic tissues are related with the inflammation and cancer (Balkwill, Mantovani 2001). In contrast, the concept of immune system to control the tumor progression was demonstrated later in 1909, when Paul Ehrlich postulated that cancer occurs spontaneously in vivo and that the immune system is able to both recognize and protect against it. In the late 1950s, Lewis Thomas introduced the theory of immunosurveillance, which was subsequently developed by Sir MacFarlane Burnet in 1964.

With the functional demonstration of mouse tumor-specific antigens supporting the ideas of all these scientists the cancer immunosurveillance hypothesis was put forth. It stated that protective thymus-dependent cells of the body constantly survey host tissues for transformed cells. Despite subsequent challenges to this hypothesis over the next several decades, new studies in the 1990s validated the cancer immunosurveillance concept and expanded it to include the contributions of both innate and adaptive immunity (Smyth, Trapani 2001; Dunn et al. 2002; Dunn et al. 2004).

In 1990’s improved mouse models of immunodeficiency allowed the scientists to reassess the role of immunity in controlling the cancer. Along with lymphocytes, IFN-γ was also found to be promoting immunological rejection of the transplanted tumor cells. Experiments in mice lacking IFN-γ receptors or STAT-1 transcription factor which is required for the IFN receptor signaling or mice lacking adaptive immunity were susceptible for the spontaneous formation of the primary tumors. Soon other laboratories started reporting similar findings which supported the concept that immune system functions as an intrinsic tumor suppressor (Shankaran et al. 2001; Schreiber et al. 2011).
1.2.1. Immunoediting: 3 ‘E’ concept

The immune system plays three primary roles in the prevention of tumors. First, it can protect the host from virus-induced tumors by eliminating or suppressing viral infection. This prevents the inflammatory environment which may be lead to tumor formation. Second, the immune system can specifically identify and eliminate tumor cells expressing tumor-specific antigens or stress molecules. The third process is tumor immune surveillance, where the immune system identifies cancerous and/or precancerous cells and eliminates them before they can cause harm (Swann, Smyth 2007). Despite this, tumors do develop in the presence of immune system, and therefore the updated concept of tumor immunoediting was discovered to explain the role of the immune system in tumor development.

The discovery in 2001, that immune system controls not only the quantity, but also the quality (immunogenicity) of tumors gave rise to revision to the cancer immunosurveillance theory. The study in Schreiber’s laboratory revealed that the tumors growing in the mice that lacked an intact immune system were more immunogenic (unedited) whereas, the tumors from immunocompetent mice were less immunogenic (edited). This notion of, immune system protects against tumors and also shapes tumor immunogenicity is the basis of the immunoediting theory. Cancer immunoediting passed through the three different “E” phases: elimination, equilibrium, and escape (Schreiber et al. 2011) (Fig 2).

**Elimination:** in elimination phase immune system recognizes and destroys the tumor. Initiation of the antitumor response occurs when the innate immune cells first receive alert signal due to the presence of the tumor cells. Pro-inflammatory molecules secreted by tumor cell itself act as danger signal for the innate immune cells. In the second step, innate immune response is amplified and more cells are recruited in the affected site due to the chemokine milieu produced locally. Tumor-infiltrating macrophages produce IL-12 which stimulates infiltration of NK cells. Chemokine production is amplified by positive feedback loop and results into the IFN-γ dependent killing of tumor. In the third step, DC comes into the scenario. Tumor antigens liberated by effects of innate immunity are engulfed by the
immature DC’s recruited at tumor site. Activated, antigen bearing DC’s migrated to the draining LN and induces the activation of the naïve tumor specific CD4+ Th1 cells. Th1 facilitate the development of the tumor specific CD8+ T cells via cross-presentation of the antigenic tumor peptides on DC MHC class I molecules. These tumor specific CD4+ and CD8+ T cells then home in the tumor site and participate in the killing of the antigen positive tumor cells.

**Equilibrium:** In this phase, the immune system and tumor cells that have survived the elimination phase enter into equilibrium; here lymphocytes and IFN-γ exert potent and rigorous pressure on the tumor cells that is enough to limit but not to fully quench genetically unstable and mutating tumor cells. The equilibrium phase is probably the longest phase among the three, and may occur over a period of many years in human (Dunn et al. 2004).

**Escape:** In the escape phase, tumor cell variants selected in the equilibrium phase can grow in an immunologically intact environment. This occurs when genetic and epigenetic changes in the tumor cells confer the resistance to the immune detection allowing the tumors to expand and become clinically detectable. Since, innate and adaptive immunity work hand in hand to eliminate the tumor, tumor circumvents either of the two or both arms of immunity in order to achieve the growth. It may employ the immune-evasive strategies to elude the responses against it.

It is now recognized that tumors can impede the antitumor responses through production of the immunosuppressive cytokines (such as TGF-β and IL-10) or via mechanisms involving immunoregulatory cells (i.e. Tregs and MDSC). Immune escape also occurs through loss of tumor antigen expression, loss of MHC components, shredding NKG2D ligands and development of IFN-γ insensitivity.

Discovery of the escape mechanisms by tumors gave a better knowledge of the tumor immunology to the scientists and initiated a research in several different aspects of the tumor immunology.
1.3. **Tumor microenvironment: a complex interactome**

In physiological conditions, tissues bear the large number of cells which work in harmony to perform the normal functioning of the body. Due to mutations in proto-oncogenes or tumor suppressor genes or genes related to the growth and survival of the cells, some of these normal cells lose the constraint and become cancerous cells. Although cancers have altered identity it does not loses interaction with surrounding environment. These interactions may lead to the infiltration of the different immune cells through chemokine and cytokine cocktail. Complex structure composed of the tumor cells along with the stromal components, immune cells, vascular and
lymphatic vessels results into the interacting tumor microenvironment. A continuous seesaw game between tumor and immune compartment is a characteristic of this microenvironment and it may go through changes throughout the 3 “E” phases of the immunoediting. In some cases, the defense signals exerted by the immune system is circumvented by tumors to exploit the surrounding cells, proliferate and finally invade to metastasize (Joyce, Pollard 2009).

In general, tumor microenvironment consists of immune cells with different functional properties (Fig.3). It consists of the antigen-presenting cells (B cells, DCs, and macrophages), T cell subsets, NK cells, neutrophils, and mast cells. Even within individual cell types, different subsets may have adverse functions. For example different subsets of CD4+ T cells, NK cells and macrophages may have either tumor suppressing or tumor promoting properties. From patient to patient and from tumor to tumor, heterogeneity can be observed with respect to the numbers, localization of the tumor-infiltrating immune cells. These immune cells can be located in mainly in the invasive margin compared to the center of the tumor, either in the stroma among with TLS or in the tumor nests. Analysis of this immune contexture allows the determination of the beneficial or deleterious effects on the cancer patients.
The tumor microenvironment consists of numerous cells including the tumor cells, endothelial cells of blood and lymphatic vessels, stromal fibroblasts, bone marrow-derived cells, tumor associated macrophages (TAM), and myeloid-derived suppressor cells (MDSC) (Fridman et al. 2012).

1.3.1. Characteristics of contexture

Immunohistochemistry, gene expression, and the clinical techniques have helped to study the presence of the various cell substrates infiltrating the different areas of tumors. Lymphocytes are not randomly distributed but are specifically localized in tumor microenvironment. Macrophages, granulocytes including the mast cells and MDSC are mainly found in the tumor beds and at the invasive margin. NK cells are mostly found in the stroma of the tumor (Platonova et al. 2011). Most B cells are organized in TLS which are mainly located in the invasive margin of the tumors.
(Germain et al. 2015). Cytotoxic and memory CD8+ T cells infiltrate in the stroma and sometimes in the tumor beds (Goc et al. 2014a). TLS are highly organized lymphoid follicles consisting of the B cell area and T cell area. B cells area consists of the several subsets of the B cells, Follicular DC and Follicular helper T cells whereas T cell area is composed of mature DC and T cells (Dieu-Nosjean et al. 2008). Immune cell infiltration is guided by various events in the tumor microenvironment. The chemokines, adhesion molecules and cytokines are important architects of orientation of immune cells and thus are integral part of immune contexture. Complex crosstalk among the receptors and chemokines expressed by the cells is responsible for the build of tumor microenvironment. If this balance is lost, it will result into the loss of co-ordination and inefficiency of the immune system in controlling the tumor.

1.3.2. Cancer associated TLS

1.3.2.1. General characteristics of TLS

TLS are the ectopic lymphoid structures which can be defined as the highly organized aggregates of T cells and B cells in the form of distinct zones adjacent to the HEV.PNAD+ HEV help in the extravasation of the CD62L+ immune cells from blood. The B cell area of the TLS acts as the active germinal centers with antibody producing plasma cells and memory B cells whereas the T cell area of the TLS shows the presence of the mature DC and stromal cells in T cell zone which can secrete the chemokines CCL19 and CCL21 involved in the attraction of the CCR7+ mature DC and T cells to the TLS. These structures are considered as transient structures which may develop in chronic infections, allograft rejection, autoimmune diseases and chronic inflammation like cancers and probably disappear after the infection or inflammatory conditions are resolved. TLS show considerable morphological, cellular, chemokine and vasculature resemblance to secondary lymphoid organs particularly to lymph nodes. It has been shown that same processes and molecules governing LN development are also basis of the TLS formation (Kratz et al. 1996).

The following table shows the characteristics and comparison between SLO and TLS.
### Table 1: The comparison among the general characteristics of SLO and TLS

<table>
<thead>
<tr>
<th></th>
<th>SLO</th>
<th>TLS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Localization</strong></td>
<td>Spleen, lymph nodes and MALT</td>
<td>Non lymphoid tissues like lung, pancreas, breast, thyroid, salivary glands etc.</td>
</tr>
<tr>
<td><strong>Structure</strong></td>
<td>Encapsulated (Spleen and lymph node) non encapsulated (MALT)</td>
<td>non encapsulated</td>
</tr>
<tr>
<td><strong>Ontogeny</strong></td>
<td>Detected Pre or post-nascent</td>
<td>Post-nascent</td>
</tr>
<tr>
<td><strong>Development</strong></td>
<td>Programmed during embryogenesis</td>
<td>Development during inflammatory conditions</td>
</tr>
<tr>
<td><strong>Plasticity</strong></td>
<td>Constitutive presence and remodeling during inflammatory conditions</td>
<td>Uniquely developed during chronic inflammation and may regress after termination of the same.</td>
</tr>
</tbody>
</table>

1.3.2.2. **Formation of TLS**

Although TLS resemble the SLO, it is not identical to SLO. Its organization resembles the loose non encapsulated organization of MALT. In TLS, cells like T cells, B cells and DCs are organized in various zones alongside the HEVs. Three critical events promote the TLS development: inflammatory cytokine production like TNF; lymphoid chemokine production by stromal cells, and HEV development (Drayton et al. 2006).

The cascade of events that occurs prenatally in SLO development shows similarities to the cellular interactions that are involved in the formation of the TLS. Paracrine interactions between the mesenchymal cells and hematopoietic cells are thought to be the basis of the TLS development. LTβR signaling on the endothelial cells induces the lymph node and the HEV formation (Onder et al. 2013). The interaction between LTi (lymphoid tissue inducer) cells and the LTo (lymphoid tissue organizer) cells through lymphotoxin signaling has found to be crucial in the initiation of the TLS formation. The mouse studies show that LTi cells upregulate the Id2 and RORγt which induces the lymphoid neogenesis (Cherrier et al. 2012). The fibroblasts producing the CXCL13 are thought to be attracting the LTi cells at the site of the TLS formation. Chemokines and cytokines, adhesion molecules like VCAM1, ICAM1, MADCAM1 produced locally by the LTo cells after the interaction with the LTi cells induce the TLS formation in the affected areas (in case of the inflammation) (Neyt et al. 2012). In pancreatic mouse model, it is found that LTα expressed in inflammatory
lesions promote expression of CXCL13 and CCL21, which appear to organize the B and T cell zones (Kratz et al. 1996; Hjelmström et al. 2000). In this model, it was observed that the expression of chemokines was dependent on the TNFR1 and not on the LTβR signaling. In other study, in case of LTαβ signaling it was observed that, LIGHT also signals through the LTβR and plays important role in the expression of homeostatic chemokines (Gommerman, Browning 2003). B cells, T cells, NK and DCs are found to be the important source of the LTβ.

The role of the LTi cells in the TLS formation in humans is poorly discovered. It is thought that RORC positive ILCs especially ILC3 (group 3 innate lymphoid cells) are the LTi cells. These cells induce the chemokines and adhesion molecules by stromal cells responsible for the recruitment of the immune cells in the TLS (Lochner et al. 2011; Noort et al. 2015). Mesenchymal cells also express the additional chemokines like CCL19, CCL21 and CXCL13 which attract the different lymphocytes into the T and B-cell zones of TLS, respectively. HEVS express the CCL21 which carries the circulating lymphocytes in and out of the T cell zone of the TLS (Ohmatsu et al. 2007). NK cells, naïve and memory T cells and activated DC express the CCR7 which signals through CCL19 and CCL21 for recruitment, activation and functioning through the TLS. Stromal cells, DCs and macrophages can produce the CXCL13 on lipopolysaccharide stimulations which can attract the B cells to the TLS through CXCR5 signaling (Neyt et al. 2012; van de Pavert, Serge A, Mebius 2010). CD4+ CXCR5+ Tfh cells expressing the ICOS, PD-1 and BCL-6 and producing IL-21 are considered important in the germinal center formation. Tfh cells are considered important in the plasma cell and memory B cell differentiation and thus important for the functional TLS formation (Chevalier et al. 2011).
1.3.3. TLS in anti-tumor immune response

TLS were first discovered and are extensively discussed in the infection and autoimmunity conditions. But the presence of these structures in tumors has been recently found. Reminiscent to the TLS in the autoimmunity, TLS in tumors show the well-organized follicles of B and T cells. Since along with the inflammation, tumors bear immunosuppressive environment due to the presence of the macrophages and regulatory T cells, it is thought that, this induces the formation of these follicles in invasive margins of some solid tumors.

There are several studies demonstrating the importance of the TLS in different types of primary as well as metastases for example, breast cancer, colorectal cancer, lung
cancer, lung metastasis of the renal and colorectal cancer, gastric cancer which shows that occurrence of TLS in cancer is broad phenomenon (Table 2). Along with the primary tumors, vaccination strategies also show the lymphoid neogenesis in cancer patients. In the HPV-16 vaccinated cervical cancer patients the functional TLS (containing the ki-67+ proliferating cells, CD20+ B cells and CD3+ T cells) were formed with the increased Th1 and CD8+ T cell responses (Maldonado et al. 2014). Post GVAX treatment, formation of the lymphoid aggregates and increase in the Th17 signatures with decrease in Tregs associated signature results into the better survival in pancreatic cancers (Lutz et al. 2014).

In case of tumor microenvironment, TLS formation has been found to be influenced by presence of Tregs. Tregs depleted in the mouse bearing MCA induced tumor lead to the increased development of the HEV and T cell infiltration and tumor destruction (Hindley et al. 2012). This effect was also observed in breast tumors where, it was observed that although the HEV can be developed in presence of Tregs, their density is influenced by the ratio of the Tregs to T cells infiltrating the tumors (Martinet et al. 2013). But so far, it is scarcely demonstrated that, the infiltration of Tregs in lymphoid aggregates is associated with worst clinical outcome of the cancer patients.

Although presence of TLS in tumors is associated with the large infiltration of the immune cells TLS among the tumors and patients to patient heterogeneous. Also, immune cells may found not in well-developed TLS, but in the less organized lymphoid aggregates like in the breast cancer, metastatic melanoma (Gobert et al. 2009; Cipponi et al. 2012). Until now, detailed mechanisms of the TLS formation and their maintenance have not completely discovered.

In summary, TLS formation in the tumor microenvironment represents the generation of the protective anti-tumor immune responses. TLS are involved in the infiltration, local education of the T cells and creation of the adaptive as well as humoral responses. This privileged functioning of TLS in the immunosuppressive microenvironment generated by tumors thus help in the better survival of the cancer patients.
<table>
<thead>
<tr>
<th>Criteria</th>
<th>Cancer type</th>
<th>Stages of the disease</th>
<th>No. of Patients</th>
<th>TLS detection by IHC</th>
<th>TLS detection by gene expression</th>
<th>Prognostic Value</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Breast carcinoma</td>
<td>I to III</td>
<td>146</td>
<td>HEV</td>
<td>-</td>
<td>Positive</td>
<td>Martinet et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I to III</td>
<td>146</td>
<td>mature DC</td>
<td>-</td>
<td>Positive</td>
<td>Martinet et al., 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I to III</td>
<td>794</td>
<td>-</td>
<td>T&lt;sub&gt;reg&lt;/sub&gt;, Th1, CXCL13</td>
<td>Positive</td>
<td>Gu-Trantien et al., 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I to IV</td>
<td>350</td>
<td>Lymphoidaggr.</td>
<td>-</td>
<td>Positive</td>
<td>Vayrynen et al., 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ND</td>
<td>25</td>
<td>mature DC</td>
<td>-</td>
<td>Positive</td>
<td>Remarket al., 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I to IV</td>
<td>40</td>
<td>mature DC and</td>
<td>-</td>
<td>Positive</td>
<td>McMullenn et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>185</td>
<td>T</td>
<td>-</td>
<td>Positive</td>
<td>Di Caro et al., 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>166</td>
<td>T</td>
<td>-</td>
<td>No value</td>
<td>Di Caro et al., 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 to IV-A</td>
<td>21</td>
<td>mature DC</td>
<td>-</td>
<td>12-chemokine</td>
<td>Coppola et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I to IV</td>
<td>125</td>
<td>-</td>
<td>-</td>
<td>Positive</td>
<td>Bindeaet al., 2013</td>
</tr>
<tr>
<td></td>
<td>Gastric cancer</td>
<td>all without chemo</td>
<td>82</td>
<td>B</td>
<td>both Th1 and B</td>
<td>Positive</td>
<td>Hennequinet al., 2015</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I to III</td>
<td>365</td>
<td>-</td>
<td>-</td>
<td>Positive</td>
<td>Hennequinet al., 2015</td>
</tr>
<tr>
<td></td>
<td>NSCLC</td>
<td>I to II</td>
<td>74</td>
<td>mature DC</td>
<td>-</td>
<td>Positive</td>
<td>Dieu-Nosjean et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I to IV</td>
<td>362</td>
<td>mature DC</td>
<td>-</td>
<td>Positive</td>
<td>Gocet et al., 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III with neo-adj. chemo</td>
<td>122</td>
<td>mature DC</td>
<td>-</td>
<td>Positive</td>
<td>Germain et al., 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III with neo-adj. chemo</td>
<td>122</td>
<td>mature DC and</td>
<td>-</td>
<td>Positive</td>
<td>Germain et al., 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>III with neo-adj. chemo</td>
<td>122</td>
<td>B</td>
<td>-</td>
<td>Positive</td>
<td>Germain et al., 2014</td>
</tr>
<tr>
<td></td>
<td>Melanoma</td>
<td>I-A to III-A</td>
<td>82</td>
<td>mature DC</td>
<td>-</td>
<td>Positive</td>
<td>Ladanyi et al., 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV</td>
<td>21</td>
<td>-</td>
<td>12-chemokine genes</td>
<td>Positive</td>
<td>Messina et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Oral SCC</td>
<td>all</td>
<td>80</td>
<td>B, T, GC-B, HEV, FDC</td>
<td>-</td>
<td>Positive</td>
<td>Wirsing et al., 2014</td>
</tr>
<tr>
<td></td>
<td>Pancreatic cancer</td>
<td>all</td>
<td>308+226</td>
<td>Lymphoid aggr.</td>
<td>-</td>
<td>Positive</td>
<td>Hirooka et al., 2015</td>
</tr>
<tr>
<td></td>
<td>RCC</td>
<td>all</td>
<td>135</td>
<td>mature DC</td>
<td>-</td>
<td>Positive</td>
<td>Giraldoe et al., 2015</td>
</tr>
<tr>
<td></td>
<td>CRC (lung)</td>
<td>ND</td>
<td>140</td>
<td>mature DC</td>
<td>-</td>
<td>Positive</td>
<td>Remark et al., 2013</td>
</tr>
<tr>
<td></td>
<td>RCC (lung)</td>
<td>ND</td>
<td>51</td>
<td>mature DC</td>
<td>-</td>
<td>Positive</td>
<td>Giraldoe et al., 2015</td>
</tr>
<tr>
<td></td>
<td>CIN + HPV16 DNA vaccine</td>
<td>CIN2/3</td>
<td>12</td>
<td>B, T, HEV</td>
<td>TLS neogenesis</td>
<td>Positive</td>
<td>Maldonado et al., 2014</td>
</tr>
<tr>
<td></td>
<td>PDAC + G-VAX</td>
<td>ND</td>
<td>54</td>
<td>B, T, T&lt;sub&gt;reg&lt;/sub&gt;, FDC, HEV, mature DC, myeloid cells, CCL21</td>
<td>TLS neogenesis</td>
<td>Positive</td>
<td>Lutz et al., 2014</td>
</tr>
<tr>
<td></td>
<td>PDAC + G-VAX</td>
<td>ND</td>
<td>39</td>
<td>-</td>
<td>T&lt;sub&gt;reg&lt;/sub&gt;, Th17&lt;sub&gt;high&lt;/sub&gt;, Th17&lt;sub&gt;low&lt;/sub&gt;</td>
<td>Positive</td>
<td>Lutz et al., 2014</td>
</tr>
</tbody>
</table>

*Table 2: Prognostic value of TLS-associated biomarkers in naive and vaccinated cancer patients (Dubois et al., submitted)*
1.4. Infiltration of immune cells in solid tumor: a strong prognostic marker

Distribution of the immune cells in different areas of the tumors suggests that these cells may have different role in tumor control. Correlation between the immune infiltration and clinical outcome was investigated in the large number of cases in literature. Although high infiltration of the immune cells in good for the patient’s survival, the important fact is that, different immune subsets have different impact on the prognosis of patients. It is observed that, high density of CD3+ T cells, CD8+ cytotoxic T cells and CD45RO+ memory T cells are associated with longer disease-free and overall survival (Galon et al. 2006; Goc et al. 2014b). In NSCLC patients, it has been observed that high density of the CD8+ T cells with low mature DC density is associated with high death rate compared to the high density of both CD8 and mature DC. It is also observed that high mature DC tumors bear the beneficial antitumor Th1 and cytotoxic T cell contexture than the low mature DC tumors. Mature DCs are required to license the positive prognostic value to CD8+ T cells (Goc et al. 2014b). Exception for this, is Hodgkin’s lymphoma (Scott et al. 2013), ocular melanoma and renal carcinoma (Osamu et al. 2001), where Th1 cells and CD8+ T cells are associated with poor clinical outcome respectively.

In contrast to CD8+ T cell population, effect of CD4+ T cell population on clinical outcome has contradictory results. Due to the presence of different subtypes among CD4+ T cells with opposite immune function, impact of total CD4+ T cell population is hard to describe. The striking example of this is Th2 cells, Th17 cells and Tregs. High Treg infiltrate has poor clinical outcome in ovarian (Curiel et al. 2004), whereas increase in density of Tregs is associated with good clinical outcome head and neck cancer (Badoual et al. 2006), follicular lymphoma(Carreras et al. 2006) and colorectal cancer (Salama et al. 2009b). Surprisingly, there are also cases where Tregs are not associated with clinical outcome (Grabenbauer et al. 2006; Heimberger et al. 2008; Jacobs, Joannes F M et al. 2010). The discrepancies about the prognostic importance and different theories about it are discussed in detail in the chapter about the Tregs.
Th1 cells are associated with good clinical outcome in majority of the cancer types including breast, ovarian, colorectal ad pancreatic cancers, renal and lung carcinomas (Fridman et al. 2012). Intra-tumoral number of DCs have often but not always associated with the good clinical outcome. This is especially associated with the accumulation of T cells in the esophageal cancer and melanoma. In breast and lung cancers, the DC infiltration is of independent prognostic relevance (Galluzzi et al. 2012). B cells act as antigen-presenting cells (Bergwelt-Baildon 2002; Zirakzadeh et al. 2013; Marits et al. 2013) and therefore may be important for inducing CD4+ T cell dependent CD8+ memory T cells that help to control tumor invasion. In lung cancer, the high density of TLS-B cells correlated with early-stage as well as advanced-stage chemotherapy patients in Non-small-cell lung cancer (NSCLC) (Germain et al. 2014a). The density of B cells is found to be associated with good clinical outcome in breast cancer and epithelial ovarian cancer (Coronella et al. 2001; Milne et al. 2009). Immunoglobulin K chain expression and the CD138+ plasma cells found to be associated with the good clinical outcome of the NSCLC patients (Lohr et al. 2013).

### 1.4.1. Prognostic importance of the TLS in cancer

The development of the local antitumor response is found to be associated with the presence of TLS. In primary tumors, the presence of the mature DC in the T cell zone is the bona fide feature of these TLS and is found to be a beneficial factor influencing survival of the patients with lung, breast, colorectal carcinoma, colorectal cancer lung metastases and melanoma (Dieu-Nosjean et al. 2008; Ladányi et al. 2007; Treilleux et al. 2004; Remark et al. 2013). An exception to this is renal cell carcinoma where the mature DC is not associated with well-organized lymphoid follicles and the high density of DC outside the TLS is associated with poor survival of patients (Giraldo et al. 2015). Mature DC in these TLS may involve in the education of the T cells, boosting the Th1 and cytotoxic T cell response. Center of these TLS are ruled by the B cells, follicular DC and T_{FH}. B cells in TLS are involved in generation of local humoral response to the tumor antigens (Reuschenbach et al. 2009; Germain et al. 2014a, Germain et al. 2015), But their exact role in the tumor-induced TLS and the
impact on the clinical outcome is debatable. In NSCLC, the plasma cells in TLS has found to be secreting the high levels of the IgG and IgA against the tumor antigens such as LAGE-1, p53 and NY-ESO-1 and the high density of the B cells is associated with the better survival of the early stage and late stage NSCLC patients (Germain et al. 2014a). In breast tumor-associated TLS, it has been found that CXCR5+ CXCL13 producing follicular helper T cells are involved in the formation and stability of the lymphoid follicles are associated with the better survival of the patients (Gu-Trantien et al. 2013a).

It has been observed that Tregs can infiltrate in the lymphoid aggregates. In colonic mucosa, FoxP3+ Tregs in lymphoid follicles is associated with poor clinical outcome. The size of germinal center and number of Tregs observed to be inversely correlated (Salama et al. 2012). In primary breast tumors, the CCL17/CCR4 mediated recruitment of Tregs in lymphoid aggregates is associated with poor clinical outcome whereas, presence of Tregs in the tumor beds is not well associated with the prognosis of the patients (Gobert et al. 2009). Although the infiltration of Tregs in tumor-induced lymphoid aggregates is demonstrated in few examples, the exact mechanisms of the regulation of TLS formation by Tregs is not completely clear.
Figure 5: Association of immune cell infiltrates with prognosis in various types of cancer

The analysis of 124 published articles studying the impact of cytotoxic T cells, memory T cells, Tregs and Th cell subpopulations with regard to prognosis of cancer patients (20 different cancer types were analyzed) is represented. ‘Good’ means that cells associated with good prognosis. ‘None’ means that there was no correlation and ‘poor’ means that cells are associated with poor prognosis (Fridman et al. 2012).

In summary, Presence of different immune subsets and clinical outcome in cancer patients is context dependent. Although, cancer is an unobvious outgrowth of body’s own transformed cells, it has been thought to play an important role in shaping its environment. The translation of this large study on the correlation between immune infiltrate and clinical outcome has led to the concept of the IMMUNOSCORE (Fridman et al. 2013). Since, a high density Th1 and CD8+ T cells correlate with positive prognosis thus the concept of immune score developed considering two markers among CD3, CD8 and CD45RO in two regions (tumor core and invasive margin), grading from 0-4 (Galon et al. 2006; Pagès et al. 2010). This IMMUNOSCORE appears to be strongest tool to prognosticate in colon cancer patients and now is
being studied in other cancer types worldwide (Galon et al. 2012). Defining the group of high risk patients with help of the immune infiltrate status will be eventually strong tool for treatment of these patients.

2. **Tregs: Key Regulators of anti-tumor immune response**

2.1. **Discovery and features of regulatory T cells**

For several decades, the concept of the cells which can suppress the immune responses has been debated. At present time, regulatory T cells are integral part of the immunology but their discovery in 1970’s, then fall in between time period and renaissance 20 years after has been an amusement ride.

Gershon and Kondo, in 1970, first time showed that, lymphocytes can suppress antigen specific T cell responses. They showed that, induction of tolerance and immunity in thymus dependent bone marrow derived cell population seems to require the co-operation of thymus derived cells (Gershon R.K. and Kondo K. 1970). Also, they observed that transfer of antigen encountered T cells to naïve mice can lead to antigen specific tolerance by arresting activity of T cells (Gershon R.K. and Kondo K. 1971). These observations showed that immune system not only eradicated the pathogens but simultaneously prevents the autoimmune conditions through the “suppressor cells”.

Despite great significance of this discovery, the research in this area was paused for the next 20 years when immunologists failed to define this cell population equivocally. Inability to find out markers for distinguishing suppressor T cells from other T cells, ambiguity in the molecular mechanisms of suppression and difficulty in designing antigen specific T cell clones suitable for cellular and molecular analyses were the obstacles in the study of this cell population.
In 1995, Sakaguchi and colleagues initiated the renaissance of these “suppressive cells” (Sakaguchi et al. 1995). They showed that when CD4+ cells from BALB/c\textsuperscript{nu/+} mice LNs and spleen depleted of CD25+ cells by adding mAB and inoculated into athymic nude (nu/nu) mice (BALB/c), all recipients spontaneously developed autoimmune diseases. Reconstitution of CD4+CD25+ cells maintained the self-tolerance by down regulating immune response to self and non-self-antigens in an antigen nonspecific manner. Also, the CD25 molecule was first promising candidate in phenotypic definition of suppressive cells that were further named as thymus derived naturally occurring regulatory T cells (nTregs). Approximately 10% of peripheral CD4+ cells and less than 1% CD8+ T cells in normal unimmunized adult mice expressed the IL-2 receptor α-chain (CD25) molecule (Sakaguchi et al. 1995).

In 2003, Forkhead Box P3 (FoxP3) was identified as unique marker for Tregs as it was predominantly expressed within CD25+CD4+ T cells (Sakaguchi 2003; Fontenot et al. 2003a). The majority of the CD4+FoxP3+ T cells were found to be CD25\textsuperscript{high} (Roncador et al. 2005). Natural Tregs express high affinity hetero-trimeric receptor for IL-2 composed of CD25, CD122 and CD132 chains.

Mutation in the FoxP3 gene in the CTLA-4 null mice displayed the multi-organ disease and lack of conventional CD4+CD25+ Treg cells (Schubert et al. 2001). In human, CD4+CD25+ Treg cells express FoxP3 but TCR stimulated CD4+CD25-human T cells also express FoxP3 and acquire Treg function (Walker et al. 2003). FoxP3 allele deletion, cells lose their suppressive function and acquire the ability to produce the large amount of IL-2 and Th1 pro-inflammatory cytokines. Thus, it was observed that, FoxP3 acts as a Treg lineage specification factor and mediator of the genetic mechanism of tolerance. Irrespective of CD25 expression, FoxP3 correlates to the suppressor activity (Fontenot et al. 2003b; Fontenot et al. 2005b; Fontenot, Rudensky 2005). Continuous FoxP3 expression is essential for maintenance of the developmentally established suppressive program in mature Tregs in the periphery (Williams, Rudensky 2007).

Tregs rely on the expression of the FoxP3 and availability of IL-2 for transcriptional program and functionality. By modifying signaling and cell surface molecules, and
also by repressing cyclic PDE (cyclic phosphodiesterase), FoxP3 maintain Treg lineage
stability (Gavin et al. 2007). When FoxP3 gene attenuated in Tregs in mice, showed
less FoxP3 expression and autoimmune syndrome similar to that of Scurfy mice
(Wan, Flavell 2007) which suggests the importance of the FoxP3 in maintaining the
Tregs stability. In mice expressing mutant FoxP3, it was observed that EGFP+CD4+ T
cells lacked regulatory function and the mice developed autoimmune disease (Lin et
al. 2007).

Although FoxP3 is presently considered as a reliable marker for nTregs major
concerns arose when it was found that FoxP3 is expressed in low levels on
conventional CD4+ and CD8+ T cells upon activation. Eventually, various co-
stimulatory molecules expressed on Tregs like CTLA-4 and GITR were discovered as
Treg markers (Roncador et al. 2005; Ermann, Fathman 2003). Furthermore, two
studies demonstrated that expression of IL-7R α-chain (CD127) is useful marker in
discriminating the Tconv and nTregs. Majority Tregs found to be negative or weekly
positive for CD127 whereas conventional T cells were found to be positive for CD127
upon activation (Liu et al. 2006). All these discoveries speed up the research in Treg
biology and it eventually became one of the glamorous fields of study in immunology
(Collison et al. 2007).
2.2. Regulatory T cell subsets

Although Tregs could be integrated into overall T cell population with suppressive properties, there are increasing evidences of the different subsets of this cell type with distinct development, phenotype and function. These are summarized in the Table 3.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Origin</th>
<th>Phenotype</th>
<th>Suppressive mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naturally occurring Tregs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4(^+) nTregs</td>
<td>Thymus</td>
<td>CD4(^+)CD25(^+)FoxP3(^+)CD127(^{low}) CTLA-4(^+) LAG-3(^+) GITR(^+)</td>
<td>Contact dependent, cytotoxicity mediated, IL-10, TGF-β</td>
<td>Fehérvari, Sakaguchi 2004</td>
</tr>
<tr>
<td>CD8(^+) nTregs</td>
<td>Thymus</td>
<td>CD8(^+)CD25(^+)FoxP3(^+) CTLA-4(^+) CD122(^+)</td>
<td>Contact dependent</td>
<td>Fontenot et al. 2005</td>
</tr>
<tr>
<td>Adaptive/induced Tregs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4(^+) nTreg like</td>
<td>Periphery</td>
<td>CD4(^+)CD25(^+) FoxP3(^+) CTLA-4(^+) GITR(^+) (requires IL-2 and TGF-β)</td>
<td>Contact dependent</td>
<td>Apostolou, Boehmer 2004</td>
</tr>
<tr>
<td>Tr1</td>
<td>Periphery</td>
<td>CD4(^+) CD25(^{low}) FoxP3(^{low})</td>
<td>IL-10</td>
<td>Groux et al. 1997</td>
</tr>
<tr>
<td>Th3</td>
<td>Periphery</td>
<td>CD4(^+)CD25(^+)FoxP3(^+)</td>
<td>IL-10, TGF-β</td>
<td>Chen et al. 1994</td>
</tr>
<tr>
<td>CD8(^+) iTregs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8(^+) iTreg</td>
<td>Periphery</td>
<td>CD8(^+)CD25(^+)FoxP3(^+)</td>
<td>IL-10, TGF-β</td>
<td>Chaput et al. 2009</td>
</tr>
<tr>
<td>CD8(^+) iTregs</td>
<td>Periphery</td>
<td>CD8(^+)CD25(^+)CD28(^-) FoxP3(^+) CTLA-4(^+) GITR(^+)</td>
<td>Contact dependent, IL-10, ILT3, ILT4</td>
<td>Cortesini et al. 2001</td>
</tr>
</tbody>
</table>

Table 3: The regulatory T cell subsets and their suppressive mechanisms (Mougiakakos et al. 2010)

Most CD4\(^+\) nTregs produced by the normal thymus constitutively express FoxP3 and CD25 which represent functionally mature population responsible maintenance of immune self-tolerance and homeostasis by suppressing excessive immune responses harmful for host (Ohkura et al. 2013). Expression of CTLA-4 and GITR is also important hallmark of the nTreg development (Wu et al. 2006; Bettini, Vignali, Dario A A 2009). Many co-stimulatory signals have been implicated in the development and lineage commitment of nTregs. This includes: CD28 ligation by CD80/CD86, IL-2R, thymic stromal-derived lymphopoietin receptor, CD154, GITR, and STAT5 signaling (Salomon et al. 2000; Burchill et al. 2006; Spence, Green 2008). Initial reports have demonstrated the expression of the transcription factor Helios (Ikaros family) limited to the thymic Tregs in mice and humans (Thornton et al. 2010). But further it was found that in humans natural Tregs consists of both the Helios + and –
Tregs (Himmel et al. 2013). There was no difference found in the suppressive activity and the expression of the CD39 and CTLA-4 between the Helios+ and – Tregs (Himmel et al. 2013). Although, there is a debate regarding the Helios as a marker for the thymic Tregs vs induced Tregs it has been observed that Tregs infiltrating the tumors and in periphery express high levels of the Helios (Scurr et al. 2014). Also, it is observed that in mice majority of the Tregs in the spleen and lymph node of the mice expressing Helios transcription factor also express the Neuropilin 1 and thus Neuropilin 1 can also be considered as the specific marker for the thymic Tregs (Shevach, Thornton 2014).

IL-2 is critically important cytokine for nTreg generation and normal activity in vivo, but nTregs itself do not produce IL-2 upon TCR ligation. In addition to IL-2R signaling, ligation of transforming growth (TGF-β), IL-4, IL-7, and IL-15 also appears to be involved in development, suppressive activity, and maintainace of nTregs (Bettini, Vignali, Dario A A 2009). The anti-apoptotic and pro-apoptotic molecules Bcl-2 and Bim also appear to have a hand in Treg development (Pandiyan, Lenardo 2008). Natural Tregs suppress activation and expansion of cells from adaptive as well as innate immunity affecting cellular and humoral immune responses; CD4+CD25+FoxP3+ nTregs hamper effector and memory CD4+ and CD8+ compartments, NK and NKT cells, DCs with respect to their activation, proliferation and function (Ghiringhelli 2005). Furthermore, proliferation, immunoglobulin (Ig) production and Ig class switch of B cells can be suppressed by nTregs (Iikuni et al. 2009).

While nTregs play a critical role in regulating self-tolerance, iTreg are responsible for regulating the immune response to wide variety of microbial and tissue antigens. They develop in SLO from naïve T cells, giving immune system an environmental adaptability. Antigenic stimulation insufficient for the generation of effector T cells is considered a prerequisite for iTreg development after TCR triggering of naïve T cells. The circumstances under which iTregs develop are wide ranging and may include presence of certain cytokines mostly, high levels of IL-2, IL-10 or TGF-β, low dose of antigens and APC exhibiting alterations in maturation and function. The local microenvironment is stimuli to the generation of iTregs (Curotto de Lafaille, Maria A,
The iTreg cell appears in the mesenteric LNs during induction of oral tolerance (Mucida et al. 2005), in the lamina propria of the gut in response to microbiota and food antigens. They are also generated in chronic inflammation (Curotto de Lafaille, Maria A et al. 2008), tumor and transplanted tissues (Cobbold et al. 2004). Tumors can directly stimulate iTregs through several factors like CD70, Cox-2, IDO, IL-10, and TGF-β (Bergmann et al. 2007; Curti et al. 2007; Li et al. 2007).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>nTreg</th>
<th>iTreg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>Thymus</td>
<td>GALT, spleen, LN, inflamed tissue</td>
</tr>
<tr>
<td>Co-stimulatory requirement</td>
<td>CD28</td>
<td>CTLA-4</td>
</tr>
<tr>
<td>Cytokine requirement</td>
<td>TGF-β, IL-2, IL-15</td>
<td>TGF-β, IL-2</td>
</tr>
<tr>
<td>Specificity</td>
<td>Self-antigens</td>
<td>Allergens, commensal microbiota, neoantigens (tumor), alloantigens</td>
</tr>
</tbody>
</table>

Table 4: Main differences between nTreg and iTreg cells

Although, CD4+ Tregs have been a main focus of Treg research, CD8+ Tregs are also increasingly emerging as a crucial component of immune regulation. Besides initial difficulties in identifying CD8+ Tregs, these types of cells were outshone by the discovery of CD4+CD25+ Tregs. This discovery of CD4+ Tregs by Sakaguchi et al. kick-off the intense research in this domain and thus CD8+ Tregs remained a little ignored. The number of CD8+ Tregs are relatively small, <1% in peripheral circulation, though much higher in intestine. A number of markers of CD8+ Tregs have been studied including CD28\textsuperscript{low}, CD122+ and CD8αα expression compared with the normal CD8αβ (Smith, Trevor R F, Kumar 2008). Similar to CD4+ Tregs, CD8+ Tregs may develop in thymus as well as in peripheral tissue. CD8+CD25+FoxP3+CTLA-4+ nTregs have been identified in several studies in rodents and humans and which may act in cell to cell contact dependent manner (Cosmi 2003). CD8+ Tregs are also reported in the cancer patients. In prostate cancer patients, CD8+ Tregs were described to be CD25+ CD122+ FoxP3+ and partly GITR+. Their activity was found to be IL-10 and TGF-β dependent (Kiniwa et al. 2007). CD8+ CD25+ FoxP3+ TGF-β expressing Tregs are observed in colorectal cancer and these cells demonstrated the immunosuppressive function \textit{in vitro} (Chaput et al. 2009).
2.3. Regulatory mechanisms exerted by Tregs

Either nTreg or iTreg, they are critical in maintaining the steady state condition in normal physiological situation to avoid the autoimmunity. Understanding Treg function is to determine how they suppress other lymphocytes at the molecular level. These suppressive mechanisms act together depending on the pathogenic consequences and type of immune response. Based on functional aspect, the various mechanisms used by Treg cells can be grouped into four basic “modes of action”:

1. Suppression by inhibitory cytokines
2. Suppression by cytolysis
3. Suppression by metabolic disruption
4. Suppression by contact dependent mechanisms

![Figure 6: Basic immunoregulatory mechanisms used by Tregs.](image)

Depiction of the various regulatory T (Treg)-cell mechanisms centered around four basic modes of action (Vignali, Dario A A et al. 2008).
2.3.1. Inhibitory cytokines

Inhibitory cytokines such as IL-10 and TGF-β have considerable attention as mediators of Treg cell induced suppression. IL-10 suppresses T cell proliferation and cytokine production and maintains peripheral tolerance to allergens, auto-antigens, transplantation and tumor antigens. It can suppress proliferation of Th1 and Th2 cells (Groux et al. 1997; Cottrez et al. 2000). It is not only produced by Tr1 cells but also by Th1 and Th2 cells (Cottrez et al. 2000). It acts on DC, monocytes and macrophages by inhibiting production of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-12, IL18, GM-CSF, TNF, and IL-12 and blocking cell maturation and up regulation of co-stimulatory molecules (Trinchieri et al. 1993).

On one hand, the capacity of IL-10 and Tregs in the inflammatory tumor microenvironment to impair anticancer Th1 immunity makes them attractive target for cancer immunity, but, paradoxically, IL-10 and Tregs also suppress Th17 activity, inflammation and anticancer response. It has been shown that IL-10 and Type I IFN signaling play overlapping role in limiting Th17 inflammation that may otherwise drive tumor growth and autoimmunity (Wilke et al. 2011; Zhang et al. 2011; Stewart et al. 2013).

Along with IL-10, TGF-β is another member of immunosuppressive cytokine which play important role in regulation of cell proliferation, differentiation. TGF-β is believed to be important in regulation of the immune system by FoxP3+ Tregs and the differentiation of both FoxP3+ Tregs and Th17 cells (Yamagiwa et al. 2001). It blocks the activation of lymphocytes and monocytes. TGF-β is expressed on the surface of the murine and human Tregs making it possible to reconcile TGF-β expression with cell contact dependent suppression (Shevach et al. 2008). Treg membrane TGF-β is also involved in NK cell suppression. TGF-β mediated suppression of NK cells leads to lower NKG2D expression, lower NK cell cytotoxicity and NK sensitive tumor growth (Ghiringhelli et al. 2005). NOTCH is involved in the immunosuppressive mechanism mediated by TGF-β. Membrane expression of TGF-β
is required for the expression of NOTCH ligands on Tregs. Membranous TGF-β can activate NOTCH-1 HES-1 axis in activated T cells which suppresses them and upon inhibition of this NOTCH-1 pathway Treg mediated suppression is abrogated in allergic airway inflammation (Ostroukhova et al. 2006). It is found that IL-10 acts on the Th1 cells whereas TGF-β acts on the Th2 cells. In Th2 cells, TGF-β suppresses the GATA-3 expression (Heath V. et al. 2000).

It is observed that cancer cells can produce and secrete TGF-β which allows it to escape immunosurveillance (Elliott, Blobe 2005). Along with the possible role of TGF-β in immunosuppression, there is strong evidence that TGF-β is required for the maintenance of nTregs and for the induction of iTregs (Huber et al. 2004) and in vivo expansion and immunosuppressive capacity of CD4+CD25+ T cells (Taylor et al. 2006).

IL-35 is recently discovered heterodimeric cytokine that is produced by FoxP3+ Tregs and contributes to their suppressive function (Collison et al. 2007). IL-35 is required for maximal regulatory function in vivo as Tregs deficient in either IL-12α or IL-27β chain are unable to control homeostatic T cell expansion whether IL-35 can inhibit all T helper subsets and other cellular populations such as B cells, macrophages or DCs (Bettini, Vignali, Dario A A 2009).

2.3.2. Suppression by cytolysis

It is very well established now that CD8+ cytotoxic T cells and NK cells execute cytolysis through secretion of granzymes (Lieberman 2003). However, subset of human CD4+ T cells exhibit cytotoxic activity. Activated human nTregs have been shown to produce Granzyme A. Through the action of perforin, these secreted granzymes enter the cytoplasm of the target cell and their serine protease function triggers the caspase cascade, eventually lead to apoptosis (Programmed cell death). The mechanism of cytotoxicity dependent Treg cell suppression has been proposed in several studies. Granzyme B (GrB) production in Tregs is mediated by CD28/TCR ligation through PI3K/mTOR pathway (Efimova, Kelley 2009).
FoxP3+ T cells are cytotoxic to different cell types, including CD8+ T, NK, DC and B cells, through GrB, perforin or Fas-FasL dependent cytotoxic pathways (Janssens et al. 2003). Tregs mediate target cell killing by Granzyme A and Perforin through adhesion of CD18 (Vignali, Dario A A et al. 2008). Noelle and coworkers first reported the GrB production in mouse Tregs. This GrB-dependent suppression was found to be independent of perforin resulting in Treg cell induced apoptosis of effector T cells, but the exact mechanism of action of Tregs in perforin -/- mice found to be unclear (Gondek et al. 2005). In lung inflammation during acute viral infections, GrB+ Tregs play important role in regulation of the immune response (Loebbermann et al. 2012). In tumor microenvironment Tregs suppress the NK cell and CD8+ T cell mediated tumor clearance via GrB-perforin pathway (Cao et al. 2007). Tumor-induced Tregs also expressed the perforin and GrB in tumor bearing animals and selectively targeted the MHC II expressing APC. FoxP3+ T cells possibly regulate the CD8+ T cell priming via perforin-dependent killing of the antigen-presenting DC in tumor-draining LNs (Boissonnas et al. 2010).

### 2.3.3. Suppression by metabolic disruption

There are several mechanisms discovered which are referred to as mechanisms that mediate ‘metabolic disruption’ of the effector T cell target. One of these mechanisms suggested is high expression of CD25 on the surface of Tregs. This enables Tregs to ‘consume’ local IL-2 and therefore starve actively dividing effector T cells by their survival (Thornton, Shevach 1998). Another mechanism includes the intracellular or extracellular release of adenosine nucleotides. Molecules like CD39 and CD73 concordantly expressed on the surface of Tregs for adenosine generation. This suppresses the effector T cell function through activation of the adenosine receptor 2A (A2AR) (Deaglio et al. 2007). Tregs also suppress the effector T cell function directly by transferring the potent inhibitory cAMP into effector T cells (Bopp et al. 2007).
2.3.4. Cell to cell contact dependent suppression: Involvement of co-stimulatory and co-inhibitory signals

The antigen recognition by T cells is initiated through the TCR engagement but its amplitude is regulated by the balance between the co-stimulatory and inhibitory signal strength (these co-stimulatory and inhibitory molecules known as ICPs) (Zou, Chen 2008). In normal physiological conditions, the expression of these ICPs (ICP) maintains the self-tolerance and prevents the host from autoimmunity in case of pathogenic infections, but tumor cells can deregulate expression of the ICP by immune cells through various mechanisms. One of the mechanisms includes the recruitment of Tregs and their activation in tumor microenvironment via interaction of tumor cells or immune cells and Tregs through the ICPs expression.

Along with cytokines expressed in the environment, Tregs also suppress the T cells and DCs by expression of ICP molecules on their surface. With the help of in vitro suppression assay, many molecules are discovered participating in cell to cell contact-dependent suppression.

One of the important negative regulator molecules among this is CTLA-4 (CD152). CTLA-4 is co-expressed with CD28 on the TCR engagement by T cells. CD28 and CTLA-4 share common ligands i.e. CD80 and CD86 (on APC) and it have been proposed that the CTLA-4 with stronger affinity to these ligands out-compete the CD28 interaction and thus induce inhibitory signal to T cells. Tregs in thymus and periphery constitutively express CTLA-4 whereas upon activation naïve Tregs may express CTLA-4 (Miyara, Sakaguchi 2007). Expression of CTLA-4 on peripheral Tregs is associated with the rapid homeostasis, and inhibition by soluble CTLA-4 (CTLA-4 Ig) leads to the decrease in the numbers of the Tregs and CTLA-4 expression in vivo (Tang et al. 2008). Blockade of CTLA-4 by Fab fragments of anti-CTLA-4 monoclonal antibody and experiment in CTLA-4 deficient mice shows abrogation of CD4+CD25+Treg-mediated suppression (Sakaguchi S. 2004, Read et al. 2006). It has been suggested that Tregs might interact with CD80 and CD86 molecules on APC via CTLA-4 and transduce a co-stimulatory signal to Tregs i.e. signals via both CTLA-4 and
TCR might interact and activate Tregs to exert suppression (Wing et al. 2008). Another possible role of CTLA-4 for Treg function is that it might trigger induction of the enzyme IDO in DC by interacting with their CD80 and CD86 (Onodera et al. 2009). Anti-CTLA-4 blockade on both effector cells as well as Tregs is found to be effective to improve the anti-tumor activity in melanoma (Peggs et al. 2009). It has been found that along with the suppression of the effector function and proliferation of the CD8+ T cells, Tregs participate in the effector to memory transition of CD8+ T cells through CTLA-4 signaling during LCMV viral infection in mice (Kalia et al. 2015).

Ivars and colleagues first reported that Tregs could down regulate the expression of co-stimulatory molecules CD80 and CD86 by DC in vitro (Lukas Cederbom, Håkan Hall and Fredrik Ivars 2000). Moreover, studies show that, LAG-3 may block DC maturation. Binding of LAG-3 to MHC-II molecules (Liang et al. 2008) expressed by immature DC induces an immunoreceptor tyrosine-based inhibition motif (ITIM)-mediated inhibitory signaling pathway which involves FCyR and extracellular signal regulated kinase (ERK)-mediated recruitment of SHP-1 that suppresses DC maturation and their immunostimulatory capacity (Vignali, Dario A A et al. 2008). Tregs from LAG3−/− mice showed reduced immunosuppression (Huang et al. 2004). Along with LAG-3, a molecule Neurophilin-1 promotes prolonged interactions with Tregs and immature DC (Sarris et al. 2008).

Programmed cell death -1 (PD-1; gene pdcd1) a molecule of immunoglobulin superfamily has been found a second promising molecule after CTLA-4 implicated in the immunotherapy for cancer patients. A CD28 family molecule and is expressed by activated T cells, B cells and NK cells. It binds to the ligands PD-L1 (CD274) and PD-L2 (CD273). PD-1 and PD-L1 interaction leads to the blocking of the stop signal after TCR ligation and thus promotes the tolerance (Fife et al. 2009). CTLA-4 blocks the activation of T cells whereas the PD-1 blocks the effector T cell functions in tumors and inflammatory tissues. PD-1 induces the cell death of antigen specific T cells and thus leading to the prevention of autoimmunity simultaneously reducing the apoptosis on the Tregs. PD-L1 expression by the cancer cells leads to the increase in the apoptosis of the effector T cells and thus thought to be an evasion mechanism manifested by the tumors to escape the immune responses (Chen et al 2002). PD-1
pathway blockade led to the reversal of exhaustion of the CD8+ T cells in LCMV infection (Barber et al. 2006). PD-L1 has showed important role in the Tregs development. PDL1−/−antigen presenting cells minimally induced the conversion of CD4+ T cells in iTregs. PD-L1 enhances the expression of the FoxP3 and suppression potential via down regulation of phosphorylation of Akt, mTOR and ERK2 and up-regulation of the PTEN which are key signaling molecules in the development of Tregs (Francisco et al. 2009).
Figure 7: Immune checkpoints in regulating T cell responses

Several co-stimulatory and co-inhibitory molecules are involved in the regulation of the T cell and APC interaction after the first TCR MHC interactions deliver the activating signal to the T cells. + In green circle represents the positive signal to the T cell or APC whereas – in red circle represents the negative signal to T cells.
Like PD-1, Tim-3 has been found as negative regulator molecule and is expressed on CD4+ and CD8+ T cells in infection and tumor. Interaction of Tim-3 on Tregs and Tim-3L on the T H1 cells (Wang et al. 2009) leads to the tolerance of auto and allo-immune responses in diabetes mice model (Sánchez-Fueyo et al. 2003). Expression of Tim-3 on activated CD4+ T cells lead to the negative regulation of cytokine production by Th1 and Th17 cells (Hastings et al. 2009). It has been found that Tim-3 expression on the CD4+ TIL in lung cancer is associated with the worst pathological parameters (Gao et al. 2012).

A recently emerging molecule TIGIT (expressed by T and NK cells) has been found to play important role in immune-regulation. The TIGIT/CD226 pathway has been discovered to be operational between the Tregs and DCs. This pathway acts similarly the CD28/CTLA-4 co-stimulatory pathway (Joller et al. 2011). TIGIT (T cell Ig and ITIM domain) is a trans-membrane glycoprotein which can bind with high affinity receptor CD155 on monocytes and CD11c+ human DC. TIGIT and CD226 share a common receptor CD155 and thus have a competition for the binding. It has been observed that TIGIT engagement with CD155 on DC leads to the IL-10 production by DC and diminished production of IL-12p40 (Yu et al. 2009). Ligation of TIGIT on Tregs induced the expression of fibrinogen-like protein 2 (Fgl2) (a molecule involved in the cytokine production by Th2) and inhibited pro-inflammatory responses by Th1 and Th17 (Joller et al. 2014). It is also observed now that Helios+ memory Tregs expressing TIGIT and FCRL3 are highly suppressive Tregs (Bin Dhuban et al. 2015; Fuhrman et al. 2015).

ICOS (CD278) is another CD28 super family member which enhances T cell responses to foreign antigen. ICOS was found to be expressed by the T cells which are closely associated with B cells in germinal centers (Hutloff et al. 1999). ICOS expression is considered to be important in CD4+ T cells for Th1 or Th2 polarization but not involved in CTL responses in viral infections (Kopf et al. 2000). Intermediate ICOS expression on the CD4+ T cells is associated with pro-inflammatory Th2 cytokines like IL-4, IL-5 and IL-13 whereas, ICOS high T cells are associated with the anti-inflammatory cytokine IL-10 (Lohning et al. 2003). ICOS-L expression by melanoma cells found to promote the activation of Tregs (Martin-Orozco et al. 2010). In tumor
microenvironment, ICOS-L expression by plasmocytoid DCs results into infiltration of Tregs and leads to immunosuppression (Faget et al. 2012b; Conrad et al. 2012).

Presence of TNF-α during the pathological consequences leads to the expression of TNF superfamily receptors on T cells especially Tregs. Neonatal administration of the anti-TNF-α increased the Tregs in NOD mice (McDevitt et al. 2002). In memory Tregs (CD45ra-) TNF-a induces NF-kB pathway in the Tregs but not in CD25- conventional T cells and leads to the expression of FAS, TNFR-2, 4-1BB and OX-40 on Tregs, which decreases their suppressive potential but the effect was reversed when the anti-TNFR2 mAB treatment was carried out during the in vitro cultures of T cells (Nagar et al. 2010). But contradictory studies also show that TNFR2 expression augments the Treg activity in ovarian cancer (Govindaraj et al. 2013) and their function in inflammatory responses (Hamano et al. 2011; Chen et al. 2013). Co-expression of GITR, OX-40 and TNFR2 along with TCR signaling has been found to favor the thymic differentiation of Tregs (Mahmud et al. 2014).

CD40-CD40L interaction helps primary CD8+ T cell responses via several mechanisms. Engagement of CD4+ T cells to DC via CD40-CD40L licenses antigen presentation potential to DC for activation of the CD8+ T cells (Bennett et al. 1998). Memory T cell generation is dependent on the CD40 and ligand interaction (Bachmann et al. 2004; Shugart et al. 2013).CD40L has been found to be an important molecule in CD8+ T cells, involved in the overcoming Tregs mediated tolerance during the viral infections (Ballesteros-Tato et al. 2013), and tumorigenesis (Soong et al. 2014) and thus, has been considered as promising tool in immunotherapy.

OX-40 is another co-stimulatory molecule involved in the maintenance of the long-term memory T cells. It was found that OX-40/- T cells produced less IL-2 and Bcl-2 and undergoes apoptosis after 2-4 days of activation. Thus, OX-40 was considered as important signal, as CD28 in T cells (Rogers et al. 2001). OX-40L deficient DCs are found to be defective in inducing co-stimulatory signal to T cells (Chen et al. 1999).OX-40-induced Survivin plays important role in clonal expansion of T cells (Song et al. 2005). Surprisingly the expression of OX-40 was found to have opposite
effect on the Tregs. It is found that Ox-40 expression on Tregs turns off their ability to suppress T cell proliferation, IFN-γ production (Vu et al. 2007) and facilitates the tumor rejection (Piconese et al. 2008).

Role of 4-1BB (CD137) in Treg immunity is unclear. Tregs express 4-1BB in response to the IL-2 and CD28 (Elpek et al. 2007b). It has been found that signaling via 4-1BB pathway inhibits the suppressive function of Tregs (Choi et al. 2004).

In addition to CTLA-4, GITR is constitutively expressed in Tregs in higher levels than other T cell subsets, although activated T cells also express GITR (McHugh et al. 2002). GITR ligand is expressed on mouse and human mature DC and pDC and it enhances the immunostimulatory function of DC but do not affect the suppressive function of the Tregs(Tuyaerts et al. 2007). GITR-L expression on CD25- T cells at initiation of immune response renders the resistance to immune regulation but, down-regulation of GITR-L by inflammatory stimuli may enhance the susceptibility of T cells to suppressor activity (Stephens et al. 2004).

In summary, Tregs require the co stimulatory molecules after TCR ligation to become fully functional. As, the immunosuppressive ability of Tregs is considered favorable for autoimmunity prevention and their immunosuppression capacity in the tumor microenvironment is considered as a curse, in the same manner, phenomenon of their ICP expression can be also considered as good or bad in different pathological contexts. Expression of the immune-co-stimulatory and regulatory molecules by Tregs in chronic inflammation like tumors has been and is still being studied largely. It should be noted that none of these pathways only affect the Treg function; simultaneously they also promote or attenuate the effector T cell functions. Nevertheless, the discovery of ICP expression on T cells uncovered the vast field of tumor immunology and is proving to be a phenomenal way to treat patients with immunotherapies. The several ICP markers exploited in the immunotherapy trials in cancer patients is discussed further in the “Tregs and immunotherapy: Blessing in disguise?”

2.4. How many mechanisms do Tregs need? Treg plasticity
Presence of Tregs in the pathogenic and inflammatory conditions and its positive or negative consequences depends on the phenotypic and functional status of Tregs in these situations. Since Tregs are flexible with the expression of repertoire of the molecules shared with the other CD4+ T cells; it reflects their functional potential in vivo. Considerable research has been carried over the past few decades in understanding the molecular basis underlying the immune regulation by Tregs. But still the questions remains that how many mechanisms are involved at the same time in the tolerance mediated by Tregs? Consequences of autoimmunity arise due to disruption of any one or more than one immunosuppression mechanism(s)?

This suggests that either key mechanisms of immunosuppression have yet to be identified or multiple mechanisms work in the concert to mediate Treg function. It is observed that in absence of IL-10/IL-35, the Tregs are still functional in vitro and in vivo and express cathepsin E (CTSE) which is required for expression or release of TNFR member TRAIL, mediating apoptosis (Pillai et al. 2011). This suggests that loss of certain regulatory mechanisms may result into forced molecular changes that are compensated by “switch on” inhibitory mechanisms. Secondly, it also suggests the existence of cross regulatory pathways which may operate in utilization of certain immunosuppression mechanisms. Collectively, it may serve to facilitate Treg plasticity (Sawant, Vignali, Dario A A 2014).

In most scenarios, the primary mechanism employed by the Tregs depends on the disease, the target cell type, the local inflammatory environment and anatomical location. It is considered now that Tregs are not terminally differentiated but have the developmental plasticity to differentiate into special subsets with their local milieu for effective control of immune regulation. This notion is synonymous with the observation that FoxP3 encodes the expression of the core Treg suppressor module (increased CD25, CTLA-4 expression) while their adaptability to the changing environment leads to induction of additional suppressive modules (transcriptional factors, miRNA, suppressive pathways) for optional regulation (Wing, Sakaguchi 2012).
It is observed that Tregs undergo differentiation in parallel with effector T cells. This indicates that Tregs exhibit functional specialization in periphery by adapting the transcriptional program of specific effector T cells they suppress (Duhen et al. 2012). The T-bet$^+$ Tregs that potently inhibit Th1 cell responses are dependent on the transcriptional factor STAT-1 and occurred directly in response to IFN-γ produced by effector T cells (Koch et al. 2012). Similar to T-bet, expression of the Th2 differentiation factor, IRF-4 endows Tregs with ability to control Th2 responses (Zheng et al. 2009). The transcription factor GATA-3 is also highly expressed in the Tregs which may help Tregs to suppress Th2 cells (Wohlfert et al. 2011). Similar to Th1 and Th2, Th17 cells are regulated by the Tregs expressing STAT-3 (Hossain, Dewan Md Sakib et al. 2013). Also, co-expression of other Th17 transcription marker like RORγt is also reported in humans (Ayyoub et al. 2009). Expression of T$_{FH}$ transcription factor Bcl-6 in Tregs has been demonstrated to be essential for Tregs control of germinal center responses (Chung et al. 2011a). Thus, variable expression of transcriptional markers defines functionally specialized sub-phenotypes of Tregs that each control distinct immune responses.

Along with transcription factors, several other mediators of the immune regulation like microRNA, chemokine receptors, and cytokines are used by Tregs to compensate for loss of key modules. Additional studies will clearly be required to determine the prevalence of Treg functional plasticity caused by divergent genetic backgrounds and/or altered environmental circumstances (Cretney et al. 2013).

2.5. Infiltration, differentiation and activation of Tregs in tumor microenvironment

2.5.1. Infiltration of Tregs in tumor microenvironment

Migration of Tregs in tumor microenvironment is well documented in the literature. There are several ways by which Tregs can migrate to tumor microenvironment. The CCL17 and CCL22 produced by plasma cells and macrophages attract the T helper and importantly Tregs in the tumor microenvironment. Major sources of CCL17 and
CCL22 like tolerogenic DCs, cells and TAM’s can be found in different tumor microenvironments. Expression of CCL17 and CCL22 in tumors is thought to be responsible for the strong influx of Tregs in several tumor models. In CT26 colon carcinoma mouse model, the CCL17 gene therapy leads to the recruitment of Tregs and thus tumor regression (Kanagawa et al. 2007). When CCR4 antagonist was used in tumor bearing mice, the CD44 \textsuperscript{hi} \text{ICOS}^{+} Tregs were targeted with this treatment, while increasing the antigen specific CD8+ T cell accumulation (Pere et al. 2011).

In human tumors also it is observed that Tregs are recruited in tumor microenvironment via CCL17/CCL22 and CCR4 interaction. Expression of the CCR4 and CCR8 allows Tregs to competitively bind the APC over the conventional T cells and thus affect the activation of the conventional T cells and it is found that CCR8 is more restricted to the Tregs (Iellem et al. 2001). Not only CCR7 but the CCR8 and CXCR4 has also been found to be important for trafficking of the Tregs to the tumors (Wang et al. 2012). Blockade of CCL1 by CpG-ODN and anti-CCL1 led to the reduced number of the Tregs and CD8+ T cells mediated rejection of tumors in mice (Hoelzinger et al. 2010). In pancreatic cancer, disruption of the CCR5 mediated infiltration of Tregs leads to reduced growth of tumors in mice (Tan et al. 2009). IL-6 induced CXCR1 was found to be important in IL-8 expressing lung cancers and melanoma (Eikawa et al. 2010).

Expression of ICOSL and OX-40 by pDC is responsible for recruitment of Tregs in melanoma and breast cancer (Faget et al. 2012a; Aspord et al. 2013). IL-27 produced by DCs also seems to be important for recruitment of Tregs in mouse tumor models (Xia et al. 2014).

Along with the immune cells, the molecules expressed by the tumor cells recruit Tregs in tumor microenvironment. It has been found that VEGF and receptors pathway is involved in the recruitment of the Tregs in tumors. CD4+FoxP3+ Tregs express VEGFR2 and produced high levels of TGF-\beta (Suzuki et al 2010). In CT-26 bearing colon cancer mouse model, anti-VEGF A and Sunitinib (tyrosine kinase receptor inhibitor) treatment remarkably decreased the Tregs in spleen and tumor (Terme et al. 2013).
2.5.2. Expansion and activation of Tregs

In tumor microenvironment, several cues are responsible for the differentiation and activation of Tregs apart from the TCR/CD28 signaling. Besides recruitment via chemotactic gradients, the tumor microenvironment promotes the expansion of nTregs as well as the generation of iTregs in situ due to the abundance of IL-10, TGF-β and adenosine which is produced by both tumor cells and MDSC (Rabinovich et al. 2007). CD40 and IL-4 produced by MDSC also help the recruitment and proliferation of Tregs in tumor microenvironment (Pan et al. 2010). Up-regulation of IDO by APC has been reported to activate Tregs and promote their proliferation (Sharma et al. 2007). CD80 and CD86 by CTLA-4 constitutively expressed on Treg increases the functional activity of IDO by DC forming a positively feedback loop. IL-2 produced by NK cells and T cells in tumor microenvironment seems to be nourishing Tregs and their expansion in tumor microenvironment (Martin et al. 2010). In IL-2−/− or CD25−/− foxp3 GFP knock-in allele mice, it was observed that IL-2 signaling was required for maintenance of the expression of genes involved in regulation of cell growth and metabolism (Fontenot et al. 2005a). It is now showed in mice that in SLO, Tregs gain access through expression of CCR7 whereas the CCR7lo Tregs localize in the non-lymphoid tissues and are insensitive to IL-2 blockade and continue signaling through ICOS (Smigiel et al. 2014). Treatment with IL-2 commonly used for melanomas has found to be expanding the ICOS+ Treg expansion (Sim et al. 2014). TGF-β, an autonomous regulator of tumor initiation, progression, immune escape and metastases in epithelial cells has been observed to play a central role for peripheral expansion of Tregs (Yang et al. 2008). Tumor cells capable of producing TGF-β and in addition can modulate MDSC’s and immature DC to become major sources of TGF-β (Huang et al. 2006).

2.5.3. Antigen specificity of Tregs in cancer

Tregs have an important immuno-pathological role in human cancer by lowering the TAA-specific T cell immunity contributing to tumor growth. First target antigen of Tregs to be reported in human was LAGE-1, a family member of NY-ESO-1. It was
described as a candidate for direct recognition by Tregs from clones derived from TILs of melanoma patients (Wang et al. 2004). Targets of Tregs are generally believed to be self-antigens which may require to be expressed in the thymus. Tumor antigens are mostly aberrantly expressed by normal cells or so called oncofetal antigens which are self-antigens that are normally expressed in epithelial cells as well; it is likely that small part of Tregs generated in the thymus is specific for these tumor-associated antigens. Tregs specific for self-antigens LAGE-1 and ARTC-1 are present among melanoma-infiltrating lymphocytes. These Tregs have a phenotype similar to thymus-derived Tregs in terms of FoxP3, GITR, CTLA-4 and CD25 expression and cytokine production (Wang et al. 2004; Wang et al. 2005).

Most recently, Tregs specific for melanoma antigen gp100, TRP-1, NY-ESO-1 and Surviving were revealed in peripheral blood of melanoma patients (Vence et al. 2007). Although it is likely that the Tregs are educated in the thymus, the Tregs generated in periphery cannot be excluded. The nature of tumor-specific antigens (TSA) dictates that TSA-specific Tregs must be induced in periphery.

All these examples suggest that tumor-infiltrating Tregs may recognize self as well as foreign proteins expressed by tumor cells. And both of these types render the tumor specific tolerance.

2.6. Tregs in cancer: ambiguity in prognostic importance

Although Tregs act as regulators of the harmful inflammatory responses in autoimmune conditions, their role of in cancer is a matter of debate. There are several examples in the literature which demonstrate this ambiguity in predicting clinical outcome of the cancer patients.

Many publications reported that the density of CD4\(^+\)FoxP3\(^+\) T cells is associated with short-term survival of cancer patients, whereas others reported an absence of correlation with clinical outcome. High number of FoxP3\(^+\) Tregs is associated with the improved overall survival in follicular lymphoma (Carreras et al. 2006), in ER\(^-\)
breast cancer (West et al. 2013). In case of Head and neck cancer, tumor-infiltrating Tregs were found to be associated with good clinical outcome possibly because of controlling the harmful inflammatory reaction which otherwise may lead to tumor progression (Badoual 2006). Whereas, recruitment of Tregs in ovarian carcinoma (Curiel et al. 2004), hepato-cellular carcinoma (Gao et al. 2007), NSCLC stage I patients (Petersen et al. 2006), prostate cancer (Knutson et al. 2006) is associated with poor clinical outcome of patients. In some specific cases, the prognostic value of Tregs is dependent on infiltration in different areas of the tumors. For example, the high density of Ti-Treg in lymphoid aggregates is related with higher risk of relapse and a shorter relapse-free survival whereas the presence of Ti-Treg within tumor beds is not associated with the clinical outcome in primary breast cancers (Gobert et al. 2009). Surprisingly, there are also cases in which Tregs are not associated with the clinical outcome of the cancer patients. For example, in glioblastoma patients, Tregs do not have strong prognostic significance (Jacobs, Joannes F M et al. 2010; Heimberger et al. 2008). In anal squamous cell carcinoma, Tregs were not associated with clinical outcome of patients (Grabenbauer et al. 2006).

Several factors can be considered responsible for these discrepancies. An important issue can be the inability to identify individual functional Tregs easily. Only subsets of the T cells identified as CD4+CD25+ and are functionally suppressive Tregs. Remaining are mostly activated effector T cells. Confirmation of analysis of Tregs needs more markers other than FoxP3 like CTLA-4, GITR which are also expressed by the activated effector cells (Shevach 2002; Zou 2006; Bach, François Bach 2003). Devoid of the strong identity marker or unique set of markers makes it unable to detect the exact effect of the Tregs on clinical outcome of cancer patients. Markers CD25 and FoxP3 which are known to be expressed by Tregs, are also expressed in lower levels by the conventional CD4+ T cells upon activation. Based on the expression of the markers, the subset of Tregs may have different impact on the prognostic importance. The coexistence of the Tregs and PD-1+ TILs was found to be associated with poor survival in breast cancer (Ghebeh et al. 2008). High expression of PD-1 on lymphocytes and FoxP3+ Tregs has found to be deleterious for the patients with ccRCC (Kang et al. 2013).
Second, the function of Tregs seems to be different according to the cancer type. Tregs are thought to be preventing harmful inflammatory reaction and cancer progression and thus are responsible for good prognosis but in ovarian carcinoma Tregs are linked to the suppression of anti-tumor immune response and thus account for poor survival of patients (Curiel et al. 2004). Location in the tumor tissue and stage of cancer are also important factors determining the prognostic importance of Tregs in cancer. This can be observed in early-stage B cell lymphoma (Elpek et al. 2007a), advanced stage ovarian carcinoma (Leffers et al. 2009), early stage NSCLC patients (Petersen et al. 2006).

The prognostic importance of Tregs also seems to be dependent on the ratio with different T cells which is reasonable because, subset of CD8+ T cells also express transcription factor FoxP3 as in CD4+CD25+Tregs (Cosmi 2003). It has observed that high ratio of CD8+ T cells/Tregs is associated with good clinical outcome in ovarian cancer patients (Eiichi Sato et al. 2005). But in another study with serious ovarian cancer, it was found that less ratio of CD8+ T cells to the CD4+ FoxP3- cells and less ratio of CD8+ T cells to the FoxP3+ cells was associated with poor clinical outcome (Preston et al. 2013). In osteosarcoma, patients with high CD8/FoxP3 ratio have high median survival compared to the low CD8/FoxP3 ratio (Fritzsching et al. 2015).

Thus, it is very important to take into consideration all the different parameters while interpreting the prognostic importance of Tregs in different cancer settings. Table 5 summarizes the several studies carried out in the literature regarding the prognostic importance of Tregs in different cancer types:
<table>
<thead>
<tr>
<th>Type of Cancer</th>
<th>Histological type</th>
<th>Phenotyp e</th>
<th>Stage</th>
<th>No. of patients</th>
<th>Prognosis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophageal cancer</td>
<td>Esophageal cancer</td>
<td>CD4+CD2 Shigh</td>
<td>I to IV</td>
<td>42</td>
<td>Poor</td>
<td>(Kono et al. 2006)</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>NSCLC</td>
<td>FoxP3+</td>
<td>I</td>
<td>64</td>
<td>Poor</td>
<td>(Petersen et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>NSCLC (chemotherapy treated)</td>
<td>FoxP3+</td>
<td>III and IV</td>
<td>159</td>
<td>Poor</td>
<td>(Liu et al. 2012b)</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>NA</td>
<td>FoxP3+</td>
<td>II and III</td>
<td>967</td>
<td>Good</td>
<td>(Salama et al. 2009a)</td>
</tr>
<tr>
<td></td>
<td>CRC</td>
<td>FoxP3+</td>
<td>I to III</td>
<td>94</td>
<td>Good</td>
<td>(Suzuki et al. 2010)</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>NA</td>
<td>CD4+CD2 Shigh (Flow cytometry)</td>
<td>I to III</td>
<td>72</td>
<td>Poor</td>
<td>(Kono et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>Radically resected</td>
<td>FoxP3+</td>
<td>I to III</td>
<td>110</td>
<td>Poor</td>
<td>(Perrone et al. 2008)</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>Pancreatic ductal adenocarcinoma</td>
<td>FoxP3+</td>
<td>I to IV</td>
<td>198</td>
<td>Poor</td>
<td>(Hiraoka et al. 2006)</td>
</tr>
<tr>
<td>Liver cancer</td>
<td>Hepatocellular carcinoma</td>
<td>FoxP3+</td>
<td>I to III</td>
<td>323</td>
<td>Poor</td>
<td>(Kobayashi et al. 2007)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>breast cancer (chemotherapy treated)</td>
<td>FoxP3+</td>
<td>I lillatolic</td>
<td>101</td>
<td>Poor</td>
<td>(Demir et al. 2013)</td>
</tr>
<tr>
<td></td>
<td>ER- breast cancer</td>
<td>FoxP3+</td>
<td>Advanced</td>
<td>175</td>
<td>Good</td>
<td>(West et al. 2013)</td>
</tr>
<tr>
<td>Breast tumors</td>
<td>FoxP3+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>Ovarian carcinoma</td>
<td>FoxP3+</td>
<td>I-III</td>
<td>99</td>
<td>Poor</td>
<td>(Wolf et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>Ovarian carcinoma</td>
<td>CD4+CD2 5+FoxP3+ (IFC)</td>
<td>II to IV</td>
<td>104</td>
<td>Poor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ovarian carcinoma</td>
<td>CD4+CD25 &quot;FOX3&quot; (IFC)</td>
<td>NA</td>
<td>52</td>
<td>Poor</td>
<td>(Preston et al. 2013)</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>Cervical cancer</td>
<td>NA</td>
<td>I to II</td>
<td>115</td>
<td>Poor</td>
<td>(Jordanova et al. 2008)</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>Prostate cancer</td>
<td>FoxP3+</td>
<td>Advanced</td>
<td>1463</td>
<td>Poor</td>
<td>(Flammiger et al. 2013)</td>
</tr>
<tr>
<td>Kidney cancer</td>
<td>Clear cell Renal cell carcinoma</td>
<td>FoxP3+</td>
<td>I to IV</td>
<td>125</td>
<td>Peritumora-Poor; Tumor beds no association</td>
<td>(Li et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>CRCC</td>
<td>CD25+Fox P3+ (flow cytometry)</td>
<td>NA</td>
<td>30</td>
<td>Poor</td>
<td>(Liotta et al. 2011)</td>
</tr>
<tr>
<td>Melanoma tumors</td>
<td>Cutaneous malignant melanoma</td>
<td>FoxP3+</td>
<td>I to IV</td>
<td>97</td>
<td>No association</td>
<td>(Ladányi et al. 2010)</td>
</tr>
<tr>
<td>Brain tumors</td>
<td>Glioma</td>
<td>FoxP3+</td>
<td>II to IV</td>
<td>135</td>
<td>No association</td>
<td>(Heimberger et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>All brain tumor types including Glioblastoma</td>
<td>FoxP3+</td>
<td>I to IV</td>
<td>83</td>
<td>All tumor types- Poor; Glioblastoma-no association</td>
<td>(Jacobs, Joannes F M et al. 2010)</td>
</tr>
<tr>
<td>Head and Neck cancer</td>
<td>HNSCC</td>
<td>CD4+CD2</td>
<td>I to IV</td>
<td>84</td>
<td>Good</td>
<td>(Badoual et al. 2006)</td>
</tr>
</tbody>
</table>
Table 5: Study of prognostic importance of Tregs in types of cancers

<table>
<thead>
<tr>
<th>Type</th>
<th>Subtype</th>
<th>FoxP3+ (Flow cytometry)</th>
<th>I to IV</th>
<th>FoxP3+ Tumor Cells</th>
<th>FoxP3+ Til-</th>
<th>FoxP3+</th>
<th>I to IV</th>
<th>No association</th>
<th>Poor</th>
<th>Good</th>
<th>(Year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNSCC</td>
<td>CD45RA- FoxP3high</td>
<td>I to III</td>
<td>112</td>
<td>No association</td>
<td>Poor</td>
<td>(Sun et al. 2014)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anal cancer</td>
<td>Anal squamous cell carcinoma</td>
<td>FoxP3+</td>
<td>I to IV</td>
<td>FoxP3+ Tumor Cells</td>
<td>FoxP3+ Til-</td>
<td>Good</td>
<td>(Grabenbauer et al. 2006)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectal cancer</td>
<td>NA</td>
<td>FoxP3+</td>
<td>I to IV</td>
<td>495</td>
<td>Good</td>
<td>(Reimers et al. 2014)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>Urinary bladder cancer</td>
<td>FoxP3 expression on UBC cells and lymphocytes</td>
<td>I to IV</td>
<td>37</td>
<td>FoxP3+ Tumor Cells-Poor- FoxP3+ Til-Good</td>
<td>(Winerdal et al. 2011)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphoma</td>
<td>DLBCL</td>
<td>CD4+ FoxP3high CD25+ (Flow cytometry)</td>
<td>NA</td>
<td>27</td>
<td>Good</td>
<td>(Glowala-Kosińska et al. 2013)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DLBCL</td>
<td>FoxP3+</td>
<td>NA</td>
<td>96</td>
<td>Good</td>
<td>(Lee et al. 2008)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Follicular</td>
<td>FoxP3+</td>
<td>NA</td>
<td>97</td>
<td>Good</td>
<td>(Carreras et al. 2006)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. Tregs and immunotherapy: a blessing in disguise?

Chemotherapy has been considered as a common way of treatment for cancer patients so far, but, it has come with its price by causing various toxic side effects. Since many decades, efforts are done to use cellular or humoral elements of immune system as a tool for fighting against cancer. Immunotherapy has shown promising results by increasing the overall and disease-free survival in various cancer types. A2011 FDA approval to the ipilimumab (humanized CTLA-4 antibody) in treatment of the metastatic melanoma patients has showed that this field is coming to its adolescence phase (Hodi et al. 2010). Ipilimumab treatment improved the overall survival of the stage III and IV melanoma patients which were ineligible for the surgical resection, although few patients suffered with the side effects like severe colitis. It was observed that anti-CTLA-4 treatment leads the high IFN-γ producing NY-ESO specific CD4⁺ICOS⁺ hi T cells and altered the effector T cell to Treg ratio which can be beneficial to patients by generation of the anti-tumor response (Liakou et al. 2008). ICOS expression on CD4⁺ cells has been found to be beneficial (Fan et al.
ICOS expression leads to PI3K signaling important for T-Bet expression by T cells during anti-CTLA-4 therapy (Chen et al. 2014).

Increasing the efficacy of immune response and limiting the potential immunosuppressive factors involved in the successful immune response is the key agenda of the immunotherapy in cancer treatment. Since Tregs are thought to be suppressing immune responses in many solid cancers, reverting this effect by using immunotherapeutic mode is considered to be promising way. Modulating the surface or intracellular molecules expressed by the Tregs has been tasted in many immunotherapy targets. Exploitation of these molecules for depletion, targeting proliferation, activation and function of Tregs and disrupting their intratumoral homing is being tasted since several decades in many cancer types. Monoclonal antibodies are serving a great purpose for this. Molecules like CTLA-4, GITR, IL-10 and TGF-β which are predominantly produced by Tregs are exploited for the various immunotherapy trials.

Success of CTLA-4 in melanoma led to the further use of the other checkpoints in melanoma and other cancers also. Anti-PD-1 (Brahmer et al. 2010; Lipson et al. 2013; Ansell et al. 2015; Topalian et al. 2014) and PD-L1 (Brahmer et al. 2012) have shown promising results in the Phase III trial in advanced metastatic melanoma, Hodgkin lymphoma and Phase II trial in RCC (Motzer et al. 2015) and anti-PD-1 has been approved by FDA for treatment in the metastatic melanoma. LAG-3 has been tested in phase III trial for breast cancer. Use of other inhibitory checkpoints like B7-H3, B7-H4, BTLA-4, and Tim-3 are still in the preclinical phase of testing.

The different clinical trials going on using the monoclonal antibodies against different co-stimulatory and inhibitory molecules, cytokines and chemokines expressed by Tregs, in several phases from preclinical to the approval phase are listed in the table 6.

Regarding the co-stimulatory molecules from the TNFR superfamily members, the preclinical and phase I trials have given the good results, but therapeutic antibodies against some molecules has shown some toxic effects in patients. The Urelumab,
anti-CD137 (4-1BB) is one of the leading molecule which was being tested for melanoma but has stopped the phase II trials in 2009 due to toxic effects on liver with increased enzyme levels in patients treated (Gangadhar, Vonderheide 2014). Another drug is Lucatumumab (anti-CD40) which is being tested in phase I trials but has side effects with liver toxicity.

Figure 8: Molecules expressed by the Tregs used for the immunotherapy trials in cancer patients

The figure depicts the immune-co-stimulatory and inhibitory molecules (CTLA-4, PD-1, PDL1, Tim-3, LAG-3, and GITR), cytokines (IL-10, TGF-β) and other molecules like CD39 expressed by the Tregs and Tconv cells used for trials of the monoclonal antibody immunotherapy treatment in cancer patients. The Ipilimumab (anti-CTLA4) and Nivolumab and Pembrolimumab (anti-PD1) (high lightened in the blue color) has been approved by the FDA for the treatment in the melanoma patients whereas, the other antibodies are still being tested in the phase I or II clinical trials (high lightened in the red color).

Anti-TGFβ1, anti-IL10 and anti-IDO are also being tested in the phase I trials which can directly affect the Treg mediated immunosuppression (Lacouture et al. 2015;
Morris et al. 2014). Recruitment of Tregs by CCR4 chemokine receptor has been tried to block by the Mogamulizumab (anti-CCR4) in T cell leukemia/lymphoma and found to be promising in the Phase II trials in patients (Ogura et al. 2014).

In summary, using the mAb’s against surface molecules, cytokines and chemokine receptor expressed by Tregs can be useful in treating cancer patients. But, it should be considered their overall efficacy on long-term survival of patients and its impact on the immune environment modulation. It’s very important to take into account the balance between the ratio of the effector and regulator cells in the patients treated. The most important issue yet to be solved is the hefty cost of immunotherapy treatments (four cycles of the Ipilimumab treatment cost $120,000) and their toxic side effects on the patients. On one side, although controlling the negative regulation of immune response can be blessing for cancer patients it’s just a start of this voyage, we need to go still farther.

Not only monoclonal antibodies but the recombinant cytokines are also in trials to boost the functioning of the conventional T cells while inhibiting the proliferation of the Tregs in cancer. Since Tregs express high levels of the IL-2Rα (Takahashi et al. 1998), the injection of the IL-2 in patients did not benefitted and thus the phase II trials were stopped. Now the efforts are taken to construct the recombinant IL-2 (mutein IL-2) which will activate the T cells and NK cells in tumors but not boost the Treg proliferation (Carmenate et al. 2013). Pegylated IL-10 (PEG-IL-10) has found to be increasing the number of the CD8+ T cells, IFN-γ production and expression of granzyme on the cells in the tumors (Mumm, Oft 2013).
<table>
<thead>
<tr>
<th>Target molecule</th>
<th>Commercial product description</th>
<th>Year</th>
<th>Type of cancer</th>
<th>status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Affecting activation of the Tregs and reverting exhaustion of effector T cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD25</td>
<td>PC-61 (Rat IgG1)</td>
<td>1999</td>
<td>Myeloma, sarcoma, leukemia</td>
<td>Preclinical</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Ipilimumab (humanized, IgG1)</td>
<td>2011</td>
<td>Melanoma</td>
<td>FDA approved</td>
</tr>
<tr>
<td></td>
<td>Ipilimumab (humanized, IgG1)</td>
<td>2014</td>
<td>NSCLC</td>
<td>Phase III</td>
</tr>
<tr>
<td></td>
<td>Tremelimumab</td>
<td>2015</td>
<td>mesothelioma</td>
<td>Phase II</td>
</tr>
<tr>
<td>PD-1</td>
<td>Nivolumab (humanized, IgG4)</td>
<td>2014</td>
<td>Metastatic melanoma, Lung carcinoma</td>
<td>FDA approved</td>
</tr>
<tr>
<td></td>
<td>Pembrolizumab (Humanized, IgG4)</td>
<td>2014</td>
<td>Metastatic melanoma</td>
<td>FDA approved</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2015</td>
<td>PDL1 positive NSCLC</td>
<td>Phase III</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2015</td>
<td>Microsatellite unstable tumors including Colorectal cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td></td>
<td>Pidilizumab (CT-011)</td>
<td>2015</td>
<td>Multiple myeloma</td>
<td>Phase I and II</td>
</tr>
<tr>
<td>PDL1</td>
<td>Atezolizumab (MPDL3280A)</td>
<td>2015</td>
<td>Metastatic NSCLC, prostate cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td></td>
<td>MEDI4736</td>
<td>2015</td>
<td>Metastatic NSCLC</td>
<td>Phase II</td>
</tr>
<tr>
<td>B7-H3</td>
<td>MGA217</td>
<td>2015</td>
<td>Refractory cancers including the renal carcinoma</td>
<td>Phase I</td>
</tr>
<tr>
<td>LAG-3</td>
<td>IMP321 (LAG-3 Ig fusion protein)</td>
<td></td>
<td>Breast cancer</td>
<td>Phase III</td>
</tr>
<tr>
<td></td>
<td>C9B7W (Rat IgG 1)</td>
<td>2012</td>
<td>Melanoma, colon adenocarcinoma, Fibrosarcoma</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Tim-3</td>
<td>RMT3-23 (Rat IgG2a)</td>
<td>2011</td>
<td>Carcinogen induced tumors</td>
<td>Preclinical</td>
</tr>
<tr>
<td>4-1BB (CD137)</td>
<td>Urelumab (humanized, IgG4)</td>
<td>2010</td>
<td>Melanoma</td>
<td>Phase II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2015</td>
<td>Colorectal cancer, head and neck cancer</td>
<td>Phase I</td>
</tr>
<tr>
<td>KIR</td>
<td>Lirilumab</td>
<td>2014</td>
<td>Multiple myeloma, NSCLC, solid tumors</td>
<td>Phase I and II</td>
</tr>
<tr>
<td>CD40</td>
<td>Dacetuzumab</td>
<td></td>
<td>Chronic lymphatic leukemia (CML), Metastatic Myeloma, non Hodgkins lymphoma</td>
<td>Phase I</td>
</tr>
<tr>
<td></td>
<td>Lucatumumab</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chilob 7/4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD27</td>
<td>Varilumab (CDX-1127, humanized IgG1)</td>
<td>2014</td>
<td>Leukemia, lymphoma</td>
<td>Preclinical</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2015</td>
<td>Solid cancers including colorectal cancer</td>
<td>Phase I</td>
</tr>
<tr>
<td>GITR</td>
<td>DTA-1 (Rat IgG2a)</td>
<td>2010</td>
<td>Fibrosarcoma</td>
<td>Preclinical</td>
</tr>
<tr>
<td></td>
<td>MK-4166</td>
<td>2015</td>
<td>Cervical cancer</td>
<td>Phase I</td>
</tr>
<tr>
<td></td>
<td>TRX518</td>
<td></td>
<td>Advanced cancers</td>
<td></td>
</tr>
<tr>
<td>Ox-40</td>
<td>9B12 murine agonistic anti-human, IgG1</td>
<td>2013</td>
<td>Metastatic solid malignancies</td>
<td>Phase I</td>
</tr>
<tr>
<td></td>
<td>Anti OX-40</td>
<td>2015</td>
<td>Stage IV breast cancer</td>
<td>Phase I and II</td>
</tr>
<tr>
<td>Co-stimulatory and Co-inhibitory Molecules</td>
<td>Year</td>
<td>Indication</td>
<td>Phase</td>
<td></td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>------</td>
<td>------------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>MOXR0916, MEDI6383</td>
<td>2015</td>
<td>Locallay advanced and metastatic cervical cancer</td>
<td>Phase I</td>
<td></td>
</tr>
<tr>
<td>CD70</td>
<td>2015</td>
<td>Rencal cell carcinoma</td>
<td>Phase I</td>
<td></td>
</tr>
<tr>
<td>TGFβ1</td>
<td>2014</td>
<td>Advanced metastatic melanoma, RCC</td>
<td>Phase I</td>
<td></td>
</tr>
<tr>
<td>IDO</td>
<td>2015</td>
<td>Stage III-IV Epithelial ovarian, fallopian tube or primary peritoneal cancer</td>
<td>Phase I</td>
<td></td>
</tr>
<tr>
<td>Indoximod</td>
<td>2015</td>
<td>Breast cancer</td>
<td>Phase II</td>
<td></td>
</tr>
<tr>
<td>CCR4</td>
<td>2014</td>
<td>Acute T cell lymphoma /leukemia</td>
<td>Phase II</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>Solid cancers</td>
<td>Phase I</td>
<td></td>
</tr>
</tbody>
</table>

**Table 6: Co-stimulatory and co-inhibitory molecules, activating or blocking antibodies in anti-cancer treatment**

Abbreviation: KIR, killer cell immunoglobulin like receptor.
4. Lung cancer: a study model

Cancer is one of the threatening cause of death in economically developed countries and the second leading cause of death in developing countries. In 2012, 355000 new cases of cancer (excluding non-melanoma skin cancer) and 148000 deaths from cancer occurred in France (Binder-Foucard et al. 2014). Among these cases, lung cancer is most commonly diagnosed disease (Jemal et al. 2011). Worldwide, it severely affects males and fourth cause of death in females. It has been estimated that in 2008, 18% i.e. 1.4 million deaths occurred due to lung cancer. In France, with 30000 deaths occurred in 2012 of which 71% occurred in men, lung cancer caused highest number of deaths (Binder-Foucard et al. 2014).

![Figure 9: Age-standardized lung cancer incidence rates by sex and world area. Source: GLOBOCAN 2012.](image)
4.1. Etiology and histology of the lung cancer

The major cause of the lung cancer is found to be smoking. Eighty percent of the patients are smokers and 15% of the patients are long-term smokers (Dubey, Powell 2009). Variation in lung cancer rates among men and women within each country largely reflect differences in stage and degree of tobacco epidemic (Jemal et al. 2011). Although most lung cancers are a result of smoking, approximately 25% of lung cancer cases worldwide are not attributable to tobacco use. To the date, epidemiological studies have identified several environmental, genetic, hormonal and viral factors associated with lung cancer risk (Sun et al. 2007). Others include exposure to several occupational and environmental carcinogens such as asbestos, arsenic, radon and polycyclic aromatic hydrocarbons (Jemal et al. 2011).

Also, it is noteworthy that several genetic alterations are important cause of the lung cancer etiology. In non-small cell lung cancer (NSCLC), KRAF mutations and EGFR kinase domain mutations are associated with the adenocarcinoma (ADC) subtype, whereas p53 mutations are known to be associated with squamous-cell carcinoma (SCC). Over-expression of the c-MET is associated with the small cell lung cancer type (Herbst et al. 2008).

In 1924, Marchesani first proposed a classification system for lung cancer. He described four histological subtypes on which the current WHO classification system is still based: Squamous cell carcinoma, small cell carcinoma, large cell carcinoma, and adenocarcinoma. Two prominent types of lung cancer are NSCLC (85% of all lung cancer) and small cell lung cancer (SCLC) (15%of all lung cancer). NSCLC can be divided largely into three major histological subtypes: SCC (30%), ADC(31%) and large cell lung carcinoma (9%)(Beadsmoore, Screaton 2003).Smoking causes mostly small cell lung cancer and squamous cell carcinoma (Herbst et al. 2008). Adenocarcinoma further can be divided into adenosquamous carcinoma and bronchoalveolar carcinoma which comprise 2% and 2-10% of all lung cancers respectively.
Figure 10: Histological types of lung cancer.

A: hematoxylin-eosin staining shows the different histological subtypes of the lung cancer; (40x) and B: (20x) Adenocarcinoma; C: (40x) and D: (20x) Squamous cell carcinoma.

4.2. TNM classification and survival of patients

Definition of stage is essential for accurate diagnosis of patients with cancer and it has led to the development of a universally accepted classification systems in most solid tumors. The “Union Internationale Contre le Cancer” (UICC) and the “American Joint Committee on Cancer” (AJCC) officially define, periodically review and rectify the stage classification system. The seventh edition of TNM classification for lung cancer was published in 2009, which is illustrated in the following Table (Detterbeck et al. 2009b).
<table>
<thead>
<tr>
<th>Stage</th>
<th>Tumor</th>
<th>Lymph node invasion</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occult carcinoma</td>
<td>TX</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IA</td>
<td>T1a</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1b</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IB</td>
<td>T2a</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IIA</td>
<td>T2b</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1a</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1b</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2a</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td>IIB</td>
<td>T2b</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>IIIA</td>
<td>T1a</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1b</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2a</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2b</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td>IIIB</td>
<td>T1a</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1b</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2a</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2b</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td>IV</td>
<td>Any T</td>
<td>Any N</td>
<td>M1a or M1b</td>
</tr>
</tbody>
</table>

Table 7: TNM classification in human lung cancer (7th edition)
<table>
<thead>
<tr>
<th>TNM</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>No evidence of primary tumor.</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ.</td>
</tr>
<tr>
<td>T1</td>
<td>Tumor ≤ 3 cm in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus (i.e., not in the main bronchus).</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor &gt; 3 cm but ≤ 7 cm in greatest dimension, or tumor with any of the following features (T2 tumors with these features are classified T2a if ≤ 5 cm): involves main bronchus, ≥ 2 cm distal to the carina; invades visceral pleura (PL1 or PL2); or is associated with a telectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung.</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor &gt; 7 cm or one that directly invades any of the following: parietal pleural (PL3) chest wall (including superior sulcus tumors), diaphragm, phrenic nerve, mediastinal pleura, or parietal pericardium tumor in the main bronchus (&lt; 2 cm distal to the carina, but without involvement of the carina) or associated a telectasis or obstructive pneumonitis of the entire lung or separate tumor nodule(s) in the same lobe.</td>
</tr>
<tr>
<td>T4</td>
<td>Tumor of any size that invades any of the following: mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, carina, or separate tumor nodule(s) in a different ipsilateral lobe.</td>
</tr>
</tbody>
</table>

**Lymph node metastasis**

| NX  | Regional lymph nodes cannot be assessed. |
| N0  | No regional lymph node metastasis. |
| N1  | Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension. |
| N2  | Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s). |
| N3  | Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s). |

**Distant metastasis**

| M0  | No distant metastasis. |
| M1  | Distant metastasis. |
| M1a | Separate tumor nodule(s) in a contralateral lobe tumor with pleural nodules or malignant pleural (or pericardial) effusion. |
| M1b | Distant metastasis (in extrathoracic organs). |

*Table 8: TNM classification and characteristics of each stage*

The TNM classification applies to the four major histological types of lung cancer including both NSCLC and SCLC through simpler classification is commonly used in small cell carcinoma. The T component of classification represents the extent of the primary tumor both in terms of the size and local invasion. N describes regional LN
involvement. The M component describes the presence or absence of metastases (Beadsmoore, Screaton 2003).

Prognostic indicator of survivorship in lung cancer include tumor cell grade of differentiation, smoking cessation, dietary supplements, tumor molecular markers and pharmacogenomics and treatment outcomes. Outcome of lung cancer varies among patients even with a given histologic group that have the same stage at the time of diagnosis and who are treated in similar ways. People who are live 5 years after the diagnosis of primary lung cancer are referred as long-term lung cancer survivors (LTLC). Only 15% lung cancer patients in United States are LTLC (Molina et al. 2008).

4.3. Treatment of lung cancer patients

The standard treatment for lung cancer upto stage IIIB (stage IV is not operable) is surgical resection of the tumor. This concerns the patients with a tumor of early-stage (approximately 35% of patients) associated with low risk of metastasis. In complement to this, patients with tumor from stage II are treated with a platinum based chemotherapy; which is supposed to eliminate the proliferating tumor cells and generally used after tumor resection (it is called as adjuvant chemotherapy). In most countries, two drug therapy using platinum based chemotherapy drugs like carboplatin (Paraplatin), paclitaxel (Taxol), gemcitabine (Gemzar) or Vinorelbine (Navelbine). Stage II to IIIA NSCLC patients receive only adjuvant chemotherapy but stage IIIB NSCLC patients receive neoadjuvant chemotherapy and adjuvant chemotherapy treatment. Adjuvant chemotherapy after definitive surgery is the standard of care for patients with early-stage NSCLC. The international adjuvant lung trial (LALT) had demonstrated that adjuvant cisplatin based chemotherapy improved 5 year survival by 4%. Metastatic NSCLC patients are treated with a platinum based chemotherapy doublet regimen (carboplatin/paclitaxel). The standard of care for metastatic NSCLC is four to six cycles of platinum based chemotherapy with actual number of chemotherapy cycles based on the best response to chemotherapy (Dubey, Powell 2009). Along with adjuvant chemotherapy, there is another
treatment which is called neo-adjuvant chemotherapy, which is pre-surgical. The objective of this therapy is to reduce the tumor burden and facilitate the surgery.

Small cell lung cancer (SCLC) is well known to be exquisitely chemo responsive; the newly diagnosed patients are treated with platinum based chemotherapy regimens most commonly in combination with etoposide.

4.4. Era of combined therapies: promising for NSCLC

Chemotherapy treatments are used in early stages of cancer. Advanced NSCLC patients cannot be treated by surgical resection or chemotherapy treatments and so the alternative therapeutic approaches need to be implied, especially in the case of immunotherapies. The mouse model and the human studies with the combination of the chemo-immunotherapy have shown promising results. In a mouse model of lung carcinoma, it has been observed that depletion of Tregs by an anti-25 mAb treatment leads to the tumor cell death and improved CD8+ T cell mediated granzyme, perforin and IFN-γ production. The combination of the mAb and carboplatin treatment increased the survival of the mice comparably better than the single treatment (Ganesan et al 2013).

ICP blockade is showing promising results in treated NSLCL patients. The Phase II study, treatment with Ipilimumab (anti-CTLA-4) on 204 patients with advanced-stage NSCLC with chemotherapy alone or in combination with paclitaxel or carboplatin has shown the improvement in the PFS and OS is 1 and 3 months respectively (Lynch et al. 2012). The size of the tumor observed to be regressed and increased infiltration of lymphocytes was observed in NSCLC patients with improved survival when treated with Ipilimumab in combination with radiotherapy (Golden et al. 2013).

In case of PD-1, there are several studies going on to test the efficacy in the NSCLC. In phase I trial, out of 129 patients (24% patients) responded well to treatment with 3mg/kg of the Nivolumab (Brahmer et al. 2012) . In similar lines, 30% patients responded well (of 20 patients in cohort of Phase I trial) when treated with Nivolumab plus chemotherapy combination (Gettinger et al. 2015). In Phase II, in 117 patients with advanced squamous NSCLC, 15% of patients responded to the
3mg/kg dose of Nivolumab (Rizvi et al. 2015). The Pembrolizumab treatment in the Phase I trial has also showed the 19.2% patients response in NSCLC (Garon et al. 2015). In breast carcinoma, anti-LAG3 (IMP321) shows promising results in combination with paclitaxel in phase I trial, thus it is thought to be useful to test in the NSCLC patients also (Brignone et al. 2010).

Although the treatments have shown efficacy in cancer patients, some toxic side effects like fatigue, nausea, diarrhea and skin fissures are also observed in many patients. In summary, with the encouraging results of the immunotherapies, door of the personalized medicine has been opened for the NSCLC patients. It seems important to consider possible combinations of the chemo-immunotherapies which can increase the survival of the patients with less toxic side effects.
Hypothesis and objectives
B. Hypothesis and objectives

The density of immune infiltrate, especially CD8+ memory T cells, Th1 cells is associated with the good clinical outcome of the patients in many solid cancer types (Fridman et al. 2012). In contrast, the prognostic impact of the organization of this immune infiltrate in the tumor was not previously investigated. When I arrived in the team, it was already demonstrated by Dr. Marie-Caroline Dieu-Nosjean and colleagues, a first time evidence of the presence of TLS in certain NSCLC tumors in humans (Dieu-Nosjean et al. 2008). These structures are composed of T-cell zone rich with T lymphocytes adjacent to dense B-cell follicle. DC-LAMP+ mature DCs are selectively localized in the T cell zone consecutively a specific marker for the TLS for lung tumors. The B-cell zone, which consists of the CD21+ FDC and Ki-67 + proliferating B cells, is suggestive of the reactive germinal centre. PNAD+ HEV is the hallmark of the TLS responsible for the recruitment of the CD62L+ T cells and B cells and thus CD62L is a marker of TLS B and T cells (except germinal centre B cells). Lymphoid structures induced by tumors are the indicator of the anti-tumor immune response since it exists in the invasive margin of the tumors and not at the distance. Our team thus initially proposed the terminology “Ti-BALT” (Tumor-induced Bronchus associated lymphoid tissues) for TLS in NSCLC (Fig. 11).
Figure 11: Characterization of Ti-BALT in NSCLC.

(A, B) Presence of Ti-BALT (arrow in A or as limited by dashed line in B) in lung tumor section counterstained with hematoxylin and eosin. (C) DC-Lamp+ mature DCs (red) home exclusively into CD3+ T-cell clusters (blue). (D) Presence of adjacent CD20+ B (red) and CD3+ T (blue)-cell rich areas of Ti-BALT. (E) CD20+ B-cell follicle (red) is characterized by the presence of a CD21+ follicular dendritic cell network (blue). (F) Some CD20+ B-cell follicles (blue) contained Ki67+ proliferating germinal center B cells (brown). Original magnification: A, ×50; B, ×100; C, D, F, ×400; E, ×200. T, tumor nest. (Dieu-Nosjean et al. 2008)

Along with this, it was demonstrated that density of the DC-LAMP+ mature DC is a very good prognostic marker of overall survival (OS), disease specific survival (DSS) and disease free survival (DFS) for NSCLC patients (Dieu-Nosjean et al. 2008) (fig 12).
Figure 12: Evaluation of DC-Lamp as a marker of Ti-BALT, and its prognostic value.

Evaluation of DC-Lamp+ mature DC, CD20+ B cells and CD8+ T cells as a marker of tumor-induced TLS and its prognostic value. (A) Kaplan-Meier curves of disease-free survival for 74 patients with non-small-cell lung cancer depending on the density of tumor-infiltrating DC-Lamp+ mature DCs and (B) the pathologic TNM stage. (C) Kaplan-Meier curves of DSS for 74 patients with early-stage NSCLC depending on (c) densities of CD20+ follicular B cells (Foll-B), DC-Lamp+ mature DCs, and the combination of both cell populations. Kaplan-Meier curves of OS for 362 patients with early-stage NSCLC depending on (D) the density of tumor-infiltrating DC-Lamp+ mature DCs (E) the density of CD8+ T cells in stroma (F) the density of CD8+ T cells in tumor nest. The horizontal dashed lines represent the median survivals. $P$ values were determined using the log-rank test and corrected according to the formula proposed by Altman et al. (Dieu-Nosjean et al. 2008; Goc et al. 2014b; Germain et al. 2014a)
Further, it was also demonstrated in our team that, high density of mature DC correlated to a strong infiltration of Effector-memory T cells. Moreover, mature DC density correlated with expression of genes related to T-cell activation, T-helper 1 (Th1) phenotype, and cytotoxic orientation (Goc et al. 2014b).

These results suggest that NSCLC-associated TLS are involved in the generation of the protective anti-tumor response. TLS resembling SLO, are responsible for the recruitment from blood via HEV, differentiation and activation of the different immune cells especially T cells in the tumor microenvironment. A specific gene expression signature is associated with T cell presence in TLS. It includes chemokines (CCL19, CCL21, CXCL13, CCL17, CCL22, and IL16), adhesion molecules (ICAM-2, ICAM-3, VCAM-1, and MAdCAM-1) and integrins (alphaL, alpha4, and alphaD) (Chaisemartin et al. 2011).

Since the tumor microenvironment is highly immuno-suppressive and tumor cells overcome the immune response through recruitment of suppressive cells, it can be hypothesised that along with CD8+ T cells and Th subsets, suppressor cells like regulatory T cells are also recruited in the tumor microenvironment through chemokines and receptors like CCL17/CCL22 and CCR4 axis. Thus, based on these results, I hypothesised that:

Tregs exert various mechanisms to regulate the immune responses. It has been reported that Tregs can kill DC in tumor-draining LNs in order to limit the onset of CD8+ T-cell responses (Boissonnas et al. 2010). Moreover, Tregs can directly decrease effector T-cell functions. Thus, it is tempting to speculate that Ti-Tregs would exert distinct functions into distinct areas of the tumor, in particular in TLS, where they may interact with mature DC and naïve T cells versus elsewhere in the tumor, where they may encounter CTL. This may explain the discrepancy about the prognostic value of FoxP3+ cells by IHC in the literature. Thus, it is important to determine the functional status of Tregs in different areas of the lung tumor, where, in same environment, CD4+ T cell subsets with opposite functions can interfere in determining the prognosis of the cancer patients.
The main objectives of this study are as follows:

1. To study the phenotype of the Tregs infiltrating different areas of lung tumors and non-tumoral tissues from NSCLC patients.

For this, I determined the differentiation, activation, and immunoregulatory status of the Tregs by flow cytometry experiments fresh tumor specimen from patients treated without neoadjuvant chemotherapy (n=34) patients with advanced-stage lung cancer and treated with neoadjuvant chemotherapy (n=20). Expression of
several differentiation and activation markers were tested on the cells enriched from the fresh tumor specimens.

2. To study the expression of genes by tumor-infiltrating Tregs to decipher the functional status of Tregs in lung cancer

Gene expression studies carried out to study large panel of molecules like cytokines, chemokines and receptors, cytotoxic molecules, transcription factors, TCR signaling pathway molecules, based on the *in situ* localization of Tregs in TLS and non-TLS area of tumor. This was helpful to screen large panel of genes which may help to decipher exact phenotype of Tregs in lung tumors and also their functional potential. For this the four different subsets of Tregs (CD62L+ and CD62L-) and Tconv CD4+ T cells (CD62L+ and CD62L-) were sorted from the fresh tumor specimen (n=20).

3. To study the prognostic impact of Tregs in lung cancer patients

The role of Treg in cancer is a matter of debate. Many publications reported that the density of CD4+FoxP3+ T cells is associated with short-term survival of cancer patients, whereas others reported an absence, or even positive correlation with clinical outcome. Their localization into the tumor seems of importance.

In this study, I determined an impact of density of CD3+Foxp3+ Tregs on the clinical outcome of the patients. N=243 NSCLC patients from stage I to IV without neoadjuvant chemotherapy treatment were included in this study. With the help of immunohistochemistry staining for CD3/FoxP3 on paraffin-embedded NSCLC tumor tissues, I determined the density of CD3+FoxP3+ Tregs and CD3+ T cells. In parallel, I also measured the density of Tregs infiltrating the TLS using DC-Lamp, a specific marker of mature DC which home exclusively in these lymphoid structures (Dieu-Nosjean et al. 2008). The clinical outcome of NSCLC patients was determined by stratification of patients based on CD3+FoxP3+ Tregs density, mature DC density and CD8+ T cell density and TLS-B cells. For this the densities of the mature DC, CD8+ T cells and TLS-B cells was acquired from the different studies carried out by colleagues in the team.

C. Results
Results
Article << Regulatory T cells create immunosuppressive environment infiltrating the tumor induced TLS and impose a poor clinical outcome of patients in lung cancer>>
Regulatory T cells create immunosuppressive environment infiltrating the tumor induced TLS and impose a poor clinical outcome of patients in lung cancer

Priyanka Devi\textsuperscript{1,2,3}, Sylvain Leveugle\textsuperscript{4}, Jérémy Goc\textsuperscript{1,2,3}, Claire Germain\textsuperscript{1,2,3}, Samantha Knockaert\textsuperscript{1,2,3}, Diane Damotte\textsuperscript{1,2,3,5}, Audrey Mansuet-Lupo\textsuperscript{5}, Pierre Validire\textsuperscript{1,6}, Marco Alifano\textsuperscript{7}, Hélène Fohrer-Ting\textsuperscript{8}, Hélène Kaplon\textsuperscript{1,2,3}, Myriam Lawand\textsuperscript{1,2,3}, Sandrine Katsahian\textsuperscript{4}, Isabelle Cremer\textsuperscript{1,2,3}, Wolf Herman Fridman\textsuperscript{1,2,3}, Catherine Sautes-Fridman\textsuperscript{1,2,3}, and Marie-Caroline Dieu-Nosjean\textsuperscript{1,2,3*}

1, Laboratory “Cancer, Immune Control, and Escape”, INSERM UMR_S 1138, Cordeliers Research Center, F-75006, Paris, France

2, Sorbonne University, Pierre and Marie Curie University Paris 06, UMR_S 1138, Cordeliers Research Center, F-75006, Paris, France

3, Paris Descartes University, Sorbonne Paris Cité, UMR_S 1138, Cordeliers Research Center, F-75006, Paris, France

4, Laboratory “Information sciences to support personalized medicine”, INSERM UMR_S 1138, Cordeliers Research Center, F-75006, Paris, France

5, Department of Pathology, Groupe hospitalier Cochin-Hôtel Dieu, AP-HP, F-75006, Paris, France

6, Department of Pathology, Institut Mutualiste Montsouris, F-75006, Paris, France
7, Department of Thoracic Surgery, Groupe hospitalier Cochin-Hôtel Dieu, AP-HP, F-75006, Paris, France

8, Center of the cellular imaging and flow cytometry, INSERM UMR_S 1138, Cordeliers Research Center, F-75006, Paris, France

* Corresponding author:

Dr. Marie-Caroline Dieu-Nosjean

Laboratory “Cancer, Immune Control, and Escape”

INSERM UMR_S 1138

Cordeliers Research Center

15, rue de l’Ecole de Médecine

F-75006 Paris Cedex 06

France

Phone: +33-1-44-27-90-86

Fax: +33-1-44-27-81-17

E-mail: mc.dieu-nosjean@crc.jussieu.fr
Abstract

Introduction: It has been established that the immune system plays an important role in tumor rejection. The density of the immune cells with effector functions is related with good clinical outcome. Our lab has previously shown that the presence of TLS is associated with good clinical outcome in lung cancer patients. TLS composed of mature DC/T-cell clusters adjacent to B-cell follicles are centers of an on-going immune response. To escape immune response, tumor can develop different mechanism to regulate the immune system by the means of regulatory T cells (Tregs). The prognostic importance of Tregs in different tumor types has found to be heterogeneous, and they can use different strategies to immunosuppress immune cells. Thus, it becomes important to decipher the role and mechanisms of Tregs in human cancers. The goal of this study was to analyze their stage of differentiation, the expression of activation, immunosuppressive and immune checkpoint molecules (n= 55 patients), and correlation with clinical outcome (n=243 patients) according to their spatial localization in lung tumor including TLS and distant sites.

Results: Tregs migrate to different areas of tumor particularly in stroma and TLS, and rarely in tumor nests. Tregs exhibit mainly central and effector memory phenotype and express vast repertoire of activation markers and immune checkpoint molecules. In general, Tregs show similar gene expression in TLS and non-TLS areas but show remarkably different gene expression phenotype than the CD4+ T conventional cells. The high density of Tregs in whole tumor i.e. TLS and non-TLS is associated with the poor clinical
outcome of NSCLC patients. The combination of Treg with TLS-immune cells (mature DC or B cells) or CD8+ T cells provides an appropriate overall survival discrimination, as compared to each variable alone.

**Conclusion:** Tregs are present in different areas of tumor, and therefore are in contact with different types of immune cells. Expression of the activation and immune checkpoint molecules suggest their potential capability to suppress the anti-tumor responses. High density of Tregs is associated with the reduced survival of lung cancer patients, and the combination of Tregs with TLS cells (DC or B cells) or effector CD8+ T cells allows the identification of NSCLC patients with the highest risk of death.

**Keywords:**

Regulatory T cell, tumor microenvironment, tertiary lymphoid structure, differentiation stage, functional orientation, immune checkpoint, biomarker, dendritic cell

**Abbreviations:**

Introduction

The tumors are sustained by a complex network of interactions between cell components like tumor, stromal, and immune cells. The immune system can detect the tumor cells, and plays an important role in tumor control (Schreiber et al. 2011). The composition and density of intra-tumoral immune cells are highly heterogeneous from patient to patient, and have influence on the disease outcome of cancer patients (Pagès et al. 2010; Fridman et al. 2012). Not only the infiltration but the organization of cells in tumor microenvironment in form of tertiary lymphoid structures (TLS) is a major phenomenon for the long-term survival of cancer patients (Dieu-Nosjean et al. 2008; Dieu-Nosjean et al. 2014; Germain et al. 2015). In particular, tumor-associated TLS may represent a privileged site for T cell differentiation and activation. A high density of TLS is associated with a Th1 and cytotoxic immune signature in lung (Goc et al. 2014b), breast (Gu-Trantien et al. 2013b) and gastric cancers (Hennequin et al. 2015), indicating that TLS may imprint the local immune microenvironment.

To escape immune responses, tumor can develop several mechanisms to regulate the immune system via for instance the recruitment of regulatory T cells (Tregs) (Ghiringhelli 2005). In human tumors, Tregs are recruited via CCL17/CCL22 and CCR4 interaction (Mizukami et al. 2008; Qin et al. 2009; Toulza et al. 2010). Tregs suppress dendritic cell (DC) and effector T cell functions via different mechanisms like the production of cytotoxic molecules (granzymes A and B, perforin), the secretion of immunosuppressive cytokines (i.e. IL10 and TGF-β), and/or the expression of immunoregulatory receptors.
(i.e. LAG-3, CTLA-4) (Vignali, Dario A A et al. 2008). The prognostic value of Tregs in cancer patients is a matter of debate (Badoual et al. 2006). It is highly influenced by the phenotype, histological parameters, different areas in tumor (Gobert et al. 2009), and cancer type. Also, the ratio of cytotoxic T cells to Tregs is considered as a strong prognostic parameter than only Tregs (Eiichi Sato et al. 2005; Preston et al. 2013).

Considerable research has been carried over the past few decades in understanding the molecular basis underlying the immune regulation by Tregs. But still the questions remain that how many mechanisms are involved at the same time in the tolerance mediated by Tregs? In most scenarios, the primary mechanism employed by the Tregs likely depends on the disease settings, the target cell type, the local inflammatory environment and anatomical location. Also, it is observed that Tregs undergo paired differentiation with effector T cells. This indicates that Tregs undergo functional specialization in periphery by co-opting the transcriptional program of specific effector T cells they suppress (Duhen et al. 2012). Also, the presence of Tregs has been found to be influential on the formation of the TLS in tumors (Hindley et al. 2012; Martinet et al. 2013). Thus, it becomes important to understand the qualitative and quantitative role of Tregs in shaping the anti-tumor immune response.

In this study, we observed the presence of CD4+CD25hiFoxP3+ Tregs in different lung tumor areas along with TLS present in the stroma of the tumors. By flow cytometry and gene expression analysis, we observed that Tregs infiltrating lung tumors are activated and expressed several immunoregulatory molecules suggesting a potential immunosuppressive function of
Tregs in the tumor microenvironment. Tumor-infiltrating TLS Tregs showed specific gene signature compared to non-TLS Tregs, and conventional CD4+ T cells. In a retrospective cohort of 243 of NSCLC patients, we demonstrated for the first time that high Treg infiltrate is associated with a poor clinical outcome, and in combination with TLS-mature DC, TLS-B cells or CD8+ T cells allows the identification of patients with the highest risk of death. A deeper analysis of Treg localization showed that Tregs homing either in TLS, either in non-TLS areas were found to be associated with the bad clinical outcome of lung cancer patients. Altogether, these data suggest that Tregs regulate cells either present in TLS, a site where the initiation of adaptive immune response takes place, and either at the effector site, respectively.

Methods

Patients

After complete surgical resection, primary lung tumor samples were obtained from the NSCLC patients at Institut Mutualiste Montsouris, Hotel Dieu and Cochin hospitals (Paris, France). A retrospective cohort of 243 NSCLC patients from Hotel Dieu Hospital (Paris) operated between year 2001 to 2005 was enrolled in this study. Patients treated with neoadjuvant chemotherapy and radiotherapy was excluded from this cohort. A time between the surgery and last follow up or death is considered as the observation time for this cohort. The data on long-term outcomes were obtained after interaction from municipality registers or the family of the patient. This protocol was approved by the local ethical committee (n° 2008-133 and n° 2012-0612) an application with the article L.1121-1 of French law. For prospective cohort, the fresh
tumor biopsies were obtained from 55 NSCLC patients. The non-tumoral distant lung specimen (NTDL) and lymph node (LN) specimen were also obtained from the patients undergoing surgery. Samples were obtained from patients with written consent. The main clinical and pathological features of the patients for retrospective and prospective cohorts are presented in the Supplementary Tables 1 and 2, respectively.

**Immunohistochemistry**

Formalin fixed, paraffin-embedded tissue serial sections with 5 µm thickness were used for immunohistochemistry double staining for CD3, FoxP3, DC-Lamp, CD8, CD20, CD21, and pan-cytokeratins. Briefly, tissue sections were deparaffinized, rehydrated and treated with the antigen retrieval buffer TRS (Dako). The sections were incubated in the protein bloc (Dako) for 30 min before the addition of the appropriate primary and secondary antibodies. The antibodies and reagents are listed in the Supplementary Table 3. The enzymatic activity was performed using substrate kits. Images were acquired using Nanozoomer (Hamamatsu) with NDPview software.

**Cell quantification**

Immune cells were quantified in the whole tumor section using Calopix software (Tribvn), and expressed as a number of cells/mm² of the areas of interest. The surface area of the region of interest was also determined using the same software. The region of TLS was determined manually referring the double DC-Lamp/CD3 (T-cell zone of TLS) and CD20/CD21 (B-cell zone of TLS) staining. The density of CD3⁺FoxP3⁺ cells in TLS was determined with automatic counting. The quantification of the TLS-DC-Lamp⁺ DC, CD3⁺ T
cells, CD8\(^+\) T cells, TLS-CD20\(^+\) B cells was determined, as previously described (Goc et al. 2014b; Germain et al. 2014b).

**Flow cytometry**

A total of 34 NSCLC fresh tumor samples were enrolled in this study. Tumoral and non-tumoral tissue specimens were mechanically dilacerated and digested in a non-enzymatic solution (cell recovery solution, BD Biosciences). The total mononuclear cells were obtained after a ficoll gradient. Mononuclear cells were stained with multiple panels of the fluorescently conjugated antibodies and their matched isotype controls (Supplementary Table 4). Further, cells were fixed and permeabilized using fix/perm kit (ebioscience) for intracellular stainings. Cells were washed, and data acquired on the Fortessa cytometer (BD Biosciences). Data were analyzed using flow Jo 9.7.6 (Tree Star Inc, Ashland, OR) and Spice 5.3.5 (developed by Mario Roederer, Vaccine Research Center, NIAID, NIH) software programs. The gating strategies are explained in the Supplementary Fig. 1.

**Cell sorting**

Four populations of cells, namely CD62L\(^+\) and CD62L\(^-\) Tregs, CD62L\(^+\) and CD62L\(^-\)CD4\(^+\) conventional T cells (Tconv) were sorted from fresh tumor and non-tumoral tissue specimens (n=20) using the in house designed protocol. Briefly, the combinations of Easysep\(^\text{TM}\) untouched human CD4\(^+\) T cell kit (stem cell technologies Ref. No.) and flow cytometry cell sorting were used to achieve the high purity of the cell subsets. The antibodies and reagents used are listed in the Supplementary Table 4. The cells were sorted directly into
vials containing RLT+ 10% β-mercaptoethanol in order to obtain the best quality and quantity of the mRNA.

**RNA extraction and reverse transcription**

Total mRNA from the sorted cells was extracted with the RNeasy micro kit (Qiagen) according to manufacturer’s instructions, and RNA quantity and quality were determined using the 2100 Bioanalyzer (Agilent Technologies). The mRNA was reverse transcribed to cDNA using a superscript VILO kit (Life Technologies). The samples below 1 ng of mRNA were amplified by 9 cycles, and samples with more than 1 ng of mRNA were amplified by 7 cycles of PCR using Taqman PreAmp 2x and MTE primers (NanoString technologies, Seattle, USA).

**Gene expression analysis**

The gene expression was performed using the nCounter analysis system (Nanostring Technologies). Two specific probes (capture and reporter) for each gene of interest were applied. The customized reporter probe and capture probe code-set of selected 125 genes, including 5 housekeeping controls (β-actin, GAPDH, EEF1G, OAZ1 and RPL19) and cell lineage controls (CD3, CD4, CD8, CD19, CD138 and EpCAM) were used for the hybridization according to the manufacturer’s instructions (Nanostring Technologies, Seattle, USA). Water was used as a negative control to check the background noise. The hybridized samples were recovered using the NanoString Prep-station and the mRNA molecules counted with the digital nCounter. The number of counts represented the expression of genes. The positive and negative controls, and one patients RNA sample as internal
control, were used to check the technical consistency during different batch of experiments.

**Statistical analysis**

Mann-Whitney U test and Wilcoxon Rank test (P<0.05*, P<0.01**, P<0.001***) was used to compare the density of cells in the different tumors. The overall survival (OS) curves were estimated by the Kaplan-Meier method, and differences between groups of patients were calculated using log-rank test. Patients were stratified into two groups according to the high and low densities of immune cells using “minimum P-value” approach, as previously published(Goc et al. 2014b; Germain et al. 2014b). Optimal cut-off values are 898.793 CD3⁺FoxP3⁻ Tconv, 21.997 CD3⁺FoxP3⁺ Tregs, 127.0348 TLS Tregs, 1.248 DC-Lamp⁺ DC, 226.5 CD8⁺ T cells/mm², and 0.3256% TLS-CD20⁺ B cells of tumor areas (Supplementary Fig. 2). All analyses were performed with Prism 5 (GraphPad), Statview (Abacus system) and R (http://www.r-project.org/) softwares. For gene expression study and heatmap demonstrations, the softwares ‘nSolver’ (Nanostring Technologies) and R were used. The Raw data were normalized with an average count of the 5 housekeeping genes using “nSolver” software. The Student T test and ANOVA test were used to compare the gene expression among the groups of data, respectively. To avoid the inclusion of the false positive results, we computed the P-values with false discovery rate (FDR) method. The data were represented in the Heatmap, volcano plot, and correlation matrix format.
Results

Tregs infiltrate different areas of lung tumors comprising TLS, and exhibit an activated memory phenotype

We evaluated the presence, localization, and frequency of tumor-infiltrating Tregs in NSCLC patients. The presence of CD3⁺FoxP3⁺ T cells was detected in different tumor areas by immunohistochemistry. Rare CD3⁺FoxP3⁺ T cells were observed in tumor beds (Fig. 1A), as for the other T cell subsets like CD8⁺ T cells (Fig. 1D, serial section of panel 1A). Indeed, the majority of T cells among with CD3⁺FoxP3⁺ (Fig. 1B) and CD8⁺ T (Fig. 1E, serial section of panel 1B) cells were detected in the tumor stroma. A deeper characterization of the stroma reaction allowed us to visualize Tregs in the T-cell rich areas of TLS (Fig. 1C), as demonstrated by the presence of DC-Lamp⁺ mature DC and CD3⁺ T cell clusters (Fig. 1F, serial section of panel 1C).

Next, we deciphered the phenotype of CD3⁺FoxP3⁺ T cells infiltrating tumors as well as non-tumoral distant sites by multicolor flow cytometry. We observed that this population exclusively expressed CD4 (not CD8), high level of CD25, and were CD127⁻/Lo (Fig. 1G). This observation was concordant to the phenotype of human natural CD4⁺Tregs demonstrated in the literature(Fontenot et al. 2005a; Fehérvari, Sakaguchi 2004). Even with a heterogeneity of CD3⁺FoxP3⁺ Tregs among NSCLC patients, the percentage of Tregs among total CD4⁺ T cells was always higher in tumor (14.49± 1.34%) compared to their percentage in NTDL (4.98± 0.63%), LN (8.24± 0.95%), and peripheral blood (6.26±1.48%; Fig 1H) suggesting an active recruitment of this
T cell subset in tumor. Since CD62L is a specific marker of TLS-T cells (Girard, Springer 1995; Goc et al. 2014b), we next studied and compared the stage of differentiation of Tregs versus CD4+ Tconv in TLS (CD62L+) versus non-TLS areas (CD62L-) of the tumor. A minority of Tregs home into tumor-induced TLS (28.90±4.58% of CD3+CD4+CD25++FoxP3+CD62L+/total Tregs as observed for CD4+ Tconv (16.07±4.58 % of CD3+CD4+CD62L+/total CD4+ Tconv (excluding Tregs) Fig. 1I). Based on the differential expression of CCR7, CD45ra, CD27, and CD28 (Supplementary Fig. 3), TLS-Tregs were mainly of central memory (CM) and effector-memory type 1 (EM1) phenotype; and even rare, all naïve Tregs were detected in TLS, as observed for CD4+ Tconv (Fig. 1J, upper panel; see Supplementary Table 5 for details). However, substantial differences were observed regarding the frequency of Tregs compared to CD4+ Tconv with less naïve and CM, and more EM1 Tregs than CD4+ Tconv in TLS. Same predominant stages of differentiation were detected for Tregs and CD4+ Tconv in non-TLS areas but with a different distribution (Fig. 1J, lower panel). Indeed, non-TLS Tregs were mainly of EM1 phenotype, and to a lesser extent, CM and EM4 whereas these three stages were equally distributed among CD4+ T conv. Of note, no terminal EM (TEMRA) Tregs (CCR7- CD45ra+) were detected in the tumor. Interestingly, the analysis of the distribution of the four T cell stages in tumor and nontumoral distant sites (NTDL, LN, and blood) indicated that the phenotype of Tregs is the same in tumor and NTDL which is distinct to LN and blood, the main difference was the frequency of CM and EM populations (Fig. 1K).
Altogether, Tregs infiltrate different tumor areas along with TLS, with distinct stages of differentiation suggesting that they may exhibit distinct function, accordingly.

**Tregs exhibit an activated phenotype and a distinct gene signature compared to the conventional CD4⁺ T cells in tumor**

The presence of different stages of Treg differentiation in distinct tumor areas led us to investigate the nature of their activation, immunosuppression, and immune checkpoint (ICP) status. Thus, the expression of activation and immunoregulation markers was investigated at the molecular (n=169) and protein (n=14) level on Tregs, and compared their phenotype to the one of CD4⁺ Tconv in tumors. Along with the FoxP3, IL2Rα and IL2Rβ, tumor-infiltrating Tregs significantly over-expressed some transcription factors (Helios and IRF4), chemokine (CCL22) and receptors (CXCR3, CCR4, and CCR8), cytokines (IL10, IL27, and IFNα) and receptors (TNFR2, IL1R1, and IL1R2), activation receptors (GITR, 4-1BB, ICOS and OX40), and several ICP molecules (membrane and soluble CTLA-4, LAG-3, Tim-3, TIGIT, CD39, B7H3, GARP, and PDL2) compared to the CD4⁺ Tconv (Fig. 2A). In accordance with the gene expression level, the percentage of Tregs positive for GITR, ICOS, 4-1BB, OX40, CTLA-4, Tim-3, and TIGIT at the protein level was remarkably higher than the CD4⁺ Tconv whereas no statistical differences was measured for LAG-3 between the two T cell subsets (Fig. 2B). Flow cytometry data dot plots depict the expression of activation and ICP molecules on Tregs (Supplementary Fig. 4).
Because, TLS is considered as the privileged sites for the activation of the T cells, we next compared the gene expression profile of Tregs versus CD4+ Tconv, according to TLS presence. Genes significantly over-expressed on Tregs were similar in TLS (Fig. 3A) and non-TLS (Fig. 3B). However, few specificities can be noticed in TLS (Tim-3, IL6, CCR5, and CXCR3), and non-TLS (ICOS-L, PDL2, B7H3, GATA3, and FoxA1) suggesting that Tregs in TLS and non-TLS may share common regulatory functions.

In conclusion, Tregs exhibit a specific molecular pattern including activation and ICP molecules compared to CD4+ Tconv in tumor.

**Functional orientation of tumor-infiltrating Tregs is remarkably different than their counterparts from blood but not from NTDL**

Because most Tregs were of memory phenotype with putative effector function in the different sites but they prevalently infiltrated tumors, we determined whether they present the same molecular pattern whatever their localization. As previously performed, we compared the gene and protein expression level of activation, immunosuppressive and ICP markers on Tregs infiltrating tumors, NTDL, LN, and blood of NSCLC patients. Surprisingly, very few genes (11 out of 120 genes tested) were differentially expressed by Tregs in tumors versus NTDL (Fig. 4A, top panel) indicating that they present a similar gene expression signature in tumoral and non-tumoral lung. Most of these genes are related to chemotaxis and ICP (PD-1, BTLA, B7-H3, IL-27, BCL6, STAT4, CD44). When comparing Tregs in tumors versus LN, we observed more over-expressed genes (n=21 among with 17 in tumors and 4 in LN, Fig. 4A, middle panel), and most of them was already identified when
comparing Tregs versus CD4\(^+\) Tconv in tumors (Fig. 2A). The most important number of genes differentially expressed on Tregs was between tumors versus blood. Except one, all of them were over-expressed in tumors (Fig. 4A, bottom panel), and include the previous highlighted genes (tumors versus LN). The tumor-associated genes in tumoral Tregs are related to transcription factors (FoxP3, FoxA1, STAT4), activation (ICOS, GITR, Ox-40, 4-1BB, TNFR2, CD26), ICP (mCTLA4, PD1, PDL1, B7-H3, BTLA, TIGIT,Tim3, LAG-3), chemotaxis (CCL20, CCL22, CXCL5, CX3CL1, CXCR3, CD200, CX3CR1,LT\(\beta\)R), immunosuppression (IL-10, CD39, GARP), and cytotoxic (granzyme B, granulysine, FasL) molecules. At the protein level, we confirmed that Tregs in the different sites did not express the same set of activation and ICP molecules, blood showing the most important difference with tumors (Fig. 4B-D). Flow cytometry analysis allowed us the study the potential concomitant expression of the studied markers. Thus, the multi-analysis of CD38, CD40L, CD69, and GITR expression shows that the percentage of Tregs negative for all these molecules strongly decreased from 80\% in blood to 8\% in the tumors (Fig. 4B). The percentage of Tregs expressing 1 or 2 markers was quite stable in all sites (around 60\%), except in blood where it dropped to 20\%. Thus, the frequency of Tregs positives for 3 to 4 markers dramatically increased from 0\% in blood to 35\% in tumors. Analysis of cells expressing at least 2 molecules indicates that, in most cases, single positive cells are mainly CD69 (except for blood with a preferential CD38 expression), double positive cells are CD69 and GITR, and triple positive cells are CD69, GITR and CD38. The same scenario occurred for the expression of 4.1BB, ICOS, and OX40 (Fig. 4C). The proportion of triple negative Tregs significantly decreased from
65% in blood to 26% in tumors whereas it increased from 2% to 22% for triple positive Tregs. In most cases, ICOS was expressed first, followed by OX40 and then 4.1BB from single to triple positive cells. As for activation markers, the percentage of Tregs expressing none of the ICP dramatically dropped from 56% in blood to 10% in tumors (Fig. 4D). Same results for Tregs expressing one marker only. In contrast, the frequency of cells positive for at least 2 markers significantly increased from 8% in blood to 70% in tumors. TIGIT, then Tim-3 or CTLA-4 was sequentially expressed by Tregs.

Altogether, lung Tregs exhibit a specific gene pattern compared to distant sites i.e. LN and blood. And, lung cancer microenvironment is mainly infiltrated by Tregs expressing activation and ICP molecules whereas the blood Tregs rather shows a resting state.

High density of tumor-infiltrating Tregs is associated with short-term survival, and combination with CD8⁺ T cells, TLS-mature DC or TLS-B cells allowed the identification of patients with the worst clinical outcome

The Kaplan Meier curves depict that the high density of the intra-tumoral Tregs correlated with the poor clinical outcome of NSCLC patients (median OS was not reached for “Tregs Low” whereas it was 51 months for “Tregs High” patients, P=0.0049, Fig. 5A). Since the high densities of TLS-mature DC, TLS-B cells, and effector CD8⁺ T cells were associated with long-term survival of patients with NSCLC (Dieu-Nosjean et al. 2008; Goc et al. 2014b; Germain et al. 2014b), Fig. 5C,E,G), we further tested the combined impact of these immune cells with Tregs on patient’s survival. We observed that low
density of the Tregs was associated with the favorable clinical outcome whatever the density of the mature DC, TLS-B cells or CD8\(^+\) T cells. In contrast, patients having high Treg density and low mature DC (median OS=25 months; \(P<0.0001\), Fig. 5D), TLS-B cells (median OS=24 months; \(P<0.0001\), Fig. 5F), or CD8\(^+\) T cells (median OS=40 months; \(P=0.0002\), Fig. 5H) density had the worst outcome with the shortest median survival compared to each variable alone. “Tregs High/mature DC, TLS-B or CD8\(^+\) T high” patients were at intermediate risk of death suggesting that, on one hand mature DC, TLS-B and CD8\(^+\) T cells, and on the other hand Tregs have a dual impact on the outcome of NSCLC patients. Since, Tregs in the different areas of the tumor have been shown to have different impact on the survival of breast cancer patients(Gobert et al. 2009), we counted the Tregs in TLS and non-TLS areas separately in 100 NSCLC patients. It was observed that high densities of TLS-Tregs and non-TLS-Tregs correlated with poor clinical outcome, similar to the total Tregs (median OS=57 versus 34 months for “TLS-Tregs Low” and “TLS-Tregs High” patients, respectively; \(P=0.0245\), Fig. 5B). We also determined the ratio of total CD3\(^+\) T cells or CD8\(^+\) T cells or mature DC/Treg densities, and it was found that “ratio High” of all these markers was beneficial for NSCLC patients compared to “ratio Low” (Supplementary Fig. 5)

In conclusion, the balance between Tregs and other immune cells like mature DC, TLS-B cells, CD8\(^+\) T cells is critical for the behavior of NSCLC patients. Combination of Tregs with one of the other immune subsets allows a better stratification of patients for survival than each subset alone, with the identification of a group of NSCLC patients with a high risk of death.
Discussion

There are many studies in the literature which discuss the prognostic relevance of the Tregs in different solid cancers, but it has been always debatable due to various factors responsible for the discrepancy. Depending on the tumor type, stage and histological type, the prognostic importance of Tregs was found to be different (Badoual et al. 2006). Based on their localization, Tregs either may suppress or help the anti-tumor responses. In germinal centers, it has been recently observed that Tregs may help the Tfh differentiation (León et al. 2014). Thus, it was interesting to speculate the different roles and phenotypes of the Tregs in different areas of the tumors. With the advanced techniques available, we re-addressed the prognostic value of Tregs as a total population, and also depending on their localization in the different subareas of lung tumors.

We first time demonstrated the presence of CD3⁺FoxP3⁺ T cells in the different areas of NSCLC tumors. We observed that CD3⁺FoxP3⁺ T cells are mainly found in the stroma of the tumor, and particularly TLS in the stroma; there are few CD3⁺FoxP3⁺ T cells in the tumor nests. We confirmed the CD3⁺FoxP3⁺ T cells as Tregs according to their CD4⁺CD25hiCD127⁻/loFoxP3⁺ phenotype by flow cytometry, as mentioned in the literature (Roncador et al. 2005; Liu et al. 2006).

Since, we observed that Tregs abundantly infiltrate the lung tumors (both TLS and non-TLS areas) compared to non-tumoral tissues, we first time studied their phenotype in detail. We observed that in general, Tregs bear the same differentiation status when compared to CD4⁺ Tconv (Goc et al. 2014b).
Tumor-infiltrating Tregs predominantly show the CM and EM phenotypes. Very few naïve Tregs infiltrate tumors but interestingly, all of them home in TLS. TLS Tregs are importantly CM and EM1; whereas non-TLS Tregs showed the further differentiation phases like EM1 and EM4. Differentiation status of Tregs was not the same in tumor compared to blood or lymph node. Although Tregs exhibited similar phenotype in distant lung and tumor, there were less effector memory cells in blood and lymph node. These results initiated us to further study the activation status of the Tregs.

High infiltration of the Tregs in lung tumors (compared to the NTDL, LN and blood) is reflected by the high expression of the chemokine CCL22 and receptors like CCR8, CCR4, and CX3CL1 by intra-tumoral Tregs. We also observed the higher expression of the CXCR3 in TLS Tregs which could be possible way of their recruitment to the inflammatory site (Redjimi et al. 2012). Mature DC can produce CCL22 (Vulcano et al. 2001) which can recruit CCR4+ Tregs. The role of CCR4, CCL17, and CCL22 in chemoattraction of Tregs to the inflamed sites is enough discussed in mouse and human studies (Chaisemartin et al. 2011; Gobert et al. 2009).

Since, the around 25% of the total intratumoral Tregs infiltrate TLS, it can be speculated that TLS-Tregs can suppress the TAA-specific T cells in the TLS as well as non-TLS areas of the tumors. When gene expression of Tregs in TLS and non-TLS areas of tumors was compared, we found a similar profile, as for total Tregs. TLS Tregs selectively expressed the molecules like Tim3, IL6, CCR5, and CXCR3 in comparison to the non-TLS Tregs which overexpressed more transcription factors GATA3, PDL2, B7H3, and ICOSL. Surprisingly, we observed an overexpression of IL-2 Rα and IL-2Rβ, co-
stimulatory (ICOS, OX40, 4-1BB and GITR) and ICP (CTLA4, TIGIT, PD1 and PDL2) molecules in Tregs compared to CD4⁺ Tconv. This was confirmed at protein level. In last few years, it has been demonstrated the role of ICOS in the IL-10 production by Tregs(Lohning et al. 2003). Expression of ICOSL and OX-40 by plasmacytoid DC is responsible for recruitment of Tregs in melanoma and breast cancer(Faget et al. 2012a; Aspord et al. 2013). Treatment with IL-2 in melanoma was found to be expanding the ICOS⁺ Treg population(Sim et al. 2014). Although the role of GITR in Tregs has been controversial, it has been found that Tregs constitutively express GITR. All Ti-Tregs expressed HLA-DR (data not shown) which suggests the contact dependent suppression mechanism used by Tregs. The TNF superfamily receptors like TNFR2, 4-1BB, and Ox-40 may help in their suppressive ability. Co-expression of GITR, OX-40, and TNFR2 along with TCR signaling has been found to favor the thymic differentiation of Tregs(Mahmud et al. 2014).

Most importantly, a proportion of Tregs express ICP. We found that Tregs in tumors express high levels of the CTLA4, TIGIT, Tim-3, B7-H3, PD1, and its ligands PDL1 and PDL2. Expression of the CTLA4 on Tregs, and its interaction with CD80 and CD86 molecules on APC (Wing et al. 2008), is greatly discussed. CTLA4 might trigger induction of enzyme IDO in DC’s by interacting with their CD80 and CD86 (Onodera et al. 2009). CTLA4 and PD1 blockades has found to be effective in the melanoma(Peggs et al. 2009), and is also showing the promising results in NSCLC probably due to its action on the Tregs expressing a higher level in comparison to the CD4⁺ Tconv. In our study, expression of the CTLA4 and TIGIT is found to be always correlated in the Ti-Tregs. Ligation of TIGIT on Tregs may inhibit pro-inflammatory
responses by Th1 and Th17 (Joller et al. 2014). It is also observed now that Helios⁺ memory Tregs expressing TIGIT and FCRL3 are highly suppressive Tregs (Bin Dhuban et al. 2015; Fuhrman et al. 2015). We observed that Tregs in lung tumors are Helios positive and they also express other transcription factors like FoxA1 and GATA3. IRF4 expression of Tregs may suggest its role in the maintenance of the proliferation and activation of Tregs, as observed in CD8⁺ T cells (Man et al. 2013).

We observed an overexpression of CD40L by CD4⁺ Tconv versus Tregs, especially by TLS CD4⁺ Tconv which can be possible consequences of T cell activation after interaction with mature DC in TLS, counteracting immunosuppression by Tregs. Surprisingly, we found an PDL2 overexpression by Ti-Tregs, especially TLS Tregs, compared to the lymph node Tregs but not differently from the NTDL and blood. It has been found that in germinal center, PD1-PDL1 ligation inhibits the CXCR5⁺ follicular Tregs and antibody production in vivo (Sage et al. 2013) but the regulation via PDL2 is poorly studied.

Intratumoral Tregs express the IL-6, IL-10, and TGF-β which may lead to the suppression of T cell function and DC differentiation and activation. We observed that Tregs highly express the IL-27 which was speculated to be a negative regulator of Th17 differentiation in the tumor microenvironment. In another study in NSCLC patients, it is observed that IL-27 was negatively correlated with the Th17 cells and RORγt expression (Duan et al. 2015).

We observed that high Treg density correlated with shorter overall survival in NSCLC patients. Literature shows that in few cases, Tregs are associated
with good, bad or no clinical outcome. In breast cancer patients, the presence of Tregs in the lymphoid aggregates is associated with the poor survival, whereas their presence in the tumors is not associated with the survival of the patients (Gobert et al. 2009), but the evidences lack to show that these lymphoid aggregates are the functional TLS. We show that the presence of a high number of Tregs in TLS and whole tumor negatively impacts the survival of patients, which could be due to exclusive infiltration of Tregs in different areas of tumors mainly TLS and stroma region, the differentiation and activation of Tregs in the TLS and their immunosuppressive function against Tconv and APC. We provide the evidence of Tregs being activated in the tumors compared to the NTDL, LN and blood.

We have already demonstrated the presence and favorable role of TLS in the anti-immune response generation in lung cancer patients (Dieu-Nosjean et al. 2008; Goc et al. 2014b; Germain et al. 2014b). The HEV have found to be involved in the recruitment of T cells, and mature DC have been found to be involved with activation of T cells in TLS. TLS are found to be participating in orchestrating the Th1 and cytotoxic oriented gene signature. We observed that, Tregs express T-bet probably to co-opt the transcription factor expression of the Th1 cells and may suppress the Th1 responses.

When the density of Tregs was combined with DC-Lamp\(^+\) mature DC, CD20\(^+\) TLS-B cells and CD8\(^+\) T cells, the stratification of the group with the highest survival could be determined. The ratio of the CD3\(^+\) T cells or mature DC or CD8\(^+\) T cells with Tregs found to be stronger prognosticator than each variable alone, it showed that a high proportion of T cells or mature DC compared to Tregs imprints the better survival for lung cancer patients.
The protein level expression of several activation and ICP markers confirmed these results. High expression of co-stimulatory molecules on TLS Tregs suggests their putative competition with T conv. for the interaction with the antigen-presenting mature DC, and finally the immunosuppression via the different mechanisms. In the non-TLS region of the tumors, Tregs have the potential to suppress cytotoxic CD8⁺ T cell functions. Finally, the high density of Tregs in whole tumor, and in particular in TLS, is associated with reduced survival of NSCLC patients, and thus exploiting markers like ICOS, CTLA4, TIGIT can be of potential therapeutic tools for the treatment of the lung cancer patients.

References


Acknowledgment:

The authors thank Ms. E. Devevre and C. Klein from the platform “Centre d’Imagerie Cellulaire et de Cytométrie” (Cordeliers Research Center, Paris, France) for great technical support and assistance in cell sorting, and cell quantification from digital slides, respectively. We also thank Mrs. D. Gentien and B. Albaud from the Genomic platform (Department of Translational Research of Curie Institute, Paris, France) for technical support in the gene expression. Finally, we thank Ms. P. Bonjour for help in tissue section.

Grant support:

This work was supported by the “Institut National de la Santé et de la Recherche Médicale (INSERM), the “Cancéropôle Ile-de-France”, the “Institut National du Cancer” (INCa, n°2010-1-PL-BIO03), Pierre and Marie Curie University, Paris-Descartes University, the “Cancer Research for Personalized Medicine” (CARPEM), the Labex Immuno-Oncology (LAXE62_9UMRS872 Fridman), and the Fondation ARC pour la Recherche sur le cancer” (SL220110603483). Ms. P. Devi received a doctoral fellowship from “Fondation ARC pour la Recherche sur le cancer”.
Figures

Fig. 1

A. CD3/FoxP3 (Tumor bed)
B. CD3/FoxP3 (stroma)
C. CD3/FoxP3 (TLS)
D. CD8/AE1AE3 (Tumor bed)
E. CD8/AE1AE3 (stroma)
F. CD3/DC-Lamp (TLS)
G. Gated on the total CD3+ T cell population
H. %Tregs/total CD4+ T cells
% T cell subset

CD4 Tconv and Tregs

I

K

CD62L+ (TLS cells) and CD62L- (nonTLS cells)

J

CD45RA + - - - - + + +
CCR7 + + - - - - - -
CD27 + + + + - + + -
CD28 + + + + - + - -
Figure 1: Identification of distinct memory Treg subsets according to lung tumor areas. (A-F) Double immunohistochemistry on paraffin-embedded lung tumor sections (serial sections: A-D, B-E, and C-F). Presence of CD3\(^+\) (red) FoxP3\(^+\) (blue) T cells in tumor nests (A), tumor stroma (B) including TLS (C). Presence of CD8\(^+\) (red) T cells infiltrating the pan-cytokeratins\(^+\) (blue) tumor beds (D) and the stroma (E). Detection of DC-Lamp\(^+\) (red) mature DC and CD3\(^+\) (blue) T-cell rich areas of TLS (F). Original magnifications: A-F, 20x. Characterization of CD3\(^+\) FoxP3\(^+\) cells according to the expression of CD4, CD8, CD25, CD127, and CD45ra by flow cytometry (G). Frequency of CD3\(^+\)CD25\(^+\)FoxP3\(^+\) cells among total CD4\(^+\) T cells in tumor (n=31), non-tumoral distant lung (NTDL, n=30), LN (n=23), and peripheral blood (n=6) (H). Percentage of CD3\(^-\)CD25\(^-\)FoxP3\(^+\) Tregs and CD4\(^+\) Tconv in TLS and non-TLS areas (n=21) according to the differential expression of CD62L (I). Stages of differentiation of both CD3\(^+\)CD25\(^-\)FoxP3\(^+\) Tregs and CD4\(^+\) Tconv according to the differential expression of CD45ra, CCR7, CD27, and CD28 in TLS (CD62L\(^+\), top panel) and non-TLS (CD62L\(^-\), bottom panel) areas (n=21) (J). Representation of the main four stages of differentiation of Tregs in the different anatomical sites (n=21 patients) (K). Abbreviations: CM, central-memory; E, exhausted; EM, effector-memory; LN, lymph node; N, naïve; NTDL, non-tumoral distant lung; S, stroma; T, tumor bed; TEMRA, terminally effector-memory CD45ra\(^+\) T cell.
Fig. 2

A

B

Ti CD4+ Tconv  Ti-Tregs

CD40LG  CD127  CD40L  GITR  ICOS  TIGIT  4-1BB  CTLA4  IL2RA  IL2RB  IL4  IFNγ  Tim3  ICOS  CTLA4  IL2RB  IL2RA  IFNG  Helios  FOXP3  CD127  CD39  CD73  CD103  B7H3  TIM3  IFNα  GITR  CD127  CD39  CD73  CD103  B7H3  TIM3  IFNα  ICOS  GITR  CD127  CD39  CD73  CD103  B7H3  TIM3  IFNα

-log10(pValue)  log2(foldChange)

0  5  10  15

-2  0  2  4

ns *** * ** ** ***

% T cell subset

CD40L  GITR  ICOS  4-1BB  Ox-40  CD127

CD4+ Tconv Ti-Tregs

CTLA-4  LAG-3  PD-1  Tim-3  TIGIT

*** ns * * **

% T cell subset

CTLA-4  LAG-3  PD-1  Tim-3  TIGIT

*** ns * * **
Figure 2: Selective molecular pattern of Tregs versus CD4⁺ Tconv infiltrating NSCLC tumors

(A) The volcano plot shows genes over-expressed in sorted Tregs (right) in comparison to sorted CD4⁺ Tconv (left) in the lung tumors. The X-axis shows the log2 fold change values for each gene, and the Y-axis shows the –log10 (P-values). The P value<0.05 is considered significant, and genes showing the significant differential expression are highlighted in red dots. The gene names showing the fold change expression higher than 1.3, are highlighted in the blue color. Student T test (P<0.05*). (B) Percentage of Tregs (black circles) and CD4⁺ Tconv (grey circles) expressing activation markers (n=18 tumors) and ICP (n=14 tumors) at the protein level. Mann-Whitney U test was used. *: P<0.05, **: P<0.01, ***: P<0.001.
Figure 3: TLS Tregs exhibit distinct gene expression signature compared to the non-TLS Tregs and CD4+ Tconv cells

The volcano plots show genes over-expressed in TLS-Tregs isolated from tumor versus TLS CD4 Tconv cells (panel A) and genes over-expressed in nonTLS Tregs isolated from tumor versus nonTLS CD4 Tconv (panel B) of NSCLC patients (n=20). The X-axis shows the log2 fold change values for each gene, and the Y-axis shows the –log10 (P-values). The P value <0.05 is considered significant, and genes showing the significant differential expression are highlighted in red dots. The gene names showing the fold change expression higher than 1.3, are highlighted in the blue color. Student T test (P<0.05*).
Fig. 4

A

NTDL

-\log_{10}(p\text{Value})

\log_2(\text{foldChange})

C

CL22

CCR7

B7H3

CX3CL1

NTDL Tumor

LN

-2 -1 0 1 2 3

0.0 0.5 1.0 1.5 2.0 2.5

\log_2(\text{foldChange})

-\log_{10}(p\text{Value})

C

CL22

CCR7

B7H3

CD39

CXCR3

IL27

FoxA1

GITR

PDL2

RANKL

SELL OX40

4-1BB

Tumor
Figure 4: Selective molecular pattern of Tregs infiltrating tumors versus non-tumoral sites (distant lung, LN, and blood).

A; The volcano plots show genes over-expressed in Tregs isolated from tumor versus NTDL (top), tumor versus LN (middle), and tumor versus blood (bottom) of NSCLC patients (n=20). The X-axis shows the log2 fold change values for each gene, and the Y-axis shows the –log10(P-values). The P value <0.05 is considered significant, and genes showing the significant differential expression are highlighted in red dots. The gene names showing the fold change expression higher than 1.3, are highlighted in the blue color. Student T test (P<0.05*).

B, C and D; Protein level expression of the activation and immunosuppressive markers was tested by flow cytometry. The percentage of the Tregs expressing the combination of 1 to 4 different activation markers (CD38, CD40L, CD69, and GITR; panel B), the combination of 1 to 3 other activation markers (4-1BB, ICOS, and OX40; panel C), and the combination of 1 to 5 different ICP (CTLA4, LAG3, PD1, TIGIT, and Tim3; panel D) or no expression of any of these markers is shown by the each pie chart for different tissues (n=18). The gray colored potions of the pie chart represents the different combinations of the markers expressed by the proportion of Tregs ranging from 5 (expression of all markers) to 0 (no expression of any marker) marker. Each colored arc represents the single marker, and thus different colored arcs on the top of the pie chart show the distribution of the cells expressing different markers in the combinations.
Fig. 5

A. 

B. 

C. 

D. 

E. 

F. 

G. 

H. 

Overall Survival

DC lo (n=105)
DC Hi (n=138)
P=0.0002

Lo DC/Lo Tregs (n=27)
Lo DC/Hi Tregs (n=78)
P<0.0001

B cell lo (n=71)
B cell Hi (n=172)
P=0.0020

Hi B cells/lo Tregs (n=31)
Hi B cells/Hi Tregs (n=141)
Lo B cells/Lo Tregs (n=22)
Lo B cells/Hi Tregs (n=49)
P<0.0001

CD8 lo (n=63)
CD8 Hi (n=178)
P=0.0027

Hi CD8/lo Tregs (n=37)
Hi CD8/Hi Tregs (n=141)
Lo CD8/Lo Tregs (n=16)
Lo CD8/Hi Tregs (n=47)
P=0.0002
Figure 5: High density of tumor infiltrating Tregs is associated with poor clinical outcome of the lung cancer patients

Immunostainings were performed on the 243 paraffin-embedded NSCLC tumor sections. The automatic countings performed on the stained and scanned tissue images. The Kaplan-Meier survival graphs plotted for the determination of the percentage OS of NSCLC patients. The Log-rank test used to determine the statistical significance of the data. A: The graph shows the survival curve based on the density of the FoxP3\(^+\) Tregs. High density of the Tregs is related to the poor survival of the patients (P=0.0049). B: for n=100 patients, the FoxP3\(^+\) Tregs counted in the TLS areas of the tumors separately. The patients with Tregs in the TLS areas stratified according to the, hi and low group and survival determined. The high density of the Tregs in TLS is associated with the poor survival of the patients (P= 0.0245 ). C,E,G; The density of the Dc-lamp\(^+\) mature DC, CD20\(^+\) B cells, and CD8\(^+\) T cells determined using the serial sections of the tissues and the density was found to be associated with good clinical outcome of the patients (P=0.0002, P=0.0020, and P=0.0027, respectively). D, F,H; when the density of the DC-Lamp\(^+\) mature DC, CD20\(^+\) B cells and CD8\(^+\) T cells combined with the density of the FoxP3\(^+\) Tregs, the patients could be stratified into 4 different groups (P<0.0001, P<0.0001, and P=0.0002, respectively).
Supplementary figures and tables:

**Supplementary Fig. 1:** Gating strategy for the characterization of T cell phenotype by flow cytometry.

Lymphocytes were defined according to the size and granularity (SSC and FSC). Doublets (FSC-A versus FSC-H, and SSC-A versus SSC-H) and dead cells (using Live Dead Cell marker) were excluded while CD3$^+$ T cell gating. The results from one patient are representative of 34 patients.
A

Density of CD3+ T cells (cells/mm²)

Optimal cut-off value

898.7939 cells/mm²

B

Density of FoxP3+ Treg cells (cells/mm²)

Optimal cut-off value

21.99746 cells/mm²
Optimal cut-off value

Density of the CD3+T cells: Tregs ratio (cells/mm²)

- C

Density of the CD8+T cells: Tregs ratio (cells/mm²)

- D

17.94117 cells/mm²

12.41506 cells/mm²
Density of mDC cells : Treg cells (cells/mm²)

Optimal cut-off value

0.05

0.06297162 cells/mm²

Density of the Tregs in TLS (cells/mm²)

Optimal cut-off value

0.05

127.0348 cells/mm²
Supplementary Fig. 2: Determination of optimal cutoff values for the discrimination of the high and low groups based on densities of the CD3\(^+\) T cells, FoxP3\(^+\) Tregs, ratio of CD3\(^+\) T cells or CD8\(^+\) T cells or mature DC with the Tregs, and finally the density of TLS-Tregs.

Optimal cut-off values are 898.793 CD3\(^-\)FoxP3\(^-\) Tconv (A), 21.997 CD3\(^+\)FoxP3\(^+\) Tregs (B), 17.94117 CD3\(^+\) Tconv/Tregs (C), 12.41506 CD8\(^+\) T cells/Tregs (D), 0.06297162 DC-Lamp\(^+\) mature DC/Tregs (E), and 127.0348 TLS-Tregs (F).
Supplementary Fig. 3: Discrimination of the main four stages of differentiation of Tregs and CD4$^+$ Tconv in tumor and non-tumoral sites of NSCLC patients.

Analysis of naïve (N, CCR7$^+$CD45ra$^+$), central memory (CM, CCR7$^+$CD45ra$^-$), effector memory EM, CCR7$^-$CD45ra$^-$) and terminal effector memory (TEMRA, CCR7$^-$CD45ra$^+$) based on the differential expression of CCR7 and CD45ra in Tregs and CD4$^+$ Tconv isolated from tumor, non-tumoral distant lung (NTDL), lymph node (LN), and blood. The data from one patient are representative of 21 patients.
Activation Panel 1

Activation Panel 2

Immunosuppression panel
Supplementary Fig. 4: Expression of activation and immunosuppressive molecules on Tregs and CD4+ Tconv infiltrating tumor.

The dot plots show the expression of activation and immunosuppressive markers. Positivity (red rectangle) was determined based on the isotype control. The data from one patient are representative of 18 patients.
Supplementary Fig. 5: Kaplan Meier curves for the total CD3$^+$ T cells and the ratio of the CD3$^+$ T cells, CD8$^+$ T cells and mature DC with Tregs, respectively.

Immunostainings were performed on the 243 paraffin embedded NSCLC tumor sections. The automatic countings performed on the stained and scanned tissue images. The Kaplan-Meier survival graphs plotted for the determination of the percentage OS of the patients. The Log-rank test used to determine the statistical significance of the data. **A:** The graph shows the survival curve based on the density of the total CD3$^+$ T cells. High density of
the CD3⁺ T cells is related to the good survival of the patients (OS, CD3 Hi 92 months versus OS CD3 low 41 months P=0.0073). B: The high ratio of the CD3⁺ T cells / FoxP3⁺ Tregs is beneficial for the patients (OS=92 months) versus the low ratio (OS=40 months, P=0.0008) C: The high ratio of the mature DC cells / FoxP3⁺ Tregs is gives good prognosis for the patients versus the low ratio (OS=46 months, P=0.0003), D: The high ratio of the CD8⁺ T cells / FoxP3⁺ Tregs is gives good prognosis for the patients versus the low ratio (OS=41 months, P=0.0005),
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>191</td>
<td>79</td>
</tr>
<tr>
<td>Male/female</td>
<td>52</td>
<td>21</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (years)+/- SEM</td>
<td>62.6+/-0.7</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>19-83</td>
<td></td>
</tr>
<tr>
<td><strong>Smoking history</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>200</td>
<td>82</td>
</tr>
<tr>
<td>Never smokers</td>
<td>31</td>
<td>13</td>
</tr>
<tr>
<td>ND</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Pack-years (years)+/-SEM</td>
<td>42.5+/-1.6</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0-120</td>
<td></td>
</tr>
<tr>
<td><strong>Histological type</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADC</td>
<td>141</td>
<td>58</td>
</tr>
<tr>
<td>SCC</td>
<td>61</td>
<td>25</td>
</tr>
<tr>
<td>Others</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>ND</td>
<td>33</td>
<td>14</td>
</tr>
<tr>
<td><strong>Emboli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>77</td>
<td>32</td>
</tr>
<tr>
<td>Yes</td>
<td>125</td>
<td>51</td>
</tr>
<tr>
<td>ND</td>
<td>41</td>
<td>17</td>
</tr>
<tr>
<td><strong>pT stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>52</td>
<td>21</td>
</tr>
<tr>
<td>T2</td>
<td>118</td>
<td>49</td>
</tr>
<tr>
<td>T3</td>
<td>56</td>
<td>23</td>
</tr>
<tr>
<td>T4</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>pN stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>156</td>
<td>64</td>
</tr>
<tr>
<td>N1</td>
<td>44</td>
<td>18</td>
</tr>
<tr>
<td>N2</td>
<td>43</td>
<td>18</td>
</tr>
<tr>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>pM stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>242</td>
<td>100</td>
</tr>
<tr>
<td>M1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>pTNM stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>108</td>
<td>44</td>
</tr>
<tr>
<td>II</td>
<td>63</td>
<td>26</td>
</tr>
<tr>
<td>III</td>
<td>71</td>
<td>29</td>
</tr>
<tr>
<td>IV</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Vital status of patients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>95</td>
<td>39</td>
</tr>
<tr>
<td>Dead</td>
<td>148</td>
<td>61</td>
</tr>
</tbody>
</table>
Supplementary Table 1: Clinical and pathological characteristics of the retrospective cohort of NSCLC patients

All parameters were evaluated among 243 NSCLC patients. Pathologic staging of lung cancer was determined according to the new TNM staging classification (Detterbeck et al. 2009a). Histological subtypes were determined according to the classification of the WHO(Brambilla et al. 2001) . Abbreviations: ADC, adenocarcinoma; ND, not determined; SCC, squamous cell carcinoma.
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>33/22</td>
<td>60/40</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (years)+/- SEM</td>
<td>67.5+/-1.3</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>48-91</td>
<td></td>
</tr>
<tr>
<td><strong>Smoking history</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>32</td>
<td>58</td>
</tr>
<tr>
<td>Never smokers</td>
<td>13</td>
<td>24</td>
</tr>
<tr>
<td>ND</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>Pack-years (years)+/-SEM</td>
<td>37.3+/-4.5</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0-140</td>
<td></td>
</tr>
<tr>
<td><strong>Histological type</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADC</td>
<td>33</td>
<td>60</td>
</tr>
<tr>
<td>SCC</td>
<td>13</td>
<td>24</td>
</tr>
<tr>
<td>Others</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>ND</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td><strong>pT stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>T2</td>
<td>36</td>
<td>65</td>
</tr>
<tr>
<td>T3</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>T4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>ND</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>pN stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>33</td>
<td>60</td>
</tr>
<tr>
<td>N1</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>N2</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>ND</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td><strong>pTNM stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>23</td>
<td>42</td>
</tr>
<tr>
<td>II</td>
<td>17</td>
<td>31</td>
</tr>
<tr>
<td>III</td>
<td>12</td>
<td>22</td>
</tr>
<tr>
<td>IV</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ND</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

**Supplementary Table 2: Clinical and pathological characteristics of the prospective cohort of NSCLC patients.**

All parameters were evaluated among 55 NSCLC patients. Pathologic staging of lung cancer was determined according to the new TNM staging classification (Detterbeck et al. 2009a) Histological subtypes were determined according to the classification of the WHO (Brambilla et al. 2001).
Abbreviations: ADC, adenocarcinoma; ND, not determined; SCC, squamous cell carcinoma.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Host</th>
<th>Isotype</th>
<th>Clone</th>
<th>company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>Rabbit</td>
<td>IgG polyclonal</td>
<td>NA</td>
<td>Dako</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Mouse</td>
<td>IgG1</td>
<td>236A/E7</td>
<td>Abcam</td>
</tr>
<tr>
<td>CD8</td>
<td>Rabbit</td>
<td>IgG polyclonal</td>
<td>NA</td>
<td>Spring biosciences</td>
</tr>
<tr>
<td>Dc-Lamp</td>
<td>Rat</td>
<td>IgG2b</td>
<td>1010E1.01</td>
<td>Dendritics</td>
</tr>
<tr>
<td>Cytokeratines</td>
<td>Mouse</td>
<td>IgG</td>
<td>AE1/AE3</td>
<td>Dako</td>
</tr>
<tr>
<td>Secondary Antibody</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-rabbit – biot</td>
<td>Donkey</td>
<td>IgG polyclonal</td>
<td>NA</td>
<td>JIR</td>
</tr>
<tr>
<td>anti-mouse IgG-AP</td>
<td>Goat</td>
<td>IgG polyclonal</td>
<td>NA</td>
<td>JIR</td>
</tr>
<tr>
<td>Donkey anti-rat IgG-BIOT</td>
<td>Donkey IgG polyclonal</td>
<td>NA</td>
<td>JIR</td>
<td></td>
</tr>
<tr>
<td>anti mouse- non coupled</td>
<td>Sheep</td>
<td>IgG1</td>
<td>NA</td>
<td>The binding site</td>
</tr>
<tr>
<td>Streptavidin-HRP</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Dako</td>
</tr>
<tr>
<td>APAAP</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Dako</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrates</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AEC</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Vector laboratories</td>
</tr>
<tr>
<td>SAP</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Vector laboratories</td>
</tr>
</tbody>
</table>

**Supplementary Table 3: Antibodies and reagents used for immunohistochemistry.**

Abbreviation: AEC, 3-Amino-9-ethylcarbazole substrate; AP, Alkaline phosphatase; APAAP, alkaline phosphatase anti-alkaline phosphatase; HRP, Horseradish peroxidase; NA, not applicable; SAP, substrate of alkaline phosphatase.
<table>
<thead>
<tr>
<th>Antibodies or reagents</th>
<th>Conjugate</th>
<th>Host</th>
<th>Isotype</th>
<th>Clone</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>AF700</td>
<td>Mouse</td>
<td>IgG1k</td>
<td>UCHT1</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>CD4</td>
<td>BV605</td>
<td>Mouse</td>
<td>IgG2bk</td>
<td>OKT4</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD8</td>
<td>BV650</td>
<td>Mouse</td>
<td>IgG1k</td>
<td>RPA-T8</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD25</td>
<td>PE-cy7</td>
<td>Mouse</td>
<td>IgG1k</td>
<td>BD+</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>FoxP3</td>
<td>PE</td>
<td>Rat</td>
<td>IgG2a</td>
<td>PCH101</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD62L</td>
<td>APC</td>
<td>Mouse</td>
<td>IgG2a</td>
<td>DREG-56</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CCR7</td>
<td>AF488</td>
<td>Mouse</td>
<td>IgG1k</td>
<td>G043H7</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD45RA</td>
<td>ECD</td>
<td>Mouse</td>
<td>IgG1</td>
<td>2H4</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>CD28</td>
<td>PerCPcy5 .5</td>
<td>Mouse</td>
<td>IgG1</td>
<td>CD28.2</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD27</td>
<td>APC-A750</td>
<td>Mouse</td>
<td>IgG2a</td>
<td>CLB-27/1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>GITR</td>
<td>AF488</td>
<td>Mouse</td>
<td>IgG1</td>
<td>eBioAIT R</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD69</td>
<td>ECD</td>
<td>Mouse</td>
<td>IgG2b</td>
<td>TP1.55.3</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>CD38</td>
<td>PerCP-eF710</td>
<td>Mouse</td>
<td>IgG1k</td>
<td>HB7</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD40L</td>
<td>APC-cy7</td>
<td>Mouse</td>
<td>IgG1k</td>
<td>24-31</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD147</td>
<td>APC</td>
<td>Mouse</td>
<td>IgG1k</td>
<td>8D12</td>
<td>eBioscience</td>
</tr>
<tr>
<td>OX-40</td>
<td>FITC</td>
<td>Mouse</td>
<td>IgG1k</td>
<td>ACT35</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>4-1BB</td>
<td>BV421</td>
<td>Mouse</td>
<td>IgG1k</td>
<td>4B4-1</td>
<td>BioLegend</td>
</tr>
<tr>
<td>ICOS</td>
<td>PerCP-eF710</td>
<td>Mouse</td>
<td>IgG1k</td>
<td>ISA-3</td>
<td>eBioscience</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>APC-cy7</td>
<td>Mouse</td>
<td>IgG1k</td>
<td>L243</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD127</td>
<td>BV650</td>
<td>Mouse</td>
<td>IgG2ak</td>
<td>A019D5</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>APC</td>
<td>Mouse</td>
<td>IgG1k</td>
<td>L3D10</td>
<td>BioLegend</td>
</tr>
<tr>
<td>LAG-3</td>
<td>FITC</td>
<td>Goat</td>
<td>polyclonal gG</td>
<td>NA</td>
<td>R&amp;D systems</td>
</tr>
<tr>
<td>TIM-3</td>
<td>BV421</td>
<td>Mouse</td>
<td>IgG1k</td>
<td>F38-2E2</td>
<td>BioLegend</td>
</tr>
<tr>
<td>TIGIT</td>
<td>PerCP-eF710</td>
<td>Mouse</td>
<td>IgG1k</td>
<td>MBSA43</td>
<td>eBioscience</td>
</tr>
<tr>
<td>PD-1</td>
<td>APC-cy7</td>
<td>Mouse</td>
<td>IgG1k</td>
<td>EH12.2 H7</td>
<td>BioLegend</td>
</tr>
</tbody>
</table>

**Supplementary Table 4: Antibodies and reagents used for flow cytometry.**

Abbreviation: NA, not applicable.
<table>
<thead>
<tr>
<th>T cell differentiation stages</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor (n=21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs</td>
<td>1.9+0.7</td>
<td>41.9+5.5</td>
<td>55.1+5.5</td>
<td>1.3+0.3</td>
</tr>
<tr>
<td>CD4^+Tconv</td>
<td>5.5+1.4</td>
<td>52.1+4.5</td>
<td>41.4+4.9</td>
<td>0.8+0.3</td>
</tr>
<tr>
<td>P-value</td>
<td><strong>0.043</strong></td>
<td><strong>0.080</strong></td>
<td><strong>0.061</strong></td>
<td>0.529</td>
</tr>
<tr>
<td>Non-tumoral distant lung (n=21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs</td>
<td>4.9+2.4</td>
<td>39.2+4.9</td>
<td>55.1+5.8</td>
<td>1.1+0.3</td>
</tr>
<tr>
<td>CD4^+Tconv</td>
<td>6.7+1.8</td>
<td>36.3+5.0</td>
<td>55.8+5.9</td>
<td>1.2+0.4</td>
</tr>
<tr>
<td>P-value</td>
<td>0.237</td>
<td>0.678</td>
<td>0.93</td>
<td>0.86</td>
</tr>
<tr>
<td>Lymph node (n=19)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs</td>
<td>8.5+3.1</td>
<td>50.7+5.6</td>
<td>40.6+6.9</td>
<td>0.5+0.12</td>
</tr>
<tr>
<td>CD4^+Tconv</td>
<td>17.5+4.2</td>
<td>45.4+6.2</td>
<td>36.5+8.9</td>
<td>0.5+0.18</td>
</tr>
<tr>
<td>P-value</td>
<td>0.174</td>
<td>0.591</td>
<td>0.268</td>
<td>0.728</td>
</tr>
<tr>
<td>Blood (n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs</td>
<td>7.9+3.8</td>
<td>66.4+6.4</td>
<td>25.4+3.5</td>
<td>0.5+0.3</td>
</tr>
<tr>
<td>CD4^+Tconv</td>
<td>41.7+10.3</td>
<td>46.4+11.8</td>
<td>11.9+3.8</td>
<td>0.8+0.4</td>
</tr>
<tr>
<td>P-value</td>
<td><strong>0.049</strong></td>
<td>0.127</td>
<td><strong>0.049</strong></td>
<td>0.827</td>
</tr>
<tr>
<td>Activation status (panel 1)</td>
<td>CD38</td>
<td>CD40L</td>
<td>CD69</td>
<td>GITR</td>
</tr>
<tr>
<td>Tumor (n=18)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs</td>
<td>47.6+6.1</td>
<td>19.4+7</td>
<td>81.4+2.3</td>
<td>60.3+5.4</td>
</tr>
<tr>
<td>CD4^+Tconv</td>
<td>41.5+4.1</td>
<td>24.8+6.9</td>
<td>74.0+2.6</td>
<td>28.1+3.9</td>
</tr>
<tr>
<td>P-value</td>
<td>0.393</td>
<td>0.174</td>
<td><strong>0.05</strong></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Non-tumoral distant lung (n=18)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs</td>
<td>25.2+4.8</td>
<td>19.3+7.9</td>
<td>51.8+4.3</td>
<td>34.4+5.7</td>
</tr>
<tr>
<td>CD4^+Tconv</td>
<td>21.1+3.2</td>
<td>23.5+7.5</td>
<td>47.2+5.4</td>
<td>8.3+2.5</td>
</tr>
<tr>
<td>P-value</td>
<td>0.8</td>
<td>0.359</td>
<td>0.393</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>Lymph node (n=12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs</td>
<td>44.7+6.3</td>
<td>13.2+8.0</td>
<td>73.7+2.4</td>
<td>26.6+4.0</td>
</tr>
<tr>
<td>CD4^+Tconv</td>
<td>42.8+5.4</td>
<td>14.2+7.8</td>
<td>48.5+3.2</td>
<td>4.4+1.2</td>
</tr>
<tr>
<td>P-value</td>
<td>0.817</td>
<td>0.564</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs</td>
<td>10.9+1.9</td>
<td>1.4+0.5</td>
<td>6.6+2.4</td>
<td>5.7+2.3</td>
</tr>
<tr>
<td>CD4^+Tconv</td>
<td>21.8+4.6</td>
<td>2.5+1.4</td>
<td>6.5+2.5</td>
<td>1.6+0.4</td>
</tr>
<tr>
<td>P-value</td>
<td><strong>0.05</strong></td>
<td>0.602</td>
<td>0.754</td>
<td>0.175</td>
</tr>
<tr>
<td>Activation status (panel 2)</td>
<td>4-1BB</td>
<td>CD127</td>
<td>ICOS</td>
<td>Ox-40</td>
</tr>
<tr>
<td>Tumor (n=18)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs</td>
<td>33.2+5.6</td>
<td>3.9+1.3</td>
<td>64.9+7.1</td>
<td>45.4+5.1</td>
</tr>
<tr>
<td>CD4^+Tconv</td>
<td>8.6+2.4</td>
<td>24.7+2.9</td>
<td>45.9+6.1</td>
<td>27.3+4.5</td>
</tr>
<tr>
<td>P-value</td>
<td><strong>0.001</strong></td>
<td>&lt;0.001</td>
<td>0.029</td>
<td><strong>0.007</strong></td>
</tr>
<tr>
<td>Non-tumoral distant lung (n=17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs</td>
<td>27.0+5.5</td>
<td>2.5+1.5</td>
<td>40.7+6.9</td>
<td>34.4+5.5</td>
</tr>
<tr>
<td>CD4^+Tconv</td>
<td>2.9+1.1</td>
<td>27.2+4.9</td>
<td>15.4+3.6</td>
<td>9.4+2.5</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.009</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lymph node (n=11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tregs</td>
<td>7.3+2.0</td>
<td>1.1+0.4</td>
<td>43.8+7.2</td>
<td>17.5+2.5</td>
</tr>
<tr>
<td>CD4^+Tconv</td>
<td>0.6+0.2</td>
<td>28.1+4.9</td>
<td>16.5+6.4</td>
<td>11.4+5.7</td>
</tr>
<tr>
<td>P-value</td>
<td><strong>0.001</strong></td>
<td>0.001</td>
<td>0.02</td>
<td>0.061</td>
</tr>
</tbody>
</table>
Supplementary Table 5: Frequency of the main stages of differentiation of Tregs and CD4⁺ Tconv in different sites of NSCLC patients.

Fresh tumor and non-tumoral tissue specimen were obtained from a total 34 NSCLC patients. The cells were used for the four different multicolor flow cytometry panels. The stage of T cell differentiation was determined based on the differential expression of CD45ra, CCR7, CD27, and CD28 (naïve T cells: CD45ra⁺CCR7⁺, CM T cells: CD45ra⁻CCR7⁺, EM T cells: CD45ra⁻CCR7⁻ and TEMRA T cells: CD45ra⁺CCR7⁻). The results are expressed as a percentage (mean±SEM) of Tregs positive for a giving marker/total Tregs, and a percentage of CD4⁺ Tconv positive for a marker/total CD4⁺ Tconv. Wilcoxon Rank test. *: P<0.05, **: P<0.01, ***: P<0.001. P values significant are highlighted and underlined in the table. Abbreviations: CM, central-memory; EM, effector-memory; TEMRA, terminally effector-memory CD45ra⁺ T cell.
Tregs in advanced stage chemotherapy treated lung cancer patients

It has been found that the immune microenvironment is modulated in cancer patients treated with chemotherapy. Chemotherapy may work using three mechanisms that is first by targeting the immune system to reduce tumor induced immune suppressive cells; targeting the tumor to increase immunogenicity via increasing the expression of the antigens or MHC and the last is by directly stimulating the effector T cell responses by activating the T cells. Drugs like cyclophosphamide had showed the inhibitory effects on Tregs (Ghiringhelli et al. 2004; Lutsiak et al. 2005; Becker, Schrama 2013). Also it has been found in the colon carcinoma mouse model that the low dose of the gemcitabine depletes the Tregs and improve the T cell mediated anti-tumor immunity (Tongu et al. 2013).

In parallel to the early stage lung cancer patients, we also studied the phenotypic status of the Tregs in the neo-adjuvant chemotherapy (NAC) treated advanced lung cancer patients (n=22). Patients with advanced NSCLC (stage IIIB-IV), receive a NAC (Cisplatin plus gemcitabine or carboplatin plus paclitaxel).

Method:

For the flow cytometry experiments please check the methods section of the manuscript attached before. The antibodies used for the flow cytometry are mentioned in the supplementary table 4

Results:

No Significant difference observed between the percentage of Tregs infiltrating the NAC treated and untreated lung tumors

Although the percentage of Tregs among total CD4+ T cells was heterogenous from patient to patient in NAC treated patients (same as observed in the NAC untreated patients), the percentage of Tregs was found to be always higher in the tumors in comparison to the NTDL, LN and blood (Fig 14).
We observed that, eventhough the tendency of the percentage of Tregs infiltrating the NAC treated lung tumors was lower (n= 16; 9.335 %) than the NAC untreated patients (n=31; 14.2%); there was no statistically significant difference between the these two conditions tested. Also, the similar tendency observed in case of the lymph node of the NAC treated patients with less percentage of Tregs (n=23; 4.04%) compared to NAC untreated patients (n=5; 5.9%). The % Mean ± SEM values also tested but observed to be insignificant.

**NAC treated lung tumor infiltrating Tregs bear similar activation and immunosuppression profile to the Tregs from NAC untreated lung tumors**

Flow cytometry analysis allowed us the study the potential concomitant expression of the studied markers and their comparison with the NAC treated and untreated patients. Thus, the multi-analysis of CD38, CD40L, CD69, and GITR expression shows that the percentage of Tregs, NAC treated patients negative for all these molecules found to be 12.5 % in the NAC treated patients in comparison to the 8% in the NAC untreated patients (Fig.15A) The percentage of Tregs expressing more than two markers out of all these markers (CD38, CD40L, CD69, and GITR) were similar (60%) in the NAC treated and untreated patients. The most preferentially expressed combinations in both the cases were CD69 and GITR. Tregs expressing the more than one marker among 4.1BB, OX-40 and ICOS were 75% in NAC treated and untreated lung tumors. ICOS was the predominantly expressed marker in all the combinations in NAC treated and untreated patients (Fig.15B). The proportion of the Tregs expressing the combination of 5 or 4 ICP molecules (CTLA-4, TIGIT, PD1, Tim3 and LAG3) found to be increased from 15% in NAC untreated patients to the 25% in the NAC treated patients. Percentage of the LAG-3 could be found as the influencing this increase. TIGIT, then Tim-3 or CTLA4 was found to be sequentially expressed by the Tregs in NAC treated and untreated patients (Fig15C).

**Tregs circulating the NAC treated patients blood show significant difference in activation status than the NAC untreated patients**

Although the percentage of activated Tregs in the blood were found to be always less than the tumor in NAC untreated patients, we observed significantly different
scenario in the blood of the NAC treated patients. The multi-analysis of CD38, CD40L, CD69, and GITR expression shows that the 45% of Tregs in blood of NAC treated patients expressed one or more activation marker with respect to the 22% of the Tregs from blood of the NAC untreated patients. Predominantly, CD38 expression was followed by the CD69 and GITR (Fig. 16A). Expression of the OX-40, ICOS and 4.1BB was remarkably higher in the blood Tregs from NAC treated patients (20% all positive) versus NAC untreated patients (5% all positive). Like in tumor ICOS+ Tregs were dominantly present in the blood of the NAC treated patients (Fig.16B). Percentage of the negative Tregs for ICP expression observed to be 55% in NAC untreated patients in contrast to the 22% in the NAC treated patients. And the TIGIT was the dominant molecule expressed by Tregs, followed by the CTLA-4 and LAG-3 in the NAC treated patient’s blood.

Figure 14: comparison of the Tregs in NAC treated and NAC untreated patients

Frequency of CD3⁺CD25⁺FoxP3⁺ cells among total CD4⁺ T cells in tumor (n=16), non-tumoral distant lung (NTDL, n=16), LN (n=5), and peripheral blood (n=9). The data represented as the median % of the Tregs in each tissue. Mann-Whitney U test was used. *: P<0.05, **: P<0.01, ***: P<0.001, ns –non significant.
Figure 15: Activation and immunosuppression profile of the Tumor Tregs in the NAC treated and untreated patients

Protein level expression of the activation and immunosuppressive markers was tested by flow cytometry. The percentage of the Tregs expressing the combination of 1 to 4 different activation markers (CD38, CD40L, CD69, and GITR; panel A), the combination of 1 to 3 other activation markers (4-1BB, ICOS, and OX40; panel B), and the combination of 1 to 5 different ICP (CTLA4, LAG3, PD1, TIGIT, and Tim3; panel C) or no expression of any of these markers is shown by the each pie chart for lung tumors from NAC treated patients (n=16) and NAC untreated patients (n=18). The gray colored potions of the pie chart represents the different combinations of the markers expressed by the proportion of Tregs ranging from 5 (expression of all markers) to 0 (no expression of any marker) marker. Each colored arc represents the single marker, and thus different colored arcs on the top of the pie chart show the distribution of the cells expressing different markers in the combinations.
Figure 16: Activation and immunosuppression profile of the Blood circulating Tregs in the NAC treated and untreated patients

Protein level expression of the activation and immunosuppressive markers was tested by flow cytometry. The percentage of the Tregs expressing the combination of 1 to 4 different activation markers (CD38, CD40L, CD69, and GITR; panel A), the combination of 1 to 3 other activation markers (4-1BB, ICOS, and OX40; panel B), and the combination of 1 to 5 different ICP (CTLA4, LAG3, PD1, TIGIT, and Tim3; panel C) or no expression of any of these markers is shown by the each pie chart for lung tumors from NAC treated patients (n=9) and NAC untreated patients (n=12). The gray colored portions of the pie chart represents the different combinations of the markers expressed by the proportion of Tregs ranging from 5 (expression of all markers) to 0 (no expression of any marker) marker. Each colored arc represents the single marker, and thus different colored arcs on the top of the pie chart show the distribution of the cells expressing different markers in the combinations.
D. Discussion

The tumor microenvironment is a complex network which plays important role in the growth of cancer cells. Humans develop spontaneous immune responses against the tumors (Schreiber et al. 2011). Immune compartment of the tumors is composed of several components with the different functions. In solid cancers, the different types of the immune cells interacting with each other as well as tumor cells play important role in the shaping of the tumor microenvironment. It has been demonstrated that the memory T cells, CD8\(^+\) T cells, and Th1 T cells are crucial for the defense against the tumor development (Fridman et al. 2012). In our team, we have strong arguments in NSCLC suggesting that the anti-tumor responses can be generated outside the lymph nodes, in the tumor microenvironment in ectopic lymphoid In particular, the presence of mature DC and B cells in TLS, and CD8\(^+\) T cells is beneficial for the survival of the lung cancer patients (Dieu-Nosjean et al. 2008; Goc et al. 2014b; Germain et al. 2014a).

In my study, I demonstrated the presence of the Tregs in TLS and non-TLS, and I clearly deciphered the phenotype of these cells infiltrating the lung tumors. I provide hereby an original evidence of the recruitment of the Tregs in TLS and non-TLS areas of the lung tumors. I showed that Tregs express several activation, immunoregulatory, and ICP molecules in the lung cancer microenvironment, and thus may affect the anti-tumor responses generated in TLS.

TLS in lung tumors: centers of the protective immune responses

The current hypothesis is that the immune responses in the lung tumors are generated in TLS. The B-cell zone of TLS is composed of the proliferating B cells and FDC, and represents an active area of the ongoing humoral response. Interestingly, the density of TLS-B cells correlates with density of plasma cells, the later producing antibodies IgA and IgG against tumor antigens in lung cancers (Germain et al. 2014a). The T cell zone of the lung tumors is composed of the large density of the CD3\(^+\) T cells, especially CD4\(^+\) T cells and exclusively infiltrating mature DC. Thus, TLS-mature
DC may act as the antigen-presenting cells and play important role in the education of the T cells infiltrating tumors. These mature DC express at least CCL19 which recruits CCR7$^+$ T cells to the lung tumors. The HEV co-localized with the TLS give an evidence of the recruitment of the different immune cells via CD62L-PNAd interaction (Chaisemartin et al. 2011). In our team, we have observed that TLS-infiltrating T cells express exclusively the CD62L marker. Further, it was observed that the TLS play important role in the shaping of the cytotoxic CD8$^+$ T cell and Th1 responses, and correlate with high density of the CD8$^+$ T cells (Goc et al. 2014b).

**Infiltration, activation of Tregs in cancer microenvironment**

The cytokine and chemokine milieu in the tumor microenvironment is responsible for the infiltration and the activation of the different immune cells and in this case, Tregs in cancer microenvironment. First with the help of the immunohistochemistry staining, we observed that CD3$^+$FoxP3$^+$ T cells infiltrate different areas of the tumors. Further, with the help of the flow cytometry, we confirmed the CD3$^+$FoxP3$^+$ cells as the Tregs described in the literature (Sakaguchi 2003; Fontenot et al. 2005a; Wu et al. 2006; Liu et al. 2006), carrying the phenotype CD3$^+$CD4$^+$CD25$^{hi}$ FoxP3$^+$CD127$^{low}$. We observed that Tregs infiltrate importantly in the stroma and the TLS of the lung tumors. Also, we observed that Tregs infiltrate predominantly in the tumors in comparison to the other non-tumoral tissues of the lung cancer patients. The number of Tregs has been found to be increased in several malignancies (Hiraoka et al. 2006; Jang 2010; Kono et al. 2006).

Thus, it can be speculated that the different chemokines expressed by the other immune cells and the augmentation of the chemokine production in tumors, could be responsible for the infiltration of the high number of the Tregs. In our team, gene expression data showed that CCL9, CXCL13, CCL21, IL-16, CCL22, and CCL17 are overexpressed in TLS compared to tumor nests which may be involved in the recruitment of naïve and memory T cells including Tregs from blood to TLS through PNAd+ HEV (Chaisemartin et al. 2011; Goc et al. 2014b; Girard, Springer 1995). The CCL17 and CCL22 produced by plasma cells and macrophages attract the T helper and importantly Tregs in the tumor microenvironment (Fridman et al. 2012).
Here, we confirmed that the Tregs in lung tumors express the chemokine CCL22 and receptors like CCR4, CCR5, CCR8 which could be their potential way to infiltrate the tumors. We observed expression of the CXCR3 on the TLS Tregs which could be responsible for their infiltration in the inflammatory site. It has been observed that CXCR3+ T-bet+ Tregs selectively accumulate in the ovarian tumors and suppress the type I responses (Redjimi et al. 2012). Although there are several studies carried out in mice and humans regarding the expression of the CCR4 by Tregs (Kang et al. 2010; Qin et al. 2009; Toulza et al. 2010; Zielinski et al. 2011). Interestingly, we observed that Tregs express high fold CCR8 in comparison of the CCR4. There are evidences showing the expression of the CCR8 linked to the migration of Tregs to inflammatory sites (Iellem et al. 2001; Wang et al. 2012; Soler et al. 2006). There are other chemokines involved in the recruitment of the Tregs in tumors but expression of the chemokine receptor by Tregs ultimately depends on the presence of attracting ligands in the scenario. The CXCL12 produced in the tumor microenvironment possibly attracts the CXCR4+ Tregs (Wei et al. 2007; Jaafar et al. 2009). Hypoxic tumor microenvironment with presence of the CCL28 also found to be attracting the CCR10+ Tregs (Facciabene et al. 2011).

We observed that, around quarter of the total Tregs express CD62L, and thus infiltrate the TLS of the lung tumors. As CD62L was found to be the specific marker of immune cells infiltrating TLS (except for germinal B cells) via the PNdod+ HEVs, further we used this marker to discriminate Tregs in TLS and non-TLS Tregs. Based on the differential expression of the CCR7 and CD45ra, Tregs infiltrating tumors mainly showed the central memory and effector memory phenotype, this was also found to be similar to the Tregs in distant lung. There are very few naïve Tregs observed in the lung tumors in comparison to CD4+ Tconv cells. Whereas in draining lymph node and the blood of NSCLC patients, there were comparatively more naïve Tregs observed. In TLS and non-TLS areas of the lung tumors, we observed that Tregs are mainly CM and EM1 (CCR7+CD45ra-CD27+CD28+), thus it can be said that Tregs are in early differentiation phase compared to the CD4+ Tconv cells but when further we observed the expression of the different activation and ICP expressed by the Tregs, it confirmed that Tregs are activated and may exhibit the immunosuppressive
functions. Since it has been observed that constant TCR signaling is important for the maintainance, differentiation, and activation of the Tregs thus it could be guessed why possible the tumor Tregs constitutively express the CD28 (Tang et al. 2003; Josefowicz, Rudensky 2009). It has been also observed that TCR/CD28 signaling is important for the expression of the transcription factor FoxP3 which is important for the lineage stability of the Tregs (Zhang et al. 2013). In periphery, Ly-6C negative Tregs rely on the strong TCR signaling for their immunosuppressive functions (Delpoux et al. 2014).

The Genetic and protein level expression of the activation markers like CD25, CD38, CD69 and GITR by Tregs suggests that Tregs in TLS and nonTLS areas of the tumors are activated and they may exhibit contact dependent immunosuppression by expression of the ICP like CTLA-4, PD1, Tim3, LAG-3 and TIGIT.

We observed high expression of the membranous and soluble form of CTLA-4 by Tregs in comparison to the CD4+ Tconv cells in TLS as well as nonTLS areas of the tumor. There are two ways by which CTLA-4 can down regulate the T cell activation. Inhibition of the IL-2 production by CTLA-4 is driven by the soluble form whereas the B7 mediated co-inhibition requires the membranous CTLA-4 expression (Carreno et al. 2000). Deletion of the clones of activated T cells by CTLA-4 requires the diminished TCR signaling and less IL-2 production (McCoy, Le Gros 1999). Membranous CTLA-4 expression by T cells acts as the physical barrier for the TCR mediated T cell activation because of the high affinity of the CTLA-4 than the CD28. Also it has been demonstrated that CTLA-4 ligation with CD80 or CD86 on the APC leads to the IDO expression by the APC indirectly inhibiting the conventional T cells (Onodera et al. 2009). Thus it be predicted that the Tregs expressing the high levels of the CTLA-4 may use this mechanism of action against the APC and Tconv in the lung tumors.

PD-1 and its ligands, PDL1 and PDL2 are up regulated in Tregs infiltrating the lung tumors. In literature it has been found that PD-1 is highly expressed by the Tregs which could be possible mechanism of their activation and enhancing their proliferation when engagement with the PD1 ligands (Freeman 2000). SHP2
mediated inhibition of the kinases upon activation and PD-1 expression by the T cells is the key mechanism of the intrinsic regulation. The expression of the PD-1 and Tim-3 by the Tregs led to the antigen dependent interaction with APC and down-regulation of the CD80 and CD86 on the APC in colorectal cancer mouse model (Bauer et al. 2014). PD-1 is not only expressed by the T cells but other immune cells like B cells (Velu et al. 2009) and also it is observed to be expressed by the tumor cells (Dong et al. 2002; Zou, Chen 2008) thus suggesting the interaction with ligands expressed on T cells and attracting the T cells to the tumor. This could be possible explanation why along with the PD-1 expression we observe the high expression of the PDL2 by Tregs in the tumors in comparison to the lymph node of the lung cancer patients.

Several co-stimulatory markers were also observed to be expressed by the Tregs like TNFR2, 4.1BB, Ox-40 and ICOS which suggests their strong activation in the tumor microenvironment. Since expression of these ICP markers was found to be exclusively high in the tumor Tregs compared to the blood Tregs. It can be imagined that Tregs exclusively get activated and functional in tumor microenvironment. We could not found the difference in the expression of genes in Tregs from tumor and non-tumoral distant lung. Since tumor induced Tregs may circulate in the distant lung nearby it can possible reason why we could not see the difference in the gene expression.

**Anti-inflammatory role of Tregs in lung cancer**

The gene expression study showed us different set of genes overexpressed by Tregs compared to CD4+ T conv. cells, and possibly involved in the regulation of the inflammation in tumors.

We observed that, Ti-Tregs expressed the immunosuppressive cytokines IL-10 and TGF-β which could regulate the Th1 and Th2 responses. Remarkably, expression of IL-10 was significantly higher than the TGF-β. Expression of the latency associated peptide (LAP) was found to be higher in the CD4+ Tconv cells in comparison to the Tregs showing the possibility latent inactive form of the TGF-β in conv T cells.
Expression of the IL-10 by Tregs was also found to be suppressing the Th17 activity which could possibly inhibit anti-inflammatory responses (Stewart et al. 2013). CD39+ Tregs expressing the IL-6 and LAP were found to be suppressing the Th17 cells in pleural infusion (Ye et al. 2011). Thus this could suggest the role of these cytokines in the control of inflammation in the lung tumor microenvironment.

TLS Tregs expressed high levels of the IL-6 which could be playing anti-inflammatory role leading to the expression of the IL-1rα and IL-10 by Tregs. Along with the expression of the IL-6 in TLS Tregs, we also observed the expression of the IL-10, IFN-α, IL-27. Also it has been observed that IL-27 expression by Tregs limits the IL-17 production by the Th17 cells in the infection circumstances and the mucosal immunity (Hirahara et al. 2012; Do et al. 2015). IL-27 has found to be developing the Tregs for specialized anti-Th1 functions at local infection sites (Hall et al. 2012).

We observed that Tregs express high levels of the IRF4, GATA-3, IL-27, IL10, and ILRα which possibly could explain their anti-Th17 role in the lung cancer microenvironment.

Along with FoxP3, different transcription factors are demonstrated playing important role in the immunosuppressive functioning of the Tregs. IRF4 is involved in the optimum suppressive function of the Tregs in mice model (Levine et al. 2014). Blimp1 mediated expression of the IRF4 induces the IL10 production and function of the effector Tregs (Cretney et al. 2011). IRF4 plays important role in the regulation of the differentiation of Th2 and Th17 and thus expressed at high levels by FoxP3+ Tregs. Another transcription factor GATA3 has role in the Tregs homeostasis in the inflammatory sites (Wang et al. 2011) (Wohlfert et al. 2011). It has been observed that Tregs expressing the T-bet and Stat-3 are involved in the suppression of the Th1 and Th17 immunity (Chaudhry et al. 2009; Chaudhry, Rudensky 2013; Koch et al. 2009). We observed expression of the T-bet and GATA-3 by Tregs which is possible because Tregs co-opt the transcriptional program of the Th1 and Th17 cells to inhibit inflammatory environment to reduce the anti-tumor responses.

Surprisingly, we observed that conventional T cells in the TLS and nonTLS areas of tumor, express high levels of the CD40L and thus it can be suspected that Tconv cells...
may use the CD40- CD40L interaction to ligate with the APC in order to get the co-
stimulatory signal for activation. This can be an escape mechanism used by T cells to
avoid the Treg mediated suppression like it has been demonstrated by the CD4 T
cells in the influenza infection (Ballesteros-Tato et al. 2013).

**Tregs in TLS: Proponents or opponents?**

A critical issue as yet inadequately addressed is where Tregs exert their
immunosuppressive function. Is the presence of Tregs in TLS regulates immune
responses at initial stages of T cell activation? And/or is it at the effector site?

Some data suggests that Tregs efficiently prevent priming of naïve T cells by their
presence in lymphoid tissue (Annacker et al. 2001). Since DC contribute to the
formation and maintenance of TLS (GeurtsvanKessel et al. 2009; Halle et al. 2009)
education of the T cells, probably alteration in Treg migration may affect TLS
formation. Differential expression of chemokine receptors, not only affect the tissue
or inflammation specific migration but also direct Tregs to different types of APC. B
cells can recruit Tregs via CCL4 expression and depletion of CCL4 or Tregs in mice can
lead to the formation of the autoantibody to IgG (Bystry et al. 2001).

The first study to directly implicate the role of Tregs in lymphoid structure arose
from the observation that CCR7-/- mice spontaneously develop BALT (Bronchus-
Associated Lymphoid Tissues) from age of day 5 in absence of infection. BALT
development was caused by alterations of hematopoietic system in CCR7 deficient
mice. Along with this, the number of Tregs in draining lymph node was observed to
be dramatically reduced indicating that BALT formation caused by disabled *in situ*
function of Tregs (Kocks et al. 2007).

The poor Treg activity or low Treg numbers results in the formation of ectopic
lymphoid tissue in the autoimmune systems. In GVHD and bone marrow
transplantation, CD62L⁺ Tregs were proved to be more suppressive in the secondary
lymphoid organs than CD62L⁻ Tregs (Taylor et al. 2004). However, the role of Tregs in
the prevention of autoimmunity as well as in TLS development is still unclear (Foo,
Phipps 2010; Carragher et al. 2008).
In case of tumor microenvironment, TLS formation has been found to be influenced by the presence of Tregs. Tregs depletion in the mouse bearing MCA-induced tumor leads to the increased development of the HEV, T cell infiltration, and tumor destruction (Hindley et al. 2012). This effect was also observed in breast tumors where, it was observed that although the HEV can be developed in presence of Tregs, their density is influenced by the ratio of the Tregs to T cells infiltrating the tumors (Martinet et al. 2013). But so far, it is scarcely demonstrated that, the infiltration of Tregs in lymphoid follicles is associated with the clinical outcome of the cancer patients. In colonic mucosa, FoxP3+ Tregs in lymphoid follicles is associated with poor clinical outcome. The size of germinal centers and number of Tregs is inversely correlated (Salama et al. 2012). In primary breast tumors, the CCL17/CCR4 mediated recruitment of Tregs in lymphoid aggregates is associated with poor clinical outcome whereas, presence of Tregs in the tumor beds is not well associated with the prognosis of the patients (Gobert et al. 2009).

It has been demonstrated that Tregs migrate to the germinal centers upon T cell activation and suppress the Th and B responses leading to decreased CXCL13 and Ig production (Lim et al. 2004). A subset of Tregs called “follicular Tregs” expressing FoxP3 and Blimp was found to regulate the germinal center response by suppressing the T\(_{FH}\) cells and B cells (Linterman et al. 2011). These follicular Tregs express the BCL-6 transcription factor which is also needed for the developmental program of the T\(_{FH}\) cells. It has been found that the precursors of these cells are the natural CXCR5- FoxP3+Tregs (Chung et al. 2011b; Wollenberg et al. 2011). We observed very few Tregs infiltrating the germinal centers of the TLS and thus the main immunosuppressive function of Treg may not be via a direct cell to cell contact in B cell follicles.

But contrasting to the idea of immunosuppression, it has found in few cases that Tregs helps functioning of the lymphoid aggregates and mounting of the humoral responses. In recent study it has been demonstrated that Tregs regulate the T\(_{FH}\) cells and germinal center responses in influenza infection. Treg depletion prevents formation of GC and it is thought to be due to the increased availability of IL-2 (otherwise consumed by Tregs) which suppress the T\(_{FH}\) differentiation and as a
consequence the germinal center B cell response (León et al. 2014). In another study, it's observed that CD4⁺FoxP3⁺ Tregs differentiate into T_{FH} cells and induce GC formation and local IgA synthesis in gut Peyers patches (Tsuji et al. 2009). In mucosal immunity it is observed that Tregs help in mounting the cholera toxin specific antibody production by B cells (Vendetti et al. 2010).

All these findings in the lymph nodes, inflammation-induced lymphoid aggregates generated several new questions regarding the role of Tregs in TLS. It has not addressed so far the role of the Tregs in the tumor-induced functional TLS.

In a very recent study Joshi and Colleagues from Tyler Jacks laboratory used an impressive genetically engineered mouse model forming lung adenocarcinomas to demonstrate the immunosuppressive role of the Tregs in lung tumor associated TLS. This study shows that Treg depletion in the lung tumor bearing mice, improves the anti-tumor response and infiltration of the tumor antigen specific T cells in the tumor and thus destruction of the tumors via the protective response generated in the TLS (Joshi et al. 2015).

Although this study directly provides the evidence of benefit of depletion of Tregs during the formation of the protective anti-tumor responses, in literature, there are not strong evidences about the role of Tregs in the human lung cancer patients. The questions needed to be addressed in human lung cancer patients, are, do Tregs suppress the anti-tumor responses generated in the TLS? Does the presence of Tregs reflect into impaired B cell responses or well-functioning of the germinal centers? Does Treg and DC interaction affect the antigen presentation in TLS, and in consequence inadequate education of the antigen-specific anti-tumor T cells?

To address these questions in lung cancer, we hypothesised that, A comprehensive analysis of the balance between several cytokine patterns, combinations of chemokines and receptors expressed by tumor infiltrating Tregs may help us to address some of unanswered questions about this split personality of Tregs in TLS. And thus, we determined the 120 selected set of the genes expressed by the Treg in TLS and nonTLS areas of the tumor. For this, we used an advanced technology called nCounter technology which is developed by the Seatle, USA based company named
Nanostring technologies. This technique is based on the principle of the reading the molecular barcodes and imaging the single molecules to count the hundreds of the transcripts in the given set of the reactions. I found this technique appropriate for our set of experiments due to its sensitiveness and easy to use methodology. The amount of the RNA acquired from the Tregs was in limited quantities due to the less numbers of Tregs in TLS and nonTLS areas of the tumors. Thus, this technique helped to perform the expression quantification in the small quantities of RNA using single cell quantification approach. Also the primers used for this technique were closely binding long length primers and thus very useful for even the degraded RNA to analyze the gene expression. This technique has also proven to be useful for the retrospective studies using FFPE tissue samples.

Since, the majority of the T cells infiltrating the TLS were observed to be CD62L+ in the Immunofluorescence staining performed on paraffin embedded NSCLC tissues (Chaisemartin et al. 2011), it is considered the CD62L as specific marker for the T cells infiltrating the TLS. Thus, the Tregs and Tconv cells sorted based on their location inside (CD62L+) and outside (CD62L-) TLS.

Depending on the expression of the CD62L marker on the Tregs we observed that Tregs express the few genes differentially. Since Tregs express CXCR3, CCR8, CCR4 and CCL22 chemokines, we speculate that, Tregs may get recruited in the tumor microenvironment and especially in TLS via these chemokines and ligands interactions.

Tregs in TLS expressed high levels of the IL-6, IL10 and IL-27 in comparison to the Tconv cells. It has been found that IL-6 and IL-21 are important cytokines involved in the differentiation of the Tfh cells (Eto et al. 2011). IL-27 has found to be negatively regulating the Th1 responses. We observed the Tregs in TLS express markers mCTLA4, sCTLA4, GITR, TNFR2,Helios, OX-40, CD39, LAG-3, IL-10, IFN-α, IL-6, Tim-3 more than the TLS conventional CD4+ T cells. Whereas the nonTLS Tregs highly express the molecules GATA-3, PDL2, ICOSLG, FoxA1, IL-10, LAG-3, GARP, 4-1BB in comparison to the nonTLS CD4+ Tconv cells. We noticed less difference in general in gene expression profile of the TLS Tregs versus non TLS Tregs. This means that
cytokines, co-stimulatory and co-inhibitory molecules like IL10, TGF-β, 4-1BB, Ox-40, TNFR2, PDL1, CTLA-4 and TIGIT are constitutively expressed by Tregs in the TLS and nonTLS sites of the tumor. In contrast, we observed that conventional CD4 T cells in tumor expressed more number of genes differentially in TLS vs nonTLS areas of tumors, in comparison to the Tregs. There is significant difference in the set of genes highly expressed by conv CD4 T cells outside the TLS (IFNγ, granzymes A and B, PD1, FASL, Tim3, LAG3) than the genes expressed by CD4 T cells in the TLS (ICOSL, BCL2, STAT6, FOXO1, SOCS3, SATB, CCR7, CD62L).

Thus, it shows that Tregs may potentially get sooner activated with expression of the vast repertoire of activation markers and may potentially compete with the CD4 T cells in TLS for their activation.

**Expansion of the specific subsets of Tregs in TLS**

In a recent collaborative study between MedImmune and our team, with the help of the new generation sequencing (NGS), we also demonstrated that lung tumors bear the highly diverse subsets (less clonality) of the CD4\(^+\) T cells and CD8\(^+\) T cell subsets. The TCR repertoires of the CD4\(^+\) T cells and CD8\(^+\) T cells correlated with the density of B cells and mature DC in TLS. Since CD4\(^+\) T cells in TLS are in close contact with the mature DC and thus may provide the active site for the priming, differentiation, and proliferation of naïve T cells into well differentiated subsets, which could be the reason why the clonality (and thus less diversity) of the CD4\(^+\) T cells in the tumors compared to the peripheral compartment (Zhu et al. 2015). The similar results has been also demonstrated in the other cancer types also. Heterogeneous expansion of CD4\(^+\) T cell subsets has been also observed in the ccRCC (Zhang et al. 2015) and the gastric cancer (Jia et al. 2015).

Although the TLS help in the generation of the anti-tumor responses the infiltration of the Tregs in the TLS may affect this function. Since, we observed that Tregs infiltrating in the lung cancer and especially in the TLS, are mainly the CD4\(^+\) Tregs and not CD8\(^+\) T cells, we can suspect that there can be specific clones of the Tregs
existing in the tumors. Tregs may compete to the other CD4\(^+\) T cells during the priming with the APC especially mature DC in the TLS, and specific clones of Tregs may emerge through antigen driven expansion.

**Modulation of the Treg phenotype in the neoadjuvant chemotherapy treated lung cancer**

It first time observed in the years of the 1980’s that the chemotherapy treatment (cyclophosphamide) affects the suppressor cell population in the patients (Berd, Mastrangelo 1988). Further, it was specifically found that chemotherapy affects the CD4+ CD25+ Tregs. Several *in vitro* and *in vivo* studies carried out with administration of the cyclophosphamide, gemcitabine or combination, demonstrated concondant effect of the depletion of Tregs with increased anti-tumor immunity (Ghiringhelli et al. 2004; Liu et al. 2010; Weir et al. 2011). Since the alkylating agents and platinum compounds directly affect the DNA synthesis, replication and transcription it leads to the cell death. But the exact mechanisms behind the decrease of Tregs are not completely clear. It has been observed that decreased number of Tregs due to neoadjuvant chemotherapy treatment and increased dendritic cells have found to be favorable for the survival of the gastric cancer patients (Min et al. 2014). In breast cancer patients it has been observed that chemotherapy affects the peritumoral Tregs but does not affect the intratumoral Tregs and thus serves the prognosticator in these patients (Liu et al. 2012a).

In my preliminary observations, no significant difference was observed in the % of the Tregs in total CD4 cells in the lung tumors of the NAC treated patients. The cohort of these patients underwent the surgery after 3-6 weeks of the NAC treatment. Since, in this period the immune system possibly reconstitutes its unbalanced composition, it could be possible reason why the NAC treated patients do not show change in the percentage of the Tregs in the tumors as well as the non tumoral tissue compartments. Also this was true regarding the phenotype of the Tregs in the tumor. Tregs infiltrating the tumors of the NAC treated patients do not show significantly different profile than the Tregs infiltrating the tumors from the NAC untreated lung cancer patients. It could be imagined that after the first dose of
the chemotherapy treatment, the window period of 3-6 weeks may allow Tregs in the tumors to get activated and express ICP markers and thus possibly suppress the anti-tumor functions.

Surprisingly, in few NAC treated cancer patients blood we observed significant difference in the activation status of the Tregs than the Tregs circulating the blood of the NAC untreated patient. I observed that Tregs in treated patient express more CD38, CD69, GITR, ICOS and TIGIT.

Since the chemotherapy induces the apoptosis and necrosis of the tumor cells, the debris of the cells circulating in the blood may strongly activate the newly reconstituted T cell population in the blood which could be the possible reason of the presence of activated Tregs in the NAC treated patients blood.

These are the preliminary observations and thus extended study needs with more number of the patients to confirm these results. Also, the prognostic significance of the Tregs in NSCLC patients needs to be determined.

**Prognostic outcome of Tregs in lung cancer patients**

Prognostic importance of the Tregs in cancer has been a debatable issue so far. The phenotype of Tregs, the tumor tissue type, stage of the cancer and most importantly the location of the Tregs in tumor microenvironment may affect the prognostic value of the Tregs in different solid cancers (Badoual et al. 2009; Demir et al. 2013; West et al. 2013).

Prognostic importance of Tregs depending on their location in tumor is rarely described. In ccRCC patients, Li et al. observed that Tregs localized in the peritumoral region are associated with the poor outcome of the patients whereas, intratumoral Tregs have no impact on the survival (Li et al. 2009). In another example, Gobert et al. demonstrated that Tregs in the lymphoid aggregates recruited via CCL22 axis are associated with the poor survival of the patients whereas Tregs in whole tumor didn’t had effect on the survival (Gobert et al. 2009). In colorectal cancer, Tregs in lymphoid follicles were associated with the good survival of the
patients (Salama et al. 2009b). But none of the examples so far in literature
demonstrated the impact of the high density of Tregs in the functional tumor-
induced TLS.

There are two evidences showing the prognostic importance of Tregs in NSCLC.
Petersen and colleagues showed that, in 64 patients of the stage I NSCLC, the high
index of the Tregs is associated with less survival of the patients. With the single
staining of the CD3 and FoxP3 separately, they assigned the score value 0 to 3 based
on the staining intensity and they observed that patients with higher proportion of
the Tregs to Tconv cells had higher risk of the recurrence (Petersen et al. 2006). The
another study in stage III and IV chemotherapy-treated lung cancer patients, it was
observed that high ratio of the CD8+/FoxP3+ tumor-infiltrating T cells was
associated with the better response to the chemotherapy treatment (Liu et al.
2012b).

In this study, not only we had the similar findings but further, we deciphered the
phenotypic status of the Tregs and also their prognostic significance in the tumor-
induced TLS. Here, we show that the Tregs in lung tumor-induced TLS and in whole
tumors, are associated with the poor survival of NSCLC patients. For localization of
the Tregs in TLS, we used the double staining for DC-Lamp/CD3 (to detect the TLS
mature DC) and CD20/CD21 (TLS B cells), which was performed on the serial sections
from same patients. The CD3 T cell zone near the B cell follicles helped us to
determine the zone of the TLS in the invasive margin of the tumors and thus to count
the Tregs (CD3+FoxP3+ T cells) with the help of the software Calopix (developed by
Tribvn, France). This helped to count the number of Tregs inside and outside the TLS.

We also showed that Tregs infiltrate mainly in the stroma among with TLS where
they can be in contact of the different immune cells. When we combined the
densities of the Tregs with TLS-mature DC, TLS-B cells, CD3+ Tconv cells, and CD8+ T
cells, we observed that a group of patients with low density of the TLS mature DC or
TLS-B or CD8+ T cells and the high density of the Tregs had the worst clinical
outcome. Whereas the group of patients with high density of the TLS-mature DC,
TLS-B cells, CD3+ Tconv cells, and CD8+ T cells with low density of the Tregs was
observed to be with a best survival. We also tested the significance of the ratio of the CD3$^+$ T cells, CD8$^+$ T cells and mature DC with Tregs in lung cancer. It was found that the high ratio of the mature DC or total CD3$^+$ T cells or CD8$^+$ T cells was always beneficial for the patients. The median overall survival was always much higher than the single Tregs alone. We found that Group of patients with High or low density of the B cells but with low density of the Tregs had always better clinical outcome in lung cancer patients. Although we observed very few Tregs in the B follicles, and were mainly observed in the TLS T cell areas, it could be speculated that Tregs in these T cell areas regulate the Tfh cells which indirectly affects the humoral responses. The groups with High mDC, B cells and CD8$^+$ T cells with high Tregs showed the intermediate survival because of the presence of the TLS as site for priming the T cells even though the Tregs may exert the putative immunosuppressive mechanisms.

This is significant because the immunotherapy trials showing success in cancer patients due of their dual effect (Pardoll 2012). Modulating the tumor environment by increasing the proportion of the effector T cells, and at the same time decreasing the numbers of Tregs has been shown the promising results so far. Thus, it could be imagined that, the same mechanism could work in the NSCLC patients also.
E. Limitations of this study

It is very important to mention here the limitations of this study to help the appropriate interpretation of the results.

First, the immunostainings or the flow cytometry experiments or the gene expression studies are performed on the tumor specimen resected from lung cancer patients after surgery, thus we cannot determine the impact of the immunoregulation by Tregs during the progression of the disease. We are unable to determine the time point in the tumor progression when the Tregs start infiltrating the tumors and gets activated.

Since, Tregs are very few in the numbers compared to the other T cell subsets in the tumoral and non-tumoral tissues, we could not perform the flow cytometry and gene expression studies in parallel on the same set of patient’s specimen. Thus, the different activation and immunosuppression markers expressed on the Tregs at gene expression and protein level belong to the independent set of the patients. In one way, it is a limitation but in another way, we also confirm our results with two different methods and in two different set of patients.

It has been found that Tregs can also be tumor-antigen specific, and thus may suppress the antigen specific conventional T cells. But we have not yet investigated the antigen specificity of the T cells in the lung tumors although we know the antigen specific antibody production by the TLS-B cells.

Regarding the data about the prognostic impact of the Tregs, we have the data about the overall survival of the patient but we do not have the information about the deaths occurred due to the cancer in patients (disease-specific survival of the patients) and also due to the other causes of the deaths without any residues of the tumor cells (disease-free survival). But collection of these data is in continuation in the team.
F. Conclusion and perspectives

The study carried out in this thesis gives a new aspect of the tumor microenvironment in lung cancer. It gives an immunoregulatory aspect of the Tregs in lung cancer and especially in the tumor-induced TLS. This study suggests that Tregs infiltrating the TLS negatively regulate the protective immune responses generated in the TLS, and thus lead to the poor survival of the lung cancer patients.

It is known that Tregs have an ambiguous role in cancer. Presence of Tregs in the lymphoid aggregates has been found to have a negative impact on the survival of the lung cancer patients, which suggests that Tregs may regulate different immune cells in different areas of tumors.

Since, Tregs express the chemokines CCR8, CCR4 and CCL22 remarkably higher than the conventional CD4$^+$ T cells; it will be interesting to see the effect of the blocking antibodies against these chemokines on the infiltration of the Tregs in tumors among with TLS. It will be interesting to look at the migration of the Tregs in tumors at ex vivo (using the fresh tumor slices migration assay developed by the Emmanuel Donnadieu’s team, Cochin institute, Paris) and also at the in vivo level, using the CCR4-/-, CCR8-/- and CCL22-/- mice models.

Also it can be interesting to investigate the specific clones of the Tregs existing in the tumor microenvironment. With the help of the tetramer staining, it can be interesting to visualize the tumor antigen specific Tregs infiltrating the lung tumors. With the help of the next generation sequencing techniques (RNAseq) the detailed genotype of the Tregs infiltrating the lung tumors can be studied. This can especially help to determine the cellular pathway alterations in the Tregs leading to their activation and immunosuppression potential in lung cancer microenvironment.

Since Tregs have been shown the impact on the TLS formation, does blocking their infiltration into tumor may enhance TLS-dependent immune responses?, is the question needs to be addressed. Experiments on the fresh tumor specimen from lung cancer patients help us to visualize at present status of the qualitative and
quantitative aspect of the immune microenvironment, but, it doesn’t allow us to monitor the different phases evolving during the disease progression. Modulation of the function of Tregs, and effects on their numbers during the disease progression can be effectively studied using the mouse models of lung cancer. Also some questions can be addressed using the mouse models like, at which time point the TLS emerge into the tumors? Does the number of TLS remain same throughout the disease progression? And blocking the infiltration of the Tregs affects the numbers, size and functionality of the Tregs?

We observed that Tregs express the cytotoxic molecules like the GrZA, GrzB, perforin and granulysin and thus it is very important to determine the target cells for the Tregs in order to understand the mechanisms of immunosuppression in the lung cancer immunity. Since, we observed high expression of the CD39 by Tregs whereas CD73 by the Tconv cells, it will be interesting to study this mechanism of the immunoregulation by the pathways in the tumor-induced TLS.

The expression of the CTLA-4 and LAG-3 by Tregs may induce IDO expression in DC, and thus leads to the indirect inhibition of the conventional T cells. It could be important to check the IDO expression by the DC in tumor microenvironment.

In summary, the presence of Tregs has been found to be deleterious for the long-term survival of the NSCLC patients. The different biomarkers studied in the lung cancers like TLS mature DC, TLS-B cells, CD8+ T cells and the Tregs in combinations can be used to stratify the groups based on the high risk of the death. The scoring based on the different combinations may guide the clinicians, surgeons and the therapists to decide the possible best treatment with this group of patients. For this, we need to include more number of patients in this cohort to study the stratification of patients based on proportion of tumor infiltrating TLS mature DC, TLS-B cells, CD8+ T cells and the Tregs in lung cancer.

Blocking the co-stimulatory and co-inhibitory markers using monoclonal antibodies has been rapidly progressed field of the immunotherapy for different solid cancer patients. The molecules like CTLA4, PD-1 are being targeted to modulate and to revert the immunosuppressive effects imposed by the Tregs. In our study, we have
found the high expression of the molecules TIGIT, CTLA4, PDL2, and GITR by Tregs in comparison to the Tconv cells. Thus, it will be interesting to check the effect of blocking these markers on the Tregs and effect on the lung tumor regression.

In future it needs to be determined the possible biomarker(s) which can be used to modulate Treg immunosuppressive functions qualitatively and quantitatively in cancers but also on other diseases.
G. References

Publication bibliography


Aspord, Caroline; Leccia, Marie-Therese; Charles, Julie; Plumas, Joel (2013): Plasmacytoid dendritic cells support melanoma progression by promoting Th2 and regulatory immunity through OX40L and ICOSL. In Cancer Immunol Res 1 (6), pp. 402–415. DOI: 10.1158/2326-6066.CIR-13-0114-T.


Balkwill, Fran; Mantovani, Alberto (2001): Inflammation and cancer: back to Virchow? In The Lancet 357 (9255), pp. 539–545. DOI: 10.1016/S0140-6736(00)04046-0.

Barber, Daniel L.; Wherry, E. John; Masopust, David; Zhu, Baogong; Allison, James P.; Sharpe, Arlene H. et al. (2006): Restoring function in exhausted CD8 T cells during chronic viral infection. In Nature 439 (7077), pp. 682–687. DOI: 10.1038/nature04444.


Bergmann, Christoph; Strauss, Laura; Zeidler, Reinhard; Lang, Stephan; Whiteside, Theresa L. (2007): Expansion of human T regulatory type 1 cells in the microenvironment of cyclooxygenase 2 overexpressing head and neck squamous cell carcinoma. In Cancer Res. 67 (18), pp. 8865–8873. DOI: 10.1158/0008-5472.CAN-07-0767.


Carmenate, Tania; Pacios, Anabel; Enamorado, Michel; Moreno, Ernesto; Garcia-Martínez, Karina; Fuente, Dasha; León, Kalet (2013): Human IL-2 mutein with higher antitumor efficacy than wild type


Cheung, Yeonseok; Tanaka, Shinya; Chu, Fuliang; Nurieva, Roza I.; Martinez, Gustavo J.; Rawal, Seema et al. (2011a): Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. In *Nat. Med.* 17 (8), pp. 983–988. DOI: 10.1038/nm.2426.

Cheung, Yeonseok; Tanaka, Shinya; Chu, Fuliang; Nurieva, Roza I.; Martinez, Gustavo J.; Rawal, Seema et al. (2011b): Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. In *Nature medicine* 17 (8), pp. 983–988. DOI: 10.1038/nm.2426.

Cipponi, Arcadi; Mercier, Marjorie; Seremet, Teofila; Baurain, Jean-François; Théate, Ivan; van den Oord, Joost et al. (2012): Neogenesis of lymphoid structures and antibody responses occur in human melanoma metastases. In *Cancer research* 72 (16), pp. 3997–4007. DOI: 10.1158/0008-5472.CAN-12-1377.


Conrad, Curdin; Gregorio, Josh; Wang, Yi-Hong; Ito, Tomoki; Meller, Stephan; Hanabuchi, Shino et al. (2012): Plasmacytoid dendritic cells promote immunosuppression in ovarian cancer via ICOS costimulation of Foxp3(+) T-regulatory cells. In *Cancer Res.* 72 (20), pp. 5240–5249. DOI: 10.1158/0008-5472.CAN-12-2271.


Cretney, Erika; Xin, Annie; Shi, Wei; Minnich, Martina; Masson, Frederick; Miasari, Maria et al. (2011): The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. In *Nature immunology* 12 (4), pp. 304–311. DOI: 10.1038/ni.2006.

Curiel, Tyler J.; Coukos, George; Zou, Linhua; Alvarez, Xavier; Cheng, Pui; Mottram, Peter et al. (2004): Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. In *Nat. Med.* 10 (9), pp. 942–949. DOI: 10.1038/nm1093.

Curotto de Lafaille, Maria A; Kutchukhidze, Nino; Shen, Shiqian; Ding, Yi; Yee, Herman; Lafaille, Juan J. (2008): Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. In *Immunity* 29 (1), pp. 114–126. DOI: 10.1016/j.immuni.2008.05.010.

Curotto de Lafaille, Maria A; Lafaille, Juan J. (2009): Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? In *Immunity* 30 (5), pp. 626–635. DOI: 10.1016/j.immuni.2009.05.002.


Deaglio, Silvia; Dwyer, Karen M.; Gao, Wenda; Friedman, David; Usheva, Anny; Erat, Anna et al. (2007): Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. In *J. Exp. Med.* 204 (6), pp. 1257–1265. DOI: 10.1084/jem.20062512.


Duan, Minchao; Ning, Zhengqing; Fu, Zhijun; Zhang, Jianquan; Liu, Guangnan; Wei, Qiu; Zheng, Xiaoyu (2015): Decreased IL-27 Negatively Correlated with Th17 Cells in Non-Small-Cell Lung Cancer Patients. In *Mediators of inflammation* 2015, p. 802939. DOI: 10.1155/2015/802939.


Eiichi Sato; Sara H. Olson; Jiyoung Ahn; Brian Bundy; Hiroyoshi Nishikawa; Feng Qian et al. (2005): Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. In PNAS 102 (51), pp. 18538–18543.

Eikawa, Shingo; Ohue, Yoshihiro; Kitaoka, Kenta; Aji, Toshiki; Uenaka, Akiko; Oka, Mikio; Nakayama, Eiichi (2010): Enrichment of Foxp3+ CD4 regulatory T cells in migrated T cells to IL-6- and IL-8-expressing tumors through predominant induction of CXCR1 by IL-6. In Journal of immunology (Baltimore, Md. : 1950) 185 (11), pp. 6734–6740. DOI: 10.4049/jimmunol.1000225.


Eto, Danelle; Lao, Christopher; DiToro, Daniel; Barnett, Burton; Escobar, Tania C.; Kageyama, Robin et al. (2011): IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. In PLoS ONE 6 (3), pp. e17739. DOI: 10.1371/journal.pone.0017739.

Facciabene, Andrea; Peng, Xiaohui; Hagemann, Ian S.; Balint, Klara; Barchetti, Andrea; Wang, Li-Ping et al. (2011): Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T(reg) cells. In Nature 475 (7355), pp. 226–230. DOI: 10.1038/nature10169.
Faget, Julien; Bendriss-Vermare, Nathalie; Gobert, Michael; Durand, Isabelle; Olive, Daniel; Biota, Cathy et al. (2012a): ICOS-ligand expression on plasmacytoid dendritic cells supports breast cancer progression by promoting the accumulation of immunosuppressive CD4+ T cells. In Cancer Res. 72 (23), pp. 6130–6141. DOI: 10.1158/0008-5472.CAN-12-2409.

Faget, Julien; Bendriss-Vermare, Nathalie; Gobert, Michael; Durand, Isabelle; Olive, Daniel; Biota, Cathy et al. (2012b): ICOS-ligand expression on plasmacytoid dendritic cells supports breast cancer progression by promoting the accumulation of immunosuppressive CD4+ T cells. In Cancer Res. 72 (23), pp. 6130–6141. DOI: 10.1158/0008-5472.CAN-12-2409.


Fife, Brian T.; Pauken, Kristen E.; Eagar, Todd N.; Obu, Takashi; Wu, Jenny; Tang, Qizhi et al. (2009): Interactions between PD-1 and PD-L1 promote tolerance by blocking the TCR-induced stop signal. In Nat. Immunol. 10 (11), pp. 1185–1192. DOI: 10.1038/ni.1790.


Galon, Jérôme; Costes, Anne; Sanchez-Cabo, Fatima; Kirilovsky, Amos; Mlecnik, Bernhard; Lagorce-Pagès, Christine et al. (2006): Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. In Science 313 (5795), pp. 1960–1964. DOI: 10.1126/science.1129139.


Ghebeh, Hazem; Barhoush, Eman; Tulbah, Asma; Elkum, Naser; Al-Tweigeri, Taher; Dermime, Said (2008): FOXP3+ Tregs and B7-H1+/PD-1+ T lymphocytes co-infiltrate the tumor tissues of high-risk breast cancer patients: Implication for immunotherapy. In BMC Cancer 8, p. 57. DOI: 10.1186/1471-2407-8-57.


Ghiringhelli, François; Larmonier, Nicolas; Schmitt, Elise; Parcellier, Arnaud; Cathelin, Dominique; Garrido, Carmen et al. (2004): CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. In European Journal of Immunology 34 (2), pp. 336–344. DOI: 10.1002/eji.200324181.

Ghiringhelli, François; Ménard, Cédric; Terme, Magali; Flament, Caroline; Taieb, Julien; Chaput, Nathalie et al. (2005): CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. In The Journal of experimental medicine 202 (8), pp. 1075–1085. DOI: 10.1084/jem.20051511.


Głowala-Kosińska, Magdalena; Chwieduk, Agata; Nieckula, Jarosław; Saduś-Wojciechowska, Maria; Grosicki, Sebastian; Rusin, Aleksandra et al. (2013): Association of circulating regulatory T cell number with the incidence and prognosis of diffuse large B-cell lymphoma. In Eur. J. Haematol. 91 (2), pp. 122–128. DOI: 10.1111/ejh.12144.

Gobert, Michael; Treilleux, Isabelle; Bendriss-Vermare, Nathalie; Bachelot, Thomas; Goddard-Leon, Sophie; Arfi, Vanessa et al. (2009): Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome. In Cancer research 69 (5), pp. 2000–2009. DOI: 10.1158/0008-5472.CAN-08-2360.


Goc, Jérémy; Germain, Claire; Vo-Bourgais, Thi Kim Duy; Lupo, Audrey; Klein, Christophe; Knockaert, Samantha et al. (2014b): Dendritic cells in tumor-associated tertiary lymphoid structures signal a Th1


Govindaraj, Chindu; Scalzo-Inguanti, Karen; Madondo, Mutsa; Hallo, Julene; Flanagan, Katie; Quinn, Michael; Plebanski, Magdalena (2013): Impaired Th1 immunity in ovarian cancer patients is mediated by TNFR2+ Tregs within the tumor microenvironment. In *Clin. Immunol.* 149 (1), pp. 97–110. DOI: 10.1016/j.clim.2013.07.003.


Gu-Trantien, Chunyan; Loi, Sherene; Garaud, Soizic; Equeter, Carole; Libin, Myriam; Wind, Alexandre de et al. (2013a): CD4+ follicular helper T cell infiltration predicts breast cancer survival. In *J. Clin. Invest.* 123 (7), pp. 2873–2892. DOI: 10.1172/JCI67428.

Gu-Trantien, Chunyan; Loi, Sherene; Garaud, Soizic; Equeter, Carole; Libin, Myriam; Wind, Alexandre de et al. (2013b): CD4+ follicular helper T cell infiltration predicts breast cancer survival. In *J. Clin. Invest.* 123 (7), pp. 2873–2892. DOI: 10.1172/JCI67428.

Hall, Aisling O'Hara; Beiting, Daniel P.; Tato, Cristina; John, Beena; Oldenhove, Guillaume; Lombana, Claudia Gonzalez et al. (2012): The cytokines interleukin 27 and interferon-γ promote distinct Treg cell populations required to limit infection-induced pathology. In *Immunity* 37 (3), pp. 511–523. DOI: 10.1016/j.immuni.2012.06.014.

Halle, Stephan; Dujardin, Hélène C.; Bakocevic, Nadja; Fleige, Henrik; Danzer, Heike; Willenzon, Stefanie et al. (2009): Induced bronchus-associated lymphoid tissue serves as a general priming site


Hennequin, Audrey; Derangère, Valentin; Boidot, Romain; Apetoh, Lionel; Vincent, Julie; Orry, David et al. (2015): Tumor infiltration by Tbet+ effector T cells and CD20+B cells is associated with survival in gastric cancer patients. In *Oncoimmunology*, p. 0. DOI: 10.1080/2162402X.2015.1054598.


Hindley, James P.; Jones, Emma; Smart, Kathryn; Bridgeman, Hayley; Lauder, Sarah N.; Ondondo, Beatrice et al. (2012): T-cell trafficking facilitated by high endothelial venules is required for tumor control after regulatory T-cell depletion. In *Cancer Res.* 72 (21), pp. 5473–5482. DOI: 10.1158/0008-5472.CAN-12-1912.

Hirahara, Kiyoshi; Ghoreschi, Kamran; Yang, Xiang-Ping; Takahashi, Hayato; Laurence, Arian; Vahedi, Golnaz et al. (2012): Interleukin-27 priming of T cells controls IL-17 production in trans via induction of the ligand PD-L1. In *Immunity* 36 (6), pp. 1017–1030. DOI: 10.1016/j.immuni.2012.03.024.


Hossain, Dewan Md Sakib; Panda, Abir K.; Manna, Argha; Mohanty, Suchismita; Bhattacharjee, Pushpak; Bhattacharyya, Sankar et al. (2013): FoxP3 acts as a cotranscription factor with STAT3 in tumor-induced regulatory T cells. In Immunity 39 (6), pp. 1057–1069. DOI: 10.1016/j.immuni.2013.11.005.

Huang, Bo; Pan, Ping-Ying; Li, Qingsheng; Sato, Alice I.; Levy, David E.; Bromberg, Jonathan et al. (2006): Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. In Cancer research 66 (2), pp. 1123–1131. DOI: 10.1158/0008-5472.CAN-05-1299.


Joller, Nicole; Hafler, Jason P.; Brynedal, Boel; Kassam, Nasim; Spoerl, Silvia; Levin, Steven D. et al. (2011): Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. In J. Immunol. 186 (3), pp. 1338–1342. DOI: 10.4049/jimmunol.1003081.


Kalia, Vandana; Penny, Laura Anne; Yuzefpolskiy, Yevgeniy; Baumann, Florian Martin; Sarkar, Surojit (2015): Quiescence of Memory CD8(+) T Cells Is Mediated by Regulatory T Cells through Inhibitory Receptor CTLA-4. In *Immunity* 42 (6), pp. 1116–1129. DOI: 10.1016/j.immuni.2015.05.023.


Kang, Myoung Jae; Kim, Kyoung Min; Bae, Jun Sang; Park, Ho Sung; Lee, Ho; Chung, Myoung Ja et al. (2013): Tumor-infiltrating PD1-Positive Lymphocytes and FoxP3-Positive Regulatory T Cells Predict Distant Metastatic Relapse and Survival of Clear Cell Renal Cell Carcinoma. In *Translational Oncology* 6 (3), pp. 282–289. DOI: 10.1593/tlo.13256.

Kang, Shijun; Xie, Jianmin; Ma, Shudong; Liao, Wangjun; Zhang, Junyi; Luo, Rongcheng (2010): Targeted knock down of CCL22 and CCL17 by siRNA during DC differentiation and maturation affects the recruitment of T subsets. In *Immunobiology* 215 (2), pp. 153–162. DOI: 10.1016/j.imbio.2009.03.001.


Ladányi, Andrea; Kiss, Judit; Somlai, Beáta; Gilde, Katalin; Fejos, Zsuzsanna; Mohos, Anita et al. (2007): Density of DC-LAMP(+) mature dendritic cells in combination with activated T lymphocytes infiltrating primary cutaneous melanoma is a strong independent prognostic factor. In Cancer Immunol. Immunother. 56 (9), pp. 1459–1469. DOI: 10.1007/s00262-007-0286-3.

Ladányi, Andrea; Mohos, Anita; Somlai, Beáta; Liszkay, Gabriella; Gilde, Katalin; Fejos, Zsuzsanna et al. (2010): FOXP3+ cell density in primary tumor has no prognostic impact in patients with cutaneous malignant melanoma. In Pathol. Oncol. Res. 16 (3), pp. 303–309. DOI: 10.1007/s12253-010-9254-x.


Li, Jin F.; Chu, Yi W.; Wang, Guo M.; Zhu, Tong Y.; Rong, Rui M.; Hou, Jun; Xu, Ming (2009): The prognostic value of peritumoral regulatory T cells and its correlation with intratumoral


Li, Yaqing; Ren, Meijing; Zhang, Xinmin; Guo, Xiaojing; Lang, Ronggang et al. (2012a): Peritumoral FOXP3⁺ regulatory T cell is sensitive to chemotherapy while intratumoral FOXP3⁺ regulatory T cell is prognostic predictor of breast cancer patients. In *Breast cancer research and treatment* 135 (2), pp. 459–467. DOI: 10.1007/s10549-012-2132-3.


Lohr, Miriam; Edlund, Karolina; Botling, Johan; Hammad, Seddik; Hellwig, Birte; Othman, Amnah et al. (2013): The prognostic relevance of tumour-infiltrating plasma cells and immunoglobulin kappa C indicates an important role of the humoral immune response in non-small cell lung cancer. In Cancer letters 333 (2), pp. 222–228. DOI: 10.1016/j.canlet.2013.01.036.


Man, Kevin; Miasari, Maria; Shi, Wei; Xin, Annie; Henstridge, Darren C.; Preston, Simon et al. (2013): The transcription factor IRF4 is essential for TCR affinity-mediated metabolic programming and clonal expansion of T cells. In Nature immunology 14 (11), pp. 1155–1165. DOI: 10.1038/ni.2710.


Martin-Orozco, Natalia; Li, Yufeng; Wang, Yijun; Liu, Shijuan; Hwu, Patrick; Liu, Yong-Jun et al. (2010): Melanoma cells express ICOS ligand to promote the activation and expansion of T-regulatory cells. In Cancer Res. 70 (23), pp. 9581–9590. DOI: 10.1158/0008-5472.CAN-10-1379.


McHugh, Rebecca S.; Whitters, Matthew J.; Piccirillo, Ciriaco A.; Young, Deborah A.; Shevach, Ethan M.; Collins, Mary; Byrne, Michael C. (2002): CD4+CD25+ Immunoregulatory T Cells. In Immunity 16 (2), pp. 311–323. DOI: 10.1016/S1074-7613(02)00280-7.
Milne, Katy; Köbel, Martin; Kalloger, Steven E.; Barnes, Rebecca O.; Gao, Dongxia; Gilks, C. Blake et al. (2009): Systematic analysis of immune infiltrates in high-grade serous ovarian cancer reveals CD20, FoxP3 and TIA-1 as positive prognostic factors. In PLoS ONE 4 (7), pp. e6412. DOI: 10.1371/journal.pone.0006412.

Min, Hu; Kai, Li; Ninu, Maskey; Zhigao, Xu; Chunwei, Peng; Bicheng, Wang et al. (2014): Decreased intratumoral Foxp3 Tregs and increased dendritic cell density by neoadjuvant chemotherapy associated with favorable prognosis in advanced gastric cancer. In International Journal of Clinical and Experimental Pathology 7 (8), p. 4685.


Mizukami, Yoshiki; Kono, Koji; Kawaguchi, Yoshihiko; Akaike, Hidenori; Kaminura, Kazuyasu; Sugai, Hidemitsu; Fujii, Hideki (2008): CCL17 and CCL22 chemokines within tumor microenvironment are related to accumulation of Foxp3+ regulatory T cells in gastric cancer. In Int. J. Cancer 122 (10), pp. 2286–2293. DOI: 10.1002/ijc.23392.


Nagar, Meital; Jacob-Hirsch, Jasmine; Vernitsky, Helly; Berkun, Yackov; Ben-Horin, Shomron; Amariglio, Ninette et al. (2010): TNF activates a NF-kappaB-regulated cellular program in human
CD45RA- regulatory T cells that modulates their suppressive function. In *J. Immunol.* 184 (7), pp. 3570–3581. DOI: 10.4049/jimmunol.0902070.


Onder, Lucas; Danuser, Renzo; Scandella, Elke; Firner, Sonja; Chai, Qian; Hehlgans, Thomas et al. (2013): Endothelial cell-specific lymphotoxin-β receptor signaling is critical for lymph node and high endothelial venule formation. In *The Journal of experimental medicine* 210 (3), pp. 465–473. DOI: 10.1084/jem.20121462.


Osamu, Nakano; Makoto, Sato; Yoshitaka, Naito; Kenichi, Suzuki; Seiichi, Orikasa; Masataka, Aizawa (2001): Proliferative Activity of Intratumoral CD8 T-Lymphocytes As a Prognostic Factor in Human Renal Cell Carcinoma: Clinicopathologic Demonstration of Antitumor Immunity 61, pp. 5132-5136.


Perrone, Giuseppe; Ruffini, Pier Adelchi; Catalano, Vincenzo; Spino, Cathie; Santini, Daniele; Muretto, Pietro et al. (2008): Intratumoural FOXP3-positive regulatory T cells are associated with adverse prognosis in radically resected gastric cancer. In Eur. J. Cancer 44 (13), pp. 1875–1882. DOI: 10.1016/j.ejca.2008.05.017.

Petersen, Rebecca P.; Campa, Michael J.; Sperlazza, Justin; Conlon, Debbi; Joshi, Mary-Beth; Harpole, David H.; Patz, Edward F. (2006): Tumor infiltrating Foxp3+ regulatory T-cells are associated with recurrence in pathologic stage I NSCLC patients. In Cancer 107 (12), pp. 2866–2872. DOI: 10.1002/cncr.22282.


Platonova, Sophia; Cherfils-Vicini, Julien; Damotte, Diane; Crozet, Lucile; Vieillard, Vincent; Validire, Pierre et al. (2011): Profound coordinated alterations of intratumoral NK cell phenotype and function in lung carcinoma. In Cancer Res. 71 (16), pp. 5412–5422. DOI: 10.1158/0008-5472.CAN-10-4179.


Redjimi, Nassima; Raffin, Caroline; Raimbaud, Isabelle; Pignon, Pascale; Matsuzaki, Junko; Odunsi, Kunle et al. (2012): CXCR3+ T regulatory cells selectively accumulate in human ovarian carcinomas to limit type I immunity. In Cancer research 72 (17), pp. 4351–4360. DOI: 10.1158/0008-5472.CAN-12-0579.


Rizvi, Naiyer A.; Mazieres, Julien; Planchard, David; Stinchcombe, Thomas E.; Dy, Grace K.; Antonia, Scott J. et al. (2015): Activity and safety of nivolumab, an anti-PD-1 immune checkpoint inhibitor, for


Shugart, Jessica A.; Bambina, Shelly; Alice, Alejandro F.; Montler, Ryan; Bahjat, Keith S. (2013): A self-help program for memory CD8+ T cells: positive feedback via CD40-CD40L signaling as a critical


Soong, Ruey-Shyang; Song, Liwen; Trieu, Janson; Lee, Sung Yong; He, Liangmei; Tsai, Ya-Chea et al. (2014): Direct T cell activation via CD40 ligand generates high avidity CD8+ T cells capable of breaking immunological tolerance for the control of tumors. In *PLoS ONE* 9 (3), pp. e93162. DOI: 10.1371/journal.pone.0093162.


Stewart, C. Andrew; Metheny, Hannah; iida, Noriho; Smith, Loretta; Hanson, Miranda; Steinhagen, Folkert et al. (2013): Interferon-dependent IL-10 production by Tregs limits tumor Th17 inflammation. In *J. Clin. Invest.* 123 (11), pp. 4859–4874. DOI: 10.1172/JCI65180.


Terme, Magali; Pernot, Simon; Marcheteau, Elie; Sandoval, Federico; Benhamouda, Nadine; Colussi, Orianne et al. (2013): VEGFA-VEGFR pathway blockade inhibits tumor-induced regulatory T-cell proliferation in colorectal cancer. In Cancer research 73 (2), pp. 539–549. DOI: 10.1158/0008-5472.CAN-12-2325.

Thornton, A. M.; Korty, P. E.; Tran, D. Q.; Wohlfert, E. A.; Murray, P. E.; Belkaid, Y.; Shevach, E. M. (2010): Expression of Helios, an Ikaros Transcription Factor Family Member, Differentiates Thymic-

Vendetti, Silvia; Davidson, Todd S.; Veglia, Filippo; Riccomi, Antonella; Negri, Donatella; Lindstedt, Ragnar et al. (2010): Polyclonal Treg cells enhance the activity of a mucosal adjuvant. In *Immunology and cell biology* 88 (7), pp. 698–706. DOI: 10.1038/icb.2010.76.


Vulcano, Marisa; Albanesi, Cristina; Stoppacciaro, Antonella; Bagnati, Renzo; D’Amico, Giovanna; Struyf, Sofie et al. (2001): Dendritic cells as a major source of macrophage-derived chemokine/CCL22 in vitro and in vivo. In *European Journal of Immunology* 31. DOI: 10.1002/1521-4141(200103)31:3<812::AID-IMMU812>3.0.CO;2-L.


Wei, Shuang; Kryczek, Ilona; Edwards, Robert P.; Zou, Linhua; Széliga, Wojciech; Banerjee, Mousumi et al. (2007): Interleukin-2 administration alters the CD4+FOXP3+ T-cell pool and tumor trafficking in patients with ovarian carcinoma. In *Cancer research* 67 (15), pp. 7487–7494. DOI: 10.1158/0008-5472.CAN-07-0565.


Wing, Kaja; Onishi, Yasushi; Prieto-Martin, Paz; Yamaguchi, Tomoyuki; Miyara, Makoto; Fehervari, Zoltan et al. (2008): CTLA-4 control over Foxp3+ regulatory T cell function. In *Science* 322 (5899), pp. 271–275. DOI: 10.1126/science.1160062.


Wollenberg, Ivonne; Agua-Doce, Ana; Hernández, Andrea; Almeida, Catarina; Oliveira, Vanessa G.; Faro, Jose; Graca, Luis (2011): Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. In *Journal of immunology (Baltimore, Md. : 1950)* 187 (9), pp. 4553–4560. DOI: 10.4049/jimmunol.1101328.

Wu, Yongqing; Borde, Madhuri; Heissmeyer, Vigo; Feuerer, Markus; Lapan, Ariya D.; Stroud, James C. et al. (2006): FOXP3 controls regulatory T cell function through cooperation with NFAT. In *Cell* 126 (2), pp. 375–387. DOI: 10.1016/j.cell.2006.05.042.


Yang, Li; Huang, Jianhua; Ren, Xiubao; Gorska, Agnieszka E.; Chytíl, Anna; Aakre, Mary et al. (2008): Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. In *Cancer cell* 13 (1), pp. 23–35. DOI: 10.1016/j.ccr.2007.12.004.

Ye, Zhi-Jian; Zhou, Qiong; Zhang, Jian-Chu; Li, Xiao; Wu, Cong; Qin, Shou-Ming et al. (2011): CD39+ regulatory T cells suppress generation and differentiation of Th17 cells in human malignant pleural effusion via a LAP-dependent mechanism. In *Respiratory research* 12, p. 77. DOI: 10.1186/1465-9921-12-77.


Zhang, Lixia; Yuan, Shunzong; Cheng, Genhong; Guo, Beichu (2011): Type I IFN promotes IL-10 production from T cells to suppress Th17 cells and Th17-associated autoimmune inflammation. In *PLoS ONE* 6 (12), pp. e28432. DOI: 10.1371/journal.pone.0028432.

Zhang, Qian; Jia, Qingzhu; Deng, Tianxing; Song, Bo; Li, Longkun (2015): Heterogeneous expansion of CD4+ tumor-infiltrating T-lymphocytes in clear cell renal cell carcinomas. In *Biochemical and biophysical research communications* 458 (1), pp. 70–76. DOI: 10.1016/j.bbrc.2015.01.069.


Zhu, Wei; Germain, Claire; Liu, Zheng; Sebastian, Yinong; Devi, Priyanka; Knockaert, Samantha et al. (2015): A high density of tertiary lymphoid structure B cells in lung tumors is associated with increased CD4 + T cell receptor repertoire clonality. In Oncoimmunology, p. 0. DOI: 10.1080/2162402X.2015.1051922.

Zielinski, Christina E.; Corti, Davide; Mele, Federico; Pinto, Dora; Lanzavecchia, Antonio; Sallusto, Federica (2011): Dissecting the human immunologic memory for pathogens. In Immunological reviews 240 (1), pp. 40–51. DOI: 10.1111/j.1600-065X.2010.01000.x.


1. Calreticulin expression by non-small-cell lung cancer correlates with signs of immunosurveillance and favorable prognosis

J. Fucikova¹,², E. Becht³,⁴,⁵, J. Goc³,⁴,⁵, K. Iribarren³,⁴,⁵, R. Remark³,⁴,⁵, D. Damotte³,⁴,⁵,⁶, M. Alifano⁷, P. Devi³,⁴,⁵, W. H. Fridman³,⁴,⁵, M-C. Dieu-Nosjean³,⁴,⁵, G. Kroemer³,⁴,⁵,⁸,⁹,¹⁰, C. Sautès-Fridman³,⁴,⁵, I. Cremer³,⁴,⁵*  

2. A high density of tertiary lymphoid structures B cells in lung tumors is associated with increased CD4⁺ T cell receptor repertoire clonality

Wei Zhu¹, Claire Germain²,³, ⁴, Zheng Liu¹, Yinong Sebastian¹, Priyanka Devi²,³, ⁴, Samantha Knockaert²,³, ⁴, Philip Brohawn¹, Kim Lehmann¹, Diane Damotte²,³, ⁴, ⁵, Pierre Validire²,³, ⁴, ⁶, Yihong Yao¹, Viia Valge-Archer⁷, Scott A. Hammond⁸, Marie-Caroline Dieu-Nosjean²,³, ⁴, and Brandn W. Higgs¹
Fucikova et al., 2015

Title: Calreticulin expression by non-small-cell lung cancer correlates with signs of immunosurveillance and favorable prognosis


Affiliations:
1 Department of Immunology, Charles University, 2nd Faculty of Medicine and University Hospital Motol, Prague, Czech Republic.
2 Sotio, Prague, Czech Republic.
3 Institut National de la Santé et de la Recherche Médicale (INSERM), UMRS1138, Centre de Recherche des Cordeliers, Paris, F-75006, France.
4 Université Pierre et Marie Curie-Paris 6, UMRS1138, Paris, F-75006, France.
5 Université Paris Descartes-Paris 5, UMRS1138, Paris, F-75006, France.
7 Service de chirurgie thoracique, Hôpital Cochin, AP-HP, Paris, France.
8 Metabolomics and Cell Biology Platforms, Gustave Roussy, Villejuif, France.
9 Equipe 11 labellisée Ligue contre le Cancer, Centre de Recherche des Cordeliers, INSERM UMRS 1138, Paris, France.
10 Pôle de Biologie, Hôpital Européen Georges Pompidou, AP-HP, Paris, France

Corresponding author. Email: isabelle.cremer@crc.jussieu.fr

Running title: Prognostic impact of calreticulin in NSCLC patients

Keywords: calreticulin, mature dendritic cells, CD8$^+$ T cells, NSCLC, prognosis

No potential conflicts of interest were disclosed by the authors.
Abstract
A high density of tumor infiltrating mature dendritic cells and CD8$^+$ T cells correlates with a positive prognostic value in a majority of human cancers. The presence of activated lymphocytes in the tumor microenvironment, primed to recognize tumor-associated antigens can result from immunogenic cell death (ICD) of tumor cells. ICD is characterized by the pre-apoptotic translocation of calreticulin (CRT) from the endoplasmic reticulum (ER) to the cell surface, as a result of an ER stress response accompanied by the phosphorylation of eukaryotic initiation factor 2α (eIF2α). We report that the level of CRT expression by tumor cells, which correlates with eIF2α phosphorylation, positively influences clinical outcome of non-small cell lung cancer patients. High CRT expression on tumor cells correlates with a high density of infiltrating mature DCs, and effector memory T cell subsets, suggesting that CRT triggers the activation of adaptive immune responses in the tumor microenvironment. Finally, patients with elevated CRT expression and dense infiltration by DCs or CD8$^+$ T lymphocytes infiltration have the best prognosis. We conclude that CRT expression constitutes a new powerful prognostic biomarker that may enhance local antitumor immune responses in the lung.
Introduction
Recent advances in tumor biology have highlighted the complex interplay between the immune system and tumor, and have revealed the major role of the adaptive immune system in the control of tumor growth (1, 2). The tumor microenvironment consists in a complex network of malignant, stromal and immune cells. Tumor-infiltrating immune cells have been described in most solid tumors (3, 4). The type, the density and the location of immune cells in the tumor microenvironment, defined as immune contexture strongly affect the prognosis of cancer patients (4-7). In non-small cell lung cancer (NSCLC), a high density of mature dendritic cells (DCs) correlates with long-term patients survival (9) and with a strong infiltration by CD8⁺ T cells that are predominantly of the effector-memory phenotype (8). Within the group of patients with high densities of CD8⁺ T cells, those with few mature DCs exhibit lower overall survival (OS) than those with high densities of mature DCs, suggesting that tumor-infiltrating CD8⁺T cells may be functionally heterogenous (8, 10).

The immunogenicity of cancer cells results from their antigenicity, (i.e. the expression of specific tumor antigens) and their adjuvanticity, (i.e. the expression or release of danger-associated molecular patterns or DAMPs). One particular way to deliver DAMPs into the tumor microenvironment is immunogenic cell death (ICD), a functionally peculiar type of apoptosis that stimulates tumor-specific immune responses (11-14). The immunogenicity of cell death relies on at least three independent events, namely (i) the pre-apoptotic exposure of the endoplasmic reticulum (ER) chaperone protein calreticulin (CRT) (15, 16) and perhaps other chaperones such as HSP70 and HSP90 (17), at the cell surface, (ii) the subsequent autophagy-dependent active secretion of adenosine triphosphate (ATP) (3, 18, 19) and (iii) the post-apoptotic release of the nuclear non-histone chromatin-binding protein high mobility group box 1 (HMGB1) (20, 21). By binding to CD91 on the surface of DCs, CRT functions as an “eat-me” signal, thus promoting the engulfment of apoptotic bodies by DCs (22).
Knockdown of CRT, blockade of ecto-CRT or inhibition of the pathway leading to CRT exposure abolishes the immunogenicity of cell death (19, 23). These findings suggest that DAMPs can stimulate antigen presenting cells (APCs), particularly DCs, to efficiently engulf dying cells, process antigens, mature and induces an efficient immune response.

Most studies on ICD have focused on experimental mouse models (19, 20, 23-28) and *in vitro* human studies (15, 17, 19, 20, 23-33), but large-scale studies are necessary to determine the prognostic value of ICD-associated DAMPs expression, as well as their role in cancer immunosurveillance in patients. Here we investigated the clinical impact of CRT expression on two independent cohorts of operable NSCLC patients having received or not neo-adjuvant chemotherapy. Using immunohistochemistry we demonstrate that CRT is a powerful prognostic factor for overall survival (OS) of NSCLC patients, even after correcting for pathological stage. Furthermore we evaluated whether expression of CRT is associated with an enhanced cellular immune response. We identified a statistically significant correlation between expression of CRT and density of mature DCs. We showed on fresh resected tumors that exposure of CRT occurs specifically at the cell surface of tumor cells and is strongly associated with the presence of effector memory T cells in the tumor microenvironment.
Materials and Methods

Patients

Study group 1. A retrospective series of 270 stage I to III-IV NSCLC patients who underwent primary surgery (without neo-adjuvant chemotherapy) and who were operated between 2001 and 2005 was obtained from Hotel-Dieu hospital (Paris, France). Baseline characteristics of these patients are summarized in Table 1.

Study group 2. A retrospective cohort of 125 stage III-N2 NSCLC patients who received neo-adjuvant chemotherapy followed by curative resection between 2000 and 2007 was obtained from Hotel-Dieu hospital. Baseline characteristics of these patients are summarized in Supplementary Table 1.

Pathological staging of lung cancer was reviewed and classified according to the new TNM classification 2009 (34), and histological types were determined according to the classification of the WHO (35).

Study group 3. A prospective series of 50 NSCLC patients were obtained from Cochin-Hotel-Dieu Hospital and Institute Mutualiste Montsouris (Paris). These patients underwent primary surgery in 2013.

Informed consent was obtained for each patient after the nature and possible consequences of the studies were explained and lung tumor samples were analyzed with the agreement of the French ethic committee (agreement 2008-13 and 2012 06-12) in accordance with article L.1121-1 of French law.

Immunohistochemistry

In the study groups 1 and 2, tumor samples were fixed in neutral buffered 10% formalin solution and paraffin-embedded. For each paraffin-embedded lung tumor, two observers (MCDN and DD) selected the tumor section containing a representative area of tumor with adjacent lung parenchyma, and the highest density of immune cells on the hematoxylin and
eosin-safran–stained tissue section. CRT, P-eIF2α, CD8 and DC-LAMP immunostaining were conducted using the avidin-biotin-peroxidase complex method. CRT staining was performed as previously described (32): paraffin section was dewaxed, followed by antigen retrieval with Target Retrieval Solution (Dako) (10μmol citrate buffer at pH 6.0) in water bath for 30 min. Sections were cooled at room temperature for 30 minutes and endogenous peroxidase was blocked with 3% hydrogen peroxide. Thereafter, sections were incubated with diluted 5% human serum for 30 minutes and incubated with mouse anti-human CRT mAb (Abcam, 5μg/mL) for 2 hours at room temperature. Peroxidase-linked secondary antibodies (Dako) and (3-amino-9-ethylcarbazole) (AEC) were used to detect specific binding. For P-eIF2α staining, each paraffin section was dewaxed, followed by antigen retrieval in TRIS EDTA pH9 in a preheated water bath (98°C, 30 minutes), and endogenous peroxidase was blocked with 3% hydrogen peroxide. After incubation with diluted 5% human serum for 30 minutes, tissue was incubated with rabbit anti-human P-eIF2α mAb (Abcam, 5μg/mL) for 2 hours at room temperature. Again, peroxidase system and AEC were used to reveal the presence of P-eIF2α.

**Methods for cell quantification**

The expression level of CRT and P-eIF2α for each patient was determined as the score of positive tumor cells. The score index was calculated for 10 different fields at 20x magnification under a light microscope (Nikon ecplipse, 80i) and classified into 5 scores (10-20% positive cells evaluated for score 1, 21-40% positive cells evaluated for score 2, 41-60% positive cells evaluated for score 3, 61-80% positive cells evaluated for score 4 and 80-100% positive cells evaluated for score 5). The quantification was done by two independent observers (JF and IC), and reviewed by an expert pathologist (DD).

The staining and quantification of DC-LAMP and CD8 was performed as previously described (8): images were acquired using Nanozoomer (Hamamatsu) operated with NDPview software, CD8^+ and DC-LAMP^+ cells were quantified in the stroma of the whole
tumor section with Calopix software (Tribvn), and expressed as an absolute number of positive cells/mm² of tumor. Both immunostaining and quantification were reviewed by at least three independent observers (JG, PD and MCDN).

**Immunofluorescence**

For the detection of CRT, the cells were placed on ice, washed twice with PBS and fixed in 0.25% paraformaldehyde in PBS for 5 min. The cells were then washed twice in PBS, and a primary anti-CRT antibody (Enzo, Farmingdale, NY) and anti-Na⁺-K⁺-ATPase alfa1 antibody (Santa Cruz, Dallas, USA) diluted in cold blocking buffer was added for 30 min. After two washes in cold PBS, the cells were incubated for 30 min with the appropriate secondary antibody Alexa Fluor 488 IgG1 (Life Technologies, Carlsbad, CA) and Alexa Fluor 594 Donkey Anti-Mouse IgG (H+L), respectively. The cells were fixed with 4% paraformaldehyde for 20 min, washed in PBS for 20 min and mounted on slides.

**Flow cytometry**

Fresh lung tumor specimens and distant non-tumoral tissues (taken at more than 10 cm of the tumor) were mechanically (manual) dissociated and digested in the presence of Cell Recovery Solution (BD Biosciences). Total live mononuclear cells were isolated from the tumors, as previously described (8), using ficoll density-gradient separation. Briefly, cells from pellet remaining after ficoll separation were incubated with primary antibodies against CD45, cytokeratin, human epithelial antigen, CD227 to distinguish the population of leukocytes, stromal and epithelial cells, and antibodies against calreticulin (Enzo), for 30 minutes at 4°C. Cells were washed and stained with Alexa 648-conjugated monoclonal secondary antibody for 30 minutes at 4°C. After washing, cell viability was assessed by AnnexinV PE (BD Pharmigen) or Live/Death Yellow staining (Life Technologies) and DAPI. For immune cell populations characterization, mononuclear cells were stained with multiple panels of antibodies conjugated to fluorescent dyes (see Supplemental Table 1). Briefly, mononuclear
cells were incubated with the primary antibodies for 30 minutes at 4°C in the dark. Cells were washed and analysed on a LSRII cytometer (BD Biosciences) and FlowJo (TreeStar, Inc.) software.

**Statistical analyses**

Survival analysis was performed using the “survival” R package, using both log-rank tests and Cox proportional-hazards regressions. When using a log-rank test, prognostic value of continuous variables was assessed using a median-based cutoff, or the optimal p-value approach (supplemental Fig. 1). In the latter case, p-values were corrected using the technique proposed by Altman et al. (48). For Cox proportional-hazards regressions, immune densities were log-transformed. In multivariate Cox regressions, variables that were not significantly associated with prognosis in univariate analysis (Wald test’s p value > 0.05) were not included, as well as variables intrinsically correlated (for instance, continuous CRT variable was prioritized over dichotomized CRT). For linear correlation, orthogonal regression was used to plot the regression line, as noise are assumed to equally affect both CRT and P-eIF2α. Fisher exact test, Student’s t-test, Wilcoxon and Mann-Whitney tests were used to test for association between variables.
Results

Prognostic impact of CRT expression in NSCLC

Tumor samples from a retrospective series of 270 patients with NSCLC (Table 1), who did not receive neo-adjuvant chemotherapy, were analyzed for CRT expression by immunohistochemistry (Fig. 1A). We observed a heterogeneous distribution of CRT expression among different patient’s tumors, with a score ranging from grade 1 to 5 (1 for weak expression and 5 for strong expression). We didn’t find significant difference of CRT expression between TNM stages (Fig. 1B). We divided the cohort into two groups, with high (CRT$^{Hi}$) and low (CRT$^{Lo}$) CRT expression on tumor cells, using the minimal p-value approach (Fig. 1C). Calculations of the optimal cutoff (stratifying according to the minimization of the p-value of a log-rank test which is corrected using the method published by Altman and colleagues (36) to control for multiple testing) set the limit of the expression score at 2.45 (supplemental Fig. 1A). This approach revealed that low CRT expression was associated to poor prognosis ($p=1.7\times10^{-14}$) and allowed for the identification of a small group of patients with low CRT expression affected by a particularly short OS (Fig. 1C). The median OS was 18 months for the CRT$^{Lo}$ group and 68 months for the CRT$^{Hi}$ group. Univariate Cox modeling confirmed the prognostic impact of CRT quantification (Table 2, $p=1.1\times10^{-16}$) and that CRT$^{Lo}$ group of patients had a higher risk of death compared to CRT$^{Hi}$ group ($p=4.04\times10^{-14}$). However, as CRT$^{Hi}$ and CRT$^{Lo}$ groups of patients are disproportionate when using the optimal cutoff, we also used the median stratification, which confirmed that high CRT expression ($n=134$ out of 270) was strongly correlated with longer OS ($p=8.17\times10^{-9}$) (Fig. 1D). The median OS was 28 months for CRT$^{Lo}$ group and increased to 100 months for the CRT$^{Hi}$ group (Fig. 1D). Kaplan-Meier survival curves were also significantly different across quartiles of CRT expression, revealing a dose-effect relationship (supplemental Fig. 2).
Fucikova et al., 2015

(p=4.35x10^{-12}). These results define CRT expression as a strong predictive marker for OS of NSCLC patients.

As CRT expression did not correlate with pathological staging (Fig. 1B), the current gold standard for lung cancer risk assessment, we investigated whether these two parameters are independently prognostic, by using a multivariate Cox model (Table 3). CRT expression, pathological staging and age turned out to be the only significant predictors of OS. Kaplan-Meier curves combining stage and CRT stratifications using the optimal p-value (Fig. 1E) and median cutoffs (Fig. 1F) were consistent with this interpretation. CRT^{Hi}/stage I patients had the best OS prognosis (median OS was 101 or 110 months using optimal or median cutoff, respectively). In contrast, patients in the pathological stage III-IV and low expression of CRT were at the highest risk of death (median OS was 11 or 14 months using optimal or median stratification, respectively) (Fig. 1E, F). Of note, when the optimal cutoff for stratifying tumors into CRT^{Lo} and CRT^{Hi} is used, it appears that CRT^{Lo}/stage I patients succumb more quickly than CRT^{Hi}/stage III-IV patients (Fig. 1E), suggesting that CRT low expression can identify patients with high risk of death even at early stages. Altogether, these data show that CRT expression and pathological stage are independent prognostic factors.

We performed CRT quantification as well as survival analyzes in an independent cohort of 125 patients that were all affected by stage III NSCLC and treated with neo-adjuvant chemotherapy (supplemental Table 2). Again, expression of CRT using the optimal p-value approach confirmed a strong prognostic impact of the CRT-based stratification (supplemental Fig. 3A, p=0.017), and CRT expression below the median level was also associated with poor prognosis (supplemental Fig. 3B, p=0.0067). Univariate Cox analysis confirmed the prognostic impact of CRT expression in this cohort of patients (HR = 0.78, p = 0.0045) (supplemental Table 3).
Correlation between eIF2α phosphorylation and CRT expression in lung tumor cells

The translocation of CRT to the cell surface is triggered in response to an ER stress that involves the phosphorylation of the eukaryotic translation initiation factor eIF2α by PKR-like ER kinase (PERK) (19, 23, 37). Using immunohistochemistry, we found that phosphorylated-eIF2α (P-eIF2α) was mostly expressed in NSCLC cells (as opposed to stromal elements), though with an interindividual variability, similarly to that found for CRT staining (Fig. 1G). We observed a statistically significant correlation between the expression of P-eIF2α and CRT in the two cohorts of NSCLC patients that were either not treated (p=2.7x10^{-10}, r=0.46, Fig. 1H) or treated with neoadjuvant chemotherapy (p=2.3x10^{-13}, r=0.70, supplemental Fig. 3E). High P-eIF2α expression was correlated with longer survival using optimal cutoff (p=0.001 Fig. 1I and p=0.0033 supplemental Fig. 3C), respectively for the cohorts of patients that were not treated and treated with neoadjuvant chemotherapy), and using median stratification (p=0.0035 Fig. 1J and p=0.0089 supplemental Fig. 3D, respectively for the cohorts of patients that were not treated and treated with neoadjuvant chemotherapy).

For both cohorts, univariate cox analysis revealed that CRT and P-eIF2α were significantly associated to OS (Table 2 and supplemental Table 3). We analyzed the prognostic value of the two parameters using multivariate cox proportional hazards regression (Table 3 and supplemental Table 4). P-eIF2α then lost its prognostic impact, which indicates that the strong correlation between the two variables prevents their concomitant use for risk assessment.

High expression of CRT is associated with an enhanced cellular immune response

CRT is best characterized for its prominent function as an eat-me signal, leading to maturation of DCs and activation of adaptive immune responses against tumors (38). Therefore, we addressed the impact of CRT expression on the density of tumor-infiltrating mature DCs (DC-LAMP^+ cells, Fig. 2A) and tumor-infiltrating CD8^+ T cells (Fig. 2B). Patients with high CRT expression exhibited significantly (p=0.042) higher densities of intratumoral mature DCs
(medians 1.1 and 0.6 cells/mm² for CRT\textsuperscript{Hi} and CRT\textsuperscript{Lo} groups respectively, Fig. 2A). However, there was no significant difference in the CD8$^+$ T-cell density between CRT\textsuperscript{Hi} and CRT\textsuperscript{Lo} tumors (p=0.17) (Fig. 2B). Confirming prior observations (8), high densities of DC-LAMP$^+$ cells and of CD8$^+$ T cells had a significant positive impact on patient survival (Supplemental Fig. 4A, B). Univariate cox analysis confirmed the prognostic value of mature DC and CD8 T cell densities (Table 2).

We evaluated the combined prognostic value of CRT plus DC-LAMP or CD8 (Fig. 2C, D). We observed that CRT\textsuperscript{Hi} patients had the best OS whatever the level of DC-LAMP. However, CRT\textsuperscript{Hi}/DC-LAMP\textsuperscript{Hi} group had the best prognosis (median OS= 101 months) compared to patients with CRT\textsuperscript{Hi}/DC-LAMP\textsuperscript{Lo}, which were at an intermediate risk of death (median OS= 54 months). The CRT\textsuperscript{Lo} group of patients (DC-LAMP\textsuperscript{Lo} or DC-LAMP\textsuperscript{Hi}) was at the highest risk of death (median OS was 13 months) (Fig. 2C). The combination of CRT expression and CD8 density yielded similar results, with the best prognosis for CRT\textsuperscript{Hi}/CD8\textsuperscript{Hi} patients, intermediate values for CRT\textsuperscript{Hi}/CD8\textsuperscript{Lo} tumors, and dismal prognosis for CRT\textsuperscript{Lo}/CD8\textsuperscript{Lo} and CRT\textsuperscript{Lo}/CD8\textsuperscript{Hi} groups (Fig. 2D). Similar results were obtained when using the median cutoff for CRT score (supplemental Fig. 4C, D).

Using multivariate cox analysis, the age, the pTNM stage and the CRT score were found to be significantly and independently associated with OS, whereas mature DC and CD8 cell densities were not found significantly associated with OS (Table 3). Altogether, these data demonstrate that CRT expression and mature DC or CD8$^+$ T cell densities are not independent prognostic factors.

**Cell surface exposure of CRT on primary tumor cells from NSCLC patients**

By using immunohistochemistry approach, the exposure of CRT expression at the cell surface of tumor cells is not easily quantifiable. We performed flow cytometry analysis on freshly resected human NSCLC patients treated by primary surgery to characterize the expression of
CRT (Fig. 3). Surface CRT expression was detected in the population of AnnexinV+/−/DAPI− cells (or alternatively live cells detected by a Live/Death staining kit) in non-tumoral and in tumoral tissues (Fig. 3A). Using an antibody against the leukocyte marker CD45 and a panel of antibodies specific for epithelial surface markers, we distinguished populations of leukocytes, stromal and epithelial cells (Fig. 3B). The percentage of CRT positive cells was evaluated among these cells in both tumoral and distant non-tumoral tissues (Fig. 3C, D). Although highly heterogeneous, the expression of membrane CRT was significantly increased (p<0.0001) on tumor epithelial cells as compared to epithelial cells from non-tumoral tissues in the majority of patients (Fig. 3D). Leukocytes and stromal cells from tumoral and non-tumoral tissues did not express CRT on their surface (Fig. 3D), although such cells did contain intracellular CRT, as revealed by immunofluorescence staining after plasma membrane permeabilization (supplemental Fig. 5) using a similar gating strategy. Confocal microscopy of epithelial tumor cells stained for ecto-CRT and the Na⁺-K⁺ pump confirmed that CRT was indeed localized at the plasma membrane of the majority of the tumor cells (Fig. 3E).

Because the expression of CRT can strongly influence the population of immune cells we evaluated the impact of membrane CRT expression by tumor cells, on the immune cell infiltrate with a focus on T lymphocytes and DCs (CD45⁺, CD3⁺, CD4⁺, CD8⁺, plasmacytoid DCs (pDCs) and myeloid DCs (cDCs) (Fig. 4A). We observed a significant lower percentage of total CD45⁺ and CD4⁺ cells and a significantly higher percentage of cDCs in CRThigh patients, compared to CRTlow patients, among total live mononuclear cells from the tumor. Furthermore we determined the frequency of the four main subpopulations of CD4⁺ and CD8⁺ T cells, related to their differentiation status. As compared with CRTlow tumors, CRThigh tumors were significantly more infiltrated by CD4⁺ and CD8⁺ effector memory T cells (CD45RA⁻/CCR7⁻) (Fig. 4B) whereas the percentage of naïve and terminally differentiated
effector-memory (also called TEM-RA) CD4$^+$ and CD8$^+$ T cells was significantly decreased (p<0.05). No difference was seen for central memory CD4$^+$ and CD8$^+$ T cells. Altogether, these results demonstrate that CRT$^{\text{high}}$ tumors have higher number of activated and effector-memory T cells, than CRT$^{\text{low}}$ tumors.
Discussion
CRT has been evaluated as a potential biomarker in several types of cancer including neuroblastoma (39), bladder (40), gastric (41), breast cancer (42) and acute myeloid leukemia (37). CRT has also been reported to be overexpressed in the cytoplasm and at the membrane level of NSCLC cells, and serum level of CRT protein was associated with tumor pathological grade (43), although the prognostic impact of circulating CRT levels has not been studied. Here, we assessed the prognostic value of CRT expression in two independent retrospective cohorts of patients with NSCLC, one treated by primary surgery without neoadjuvant chemotherapy (n=270, stage I to III-IV) and the second one treated by neoadjuvant chemotherapy and surgery (n=125, stage III N2).

In NSCLC samples we observed a major interindividual variability in CRT expression irrespective of the histological subtype of NSCLC. Nevertheless CRT expression was not affected by the stage and Cox multivariate regression analyses confirmed that CRT expression and stage are two independent prognostic factors. High levels of CRT were associated with long survival of NSCLC patients. Our data are in accordance with findings in neuroblastoma (39), in which high CRT correlates with neuroblastoma differentiation and favorable outcome. Conversely, in esophageal squamous cell carcinoma CRT expression had no impact on patients’ survival (32), suggesting that CRT levels may only predict patient survival in some cancer types. In our study, we observed that CRT levels had a strong impact on patient survival. In NSCLC samples, CRT expression strongly correlated with the constitutive phosphorylation of eIF2α. This suggests that a subgroup of NSCLC is associated with a strong constitutive ER stress response that culminates in CRT expression and exposure, facilitating anticancer immunosurveillance. To date, the precise mechanisms that explain why some but not all NSCLCs are enclined to ER stress and others not remain to be elucidated. In breast cancer, changes in ploidy have been linked to the level of eIF2α phosphorylation and
the intensity of the tumor infiltrate after anthracycline-based chemotherapy (44). Whether similar mechanisms apply to NSCLC remains to be investigated.

When present at the surface of tumor cells, CRT can stimulate antigen-presenting cells, particularly DCs, to efficiently engulf dying cells, process their antigens and prime T cell immune response (16, 19, 29). Accordingly, a high density of mature DCs closely correlated with high expression of CRT in NSCLC. Previous reports demonstrated that high levels of ecto-CRT on tumor cells positively correlated with the activation of DCs and the capacity of DCs to stimulate interferon-γ by autologous T cells (17, 19, 29). However, we did not observe a major positive correlation between the density of total CD8\(^+\) T cells and CRT expression in NSCLC. In a cohort of prospective patients treated by primary surgery without neoadjuvant chemotherapy, we have observed significantly higher percentage of cDCs and effector memory CD4\(^+\) and CD8\(^+\) T cells in CRT\(_{\text{high}}\) tumors than in CRT\(_{\text{low}}\) NSCLCs, whereas the frequency of total CD4\(^+\) and CD8\(^+\) T cells was identical. These data are in accordance with results showing that the densities of infiltrating mature DC and effector-memory T cells correlate among each other (8). These results demonstrate that high CRT expression at the cell surface of tumor cells leads to elevated frequency of mature DCs, and impact the quality of antitumor immune response, leading to the differentiation of tumor infiltrating T cells towards an effector memory phenotype. Effector memory T cells have been identified to control cancer progression. Mainly the cytotoxic and cytokinic capability of effector memory T cells may provide them the relevant weapons to control tumor progression and metastatic invasion at the primary tumor site (45). In numerous cancers, the presence of tumor infiltrating effector memory T cells correlates with reduced metastatic invasion and increased survival of the patient (46).

The most favorable prognosis was observed for the group of patients with high CRT expression and high density of DC-LAMP\(^+\) or CD8\(^+\) cells. Patients with high CRT and low
Fucikova et al., 2015

DC-LAMP$^+$ or CD8$^+$ cells were at an intermediate risk. Individuals with low expression of CRT in their NSCLC were at the highest risk of death, irrespective of the density of DC or CD8$^+$ T cells in the tumor infiltrate. This points to the possibility that the immune reaction in CRT$^{Hi}$ tumors may be particularly active in anticancer immunosurveillance, probably involving ICD leading to an effective CD8$^+$ T cell response directed against tumor-associated antigens, while that of CRT$^{Lo}$ tumors would be dysfunctional. In this case, one could speculate that in the absence of efficacious priming, the T cell response is polyclonal and not efficient. However, this conjecture requires further experimental exploration.

In conclusion, high CRT expression, which is likely driven by an ER stress response, constitutes a positive prognostic biomarker in NSCLC patients. The available data are compatible with the hypothesis that the local presence of CRT, which is constitutively expressed at the surface of transformed epithelial cells, may enable a DC-dependent anticancer immunosurveillance.
Acknowledgments

Funding: This work was supported by the “Institut National de la Santé et de la Recherche Médicale” (INSERM), Université Pierre et Marie Curie, Université Paris Descartes, the Cancer Research for Personalized Medicine (CARPEM), the Paris Alliance of Cancer Research Institutes (PACRI), the LabEx Immuno-Oncology, and the Institut National du Cancer (2011-PLBIO-06-INSERM 6-1, PLBIO09-088-IDF-KROEMER. J. Fucikova was supported by grant IGA NT 14533 and IGA NT 11404-5, Ministry of Health, Czech Republic.

References


Fucikova et al., 2015

**Tables**

**Table 1** Clinical characteristics of NSCLC patients not treated with neo-adjuvant chemotherapy, with CRT\textsuperscript{Hi} versus CRT\textsuperscript{Lo} tumors.

<table>
<thead>
<tr>
<th></th>
<th>Overall cohort ((n=270))</th>
<th>CRT\textsuperscript{Hi} ((n=239))</th>
<th>CRT\textsuperscript{Lo} ((n=31))</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>227 (84%)</td>
<td>200 (84%)</td>
<td>27 (87%)</td>
<td>0.8</td>
</tr>
<tr>
<td>Female</td>
<td>43 (16%)</td>
<td>39 (16%)</td>
<td>4 (13%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (years) +/- SEM</td>
<td>64 ± 0.04</td>
<td>64 +/- 0.04</td>
<td>64 +/- 0.4</td>
<td>0.9</td>
</tr>
<tr>
<td><strong>Smoking history</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>232 (86%)</td>
<td>205 (86%)</td>
<td>27 (87%)</td>
<td></td>
</tr>
<tr>
<td>Never smoker</td>
<td>33 (12%)</td>
<td>31 (13%)</td>
<td>2 (6.5%)</td>
<td>0.55</td>
</tr>
<tr>
<td>NA</td>
<td>5 (2%)</td>
<td>3 (1%)</td>
<td>2 (6.5%)</td>
<td></td>
</tr>
<tr>
<td><strong>Histological type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADC</td>
<td>143 (53%)</td>
<td>126 (53%)</td>
<td>17 (55%)</td>
<td></td>
</tr>
<tr>
<td>SCC</td>
<td>101</td>
<td>92 (39%)</td>
<td>9 (29%)</td>
<td>0.36</td>
</tr>
<tr>
<td>Others</td>
<td>(37.5%)</td>
<td>12 (5%)</td>
<td>3 (9.5%)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>15 (5.5%)</td>
<td>9 (3%)</td>
<td>2 (6.5%)</td>
<td></td>
</tr>
<tr>
<td><strong>Stage of disease</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>112 (41%)</td>
<td>103 (43%)</td>
<td>9 (29%)</td>
<td>0.28</td>
</tr>
<tr>
<td>Stage II</td>
<td>75 (28%)</td>
<td>64 (27%)</td>
<td>11 (35.5%)</td>
<td></td>
</tr>
<tr>
<td>Stage III-IV</td>
<td>83 (31%)</td>
<td>72 (30%)</td>
<td>11 (35.5%)</td>
<td></td>
</tr>
<tr>
<td><strong>P.eiF2α score</strong></td>
<td>4 ± 0.0058</td>
<td>4 ± 0.0058</td>
<td>2.6 ± 0.092</td>
<td>0.0067</td>
</tr>
<tr>
<td><strong>Immune infiltrate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC-LAMP density</td>
<td>1 ± 0.013</td>
<td>1.1 ± 0.016</td>
<td>0.62 ± 0.048</td>
<td>0.042</td>
</tr>
<tr>
<td>CD8 density</td>
<td>93 ± 1.1</td>
<td>97 ± 1.2</td>
<td>54 ± 6.7</td>
<td>0.17</td>
</tr>
</tbody>
</table>

All clinical parameters were evaluated among the 270 NSCLC patients. Age at the date of the surgery is reported. DC-LAMP and CD8 T cell densities were evaluated among 221 patients. P-eiF2α score was evaluated among 164 patients. Significant association to CRT stratification (based on the optimal p value approach to determine CRT\textsuperscript{Hi} and CRT\textsuperscript{Lo} groups) was assessed using Mann-Withney or Fisher exact tests. Abbreviations: ADC, adenocarcinoma; SCC, squamous cell carcinoma; NA, not available.
Table 2: Univariate cox-regression for patients without neo-adjuvant chemotherapy

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% Cl</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (F vs M)</td>
<td>1.25</td>
<td>0.83-1.91</td>
<td>0.28</td>
</tr>
<tr>
<td>Age, years</td>
<td>1.02</td>
<td>1.0-1.03</td>
<td>0.004</td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoker vs smoker</td>
<td>0.78</td>
<td>0.47-1.27</td>
<td>0.32</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADC vs SCC</td>
<td>1.18</td>
<td>0.85-1.63</td>
<td>0.31</td>
</tr>
<tr>
<td>ADC vs others</td>
<td>1.26</td>
<td>0.65-2.42</td>
<td>0.49</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I vs II</td>
<td>1.87</td>
<td>1.28-2.73</td>
<td>0.001</td>
</tr>
<tr>
<td>I vs III-IV</td>
<td>2.99</td>
<td>2.08-4.30</td>
<td>2.7e-09</td>
</tr>
<tr>
<td>CRT score</td>
<td>0.54</td>
<td>0.47-0.61</td>
<td>1.10e-16</td>
</tr>
<tr>
<td>P-eIF2α score</td>
<td>0.72</td>
<td>0.58-0.89</td>
<td>0.003</td>
</tr>
<tr>
<td>Log_{10} DC-LAMP density</td>
<td>0.72</td>
<td>0.56-0.93</td>
<td>0.01</td>
</tr>
<tr>
<td>Log_{10} CD8 density</td>
<td>0.78</td>
<td>0.69-0.89</td>
<td>2.9e-4</td>
</tr>
<tr>
<td>CRT group, Hi vs Low</td>
<td>0.20</td>
<td>0.13-0.30</td>
<td>4.04e-14</td>
</tr>
</tbody>
</table>

Univariate Cox- proportional hazards analysis for overall survival according to clinical and immune parameters.
Table 3: Multivariate cox-regression for patients without neo-adjuvant chemotherapy

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>1.02</td>
<td>1.00-1.05</td>
<td>0.027</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I vs II</td>
<td>1.92</td>
<td>1.13-3.25</td>
<td>0.015</td>
</tr>
<tr>
<td>I vs III-IV</td>
<td>2.08</td>
<td>1.23-3.50</td>
<td>0.0057</td>
</tr>
<tr>
<td>CRT score</td>
<td>0.58</td>
<td>0.47-0.73</td>
<td>1.58e-06</td>
</tr>
<tr>
<td>P-eiF2α score</td>
<td>0.89</td>
<td>0.70-1.14</td>
<td>0.37</td>
</tr>
<tr>
<td>Log₁₀ DC-LAMP density</td>
<td>0.95</td>
<td>0.69-1.31</td>
<td>0.76</td>
</tr>
<tr>
<td>Log₁₀ CD8 density</td>
<td>0.95</td>
<td>0.79-1.12</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Multivariate Cox proportional hazards analyses for overall survival according to clinical and immune parameters. Parameters identified in the univariate analysis as significantly influencing outcome were introduced in a multivariate Cox-proportional hazards regression model.
Fucikova et al., 2015

Figures

Fig. 1. Positive prognostic value conferred by tumoral CRT and P-eiF2α expression in NSCLC patients

Representative images showing low and high CRT expression in NSCLC tissue. CRT positive cells are shown in red (A). Boxplots representing the distributions of CRT score according to stages (B). Kaplan-Meier survival curves for overall survival (OS) for 270 patients with

Figure 1
NSCLC without neo-adjuvant chemotherapy according to the presence of a high or low expression of CRT evaluated using the optimal cutoff approach, $p=1.7 \times 10^{-14}$ (C), using median, $p=8.17 \times 10^{-9}$ (D) and using both CRT expression as well as stage stratification using the optimal cutoff approach, $p=3.1 \times 10^{-22}$ (E) and using median, $p=1.23 \times 10^{-18}$ (F). Representative images showing low and high P-eIF2α expression in NSCLC tissue. P-eIF2α positive cells are shown in red (G). Correlation graph for P-eIF2α and CRT expression on tumor cells in the cohort of NSCLC patients. The gray line was determined by orthogonal regression (H). Kaplan-Meier survival curves for OS for 164 NSCLC patients according to the presence of a high or low density of P-eIF2α evaluated using optimal cutoff approach, $p=0.0011$ (I) or using median, $p=0.036$ (J). P value was determined by log-rank test. Tables show the number of patients at risk in each group at several time points.
**Fig. 2.** Prognostic value conferred by tumor CRT expression combined with DC-LAMP and CD8 densities in NSCLC patients

Barplots representing the median DC-LAMP$^+$ (A) and CD8$^+$ (B) cell densities in CRT$^{Hi}$ and CRT$^{Lo}$ groups. Kaplan-Meier survival curves for OS for NSCLC patients according CRT expression combined with the presence of a high or low density of DC-LAMP$^+$ cells, $p=2.04\times10^{-15}$ (C), or CD8$^+$ T cells, $p=3.4\times10^{-14}$ (D), evaluated using the optimal cutoff approach. P value was determined by the log-rank test. Tables show the number of patients according to the expression of CRT and DC-LAMP or CD8 high and low groups.
**Figure 3:** Primary epithelial cells of NSCLC patients can expose CRT at their cell surface

Flow cytometry analysis of CRT expression on the cell surface of epithelial cells, leukocytes and stromal cells. Histograms representing strategy of gating for non-tumoral (left panel) and tumoral tissues (right panel) among NSCLC patients treated by primary surgery. Only DAPI⁻ cells or Live/Death staining yellow negative cells are selected for further analysis of CD45⁺ and cells positive for epithelial markers (EM) (A). Cells located in upper left quadrant CD45⁺/EM⁻ (R2) represent the population of leukocytes, cells located in lower left quadrant CD45⁻/EM⁻ (R3) represent the population of stromal cells and cells located in lower right
quadrant CD45⁻/EM⁺ (R1) represent the population of epithelial cells (B). Representative histograms for cell surface expression of CRT determined by cytometry on epithelial cells, leukocytes and stromal cells in both non-tumoral (left panel) and tumoral tissues (right panel) (C). CRT surface expression on epithelial cells, leukocytes and stromal cells in non-tumoral (NT) or tumoral tissues (T) in a cohort of 50 NSCLC patients. P values were calculated by t-test, ***p<0.001 (D). Representative confocal microscopy staining for CRT on the cell surface that colocalized with Na⁺-K⁺ pump surface staining (E). P values were calculated by t-test, NS non significant.
Figure 4: Phenotypic analysis of the immune cell infiltrate according to the high and low expression of CRT among prospective cohort of NSCLC patients

Large scale flow cytometry analysis of immune cell populations stratified by the expression of CRT in 25 fresh lung tumors (13 CRT$^{\text{low}}$ tumors vs. 12 CRT$^{\text{high}}$ tumors using median stratification). Expression of CRT was evaluated by flow cytometry. The percentage of different cell types among total live mononuclear cells from the tumors of CRT$^{\text{high}}$ and CRT$^{\text{low}}$ is shown. The percentage of total CD45, myeloid and plasmacytoid dendritic cells (cDCs and pDCs) and CD3 T cells and CD4 and CD8 T cell subsets is shown (A). T cell subsets with naïve, effector memory, central memory and terminally differentiated effector memory (TEM-RA) phenotype based on expression of CD45RA and CCR7 in CRT$^{\text{high}}$ and CRT$^{\text{low}}$ tumors is shown (B). P values were calculated by t-test, *p<0.05.
List of supplementary material:

**Supplemental Figure 1:** Determining the optimal cutoff for the cohort of patients not treated with neoadjuvant chemotherapy.

**Supplemental Figure 2:** Kaplan-Meier survival curves for OS for NSCLC patients across quartiles of CRT expression.

**Supplemental Figure 3:** Positive prognostic value conferred by tumoral CRT and P-eiF2α expression in NSCLC patients treated by neo-adjuvant chemotherapy.

**Supplemental Figure 4:** Prognostic value conferred by tumor CRT expression combined with DC-LAMP and CD8 densities in NSCLC patients using median stratification.

**Supplemental Figure 5:** CRT detection in primary epithelial cells of NSCLC patients after permeabilization process.

**Supplemental Table 1:** Antibodies used for FACS analysis

**Supplemental Table 2:** Clinical characteristics of stage III NSCLC patients treated by neo-adjuvant chemotherapy, with CRT^{Hi} versus CRT^{Lo} tumors.

**Supplemental Table 3:** Univariate cox-regression for patients with chemotherapy

**Supplemental Table 4:** Multivariate cox-regression for patients with chemotherapy
Supplementary data:

**Supplemental Fig. 1.** Determining the optimal cutoff for the cohort of patients not treated with neoadjuvant chemotherapy
(A) Related to Figure 1. The optimal cutoff is 24.5 for CRT expression. (B) and (C) Related to Fig. 3. The optimal cutoff is 1.25 for DC-LAMP and 64.2 cells/mm² CD8 density. Related to Fig. 2. The optimal cutoff is 2.1 for eIF2α expression (D). P-values were corrected using the method proposed by Altman. Vertical dashed bars show side cutoff values that are discarded beforehand.

**Supplemental Fig. 2.** Kaplan-Meier survival curves for OS for NSCLC patients across quartiles of CRT expression, $p=4.35\times10^{-12}$
Supplemental Fig. 3. Positive prognostic value conferred by tumoral CRT and P-eIF2α expression in NSCLC patients treated by neo-adjuvant chemotherapy

Kaplan-Meier survival curves for OS for 125 patients with NSCLC treated by neo-adjuvant chemotherapy according to the presence of a high or low expression of CRT evaluated using the optimal cutoff approach, p=0.017 (A) and using median, p=0.0067 (B). Kaplan-Meier survival curves for OS for 83 NSCLC patients treated by neo-adjuvant chemotherapy according to the presence of a high or low index of P-eIF2α using the optimal cutoff approach, p=0.033 (C) or using a median cutoff, p=0.089 (D). P values were determined by log-rank tests. Tables show the number of patients according to the CRT high and low groups.
Correlation graph for P-eIF2α and CRT expression on tumor cells in the cohort of NSCLC patients with neoadjuvant chemotherapy (E).

**Supplemental Fig. 4.** Prognostic value conferred by tumor CRT expression combined with DC-LAMP and CD8 densities in NSCLC patients using median stratification for CRT.

Supplemental figure 4

Kaplan-Meier survival curves for OS curve for 221 NSCLC patients without neoadjuvant chemotherapy according to the presence of a high or low density of DC-LAMP$^+$ cells evaluated using the optimal cutoff, $p=8.5\times10^{-5}$, $P$ values were determined by log-rank tests. Table shows the number of patients according to the DC-LAMP high and low density groups (A). Kaplan-Meier survival curves for OS curve for 221 NSCLC patients without neoadjuvant chemotherapy according to the presence of a high or low density of CD8$^+$ cells evaluated using the optimal cutoff, $p=6.2\times10^{-4}$ (B). P value was determined by the log-rank test. Table
Fucikova et al., 2015

shows the number of patients according to the CD8 high and low groups. Kaplan-Meier survival curves for OS for NSCLC patients according CRT expression combined with the presence of a high or low density of DC-LAMP$^+$ cells, $p=1.07 \times 10^{-9}$ (C), CD8$^+$ T cells, $p=2.6 \times 10^{-7}$ (D) evaluated using median approach. P value was determined by the log-rank test. Tables show the number of patients according to the expression of CRT and DC-LAMP or CD8 high and low groups.
Supplemental Fig. 5. CRT detection in primary epithelial cells of NSCLC patients after permeabilization process

Flow cytometry analysis of CRT expression on the cell surface of epithelial cells, leukocytes and stromal cells after permeabilization process. Histograms representing strategy of gating for non-tumoral (left panel) and tumoral tissues (right panel) among NSCLC patients treated by primary surgery. Only DAPI− cells or Live/Death staining yellow negative cells are
selected for further analysis of CD45$^+$ and cells positive for epithelial markers (EM) (A). Cells located in upper left quadrant CD45$^+$/EM$^-$ (R2) represent the population of leukocytes, cells located in lower left quadrant CD45$^-$/EM$^-$ (R3) represent the population of stromal cells and cells located in lower right quadrant CD45$^-$/EM$^+$ (R1) represent the population of epithelial cells (B). Representative histograms for cell surface expression of CRT determined by cytometry on epithelial cells, leukocytes and stromal cells in both non-tumoral (left panel) and tumoral tissues (right panel) (C). CRT surface expression on epithelial cells, leukocytes and stromal cells in non-tumoral (NT) or tumoral (T) tissues (D). P values were calculated by t-test, *p<0.05.
### Supplemental Table 1. Antibodies used for FACS analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Conjugate</th>
<th>Host</th>
<th>Isotype</th>
<th>Clone or reference</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>APC</td>
<td>mouse</td>
<td>IgG1</td>
<td>HI30</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD11c</td>
<td>PC 5.5</td>
<td>mouse</td>
<td>IgG1</td>
<td>BU15</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>CD86</td>
<td>PE</td>
<td>mouse</td>
<td>IgG2b</td>
<td>JT2.2</td>
<td>BioLegend</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>APC-Cy7</td>
<td>mouse</td>
<td>IgG2a</td>
<td>L243</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CCR7</td>
<td>PB</td>
<td>mouse</td>
<td>IgG2a</td>
<td>G043H7</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD123</td>
<td>PC7</td>
<td>mouse</td>
<td>IgG2a</td>
<td>7G3</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD3</td>
<td>FITC</td>
<td>mouse</td>
<td>IgG1</td>
<td>UCHT1</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD16</td>
<td>FITC</td>
<td>mouse</td>
<td>IgG1</td>
<td>3G8</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD19</td>
<td>FITC</td>
<td>mouse</td>
<td>IgG1</td>
<td>LT19</td>
<td>Exbio</td>
</tr>
<tr>
<td>CD20</td>
<td>FITC</td>
<td>mouse</td>
<td>IgG2a</td>
<td>B9E9</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>CD56</td>
<td>FITC</td>
<td>mouse</td>
<td>IgG2a</td>
<td>MEM-188</td>
<td>Exbio</td>
</tr>
<tr>
<td>CD14</td>
<td>PE-DYS90</td>
<td>mouse</td>
<td>IgG1</td>
<td>MEM-15</td>
<td>Exbio</td>
</tr>
<tr>
<td>CD3</td>
<td>Alexa 700</td>
<td>mouse</td>
<td>IgG1</td>
<td>UCHT1</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD4</td>
<td>FITC</td>
<td>mouse</td>
<td>IgG1</td>
<td>13B8.2</td>
<td>Immunotech</td>
</tr>
<tr>
<td>CD8</td>
<td>APC-H7</td>
<td>mouse</td>
<td>IgG1</td>
<td>SK1</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD27</td>
<td>APC-Alexa750</td>
<td>mouse</td>
<td>IgG2a</td>
<td>CLB-27/1</td>
<td>Caltag</td>
</tr>
<tr>
<td>CD28</td>
<td>PE</td>
<td>mouse</td>
<td>IgG1</td>
<td>CD28.2</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD56</td>
<td>APC</td>
<td>mouse</td>
<td>IgG2b</td>
<td>NCAM16.2</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD62L</td>
<td>APC</td>
<td>mouse</td>
<td>IgG1</td>
<td>Dreg-56</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD69</td>
<td>PeCy7</td>
<td>mouse</td>
<td>IgG1</td>
<td>FN50</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CCR7</td>
<td>UC</td>
<td>mouse</td>
<td>IgM</td>
<td>2H4</td>
<td>BD Pharmingen</td>
</tr>
</tbody>
</table>
**Supplemental Table 2.** Clinical characteristics of stage III NSCLC patients treated by neoadjuvant chemotherapy, with CRT\textsuperscript{Hi} versus CRT\textsuperscript{Lo} tumors.

<table>
<thead>
<tr>
<th></th>
<th>Overall cohort (n=125)</th>
<th>CRT\textsuperscript{Hi} (n=77)</th>
<th>CRT\textsuperscript{Lo} (n=48)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>102 (82%)</td>
<td>64 (83%)</td>
<td>38 (79%)</td>
<td>0.64</td>
</tr>
<tr>
<td>Female</td>
<td>23 (18%)</td>
<td>13 (17%)</td>
<td>10 (21%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (years) +/-SEM</td>
<td>57 ± 0.069</td>
<td>56 ± 0.11</td>
<td>59 ± 0.17</td>
<td>0.047</td>
</tr>
<tr>
<td><strong>Smoking history</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smoker</td>
<td>107 (86%)</td>
<td>66 (86%)</td>
<td>41 (85%)</td>
<td>1</td>
</tr>
<tr>
<td>Never smoker</td>
<td>18 (14%)</td>
<td>11 (14%)</td>
<td>7 (15%)</td>
<td></td>
</tr>
<tr>
<td><strong>Histological type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADC</td>
<td>58 (46.5%)</td>
<td>40 (52%)</td>
<td>18 (37.5%)</td>
<td>0.045</td>
</tr>
<tr>
<td>SCC</td>
<td>48 (38.5%)</td>
<td>30 (39%)</td>
<td>18 (37.5%)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>19 (15%)</td>
<td>7 (9%)</td>
<td>12 (25%)</td>
<td></td>
</tr>
<tr>
<td><strong>P-eiF2α score</strong></td>
<td>3.6 ± 0.015</td>
<td>4.4 ± 0.013</td>
<td>2.4 ± 0.03</td>
<td>2.90e-10</td>
</tr>
</tbody>
</table>

All parameters were evaluated among the 125 NSCLC patients. P-eiF2α score was evaluated among 83 patients. Age was calculated at the date of the surgery. Chi-square P values were determined using the Fisher’s and the Wilcoxon exact tests. Abbreviations: ADC, adenocarcinoma; SCC, squamous cell carcinoma.
### Supplemental Table 3: Univariate cox-regression for patients with chemotherapy

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (F vs M)</td>
<td>1.73</td>
<td>0.86-3.47</td>
<td>0.12</td>
</tr>
<tr>
<td>Age, years</td>
<td>1.01</td>
<td>0.98-1.03</td>
<td>0.51</td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoker vs smoker</td>
<td>0.84</td>
<td>0.44-1.60</td>
<td>0.61</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADC vs SCC</td>
<td>0.67</td>
<td>0.39-1.13</td>
<td>0.13</td>
</tr>
<tr>
<td>ADC vs others</td>
<td>1.32</td>
<td>0.71-2.46</td>
<td>0.37</td>
</tr>
<tr>
<td>CRT score</td>
<td>0.78</td>
<td>0.65-0.92</td>
<td>0.0045</td>
</tr>
<tr>
<td>P-eIF2α score</td>
<td>0.80</td>
<td>0.64-0.99</td>
<td>0.045</td>
</tr>
<tr>
<td>CRT group, Hi vs Low</td>
<td>0.46</td>
<td>0.28-0.73</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Univariate Cox- proportional hazards analysis for overall survival according to clinical and immune parameters.
**Supplemental Table 4:** Multivariate cox-regression for patients with chemotherapy

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRT score</td>
<td>0.75</td>
<td>0.56-1.01</td>
<td>0.06</td>
</tr>
<tr>
<td>P-eiF2α score</td>
<td>0.98</td>
<td>0.73-1.33</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Multivariate Cox proportional hazards analyses for overall survival according to clinical and immune parameters. Parameters identified in univariate analysis as significantly influencing outcome were introduced in multivariate Cox-proportional hazards regression model.
A high density of tertiary lymphoid structure B cells in lung tumors is associated with increased CD4+ T cell receptor repertoire clonality


To cite this article: Wei Zhu, Claire Germain, Zheng Liu, Yinong Sebastian, Priyanka Devi, Samantha Knockaert, Philip Brohawn, Kim Lehmann, Diane Damotte, Pierre Validire, Yihong Yao, Vilia Valge-Archer, Scott A. Hammond, Marie-Caroline Dieu-Nosjean & Brandon W. Higgs (2015): A high density of tertiary lymphoid structure B cells in lung tumors is associated with increased CD4+ T cell receptor repertoire clonality, OncoImmunology, DOI: 10.1080/2162402X.2015.1051922

To link to this article: http://dx.doi.org/10.1080/2162402X.2015.1051922
A high density of tertiary lymphoid structure B cells in lung tumors is associated with increased CD4+ T cell receptor repertoire clonality

Wei Zhu1, Claire Germain2,3,4, Zheng Liu1, Yinong Sebastian1, Priyanka Devi2,3,4, Samantha Knockaert2,3,4, Philip Brohawn1, Kim Lehmann1, Diane Damotte2,3,4,5, Pierre Validire2,3,4,6, Yihong Yao1, Viia Valge-Archer7, Scott A. Hammond8, Marie-Caroline Dieu-Nosjean2,3,4, and Brandon W. Higgs1

1 Translational Sciences, MedImmune, Gaithersburg, Maryland, USA
2 Laboratory “Cancer, Immune control, and Escape”, INSERM UMRS 1138, Cordeliers Research Center, Paris, France
3 University Sorbonne, University Pierre and Marie Curie, UMRS 1138, Paris, France
4 University Sorbonne Paris Cité, University Paris Descartes, UMRS 1138, Paris, France
5 Department of Pathology, Cochin hospital, AP-HP, Paris, France
6 Department of Pathology, Institut Mutualiste Montsouris, Paris, France
7 Oncology Research, MedImmune, Cambridge, United Kingdom
8 Oncology Research, MedImmune, Gaithersburg, Maryland, USA

Corresponding author Email: zhuw@medimmune.com

Financial support: This work was supported by the “Institut National de la Santé et de la Recherche Médicale”, University Paris-Descartes, University Pierre and Marie Curie, and MedImmune.

Keywords: TCR/BCR repertoire, tertiary lymphoid structure, non-small cell lung cancer, deep sequencing

Abbreviations: TCR=T cell receptor; BCR=B cell receptor; IgH=Immunoglobulin heavy chain; CDR3=complementarity-determining region 3; NSCLC=non-small cell lung cancer; TLS=tertiary lymphoid structure; NGS=next generation sequencing; DC=dendritic cell; ADC=adenocarcinoma; SCC=squamous cell carcinoma; LCC=large-cell carcinoma; NT=non-tumoral distant tissue; P= blood/draining lymph node; T_{RM}=tissue resident-memory T cell; Foll-B cell=Follicular B cell; T_{FH}=follicular helper T cell.
T and B cell receptor (TCR and BCR, respectively) Vβ or immunoglobulin heavy chain complementarity-determining region 3 sequencing allows monitoring of repertoire changes through recognition, clonal expansion, affinity maturation, and T or B cell activation in response to antigen. TCR and BCR repertoire analysis can advance understanding of anti-tumor immune responses in the tumor microenvironment.

TCR and BCR repertoires of sorted CD4⁺, CD8⁺ or CD19⁺ cells in tumor, non-tumoral distant tissue (NT), and peripheral compartments (blood/draining lymph node [P]) from 47 non-small cell lung cancer (NSCLC) patients (age\text{median}=68 years) were sequenced. The clonotype spectra were assessed among different tissues and correlated with clinical and immunological parameters.

In all tissues, CD4⁺ and CD8⁺ TCR repertoires had greater clonality relative to CD19⁺ BCR. CD4⁺ T cells exhibited greater clonality in NT compared to tumor (p=0.002) and P (p<0.001), concentrated among older patients (age>68). Younger patients exhibited greater CD4⁺ T cell diversity in P compared to older patients (p=0.05), and greater CD4⁺ T cell clonality in tumor relative to P (p<0.001), with fewer shared clonotypes between tumor and P than older patients (p=0.04). More interestingly, greater CD4⁺ and CD8⁺ T cell clonality in tumor and P, respectively (both p=0.05), correlated with high density of tumor-associated tertiary lymphoid structure (TLS) B cells, a biomarker of higher overall survival in NSCLC.

Results indicate distinct adaptive immune responses in NSCLC, where peripheral T cell diversity is modulated by age, and tumor T cell clonal expansion is favored by the presence of TLSs in the tumor microenvironment.
Introduction

Methods to obtain both the identity and classification of tumor antigens have become fundamental to our understanding of the host-triggered immune response in multiple cancers. When exposed to either tumor-specific or -associated antigens, the adaptive immune system reacts with a collection of antigen-specific receptors expressed by populations of B and T lymphocytes.\(^1\) This large repertoire of T or B cell receptors (TCRs or BCRs, respectively), generated by random rearrangements of the variable (V), diversity (D), and joining (J) segments during haematopoiesis, is intentionally diverse for generalized targeting of a broad range of potential antigens.\(^1\)-\(^3\) Following binding of the TCR or BCR to its cognate antigen, naïve T or B lymphocytes become activated, proliferate and may undergo somatic mutations of the V regions to improve the binding affinity of the TCR and BCR to antigen as well as class switch recombination.\(^4\),\(^5\) Clonal expansion (i.e., high clonality), or the large increase in T or B cells from a single cell, can be the interpreted as the opposite of diversity – the latter of which indicates a variety of different clones with few dominant clones at high frequencies. The expansion of clones with the highest affinity TCRs and BCRs for antigen may reduce the diversity (increase the clonality) of the responding lymphocyte populations.\(^2\) As humans age, immune responses become impaired, characterized by: (1) reduced lymphopoiesis of new naïve B and T cells, with novel BCRs\(^6\) and TCRs,\(^7\) respectively, (2) reduced dendritic cell (DC) function resulting in poor responses to inflammatory signals and lowered capacity to present antigen and to activate adaptive immune responses,\(^8\) (3) poor germinal center formation, site of B cell expansion and selection,\(^9\) and (4) decreased potential for T cell expansion, expression of activation markers, production of cytokines, diversity of TCR repertoire and delayed T cell responses to antigen.\(^7\),\(^10\) Thus, these changes during aging help to explain the increased susceptibility to infection and reduced response to vaccinations in the elderly population.\(^3\),\(^11\),\(^12\)

Previous work has characterized the presence of immune cells organized as ectopic lymphoid structures, referred as tertiary lymphoid structures (TLS), in the tumor of non-small cell lung cancer (NSCLC) patients.\(^13\),\(^14\) These TLS contain a large density of DC-Lamp\(^+\) mature DCs and CD20\(^+\) follicular B (Foll-B) cells, both of which home selectively into TLS. The densities of these mature DCs or Foll-B cells have been shown to associate with improved prognosis in NSCLC patients,\(^13\),\(^15\) anti-tumor humoral immune response and cytotoxic and Th1 T
cell differentiation. However, detailed studies to understand the role of TLS in the generation and expansion of intra-tumoral immune cells (T and B cell subsets) and shaping of TCR and BCR repertoire are lacking.

With the advancement of next generation sequencing (NGS), it is now possible to fully characterize TCR and BCR repertoires, to accurately represent the clonal diversity in humans. Primers specific for the V, D, J, and constant (C) gene segments of the CDR3 of TCRs and BCRs are used to generate millions of sequencing reads and thousands of unique clonotypes, with distributional properties that can be monitored over time or compared between the tumor or normal tissue compartments within a cancer patient. The CDR3 region of the receptor is the primary CDR that contributes most to the antigen recognition by the interaction between the TCR and its cognate peptide antigen located within the major histocompatibility complex groove, or BCR with its cognate polypeptide antigen, and is the principle component of antigen recognition specificity in both TCRs and BCRs. TCR and BCR repertoire technology can provide a robust characterization of the global receptor repertoire of particular lymphocyte populations, though within both the peripheral and disease-affected compartments, there is a mixed population of T cells that can differ by clonotype contribution, cell subtypes, and sensitivities to therapies.

Further, the frequencies of monoclonal and oligoclonal TCR Vβ families have been shown to significantly differ between CD4+ and CD8+ T cell subsets. Because it represents a challenge to discern the cellular composition within the population of tumor-infiltrating lymphocytes, sorting of cell subsets is important to further accurately distinguish contribution between the CD4+ and CD8+ adaptive response.

In this study, we used NGS to evaluate the spectra of the Vβ of TCR or immunoglobulin heavy (IgH) chain of BCR repertoires in CD4+, CD8+, or CD19+ cells from tumor (T), non-tumoral distant tissue (NT), or peripheral (peripheral blood or draining lymph node, P) compartments of NSCLC patients. We revealed the differences in immunodiversity in CD4+ and CD8+ TCR repertoires compared to CD19+ BCR repertoire, the most recurrent differences in clonality between the three compartments in CD4+ T cells across patients, as well as age-specific differences in TCR/BCR repertoires. Additionally, intratumoral or peripheral CD4+ or CD8+ TCR repertoires, respectively, were correlated with TLS B cell density, a previously characterized biomarker of higher overall survival in NSCLC patients. These findings further
our understanding of the adaptive response to tumor antigens and help to characterize the TCR and BCR diversity in the tumor microenvironment in NSCLC.

Results

Global differences in clonality index between NSCLC patients and healthy donors

In this study, nonproductive or out-of-frame rearrangement TCR Vβ or BCR IgH CDR3 sequences were not included in the analysis. Among all productive clones, the TCR and BCR repertoire clonality was evaluated among the 47 NSCLC patients across four tissue compartments: tumor, non-tumoral (normal) distant tissue, peripheral blood, or draining lymph node (LN). Few public clones were shared between patients, as expected from a previous report. In both CD4+ and CD8+ TCR and CD19+ BCR repertoires, the clonality in the peripheral blood and to a lesser extend the lymph node of NSCLC patients was generally higher than that observed in a cohort of 11 normal healthy donor blood specimens (10 BCR and 9 TCR; Figure 1A). Within both the NSCLC patient and healthy donor cohorts, the TCR repertoire had higher clonality than the BCR repertoire in the blood. As expected, in both cohorts, the clonality of the TCR and BCR repertoires was positively correlated with the maximum single clonotype frequency (Figure 1B).

Both cell counts and nucleic extraction yields for all samples (Supplementary Table 1) were compared against the various measures of immunodiversity and no correlations were identified (Supplementary Figures 1-4).

Differences in CD4+ TCR repertoire clonal expansion between the different tissue compartments of NSCLC patients

Across all four different tissue compartments, there was a general increase in average clonality index from CD19+ to CD4+ to CD8+ cells (Figure 2). Specifically, regardless of the anatomical site, the average CD8+ TCR clonality is much higher than both the CD4+ T and B cells. This pattern is most consistent in the tumor, non-tumoral distant tissue, and peripheral
blood compartments and less consistent in CD4+ cells in the draining lymph node (data not shown). Subjects had either matched peripheral blood or draining lymph node specimens, but not both. To maximize the number of patient samples with matched tumor and matched non-tumoral distant tissue, peripheral blood or draining lymph node specimens, the two peripheral compartments were combined into a single measurement (peripheral blood/draining lymph node). Then the clonality was compared between the tumor (T), non-tumoral distant tissue (NT) and peripheral blood/draining lymph node (P) for each patient within the CD19+, CD4+, or CD8+ cell populations. Within the CD4+ T cells specifically, the non-tumoral distant tissue specimens had a significantly higher average TCR repertoire clonality index than both the peripheral compartment (p<0.001) and the tumor (p=0.002), while the tumor had a significantly higher average clonality index compared to the peripheral compartment (p<0.001; Figure 3). No significant differences in clonality were observed between the tumor, the non-tumoral distant tissue, or the peripheral compartments in either the CD19+ or CD8+ cell populations (Figure 3).

Younger NSCLC patients have higher CD4+ clonal diversity in the peripheral compartment and older NSCLC patients have highest clonality in the non-tumoral distant tissue

The patient population was divided into two age groups at the median of 68 years. Within the CD4+ cell population, the average clonality index was significantly higher in the patients age>68 compared to patients age≤68 in the peripheral compartment (p=0.05) or stated alternatively, younger patients (age≤68) exhibited higher immunodiversity in the peripheral compartment (Table 1 and Figure 4A). The same trend (though not statistically significant) was also observed for CD4+ T cells in non-tumoral distant lung. The younger patient cohort had a significantly higher average clonality index in the tumor compared to the peripheral compartment (p<0.001; Figure 4B), while the older patient cohort (age>68) had a significantly lower average clonality index in the tumor compared to the non-tumoral distant tissue (p<0.001; Figure 4B). This age stratification demonstrates the bias in the older patient cohort for driving the observation reported previously in this study regarding higher average CD4+ TCR clonality index observed in the non-tumoral distant tissue compared to the two other compartments. No significant difference in clonality was observed between the peripheral compartment and tumor in the older patient cohort or between the non-tumoral distant tissue and tumor in the younger
patient cohort. The non-tumoral distant tissue in both the younger and older patient cohorts had higher average CD4\(^+\) TCR clonality indices compared to the peripheral compartment (p<0.001; Figure 4B). Though statistical significance was not observed, this similar trend of increased average clonality in older patients in the non-tumoral distant tissue and peripheral compartment was observed for the CD8\(^+\) TCR repertoire, noting that the CD8\(^+\) T cells showed the highest overall clonality index (Figure 2).

When evaluating the prevalence of shared clonotypes (Horn index) between pairs of each of the three tissue compartments for a patient, there was a higher average prevalence of shared CD4\(^+\) clonotypes between the peripheral compartment and tumor in the older patient cohort compared to the younger patient cohort (p=0.04; Figure 5). A similar trend in shared CD4\(^+\) clonotypes was also observed between the non-tumoral distant tissue and peripheral compartment, though not statistically significant (p=0.08). No difference in prevalence of shared clonotypes between the tumor and non-tumoral distant tissue was observed between the age groups.

Also within CD4\(^+\) cells, female NSCLC patients exhibited a higher average clonality index in the non-tumoral distant tissue compared to males (p=0.05; Table 1), though across tissue compartments, there was no significant association observed with gender, in contrast to that observed with age. No significant difference in comparisons of clonotypes with either the highest frequency or richness value (R) between age groups was observed (Supplementary Figure 5).

CD4\(^+\) TCR repertoire clonal expansion correlates with high TLS B cell density in the tumor

A previous study has demonstrated a correlation between TLS CD20\(^+\) follicular B-cell density and patient survival in NSCLC, thus connecting TLS to a protective B cell–mediated immunity (Supplementary Figure 6).\(^{15}\) Among the NSCLC patients, 30 were determined to be Follicular B cell (Foll-B cell) high and 17 were Foll-B cell low (Table 1). Using this population of 47 patients, the CD4\(^+\) and CD8\(^+\) TCR repertoires had higher average clonality indices in the Foll-B cell high group (p=0.05) compared to the Foll-B cell low group in the tumor (CD4\(^+\)) and in the peripheral (CD8\(^+\)) compartment, respectively (Figure 6). In contrast, neither CD8\(^+\) nor
CD19+ cells demonstrated a significant difference between Foll-B cell high or low groups in the
tumor and no difference between Foll-B cell high or low groups was correlated with average
clonality for CD8+, CD4+, or CD19+ cells in the non-tumoral distant lung.

The prevalence of shared clonotypes between pairs of each of the three compartments for
a patient was evaluated between the Foll-B cell high or low groups and no significant difference
was observed for any compartment pairing or cell population (data not shown). Similarly, no
difference in richness was observed between Foll-B cell high or low groups (data not shown).

Discussion

Here we characterized the TCR and BCR repertoires in NSCLC patients in sorted CD4+, 
CD8+, or CD19+ cell subsets to understand the clonotype spectra differences between the tumor,
non-tumoral distant tissue, and peripheral compartments. The greatest average clonality was
observed in CD4+ and CD8+ TCRs, with CD4+ TCRs having greatest average clonal expansion
in the non-tumoral distant tissue, though this was shown to be driven by the clonal expansion in
the older patient cohort. The tumor exhibited greatest average clonotype expansion compared to
peripheral compartments, but only in the younger patient cohort. High immunodiversity was
observed in CD4+ TCRs within the peripheral compartment in the young patient cohort relative
to old patient cohort. Few public clonotypes were shared between patients, though the highest
clonotype commonality occurred between compartments within the same patient. Specifically,
among common clonotypes for a patient between tissue compartments, the older patient cohort
had significantly more CD4+ clonotypes shared between the tumor and peripheral compartment
as compared to the younger patient cohort; a similar trend with age groups was observed
between the peripheral compartment and non-tumoral distant tissue. Further, among variables
with at least 20% patients in one group, there was no correlation observed in any compartment or
cell type between clonality index and T stage, N stage, histological type, or density of CD8.
Smoking status was highly skewed towards smokers in this study (77% smokers vs. 13% non-
smokers), thus no association with clonality was calculated with this variable.

Intrinsic and extrinsic factors that define the diversity of different T cell subsets within
the tumor have been previously documented,26 in addition to the correlation between the
intratumoral prevalence of certain subsets such as CD8+ or regulatory T cells (Tregs) and clinical
prognosis in specific cancers.\textsuperscript{27-29} Among the different T cell subsets, both the intratumoral and inter-compartmental heterogeneity of the TCR repertoire has been reported in studies in many types of cancer including colorectal, renal cell, and ovarian cancer.\textsuperscript{23,30,31} Even within CD8\textsuperscript{+} T cells, subsets with expression of different surface markers have revealed differences in oligoclonal expansion in TCRβ clonotypes.\textsuperscript{32} These results underscore the importance of appropriate characterization of the activation and infiltration status within tumor or normal compartments with a mixed population of T cell subsets that differ in complexity and diversity.\textsuperscript{3,32,33} In this study, we address this issue with sorted CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells and CD19\textsuperscript{+} B cell subsets within the tumor, non-tumoral distant tissue, or peripheral compartments of a large population of NSCLC patients.

This type of cell-specific TCR or BCR sequencing from multiple tissue compartments requires sufficient specimen and yields following cell sorting and deoxyribonucleic acid extraction. Limitations in substantial material can greatly bias sequenced read counts, which influences normalization and measures of diversity/clonality, richness, and shared clonotypes. In cases where certain specimens had insufficient read counts, these samples were removed from the analysis. Similarly, both cell counts and nucleic extraction yields for all samples were compared against the various measures of immunodiversity and no correlations were identified, thus reducing any potential technical biases in findings. Nonetheless, future studies may benefit from previous methods developed to estimate patient CD4\textsuperscript{+} or CD8\textsuperscript{+} TCR contribution from PBMC populations, rather than relying on cell sorting and potentially compromising sufficient sample yields for TCR repertoire characterization.\textsuperscript{34}

Across all NSCLC patients, the lowest average CD4\textsuperscript{+} clonality (thus the highest immunodiversity) was observed in the peripheral compartment (blood/lymph nodes) compared to both non-tumoral and tumoral lung of patients. In the peripheral compartment, naïve T cells are typically primed within the draining lymph node, antigen-specific T cells are activated and undergo clonal expansion, and then migrate to tumor sites (and distant normal); a greater number of specific T cell clones would be expected in inflammatory sites/tumor tissues. We previously reported that lung tumor associated-TLS are mainly composed of CD4\textsuperscript{+} T cell subsets among the T cell compartment.\textsuperscript{16} Even rare, tumor-infiltrating naïve T cells are selectively detected in contact with mature DC in TLS suggesting that TLS may represent an active site for the priming and the proliferation of selected CD4\textsuperscript{+} T cell clones and thus may explain why the CD4\textsuperscript{+} T cell
clonality is higher in tumor versus the peripheral compartment. As the immune infiltrate, and in particular TLS are mainly detected in the invasive margin of the tumor (i.e. areas at the interface between the non-tumoral and the tumoral tissues), TLS-derived effector T cells may also migrate to the non-tumoral distant tissue, an argument in favor of a high clonality index of CD4+ T cells also in the non-tumoral distant lung.

This pattern was exemplified in the younger patient cohort in CD4+ T cells, where the tumor exhibited higher clonality compared to the peripheral compartment, as opposed to older NSCLC patients who displayed a high CD4+ T cell clonality in the periphery. A recent study showed a similar pattern in the peripheral compartment of younger healthy subjects, with increased TCR repertoire diversity in the blood of younger subjects and significant decrease with age. Using ultra-deep sequencing, the authors revealed that diversity of T cell clonotypes decreased almost linearly with age. In addition, there was a significant negative correlation of age with the percentage of naïve T cells in peripheral blood, though this trend was most pronounced up to subjects age 70. Therefore age-related immunosenescence may be the major mechanism responsible for the difference of CD4+ T cell clonality between younger and older patients. The underlying mechanisms of immunosenescence of T cells can be traced back to thymic involution with aging. However, the immune system may also selectively purge certain T cell clonotypes while keeping others. In a recent study, it was demonstrated that in addition to repertoire richness contraction, age was associated with inequality of clonal sizes, namely the presence of large naïve T cell clones in the repertoire of elderly individuals, reflecting uneven homeostatic proliferation; this hypothesis needs further investigation.

Also relevant in the older patient cohort, is the reduced clonality within the tumor relative to the non-tumoral distant tissue potentially as a result of an immunosuppressive tumor microenvironment, the magnitude of which may further increase due to age-related immunosenescence. The promotion of a tolerant microenvironment by tumors through mechanisms of antigen-presentation impairment, defects in TCR signaling, and introduction of immunosuppressive factors has been well characterized. Within antigen-specific CD4+ T cells specifically, both in vivo and in vitro studies have demonstrated that they become tolerant during tumor growth. T cells are not primed and rendered unresponsive during tumor progression, a mechanism found in both solid and hematologic tumors. Tumor-induced tolerance in CD8+ T cells also exists, though both the definition of tolerance in the CD8+ subset
as well as the downstream mechanistic effect is not as clearly known as that reported in CD4\(^+\) T cells.\(^{42}\) Induced defects in TCR signaling is another strategy exhibited by the tumor to promote growth and progression. Studies have shown a reduction in components that drive the effector phase of T cells to induce anti-tumor responses, such as decreased expression of CD3 \(\zeta\) chain and reduced activity of the TCR-associated tyrosine kinases p56(Lck) and p59(Fyn).\(^{50,51}\)

Additional support for the increased clonality in the non-tumor relative to the tumor compartment is based on recent studies describing the presence of tissue resident-memory T (T\(_{RM}\)) cells in peripheral non-lymphoid tissues. T\(_{RM}\) cells have an important role in protective responses against infection and in the recruitment of immune cells to tissues in cancer.\(^{52-59}\) These clonally expanded memory T cells, located in peripheral tissues, can launch a rapid response to antigen following an initial antigen exposure.\(^{53}\) CD4\(^+\) T\(_{RM}\) cells reside in non-lymphoid tissues such as skin, lung, and mucosal compartments.\(^{54-59}\) Within non-inflamed normal lung tissue, distant from localized tumor in patients with various types of malignancies (localized to specific segments of the lung), V\(\beta\) segments of TCRs from CD4\(^+\) and CD8\(^+\) were measured and proliferation of these cells in response to Influenza exposure was compared between lung, skin, and blood.\(^{59}\) Results indicated that functional T\(_{RM}\) cells were much more prevalent in the lung compared to the other two compartments and enriched for T cells specific for Influenza antigens. A similar finding was identified in mice recipients of spleen- or lung-derived memory CD4\(^+\) T cells, which were compared following challenge with Influenza virus. The mice recipients of lung-derived memory T cells showed a much faster and robust protection against the virus compared to spleen-derived memory T cells, suggesting that functional T\(_{RM}\) cells have certain tissue compartmentalization properties, such that those retained in lung tissue provide increased protection to respiratory virus infection.\(^{56,59}\) These mechanisms support our observation of increased clonotype expansion in the non-tumoral distant tissues for CD4\(^+\) T cells relative to the tumor (as well as the periphery) of the NSCLC patients – a pattern driven by the older patient population.

Also in this older patient cohort, the prevalence of shared CD4\(^+\) clonotypes between the tumor and peripheral compartment is significantly higher than the younger patients – a trend that is apparent in the peripheral compartment and non-tumoral distant tissue as well. This implies that more unique CD4\(^+\) clonotypes are identified in the peripheral compartment in the younger patient cohort compared to the older patient counterpart, which might be explained by age-
related immune responses (i.e. reduced lymphopoiesis of new naïve CD4\(^+\) T cells in older patients). This concept is also supported by the lack of difference in shared clonotypes between the non-tumoral distant tissue and tumor when comparing the young and old patient cohorts. Previous reports have reinforced the concept of age-related shifts of the TCR repertoire in the peripheral compartment towards memory T cells in both mouse models and humans, with naïve T cells maintained in part by homeostatic proliferation.\(^{60-62}\) This ratio of naïve to memory T cells is reduced with age, as is TCR repertoire diversity in the naïve T cells, consistent with the CD4\(^+\) results shown here in the peripheral compartment, as well as more shared CD4\(^+\) clonotypes between the peripheral and tumor compartments. These findings, along with the aforementioned study by Qia et al,\(^3\) suggest further investigation into the relative abundance and clonotype profiles of naïve and memory CD4\(^+\) T cells and association with age.

A smaller study in 15 colorectal cancer patients has shown increased oligoclonality in tumors compared to mucosal tissue samples from the same patient,\(^{30}\) which agrees with what is reported in this study for patients less than the median age (≤68). For NSCLC patients older than the median age, a shift in adaptive response occurs, with the non-tumoral distant tissue having higher oligoclonality than the tumor. However, it is important to note that the clonality indices of CD4\(^+\), CD8\(^+\) and CD19\(^+\) cells did not differ between younger and older NSCLC patients in the tumor suggesting that an active process is involved in the selection and maintenance of specific T and B cell clones in the tumor, even in the presence of immunosenescence. One explanation would be the continuous expression of cognate tumor antigens in the tumor microenvironment over time in order to maintain clonal expansion of specific clones within the tumor relative to distant sites.

We also observed higher average clonality in female NSCLC patients, compared to males within the non-tumoral distant tissue in CD4\(^+\) T cells. This pattern was not associated with the age-related differences in clonality reported here, which dismisses any confounding associations with the age-specific findings (Fisher’s exact test \(p\) = not significant). Studies by Murray et al, have demonstrated gender-specific V(D)J recombinase-mediated TCR\(\beta\) gene usage and coding joint processing in human development that demonstrate direct effects on TCR diversity, though these studies were conducted on healthy children at a maximum age of 12.5 years.\(^{63,64}\) In this study, these gender-specific differences in clonality did not occur across compartments – an
observation that did occur with age-related associations, thus the strength of this gender-specific observation in NSCLC needs to be further evaluated in subsequent studies.

In previous work, we reported the presence of TLS consisting of Foll-B cells surrounding clusters of DC-Lamp$^+$ mature DCs and T cells in NSCLC tumors, and that a high density of mature DCs was correlated with improved patient prognosis.\(^\text{13}\) Similarly, we later showed that a high density of TLS Foll-B cells was correlated with improved survival in NSCLC patients, and that the combination of both mature DC and TLS B cell densities further improved this survival benefit.\(^\text{16}\) Here we observe a significant correlation between high TLS Foll-B cell density and high CD4$^+$ TCR repertoire clonality in the tumor, as well as high CD8$^+$ TCR repertoire clonality in the periphery. A similar trend was also shown for CD4$^+$ T cells in the non-tumoral lung and the peripheral sites suggesting that selective CD4$^+$ T cell clones may spread throughout the whole body.

It is known that B cells can act as powerful antigen-presenting cells in many diseases. In particular, Bouaziz et al. demonstrated in mouse models of autoimmune disorder that B cell depletion selectively impaired the activation and clonal expansion of autoreactive CD4$^+$ T cells but not of CD8$^+$ T cells.\(^\text{65}\) As TLS represents a privileged site for the initiation of adaptive immune responses, it is tempting to speculate that TLS B cells may play a key role in the priming and the proliferation of selected intratumoral CD4$^+$ T cell clones. In particular, among the CD4$^+$ T cell subsets, follicular helper T cells (T$_{FH}$) are selectively detected within the B cell follicle of secondary lymphoid organs as well as TLS.\(^\text{66}\) T$_{FH}$ specialize in providing cognate help to B cells in follicles, and are fundamental for germinal center formation, affinity maturation and the differentiation of naïve B cells into memory B cells and plasma cells secreting high-affinity antibodies. Thus, it is tempting to speculate that the high clonality of CD4$^+$ T cells observed in TLS Foll-B cell High group may be the result of an active cross-talk between T$_{FH}$ and follicular B cells leading to the proliferation of selective T$_{FH}$ clones. This hypothesis is in accordance with the favorable prognostic value of T$_{FH}$ reported in breast cancer patients.\(^\text{66}\)

In summary, advancements in deep sequencing have paved the way for measures of the anti-tumor immune response at the detail of the TCR and BCR involvement. This study is the first large patient study (n=47) to our knowledge to examine the TCR and BCR repertoires in sorted CD4$^+$, CD8$^+$, and CD19$^+$ cell subsets across four tissue compartments in NSCLC patients, with stringent tests of association. Here we notably present distinctive CD4$^+$ T cell involvement
in the adaptive immune response for patients with NSCLC, with a shift in age from high clonal expansion in the periphery to reduced clonal expansion in the tumor, potentially due to systemic immunosenescence and/or putative immunosuppression in tumors. Recently, clinical results for immune-mediated therapies for cancers (IMT-C) have shown encouraging results for treatment of cancers such as lung and melanoma. As these therapies generally activate T cells as a primary mechanism against tumor cells, the reduction in T cell diversity in older cancer patients may require unique personal healthcare strategies to consider potential T cell replenishment to maximize effectiveness of IMT-C in older patients. We also correlate tumoral CD4+ TCR repertoire clonality to high Foll-B cell density in the tumor microenvironment—a previously identified prognostic biomarker in NSCLC. Better understanding the mechanisms of the locally developed adaptive immune response may have important implications for the development of new drug interventions based on TCR repertoire within this cancer indication.

**Methods/Materials**

**NSCLC patients**

A prospective study was performed on fresh NSCLC samples, comprised of 47 untreated patients with NSCLC (Table 1). Tumor tissue (T), non-tumoral distant tissue (NT), peripheral blood (Bl), or draining lymph node (LN) were obtained from patients who underwent a complete surgical resection of their lung tumors at Hotel Dieu hospital, Cochin hospital or Institut Mutualiste Montsouris (Paris, France). Patients who received neoadjuvant chemotherapy or radiotherapy were ineligible. A written informed consent was obtained from patients before inclusion in the prospective study. The protocol was approved by the local ethics committee (n°2012-0612) in application with the article L.1121.1 of French law.

**Cell sorting**

Fresh tissues were mechanically (manual) dissociated and digested in the presence of DNAse I (Sigma Aldrich) and collagenase A (Roche) for 1h at 37°C. The cell suspension was then filtered through a 70-μm filter (BD Biosciences), and mononuclear cells were isolated by centrifugation over a Ficoll Hypaque density gradient. CD3+ CD4+ and CD3+ CD8+ T cells as well as CD19+ B cells were sorted using a FACS Aria III cell sorter (BD Biosciences) among
alive cells (gated DAPI), non-epithelial cells (gated CD227<sup>-</sup>, epithelial antigen<sup>-</sup>, pan-cytokeratins<sup>-</sup>), and hematopoietic cells (gated CD45<sup>+</sup>) (antibodies used are listed in Supplementary Table 2). Reanalysis of the sorted populations showed a purity >98%.

**Immunohistochemistry**

Serial sections of paraffin-embedded NSCLC tumors were stained as previously described (De Chaisemartin, Cancer Res 2011), using antigen retrieval conditions, antibodies and reagents listed in Supplementary Table 2.

**Method for immune cell quantification**

Tumor-infiltrating CD8<sup>+</sup> T cells were enumerated in the tumor stroma of the whole tumor sections. DC-Lamp<sup>+</sup> mature DCs and CD8<sup>+</sup> T cells were quantified with Calopix software (Tribvn, Paris, France), and expressed as an absolute number of positive cells per mm<sup>2</sup> of tumor. Follicular CD20<sup>+</sup> B cell quantification was performed with Calopix, and expressed as the surface of CD20<sup>+</sup> B-cell follicles (mm<sup>2</sup>) per mm<sup>2</sup> of tumor. Both immunostaining and quantification were reviewed by two independent observers (CG, SK, PD, and/or MCDN).

**TCR or BCR repertoire sequencing**

DNA was isolated from sorted cell populations utilizing the Qiagen QIAamp DNA micro kit, according to the manufacturer’s suggested protocol. Resulting DNA concentrations were assessed by Qubit<sup>®</sup> fluorometric quantitation. TCR and BCR sequencing was then performed by Adaptive Biotechnologies via the immunoSEQ service. Due to the yields obtained from the relatively low counts of sorted cells, survey level depth sequencing was performed for all samples, but sufficient number of sequenced reads were required for final inclusion in the study: specimens with read counts <5,000 were removed from all analysis.

**Statistical analysis**

The proportion of <i>U</i> productive, or in-frame rearrangement unique TCR or BCR clonotypes <i>i</i> is defined as:
where $n_i$ is the count of the productive reads of the clonotype $i = 1, 2, ..., U$. Diversity $D$ is calculated by the normalized Shannon entropy given by:

$$D = -\sum_{i=1}^{U} p_i \log_2 p_i /\log_2(U)$$

Then the clonality index ($CI$) can be defined as the complement of diversity, $CI = (1 - D)$. So as $CI$ approaches 0, this indicates a diverse TCR or BCR repertoire, or a preponderance of unique clones within the repertoire. In contrast, as $CI$ approaches 1, this indicates a clonal TCR or BCR repertoire, or increased expansion of certain clones in the repertoire. A measure of relative clonotype richness $R$ was also calculated as the average unique clonotypes per cell (assuming one unique clonotype per cell), given by:

$$R = \frac{U}{\sum_{i=1}^{U} n_i}$$

Finally, the prevalence of shared clonotypes between tissue compartments within the same patient was calculated using the Horn index, defined as:

$$H_{NT,T} = \frac{2 \sum_{i=1}^{U} p_i^{NT} p_i^T}{\sum_{i=1}^{U} (p_i^{NT})^2 + \sum_{i=1}^{U} (p_i^T)^2}$$

where $T$ and $NT$ are the tumor and non-tumoral distant tissue compartments, respectively. $H$ can range in value from 0 to 1 with values that approach 1 indicating more shared clonotypes between tissue compartments for a patient. The same calculation was conducted between the tumor and peripheral compartment as well as the non-tumoral distant tissue and peripheral compartment.

All unpaired two sample tests were conducted with a Welch’s modified t-test, while paired comparisons were calculated with a Student’s paired t-test. All p-values are adjusted for
multiple testing comparisons using the qvalue package (62 comparisons for within-compartment analyses [Table 1] and 9 or 6 comparisons in across-compartment analyses) and rounded to two decimal places, so values reported at $p=0.05$ are in fact $<0.05$ prior to rounding. To avoid biased comparisons, only variables with at least 20% of patients in one of the two groups were used in correlation analysis with clonality (those $<20\%$ denoted with NE in Table 1). All analysis was conducted in the $R$ statistical programming environment (R Development Core Team 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org).

**Acknowledgments**
We are grateful to Prs. Wolf Hervé Fridman and Catherine Fridman for helpful scientific discussions. We thank clinicians (Prs. Marco Alifano and Philippe Girard) and pathologists (Dr. Audrey Mansuet-Lupo) from Hotel Dieu hospital, Cochin hospital, and Institut Mutualiste Montsouris (Paris, France) who provided us with tumor samples. The authors also thank Hanane Ouakrim for help in clinical data collection, and Estelle Devevre and Helene Fohrer-Ting from the “Centre d’Imagerie Cellulaire et de Cytométrie” (Cordeliers Research Center, Paris, France) for excellent technical assistance and support in flow cytometry.
References


24. Xiong Y, Tan Y, Song YG. Analysis of T Cell receptor Vβ diversity in peripheral CD4+ and CD8+ T lymphocytes obtained from patients with chronic severe Hepatitis B. Hepat Mon 2014; 14.


effective T-cell activation by B-cell lymphomas leading to antitumor immunity. Blood 2006; 107: 2871–78.


Table 1. NSCLC patient baseline variables evaluated for significant differences in clonality between levels using adjusted p-values

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. (%)</th>
<th>NT CD19+</th>
<th>NT CD4+</th>
<th>NT CD8+</th>
<th>P CD19+</th>
<th>P CD4+</th>
<th>P CD8+</th>
<th>T CD19+</th>
<th>T CD4+</th>
<th>T CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td>0.20</td>
<td>0.05</td>
<td>0.57</td>
<td>0.63</td>
<td>0.35</td>
<td>0.57</td>
<td>0.57</td>
<td>0.63</td>
<td>0.49</td>
</tr>
<tr>
<td>Male</td>
<td>21 (44.7%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>26 (55.3%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at Surgery</td>
<td></td>
<td>0.54</td>
<td>0.09</td>
<td>0.19</td>
<td>0.48</td>
<td>0.05</td>
<td>0.25</td>
<td>0.41</td>
<td>0.31</td>
<td>0.57</td>
</tr>
<tr>
<td>&lt;68</td>
<td>22 (46.8%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;=68</td>
<td>25 (53.2%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological Type</td>
<td></td>
<td>0.16</td>
<td>0.19</td>
<td>0.61</td>
<td>0.31</td>
<td>0.31</td>
<td>0.63</td>
<td>0.43</td>
<td>0.31</td>
<td>0.43</td>
</tr>
<tr>
<td>ADC+neuroendocrine</td>
<td>1 (2.13%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADC</td>
<td>23 (48.93%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADC+SCC</td>
<td>1 (2.13%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCC</td>
<td>1 (2.13%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>neuroendocrine</td>
<td>1 (2.13%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCC</td>
<td>20 (42.55%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Smoking Status</strong></td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>Smoked/Current smoker</td>
<td>36 (76.6%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never Smoked</td>
<td>6 (12.8%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>5 (10.6%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dendritic cell Group</strong></td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>39 (83.0%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>8 (17.0%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD8+ T cell Group</strong></td>
<td>0.35</td>
<td>NE</td>
<td>0.09</td>
<td>0.51</td>
<td>NE</td>
<td>NE</td>
<td>0.63</td>
<td>0.09</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>36 (76.6%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>11 (23.4%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Follicular B cell Group</strong></td>
<td>0.27</td>
<td>0.35</td>
<td>0.19</td>
<td>0.35</td>
<td>0.19</td>
<td>0.05</td>
<td>0.35</td>
<td>0.05</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>--------------</td>
<td>-----------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 (63.8%)</td>
<td>17 (36.2%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>T Stage</th>
<th>0.48</th>
<th>0.43</th>
<th>0.26</th>
<th>0.46</th>
<th>0.16</th>
<th>0.31</th>
<th>0.09</th>
<th>0.63</th>
<th>0.16</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;=T2a</td>
<td>33   (70.2%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;=T2b</td>
<td>14   (29.8%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N Stage</th>
<th>0.16</th>
<th>0.25</th>
<th>0.31</th>
<th>0.35</th>
<th>0.61</th>
<th>0.31</th>
<th>0.35</th>
<th>0.35</th>
<th>0.63</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>29   (61.7%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1/2</td>
<td>18   (38.3%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Footnote: Calculations were conducted with a Welch’s two-group test, cutting each variable at the two most prevalent levels for each variable. All p-values reported are adjusted for 62 different comparisons using the qvalue method and rounded to two decimal places; NE=not evaluated (due to highly unbalanced sample sizes between variable levels [variables with <20% in one group were not included]); NT=normal distant tissue; T=tumor; P=peripheral compartment (peripheral blood/draining lymph node); ADC=adenocarcinoma; SCC=squamous cell carcinoma; LCC=large-cell carcinoma.
Figure 1: Distributional properties of clonality index, productive read count, and maximum clonotype frequency in peripheral compartments of NSCLC patients as compared to peripheral blood from normal donors. A) No bias in clonality index as a result of productive read count was observed in peripheral blood from normal donors (left), draining lymph node from NSCLC (right).
patients (middle), or peripheral blood from NSCLC patients (right), and B) Correlation observed between the maximum clonotype frequency and clonality index in peripheral blood from normal donors (left) and peripheral blood from NSCLC patients (right). LN=draining lymph node.
**Figure 2:** Increase in clonal expansion of clonotypes from CD19$^+$ cells to CD4$^+$ cells to CD8$^+$ cells in all four tissue compartments of NSCLC patients. P=peripheral compartments (peripheral blood/draining lymph node); NT= non-tumoral distant tissue; T=tumor.
Figure 3: Significantly higher clonal expansion observed in the non-tumoral distant tissue compared to both tumor and peripheral compartments in CD4$^+$ TCR repertoire from NSCLC patients. NT= non-tumoral distant tissue; P=peripheral blood/draining lymph node; T=tumor; **= p<0.01; ***=p<0.001; all p-values are adjusted for multiple comparisons.
Figure 4: Clonal expansion in the tumor and non-tumoral distant tissue/peripheral compartments correlates with age in the CD4+ TCR repertoire. A) Significantly increased diversity (decreased clonality) in the peripheral compartments of younger NSCLC patients (age≤68) compared to older NSCLC patients (age>68) in CD4+ cells, B) significantly increased clonality in the tumor compared to the peripheral compartments of young NSCLC patients in CD4+ cells, and significantly increased clonality in the non-tumoral distant tissue compared to the tumor of older NSCLC patients in CD4+ cells. In both age groups, the non-tumoral distant tissue had higher clonality CD4+ cells compared to the peripheral compartments. NT= non-tumoral distant tissue; P=peripheral blood/draining lymph node; T=tumor; *=p<0.05; **=p<0.01; ***=p<0.001; all p-values are adjusted for multiple comparisons.
Figure 5: Prevalence of shared clonotypes (Horn index, $H$) between the tumor and peripheral compartments correlates with age in the CD4$^+$ TCR repertoire. Significantly increased prevalence of shared clonotypes between the tumor and peripheral compartments in the older NSCLC patients (age>68) compared to younger NSCLC patients (age≤68) in CD4$^+$ cells. NT_P=shared between non-tumoral distant tissue and peripheral blood/draining lymph node; NT_T=shared between non-tumoral distant tissue and tumor; P_T=shared between peripheral blood/draining lymph node and tumor; *=p<0.05; **=p<0.01; ***=p<0.001; all p-values are adjusted for multiple comparisons.
Figure 6: Clonal expansion of CD8$^+$ T cells in the peripheral compartment and of CD4$^+$ T cells in the tumor is associated with increased Foll-B cell density. NT= non-tumoral distant tissue; P= peripheral blood/ draining lymph node; T= tumor; *=p<0.05; **=p<0.01; ***=p<0.001; all p-values are adjusted for multiple comparisons.