Profiling Circulating T cells and Tumor Landscape for Predicting Responses to PD-1 blockade in Patients with Non-Small Cell Lung Cancer

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Gothenburg, 2024

Cover illustration: Illustration highlighting study design and how it could be related to response to treatment. Created on biorender and additional effects by Madhab Dutta.

Gothenburg, 2024

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ISB978-91-8069-819-1 (PRINT)

ISBN978-91-8069-820-7 (PDF)

http//hdl.handle.net/2077/81804

Printed by Stema Specialtryck AB, Borås

Trycksak
3041 0234

Dedicated to my Koka.

Abstract

Immune checkpoint blockade (ICB) has significantly improved outcomes in non-small cell lung cancer (NSCLC), yet only a subset of patients achieves durable responses. This underscores the need for effective methods to assess ICB efficacy. This thesis investigates the response to PD-1 blockade in NSCLC patients across stages I to IV, utilizing blood and tissue samples.

In stage III and IV patients, we analyzed circulating immune cell subsets in blood and tumor biopsies. Flow cytometry revealed that changes in activated memory CD8+ T cells, coupled with mutations tumor-specific genes, differentiate responders from non-responders to ICB. The immune infiltration to the tumor analysed via immunohistochemistry (IHC) further identified that the presence of CD4+ and CD8+ effector T cells, along with FOXP3+ regulatory T cells, that were associated with clinical response. The findings indicate that tumor biopsies can be useful to evaluate the genetic features of the tumor and infiltration of various immune cell subsets, aiding in the identification of ICB responders.

Phenotypic and transcriptomic analysis of circulating CD8+ T cells indicated that responders show upregulation of immune checkpoint receptors such as TIGIT and PD-1. Notably, long-term responders exhibited an increase in TCF-1+PD-1+ CD8+ T cells post-treatment. In stage III and IV patients, early changes in T cells detectable at baseline and over time may contribute to the development of more precise biomarkers for response. Integrating these blood-based findings with next-generation sequencing (NGS) and IHC of tumor biopsies could enhance the biomarker specificity.

Moreover, we have developed a co-culture model of normal and tumor tissue with peripheral blood mononuclear cells (PBMCs) from stage I and II NSCLC patients that undergo surgery. The model was used to assess the T cell reactivity in individual patients, and we found that the CD8 T cells were activated in response to tissue antigens and maintained effector functions. Overall, our findings can contribute to improving patient-specific prediction of ICB response for achieving better clinical outcomes.

Sammanfattning på Svenska

Lungcancer är en av de vanligaste och dödligaste cancerformerna i världen och uppkommer när celler i lungorna börjar växa okontrollerat och bilda tumörer. Det finns två huvudtyper av lungcancer: icke-småcellig lungcancer (NSCLC) och småcellig lungcancer (SCLC). NSCLC är den vanligaste och utgör cirka 85 % av alla fall.

Behandlingen av lungcancer beror på typ, stadium och patientens allmänna hälsa. Vanliga behandlingsmetoder inkluderar kirurgi för att ta bort tumören, strålbehandling, kemoterapi, målinriktade läkemedel och immunterapi. Vid tidigare stadier av lungcancer kan kirurgi eller strålbehandling vara botande, medan senare stadier ofta kräver kombinationer av behandlingar för att förlänga livet och lindra symptom.

Under de senaste fem åren har immunterapi förbättrat överlevnaden hos lungcancerpatienter, där behandlingen aktiverar patientens eget immunsystem mot tumören. Immunterapi involverar antikroppar mot en receptor, PD-1, som finns på ytan av CD8+ T-celler, en typ av vita blodkroppar som har som uppgift att döda cancerceller. När PD-1 binder till sin ligand, PDL-1, på ytan av cancerceller, hämmas CD8+ T-cellernas funktion. Immunterapi kan blockera denna PD-1/PDL-1-interaktion i tumören, vilket aktiverar CD8+ T-celler i tumörvävnaden och i sin tur bromsar tumörtillväxten. Trots framstegen är det många patienter som inte svarar på behandlingen eller slutar svara efter en tid och biomarkörer som kan förutse detta är otillräckliga. Mekanismerna som driver resistens mot immunterapi är också i nuläget oklara. Syftet med avhandlingsarbetet har varit att identifiera biomarkörer som i ett tidigt skede av behandlingen kan associeras till god eller sämre effekt av immunterapi.

I dessa studier analyserar vi insamlat material från både blod och vävnad, från en kohort av patienter, före och efter immunterapi, med syftet att monitorera effekten av behandlingen. Omfattande cellulära och molekylärbiologiska analyser har utförts av immunceller och

tumörceller i patientmaterialet, för att förstå dels (1) hur immunförsvaret påverkar tumören vid immunterapi, dels (2) hur egenskaper hos tumören kan driva recidiv vid immunterapi.

Vi har identifierat specifika immunceller, både i blod och i vävnad, som skulle kunna förutse klinisk respons med immunterapi. Våra studier har bidragit till att identifiera potentiella biomarkörer i blod, som skulle kunna utvecklas till ett rutintest inom sjukvården i framtiden för att särskilja patienter som svarar på behandling från de som inte gör det. Dessa resultat och kunskaper kommer att vara viktiga de närmaste åren för att vidareutveckla arbetet med precisionsmedicin, vilket handlar om att ge rätt behandling till rätt patient vid rätt tidpunkt. Detta leder i sin tur till att behandlingarna blir mer effektiva, vilket ger större nytta för patienterna.

List of Papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Combinatory analysis of immune cell subsets and tumor-specific genetic variants predict clinical response to PD-1 blockade in patients with non-small cell lung cancer

Nikita Dutta, Anna Rohlin, Ella A. Eklund, Maria K. Magnusson, Frida Nilsson, Levent M. Akyürek, Per Torstensson, Volkan I. Sayin, Anna Lundgren, Andreas Hallqvist, Sukanya Raghavan

Frontiers in Oncology, Volume 12 (2022), DOI: 10.3389/fonc.2022.1073457

II. Circulating CD8 T Cells and circulating tumor DNA as prognostic biomarkers of clinical response to PD-1 blockade for patients with non-small cell lung cancer

Nikita Dutta, Johanna Svensson, George Alehandro Saad, Marielle Mello, Ella A. Eklund, Ilayda Altinönder, Per Torstensson, Volkan I. Sayin, Anna Rohlin, Hervé Luche, Andreas Hallqvist, and Sukanya Raghavan

Manuscript

III. Multiplex Immunofluorescence Analysis of Immune Subsets in Archival Biopsies to Predict Clinical Response to PD-1/PD-L1 Blockade in Non-Small Cell Lung Cancer

Nikita Dutta, Gina Vich, Linn Lund Karlsson, Johanna Svensson, Ahmed Jawad, Louis Szeponik, Ella A Eklund, Marianne Quiding-Järbrink, Volkan Sayin, Anna Rohlin, Levent M. Akyürek, Andreas Hallqvist, and Sukanya Raghavan

Manuscript

IV. An invitro coculture model to assess CD8+ T cell reactivity to tumor antigens from patients with non-small cell lung cancer

Nikita Dutta, Linn Lund Karlsson, Sama Sayin, Ella A. Eklund, Sabiha Amir, Kevin Ali, Dyar Mustafa, Levent M. Akyürek, Martin Silverborn, Anna Rohlin, Andreas Hallqvist, Johan Botling, Volkan I. Sayin, Per Lindahl, and Sukanya Raghavan

Manuscript

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Abbreviations

ADCC Antibody-dependent cell cytotoxicity
AID Activation-induced cytidine deaminase
ALK Anaplastic lymphoma kinase
APCs Antigen presenting cells
ASC Adenosquamous carcinoma
BCL6 B-cell lymphoma 6)
BCR B cell receptor
BRAF V-raf murine sarcoma viral oncogene homolog B1
CCL5 Chemokine ligand 5
CRC Colorectal cancer
ctDNA circulating tumor DNA
CTLA-4 Cytotoxic T-lymphocyte associated protein 4
DAMPs Damage-associated molecular patterns
DC Dendritic cells
EGFR epidermal growth factor receptor
EMA European Medicines Agency
EOMES Eomesodermin
FFPE Formalin-Fixed Paraffin-Embedded
GC Germinal centers
H&E Hematoxylin and eosin
HLA Human leukocyte antigen
ICOS Inducible T-cell costimulator
IFNɣ Interferon ɣ
IgG Immunoglobulin
IL-2 Interleukin-2
Immunohistochemistry (IHC)
KEAP1 Kelch-like ECH-associated protein 1
KRAS Kirsten rat sarcoma virus
LAG-3 Lymphocyte Activation Gene 3
LUAD lung adenocarcinoma
MHC Major histocompatibility complex
NGS Next generation sequencing
NK cells Natural Killer cells
NSCLC Non-small cell lung cancer
PD-1 Programmed Cell Death Protein-1
PD-L1 Programmed Cell Death Protein Ligand-1
PFS Progression free survival
PRR pattern recognition receptors
RET Rearranged during transfection
ROS1 Proto-oncogene tyrosine-protein kinase

SCLC Small cell lung cancer
STK 11 Serine/threonine kinase 11
TAM Tumor associated macrophages
TCF1 T cell factor 1
Tcm Central memory T cells
TCR T cell receptor
Tem Effector memory T cells
TGF-β Transforming growth factor beta
TIGIT T cell immunoreceptor with Ig and ITIM domains
TIM-3 T-cell immunoglobulin and mucin domain 3
TLR Toll-like receptors
TLS Tertiary lymphoid structures
TMB Tumor mutational burden
TNFα Tumor necrosis factor α
TOX Thymocyte selection-associated high mobility group box protein
TP53 Tumor Protein p53
Treg Regulatory T cells
Trm Tissue resident memory T cells
VEGF Vascular endothelial growth factor
VISTA V-domain immunoglobulin suppressor of T cell activation
β2M Beta2 Microglobulin

Introduction

Hallmarks of Cancer

As defined by the National Cancer Institute, cancer is a disease in which cells grow uncontrollably and spread to other areas of the body. There are about 100 types of known diagnosed cancers. They are named based on the organ they originate from; such as lung or brain or from the cell type; epithelial cell or squamous cell. [1] On the basis of cell type, cancer can be categorized into (1) carcinoma, formed by epithelial cells, which can be further divided into Adenocarcinoma, Squamous cell carcinoma, Basal cell carcinoma and transitional cell carcinoma; (2) Sarcoma, these are formed in the bone and soft tissue; (3) Leukemia, which forms in the bone marrow; (4) Lymphomas, which is a cancer of the T and B cell lymphocytes, (5) Multiple Myeloma, cancer which begins in the plasma cells; (6) Melanoma affecting melanocytes in the skin and finally, (7) Brain and Spinal Cord Tumors. Some other types, include Neuroendocrine, Germ Cell and Carcinoid Tumors. [1] In 2022, there were globally 20 million new cancer cases and 9.7 cancer related death. [2] In 2000, Hanahan and Weinberg wrote a seminal paper on The Hallmarks of Cancer. Tumors are a result of 6 alterations in the physiology of the cell which include (1) resisting growth suppressors, (2) endless replication of cells, (3) resisting apoptosis, (4) endless replicative potential, (5) continued angiogenesis, and (6) invading tissue and metastasis. [3] Two more emerging factors were added to the initial 6, immune evasion and reprograming cellular metabolism and it was later confirmed that they were indeed hallmarks of cancer. [4, 5]

Immunity in Cancer

The cancer immunity cycle proposed 1st by Chen and Mellman includes a multistep process (Figure 1) starting with release of antigen when cancer cells die which are taken up by dendritic cells (Step 1) which then present tumor antigens to the T cells

in the lymph node where T cells are primed and activated (Step 2). The tumor specific activated T cells traffic to the tumor via blood vessels (Step 3, figure 1). T cells then infiltrate the tumor (Step 4), where T cells can recognize the tumor cells via presentation on MHC and finally, kill the tumor cells and so the cycle begins again. [6] The Cancer Immunity Cycle is so named because it is a cyclic process influenced by immune regulatory mechanisms. Each step includes a balance of stimulation and inhibition of the immune cells. The cycle contributes to immune surveillance unless it is dampened by the interaction of Programmed Cell Death Protein ligand-1 (PD-L1) expressed on tumor cells with Programmed Cell Death Protein-1 (PD-1) expressed on T cells. Another immune dampening interaction is between CTLA-4 expressed on T cells which competes with CD28 on T cells for binding to CD80/CD86 on antigen presenting cells (APCs) thus blocking the co-stimulatory signal, inhibiting T cell activation (Step 5). [6] However, when interactions with immune checkpoint receptors are blocked, T cells are able to regain their effector functions (Step 6). [7] It is also known that the immune response against tumors is not solely blocked by the PD-1 and CTLA-4 signaling pathways. There are several additional immune checkpoint receptors such as TIGIT, LAG-3, TIM-3, VISTA which has a role in dampening T cell activation. Tolerogenic cell death or the tumor microenvironment could also be suppressive. Chemotherapy, radiation therapy and targeted therapy among others aid in cancer cell death. Cancer cell death releases antigens, which are taken up by APCs such as dendritic cells (DCs). [8] Antibodies blocking immune checkpoint receptors PD-1 or CTLA-4, or agonistic antibodies binding co-stimulatory molecule 41BB (CD137), or cytokines IL-2, IL-15, IL-21, IL-18 can aid in T cell priming in the lymph node. Antibodies to VEGF can promote the infiltration of tumor specific T cells into tumors and the stroma. [6, 8] Although the cancer immunity cycle is well accepted in the literature, Mellman *et al*, underscore the importance of understanding the tumor microenvironment to better understand the immune responses. Depending on the cancer indication, the tumor can be classified into immune inflamed “hot”, or immune excluded or immune dessert “cold” based on the

immune cell infiltration. Exclusion of T cells from the tumor core may occur as a result of endothelial dysfunction, cancer-associated fibroblast (CAF) activity, or dysfunctional chemokine signaling which is governed by mutations in genes that control these processes. [9] In addition to the immunotype, the genomic features or genotype of the tumor is important to understand the evolution of the tumor so that the correct treatment can be offered to the patient. [8]

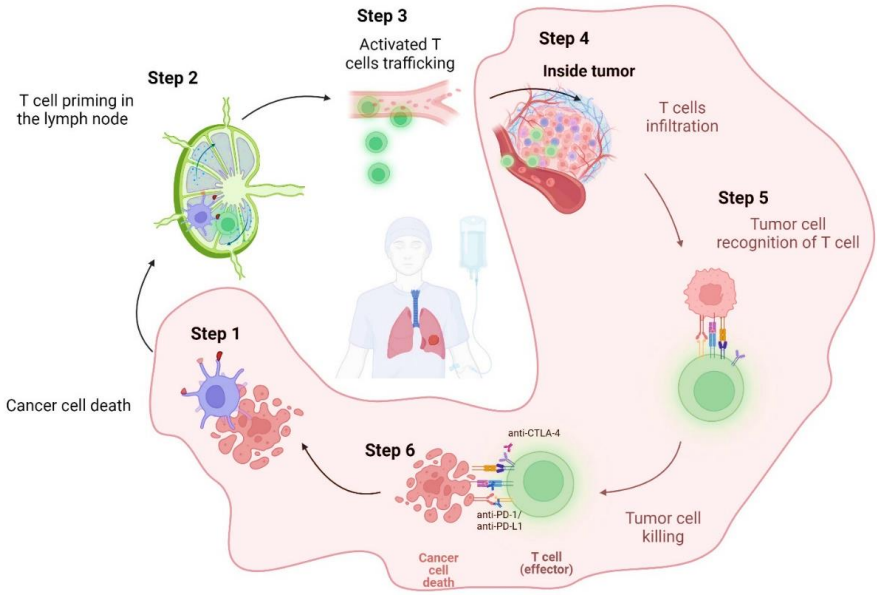

Figure 1: Immune processes during an anti-tumor response. Created on Biorender.

Innate immune responses in cancer

Innate immunity is the first response against pathogens and abnormal tissue antigens. When a body encounters an infection or a wound, the innate immune system recruits immune cells such as macrophages, mast cells, neutrophils, NK cells to respond to it. Tissue resident macrophages recognize the pathogen, release cytokines and recruits' neutrophils to the tissue. NK cells are a type of innate immune

cell that function to promote anti-tumor immunity via expression of activating and inhibitory receptors. NK cells can kill tumor cells and release cytokines such IFNɣ and TNFα. NK cells also contribute to the infiltration of cDC1 into tumors via the release of CCL5, XCL1 and FLT3LG. [10] Dying tumor cells release damage-associated molecular patterns (DAMPs) and tumor associated antigens which are then taken up by dendritic cells. DAMP sensing increases phagocytosis of necrotic debris and activates the adaptive immune system. DAMPs are also recognized by pattern recognition receptors (PRRs) such as TLRs. [11] Dendritic cells play a vital role as a bridge between the innate and adaptive immune system. Once DCs take up the antigen, DCs get activated, migrate to the lymphoid tissues where antigens are presented to the T and B cells via MHC class I and II. DCs are important in the generation of T cell mediated antitumor responses, due to the ability to cross-present tumor antigens to naïve T cells. [12]

Adaptive immune responses in Cancer

Adaptive immunity involves an interplay between antigen-presenting cells, T and B lymphocytes and facilitates antigen-specific responses, generation of immunological memory and maintains host immune homeostasis. [13] Adaptive immune responses are broadly carried out by B cells by means of antibody responses and T cells by means of cell-mediated immune responses. After an initial infection, epithelial and innate immune cells respond to control the pathogen. The lymphocytes which will target the pathogen circulate through the blood and lymphatics. As mentioned previously, DCs traffic antigens into the local lymph nodes for presentation to naïve T cells. With the help of cytokines and chemokines, naïve T cells recirculate between the lymph nodes and here, T cells interact with the DC with via TCR-MHC interaction. A naïve T cell required 3 signals to be activated: antigen recognition, co-stimulatory signals and cytokine signals. First, T cells must recognize a foreign peptide bound to an MHC molecule via its TCR. Additionally, activation requires a costimulatory signal from APCs. Finally, it requires cytokine signals for full T cell activation. B cells and DCs are both antigen presenting cells that can present

an antigen to a T cell. Additionally, B cells express many of the same co-stimulatory molecules expressed on DCs, for example; CD40, CD80 and CD86. [13]

In the same lymph node, upon antigen encounter via their BCRs, B cells become activated and undergo BCR induced signaling and B cells are activated for further differentiation. Activated B cells can then interact with CD4+ T cells at the interface of T cell and B cell zones. [14] B cells present processed antigens to CD4 T cells via MHC II which further help in B cell differentiation via CD40-CD40L and ICOS-ICOSL interactions and secretion of IL-4 and IL-21. B cells have a choice of two fates; either to become short lived antibody secreting cells called plasma blasts and others enter germinal centers (GC). [15] In GCs, B cells will undergo somatic hypermutation where BCRs diversify via mutations that are introduced by the enzyme activation-induced cytidine deaminase (AID). BCRs then undergo selection and demonstrate the ability to bind to CD4 T cells. The selected B cells become memory B cells and others become long lived antibody secreting IgM-, IgG-, IgA-, or IgE plasma cells. When plasma cells are activated, thousands of antibodies are secreted per second. [14, 16] B cells and plasma cells have been known to support antitumor immune responses by various mechanisms. Plasma cells support anti-tumor response by secreting tumor cell-specific IgG1 antibodies that can mediate antibody-dependent cell cytotoxicity (ADCC) and phagocytosis of tumor cells. [17] The role of B cells in human cancers have been widely studied. However, there were several studies that have shown that the intratumoral accumulation of B cells was associated with good prognosis in breast cancer, CRC, melanoma and NSCLC. [18-22] In cancers, a subset of B cells that produce IL-10 are known as regulatory B cells, present in murine and human tumors. Regulatory B cells have the capacity to convert conventional CD4+ T cells to regulatory T cells. [23] Additionally, B cells are located in tertiary lymphoid structures (TLS) which are defined as dense lymphoid aggregates with at least one germinal center. [24] TLS resemble secondary lymphoid structure and are characterized by CD20 B cells surrounded by CD3 T cells. B cells and T cells make up

the bulk of the TLS, but also have CD21+ follicular dendritic cells. In melanoma, TLS presence was associated with improved survival in patients. [25] There are three classes of TLS, that is immature TLS, wherein there are aggregates of T and B cells but few DCs. There is little evidence of immune reaction here. Second, mature TLS which include a B cell area forming primary follicle (B cells surrounded by T cells) or third type, secondary follicle (contains a germinal centre). TLS present in the tumor margin or invasive margin are sites of tumor associated immune response. [26] TLS present both in mouse and human lung adenocarcinomas are known to produce antibodies which promote tumor immunity. [27]

Immune editing in Cancer

The immune system constantly scans for precancerous cells, a concept known as cancer immunosurveillance. Robert E. Schreiber and colleagues introduced the major concept in cancer immune surveillance, the 3Es in immune editing, Elimination, Equilibrium, Escape. [28] In the elimination phase, the immune system can target and destroy transformed cells before they become a tumor. The innate immune system and adaptive immune system both play a role in the elimination of the tumor. In the equilibrium phase, the host immune system and the tumor cell which were not eliminated, are in a dynamic equilibrium. In this phase, the lymphocytes and IFNγ exert a selective pressure on the tumor cells. This is however, not enough to completely to eliminate them. Many of the original escape variants of the tumor cells are eliminated, however but new variants can arise. The tumor cells emerging from a new round of selection could be even more resistant than the previous one, for elimination by the immune system by for example evading recognition by the immune system. In the escape phase, the variants that are resistant to immune attack/elimination via (1) loss of MHC I, (2) loss of neoantigens (3) downregulation of costimulatory molecules, (4) increase in tumor suppressive mechanisms such as by expression of PD-L1, will expand rapidly and proliferate in an uncontrolled manner. [29, 30] Immune surveillance concepts have become

increasingly important to consider when studying biomarkers of response and importance of capturing the tumor evolution and immune evasion by cancer cells. [31]

Lung Cancer

Molecular Pathology

Lung cancer is the one of the most diagnosed cancer types in 2022 with about 2.5 million cases worldwide. It was also a leading cause of cancer related death, with an estimated 1.8 million deaths per year. [32] The primary form of lung cancer is non-small cell lung cancer (NSCLC) (~ 85%) and small cell lung cancer (SCLC) (~15%). NSCLC is heterogenous and includes lung adenocarcinoma (LUAD) as the most common histological group (~50%), squamous cell carcinoma (~30%), large-cell lung carcinoma (LCLC) (~10%) and adenosquamous carcinoma (ASC). There are also rarer subtypes histologically, atypical carcinoid tumor, bronchial gland carcinoma, and sarcomatoid carcinoma, collectively accounting for the remaining types of NSCLC (~10%). [33] After an initial lung biopsy is obtained from the patient at diagnosis, H&E staining is performed, and the pathologist determines the subtype of NSCLC. Immunohistochemistry (IHC) staining of tumor biopsies positive for Thyroid Transcription Factor-1 (TTF-1) and Cytokeratin 7 (CK7) indicates lung adenocarcinoma diagnosis and positive IHC staining of tumor tissue for Tumor Protein 63 (p63), Cytrokeratin 5/6 (CK5/6) and p40 indicates a squamous cell carcinoma diagnosis. LUAD tissue would normally be negative for squamous cell carcinoma markers and vice versa. [33]

Stages of NSCLC

The staging of the tumor at the time of diagnosis defines the anatomic extent of cancer including the tumor (T) to describe the size of the tumor, nodal (N) to describe the regional lymph node involvement and metastases (M) for distant metastases beyond the regional lymph nodes, also known as TNM staging. Approximately 60% of

patients will have distant metastases by the time they are diagnosed at stage IV. At stage III, 25% of the patients would have tumor that is locally advanced or metastasized to the regional lymph nodes. The remaining 15% with stages I-II would have a tumor confined to the lung or with limited regional lymph node involvement. [34]

Treatment of NSCLC

Patients diagnosed with stage I-II disease are in most cases advised surgery as the current standard of care. For stage I-II, if they are medically unfit for surgery due to age or comorbidities [34], they are given stereotactic body radiotherapy (SBRT). SBRT is the precise delivery of high doses of radiation in a short period of time. Post-surgery, stage I-III patients receive adjuvant chemotherapy and adjuvant PD-L1 inhibition in case of PD-L1 expression is >50%. [33] The majority of stage III patients receive chemoradiotherapy, comprising of a platinum treatment with radiotherapy dose of 60-68 Gy. Stage III patients are also offered PD-L1 inhibitor, Durvalumab after chemoradiotherapy and this has been shown to improve survival. [33, 35] Stage IV patients that do not have any oncogenic driver mutations are offered immune checkpoint blockade such as PD-1 and PD-L1 blockade as part of their first line treatment. [33] The tumor biopsies obtained at the time of diagnosis are assessed for genomic alterations by next-generation sequencing (NGS) in EGFR, ALK, BRAF, RET and ROS1 oncogenes. This is important because patients receive small molecule kinase inhibitors based on the mutations detected. [36] PD-L1 staining is performed for all patients and stage IV patients may receive immune checkpoint blockade, namely antibodies to PD-1 or PD-L1 as monotherapy when the PD-L1 expression is >50%. If the PD-L1 expression of the tumor is <50%, patients may receive a combination therapy of PD-1/PD-L1 blockade with chemotherapy. [37] or CTLA4-blockade.

Immune Checkpoint Blockade

Immune checkpoint blockade (ICB) with antibodies targets immunosuppressive molecules expressed on T cells and trigger suppression of T cell activation. Immune checkpoint inhibitors allow tumor specific T cells to overcome their inhibitory mechanisms and mount an anti-tumor response. [35, 36] Ipilimumab (anti-CTLA-4) was approved by the FDA for metastatic melanoma in 2011 and 5 additional immune checkpoint blockades targeting the PD-1-PD-L1 axis were approved in the next few years. These were Pembrolizumab, Nivolumab, Dostarlimab, Cemiplimab, Retifanlimab and Toripalimab (anti-PD-1) and Atezolizumab, Avelumab and Durvalumab (anti-PD-L1). CTLA-4 is expressed after TCR engagement and dampens TCR signaling by competing with the costimulatory molecule CD28 for B7-1 (CD80) and B7-2 (CD86) for which CTLA-4 has higher avidity. [37] PD-1 is expressed on T cells and binds to its two ligands, PD-L1 (B7-H1 or CD274) and PD-L2 (B7-DC or CD273) which is expressed on the tumor cells and hematopoietic cells such as macrophages, DCs, T cells, B cells and mast cells. PD-1 is also expressed upon activation on B lymphocytes, NK cells and activated monocytes. [38] As the ligands for PD-1 are expressed on non-lymphoid tissue, PD-1 signaling dampens T activation mainly in the periphery (Figure 2). Upon engagement with its ligand, phosphorylation of PD-1 takes place at immune receptor tyrosine–based inhibitory motif (ITIM) and immune receptor tyrosine–based switch motif (ITSM), followed by recruitment of the SRC homology 2 (SH2) domains of SH2-containing phosphatase (SHP2). The SHP2 phosphatase dephosphorylates molecules downstream of TcR signaling leading to a block in T-cell proliferation, cytokine production and cytolytic function of CD8 T cells. [35, 39, 40]

In advanced NSCLC patients, based on the PD-L1 staining as a biomarker, when patients had a tumor proportion score >50%, overall survival was significantly longer in the pembrolizumab groups than chemotherapy alone. [41, 42] Advanced NSCLC patients who had TPS<1%, the combination of pembrolizumab with chemotherapy

was better for overall survival than chemotherapy alone. [43] In advanced NSCLC, overall survival and PFS was significantly higher with nivolumab and docetaxel, a chemotherapy drug treatment compared to docetaxel alone regardless of PD-L1 status. [44] Additionally, in metastatic NSCLC, when pembrolizumab was administered to patients in combination with chemotherapy, the results from clinical trials revealed improved response rates and longer progression-free survival. [45]

Next generation of immune checkpoint receptors include lymphocyte activation gene 3 (LAG-3), T cell immunoglobulin and mucin domain containing-3 (TIM-3), and T cell immunoreceptor with Ig and ITIM domains (TIGIT). LAG-3 is expressed on T cells, particularly Tregs, NK cells, B cells and plasmacytoid DCs. TIM-3 is expressed on CD4 and CD8 T cells and regulates type I immunity. TIM-3 is also expressed on NK cells, macrophages and DCs, mast cells and B cells. TIM-3 has 4 ligands, GAL-9, phosphatidylserine, high mobility group box 1 (HMGB1) and carcinoemryonic antigen-related cell adhesion molecule 1 (CEACAM-1). Initially, TIM-3 was studied in the context of autoimmunity, and it was found that TIM-3 was lowly expressed on T cells in patients with multiple sclerosis, psoriasis, rheumatoid arthritis and colitis. Recently, TIM-3 has been found to be a marker of terminally dysfunctional CD8 T cells even more than PD-1 and has a low expression of TCF-1, which is a marker of cell that stem-like features, which are characterized by the ability to rapidly generate progeny cells which produce cytokines and have cytotoxic functions. Stem-like T cells also retain the ability to generate more differentiated cells and harbor properties of long-term persistence. [46, 47] A immune checkpoint molecule gaining attention is TIGIT, which is expressed on T cell populations such as Tregs, dysfunctional T cells and follicular T helper cells. It was reported that TIGIT was also expressed on B cells with regulatory properties and on innate immune cells such as innate lymphoid cells. TIGIT, when it binds to its ligands CD155 and CD112, expressed on APCs and tumor cells, suppresses T cell activity. It competes with CD226 (DNAM-1) to bind to these ligands, where CD226 promotes T cell activation.

TIGIT can inhibit both T cell activation and NK cell function. Additionally, via CD155 TIGIT can also inhibit APCs and enhances Treg function via the suppressive mediator Fibrinogen-like protein 2 (Fgl2). [48] Tregs expressing TIGIT can suppress proinflammatory Th1 and Th17 responses. Inhibitors for immune checkpoints are currently in trial for different cancers. Targeting immune checkpoints with checkpoint inhibitors/checkpoint blockade has been promising, and several trials are currently ongoing for solid tumors, investigating their efficacy both as monotherapy and in combination with anti-PD-1 therapy. [49]

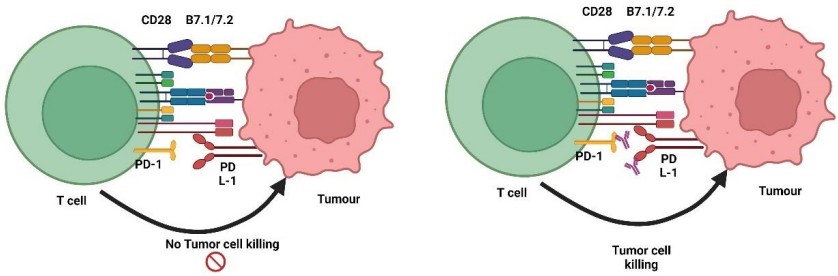

Figure 2: PD-1 and PD-L1 blockade When PD-1 binds to PD-L1, T cell activity is suppressed. Introducing antibodies to block this interaction releases the inhibition, allowing T cells to function effectively. Created on Biorender.

Resistance to Immune checkpoint blockade

Resistance mechanisms to ICB are of three types: Primary, adaptive and acquired. Patients who have primary resistance to ICB quickly relapse within 3 months of therapy start. The most common mechanism of resistance development can be due to lack of tumor neoantigens for T cells to recognize. Alternatively, in adaptive resistance, the immune system can recognize cancer cells, but the cancer cell adapts itself from immune attack. For example, cancer cells develop immune evasion mechanisms such as alterations in the antigen presenting machinery.

Acquired resistance mechanisms is a scenario wherein cancer cells initially respond to the ICB but after a period, the cancer progresses, and the patients stops responding to ICB. [50] Primary and adaptive resistance can be tumor-cell intrinsic. Mechanisms that comprise tumor intrinsic properties include genetic and epigenetic factors. For example, an aberrant mutation gives rise neoantigens and self-peptides that attract T cells, but an absence of neoantigenic peptides, cancer antigens or a low TMB could lead to resistance. Tumor-cell extrinsic mechanisms include a lack of infiltration of T cells with tumor antigen specific TCRs and upregulation of inhibitory immune checkpoints such as TIM-3 or LAG-3. Finally, the presence of immunosuppressive cells in the tumor microenvironment such as TAMs and Tregs can lead to resistance as well. [50] Acquired mechanisms of resistance comprises of loss of antigen expression, β2M defects leading to loss of HLA and mutations in IFNɣ signaling pathways that can dampen T cell activity in the tumor.

Biomarkers

Immune checkpoint blockade are quite expensive treatments and can sometimes cause adverse side effects and benefit a small number of patients. It is therefore important to monitor patients after treatment to identify biomarkers of clinical response, to differentiate a responder from a nonresponder. [51, 52] Biomarkers can be either prognostic or predictive. A *prognostic* biomarker is used to identify patients and classify them based on the risk of the outcome of disease regardless of the treatment (such as disease recurrence, progression, or death). A *predictive* biomarker is useful to identify patients to predict the outcome from the treatment. [53]

Prognostic biomarkers for immunotherapy in lung cancer are tumor-infiltrating lymphocytes, LIPI (lung immune prognostic index), NLR (neutrophil to lymphocyte ratio) and radiomics biomarkers which utilizes artificial intelligence tools to quantify

radiographic pictures of the tumor or areas to which it has metastasized to. In a study where NSCLC and melanoma patients were undergoing anti-PD-1 treatment, the lesions from baseline CT scan images were analyzed by using machine learning algorithms to evaluate and quantify radiographic characteristics to predict response to immunotherapy. [54] Some of the predictive biomarkers of response to PD-1/PD-L1 blockade PD-L1 expression by tumor and immune cells, tumor mutational burden and mutations, microsatellite instability, intracellular cytokine signaling pathways and gut microbiota. [52]

Tissue based predictive or prognostic biomarkers

Currently, the only predictive biomarkers approved to select patients for immunotherapy is % staining for PD-L1 and Tumor mutational burden (TMB) (approved by FDA not EMA). PD-L1 expression by tumor or immune cells within the tumor is a biomarker to predict clinical response to immune checkpoint blockade. In advanced NSCLC, PD-L1 expression is measured by Tumor Proportion Score (TPS) and is classified into TPS <1%, TPS 1 to 49% and TPS ≥50% and is estimated by a pathologist. [55] It was thought that high PD-L1 expression on the tumor would allow it to be more targetable as many cancers use the PD1/PD-L1 interaction to evade the immune system. However, it is now clear that PD-L1 expression as a biomarker has limitations. The challenge lies in the variability of the cut off value of the PD-L1 expression and on the expression itself on the tumor cells and the infiltrating immune cells. Variations in cut-off values make the staining very heterogeneous. [56] In a randomized phase 3 clinical trial (OAK) that studied the effects of PD-L1 blockade therapy (Atezolizumab) vs docetaxel reported that there was overall survival improvement in NSCLC patients with Atezolizumab regardless of PD-L1 expression which is interesting considering the patients were on PD-L1 blockade. [57]

TMB is defined as the total number of somatic mutations per megabase of the tumor genome and has been utilized to predict clinical response to PD-1 and PD-L1 inhibitors. [58] It is reasoned that a high TMB would indicate the presence of neoantigens which could be presented to T cells. In the clinical trial (KEYSTONE 158), patients with advanced solid tumors who had high TMB (≥ 10 mutations per mega base pair) responded better to Pembrolizumab than patients with a low TMB classified as <10 mutations per mega base pair. [59] However, there is a lack of a standardized gene panel to quantify TMB and a good cut off value [59, 60]. In a phase 3 randomized clinical trial (CheckMate 227) to evaluate Nivolumab (anti-PD-1) and Ipilimumab (anti-CTLA-4) vs chemotherapy, it was found that overall survival was similar among patients who had a high TMB (≥ 10 mutations per mega base pair) or low TMB (<10 mutations per mega base pair). In the same study, it was reported that a combination of both high PD-L1 expression (≥50%) and high TMB did not identify patients with better overall survival, and that it was similar to patients with low PD-L1 expression (<1%) and low TMB. [61]

Patients are routinely assessed for a panel of genomic alterations such as KRAS, EGFR, ALK, BRAF, RET and ROS1 genes. Based on specific genomic alterations, clinicians can decide which treatment patients receive. However, this panel could be broadened to evaluate response to PD-1/PD-L1 blockade. [62] Kirsten rat sarcoma virus (KRAS) is a frequently mutated oncogene in NSCLC containing 21 missense mutations, with G12D (29.19%), G12V (22.97%), and G12C (13.43%). It has been reported that lung adenocarcinoma patients that had mutant KRAS tumors achieved durable clinical benefit post anti-PD-1/anti-PD-L1 treatment. [63, 64] Additionally, KRAS-mutant tumors are mostly found among smokers or heavy smokers and only 5-10% of nonsmokers have KRAS mutations. [65] In NSCLC, patients who had a high TMB also had the KRAS-G12C variant. [66] Progress has been made in targeting the KRAS-G12C mutant with drugs approved by the FDA such as Sotorasib [67], but not for KRAS-G12D. [68, 69] Co-mutations in KRAS, STK-11, KEAP1, TP53 have been

reported to be associated with improved response rates in NSCLC after ICB. [70] Mutations in KEAP1 which is a tumor suppressor gene, in NSCLC patients is associated with resistance to treatment and worse prognosis. It has been estimated that 92% of the missense variants in *KEAP1* and *STK11* were loss-of-function variant. [71] Mutations in other tumor suppressor gene, STK11 inactivation is associated with a cold tumor phenotype, where there is less infiltration of CD8 T cells. In mouse NSCLC models, ablation of STK11 resulted in the recruitment of neutrophils and suppressive T cells. [72] Mutations in TP53 and KRAS increased PD-L1 expression on tumors on an mRNA level and mutation in TP53 also led to higher expression of immune checkpoint receptors on T cells. Whereas a mutation in KRAS led to a lower expression of immune checkpoints on T cells in the tumor. [63] TP53 mutations were also associated with high infiltration of T cells in tumors. These studies report that a patient with TP53 and KRAS mutation in a tumor have a higher tendency to respond to PD-1 blockade. [63, 73] Thus, the mutational landscape and its relationship to the immune landscape is important in the search for biomarkers. Finding genetic "hot spots" in patients who have various co-mutations would increase the likelihood of generating neoantigens that would be recognized by T cells in the tumor. Neoantigen specific T cells could respond to PD-1 blockade thereby suggesting that a heterogeneous mutational landscape can determine the response to PD-1 blockade treatment. [74, 75]

An emerging tissue-based biomarker is Tertiary Lymphoid Structures (TLS), which are defined as dense lymphoid aggregates with at least one germinal center [24]. TLS resemble secondary lymphoid structures and are characterized by CD20 B cells surrounded by CD3 T cells. B cells and T cells make up the bulk of the TLS, but CD21+ follicular DCs are also present. In cutaneous melanoma, the presence of TLS in the tumor tissue was associated with improved survival in patients. In three retrospective studies involving patients with solid tumor, including NSCLC, Vanhersecke *et al* report that patients with TLS by calculating TLS density (number of TLSs per mm^2 of

tumor surface) treated with PD-1/PD-L1 antibodies, had an improved overall survival and progression free survival in several cancers such as NSCLC, sarcoma and melanoma. [25, 76, 77] TLS do not form under normal physiological conditions but develop in chronically inflamed environments, such as in autoimmune diseases, chronic inflammation, and cancer. [77] There are three classes of TLS, based on the composition of the immune cells namely, immature TLS, with aggregates of T and B cells and few DCs. There is little evidence of immune reaction here. Second, mature TLS which include a B cell area forming primary follicle (B cells surrounded by T cells) or third type, secondary follicle (contains a germinal centre). TLS present in the tumor margin or invasive margin are sites of tumor associated immune response. [26] TLS present both in mouse and human lung adenocarcinomas are known to produce antibodies which promote tumor immunity. [27]

Blood based biomarkers

There has been growing interest in blood-based biomarkers such as immune cell subsets to predict response to immunotherapy. In blood, pretreatment high neutrophil to lymphocyte ratio (NLR) and low TMB has been shown to be associated with a poorer survival and a lower probability of response to immunotherapy compared to patients with low NLR and high TMB. [78] Several studies have analyzed various T cell subsets in patients undergoing immune checkpoint blockade. In melanoma and lung cancer, proliferation of PD-1+CD8 T cells in the blood were associated with clinical benefit. [79] In advanced NSCLC patients treated with Nivolumab, a study Ottonello *et al* found that responders who had a longer overall survival and progression free survival had high frequencies of baseline CD3, CD4, CD8 T cells and low NK cell frequencies compared to nonresponders who had low frequencies of baseline CD3, CD4 and CD8. The high and low groups of CD3, CD4, CD8 T cells were decided based on the median fluorescence intensity of the marker. [80] This study also reported that a high baseline PD-1 expression on CD3 and CD8 T cells was associated to progressive disease. In advanced NSCLC patients who received anti-PD-1, high baseline levels of circulating CD4+CCR9+, CD4+CCR10 or

CD8+CXCR4+ T cells was associated with a reduced overall survival rate. [80-82] An increased frequency of circulating CX3CR1^{+} CD8 T cells post treatment anti-PD-1 blockade correlated with circulating CD8^{+} T cells. Presence of CX3CR1+ CD8 T cells correlate with clinical response and survival in patients with NSCLC. [83] In another study, in a cohort of 14 NSCLC patients that were undergoing PD-L1 blockade, PBMCs were collected pre and post treatment. Via whole exome sequencing and RNA sequencing, tumor neoantigens were identified which are predicted to bind to major histocompatibility complex class I (MHC-I). Overall, neoantigen-specific T cells was detected in the peripheral blood via tetramer staining of patients who underwent PD-L1 blockade. Responders had neoantigen-specific T cells that were of a highly differentiated phenotype expressing TIGIT, CD25, CD57, KLRG-1, 2B4 and CD161 and in contrast, nonresponders had neoantigen specific T cells that were of memory phenotypes, expressing CD127, CD28, CD27 and CCR7. [84] In another study, CD8+PD-1^{hi} T cells in baseline tumor biopsies were predictive of response to PD-1 blockade in NSCLC patients and was tumor reactive compared to CD8+PD-1^{neg}. [85] In study of 4 cancer types, including NSCLC, T cell clones that were found in the tumor were also found in the normal adjacent tissue. The same T cell clones were found to expand in the blood. Responders to ICB, had T cells in tumor were replenished with T cell clones from the blood suggesting a role of periphery in tumor immunity. [86]

Cells that undergo apoptosis, necrosis or other forms of cellular turnover, small double-stranded DNA fragments are released which are less than 200 base pairs into the bloodstream. The double-stranded DNA fragments are then detected in the plasma or serum. In cancer patients, some portion of them are circulating tumor DNA (ctDNA) and which can reflect the genetic and epigenetic changes of the tissue. [82, 87] ctDNA can be used as a biomarker of the tumor burden and provide insights into the tumor landscape which is evolving under the pressure of ICB. [31] ctDNA analysis would allow for the identification of patients who have become resistant to ICB so

that alternate treatment could be suggested. In advanced NSCLC, a drop in ctDNA was associated with response to ICB and prolonged survival in patients. [88] There is, however, a growing need to combine ctDNA analyses with immune cell analyses from the blood to understand the effect of ICB on immune cell subsets. [89, 90]

Recent studies have shown that current biomarkers for immune checkpoint therapy have limitations. To maximize patient benefits and improve healthcare outcomes, it is essential to use a combination of biomarkers to predict clinical responses to PD-1 blockade. For patients with advanced NSCLC, a combined approach using multiple markers may be the most effective way to predict therapy outcomes.

In vitro models

Patient specific models to evaluate tumor immune responses and to test the sensitivity to different immunotherapies would be the useful. [91] Dijkstra *et al* establish a 3D organoid NSCLC model and coculture PBMCs to create a system where they could study and expand tumor reactive T cells. This could be used to assess and predict drug sensitivity. [91, 92] Such models could be used to understand resistance mechanism in a patient specific manner. Organoids are three-dimensional versions of organs grown in vitro can mimic the complexity of an organ or a tumor in case of a tumor organoid. It is beneficial to study and understand a disease. T cells expanded in a tumor organoid culture can be expanded and use to kill cancer cells. [93] However, setting up an organoid culture is time consuming and expensive. Additionally, an organoid culture is devoid of cancer associated fibroblasts and their unique cytokine profile and stromal components which are key to understanding the complexity of the tumor microenvironment. Although, the stromal compartment can be partially restored, the immune microenvironment in the tumor is also missing in organoids which are important for assessing immune-tumor interaction. [94] Models that preserve tumor heterogeneity might be the best way to overcome these issues. In another study, tumor slices were grown on membrane inserts ex-vivo and drug sensitivity to chemotherapeutic agents and nivolumab (anti-

PD-1) was assessed. T cell reactivity was assessed and response to nivolumab was predicted in 2 out 12 patients. [95] Tumor digests preserve tumor heterogeneity although may have a larger number of dead or dying cells. For example, in assays to study T cell reactivity, T cells were reactive to tumor organoids with the highest expression of IFNɣ and CD107a but they were also reactive to tumor digests but with lesser expression levels IFNɣ and CD107a. [92] The comparison co-culture of PBMCs with organoids versus tumor digests suggested that a tumor digest in coculture with T cells could also allow for the expansion of tumor reactive T cells. [92, 96] With growing evidence of tumor specific T cells in the blood, it has become desirable to expand CD8 T cells in vitro in the presence of antigens from the tumor. [92]

T cells in cancer

Memory T cell subsets

Long-lived memory T cells play an important role in protecting against pathogens and cancer. The memory T cell population includes central memory (Tcm), effector memory (Tem) and tissue resident memory T cells (Trm). Memory T cells either circulate through blood, lymphoid and peripheral organs, or reside in tissues where tumors can develop. [97] Trm are the first response to infections at the tissue sites and clear pathogens. They are transcriptionally, phenotypically and functionally different from central and effector memory T cells and do not circulate in the blood. Tumor infiltrating lymphocytes include Trm that may be tumor specific T cells. TGF-β is essential for the formation and maintenance of Trm cells within the tumor. [98] Trms are identified by CD103 and/or CD49a integrins and c-type lectin, CD69 which are required in tissue residency. Trm express several inhibitory molecules, however, can still retain cytotoxic activity. [99] Recent studies into Trm function showed that it is not just important for the eradication of infections but also an important cell subset for tumor immunity. [100] In melanoma survivors who responded to immunotherapy, the clonotypes with high IFNγ and TNFα signature was shared between Trm cells and

effector memory T cells in the blood. The clonotypes also persisted for years in the blood and skin of the survivors [101] CD103+ Trm cells are known to have a role in antitumor immunity in lung cancer and are retained in the tumor by binding to E-cadherin and secrete cytotoxic molecules, express immune checkpoints and release cytokines in the tumor microenvironment. [102, 103] A high infiltration of CD8+CD103+ Trm in NSCLC tumors and expression of PD-1 and TIM-3 was associated with patient survival. Hence, it would be beneficial to explore the role of Trm CD103+ as a potential biomarker of response to PD-1 blockade. [104]

Sallusto *et al* in a seminal paper show that naïve T cells are differentiated into central memory T cells (Tcm; CD45RA-CCR7+) which home to the lymph nodes, and effector memory T cells (Tem; CD45RA-CCR7-) which migrate to peripheral tissue (Figure 3). Tcm cells have little or no effector properties unlike Tem but have a higher proliferative capacity and constitute different clones of T cells. Tcm give rise to effector cells upon secondary challenge and Tem. [105] The Tem are antigen specific, are high in perforin and provide effector protection. Depending on the strength of the TCR stimulation, engagement and cytokine expression, naïve T cell can give rise to T effector cells, among which some are Tem. [106, 107] There are difference in relative proportions of Tcm and Tem in CD4 T cells. Tcm is predominant among CD4 T cells, and Tem is predominant in the CD8 compartment. Activated effector memory T cells upregulate HLA-DR and CD38. [106, 108]. Understanding of the effects of PD-1 blockade on T cell memory subsets could be used to predict clinical response to PD-1 blockade. [109, 110]

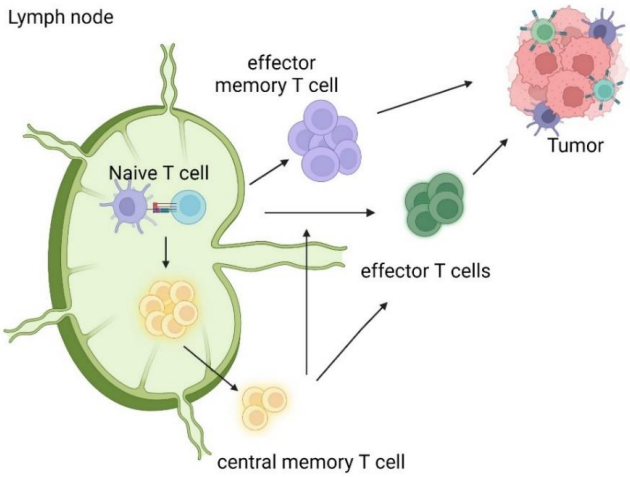

Figure 3: Memory T cell differentiation: T cell differentiation of naïve T cells into effector memory T cells and central memory T cells. Created on biorender.

T cell exhaustion

T cell exhaustion is defined as a loss of effector and memory functions due to persistent exposure to chronic inflammation such as in cancer or infections. It is well known that T cell dysfunction and exhaustion is characterized by the expression of several inhibitory immune checkpoints. Due to chronic exposure to antigens, the effector T cells, and memory T cells become exhausted, and lose their functional properties [111, 112]. T cells are also known to undergo states of differentiation. The CD8 T cell differentiation leading to exhaustion can be heterogeneous and can have mix of differentiation states. In parallel, T cells also differentiate to effector and memory T cell states. [113] Naïve T cells that are $TCF1^{hi}$ differentiate to T cells that express TCF1, BCL6, EOMES, thymocyte selection-associated high mobility group box protein (TOX) and express markers such as PD-1, CD28, CXCR5 and ICOS. They are defined as precursor or progenitor exhausted T cells. [113, 114] Precursor exhausted T cells or progenitor exhausted T cells also harbor self-renewal and proliferative capacity and capacity to kill target cells (tumor). They are also TIM-3

negative. [115] Upregulation of TOX occurs when there is a chronic antigen stimulation, and progenitor exhausted T cells can undergo TOX-driven epigenetic changes. During differentiation, the precursor exhausted generate effector-like intermediate exhausted T cells, that are TCF1 negative and Tbet high. Intermediate exhausted T cells express PD-1, TIM-3 and eventually lose function and become terminally exhausted which are TCF negative, but TOX high and EOMES+ and express several exhaustion markers such as PD-1, TIM3, CD39, LAG-3, CD101 and CTLA4. [113, 114]

There is growing evidence that stem-like TCF-1+PD-1+CD8 T cells are the subtype of T cells that can be reinvigorated post PD-1 blockade and represent progenitor exhausted T cells. [116] They can self-renew and experience a proliferative burst post PD-1 blockade. [117] T cell factor 1 (Tcf1) which is encoded by *Tcf7* is a key transcription factor in the canonical Wnt signaling pathway and has a role in generating T cell memory formation. [114, 118] Intratumorally TCF1+PD-1+CD8 T cells harbor stem-like properties and are required for maintenance of immune response against the tumor and for efficacy of ICB. [119, 120] Additionally, it was also found that draining lymph node is a reservoir of TCF-1+PD-1+CD8 T cells in mice which continuously migrated into the tumor. [120] TCF-1+PD-1+CD8 T cells are maintained in tertiary lymphoid structures which are located within and in the periphery of the tumor. [117]

Aims

1. To identify a combination of biomarkers that would improve the ability to predict clinical response to PD-1 blockade in NSCLC patients.
2. To explore baseline differences in T-cell phenotype and transcriptome to differentiate responders from non-responders to PD-1/PD-L1 blockade.
3. To investigate effector and regulatory immune cell subsets in biopsies from NSCLC patients that may be predictive of clinical response
4. To develop a personalized model for coculturing T cells derived from blood with tissue derived cells, with the goal of assessing T cell reactivity and evaluating their sensitivity to PD-1 blockade.

Methods

Patient cohorts

In paper I, II and III, Stage III and IV NSCLC patients were included from a larger cohort, "Biolung cohort" and the inclusion of the patients to study I, II and III was based on the treatment, PD-1/PD-L1 blockade or in combination with chemotherapy. In paper I, patients were recruited consecutively and prospectively based on monotherapy, Pembrolizumab, Nivolumab or Durvalumab treatment. In paper II, patients received PD-1/PD-L1 blockade alone or in combination with chemotherapy. In paper II, samples and analysis of 4 out of 36 patients overlapped. Blood was collected in EDTA tubes and Streck tubes at 5 timepoints at baseline and after each treatment cycle for up to 4 cycles (Figure 4). In paper I and II the patients recruited to the study served as their own controls and were followed longitudinally for treatment response to PD-1 blockade. In paper III, patients from the Biolung cohort were selected based on H&E staining evaluated by a pathologist. Assessment was made regarding the size and presence of tumor cells and immune cells. In paper III, 11 patients overlapped out of 18 with patients in paper I and II.

In paper IV, n=8, stage I and II NSCLC patients were included. The inclusion patients to study IV was based on the diagnosis, histology and tumor size $\geq$2cms. Patients had adenocarcinoma (n=7) and squamous cell carcinoma (n=1). All patients were treatment naive.

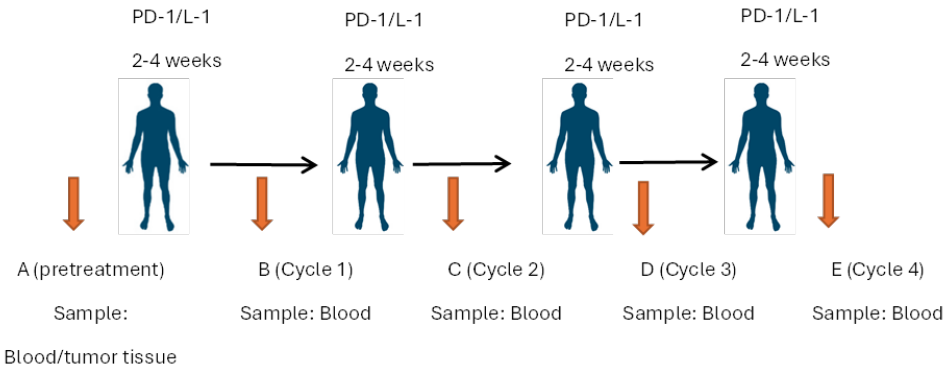

Figure 4: Blood sampling of biolung cohort. Created on biorender.

Multicolor Flow cytometry

Flow cytometry instruments measure characteristics size and granularity of cells. Cells are stained with antibodies tagged to a fluorescent dye targeting extracellular molecules on the surface of the cells or intracellular molecules inside the cell. With the help of a flow cytometer, we can analyze different immune cell populations. The main principle of a flow cytometer is light emitted by a laser, illuminates the cell, the light scatters based on the structural and morphological properties of the cell, and the fluorescence emission from a probe is directly proportional to the amount of probe bound to the cell. [121] When staining cells with different antibodies tagged with fluorescent dyes, one must carefully choose the right dyes in the panel. Essentially, panel design involves selecting bright fluorophores for weakly expressed markers and intracellular targets. Whereas dim fluorophores are chosen for strongly expressed markers. Additionally, fluorophores are chosen in a manner to avoid emission spectra overlap which can affect the resolution in differentiating the cell populations. All antibodies included in a panel need to be titrated and the concentration of antibody that provides the highest signal without non-specific binding is chosen to get the best signal to noise ratio. It is also important to avoid

influence of the antibody concentration on the compensation. In paper I, II and IV, we have utilized flow cytometry to analyze immune subsets in circulation of patients and in cell cultures. Cells were stained with fluorescently labeled antibodies for cell surface markers. In paper I, whole blood samples from patients were stained with surface antibodies and acquired on BD Canto or Lyric. In paper II and IV, extensive panels were designed to analyze (1) T cell subsets based on the immune phenotyping of human cells [108], (2) function comprising of intracellular cytokines, (3) phenotypes of NK cells, (4) regulatory T cells, (5) exhaustion and costimulatory molecules on T cells. Cryopreserved PBMCs were thawed and stained with cell surface markers. Further, cells were fixed and permeabilized with a fixation and permeabilization buffer to detect intracellular targets. Samples were then acquired on BD fortessa X20, which allowed the analysis of 14-16 fluorophores simultaneously. In every experiment conducted, dead cell staining and fluorescent minus one controls (FMOs) were included to determine the gating of the cells during analysis.

Isolation of DNA and sequencing

Genomic DNA was extracted from both the blood collected in K2EDTA tubes and tumor from formalin fixed paraffin embedded (FFPE) sections. Genetic analysis was performed on 15 patients from tumor tissue biopsies. Control for tumor tissue biopsies was genomic DNA control derived from the blood. The Oncopanel All in One v2.8, Eurofins Genomics NGS panel included 597 genes (Europe Sequencing GmbH, Germany) and the design covered 10bp flanking regions of all exons. The steps involved in extraction, quantification, library preparation (Agilent , SureSelect Technologies Santa Clara, CA, USA) and sequencing was performed at Eurofins Genomics, using optimized in house protocols. Sequencing was carried out on the Illumina NovaSeq 6000 platform (llumina, San Diego, CA, USA). Due to the restricted material available, 10-15 sections/patient, we chose to send the samples for DNA isolation and sequencing to Eurofines.

Bioinformatics analysis pipeline for tumor tissue

Variant calling and data quality assessment was performed at Eurofins Genomics (Europe Sequencing Gmb, Germany). A cutoff of 5% allele frequency was used in all filtrations. Alamut Visual (version 2.15; Sophia Genetics, Lausanne, Switzerland) and was used for the interpretation of the variants found, including Tumor mutation burden. For mutational signature analysis, software FastQC was used to assess quality of the data and SAMtools was used to sort, index and assess mapping statistics. The bioinformatics data was quality checked and the analysis pipeline was performed at Eurofins. Clinical NGS analysis of patient tumor is included in Paper I, II, III and IV.

Immunohistochemistry

Multiparameter immunohistochemistry using TSA method

Formalin Fixed Paraffin Embedded archival biopsy tissue was used immunohistochemistry staining method. IHC staining was set up using Akoya biosciences tyramide signal amplication (TSA) method [122]. This method is beneficial as it allows for detection of multiple cell types on the same tissue. Once the primary antibody has bound to its antigen, a secondary antibody conjugated with horseradish peroxidase (HRP) molecule is added. When the tyramide linked to an opal dye (fluorophore) is added, it is activated by the HRP and via covalent binding, it binds close to where the antigen is expressed on the tissue. Once this was performed for the first primary antibody, it is then repeated. To make way for the second primary antibody, the first primary antibody is stripped off using microwave treatment. The first covalently bound fluorophore is not affected by the microwave treatment. This method avoids the problems faced with conventional IHC such as cross species reactivity of antibodies and a restriction in choosing primary antibody from several different species.

Three panels were set up to study T cells and granzyme B expression, ICOS expression of T cells and tertiary lymphoid-like structures. Panel 1 comprised of Granzyme B, CD3, CD8 and CD4. Panel 2 comprised of ICOS, CD3, CD4, CD8. Panel 3 comprised

of CD20, CD3 and CD4. It is very important that several control experiments are performed, and the protocol is optimized. The experiments were first performed on tonsil and lobectomy tissue. Each antibody was titrated before deciding on a concentration to use. Several rounds of microwave treatments were carried out to determine the position of each primary antibody.

Additionally, to make sure that the antibody had been stripped off entirely after microwave treatment and there is no left-over antibody, experiments were performed wherein primary antibody, secondary antibody and then TSA/OPAL dye is added for a marker on tonsil slide. After first round of staining, it is subjected to a microwave treatment, at this stage a primary antibody isn't added but secondary and the next corresponding TSA/OPAL dye (not the previous) is added and then the protocol continues. When looked under the microscope, the first TSA/OPAL dye detected the first primary antibody, but the second TSA/OPAL dye added gave out no signal in its corresponding filter. This suggested that the removal of the antibody had taken place and that the secondary antibody in the second step did not have the primary antibody to bind to from the 1st round of staining (Figure 5).

The technique is very useful to carry out multi-parameter antibody analysis on the tissue as we are not restricted by species of the antibody, and we design extensive panels. However, it is still limited to maximum of 6 colors including DAPI staining.

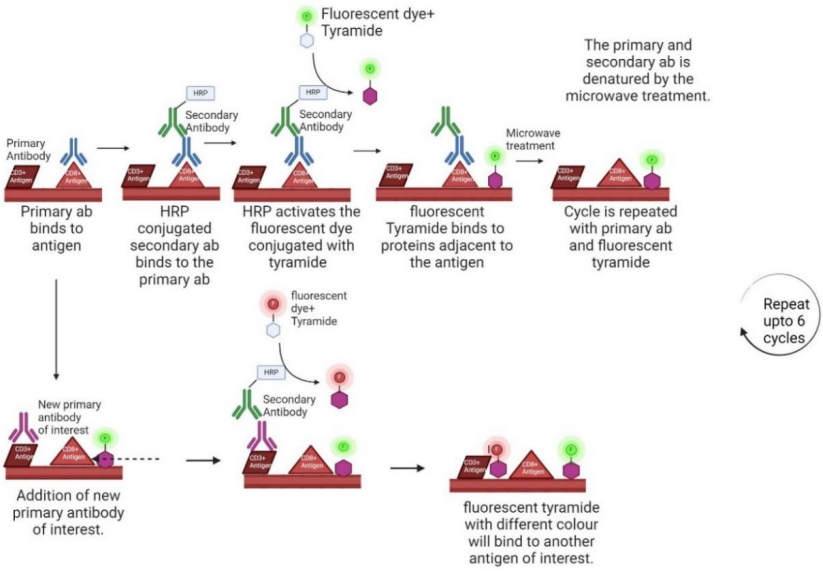

Figure 5: IHC staining using TSA method Different steps involved in the staining process. Created on biorender

CITE sequencing

CITE-seq (cellular indexing of transcriptomes and epitopes by sequencing) is a single cell multi-omics technology that measure RNA transcripts and proteins simultaneously. It combines single-cell RNA-sequencing (scRNA-seq) approaches to measure gene expression and cell-surface proteins in individual cells. CITE seq allows us to understand cellular heterogeneity. [123] The rationale for using it in our study was that we wanted to identify specific immune subsets that could distinguish a responder from a nonresponder. To deepen our understanding, we explore the differential gene expression within these circulating subsets. CITE seq allowed us to simultaneously analyze cell populations both with surface markers and their gene expression profiles. PBMCs were thawed carefully and washed on curiox laminar wash. To achieve the maximum number of CD8 T cells, we sorted live cells on a flow

cytometer and use a negative selection kit to isolate CD8 T cells. Careful steps were taken to minimize dead cells or dying cells as RNA is release, which can increase background noise in sequencing data. This noise can falsely affect biological signals and compromise the overall data quality.

We utilize Uniform Manifold Approximation and Projection (UMAP), graph based clustering and differential gene expression to visualize the data. We utilize UMAP to visualize T cell clustering. UMAP is considered as a dimensionality reduction tool to display different types of data. Dimensionality reduction helps represent multi-dimensional data in two or three dimensions. Using UMAP, we were able to identify cell populations that were present or absent in the two response groups. Some of the issues we faced was in some cell populations, we were not able to achieve a cell number from which we could further analyze the data set. We chose the surface antibodies based on the flow cytometry data performed earlier in the study. Gene ontology data sets were used to find the role of genes that were upregulated in patients. The Gene Ontology (GO) is a database, which is the world's largest source of information on the functions of genes. GO knowledgebase is developed using formal ontology by defining classes of gene function. [124]

Multivariate data analysis

In paper I, to examine the relationship between clinical responder and non-responder, a multivariate factor analysis was performed using the SIMCA-P+ software (Sartorius Stedim Data Analytics AB, Umeå, Sweden). The benefit of using SIMCA (Soft Independent Modelling of Class Analogy) in our study was that we could observe which variables strongly associate with response and additionally to check if a combination of variables could distinguish a responder from a nonresponder. The two groups, responder and non-responder (Y-variable) and the variables which are the immune cell subsets and tumor genetic signature of the patients (X-variable) were predefined. Orthogonal partial least squares discriminant analyses (OPLS-DA) identify the differences between the Y and X variable that influence the separation in

a supervised manner. OPLS-DA is also used to understand why two groups differ. [125] The score plot points out the differences between the group (horizontally across Y axis) and differences within the groups (vertically across the X axis). In score plot, the separation between patient groups (Y variables) is represented. A loadings plot is used to understand the contribution of each variable towards the separation. Here, the variables on each extreme sides of the plots are closely related to the respective patient groups. The reliability of the variables in clustering of the groups can be identified by a high confidence interval. The quality of the model is determined by R2Y and Q2. R2Y which represents the goodness of the fit of the model (R2Y ≥0.5 is a good discrimination and the best possible fit is R2Y=1) and Q2 represents the goodness of prediction of the model. Q2 is >0.4 is satisfactory. The difference between Q2-R2Y should not exceed 0.2-0.3. To assess the discriminatory power of a specific X variable and its influence on the model, a parameter called Variable influence on projection (VIP) was used.

Ethical aspects

The studies were performed in accordance with the Declaration of Helsinki and protocols have been approved by the Ethical Review Board at the GU (number 953/18). In addition, the entire procedure from collection of material from surgery for paper IV has been approved by the Swedish Ethical Review Authority (Dnr: 495-17, Dnr 2023-01574-01. A full description of the study is provided to the patients by the consulting physician during the process of informed consent. The volume of blood draw has to date not resulted in any negative impact on the wellbeing of the patient. Patients were excluded from the study if the treatment was stopped due to adverse events or changed due to progression. Standard clinical procedures were observed for the collection of patient blood and tissue samples. The sample collection will be carried out by a trained nurse. The studies are being carried out in close collaboration with oncologist Andreas Hallqvist (M.D. PhD) who is also aware of the medical risks and ethical issues of working with patients. All known precautions were taken when

handling the personal data in strict accordance with EU GDPR. All clinical data was coded such that no medical information can be traced back to an individual patient. The personal information of the patients included in the study was stored in a file which was accessible only to the study nurse and attending physicians involved in the study.

Results and Discussion

PAPER I

In paper I, we assess the power of combining immune and genetic markers to distinguish in a cohort of NSCLC patients, responders from nonresponders. Blood was collected at baseline (pretreatment) and at 4 time points after following the scheduled treatment. Patients in this cohort were treated with monotherapy, anti-PD-1 or anti-PD-L1. Our aim was to identify subsets of T cells circulating in patients pre- and post PD-1/PDL1 blockade and their relation to clinical response.

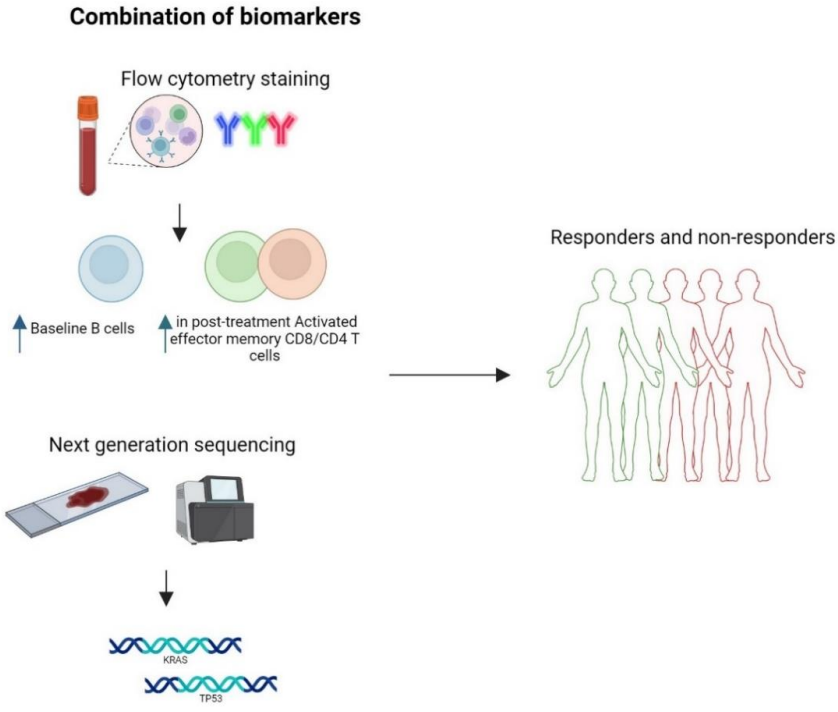

***Figure 6**:* Flow cytometry analysis of immune cell populations in the blood and next generation sequencing of tumor biopsies was performed to distinguish a responder from a nonresponder (green: responder, red:non-responder) Created on biorender.

We performed flow cytometry analysis in a discovery cohort of 15 patients. The analysis was carried out on whole blood samples of various immune subsets such as memory T cells, regulatory T cells, NK cells and B cells. A study reported that NSCLC patients who received anti-PD-1 and anti-PDL1, which report proliferating T cells more specifically, effector memory T cells and NK cells post treatment in patients who achieved both durable clinical benefit and non-durable clinical benefit. [73] However, in our cohort we did not observe a difference in frequencies of Tregs, NK cells, T cells, but observe that responders tended to have a higher B cell frequency (CD19) in the blood. In study by Xia *et al*, advanced NSCLC patients who responded to PD-1 monotherapy baseline circulating CD19+ B cells and IgM^+ memory B cells was found be higher than in nonresponders. [126]

We observed a significant upregulation of the activated effector memory CD4 and CD8 subset (CD45RA-CCR7-HLR-DR+CD38+) post-treatment in all patients. Ribas *et al* report melanoma cohort that patients who responded to Pembrolizumab (PD-1 blockade), had a significant expansion of T cells, specifically CD8 memory T cells, B cells and myeloid-derived suppressor cells but there was no difference in the frequency of Tregs and NK cells. [127] Consistent with our findings, another study also reports that in NSCLC patients, CD45RA-CD4 T cells were associated with progression free survival post immune checkpoint inhibitors. [128]

Additionally, we also observed that the frequency of central memory cells CD4 and CD8 T cells CD45RA-CCR7+ decreased after treatment in the responders although not significant. Central memory CD8 T cells decrease after the first cycle possibly due to retention in the lymph nodes and differentiate into effector cells and effector memory T cells. We further explore tumor biopsies by next generation sequencing (NGS) (Oncopanel All in One v2.8 from Eurofins Genomics), if gene variants in *TP53,* Serine/threonine kinase 11 (*STK11),* Kirsten rat sarcoma *(KRAS) and* Kelch-like ECH-associated protein 1 (*KEAP1*), associated with response to PD-1 blockade. Co-mutations of TP53, STK11, KEAP1 with KRAS are common in NSCLC. [129] A study by Skoulidis

et al showed that KRAS mutant NSCLC tumors that had mutations in STK11, had complete inactivation of KEAP 1, and had low expression of PD-L1. On the other hand, KRAS mutant tumors with TP53 mutations had an enrichment of expression of inflammation and anti-tumor immunity signatures, somatic mutations and improved relapse-free survival. [130] STK11 also known as liver kinase B1 (LKB1) is a tumor suppressor gene and mutations in STK11 in KRAS mutant lung adenocarcinoma has been reported to be a genomic driver of primary resistance to PD-1/PD-L1 blockade. [131] In our cohort of patients, in 7/10 responders and 3/4 non-responders, *TP53* variants were found. These were missense, nonsense, inframe and splice mutations. Missense variants in the *KRAS* gene were also found in 5/10 responders but none were found in the non-responders. However, in our study, missense KEAP1 mutations were observed in 2/10 responders but none in non-responders and *STK11* mutation was found in one responder and none in nonresponders. Although it is reported that STK11 and KEAP1 mutations are associated with poor response to ICB, we could not find that association in our cohort of patients. [70] Due to our cohort of patients being smaller compared to other studies in the literature, it is possible we do not observe co-mutations such as STK11 and KEAP 1 that has been reported in larger cohorts. However, patients that harbored most mutations, for example *TP53*, *KEAP1*, *KRAS*, were responders to PD-1 blockade. A gene score was devised based on the number of mutations each patient carried. A gene score of ≥ 2 was assigned if a patient had a pathogenic, likely pathogenic and variant of unknown significant (VUS) mutations in 7/10 patients in the responder groups and <1 in 5/5 patients in the nonresponder group. [66] Mutations in KRAS have been associated with better outcomes post ICB in advanced NSCLC patients as we observe in our cohort as well. [62] A panel of specific genes along with TMB should be utilized to understand a patient's response to ICB instead of TMB alone which accounts for the number of total variants in a genome.

Finally, we utilized a multivariate discriminant analysis (OPLS-DA) to investigate the relationship between the clinical response after PD-1 blockade and the parameters

studied for each patient (X-variables). In the software, we include baseline frequencies of CD3, CD4 and CD8 T cells, and Tregs. In addition, activated effector memory T cells, central memory T cells, effector T cells fold change and B and NK cells were also added. In addition to immune subsets, the clinical and genetic parameters such as gene score, PD-L1 status, and TMB score we included in the analysis. We conclude that the two clinical response groups, responder and non-responder clustered separately, distinguishing both groups based on the differences in the immune cell parameters and tumor genetic analysis, measured for each patient. Based on our study, it is important to have a combination of parameters, which could be used to monitor patient response. This would be helpful in the future to have a biomarker to predict clinical response to immunotherapy.

Paper II

In paper II, we analyzed blood samples from 37 advanced stage III and IV patients. We collected blood in lithium heparin tubes and via ficoll separation, isolated PBMCs from blood and froze it to use it at a later point. Blood was also collected in streck tubes and was isolated and analyzed. PBMCs from several timepoints for the same patients were thawed before use and stained with flow cytometry antibodies and analyzed (Figure 7). Patients were categorized as (i) early non-responders (progressive disease within 3 months), (ii) late non-responders (progressive disease within 6-9 months), (iii) responders (Partial response and Stable disease)

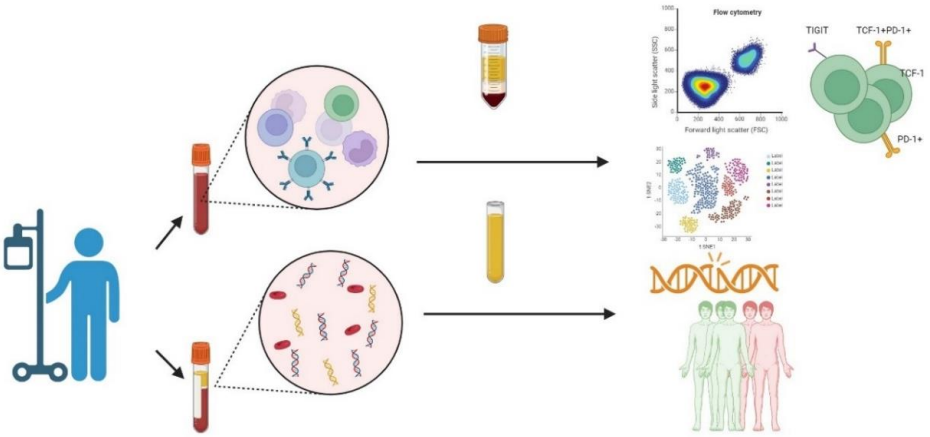

Figure 7: Blood is collected from patients undergoing treatment. PBMCs and plasma was isolated. Immune cell subsets in PBMCs were analyzed by flow cytometry, and ctDNA levels in plasma was analyzed by SimSen seq method. Created on biorender

(partial response or stable disease at 9-15 months), and (iv) long-term responders (partial response or stable disease after 21 months). In responders, our aim was to

explore differences within the group and to assess immune differences that categorized patients into >21 months responders and 9-15 responder groups. For nonresponders, we explore the differences between early nonresponders at 3 months and late nonresponders at 6-9 months. Immune monitoring allowed us to distinguish between patient groups, which may also help in identifying future biomarkers to predict clinical responses to PD-1 therapy. Although some studies show differences in circulating total CD8 T cells and CD4 T cells can differentiate a responder from a nonresponder, we do not observe such a difference. However, upon further observation, early nonresponders had T cells that expressed high IFNγ compared to late nonresponders and responders although this was not significant. It has been reported how IFNγ increased PD-L1 expression on tumor cells via JAK1/JAK2-STAT1/STAT2/STAT3-IRF1 which could be a potential reason as to why patients who expressed high IFNγ were early nonresponders. [132, 133] After performing CITE sequencing on PBMCs, using UMAP analysis pipeline, we found that MAIT cells in circulation were enriched in nonresponder in contrast to what has been reported in the literature. MAIT cells isolated from the blood of patients with NSCLC pre-treatment had an exhausted phenotype, however presence of circulating MAIT cells still predicted responders to PD-1 blockade. [134, 135] While in another study of NSCLC patients, anti-PD-1 treatment improved MAIT activation and function regardless of response. [136] Additionally, in patients with melanoma CD8+ MAIT cells in circulation was increased in responders post PD-1 blockade. [137]

Additionally, we observe the enrichment of memory T cells in responders via UMAP clustering. We report that the ratio of pre to post treatment is higher in responders to PD-1 blockade compared to nonresponders. The advantage of using CITE sequencing was that we were now able to analyze memory subsets in both nonresponder and responders. Differential gene expression revealed that effector memory CD8 T cells in responders harbored genes upregulated associated with gene ontology annotations such as T cell activation, positive regulation of T cell activation and cell-cell adhesion. On gene ontology database, positive regulation of T cell activation

includes some key genes that are positively associated with signaling such genes for CD3 complex (CD3D, CD3E, CD3G), ZAP70, LCK; Part of the T cell receptor (TCR) complex cell activation, IL-2; required for T cell activation, memory T cell activation, gamma-delta T cell activation, T cell differentiation, costimulation and proliferation. We do not observe the same trend in the non-responder. This suggested that the effector memory CD8 T cells in the blood could be antigen specific and sensitive to PD-1 blockade. Antigen specificity in the blood can be evaluated using surrogate markers when the antigen is unknown which can be achieved by analyzing the expression of PD-1, TIGIT, and TIM-3. Additionally, TCR sequencing can performed and TCR clones can be monitored in both the tumor and blood.

In our study, we find that TIGIT and PD-1 are significantly expressed on responders on CD8 T cells at baseline and we hypothesize that T cells that expressed TIGIT and PD-1 could be tumor specific. [138] However, there was no difference in TIM-3 expression. TIGIT and PD-1 are expressed early, when T cells are activated, and downregulated on effector cells. However, TIM-3 is expressed on dysfunctional T cells and perhaps it could be challenging to see differences in TIM-3 expression from peripheral T cells. However, TIM-3 with PD-1 is expressed on terminally differentiated T cells in the tumor. [117, 138]

We next assessed progenitor exhausted TCF-1+PD-1 CD8 T cells, and find that in most of the long-term responders, TCF-1+PD-1 CD8 T cells expand upon PD-1 blockade and the ratio of the frequency of TCF-1+PD-1+ of CD8 T cells post to pretreatment was significantly higher in long-term responders compared to nonresponders. The 9–15-month responders had a decrease in TCF-1+PD-1+ CD8 T cells but a drawback in our study was that we had fewer patients in 9–15-month cohort. In general, presence of TCF-1+PD-1+CD8 T cells have been associated with positive outcome with anti-PD-1 treatment. It's possible that in patients where we do not observe changes in TCF-1+PD-1+ CD8 T cells, the variation may be due to missing the optimal time window during blood sampling. In a future perspective, it would be interesting to see if

infiltrating TCF-1+PD-1+CD8 T cells in patient biopsies as it has been shown that presence of tumor infiltrating TCF-1+PD-1+CD8 T in NSCLC patients are associated to response to PD-1 blockade. [139]

The tertiary lymphoid structures can harbor TCF-1+PD-1+ CD8 T cells in a quiescent state. [117] We also observe that TIGIT+CD8 T cells, PD-1+ CD8 T cells and ratio of pre and post treatment TCF-1+PD-1+ CD8 T cells had no association with treatment given to patients, PD-L1 expression and KRAS mutation. It has been reported that in NSCLC patients, PD-L1 expression on the tumor was associated with patients harboring KRAS mutations. [140] It had the strongest correlation with response to treatment. However, it must be noted in our study that, the number of patients varied between treatments and that may affect the association of each parameter to the subset. We now know that T cells circulating in responders had a different phenotype compared to nonresponders. We hypothesize that CD8 T cells exhibit distinct transcriptional profiles in responders and non-responders to PD-1 blockade. T cells in responders have T cell activation signatures and genes such as *TCF7, JUNB, FOS* upregulated which can be associated with stem-like properties such as self-renewal and proliferative capacity and T cell activation. [141, 142]

T cells in circulation should be tracked alongside the tumor burden as the patients undergo treatment. Therapy response in patients is evaluated by clinical and radiological evaluation. However, it is challenging to standardize clinical assessments and they can be subjective. Moreover, cases of pseudo progression may be misinterpreted as tumor progression. It is therefore important to have early predictors to ICB therapy. [143] Reductions in ctDNA levels have been associated with improved overall survival in NSCLC patients who received immune checkpoint blockade. [144] Additionally, ctDNA may serve as a proxy of the patient's tumor burden and could be used to monitor treatment response. We find that in 1 long term responder, as TCF-1+PD-1+CD8 T cells increased post treatment and ctDNA levels remained non-detectable. In all 3/4 nonresponders, we observe an increase in ctDNA

levels and TCF-1+PD-1+ CD8 T cells reduced after treatment. As the tumor evolves, T cells may not be able eliminate clones that have escaped and hence it would be interesting to study how the immune responses during tumor escape.

Paper III

The purpose of this study was to investigate how the tumor microenvironment can potentially shape the clinical response to PD-1/PD-L1 blockade. Tumor biopsies taken prior to treatment were included for this study. FFPE slides with tissue was used for IHC (Figure 5) We applied immunohistochemistry staining methods utilizing Tyramide Signal Amplification (TSA) method to analyze various immune cell subsets in the tumor (Figure 6). In terms of infiltration of CD3, CD4 and CD8, we see no statistically significant difference between responders and nonresponders. Suggesting that most patients have an infiltration of T cells but the function of T cells and the spatial relationship of T cells with other immune cells would need to be further analyzed to understand its effect on the clinical response. Hematoxylin and eosin stained FFPE slides were assessed by a pathologist, where the tumor cells content in the individual sections ranged between 15%-40%. There was a significant increase in granzyme B+ T cells in the biopsies from nonresponders. The presence of high CD8 T cells expressing granzyme B may not necessarily indicate that the T cells have anti-tumor activity. In a bladder cancer model in mice, it was reported how a combination of chemotherapy drugs increases the infiltration of CD8 T cells into the tumor and expressed granzyme B. However, this subset had no effects in reducing tumor size suggesting that CD8 T cells that express granzyme B may not be functional. [145] In our cohort, nonresponders had a higher infiltration of CD8 T cells expressing granzyme B indicating that cytotoxic T cells but may not have effects on tumor shrinkage. Additionally, a suppressive tumor microenvironment could potentially influence the T cell function. Next, we analyzed ICOS+ CD8 T cells and CD4 T cells. We observed that responders had a significantly higher effector (non-Treg) $ICOS^{+}CD4$ T cells ($p=0.01$) and $ICOS^{+}CD8$ T cells ($p=0.03$). ICOS has been shown to be upregulated in the tumor tissue on both CD8 and CD4 activated T cells in a mouse model of Lewis lung cancer. [146] This could suggest that T cells we observe in the tissue are activated, however it requires further testing to know if they are cytotoxic. We hypothesize the non-Treg CD4+ICOS+ T cells could also represent a population of

T follicular helper cells (TfH) in the tissue. TfH cells that migrate to B cell follicles express CXCR5, PD-1, ICOS and BCL-6. [147] In our cohort since we have not analyzed additional markers, it would be difficult to know if the CD4 ICOS+ cells were indeed Tfh cells. We also observe CD4+FoxP3+ T cells tended to be higher in nonresponders. Additionally, T cells could be present in the tumor, yet lack anti-tumor effects due a suppressive immune microenvironment characterized by the expression of PD-L1, indoleamine-2,3-dioxygenase (IDO) and regulatory T cells in melanoma mouse models. [148]

Lastly, we analyze B cell infiltration in the tumor and TLS present in the TME. There was a tendency for higher numbers of B cells in the tissue in responders compared to nonresponders although the difference was not significant. Some of the mechanisms by which B cells aid in immune response is antibody-dependent cellular cytotoxicity, promotion of CD4+ and CD8+ T cell activation, maintenance of antitumor immune memory. Patil *et al* also report that plasma cell signatures are predictive of response to PD-L1 blockade in NSCLC tumors. Additionally, plasma cells and B cells were also associated with TLS formation. [149] Number of TLS structures counted which were similar in responders than nonresponders. Interestingly, B cells have a role in anti-tumor immunity by the production of antibodies in TLS. B ells via the BCR can present antigens to CD4 and CD8 T cells in TLS. [149] It would be interesting to further analyze B cells to investigate whether B cells in the tumor are antigen presenting cells or secrete anti-tumor antibodies or both.

A major weakness of the study was the few patients in the cohort. To have conclusive findings, we would need to analyze more patients and validate further in newer cohorts. Additionally, it would be interesting to analyze for Tfh cells in the tissue. Spatial transcriptomics would also allow for the analysis of different cellular neighborhoods to evaluate how different immune cells interact with each other. We

have a robust method which can be used for purposes of studying immune landscapes in patient biopsies and future biomarker studies.

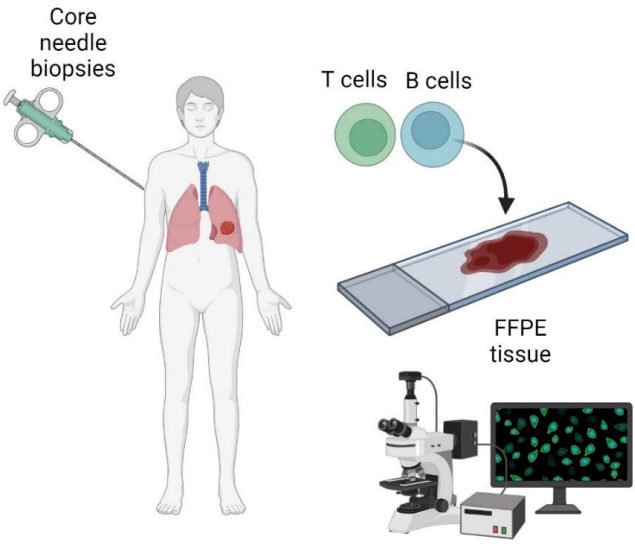

Figure 8: FFPE slides of tumor biopsies were stained with immunohistochemistry antibodies and analyzed by microscopy. Created on biorender.

Paper IV

Patients receiving anti-PD-1/PD-L1 therapies may not always respond or may develop resistance after an initial response. [150] [151] Evaluating the specific mechanisms of response requires a patient-specific approach. Dijkstra et al. developed a model expanding tumor-specific T cells from PBMCs after coculture with tumor organoids and digests. [92] Using their model as a benchmark, we set out to establish *an invitro* model with culture of normal/tissue digests with autologous T cells isolated from the peripheral blood of patients. This model analyzes CD8 T cell activation and differentiation into cytotoxic T cells with or without anti-PD-1. Other ex-vivo models, such as tumor slices are grown on membranes and tested for drug sensitivity has its advantages due to its simplicity in execution. [95] It would be interesting to compare our model with an ex-plant tumor model to evaluate the efficacy in predicting treatment response.

Treatment-naïve Stage I and II NSCLC adenocarcinoma patients were recruited between November 2023 and March 2024. Blood samples pre-surgery and tumor/normal adjacent tissue post-surgery were collected. The coculture assay spans 15 days. T cells were harvested after 7 days of culture with normal/tumor digests, then cocultured with fresh digests for another 7 days. On day 14, T cells were harvested and rechallenged with tumor digests for 24 hours (Figure 7). The principal concept of the rechallenges with tumor digests is increasing the number of tumor specific cells. We successfully set up a model after 15 days of coculture, where we could observe a pool of expanded T cells in cocultures of normal/tumor digests. First, we evaluate T cell activation via activation marker CD25. CD25, the high affinity alpha subunit of the IL-2 receptor, is a late-stage activation marker and is expressed 48 hours after stimulation. [152] CD25 expression is observed on short-lived antigen-specific effector CD8 T cells, and the expression depends on help from CD4 T cells. [153] CD8 T cells expressed significantly higher levels of CD25 when cocultured with tumor digests compared to PBMCs alone. However, CD25 expression does not necessarily indicate that the T cells are antigen specific. For identifying antigen-

specific CD8 T cells, a combination of CD69 with CD40L/PD-L1/4-1BB is recommended in flow cytometry-based AIM (Activation Induced Marker) assays, though this has been reported in infectious and autoimmune diseases [152] in which the kinetics of CD25 expression and T cell activation could vary compared to cancer. Still, AIM assays might be a useful tool for profiling tumor-specific T cells in cancer patients which needs to be further explored.

We next assessed the function of CD8 T cells by evaluation the degranulation of CD107a and cytokines IFNɣ and TNFα. In 3 out of 7 patients, CD8 T cells from normal/tumor digests expressed IFNɣ and TNFα with no cytokine induction in PBMCs alone. Cytokines play a role in tumor immunity and IFNɣ has anti-tumor and pro-apoptotic functions. However, it has also a role in the upregulation of PD-L1 expression on tumor cells which can hamper anti-tumor responses. [22] PD-L1 expression by the tumor was assessed in the clinic, and it varied between patients and tended to remain low. However, to identify the role of PD-L1 in dampening immune response in the coculture, we would need to analyze PD-L1 expression on tumor cells before the start of the coculture and at the end of the coculture to address the role of IFNɣ signaling in upregulating PD-L1 expression on tumor cells.

CD107a is a degranulation marker of NK cells and activated CD8 T cells. [154] Upon T cell activation, CD107a appears on the CD8+ T cell surface, along with intracellular IFNɣ production. CD107a is a key marker of cytotoxic T cell function. [155, 156] We observed high expression of CD107a on CD8 T cells from 3 patients cocultured with normal/tumor digests. The same trend was observed in the same 3 patients where we observed IFNɣ and TNFα induction in tissue specific manner. This indicated that the T cells in circulation had the capacity to be activated and become functional in a tissue specific manner and potentially acquire cytotoxic functions.

We next evaluated our model to test the sensitivity of T cells to anti-PD-1 when added to the coculture. We could observe anti-PD-1 decreased the frequencies of CD8 T cells expressing exhaustion markers, TIGIT+TIM-3+CD39+ in 5/7 patients. In addition,

we could identify one patient, NSCLC-2, when tumor digests were cocultured with PBMCs and anti-PD-1, CD8 T cells had a higher induction of IFNɣ, TNFα and CD107a in an antigen specific manner than in tumor cocultured with PBMCs indicating that the patient had CD8 T cells sensitive to anti-PD-1.

A coculture assay using tumor material from a patient who underwent surgery could be useful in predicting response to adjuvant treatment after the surgery. Such a model would be also useful for therapies such as adoptive T cell therapy where one can selectively propagate only antigen specific T cells.

In summary, we were able to establish a model in which T cells survived and retained their effector properties. The results showed that patients were heterogenous in their T cell response with respect to activation and cytokine secretion. We identify in some patient's tumor antigen-specific T cells that could be potentially sensitive to anti-PD-1.

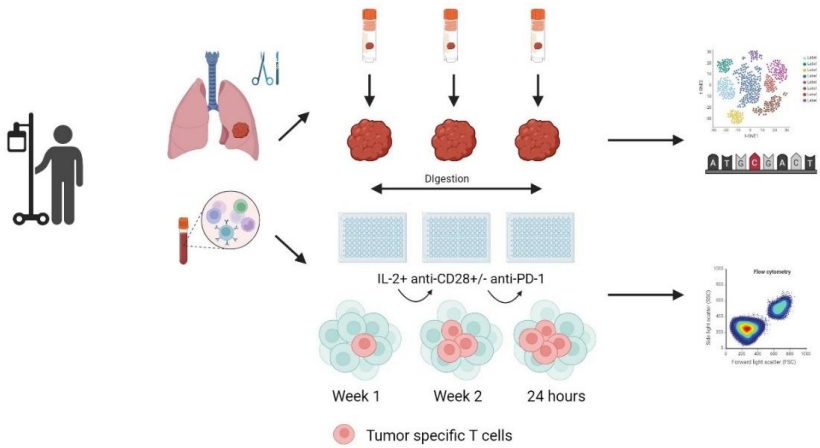

Figure 9: Tumor and normal tissue was obtained from patients undergoing surgery. Tumor and normal tissue was digested and cocultured with PBMCs for 15 days. At the end of 15 days, T cells are retrieved and analyzed with flow cytometry. RNA from tumor tissue and normal tissue was isolated and sequenced. Created on biorender.

Future perspectives and conclusions

In paper I, we show a that a combination of markers was able to distinguish between responders from nonresponders. We observe that activated effector memory CD8 T cells and activated effector memory CD4 T cells proliferate post treatment with anti-PD-1. We also report that when a patient harbors a combination of mutations in genes, had a higher gene score, they were in the responder group. This study was performed in a group of 15 patients who were on monotherapy. To further validate our findings, we would need to perform the same analysis on a new and larger cohort and compare it to existing biomarkers of clinical response to PD-1/PD-L1 blockade. In the future, we would include more cancer types and evaluate the efficiency of our model in predicting response to PD-1/PD-L1 blockade.

In paper II, we report that T cells in responders were inherently distinct from nonresponders in circulation. Responders to PD-1/PD-L1 blockade had higher expression of TIGIT and PD-1 on CD8 T cells at baseline than nonresponders which may indicate tumor specificity. Further, upregulation of *TCF7* and other genes related to T cell activation indicate that responders have an activated and progenitor exhausted gene expression compared to nonresponders. Finally, we combine ctDNA analysis with the ratio of post- to pretreatment TCF+1PD-1+ CD8 T cells and observe trends indicating that both parameters may be useful for monitoring patient response. TIGIT+CD8 T cells and PD-1+CD8+ T cells can be monitored in larger cohorts to develop as a biomarker of clinical response or to identify patients that could potentially benefit from combination therapy of anti-PD-1 and anti-TIGIT that is currently in clinical trials.

In paper III, we analyze the tumor microenvironment of stage III and IV patients using biopsy material, and we asked ourselves if the presence of effector and regulatory immune cell subsets in biopsies taken at the time of diagnosis could predict outcome to PD-1/PD-L1 blockade. We find that, nonresponder patients tended to have higher

CD4+FoxP3+ regulatory T cells in the tumor, while responders had significantly higher numbers of CD4+ICOS+ and CD8+ICOS+ T cells in the biopsies. Responders also tended to have a higher infiltration of B cells than nonresponders, with TLS observed in both responders and nonresponders. The biopsy sample is limited to be able to fully analyze the entire tumor microenvironment. However, it remains the only method to evaluate the TME in stage III and IV patients, as patients typically do not undergo surgery. To validate our findings, we need to include additional patients. Further, it would be interesting to include analysis of cellular neighborhoods with spatial relationships of T cells with immune cells such as a DCs and macrophages.

In paper IV, our goal was to set up a patient specific model to study individual patients T cell responses to tumors. We establish a model, in which normal/tumor digests are cocultured with PBMCs. From the assessment of 8 patients, we report that in our model T cells survive, expand and have functional properties from normal/tumor digests cocultured with PBMCs. Next step in the model is to develop an assay to assess the killing activity of CD8 T cells using a Incucyte® Immune Cell Killing Assay, allowing us to quantify killing activity of T cells in real-time. We know that T cells from patients are activated and express cytokines, however we would have to evaluate whether they have killing capacity. We also would need to analyze the cytokines released during cultures in supernatants that were collected at the end of week 1, week 2 and day 15.

From a mechanistic perspective, it would be valuable to compare the function and effector properties of T cells from cocultures with those infiltrating the tumor. The next step would be to determine if the same T cell clones using TcR sequencing, present in the tumor, particularly those with effector functions, are also found in the T cell coculture.

Acknowledgements

It has been 5 glorious years in the department. I am lucky to have been surrounded by fantastic colleagues who are so warm, kind and intelligent. Thank you everyone at the **Department of Microbiology and Immunology** for helping me and inspiring me in some way or the other!

I would specially like to thank all the **Patients** who were a part of this study, without whom the study wouldn't have been possible.

My supervisor, **Sukanya**, I am so grateful for the opportunity to do these exciting projects with you! Thank you for being a such a great mentor and supervisor. For the guidance and support, you have provided me, whether it has been scientific discussions or brainstorming, your door has always been open! That has helped me to grow and be better at Immunology and at science in general. Thank you especially for being so caring and warm as a person! And always going out of your way to help! I truly appreciate it so much.

Co-supervisor**, Andreas** Thank you Andreas, for all your valuable input, advice and humor at our biolung meetings, manuscript preparations and finally, defense preparation. You have been so patient with my questions and available with your prompt replies despite your busy schedule! thank you for all you have done!

Co-supervisor, **Anna R** Thank you Anna for guiding me through the complex world of genetics! I have learnt and gained so much from you in the past few years and for that I am so grateful! And for all your help during manuscript preparations and all the interesting discussions/conversations!

Co-supervisor, **Marianne** Thank you for your continued support and scientific discussions!

Thanks to **Biolung group and GLAS groups** for all the stimulating discussions and collaborations!

Special thanks to the **nurses**—**Christina, Katarina, and Emil** at **Sahlgrenska**—who have provided invaluable support over the years in organizing and collecting patient samples for the biolung study. My gratitude also extends to all the **nurses** at KPE and LONKEN (lung oncology clinic). I would like to thank **Per Torstensson** at **Skaraborgs Sjukhus**, **Skövde**, and the **nurses** there, for their efforts in sample collection and in transporting samples to Gothenburg.

A special thanks to thoracic surgeons, **Martin Silverborn, Maya Landenhed Smith** and **Andreas Westerlind** at the Department of Cardiothoracic Surgery, Sahlgrenska Hospital for assisting us patient material. I am also grateful the **nurses** at Thoraxmottagning and coordinator, **Medina Husemanovic,** who helped with administrative planning.

A big thank you to **Johanna** for your assistance with the manuscripts 😊. I am also deeply grateful to you, **Ella** for your help with all my projects and clinical inputs. **Kevin**, thank you for

your help with patient material collection and fun conversations, and **Ilayda**, thank you for your help with R!

My Thanks to **Hervé, George Alehandro, Marielle** for all the help with CITE sequencing, from the careful planning of the experiments and guiding me through the analysis; the discussions and many meetings! and of course, for my time at CIPHE

Special Thanks **to Levent, Johan B, Shahin** and **Sabiha** at Clinical Pathology for help with patient material.

Thanks to **Volkan** and **Per L,** for your invaluable support and inputs for the preparation of the manuscript! Thank you, **Sama**, for help and guidance with RNA sequencing analyses and helping us through the summer!

I also would like to thank past members in the lab, **Ahmed, Gina and Sara** who helped us with experiment set ups and patient material collection.

My thanks to **Angelica**, for assisting us with patient material preparation. I am so grateful for you **Linn**, and all that you do! Thank you for your help in the lab and all the discussions and conversations! It has been so great to work with you!

A very special thanks to you, **Davide**, for allowing me to use your microscope endlessly!

Thank you, **Anna L**, for your immunology guidance and help with Paper I, **Maria M** Thank you for teaching me how to use SIMCA and patiently explaining it so well whenever we asked silly questions!

Thank you, **Karin**, for being our go-to person for any lab-related advice.

Li Ching You were my first friend in Gothenburg!! I am forever thankful for the support I got from you! We miss you a lot here in our formerly poop office!!

Nimitha I don't know where to start! Thank you for not just being a friend but a kind and supportive and patient ear whenever I have needed to talk to you. I turn to you not just for fun conversations but a lot of scientific discussions and have received some of the best ideas and feedback!

Akshi Thank you for those homecooked comfort meals which I really love and endless conversations! You have always provided clarity when I had none!

Girls on the 6th! **Maria S, Alexandra, Bani, Najmeh,** thank you for all the fun conversations, 10 mins which become 1 hour when I am on the 6th, you guys are truly such a fun office 😉.

Frida My biggest concern while moving to Sweden was "will I make friends" How silly was I! But first, thank you for the initial help I got from you in the lab and all the organization 😉 and most importantly Thank you for being my best friend over the last few years and helping me navigate life in Sweden. There is so much more to say but you already know it! Now, I hope I can finally visit you!...(Thank yew!).

Laura Thank you for being a part of my support system in Gothenburg!! You have always been there for me, especially during the pandemic! Thank you for caring for me during my first experience with COVID when I was particularly scared and isolated! I am so glad for our movie dates, midsummers, cooking-at-home-nights. You have truly been such a massive part of my life in last 5 years 😊.

Romain Je suis si heureuse que tu sois venu dans la Suède glaciale depuis ta petite île ensoleillée!!**....**Too bad you won't understand that!! Thank you for making me laugh till my stomach hurts, for listening to me even at my most irrational self, and for making me better!

Andrew well, no thanks needed here, I guess. You owe me for 200 years. But still, thank you for being the kindest, most caring person and answering my billion questions regarding the thesis. My time here wouldn't have been the same without you and your dry-wit humor!

Hannes Thank you all the interesting conversations (and sometimes arguments 😉) I thoroughly enjoyed them and learnt new things from you!

Danica Thank you for all the laughs, pantry conversations and introducing me to your yum Filipino food 😉

Josue My 'murican friend!!! Thank you for the energy that you bring everywhere...by that I mean tequila! Your infectious laugh and fun conversations are the best and so uplifting!! And thank you **Caitlyn** for your warmth, for taking care of us and looking out for us when we are at afterworks 😉!!

Lydia I am glad I have found someone who I can discuss my training stuff with! and thank you for being a personal cheerleader 😊

Hana I wish I met you earlier, better late than never! Thank you for all the fun conversations and checking on me these last few months! Look forward to more dinners!

My **Axomiya** family in Gothenburg with special thanks to **Luna, Fardin and Pahi** who have been my home away from home!

Miriam Thank you for being a kind friend who has the most nuanced views on life (which I love) and my late-night training partner especially when I wrote my thesis!

My ex-colleagues **Rashmi, Jiju, Neelam** and ex-boss **Sandeep-** I do not think all of this would have been possible without your constant support, mentorship and encouragement I received during the 3.5 years at Aurigene. So thank you! I learnt so much there and still reflect on my initial training. I miss our lunch and coffee break conversations that often ranged from T cells/ PD-1, TIGIT, CD47, small molecules to Indian Politics and a lot of laughter 😉. I think a lot about it with fondness 😊.

My family, my pillar of strength! Words can't express enough how thankful and grateful I am for my family, my **Dad**, who has always inspired me with his hard work and sincerity. To my **Mom,** who has always provided me with comfort and positivity even though we are miles away. Finally, my little sister, **Asmita** who has been there for me through thick and thin!

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