Dissertation

Prospective Evaluation of Autoantibodies and Peripheral Blood B-cell Subtypes as Predictive Biomarkers in Cancer Patients undergoing Palliative Treatment with Immune Checkpoint Inhibitors

submitted by

Dr. med. univ. Dominik Andreas Barth

for the Academic Degree of

Doctor of Medical Science

(Dr. scient. med.)

at the

Medical University of Graz

Department of Internal Medicine

Division of Oncology

under the Supervision of

Assoc.Prof. PD Mag. Dr. Martin Pichler, MBA

2023

Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the Guidelines of the Medical University of Graz on Good Scientific Practice.

Graz, April 2023 *Dominik A. Barth eh.*

Disclosures

Parts of this thesis have been published in the following article:

Dominik A. Barth[1], Stefanie Stanzer[1], Jasmin Spiegelberg[1], Thomas Bauernhofer[1], Gudrun Absenger[1], Joanna Szkandera[1], Armin Gerger[1], Maria A. Smolle[2], Georg C. Hutterer[3], Sascha A. Ahyai[3], Tobias Madl[4,5], Florian Posch[6], Jakob M. Riedl[1], Christiane Klec[1], Philipp J. Jost[1], Julia Kargl[6,7], Martin H. Stradner[8*], Martin Pichler[1,9]

Patterns of peripheral blood B-cell subtypes are associated with treatment response in patients treated with immune checkpoint inhibitors: a prospective longitudinal pan-cancer study. Frontiers in Immunology. 2022 Apr 1;13:840207.

Access link: https://www.frontiersin.org/articles/10.3389/fimmu.2022.840207/full

[1]Division of Oncology, Department of Internal Medicine, Medical University of Graz, Graz, Austria
[2]Department of Orthopedics and Trauma, Medical University of Graz, Graz, Austria
[3]Department of Urology, Medical University of Graz, Graz, Austria
[4]Gottfried Schatz Research Center, Molecular Biology and Biochemistry, Medical University of Graz, Graz, Austria
[5]BioTechMed-Graz, Graz, Austria
[6]Division of Hematology, Department of Internal Medicine, Medical University of Graz, Graz, Austria
[7]Otto Loewi Research Center, Division of Pharmacology, Medical University of Graz, Graz, Austria
[8]Division of Rheumatology and Immunology, Department of Internal Medicine, Medical University of Graz, Graz, Austria
[8]Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

All authors agreed to the use of their data in this thesis.

Parts of this thesis have been published in the following article:

Dominik A. Barth[1], Stefanie Stanzer[1], Jasmin Spiegelberg[1], Thomas Bauernhofer[1], Gudrun Absenger[1], Florian Posch[1,2], Rainer Lipp[1], Michael Halm[1], Joanna Szkandera[1], Marija Balic[1], Armin Gerger[1], Maria A. Smolle[3], Georg C. Hutterer[4], Christiane Klec[1], Philipp J. Jost[1], Julia Kargl[5,6], Martin Stradner[7], Martin Pichler[1,8]

Evaluation of autoantibodies as predictors of treatment response and immune-related adverse events during the treatment with immune checkpoint inhibitors: A prospective longitudinal pan-cancer study. Cancer Med. 2022 Aug;11(16):3074-3083.

Access link: https://onlinelibrary.wiley.com/doi/full/10.1002/cam4.4675

[1]Division of Oncology, Department of Internal Medicine, Medical University of Graz, Graz, Austria
[2]Division of Hematology, Department of Internal Medicine, Medical University of Graz, Graz, Austria
[3]Department of Orthopedics and Trauma, Medical University of Graz, Graz, Austria
[4]Department of Urology, Medical University of Graz, Graz, Austria
[5]Otto Loewi Research Center, Division of Pharmacology, Medical University of Graz, Graz, Austria
[6]BioTechMed-Graz, Graz, Austria
[7]Division of Rheumatology, Department of Internal Medicine, Medical University of Graz, Graz, Austria
[8]Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

All authors agreed to the use of their data in this thesis.

Acknowledgements

First, I would like to express my deepest gratitude to my supervisor Professor Martin Pichler, who has been an exceptional and valuable mentor throughout the last years and has motivated and encouraged me right from the beginning. Thank you, Martin, for your mentorship, generosity and continued support.

I would further like to thank Professor Martin Stradner from the Division of Rheumatology and Immunology, for his time, expertise and support throughout my dissertation project. Without him, this cooperation project would not have been possible.

I would further like to express my thanks to Dr. Christiane Klec, who has been a valuable member or my dissertation committee and a great teacher in preparation for my research stay at the MD Anderson Cancer Center in Houston, Texas, USA.

Lastly, I want to express my love, thanks and gratitude to my partner Angelika as well as my parents, family and friends for their emotional support and motivating words during the preparation of this thesis. I am glad and lucky for having you around me.

The author received support by the Medical University of Graz through the Doctoral School Molecular Medicine and Inflammation.

Table of Contents

DISCLOSURES ..... **III**

ACKNOWLEDGEMENTS ..... **V**

TABLE OF CONTENTS ..... **VI**

ABBREVIATIONS ..... **VIII**

LIST OF FIGURES ..... **XI**

LIST OF TABLES ..... **XII**

ZUSAMMENFASSUNG ..... **XIII**

ABSTRACT ..... **XV**

INTRODUCTION **1**

1.1 CANCER: A GLOBAL HEALTH BURDEN 1
1.2 IMMUNE CHECKPOINT INHIBITORS: A GAMECHANGER IN CANCER TREATMENT 1
1.2.1 Principle of immune checkpoint inhibitors *2*
1.2.1.1 CTLA4 2
1.2.1.2 PD-(L)1 3
1.2.2 Clinical impact of checkpoint inhibitors *4*
1.2.3 Immune-related adverse events *5*
1.2.4 Biomarkers in immune checkpoint inhibitor treatment *6*
1.2.4.1 PD-L1 expression 6
1.2.4.2 Tumor mutational burden 6
1.2.4.3 Microsatellite Instability 7
1.3 ROLE OF B-CELLS IN TUMOR IMMUNOLOGY 7
1.3.1 Tumor infiltrating B-cells in cancer prognosis. *7*
1.3.2 Tertiary lymphoid structures *10*
1.3.2.1 Formation of TLS 11
1.3.2.2 TLS in immune checkpoint inhibitor treatment 14
1.3.3 Mechanisms of B-cells in tumor immunology *15*
1.3.3.1 Antigen presentation 15
1.3.3.2 Antibody production and antibody-mediated effects 16
1.3.3.3 Cytokines and chemokines 19
1.3.3.4 Complement-based 21
1.4 DELINEATING THE STUDY QUESTION AND AIMS 21

MATERIALS AND METHODS **23**

2.1 PREAMBLE 23
2.2 STUDY DESIGN 23
2.3 PATIENT RECRUITMENT 23
2.3.1 Inclusion criteria *23*
2.3.2 Exclusion criteria *24*
2.4 PATIENT CHARACTERISTICS 24
2.5 STUDY VISITS 24
2.6 SAMPLE ACQUISITION AND SAMPLE PROCESSING 25
2.7 ETHICS APPROVAL 25
2.8 STUDY 1 – AUTOANTIBODIES 25
2.8.1 Autoantibody measurement (as published in (171)) *25*
2.8.2 Statistical analysis *26*
2.9 STUDY 2 – PERIPHERAL BLOOD B-CELL SUBTYPES 27
2.9.1 Flow cytometry (as published in (172)) *27*
2.9.2 Statistical analysis *29*

RESULTS **30**

3.1 STUDY 1 – AUTOANTIBODIES 30
3.1.1 Baseline characteristics *30*
3.1.2 Autoantibodies at baseline *32*
3.1.3 Association of autoantibodies at baseline with clinical endpoints *34*
3.1.4 Subgroup analysis of autoantibodies at baseline *36*
3.1.5 Longitudinal development of autoantibody titers after 8-12 weeks of ICI treatment *38*
3.1.1 Association of autoantibodies with clinical endpoints after 8-12 weeks of ICI treatment *38*
3.1.2 Autoantibodies as predictors for immune related adverse events *40*
3.2 STUDY 2 – PERIPHERAL BLOOD B-CELL SUBTYPES 41
3.2.1 Baseline characteristics *41*
3.2.1 Association of peripheral blood B-cell subtypes at baseline with treatment response *43*
3.2.2 Change of B-cell counts and frequency during ICI-treatment *48*
3.2.1 Association of changes in peripheral blood B-cell subtypes after 8-12 weeks of ICI therapy with treatment response *50*

DISCUSSION **55**

4.1 STUDY 1 – AUTOANTIBODIES 55
4.2 STUDY 2 – PERIPHERAL BLOOD B-CELL SUBTYPES 59
4.3 CONCLUSIONS 65

REFERENCES **66**

Abbreviations

95%CI *95% confidence interval*

ACPA *cyclic citrullinated peptide antibodies*

ADCC *antibody-mediated cell-mediated cytotoxicity*

ANA *anti-nuclear antibodies*

ANCA *anti-neutrophil cytoplasmatic autoantibodies*

AP-1 *activator protein 1*

APRIL *A proliferation-inducing ligand*

BRAF *B-Raf proto-oncogene, serine/threonine*

B_{regs} *regulatory B-cells*

CCL *CC-Motif-Chemokine Ligand*

CD *cluster of differentiation*

CENPB *centromere protein B*

CI *confidence intervall*

CPS *combined positive score*

CR *complete remission*

CR2 *complement receptor 2*

CT *computer tomography*

CTAG1B Cancer-Testis antigen 1B

CTCAE *Common Terminology Criteria for Adverse Events*

CTLA4 cytotoxic T-lymphocyte associated protein 4

CXCL *CXC-Motif Chemokine Ligand*

DCR *disease controll rate*

dsDNA *double strand DNA*

EMA *European Medicines Agency*

ENA *extractable nuclear antigens*

FACS *fluorescence-activated cell sorting*

FDA *Food and Drug Administration*

GM-CSF *granulocyte-macrophage colony-stimulating factor*

HEVs *high endothelial venules*

HR *hazard ratio*

ICAM-1 *intercellular adhesion molecule 1*

ICAM-2 *intercellular adhesion molecule 2*

ICI *Immune checkpoint inhibitor*

ICIs *immune checkpoint inhibitors*

IL *interleukin*

INF *interferon*

irAEs *immune-related adverse events*

LAG3 *lymphocyte-activation gene 3*

LCK *Lymphocyte-Specific Protein Tyrosine Kinase*

LILRB1 *leukocyte immunoglobulin like receptor B1*

LTi *lymphoid tissue inducer*

LTα3 *lymphotoxin alpha 3*

MEK *mitogen-activated protein kinase 1*

MHC *major histocompatibility complex*

MLH1 *MutL protein homologue 1*

MMR *mismatch repair*

MRI *magnetic resonance imaging*

MSH2 *MutS protein homologue 2*

MSI *microsatellite instability*

MUC1 *mucin 1*

NFAT *nuclear factor of activated T cells*

NF-κB *including nuclear factor kappa-light-chain-enhancer of activated B-cells*

NSCLC *non-small cell lung cancer*

OR *odds ratio*

ORR *objective response rate*, *objective response rate*

OS *overall survival*

PBMC *peripheral blood mononuclear cells*

PD-1 *programmed cell death 1*

PD-L1 *programmed cell death ligand 1*

PR *partial remission*

RCC *renal cell carcinoma*

RCTs *randomized-controlled trials*

RD *risk difference*

RFS *recurrence-free survival*

SD *stable disease*

SLOs *secondary lymphoid organs*

SOX2 *SRY-box transcription factor 2*

TCGA *The Cancer Genome Atlas*

TGF-β *transforming growth factor β*

TIGIT *T cell immunoreceptor with Ig and ITIM domains*

TIL-Bs *tumor infiltrating B-cells*

TIL-Ts *tumor infiltrating T-lymphocytes*

TIM3 *T cell immunoglobulin domain and mucin domain 3*

TLS *tertiary lymphoid structures*

TMB *tumor mutational burden*

TME *tumor microenvironment*

TNFSL *transmembrane TNF superfamily ligands*

TNFα *tumor necrosis factor α*

TPS *tumor proportional score*

TRAIL *TNF-related apoptosis-inducing ligand*

T_{regs} *regulatory T-cells*

ULN *upper limit of normal*

List of Figures

Figure 1: Gating strategy (as published in (174)**)**..... 28

Figure 2: Progression-free survival (PFS) for positive vs. negative autoantibody screening at treatment initiation. (as published in (173)**)**..... 35

Figure 3: Progression-free survival (PFS) for positive vs. negative ANA titers at treatment initiation. 37

Figure 4: Distribution of B-cell subtypes in responders and non-responders (as published in (174)**).** 46

Figure 5: Changes in B-cell subtypes after 8-12 weeks of ICI treatment (as published in (174)**).**..... 49

Figure 6: Distribution of CD21$^-$ B-cell frequencies in responders and non-responders to ICI therapy (as published in (174)**)** 54

List of Tables

Table 1: Antigens, dye and clone used in flowcytometry analysis (as published in (174)**)** 27

Table 2: Summary table of the study population of study 1. (as published in (173)**)** 31

Table 3: Autoantibody measurements at baseline and after 8-12 weeks of ICI treatment. (as published in (173)**)** .. 33

Table 4: Univariable and multivariable analyses of autoantibody levels at treatment initiation predicting DCR and ORR. (as published in (173)**)** 34

Table 5: Uni- and multivariable Cox regression regarding PFS at baseline and after 8-12 weeks of therapy. (as published in (173)**)** 36

Table 6: Univariable and multivariable analyses of autoantibody levels at 2nd blood draw predicting DCR and ORR. (as published in (173)**)** 39

Table 7: Kidney and liver toxicity parameters at baseline and after 8-12 weeks of immunotherapy. (as published in (173)**)** 40

***Table 8: Baseline characteristics of the study population.* (as published in** (174)**)** 42

Table 9: Association of B-cell subpopulations with clinico-pathological parameters. (as published in (174)**)** 44

Table 10: Univariable logistic regression analysis of clinicopathological parameters for DCR and ORR. (as published in (174)**)** 45

Table 11: B-cell measurements and associations with disease control rate (DCR) and objective response rate (ORR) at baseline. (as published in (174)) 47

Table 12: Increase vs. Decrease or no change (reference) after 8-12 weeks of ICI treatment (as published in (174)**).** 51

Table 13: Absolute changes of B-cells after 8-12 weeks of ICI treatment and associations with disease control rate (DCR) and objective response rate (ORR) (as published in (174)**).** 52

Table 14: Relative Changes per 100% increase of B-cells after 8-12 weeks of ICI treatment and associations with disease control rate (DCR) and objective response rate (ORR) (as published in (174)**).** 53

Zusammenfassung

Hintergrund:

Die weitläufige Einführung von sogenannten Immuncheckpoint-Inhibitoren (ICIs) in der systemischen Behandlung verschiedenster solider Krebsarten hat die Krebsbehandlung nachhaltig verändert. Bisher lag der Forschungsschwerpunkt in diesem Bereich vorwiegend auf dem Verständnis der T-Zell-basierten Immunantwort, während B-Zellen, obwohl sie durch ihre Fähigkeit zur Antigenpräsentation und Antikörperproduktion ein wichtiger Bestandteil der adaptiven Immunität sind, weniger untersucht wurden. Das Auftreten von Autoantikörpern bei Patientinnen und Patienten unter einer Therapie mit ICIs wurde in der Vergangenheit als Marker für das Therapieansprechen diskutiert, jedoch reichen derzeitige Studiendaten, unter anderem aufgrund des Fehlens prospektiver Studien, nicht aus, um letztgültige Schlüsse zu ziehen. Darüber hinaus wurden verschiedene zirkulierende B-Zell-Subtypen unter der Therapie mit ICIs noch nicht ausreichend auf ihre Eignung als Biomarker untersucht. Ziel dieser Studie war es, sowohl Autoantikörper als auch zirkulierende B-Zellen als Marker für das Therapieansprechen unter einer Therapie mit ICIs im Rahmen einer prospektiven, longitudinalen Biomarkerstudie zu untersuchen.

Methoden:

Fünfundvierzig Patientinnen und Patienten, die sich zwischen Jänner 2017 und Dezember 2020 einer palliativen Mono- oder Kombinationstherapie mit ICIs unterzogen, wurden in diese prospektive single-center Kohortenstudie eingeschlossen. Autoantikörpertiter, einschließlich ANA-, ENA-, rheumatoide Arthritis-, Hepatopathie- und Myositis-assoziierte Autoantikörper, sowie sieben verschiedene B-Zell-Subtypen, die mittels fluoreszenzaktivierter Zellsortierung (FACS) quantifiziert wurden, wurden zum Zeitpunkt vor Beginn der ICI-Behandlung und nach 8-12 Wochen zum Zeitpunkt der ersten Evaluierung des Therapieansprechens bestimmt. Die Krankheitskontrollrate (DCR) und die objektive Ansprechrate (ORR) waren sowohl für die Analyse der Autoantikörper als auch der B-Zellen die primären Endpunkte, während das progressionsfreie Überleben (PFS) und das Auftreten immunvermittelter Nebenwirkungen (irAEs) co-sekundäre Endpunkte bei der Analyse der Autoantikörpertiter waren.

Ergebnisse:

Positive Autoantikörpertiter waren sowohl zu Studienbeginn als auch nach 8-12 Wochen Therapie mit ICIs nicht mit dem Therapieansprechen (DCR, ORR und PFS) assoziiert. Darüber hinaus konnte kein statistisch signifikanter Zusammenhang mit der Rate von irAEs festgestellt werden. Hinsichtlich der zirkulierenden B-Zellen gab es bei erster Messung zu Studienbeginn keinen signifikanten Zusammenhang mit den klinischen Endpunkten. Nach 8-12 Wochen ICI-basierter Therapie zeigten Patientinnen und Patienten mit einem Anstieg der Frequenz der $CD21^-$ oder der „switched memory“ B-Zellen eine geringere Wahrscheinlichkeit für ein Therapieansprechen (alle $p<0.05$). Im Gegensatz dazu waren die Ansprechraten bei Patientinnen und Patienten höher, bei denen die Häufigkeit von naiven B-Zellen nach der ICI-Behandlung zunahm ($p=0,039$).

Schlussfolgerungen:

In dieser Studie konnte weder ein signifikanter Zusammenhang zwischen Autoantikörpern und dem Ansprechen auf eine ICI-basierte Therapie hergestellt, noch eine veränderte Rate des Auftretens von irAEs je nach Antikörpertiter beobachtet werden.

Allerdings wurden signifikante Assoziationen eines Anstiegs der Häufigkeiten bestimmter Subtypen von zirkulierenden B-Zellen, nämlich CD21-, „switched memory“- und naiven B-Zellen, mit dem Therapieansprechen beobachtet. Weiters ist dies die erste Studie, die einen Zusammenhang zwischen CD21- B-Zellen, welche eine anergische und erschöpfte B-Zell-Population darstellen, und der Wirksamkeit einer ICI-basierten Therapie herstellt.

Abstract

Introduction:

Reinduction of anti-cancer immune responses by the means of immune checkpoint inhibitors (ICIs) have greatly impacted systemic cancer treatment across cancer types in the last decade. Focus in research has been primarily on T-cell responses upon ICI therapy, while B-cells, despite being an important part of adaptive immunity through their ability of antigen presentation and antibody production, have been studied to a lesser extent. The presence of autoantibodies in cancer patients undergoing ICI treatment has been suggested as markers for response prediction, yet evidence is inconclusive and prospective studies are missing. Moreover, a longitudinal characterization of circulating B-cell subtypes during ICI therapy has been missing. The aim of this study was to fill this gap, and evaluate both, autoantibodies and circulating B-cell subtypes as markers for treatment response in patients undergoing ICI therapy within a prospective longitudinal biomarker study.

Methods:

Forty-four cancer patients who were treated with mono- or combination ICI treatment were included in this prospective single-center cohort study. Autoantibody titers, including ANA, ENA, rheumatoid arthritis-, hepatopathy-, and myositis-associated autoantibodies, as well as seven different B-cell subtypes, quantified by fluorescence-activated cell sorting (FACS), were determined at the timepoint before ICI treatment start, and after 8-12 weeks of ICI therapy at the timepoint of first response evaluation. Disease-control rate (DCR) and objective response rate (ORR) were primary endpoints for the analysis of both, autoantibody and B-cells, while progression-free survival (PFS) and the development of immune-related adverse events (irAEs) were co-secondary endpoints in the evaluation of autoantibodies.

Results:

Autoantibodies at baseline as well as after 8-12 weeks of ICI therapy did not predict ICI treatment efficacy, as indicated by DCR, ORR and PFS. Furthermore, no significant association with the rate of irAEs could be observed.

As for circulating B-cell subtypes, at baseline, there was no significant relationship between different B-cell subtypes and response parameters. After 8-12 weeks of ICI therapy, an increase in the frequency of $CD21^-$ or switched memory B-cells was significantly associated with

decreased odds of response as for both, DCR and ORR (all $p<0.05$). Conversely, response rates were higher patients who and an increase in the frequency of naïve B-cells upon ICI treatment ($p=0.039$).

Conclusion:

In this study, no association of autoantibodies, neither at baseline nor after treatment start of ICIs, with parameters of response or with irAEs could be observed.

However, an increase of certain peripheral blood B-cell subtypes, namely $CD21^-$, switched memory and naïve B-cells, were associated with response. This is the first study to link $CD21^-$ B-cells, which represent an anergic and exhausted B-cell population, with ICI treatment efficacy.

Introduction

1.1 Cancer: a global health burden

Cancer is one of the leading causes of death worldwide and was responsible for approximately 10 million cancer-associated deaths worldwide in 2020 (1). According to recent reports of the Prospective Urban Rural Epidemiology (PURE) study, which is a population-based cohort study conducted in 21 countries and includes individuals who are 35-70 years of age, cancer is the leading cause of death in high- and upper middle-income countries, replacing cardiovascular diseases for the first time. However the latter remains the leading cause of death globally and in low-income countries (2). Other estimations expect cancer to become the leading cause of death worldwide as early as 2030 (3). As such, cancer is considered as a major global health care burden. In Austria, cancer prevalence was a total of 380,000 cases in 2020 and over 43,000 individuals where newly diagnosed with cancer, whereas cancer-related death was observed in 20,916 patients (4).

According to the GLOBOCAN estimates of worldwide cancer incidence over 19 million individuals were newly diagnosed with cancer in 2020 worldwide, with breast (11.7%), lung (11.4%), colorectal (10%) and prostate (7.3%) cancer being the most frequently diagnosed cancer types (1). Cancer incidence is stable in western countries such as the United States of America (1,9 million new cases expected in 2023), but cancer prevalence is still increasing as a result of declining mortality due to the broad implementation of screening programs enabling early cancer diagnosis as well as the implementation and development of novel therapeutic options in cancer care (5). In Austria, this led to an increase in cancer survival, which is reflected by an increase of the cumulative 5-year overall survival rate to 61% between 2013 and 2017 (4).

1.2 Immune checkpoint inhibitors: a gamechanger in cancer treatment

Immune checkpoint inhibitor (ICI) therapy has revolutionized systemic cancer treatment leading to significant improvements in patient's survival and quality of life, often leading to long-lasting remissions even in palliative treatment lines across several cancer entities (6–8). In 2018, James P. Allison and Tasuku Honjo were jointly awarded the Nobel Prize in Physiology or Medicine for the discovery of the immune checkpoint molecules cytotoxic T-

lymphocyte associated protein 4 (CTLA4) (9–11) and programmed cell death 1 (PD-1) (12–15) as novel therapeutic targets in order to reactivate the immune system in the fight against cancer (16). Today, ICIs are broadly implemented in the systemic treatment of cancer in palliative as well as (neo-) adjuvant treatment settings (17–19).

1.2.1 Principle of immune checkpoint inhibitors

The initiation of an effective immune response is a highly regulated process including a fine balance between both immune activating and immune suppressive inter- and intracellular signals and pathways in order to build a strong and effective immune response without risking uncontrolled inflammation or even autoimmunity.

So-called immune checkpoints are transmembrane molecules expressed on the cell surface of various immune cells including T-cells and antigen presenting cells which, when bound to their ligands, may activate or dampen T-cell activity and T-cell-mediated immune response (20,21). In cancer, these immune checkpoint molecules are central players when it comes to one of the most recognized and therapeutically targeted hallmark of cancer in the last decade: immune evasion (22).

Today, several different immune checkpoints such as but not limited to lymphocyte-activation gene 3 (LAG3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), T cell immunoglobulin domain and mucin domain 3 (TIM3), B7H7, leukocyte immunoglobulin like receptor B1 (LILRB1), and CD47 are known and extensively studied to date (20,23). Yet only the immune suppressive checkpoint molecules CTLA4 and PD-1 as well as the ligand of the latter PD-L1, have become novel and effective targets in cancer treatment (24).

1.2.1.1 CTLA4

CTLA4 is only expressed on T-cells (24). On the one hand, binding of the T-cell receptor with its cognate antigen and co-stimulation via the engagement of CD28 on the surface of T-cells with CD80 (B7.1) or CD86 (B7.2) on antigen-presenting cells leads to T-cell activation. On the other hand, it initiates the upregulation of CTLA4 and the recruitment of intracellularly stored CTLA in T-cells to the cell surface (23,25). As compared to CD28, CTLA shows a higher binding affinity to CD80 and CD86 (26) and subsequentially dampens further T-cell activation by competition for co-stimulatory factors (23,24). Additionally, engagement of CTLA4 with its

ligands CD80 and CD86 also triggers an intracellular signal cascade resulting in the suppression of T-cell-activating transcription factors including nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB), nuclear factor of activated T cells (NFAT) and activator protein 1 (AP-1) (23,27,28). Thus, therapeutically targeting CTLA4 with monoclonal antibodies enhances T-cell activation and restores anti-tumor immune responses.

CTLA4 is also highly abundant on the surface of regulatory T-cells (T_{regs}), which are associated with the promotion of tumor growth by inhibiting anti-tumor immune responses, and stimulates the expression of immunosuppressive cytokines such as interleukin (IL)-10, transforming growth factor β (TGF-β) and IL-35 (29). They also suppress the proliferation of $CD8^+$ T-cells and suppress the function of dendritic cells in the tumor microenvironment (TME) (29). Evidence suggests that monoclonal antibodies against CTLA4 also lead to the depletion of T_{regs} via cell-mediated cytotoxicity by FCγR-expressing macrophages (29–31).

1.2.1.2 PD-(L)1

Like CTLA4, PD-1 also acts as a break in T-cell activation. PD-1 is expressed on nearly all types of immune cells including dendritic cells, natural killer cells, T-cells and B-cells, yet highest expression of PD-1 is observed in exhausted T-cell populations (23,24,32). In contrast to CTLA, which expression levels increase 2-3 days after T-cell activation, enhanced PD-1 expression appears at later stages of T-cell activation (9,24). Interaction with its ligand PD-L1 (also referred to as B7H7), which is expressed on all cell types including immune cells and cancer cells, results in the intracellular phosphorylation of PD-1, thereby activating a signal cascade which suppresses activating signals of the T-cell receptor pathway (23). Consequently, T-cell migration and activation as well as the secretion of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), IL-2 and interferon (INF)-γ are suppressed (33). The inhibition of T-cell activation leads to a decreased anti-cancer immune response, a mechanism which is taken advantage of by cancer cells, which increase immune evasion by overexpression of PD-L1. The expression of PD-L1 in cancer cells is regulated and induced by various different mechanisms including genomic alteration, transcriptional and post-transcriptional regulation and protein-translational processing (32).

Targeting both, PD-1 or PD-L1, with monoclonal antibodies results in the reactivation of T-cell response and may overcome cancer immune evasion (12–15).

1.2.2 Clinical impact of checkpoint inhibitors

A detailed discussion of the clinical impact of ICIs in cancer therapy is beyond the scope of this dissertation thesis, as the topic is extensive, and indications of ICI treatment are constantly increasing with publications of new studies and trials in numerous cancer types in various treatment settings. Therefore, only a few examples are given in this chapter to illustrate the importance of ICIs in cancer therapy.

The new era of immunotherapy in cancer treatment started with the introduction of the CTLA4 inhibitor ipilimumab in metastatic melanoma patients in 2010. In a respective phase III study, patients who received ipilimumab showed a superior median overall survival (OS) as compared to patients who received gp100 alone (10.1 vs 6.4 months) (34). Just a few years later, the PD-1 inhibitor pembrolizumab showed significant efficacy in patients with melanoma refractory to ipilimumab therapy (35) and, moreover, superior response rates (33% vs 12%) and prolonged OS (hazard ratio (HR)=0.63, p=0.0005) as compared to ipilimumab (36). Another breakthrough came from the phase III CheckMate-067 trial which initiated the combination of CTLA4 and PD-L1 inhibition (ipilimumab + nivolumab) and reported encouraging and long-lasting responses. After a minimum follow-up of 48 months, median OS was still not reached in the ICI combination group whereas it was 37 and 20 months in the nivolumab- or ipilimumab-only groups respectively (37,38).

Since the introduction of ICI treatment in melanoma, it has been implemented in both palliative and (neo)adjuvant treatment settings in multiple cancer entities and greatly impacted patient survival and prognosis. For instance, in renal cell carcinoma (RCC) both, ICI combinations as well as combinations of various PD-1 and PD-L1 inhibitors such as nivolumab, pembrolizumab and avelumab with tyrosine kinase inhibitors (TKIs), significantly improved response and survival rates in the palliative setting (39–43). A recently published real-world analysis of metastatic RCC patients supported the results of randomized-controlled trials (RCTs) and reported a significant survival benefit for patients receiving ipilimumab + nivolumab (HR=0.75, p<0.001) or ICI + TKI combinations (HR=0.82, p=0.002) (44).

Similarly, in non-small cell lung cancer (NSCLC) the establishment of ICIs as first-line palliative monotherapy or in combination with chemotherapy significantly improved survival rates, yet real-world data generally indicate a smaller benefit than reported in RCTs (45).

Further indications where ICIs were implemented as an important backbone of systemic treatment include gastric cancer (46), esophageal cancer (47), non-melanoma skin cancer (48),

triple-negative breast cancer (49) and small-cell lung cancer and urothelial carcinoma (50,51), for instance.

1.2.3 Immune-related adverse events

Removing the brakes from the immune system through the administration of ICIs harbors the risk of triggering autoimmunity as class-specific adverse events of ICIs (immune-related adverse events | irAEs). Early studies in CTLA4 knockout mice revealed severe autoimmune reactions with infiltration of activated T-cells in multiple tissues leading to organ failure and premature death of CTLA4 knockout mice (52,53).

According to a meta-analysis, including 36 phase II and III studies, the overall incidence rate for the occurrence of any-grade irAEs lies between 54% and 76%, depending on the substances, the targeted checkpoint molecule, and ICI combination treatment (54). The risk of developing grade 3 or 4 irAEs according to the Common Terminology Criteria for Adverse Events (CTCAE), which are accompanied by hospital admission and represent comparably life-threatening complications, ranges from 15% to 29% (54).

Generally, irAEs can affect all tissues and organs of the body, with the most frequent being rashes (≥ grade 3: 2-10%), endocrine side effects such as thyroid dysfunction (8-14%) and hypophysitis (3%), diarrhea through ICI-mediated colitis (1-14%) and hepatitis (1-22%) (55). Rare, but non the less feared and potentially life threatening irAEs include pneumonitis, primary adrenal insufficiency and (peri)myocarditis. Other rare irAEs include type 1 diabetes, kidney injury, arthritis and other rheumatic diseases as well as ocular side effects such as sicca syndrome (55).

Depending on the grade of irAEs, ICI treatment may be continued upon close monitoring of patients (grade 1 and 2), or is to be interrupted or even terminated (≥ grade 3), with the necessity of high dose corticosteroid administration (56).

Predicting which patients are at increased risk of developing irAEs and closely monitor these patients in order to react early at the onset of side-effects is of paramount importance, yet specific biomarkers are missing. In the last years, preexisting autoantibodies, such as anti-nuclear antibodies (ANA) or anti-neutrophil cytoplasmatic autoantibodies (ANCA), or the development of autoantibodies upon ICI treatment have been suggested as irAEs specific biomarkers, yet the study landscape remains controversial in this regard (57).

1.2.4 Biomarkers in immune checkpoint inhibitor treatment

Despite the success of ICI treatment in oncology, several patients either relapse upon ICI therapy or do not show any treatment response at all due to resistance mechanisms (58). Given the comparably high financial burden of ICIs on healthcare systems, reliable predictive biomarkers to identify patients who are likely to response to ICI treatment are warranted (59). Some biomarkers are (at least partly) implemented in clinical routine, the most relevant of which in a clinical context are discussed in this section of the thesis.

1.2.4.1 PD-L1 expression

Early studies have already indicated the strong correlation between high PD-L1 expression in tumor samples and increased response rates upon anti-PD-1 treatment (60,61). In addition to the mere percentage of PD-L1 positive tumor cells (tumor proportional score | TPS), different scoring systems have been developed for the more advanced assessment of PD-L1 positivity. For instance, the combined positive score (CPS) is defined as the number of all PD-L1 positive cells divided by the number of viable tumor cells (62,63), and is determinant for ICI treatment in gastric cancer (64,65).

Today, the detection of PD-L1 expression via immunohistochemistry using standardized assays is already part in the routine diagnostic workup of several cancer entities and the PD-L1 status is determinant if patients qualify for ICI treatment in some cancer entities (63). For instance, in NSCLC only patients with PD-L1 > 50% may receive ICI monotherapy, whereas patients with a PD-L1 expression of less than 50% are recommended additional chemotherapy (66). In gastric cancer, first-line PD-1 targeted ICI treatment with nivolumab in combination with chemotherapy is approved only for patients with positive PD-L1 testing (46).

In contrast, as for example in RCC, PD-L1 status has no influence on clinical decision making and patients are offered ICI-based treatment regimens independent of high or low PD-L1 expression (67).

1.2.4.2 Tumor mutational burden

The tumor mutational burden (TMB) is considered as a marker for antigenicity of tumor and is defined as the total number of mutations of the tumor genome (68,69). The expression of neoantigens and aberrant self-antigens increases with the number of mutations within a tumor

regardless of their origin, which subsequently may enhance tumor immunogenicity. Neoantigens may be recognized by T-cells and initiate antineoplastic T-cell responses resulting in a more favorable efficacy of ICI therapy (58,69). Highly immunogenic tumors such as melanoma, non-squamous NSCLC and urothelial cancer generally show better responses to ICI treatment as compared to less immunogenic tumors including pancreatic and colorectal cancer (mismatch repair proficient) (70).

1.2.4.3 Microsatellite Instability

Cells with somatic or hereditary (e.g. Lynch Syndrome) genetic defects or mutations in mismatch repair (MMR) genes such as MutL protein homologue 1 (MLH1) and MutS protein homologue 2 (MSH2) acquire multiple frame shift mutations in microsatellites. Microsatellites are coding or non-coding DNA sections of multiple repeats of short sequences ranging from one to six base pairs. Accumulation of mutations in microsatellites subsequentially leads to microsatellite instability (MSI-high) which is associated with a high abundance of neoantigens and high TMB. Patients with MSI-high tumors, such as for instance MMR deficient colorectal cancer, respond astonishingly well to ICI treatment (70–72). This leads to the approval of the PD-1 inhibitor pembrolizumab for the site agnostic use in cancer patients with MSI-high tumors of both, the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) (58,73).

1.3 Role of B-cells in tumor immunology

Although the main focus in research aiming to gain a deeper understanding of tumor immunity, immune evasion and the role of lymphocytes in the context of immune checkpoint blockade in cancer treatment, has been on T-lymphocytes, tumor infiltrating B-cells (TIL-Bs) are emerging as new targets of research. Thus, the subsequent chapters broadly discuss and elucidate the role of B-cells and associated mechanisms in detail.

1.3.1 Tumor infiltrating B-cells in cancer prognosis.

A growing body of evidence suggests the prognostic relevance of TIL-Bs in various cancer types (74). For instance, in triple negative breast cancer, the presence and high density of

TIL-Bs in tumors was associated with a more favorable prognosis in several studies (75–77). In line with these results, a recent meta-analysis including a total of 2,628 patients with both, hormone receptor positive and triple negative breast cancer, reported TIL-Bs as significant prognosticators of OS (HR= 0.42, 95%CI 0.24-0.60) and recurrence-free survival (RFS | HR=0.41, 95%CI 0.27-0.55) (78).

Another example demonstrating the potential positive prognostic value of TIL-Bs is thyroid carcinoma, where Yang et al. (79) reported, that patients with tumor infiltrating lymphocytes and especially with high abundance of TLB-Bs showed superior survival outcomes.

Conversely, melanoma patients within the phase III COMBI-v trial, who had previously untreated B-Raf proto-oncogene, serine/threonine kinase (BRAF) V600 mutations and received treatment with the BRAF inhibitor dabrafenib and the mitogen-activated protein kinase 1 (MEK) inhibitor trametinib, had significantly shorter OS when the respective gene signatures pointed towards low T-cell and high B-cell abundance in tumor tissues (80). Corroborating these results, high levels of tumor-infiltrating $CD20^+$ cells were previously associated with shorter survival outcomes (81). However, data on the prognostic value of TIL-Bs in melanoma are still controversial (82). In contrast to the previously mentioned studies, presence and high levels of TIL-Bs were also connected to more favorable survival outcomes in melanoma patients (83–85). The opposing results may be attributed to varying definitions of B-cells between studies and the lack of more detailed B-cell phenotyping and B-cell subclassification (82).

In NSCLC, B-cells are increased in tumor tissues as compared to tumor adjacent tissues and were reported to present antigens to $CD4^+$ tumor-infiltrating lymphocytes, thereby inducing three different $CD4^+$ lymphocyte responses *in vitro*, as indicated by antigen presentation assays: activated responses, antigen-associated responses, and no responses (86). Moreover, the presence of so-called exhausted TIL-Bs ($CD69^+HLA^-DR^+CD27^-CD21^-$) correlated with a regulatory phenotype of $CD4^+$ tumor-infiltrating lymphocytes (86). T_{regs} are currently thought to attenuate antitumoral immune effects by various immune-suppressive mechanisms in NSCLC and are consequently associated with adverse prognosis (87–89). Although generally, more activated TIL-Bs were found in NSCLC tissues as compared to controls in the study by Bruno et al. (86), within the limitation of a limited sample size of 10 patient cases, evidence points in the direction of exhausted TIL-Bs being linked to adverse cancer outcomes, since patients showing an exhausted TIL-Bs phenotype either faced recurrent disease or cancer-

related death in their study (86). Of note, larger scaled clinical studies including proper survival analysis are warranted to verify these results.

In gastric cancer, high abundance of TIL-Bs as indicated by total $CD20^+$ B-cells in tertiary lymphoid structures (TLS) in the tumor microenvironment (TME) was significantly associated with OS across tumor stages (90). The analysis did not differentiate between B-cell subsets, however, more recent studies indicate that the impact of TIL-Bs on gastric cancer prognosis and progression may also depend on the subtype of TIL-Bs. A high frequency of regulatory B-cells (B_{regs}) was linked to increased immune evasion in gastric cancer by suppression of autologous $CD4^+$ T-cells and their production of INF-γ (91). Additionally, low frequency of B_{regs} in tumor tissue was significantly and independently associated with a more favorable prognosis as indicated by OS (5-year OS 65.4% vs 13.3%, $p<0.001$) (91). Apparently, the prognostic role of TIL-Bs across cancer types is not uniform and varied between tumor entities in a study performing extensive survival analysis including data of The Cancer Genome Atlas (TCGA) (92). In their study, Zheng et al. (92) analyzed the prognostic impact of tumor infiltrating T-lymphocytes (TIL-Ts) by determining the protein levels of the Lymphocyte-Specific Protein Tyrosine Kinase (LCK) and CD20, which are class-specific for T- and B-cells, by combining data from both, the TCGA and Genomic Data Commons (GDC) databases. In their analysis, the authors screened the prognostic relevance of tumor infiltrating lymphocytes in 30 different cancer entities and found favorable prognostic associations of high levels of CD20 in multiple cancers including lower grade glioma, melanoma, head and neck squamous cell carcinoma, testicular germ cell tumors, ovarian serous cystadenocarcinoma, hepatocellular carcinoma, papillary renal cell carcinoma, sarcoma, and lung squamous cell carcinoma. Adverse prognosis in patients with high CD20 levels was found amongst others in gastric cancer, diffuse large B-cell lymphoma and prostate cancer (92). Interestingly, B-cells and plasma cells may also exert protumor effects through their potential of tumor antigen-specific induction of T_{regs} via the presentation of tumor antigens by TIL-Bs as well as the release of immunosuppressive cytokines thereby promoting immunosuppressive phenotypes of myeloid cells (93). Therefore, in light of varying results by tumor type as well as the diverging prognostic impact according to different subsets of TIL-Bs, such as in gastric cancer as discussed above, prognostic conclusions depending on presence or absence, high or low abundance of TIL-Bs solely based on quantitative measurements of TIL-Bs without considering different B-cell subsets and their diverse immunological context, should be drawn with caution.

Moreover, TIL-Bs are encountered as parts of TLS in most cases, thus the immunological interplay between the different parts forming TLSs should not be left out of consideration. Thus, the immunological roles and further mechanisms of TIL-Bs in the context of cancer immunology and ICI treatment are discussed in the subsequent chapter.

1.3.2 Tertiary lymphoid structures

A growing body of evidence characterizes and describes the formation of TLS in the tumor microenvironment of solid cancers, thereby gradually revealing their roles in cancer immunity (94). The presence of TLS in the TME is associated with improved survival as, for instance, in breast cancer, where patients with the presence of TLS had a 39% risk-reduction of experiencing disease recurrence and a 40% decreased risk of death in a recent meta-analysis including 3,898 patients (95).

In contrast to secondary lymphoid organs (SLOs), such as lymph nodes, TLS are usually formed under pathophysiological conditions in the context of chronic inflammation, such as in various autoimmune diseases and cancer (94,96). They develop in the TME and stroma of solid tumors (97,98). Basically, TLS are formed by a bulk of B-cells in the center, which are mainly surrounded by varying densities of $CD4^+$ T follicular helper cells and $CD8^+$ cytotoxic T-cells, although also other T-cell subsets such as T_{regs} and $CD4^+$ T helper 1 cells may be located in the T-cell compartment. Other cell types reported include different populations of dendritic cells, such as follicular and mature dendritic cells, macrophages and $CD3^+$ T-cells (94,99,100).

Generally, TLS is not a uniformly defined term in the current contextual scientific landscape and often refers to any lymphoid structure in the stroma and tumor microenvironment of hot, solid tumors linked to response to tumor antigens and cytokine responses (97), which, as a matter of fact, is a noteworthy obstacle in comparing studies. In most cases, TLS comprise typical compartments of T-cells and dendritic cells as well as B-cells and follicular dendritic cells with the detection of specific markers such as CC-Motif-Chemokine Ligand (CCL) 19, CCL21, CXC-Motif Chemokine Ligand (CXCL) 12 and CXCL13 by immunohistochemistry (98,99,101). However, since no consensus on structural, cellular, and molecular characteristics of TLSs has been reached and established to date, comparison of studies may be impeded or at least made substantially more difficult. In order to tackle this problem, a stricter definition of

TLS was recently suggested by Laumont et al. (97) setting a minimum of requirements to be met in order to speak of TLS:

1) Clearly demarked B-cell follicles which contain $CD21^+$ follicular dendritic cells
2) Adjacent T-cell zones which contain conventional dendritic cells and high endothelial venules (HEVs)

In addition, these highly organized and mature TLS may be further characterized in primary or secondary TLS depending on the absence or presence of lymph-node like germinal centers, with follicular dendritic cells, proliferating B-cells, B-cells undergoing somatic hypermutation as well as the presence of follicular T-helper cells, respectively (97). Other lymphoid structures in the TME, which are not meeting these criteria should be referred to as lymph myeloid aggregates and may represent early stages in TLS formation or, on the contrary, are stalled or in a process of dissolution (97).

In contrast to ordinary lymph nodes, TLS do not have a capsule and, moreover, do not have histological features of dark and light zones of typical germinal centers in lymph nodes. This may enable direct contact of antigens with B-cells and highlights TLS as potential sites for antigen-specific B-cell responses including somatic hypermutation and affinity maturation (97,102). Yet, this carries a certain risk of generating autoreactive B-cells, as the cautious checks and balances involving, amongst others, a fine interplay with follicular T-helper cells and T_{regs} may not be in place in TLS as effectively as in ordinary lymph nodes (97,103,104). Interestingly, the immune checkpoints PD-1 and CTLA4 also play a role in preventing autoreactivity in B-cell maturation (105). If this brings the potential of allowing just enough autoreactivity in order to build an effective B-cell mediated anti-cancer immune response or if it harbors the risk of favoring the development of irAEs upon ICI treatment or even triggers paraneoplastic syndromes is not yet clear (97).

1.3.2.1 Formation of TLS

To date, the origin of TLS formation is unclear and is dominated by two hypotheses which include 1) the requirement of lymphoid tissue inducer cells expressing the nuclear receptor RORγt for the priming of the local mesenchyme similar to the embryogenic development of SLOs, or 2) the origin from and involvement of locally abundant immune cells in the TME (94). In the absence of lymphoid tissue inducer (LTi) cells, other immune cells may act as

substitutes for LTi cells and induce TLS formation. For instance, Peske et al. (106) reported the secretion of lymphotoxin alpha 3 (LTα3) and INF-γ by natural killer cells and effector $CD8^+$ T-cells, thereby inducing the formation of TLS in a murine melanoma and lung carcinoma model. In addition, presence of NCR^+ innate lymphoid cells (NCR^+ILC) in NSCLC tumor tissue may contribute to the formation of TLS through the production and secretion of cytokines including IL-22, IL-8, IL-2 and TNF-α upon activation by NKp44 receptor signaling, thereby activating endothelial cells and recruiting further immune cells to the tumor site (107). Only recently, Chaurio et al. (108) demonstrated the formation of TLS induced by cytokine secretion of T follicular helper cells.

TLS induction by other immune cells was also reported in diseases other than cancer, such as in autoimmunity and chronic inflammation (94,109,110). Lochner et al. (109) found that the formation of TLS was induced by B-cells expressing LTα1β2 in the absence of RORγt and LTi cells in a mouse model, thus highlighting the role of B-cell in TLS formation. However, since these results have not been acquired in a cancer model, direct conclusions on the impact of B-cells on TLS formation in cancer tissue need to be drawn with caution. Yet, recent publications also support the role of TIL-Bs in TLS formation in the TME, since in a murine melanoma model, TIL-Bs expressing LTα1β2 were required in the process of TLS formation (100).

In contrast to TLS, the formation of SLOs is far better understood, which may enable to draw conclusions and parallels to TLS formation. During the formation of SLOs, LTα1β2 and TNF, secreted by LTi cells, chemokines which bind to their respective receptors on mesenchymal lymphoid organizer (LTo) cells are important (94). This results in the induction of adhesion molecules such as amongst others Intercellular Adhesion Molecule (ICAM-1) and Vascular Cell-Adhesion Molecule 1 (VCAM-1) and the recruitment of immune cells to the lymphoid niche through the secretion of special chemokines, such as CCL19, CCL21 and CXCL13 (94). These chemokines, CCL19, CCL12, CCL21 and CXCL13 are also present in mature TLS in cancer tissues underlining the potential similarity between SLO and TLS formation (98,99,101).

The role of LTo cells in SLO formation may be substituted by cancer-associated fibroblasts in cancer as indicated by a study of Rodriguez et al. (100). In their study, the authors found that cancer-associated fibroblasts are activated by TNF-signaling and subsequently aggregate and form reticular networks mediated by $CD8^+$ T-cells. Further, through the expression of CXCL13

by cancer-associated fibroblasts, TIL-Bs are subsequentially recruited and express LTα1β2, thereby driving TLS expansion by VCAM-1 and A proliferation-inducing ligand (APRIL) expression on cancer-associated fibroblasts (100). Additionally, it is hypothesized, that besides tumor-associated stromal cells, different immune cells such as T follicular helper cells, macrophages, $CD8^+$ T-cells and B-cells may also act as LTo cells by their expression of CXCL13 (94). Yet further studies, are warranted to validate this hypothesis. The importance of CXCL13 signaling in cancer-associated TLS formation is also highlighted by other studies such as, for instance, in ovarian (111) and nasopharyngeal carcinoma (112). Corroborating the results in cancer, in other diseases associated with chronic inflammation, such rheumatoid arthritis, stromal fibroblasts were shown to secrete lymphoid chemokines, thereby acting as LTo cells (113).

In summary, both the function of lymphoid tissue inducer cells and LTos can be surrogated by other immune cells and mesenchymal cells in the TME, leading to the formation of TLS in cancer showing distinctive similarities to SLO formation (94).

Moreover, the presence of HEVs is an important premise for the recruitment of TIL-Bs in the TME (114). HEVs are postcapillary venules which facilitate the migration of lymphocytes from the blood into lymph nodes and SLOs by high expression of sulfated sialomucins and the receptors ICAM-1 and ICAM-2 (115). Interestingly, evidence suggests that T_{regs} in TLS may inhibit HEV formation (116,117).

Although the insight into the mechanisms and processes of TLS formation in cancer is gradually getting deeper by an increasing number of studies in recent years, the exact circumstances and intratumoral prerequisites creating an environment which favors and induces TLS formation by immune and stromal cells are still widely unknown to date (94). Like the formation of SLOs, in cancer TLS formations are considered to be driven by antigen responses of both, TIL-Bs and TIL-Ts. Generally, the presence and number of $CD4^+$ and $CD8^+$ TIL-Ts favors and correlates with the formation of TLS in cancer tissue (118). Moreover, a significant association of high clonality of T-cell receptors of $CD4^+$ and $CD8^+$ TIL-Ts with a high density of TIL-Bs within TLS in NSCLC indicates that T-cell antigen response may be crucial for TLS formation (119). In line with these results, Cipponi et al. (120) reported a clonal amplification, somatic mutation as well as isotype switching of B-cells in TLS in cutaneous melanoma metastases (120). These and further studies such as in ovarian (121) or esophago-gastric (122) cancer, corroborate the

hypothesis of B-cell-associated antigen-driven TLS formation in cancer and further highlight the role of B-cells in TLS formation and cancer immunology.

1.3.2.2 TLS in immune checkpoint inhibitor treatment

A growing body of evidence suggests the prognostic and potential predictive impact of TLS in the TME of cancer patients receiving ICI treatment.

For instance, in patients with advanced stage urothelial carcinoma, CXCL13 expression in tumor tissue correlated with TLS abundance and, in addition, was a significant prognosticator of survival as well as predictive for objective response rate in patients treated with ICI but not in patients treated with other treatment modalities (123). Corroborating these results, patients with advanced urothelial carcinoma and strong TLS-related gene signatures in tumor samples showed significantly higher survival and response rates to ICI treatment (124). Of note, both studies included TCGA data in their analysis, thus prospective validation and analyses in clinical cohorts are still missing. Similar results were found in patients with high-grade serous ovarian cancer, where CXCL13 expression was associated with prolonged survival, $CD20^+$ B-cell clusters as well as the presence of TLS. Of note, TLS only demonstrated a significant prognostic impact in the presence of simultaneous CXCL13 expression, indicating CXCL13 as a marker of immunologically 'hot' tumors (125). Positive significant associations of high abundance of TLS, either defined by immunohistochemistry or by gene expression signatures, including several chemokines and lymphocyte-specific receptors, and improved response rates or survival in cancer patients undergoing ICI therapy were further found in head and neck squamous cell carcinoma (HNSCC) (126) and esophageal squamous cell carcinoma (127).

The broad association of TLS with response to ICI therapy and improved survival outcomes in cancer patients gave rise to attempts to artificially induce TLS formation in cancer thereby improving or even enabling response to checkpoint inhibitors. Although no such drugs are currently available in clinical routine, the induction of TLS by therapeutically targeting HEVs in tumor tissue may be a promising strategy (128). Tumor Necrosis Factor Superfamily Member 14 (TNFSF14/CD258), also referred to as LIGHT, was targeted at tumor vessels via vascular targeting peptides (VTP), which lead to vascular normalization in tumors and subsequent formation of TLS with its associated survival benefits in combination with ICI treatment in pre-clinical murine tumor models, thus overcoming tumor-intrinsic resistance mechanisms (128). A growing body of evidence supports LIGHT-mediated transformation of immunologically

'cold' TMEs to 'hot' tumors and various delivery systems and combinations with ICIs and tumor vaccines have been investigated to date in several pre-clinical tumor models. Yet, this promising therapeutic concept still has to overcome several obstacles to be moved into humans, thus validation of this concept in clinical trials is still pending (129).

1.3.3 Mechanisms of B-cells in tumor immunology

The exact mechanisms of B-cells in tumor immunology with special emphasis on their role in ICI treatment have not yet been entirely understood and illuminated. Given the great heterogeneity of B-cells with multiple subtypes which take over different tasks in the adaptive immune system, their role in cancer and particularly ICI treatment also depends on the B-cell phenotype (130). The role of different B-cell subsets in tumor immunology with emphasis on their role in ICI treatment will be discussed in detail in the discussion section of this thesis (***see Discussion***).

Therefore, this chapter focuses on principle mechanisms of B-cell mediated immune responses in cancer. Although the exact relationships and mechanisms in cancer are not completely understood yet, based on the already uncovered and basic functions of B-cells in health and other diseases, certain mechanisms may be extrapolated in the context of B-cell mediated cancer-associated immune response, which will be discussed in this chapter in a point-by-point fashion.

1.3.3.1 Antigen presentation

Besides macrophages and dendritic cells, B-cells are the third major histocompatibility complex (MHC) class II-expressing professional antigen presenting cells in our adaptive immune system (131). B-cells may take up antigens via binding of the respective epitopes to their specific B-cell receptor and present these to $CD4^+$ T-follicular helper cells through MHC molecules, thereby sharpening adaptive immunity. This has proven to be especially effective in a setting of low antigen abundance (131).

In cancer, TIL-Bs may contribute to antigen presentation of cancer-associated (neo-) antigens and have a major impact on sufficient and effective anti-tumor T-cell responses. Bruno et al. (86) were able to deliver direct observations of freshly isolated NSCLC tumor cells from NSCLC patients acting as antigen presenting cells for autologous $CD4^+$ TIL-Ts in an *in vitro* antigen presentation assay resulting in an effector T-cell response (86). In line with these results, antigen-presenting B-cells derived from tumor-draining lymph nodes of cancer patients were

shown to increase antigen specific immune-responses of autologous T-cells to cancer testis antigens in *in vitro* experiments. In addition, antigen-presenting TIL-Bs cells of the same subtype ($CD21^-$ $CD86^+$) were shown to be highly abundant in the tumor tissues and TLS of nine different cancers, including colorectal cancer or breast cancer, suggesting TLS as the site of B-cell mediated antigen presentation (132). Moreover, activated B-cells isolated from peripheral blood mononuclear cells (PBMC) presenting Oct4 and Sox2 antigens were reported to stimulate $CD8^+$ cytotoxic T-cells leading to an effective inflammatory response and cytokine release and a consecutive enhanced anti-tumor effect against lung stem-like cancer cells *in vitro* (133). Corroborating these results, only recently, the interaction of neoantigen presenting germinal center B-cells and $CD4^+$ follicular T-helper cells were identified as a requirement for successful IL-21 mediated activation of tumor-infiltrating $CD8^+$ cytotoxic cells and associated enhanced Granzyme B expression in lung adenocarcinoma (134). Indirect evidence for antigen presentation of TIL-Bs arises from a study investigating localized ablative immunotherapy and photothermal therapy in a murine breast cancer model. In the study, treatment was associated with an increase of gene signatures associated with B-cell activation and antigen presentation (135).

It is hypothesized, that B-cells acting as antigen-presenting cells in the TME may be linked to epitope spreading, a process well-known from autoimmunity, where the reaction to a single initial epitope of an antigen subsequently spreads to other epitopes (97). By presenting MHC class II molecules to $CD4^+$ T-cells, the now activated $CD4^+$ cells may facilitate the response of other B-cells with receptors binding to a different epitope of the same antigen. This could explain the co-existence of both, mutated antigen-specific T-cells and antibodies directed against the wild-type antigen in cancer patients with simultaneous autoimmune diseases (136,137) (97). Interestingly, in a murine melanoma model, epitope spreading to wild-type tumor-lineage self-antigens was associated with effective response to ICI treatment (138).

Collectively, this underlines the important and not yet extensively defined or completely understood role of antigen-presentation by TIL-Bs in cancer immunology.

1.3.3.2 Antibody production and antibody-mediated effects

One of the main tasks of B-cells within the immune system is the production of antibodies against foreign antigens in the course of adaptive immunity, for they are the only cells which can convert into antibody-secreting plasma cells (139). This involves antigen recognition,

somatic hypermutation and affinity maturation, class switching and clonal expansion as well as the differentiation into plasma cells (140). Thus, the role of B-cell mediated antibody production in cancer is of vital interest in cancer immunology.

The intratumoral abundance of antibodies has first been reported as early as in 1977 (141) and since then the role of intratumoral and anti-tumor antibodies has been controversially discussed. Especially early reports in tumors, such as in lung cancer (142) and cancers of the genitourinary sphere (143), suggest a negative prognostic role of tumor-associated antibodies and a potential protumor effect of TIL-Bs and associated antibodies (93).

Antibodies produced by TIL-Bs and plasma cells may be directed directly against tumor antigens and several specific antibodies against widely known tumor antigens, such as for instance aberrantly glycosylated mucin 1 (MUC1, also known as CA15-3), are known to date and have been associated with cancer outcome (97). For instance, in breast cancer, of which MUC1 is one of the most specific tumor-associated antigens, high levels of IgG but not IgM subclass antibodies against MUC1 were significantly associated with patients OS (HR=0.19, p=0.002) (144). Similar results were observed in NSCLC (66) and pancreatic cancer (145). There are several other examples of common autoantibodies targeting well-known tumor-associated antigens. For example, Tp53-antibodies, which were commonly associated with adverse survival outcomes, according to a recent meta-analysis (146). Antibodies to Cancer-Testis antigen 1B (CTAG1B also referred to as NY-ESO-1) are frequently encountered in various cancers such as but not limited to colorectal cancer (147), gastric cancer (148), esophageal cancer (149) and mesenchymal tumors (150). A recent study analyzed the role of tumor-associated antibodies in the TME of breast cancer patients using a 91-antigen microarray panel, including several cancer-testis antigens such as the before mentioned CTAG1B as well as other tumor-antigens such as Tp53, MUC1, SRY-box transcription factor 2 (SOX2) or the well-known BRCA2 (151). Out of 91 antigens, eight antigens showed breast-cancer specific antibody responses, which were amongst others BRCA2 and CTAG1B. In their study, Garaud et al. (151) reported that higher levels of IgG responses but not IgA responses to cancer-associated antigens were significantly associated with shorter recurrence-free survival (RFS).

However, current evidence suggests that the majority of tumor-related antibodies are not directed against mutant and tumor-specific (neo)antigens but rather against wild-type self-antigens. This leads to the hypothesis that anti-tumor antibodies against nuclear, cytoplasmatic or extracellular compartments arise in a process of disrupted B-cell self-tolerance, rather than

representing a targeted and specific anti-tumor antibody response (97). Yet, a certain methodological bias arises from the broad investigation of wild-type antigen databases and the fact that studies aiming to analyze neoantigen-specific antibodies produced by TIL-Bs are widely missing (97). In addition, the origin of tumor-specific antibodies is not entirely clear yet and may not be dependent on TIL-Bs in the TME after all. In ovarian (152) and breast cancer (151), it is reported that tumor-specific antibody titers in the serum of patients were either not associated with the abundance of B-cells in the TME or persisted at evenly high levels even after tumor debulking, and consecutive removal of TIL-Bs. This may suggest that antibody response to tumor antigens may take place in SLOs such as lymph nodes and is not necessarily dependent on the presence and formation of TLS (97).

The impact of anti-tumor antibodies on cancer prognosis and their role in anti-tumor immune response is not entirely clear and may differ depending on cancer type or the patient's individual cancer-associated immune response (93). Thus, the abundance of such antibodies in the serum or the TME of cancer patients may either play a role in sufficient and successful anti-cancer immune responses or, on the contrary, even exert tumor promoting effects. As such, it is hypothesized, that antibody production of B-cells and plasma cells may impede an antitumor response of the adaptive immune system by the release of antibodies which are not able to induce sufficient anti-tumor T-cell response or induce tumor cell cytotoxicity. Moreover, the formation of immune complexes with (tumor) antigens may promote chronic inflammation, tissue remodeling and immunosuppressive myeloid cells (93).

On the other hand, highly specific anti-cancer antibodies secreted from TIL-Bs and plasma cells may also exert broad anti-tumor effects by facilitating the uptake of tumor antigens by tumor-associated macrophages and dendritic cells and induce antibody-mediated cell-mediated cytotoxicity (ADCC) (93). In this process, natural killer cells and myeloid cells bind to the Fc-receptor of antibody-antigen complexes on the surface of tumor cells thereby exerting ADCC. Likewise, tumor-associated macrophages may bind to the Fc-receptor domains, thus inducing Fc-receptor-mediated phagocytosis of the cancer cell (93,97). Beyond that, another hypothesis of antibody mediated anti-tumor effects of B-cells include antibody-dependent intracellular neutralization, which is supported by the astonishingly high prevalence of antibodies targeted against intracellular proteins amongst TIL-B-derived antibodies (97). Such antigens include several broadly known antigens such as amongst others CTAG1B, BRCA2, SOX2, TP53 and MAPK9. Wieland et al. (153) observed humane-papilloma-virus (HPV)-specific antibodies

against the HPV proteins E2, E6 and E7 in the TME and serum of patients with HPV^+ head and neck squamous cell cancer. These antibodies were produced in the context of ongoing germinal center reactions of TIL-Bs including different clusters of TIL-Bs, such as activated B-cells, antibody-secreting cells, germinal center B-cells and transitory B-cells. Besides adding yet another intracellular antigen targeted by tumor-associated antibodies, the results represent a direct link of active antibody production and B-cells in the TME upon prolonged antigen exposure (153).

Evidence suggests that the role of tumor-associated antibodies and the related antibody-mediated immune response may even differ by different antibody isotypes and subclasses (154). In the aforementioned study by Fremd et al. (144) IgG but not IgM was associated with patient survival. Similarly, Garaud et al. (151) reported that higher levels of IgG responses but not IgA responses to cancer-associated antigens were significantly associated with shorter recurrence-free survival (RFS). In lung cancer, Isaeva et al. (155) linked IgG4 antibodies from TIL-Bs with a more favorable prognosis, yet there was no significant association between patient outcomes and the abundance of IgG1 antibody isotypes. Conversely, in melanoma IgG4 was reported to impair the IgG1-mediated anti-tumor effects, and was additionally related to adverse prognosis (156).

In conclusion, B-cells and B-cell mediated secretion of tumor-associated antibodies are an important part of cancer immunity, as they may have both, pro- and antitumor effects. Yet, the potentially crucial role activated B-cells, hypermutation and an associated class switch to IgG secreting plasma cells during treatment with ICIs was recently underlined by an excellent study of Hollern et al. (157) in a murine model of ICI treated triple negative breast cancer. In their study, the loss of antibody secretion as well as the blockade of the Fc-receptor abolished response upon ICI therapy in their mouse model, indicates the importance of functional B-cells and antibody production during ICI therapy (157).

1.3.3.3 Cytokines and chemokines

The role of B-cells in the formation of TLS through the secretion of lymphotoxins and chemokines such as CXCL13 and LTα1β2 thereby acting as LTo cells has already been highlighted in a previous chapter (***see 1.3.2.1 Formation of TLS***). Beyond that, B-cells influence T-cell response via the secretion of a variety of other chemokines including IL-2,

IL-6, IL-17, CCL3, TNF, INF-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) (139).

In cancer, there are several examples of B-cells regulating T-cell response and shaping the inflammatory TME through the expression of cytokines and chemokines.

For instance, circulating B-cells were only recently shown to exert direct innate cytotoxic effects through the concurrent expression of transmembrane TNF superfamily ligands (TNFSL) such as TNF, Fas-ligand, LTα1β2 and TNF-related apoptosis-inducing ligand (TRAIL) and the subsequent induction of apoptosis in a study by Bratislav et al. (158). Interestingly, the study reported a decreased expression of TNFSL in cancer patients, yet TIL-Bs in the germinal centers of TLS expressed higher TNFSL levels as compared to other circulating B-cells. The authors further hypothesize that the cytotoxic fragmentation of target cells may be a link of innate and adaptive immunity as B-cells are also professionally antigen-presenting cells (158).

In gastric cancer, IL-10 expressing TIL-Bs reduced the expression of cytokines such as INF-γ, TNF and IL17 of autologous $CD4^+$ and $CD8^+$ T-cells *in vitro*, thereby inhibiting effective anti-cancer T-cell response (159). Interestingly, IL-17A was previously associated with increased B-cell migration, antibody response as well as direct B-cell mediated cytotoxicity through expression of Granzyme B and Fas-Ligand (160).

An interesting study in breast cancer revealed that TIL-Bs produced clearly and significantly higher expression levels of the so-called "Th1-effector cytokines" INF- γ and TNF-α in TIL-Bs as compared to SLOs and less diverse differences and generally lower expression of "Th2-effector cytokines" including IL-4, IL-5, IL-6 and IL-13 indicating an enhancement of cytotoxic T-cell responses via TIL-B-mediated secretion of INF-γ and TNF-α (77). Furthermore, IL-10 and TGFβ, which are known as immunosuppressant cytokines associated with a regulatory B-cell phenotype, were also expressed in TIL-Bs (77) corroborating the previously discussed study of Hu et al. (160).

In melanoma, B-cells with a plasmablast-like phenotype expressed CCL3, CCL4 and CCL5, CCL28 and CXCL16, which are chemokines linked with T-cell recruitment and were further associated with response and survival in ICI treatment. Depletion of B-cells lead to a significant decrease in tumor inflammation indicated by a decrease in $CD8^+$ TIL-Ts (161). This directly connects tumor-associated B-cell infiltration with cellular and inflammatory composition of the TME in melanoma.

However, cytokine secretion of B-cells may also be associated with cancer progression and reduced survival (139). As such, in prostate cancer, shrinking tumors in mice treated with androgen depletion where strongly infiltrated by B-cells, which stimulated cancer progression via activating the NF-kB pathway through secretion of LTα1β2. Moreover, B-cell depletion was associated with prolonged relapse-free survival in the respective study (162).

1.3.3.4 Complement-based

The complement system is a highly preserved and strictly regulated series of serine proteases including more than 50 components and is a major player in innate immunity as it is one of the first responders against invasive pathogens (163,164). Ultimately, the complement system forms the membrane attack complex leading to disruption of the cell membrane, calcium influx and lysis of the target cell, thereby exerting direct cytotoxicity (165). Beyond that, it is also linked to adaptive immunity. Three major pathways lead to the activation of the complement system: the classical pathway including antigen-antibody complexes, the alternative pathway including permissive surfaces, and the lectin pathway, where pattern-recognizing mannose-binding lectins (MBLs) bind to repetitive sugar motives (166,167). As for the classical pathway, the formation of an antigen-antibody complex and subsequent complement activation leads to the binding of C3d to the antigen. Subsequentially, C3d binds to complement receptor 2 (CR2) on the surface of B-cells in the context of co-receptors such as CD19 and CD81, thereby increasing antigen-associated signaling of B-cell receptors (167,168). A blockade of this co-receptor complex leads to a significant loss of germinal centers (168). Yet, it was previously shown that the B-cell receptor itself may generate CR ligands and the presence of soluble antibodies is not necessarily a precondition for the activation of the classical pathway, underlining the link of B-cells with the complement system (169).

1.4 Delineating the study question and aims

The broad implementation of ICIs in systemic cancer treatment has revolutionized cancer therapy, yet the majority of patients do not benefit from ICI therapy or experience recurrent or progressive disease (170). Several biomarkers such as PD-L1 expression, TMB and microsatellite instability are considered in clinical routine, however, these biomarkers are not able to perfectly predict success of ICI treatment in individual patients (58). As such, further

research is warranted to identify cost-effective and reliable biomarkers to complement established predictive markers.

Moreover, given the dangerous or even life-threatening irAEs in the course of ICI treatment, there is an urgent need for biomarkers in this regard, which are widely missing in clinical routine to date. The occurrence of autoantibodies known from rheumatic and autoimmune diseases may accompany the development of irAEs, as reported previously (57). Indeed, current evidence suggests a potential role of autoantibodies as predictors of treatment response and the development of irAEs, yet due to controversial results in multiple studies no clear conclusion may be drawn as of now and prospective evaluations were missing (57).

In the last decades, ICI research has focused primarily on T cell-mediated immune responses, but in recent years, the diverse functions and roles of B cells in cancer immunology as fundamental components of adaptive immunity, including antibody production, have gained interest. B-cells in the TME and in TLS of tumors have been linked to cancer outcome as outlined in the previous chapters, however, there is limited evidence that circulating B cells may serve as easily available and comparably cost-effective biomarkers in ICI therapy (171,172).

Thus, the aim of this dissertation project was to conduct a prospective pan-cancer observational cohort study to investigate two main aspects of the questions outlined.

1) **Autoantibodies**
   - Characterize the association of preexisting autoantibodies with response to palliative ICI therapy and the development of higher grade irAEs.
   - Evaluate the induction of autoantibodies during ICI treatment and the association with treatment response and the development of higher grade irAEs.
2) **B-cells**
   - Evaluate the predictive potential of peripheral blood B-cells on ICI efficacy under consideration of various B-cell subtypes.
   - Characterize the changes of various peripheral blood B-cell populations during ICI treatment.
   -

The results of this project were published separately in *Cancer Medicine (Barth et al., 2022)* (173) and *Frontiers in Immunology (Barth et al.2022)* (174).

Materials and Methods

2.1 Preamble

Parts of this section have previously been published in *Cancer Medicine (Barth et al., 2022)* (173) and *Frontiers in Immunology* (*Barth et al., 2022*) (174) as part of my dissertation thesis and are reproduced identically in part.

First, in this section overlapping materials and methods of both studies are jointly outlined, since the two studies were conducted within the same dissertation project and study cohort.

In a second step, study-specific methods are highlighted and further discussed.

2.2 Study design

The studies were carried out as prospective longitudinal cohort studies. Patients with cancer who received palliative ICI therapy at the Division of Oncology, Department of Internal Medicine, Medical University of Graz were included in the study. The study aimed to investigate the study questions in a pan-cancer cohort, thus patients were included regardless of their primary tumor site and cancer type.

2.3 Patient recruitment

Patients who received ICI therapy between January 2017 and December 2020 were included in the study according to predefined inclusion and exclusion criteria.

2.3.1 Inclusion criteria

- > 18 years of age
- Male or female patients
- Mono- or combination treatment with ICIs
- ICI treatment between January 1st, 2017 and December 31st, 2020
- Metastatic or locally advanced solid cancer with histologically confirmed disease, independent of primary cancer site (pan-cancer)
- Palliative treatment setting

- Pretreatment with other treatment regimens including chemotherapy, targeted therapy or radiotherapy was allowed
- Written informed consent

2.3.2 Exclusion criteria

- Patients < 18 years of age
- Patients unable to give written informed consent
- Patients with any history of preexisting autoimmune diseases

2.4 Patient characteristics

Reported and assessed characteristics of patients included sex, age, weight, height, BMI, history of smoking, and tumor specific parameters including cancer type, histology, previous treatment lines, PD-L1 expression and current ICI treatment.

2.5 Study visits

Blood draws for sample acquisition for the analysis of B-cell subtypes and autoantibodies were scheduled for the time before treatment initiation and 8-12 weeks after treatment start at the time of first response evaluation. If progressive disease was evident before first response evaluation, the second blood draw was performed at the time of progression.

Study visits were carried out by an experienced oncologist before treatment initiation as well as before every administration of ICI treatment. Patients were closely monitored for the occurrence of irAEs at every study visit which routinely included patient interviews, physical examinations as well as elaborate laboratory assessments including differential blood count, markers of inflammation (leukocytes, C-reactive protein (CRP)), liver parameters (ALT, AST, GGT, Bilirubin), kidney parameters (creatinine, eGFR, urea), muscly enzymes (creatinine kinase), lipase and endocrine markers (including TSH, fT3, fT4, Cortisol and ACTH). Adverse events were graded according to the Common Terminology Criteria for Adverse Events version 5. Patients were evaluated for treatment response every 8-12 weeks by computer tomography (CT) or magnetic resonance imaging (MRI) scans as appropriate, considering RECIST version 1.1 criteria.

2.6 Sample acquisition and sample processing

Blood draws for sample acquisition for the analysis of B-cell subtypes and autoantibodies were scheduled for the time before treatment initiation and 8-12 weeks after treatment start at the time of first response evaluation.

After sample collection, all blood samples were delivered to the Laboratory of the Division of Rheumatology and Immunology, Department of Internal Medicine, Medical University of Graz in a standardized manner after collection. All measurements were routinely performed by the Laboratory of the Division of Rheumatology and Immunology, Department of Internal Medicine, Medical University of Graz. All assays were calibrated and standardized for routine clinical sample testing.

2.7 Ethics approval

Written informed consent was obtained from each patient included in this study. This study was approved by the local ethics committee (IRB00002556) of the Medical University of Graz (29-593 ex 16/17) prior to any patient-related activity was performed.

2.8 Study 1 – Autoantibodies

2.8.1 Autoantibody measurement (as published in (173))

Antinuclear antibodies were examined by indirect immunofluorescence on Hep2 cells according to the international consensus and nomenclature (175). Antibodies to extractable nuclear antigens (ENA) (anti-centromere protein B (CENPB), anti-double strand DNA (dsDNA), anti-La, anti-PM100, anti-PM75, anti-RNP70, anti-Ro, anti-SCL70, anti-U1RNP, and anti-cyclic citrullinated peptide antibodies (ACPA)) were analyzed by fluorescence immunoassay (all Thermo Fisher) using an automated fluorescence reader (Phadia 250, Thermo Fisher). Rheumatoid factor IgA was measured by ELISA (Orgentec Diagnostika). Anti-GP210, anti-LKM1, anti-M2, anti SP100, anti-SLA-LP, anti-LC1, anti-F-Actin (LIVER PROFILE 7 Ag DOT, Alphadia) and anti-EJ, anti-JO1, anti-Ku, anti-MDA5, anti-MI2a, anti-MI2b, anti-

NXP2, anti-Oj, anti-PL-12, anti-PL-7, anti-SAE, anti-SRP, anti-TIF-1γ (EUROLINE Autoimmune Inflammatory Myopathies, Euroimmun) were analyzed using immunodot assays. Automated readout according to the manufacturer's protocol yielded semi-quantitative results.

2.8.2 Statistical analysis

For statistical analysis, the different autoantibodies were categorized into the following subgroups of autoantibodies:

- ANA
- ENA: anti-CENPB, anti-dsDNA, anti-La, anti-PM100, anti-PM75, anti-RNP70, anti-Ro, anti-SCL70, anti-U1RNP
- Rheumatoid arthritis: anti-CPA and rheumatoid factor
- Hepatopathy: Anti-GP210, anti-LKM1, anti-M2, anti SP100, anti-SLA-LP, anti-LC1, anti-F-Actin
- Myopathy: anti-EJ, anti-JO1, anti-Ku, anti-MDA5, anti-MI2a, anti-MI2b, anti-NXP2, anti-Oj, anti-PL-12, anti-PL-7, anti-SAE, anti-SRP, anti-TIF-1γ

For the means of biomarker dichotomization, patients were considered to have positive autoantibody measurements if at least one antibody titer was elevated above the upper limit of normal (ULN). As for the definition of ANA positivity, an ANA-titer ≥ 1:80 was chosen.
Disease control rate (DCR) and objective response rate (ORR) were chosen as co-primary endpoints of this study. DCR was defined as the percentage of patients who had complete remission (CR), partial remission (PR) or stable disease (SD) as best response upon ICI treatment. Accordingly, ORR was defined as the percentage of patients who had CR or PR.
Progression-free survival (PFS), defined as the time from treatment start to the date of disease progression or death of any cause, as well as the occurrence of higher grade irAEs (≥ grade 3 according to CTCAE 5.0) were considered as secondary endpoints of the study.
To assess the association of clinicopathological parameters with the autoantibody measurements $\chi 2$-tests, Fisher's exact tests and t-tests were used as appropriate.
Uni- and multivariable logistic regression models were implemented for assessment if the presence of autoantibodies at baseline were predictors of treatment response as indicated by DCR and ORR. Odds ratios (OR) as well as corresponding 95% confidence intervals (95%CI)

were reported appropriately. At the timepoint of the second blood draw absolute risk differences (RD) were estimated within a generalized linear model in order to avoid perfect prediction of the outcome due to a limited sample size at this timepoint. Multivariable analysis was empirically adjusted for cancer type only in all analysis settings, regardless of its significance in the univariable analysis in order to account for potential differences in DCR, ORR and PFS depending on the tumor type. For survival analysis PFS was estimated using Kaplan-Meier analysis and log-rank test was performed to assess for differences between groups. A two-sided p-value of <0.05 was considered significant for all statistical analyses. All statistical analyses were performed using Stata for Windows Version 16 (StataCorp LP, Collage Station, TX, USA).

2.9 Study 2 – peripheral blood B-cell subtypes

2.9.1 Flow cytometry (as published in (174))

The absolute number of $CD19^+$ B cells was determined by flow cytometry as previously described (176). B-cell subsets were analyzed in peripheral blood, mononuclear cells isolated from lithium heparin blood by Ficoll gradient density centrifugation. One million cells were incubated with antibodies against CD19, IgD, CD24, CD38, CD27, CD86, CD21, and IgM (Miltenyi Biotec, Bergisch Gladbach, Germany) (***Table 1***).

Table 1: Antigens, dye and clone used in flowcytometry analysis (as published in (174)**)**

antigen	dye	clone
CD 19	Viogreen	LT19
IgD	VioBlue	IgD26
CD 24	PerCP-Vio700	32D12
CD 27	APC	M-T271
CD38	FITC	IB6
CD86	PE-Vio770	FM95
CD 21	APC-Vio770	HB5
IgM	PE	PJ2-22H3

Samples were measured using a fluorescence-activated cell sorting (FACS) Canto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed using the FACSDiva software (BD Biosciences) using a gating strategy as published previously (177) and depicted in ***Figure 1***.

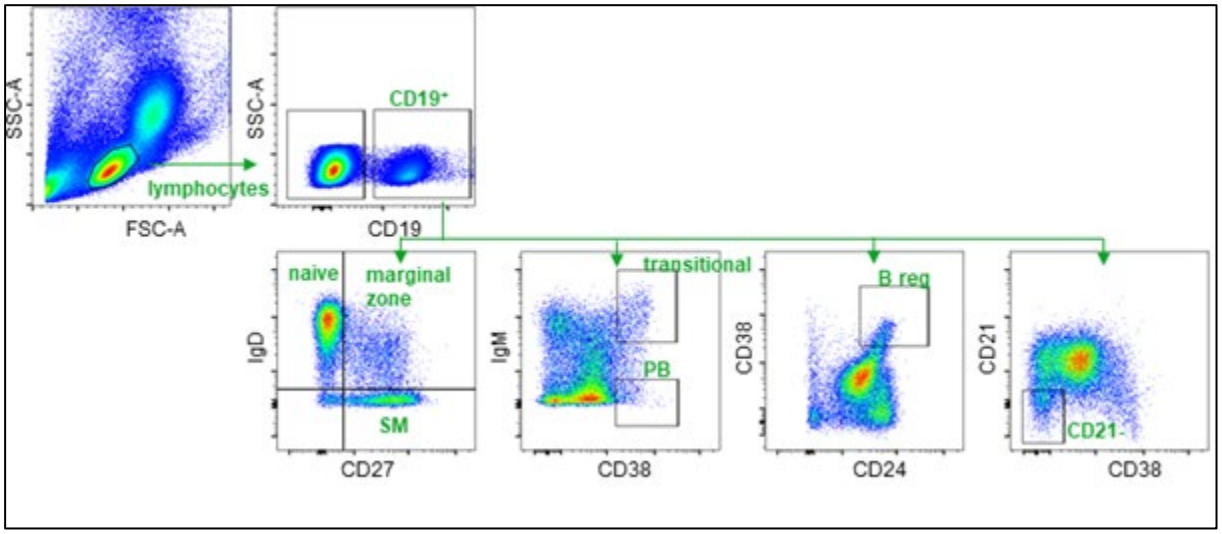

Figure 1: Gating strategy (as published in (174)**)**

B-cell subsets were classified according to the EUROclass gating strategy by Wehr et al. (178) as follows:

- $CD21^-$ ($CD19^+$ $CD38^-$ $CD21^-$)
- unswitched memory (or marginal zone) B-cells ($CD19^+$ $CD27^+$ IgD^+ IgM^+)
- naïve B-cells ($CD19^+$ $CD27^-$ IgD^+ IgM^+)
- transitional B-cells ($CD19^+$ $CD38^{++}$ IgM^{++})
- switched memory B-cells ($CD19^+$ $CD27^+$ IgD^- IgM^-)
- regulatory B-cells ($CD19^+$ $CD24^+$ $CD38^{++}$)
- plasmablasts ($CD19^+$ $CD38^{++}$ IgM^-)

Regulatory B-cells were defined as proposed by Das et al. (179) and Blair et al. (180).

2.9.2 Statistical analysis

Disease control rate and ORR were defined as co-primary endpoints of the study. DCR was defined as the percentage of patients who had complete remission (CR), partial remission (PR) or stable disease (SD) as best response upon ICI treatment. Accordingly, ORR was defined as the percentage of patients who had CR or PR.

Mann-Whitney-U-test and the Kruskal-Wallis-test were used as appropriate for evaluating the association of different B-cell subtypes at baseline with clinicopathological parameters of patients.

Uni- and multivariable logistic regression models were implemented in order to evaluate the association of B-cell subtypes with response to ICI therapy in cancer patients. Odds rations and corresponding 95%CIs were reported accordingly. Throughout the analysis, ORs for different B-cell subtypes were calculated and reported as per 1000, 100 or 10 units increase, as appropriate, in order to report clinically meaningful effects of changes in B-cell subtypes.

Considering the assessment of B-cell subtypes at the second blood draw after 8-12 weeks of ICI therapy, absolute (2^{nd} blood draw – 1^{st} blood draw) and relative change ((2^{nd} blood draw -1^{st} blood draw)/1^{st} blood draw*100) from baseline as well as a dichotomized approach (increase vs. decrease or no change) were analyzed for their association with the study endpoints. Changes of B-cell levels between baseline and after 8-12 weeks of ICI treatment were assessed using the Wilcoxon-Sign-Rank Test.

Due to the hypothesis-generating character of our study, multivariable analyses were only adjusted for tumor type and results were not adjusted for multiple testing. Tumor types were summarized as NSCLC, genitourinary (RCC and urothelial carcinoma), gastrointestinal (colorectal, gastric and cholangiocellular cancer) and head and neck squamous cell carcinoma. A two-sided p-value of <0.05 was considered statistically significant in all analyses.

All statistical analyses were performed using Stata for Windows Version 16.1 (StataCorp LP, Collage Station, TX, USA). Box plots were created using GraphPad Prism Version 9.1.2 for Windows (GraphPad Software, San Diego, California, USA).

Results

Parts of this section have previously been published in *Cancer Medicine (Barth et al., 2022)* (173) and *Frontiers in Immunology* (*Barth et al., 2022*) (174) as part of my dissertation thesis and are reproduced identically in part.

3.1 Study 1 – Autoantibodies

Parts of this section were reproduced from or similarly published in Barth et al. (173).

3.1.1 Baseline characteristics

A total of 45 cancer patients who received palliative ICI treatment were recruited in this prospective single-center cohort study. One patient was excluded due to loss of follow-up; thus 44 patients were included in the final analysis of the study.

Most patients (70%) included in the study were male (n=31), and median age at the time of study inclusion was 63.5 years. With 34% (n=15) NSCLC was the most prevalent cancer type, whereas 25% (n=11), 16% (n=7), 14% (n=6), and 11% (n=5) of patients were diagnosed with RCC, head and neck squamous cell carcinoma, urothelial carcinoma and gastrointestinal cancers, respectively. Roughly half (52%) of the study population had a history of smoking. PD-L1 expression was not available for all patients, since PD-L1 status was for the most part only routinely assessed in NSCLC and is not recommended in RCC, urothelial 2nd line treatment and head and neck cancer 2nd line treatment. Twenty-three percent (n=10) of patients had a PD-L1 expression >1%, whereas 14% were tested PD-L1 negative. Most patients were either treated with the PD-1 inhibitors nivolumab (n=22) or pembrolizumab (n=20). In addition, one patient was treated with the PD-L1-inhibitor atezolizumab, whereas one patient received an ICI combination therapy consisting of nivolumab plus ipilimumab.

Almost half of the study population (47.7%) received ICI treatment within a 2nd line treatment setting, while n=17 patients (38.6%) were treated with ICIs in the 1st line palliative setting. Together with patients in their 3rd line palliative treatment (13.6%), almost two thirds of patients (61.4%) had undergone previous palliative therapy, whereas only n=17 patients (38.6%) were treated with at least one line of chemotherapy prior to ICI treatment. See ***Table 2***.

Table 2: Summary table of the study population of study 1. (as published in (173)**)**
*Association of clinico-pathological parameters with positive autoantibody screening at baseline

		n (%miss.)	Summary measure	*p*-value*
Demographic variables				
Sex		44 (0%)		0.599
---female			13 (30%)	
---male			31 (70%)	
Age (years)		44 (0%)	63.5 [57-70.5]	0.0571
BMI (kg/m²)		44 (0%)	24.4 [21.4-26.6]	0.5435
Cancer entities		44 (0%)		0.612
Non-small cell lung cancer			15 (34%)	
---Adenocarcinoma			9	
---Squamous cell carcinoma			5	
---Large cell lung carcinoma			1	
Renal Cell Carcinoma			11 (25%)	
---clear cell			9	
---papillary			1	
---translocation RCC			1	
Head and Neck (squamous cell)			6 (14%)	
Bladder Cancer			7 (16%)	
Colorectal Cancer			3 (7%)	
Gastric Cancer (signet ring cell)			1 (2%)	
Cholangiocellular Carcinoma			1 (2%)	
History of Smoking		44 (0%)	23 (52%)	0.989
PD-L1 Expression		16 (64%)		
---positive			10 (23%)	
---negative			6 (14%)	
Treatment		44 (0%)		0.567
---Nivolumab			22 (50%)	
---Nivolumab / Ipilimumab			1 (2%)	
---Pembrolizumab			20 (46%)	
---Atezolizumab			1 (2%)	

		n (%miss.)	Summary measure	*p*-value*
Treatment line		44 (0%)		0.442
---1st line			17 (38.6%)	
---2nd line			21 (47.7%)	
---3rd line			6 (13.6%)	
Autoantibodies at baseline		44 (0%)		NA
---positive			21 (48%)	
---negative			23 (52%)	

3.1.2 Autoantibodies at baseline

At baseline n=21 patients (48%) had any elevated autoantibody titer and were considered autoantibody positive. Most of these patients (n=18 | 40.9%) had ANA titers ≥ 1:80, whereas positive ENA measurements were found in 5 (11.4%) patients. Two patients (4.5%) had positive hepatopathy antibody titers and one patient showed elevated titers of antibodies associated with myositis. There were no positive titers of antibodies associated with rheumatoid arthritis. We observed some overlaps in antibody titers as four patients had positive ANA as well as elevated ENA titers. Moreover, one patient showed both, an increase in ANA titers and antibodies associated with hepatopathy (***Table 3***).

There was no significant association of clinicopathological parameters and autoantibodies at baseline (***Table 2***). In detail, the distribution of gender, age, BMI, tumor entity, smoking status and histology did not significantly differ according to positive or negative measurements of ANA, ENA, hepatopathy- and myositis antibodies (all $p>0.05$).

Table 3: Autoantibody measurements at baseline and after 8-12 weeks of ICI treatment. (as published in (173)**)**

	Autoantibody	**1st blood draw (n=44) Median [IQR]**	**Number of Patients positive at 1st blood draw**	**2nd blood draw (n=31) Median [IQR]**	**Number of Patients positive at 2nd blood draw**
Antinuclear antibodies	ANA	0 [0-160] Maximum 5120	18	0 [0-640] Maximum 5120	14
extractable nuclear antigens antibodies	ENAscreen	0.1 [0.1-0.2]	2	0.1 [0.1-0.2]	2
	CENPB	0.2 [0.1-0.3]	1	0.3 [0.2-0.4]	1
	dsDNA	0.9 [0.5-1.5]	0	1 [0.4-1.6]	0
	anti-La	0.1 [0-0.3]	0	0.1 [0-0.3]	0
	anti-PM100	1 [0-2]	1	1 [0-2]	1
	anti-PM75	1 [1-2]	1	2 [1-2]	1
	anti-RNP70	0.1 [0-0.2]	0	0.1 [0-0.3]	0
	anti-Ro	0.2 [0.1-0.3]	1	0.3 [0.2-0.4]	1
	anti-SCL70	0.0 [0-0.8]	0	0.6 [0-0.8]	0
	anti-U1RNP	1.2 [0.7-1.6]	0	1.3 [1.0-1.8]	0
Rheumatoid arthritis associated	anti-CPA	0.8 [0.6-1.25]	0	1.0 [0.7-1.5]	0
	Rheumatoid factor IgA	0 [0-0]	0	0 [0-0]	0
Hepatopathy-associated	anti-GP210	0 [0-0]	0	0 [0-0]	0
	anti-LKM1	0 [0-0]	0	0 [0-0]	0
	anti-M2	0 [0-0]	1	0 [0-0]	1
	anti-SP100	0 [0-0]	1	0 [0-0]	1
	anti-SLA-LP	0 [0-0]	0	0 [0-0]	1
	anti-LC1	0 [0-0]	0	0 [0-0]	0
	anti-F-Actin	0 [0-0]	0	0 [0-0]	0
Myositis-associated	anti-EJ	1 [1-1]	0	1 [1-2]	0
	anti-JO1	0.1 [0-0.2]	0	0.1 [0-0.2]	0
	anti-Ku	0.5 [0-1]	0	1 [0-2]	0
	anti-MDA5	2 [1-2]	0	2 [1-2]	0
	anti-MI2a	2 [1-2]	0	2 [2-3]	0
	anti-MI2b	2 [2-3]	0	2 [2-4]	1
	anti NXP2	2 [1-2]	0	2 [1-2]	0
	anti-Oj	1 [1-2]	0	1 [1-2]	0
	anti-PL-12	1 [1-2]	1	1 [1-2]	1
	anti-PL-7	1 [1-2]	0	1 [1-2]	1
	anti-SAE	1 [1-2]	0	1 [1-3]	0
	anti-SRP	2 [1-3]	1	2 [2-4]	0
	anti-TIF-1γ	1 [1-2]	0	1 [1-2]	0

3.1.3 Association of autoantibodies at baseline with clinical endpoints

Overall, during a median follow-up time of 13.5 (IQR 2.8 – 25.1) months, best response was PR in 10 patients, while SD upon ICI treatment was observed in 10 patients. Twenty-four patients did not respond to ICI therapy and had PD at the initial response evaluation or had died of any cause before imaging could be performed. Complete remission was not achieved in any patient during the follow-up period. These data correspond with a DCR of 45.5% and an ORR of 22.7% in the overall cohort.

Disease control rate was only numerically higher in patients with positive autoantibody measurements at baseline (52.2% vs. 38.1%), yet there was no statistically significant difference (p=0.349). Likewise, there was no significant difference in ORR in patients with and without positive autoantibody titers at baseline (19.1% vs. 26.1%, p=0.578).

Next, uni- and multivariable logistic regression adjusted for tumor type was performed. In the analysis, presence of any autoantibodies at treatment initiation was no significant predictor of response as indicated by both, DCR and ORR.

Table 4: Univariable and multivariable analyses of autoantibody levels at treatment initiation predicting DCR and ORR. (as published in (173)**)**

Multivariable analysis was adjusted for tumor type. OR – odds ratio; ANA – antinuclear antibody; CI – confidence interval; DCR – disease control rate; ORR – objective response rate

	Variable	Univariable analysis		Multivariable analysis	
		OR (95% CI)	***p*-value**	**OR (95% CI)**	***p*-value**
DCR	**Any autoantibody**				
	Negative	1 (reference)		1 (reference)	
	positive	0.56 (0.17-1.88)	0.351	0.62 (0.16-2.37)	0.480
	ANA				
	<1:80 (negative)	1 (reference)		1 (reference)	
	≥1:80 (positive)	0.63 (0.19-2.16)	0.468	0.54 (0.17-2.48)	0.529
ORR	**Any autoantibody**				
	Negative	1 (reference)		1 (reference)	
	positive	0.67 (0.16-2.79)	0.579	0.79 (0.17-3.63)	0.765
	ANA				
	<1:80 (negative)	1 (reference)		1 (reference)	
	≥1:80 (positive)	0.95 (0.23-4.01)	0.947	1.11 (0.25-4.94)	0.890

Next, we evaluated the prognostic ability of preexisting autoantibodies and the association with the secondary endpoint PFS. Kaplan-Meier analysis revealed no significant difference in PFS between patients with and without any positive autoantibody titer at baseline (log-rank p=0.7151, ***Figure 2***). This was confirmed in a uni- (HR=1.131, 95%CI 0.584-2.189, p=0.715) and multivariable Cox proportional hazard analysis adjusted for tumor type (HR=0.944, 95%CI 0.457-1.950, p=0.876) (***Table 5***).

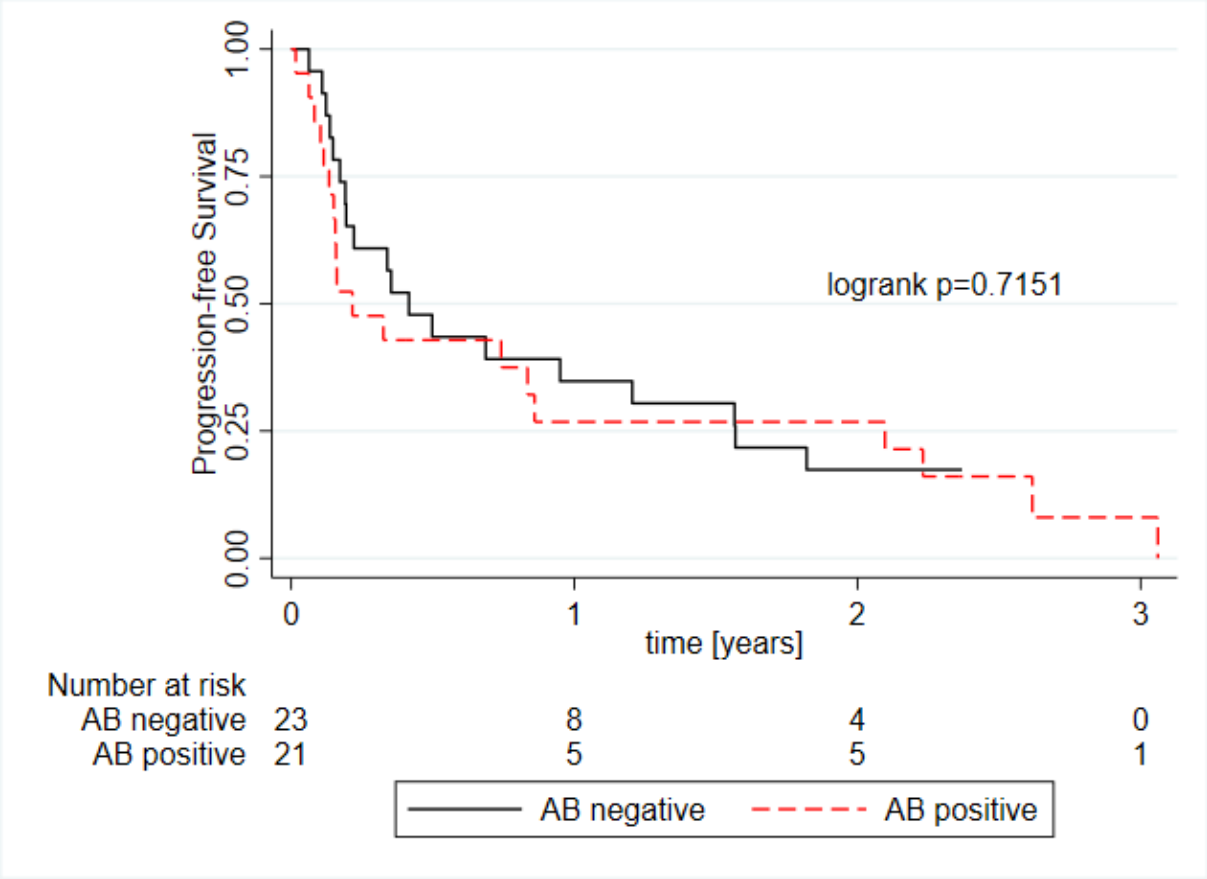

Figure 2: Progression-free survival (PFS) for positive vs. negative autoantibody screening at treatment initiation. (as published in (173)**)**

Table 5: Uni- and multivariable Cox regression regarding PFS at baseline and after 8-12 weeks of therapy. (as published in (173)**)**
Multivariable analysis was adjusted for tumor type. HR – hazard ratio; ANA – antinuclear antibody; CI – confidence interval; DCR – disease control rate; ORR – objective response rate

	Variable	Univariable analysis		Multivariable analysis	
		HR (95% CI)	***p*-value**	**HR (95% CI)**	***p*-value**
1st blood draw (baseline)	**Any autoantibody**				
	Negative	1 (reference)		1 (reference)	
	positive	1.131 (0.584-2.189)	0.715	0.944 (0.457-1.950)	0.876
	ANA				
	<1:80 (negative)	1 (reference)		1 (reference)	
	≥1:80 (positive)	0.971 (0.492-1.917)	0.933	0.801 (0.377-1.703)	0.565
2nd blood draw	**Any autoantibody**				
	Negative	1 (reference)		1 (reference)	
	positive	1.317 (0.567-3.061)	0.522	0.923 (0.343-2.482)	0.874
	ANA				
	<1:80 (negative)	1 (reference)		1 (reference)	
	≥1:80 (positive)	0.834 (0.362-1.922)	0.670	0.405 (0.145-1.138)	0.086
	ANA level change				
	No increase	1 (reference)		1 (reference)	
	increase	0.825 (0.338-2.011)	0.672	0.614 (0.245-1.540)	0.298

3.1.4 Subgroup analysis of autoantibodies at baseline

Next, subgroup analysis for different categories of autoantibodies was performed. Since only few patients had positive ENA, hepatopathy- or myositis autoantibodies, this subgroup analysis was restricted to ANA titers. ANA titers at baseline were not predictive for response to ICI therapy in uni- and multivariable logistic regression analysis adjusted for tumor type. There was neither a statistically significant association with DCR nor ORR (***Table 4***).

Likewise, there was no difference in PFS between patients with elevated ANA titers and patients without pathological increase of ANA (***Table 5*** and ***Figure 3***).

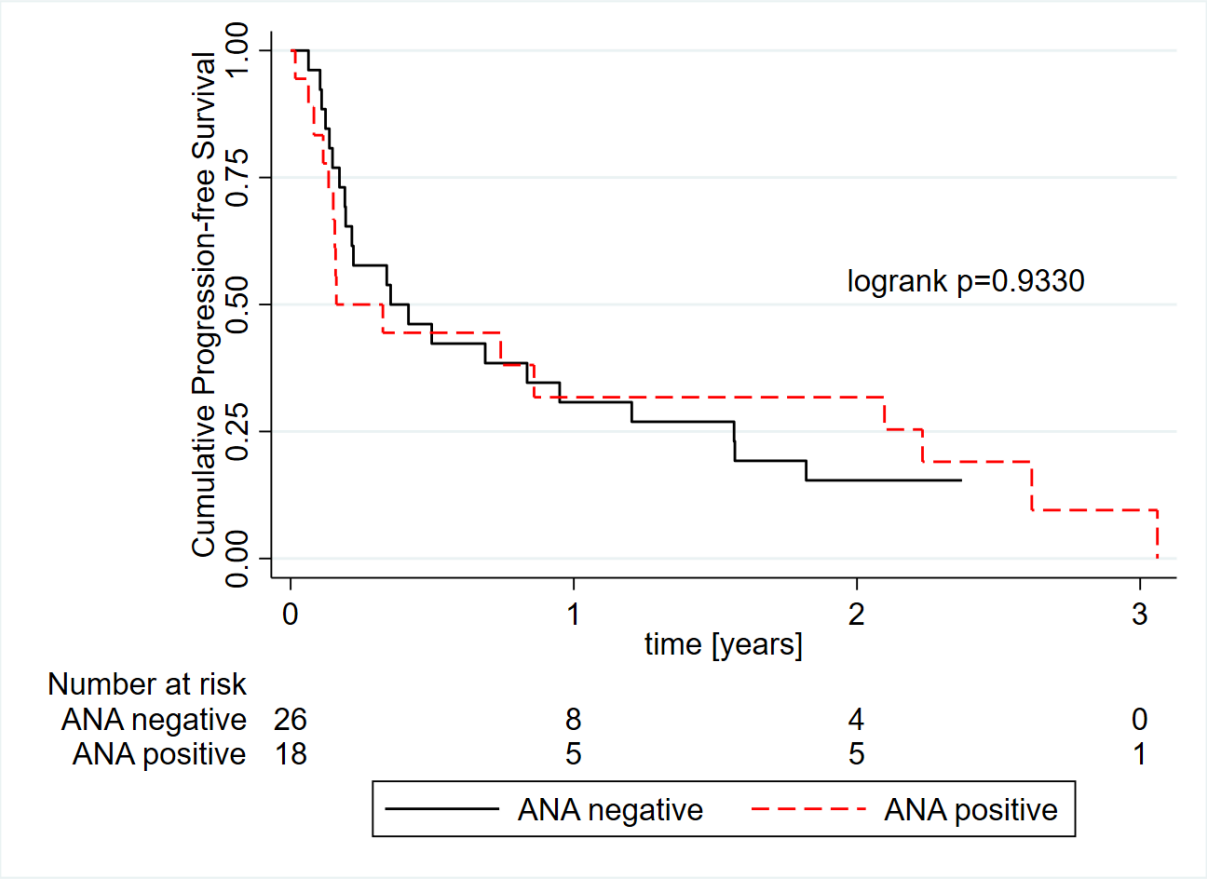

Figure 3: Progression-free survival (PFS) for positive vs. negative ANA titers at treatment initiation.

Moreover, subgroup analysis for the three most prevalent cancer types in our cohort, namely NSCLC, RCC and urothelial carcinoma of the bladder, was performed. Again, there was no significant difference in DCR in patients with vs. without any positive autoantibody titer at the timepoint of treatment initiation in NSCLC (p=0.205), RCC (p=0.898) and urothelial carcinoma (p=0.809). In addition, there was no statistically significant difference in ORR according to autoantibody titers at baseline in patients with NSCLC (p=0.792), RCC (p=0.898) and urothelial carcinoma (p=0.350).

Likewise, taking ANA titers at baseline into account only, both DCR and ORR did not significantly differ in NSCLC (p=0.189 and p=0.438), RCC (p=0.898 and p=0.898) and bladder cancer (p=0.809 and p=0.350).

3.1.5 Longitudinal development of autoantibody titers after 8-12 weeks of ICI treatment

A second blood draw after 8-12 weeks of ICI therapy was performed in 31 patients (70.5%) at the timepoint of first response evaluation. A second blood draw was not available for n=13 patients (29.5%), as they dropped out of the study due to PD and death, unfitness for further treatment, withdrawal of consent, or they had received ICI therapy within less than 8 weeks for any other reason.

After 8-12 weeks of ICI therapy, n=18 patients had at least one elevated autoantibody titer, dispersed among ANA (n=14, 45.1%), ENA (n=4, 12.9%), hepatopathy- (n=3, 9.7%) or myositis- (n=3, 9.7%) associated autoantibodies, respectively.

Sixteen patients initially had negative autoantibody titers at baseline, four of which experienced an induction of autoantibodies after 8-12 weeks of ICI treatment. As such, three patients had ANA titers ≥1:80 and one patient developed elevated hepatopathy autoantibodies by the time of the second blood draw. In contrast, one patient converted to normal ANA titers after initially showing increased ANA levels at baseline.

Focusing on the change of ANA titers, n=10 patients (32.4%) showed an increase and two patients (6.5%) had a decrease in ANA levels. Eighteen patients (58.1%) had no change in ANA titers after 8-12 weeks of ICI therapy.

3.1.1 Association of autoantibodies with clinical endpoints after 8-12 weeks of ICI treatment

There was no significant difference in DCR between patients with and without any positive autoantibody titer after 8-12 weeks of ICI treatment (55.6% vs. 76.9%, p=0.220). Likewise, uni- and multivariable analysis estimating the absolute risk difference within a generalized linear model did not reveal significant associations of autoantibody levels at the second blood draw with neither DCR nor ORR (***Table 6***). Interestingly, multivariable analysis adjusted for tumor type showed at least borderline significant results for DCR (RD=-0.332 95%CI -0.682 - 0.017, p=0.062).

Moreover, subgroup analysis considering only ANA titers at the second blood draw did not reveal any predictive ability of ANA levels as indicated by DCR and ORR. As such, there were

no significant associations of neither positive ANA levels nor the increase in ANA titers between the first and second blood draw with patients' response to ICI therapy (***Table 6***).

Table 6: Univariable and multivariable analyses of autoantibody levels at 2nd blood draw predicting DCR and ORR. (as published in (173)**)**
Multivariable analysis was adjusted for tumor type. RD – risk difference; ANA – antinuclear antibody; CI – confidence interval; DCR – disease control rate; ORR – objective response rate

	Variable	Univariable analysis		Multivariable analysis	
		RD (95% CI)	***p*-value**	**RD (95% CI)**	***p*-value**
DCR	**Any autoantibody**				
	Negative	1 (reference)		1 (reference)	
	positive	-0.214 (-0.538-0.111)	0.197	-0.332 (-0.682-0.017)	0.062
	ANA				
	<1:80 (negative)	1 (reference)		1 (reference)	
	≥1:80 (positive)	-0.004 (-0.343-0.334)	0.981	0.110 (-0.268-0.489)	0.567
	ANA titer change				
	No increase	1 (reference)		1 (reference)	
	increase	-0.05 (-0.419-0.319)	0.790	-0.147 (-527-0.233)	0.449
ORR	**Any autoantibody**				
	Negative	1 (reference)		1 (reference)	
	positive	-0.693 (-2.810-1.424)	0.521	-0.107 (-0.443-0.229)	0.533
	ANA				
	<1:80 (negative)	1 (reference)		1 (reference)	
	≥1:80 (positive)	-0.067 (-0.395-0.261)	0.688	0.078 (-0.241-0.398)	0.631
	ANA titer change				
	No increase	1 (reference)		1 (reference)	
	increase	-0.033 (-0.382-0.315)	0.851	0.042 (-0.307-0.391)	0.813

In addition, the presence of any autoantibody, positive ANA or an increase of the ANA titer were no statistically significant predictors of PFS (***Table 5***).

Due to the limited sample size at the timepoint of the second blood draw, further subgroup analysis by cancer type was restricted to NSCLC and RCC only. Subgroup analysis in patients with NSCLC revealed that patients without elevated autoantibody titers after 8-12 weeks of ICI treatment had significantly higher response rates as indicated by DCR (p=0.038), but not as indicated by ORR (p=0.490). In patients with RCC, meaningful analysis of DCR could not be performed, since all patients showed at least SD upon ICI treatment and no patient experienced

tumor progression. As for ORR, there was no significant difference between patients with or without positive autoantibody measurements 8-12 weeks after treatment initiation (p=0.850). Results were similar when the presence of positive ANA titers as well as increase vs decrease in ANA titers after 8-12 weeks of ICI therapy was evaluated. There was no significant difference in DCR and ORR in patients with NSCLC or RCC (all p>0.05).

3.1.2 Autoantibodies as predictors for immune related adverse events

Next, the association of autoantibodies with higher grade irAEs was evaluated as a secondary endpoint. Overall, five patients (11.4%) developed irAEs of grade 3 or higher during the follow-up period. Kidney and liver laboratory parameters for the overall cohort are displayed in ***Table 7***.

Table 7: Kidney and liver toxicity parameters at baseline and after 8-12 weeks of immunotherapy. (as published in (173)**)**
IQR – interquartile range; GGT – gamma glutamyl transferase; AP – alkaline phosphatase; AST – aspartate aminotransferase, ALT – alanine aminotransferase

Laboratory value	**1st blood draw** (n=44) (median [IQR])	**2nd blood draw** (n=31) (median [IQR])
Creatinine (mg/dL)	0.90 [0.75-1.17]	0.95 [0.69-1.2]
GGT (U/L)	59 [27-127]	46 [23-92]
AP (U/L)	95 [67-130]	91 [63-139]
AST (U/L)	26 [20-31]	25 [20-40]
ALT (U/L)	21 [12-36]	16 [11-28]

Of five patients who developed higher grade irAEs, two patients developed severe hepatitis, two patients were diagnosed with severe hypophysitis and one patient each developed rash, thyroiditis, and pneumonitis. Interestingly, DCR was significantly higher in patients who developed higher grade irAEs (p=0.009), while ORR was similar in patients with and without irAEs (p=877).

Positive ANA titers were present in two patients who experienced higher grade irAEs at baseline, whereas no increased levels of ENA, hepatopathy-, myositis or rheumatoid arthritis-associated autoantibodies were observed in patients who developed irAEs.
Generally, ANA positivity at baseline was not significantly associated with increased odds for the development of higher grade irAEs (OR=0.702, 95%CI 0.105-4.674, p=0.714).
There was no statistically significant difference regarding the distribution of irAEs in patients with positive ANA titers at baseline (p=0.965) and at the second blood draw (p=0.8) or in patients with increasing ANA titers 8-12 weeks after treatment start (p=0.410).

3.2 Study 2 – peripheral blood B-cell subtypes

Parts of this section were reproduced from or similarly published in Barth et al. (174).

3.2.1 Baseline characteristics

A total of 45 cancer patients who received palliative ICI treatment were recruited in this prospective single-center cohort study. One patient was excluded due to loss of follow-up: Furthermore, B-cell FACS at baseline could not be carried out in five patients for any other reason. These patients were consequently excluded from the study and a total of 39 patients was included in the final analysis.
Most patients were male (n=27, 69%), and the median age at the time of treatment initiation was 64 years. The most prevalent cancer type was NSCLC (n=14, 23%), followed by RCC (n=9, 23%), urothelial carcinoma of the bladder (n=7, 18%) and head and neck squamous cell carcinoma (n=4, 10%). One patient each (3%) had histologically verified gastric cancer or cholangiocellular carcinoma. All patients received mono- or combination ICI treatment with the most prevalent being monotherapy with either pembrolizumab (51%) or nivolumab (43%). In addition, one patient each was treated with either atezolizumab or a combination of the PD-1 inhibitor nivolumab and the CTLA4 inhibitor ipilimumab. Almost two thirds (64%) of patients had received at least one previous palliative treatment line with either chemotherapy (n=17) or targeted therapies with TKIs (n=8). Fourteen patients (36%) received ICI therapy as a palliative 1st line treatment, whereas n=20 (51%) and n=5 (13%) patients were in 2nd and 3rd line treatment. See ***Table 8*** for baseline characteristics of the study population.

Table 8: Baseline characteristics of the study population. **(as published in** (174)**)**

		n (%miss.)	**Summary measure**	
Demographic variables				
Sex		39 (0%)		
---female			12 (31%)	
---male			27 (69%)	
Age (years)		39 (0%)	64 [59-70]	
BMI (kg/m²)		39 (0%)	24.4 [21.4-27.1]	
Cancer entities		39 (0%)		
Non-small cell lung cancer			14 (36%)	
---Adenocarcinoma			8	
---Squamous cell carcinoma			5	
---Large cell lung carcinoma			1	
Renal Cell Carcinoma			9 (23%)	
---clear cell			8	
---papillary			1	
Head and Neck (squamous cell)			4 (10%)	
Bladder Cancer			7 (18%)	
Colorectal Cancer			3 (7%)	
Gastric Cancer (signet ring cell)			1 (3%)	
Cholangiocellular Carcinoma			1 (3%)	
History of Smoking		39 (0%)	19 (49%)	
Treatment		39 (0%)		
---Nivolumab			17 (43%)	
---Nivolumab / Ipilimumab			1 (3%)	
---Pembrolizumab			20 (51%)	
---Atezolizumab			1 (3%)	
Treatment line		39 (0%)		
---1st line			14 (36%)	
---2nd line			20 (51%)	
---3rd line			5 (13%)	

Absolute counts and frequencies of different B-cell subtypes were tested for associations with clinicopathological parameters. In the analysis, higher absolute counts of $CD19^+$ B-cells were observed in females (median 6826 cells/µl; IQR 1369-10,018 cells/µl) as compared to males (median 3053 cells/µl; IQR 639-5032 cells/µl) (p=0.0452). Moreover, the frequency of B-cells of all lymphocytes was significantly higher in patients with a history of smoking (median 3.25%) as compared to never-smokers (median 1.80%) (p=0.0119). Further, no other significant association with any analyzed B-cell subtype was observed.

Summary measures of the B-cell subpopulations at baseline and respective associations with clinico-pathological parameters are depicted in **Table *9***.

3.2.1 Association of peripheral blood B-cell subtypes at baseline with treatment response

Best response during the follow-up period was PR in n=10 (26%) patients, SD in n=9 (23%) and PD in n=20 (51%) patients. Complete remission upon ICI therapy was not achieved in any patients. Consequently, DCR was 48.7% and ORR was 25.6% in the study cohort.

Given the limited sample size and the hypothesis-generating character of the study, multivariable analyses were only empirically adjusted for tumor type in all further analysis. Nonetheless, sensitivity analysis for the association of clinicopathological parameters, including age, sex, tumor type, treatment line and treatment modality, with response rates was conducted using univariable logistic regression. In the analysis clinicopathological parameters were neither associated with DCR nor ORR (**Table *10***).

Table 9: Association of B-cell subpopulations with clinico-pathological parameters. (as published in (174)**)**

IQR – interquartile range, significant values are highlighted in bold. P-values were retrieved from Mann-Whitney-U-test or the Kruskal-Wallis-test as appropriate.

			***p*-values**				
		Median [IQR]	**Age (>65yrs.)**	**Gender**	**Smoker**	**Tumor type**	**Treatment line**
Lymphocytes	absolute count	145,922 [93,985 - 190,337]	0.6642	0.3745	0.8786	0.3338	0.5102
B-cells - total	absolute count	3725 [928 – 6,726]	0.4746	**0.0452**	0.0612	0.1768	0.6281
	% lymphocytes	2 [1.19 – 5.33]	0.4619	0.7348	**0.0119**	0.1249	0.5249
CD21-negative	absolute count	860 [246 – 2149]	1.0000	0.2098	0.0696	0.1477	0.7930
	% B cells	27 [21.4 – 48.4]	0.0642	0.4044	0.4234	0.5512	0.3513
unswitched memory B-cells	absolute count	241 [56 – 367]	0.8615	0.4048	0.1640	0.4200	0.8512
	% B cells	6.45 [4.31 – 9.18]	0.6043	0.1782	0.4780	0.3154	0.3909
Transitional Zone B-cells	absolute count	45 [12 – 193]	0.4876	0.9461	0.4735	0.3978	0.3483
	% B cells	1.55 [0.64 – 4.95]	0.6091	0.5732	0.8951	0.6548	0.1580
Naive B-cells	absolute count	1,818 [590 – 4,095]	0.3766	0.0606	0.2138	0.4098	0.6164
	% B cells	59.2 [48.1 – 78.3]	0.2429	0.3745	0.7919	0.5709	0.0664
Switched memory B-cells	absolute count	419 [179 – 915]	0.7421	0.0926	0.0934	0.2321	0.6357
	% B cells	16.7 [8.26 – 24.2]	0.5704	0.7584	0.8950	0.4727	0.1916
$CD24^{+}CD38^{++}$ Regulatory B-cells	absolute count	0 [0 – 0] Maximum 2	0.5198	1.0000	0.3521	0.1714	0.8889
	% B cells	0 [0 – 0] Maximum 0.33	0.4532	0.8743	0.1715	0.1329	0.9366
Plasmablasts	absolute count	78 [19 – 266]	0.9275	0.0844	0.4737	0.2484	0.7873
	% B cells	2.97 [1.48 – 5.49]	0.3615	0.2578	0.5499	0.6135	0.9364

Table 10: Univariable logistic regression analysis of clinicopathological parameters for DCR and ORR. (as published in (174)**)**
Abbreviations: DCR – disease control rate, ORR – objective response rate, OR – odds ratio, CI – confidence interval, NSCLC – non-small cell lung cancer, GU – genitourinary cancer, HNC – head and neck cancer, GI – gastrointestinal cancer

	Variable	Univariable analysis	
		OR (95% CI)	***p*-value**
DCR	**Age**	1.01 (0.95-1.07)	0.875
	Sex		
	Female	1 (reference)	
	Male	0.34 (0.08-1.43)	0.142
	Tumor entity		
	NSCLC	1 (reference)	
	GU	1.29 (0.30-5.43)	0.732
	HNC	0.33 (0.03-4.04)	0.388
	GI	0.67 (0.08-5.30)	0.702
	Treatment line	0.68 (0.26-1.79)	0.435
	Treatment modality		
	Nivolumab	1 (reference)	
	Other	0.74 (0.21-2.64)	0.643
ORR	**Age**	0.98 (0.91-1.05)	0.524
	Sex		
	Female	1 (reference)	
	Male	0.57 (0.13-2.57)	0.466
	Tumor entity		
	NSCLC	1 (reference)	
	GU	1.22 (0.22-6.73)	0.818
	HNC	1.22 (0.09-16.43)	0.880
	GI	2.44 (0.27-22.02)	0.425
	Treatment line	1.10 (0.37-3.26)	0.864
	Treatment modality		
	Nivolumab	1 (reference)	
	Other	0.71 (0.17-2.99)	0.636

Analyzing the distribution of B-cell subtypes in responders and non-responders, we found absolute counts of plasmablasts to be significantly higher in patients who had CR, PR or SD upon ICI treatment (response as indicated by DCR) (p=0.048). Besides plasmablasts, there were no further significant differences in the distribution of B-cell subtypes according to responders and non-responders (**Figure *4 A-D***).

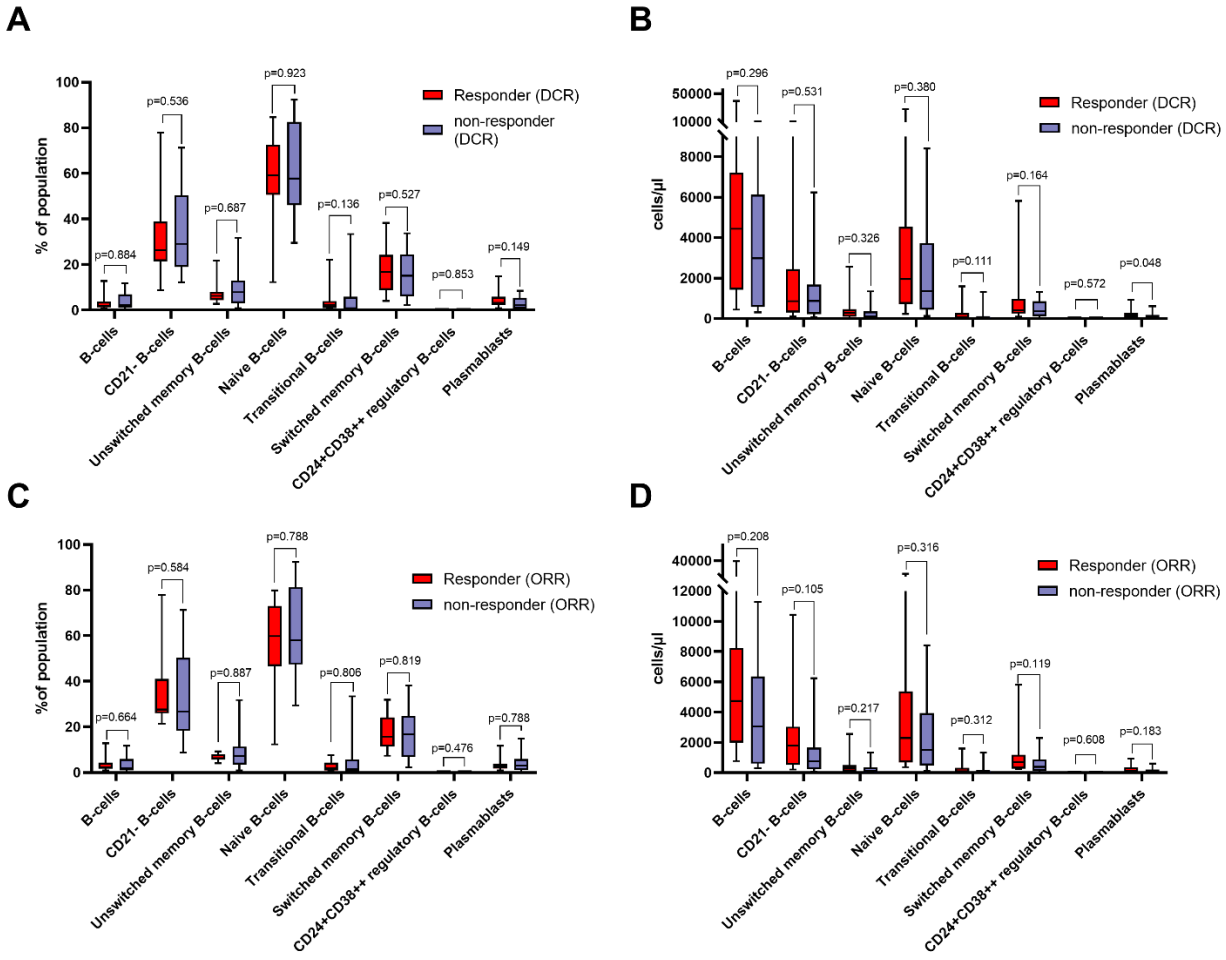

Figure 4: Distribution of B-cell subtypes in responders and non-responders (as published in (174)**).**
A) Relative distribution of different B-cell subtypes in responders and non-responders as indicated by disease control rate (DCR) at baseline. B) absolute counts of different B-cell subtypes in responders and non-responders as indicated by DCR at baseline. C) Relative distribution of different B-cell subtypes in responders and non-responders as indicated by objective response rate (ORR) at baseline. D) absolute counts of different B-cell subtypes in responders and non-responders as indicated by ORR at baseline. Values in A) and C) are percentages of total lymphocytes (for B-cells) and percentages of total B-cells (for B-cell subsets). All groups compared by Mann-Whitney-U-test.

In both uni- and multivariable logistic regression models, no significant association of total B-cells, CD21- B-cells, unswitched memory B-cells, transitional zone B-cells, naïve B-cells, switched memory B-cells, $CD24^{+}CD38^{++}$ regulatory B-cells and plasmablasts measurements with odds of response could be observed (**Table *11***).

Table 11: B-cell measurements and associations with disease control rate (DCR) and objective response rate (ORR) at baseline. (as published in (174))

*per 1000 Unit increase, **per 100 Unit increase, ***per 10 Unit increase; % lymph – percent of total lymphocytes; % B – percent of total B-cells; NA – not applicable, significant values are highlighted in bold.

		Disease Control Rate				**Objective Response Rate**			
Variable		**Univariable Analysis**		**Multivariable Analysis**		**Univariable Analysis**		**Multivariable Analysis**	
		OR (95%CI)	***p*-value**	**OR (95%CI)**	***p*-value**	**OR (95%CI)**	***p*-value**	**OR (95%CI)**	***p*-value**
Lymphocytes	count*	1.01 (0.99-1.02)	0.067	1.01 (1.00-1-02)	**0.037**	1.01 (1.00-1.02)	**0.039**	1.01 (1.00-1.02)	**0.045**
B-cells – total	count*	1.08 (0.93-1.26)	0.300	1.15 (0.92-1.43)	0.222	1.10 (0.95-1.28)	0.189	1.10 (0.95-1.29)	0.200
	% lymph	0.96 (0.79-1-17)	0.679	0.99 (0.80-1-23)	0.925	1.02 (0.80-1-31)	0.853	1.00 (0.80-1.25)	0.984
CD21- B-cells	count*	1.13 (0.81-1.59)	0.462	1.19 (0.81-1-76)	0.374	1.34 (093-1.96)	0.117	1.36 (0.92-2.02)	0.124
	% B***	0.87 (0.60-1.25)	0.441	0.86 (0.58-1.28)	0.463	1.05 (0.70-1.58)	0.803	1.05 (0.70-1.60)	0.793
Unswitched memory B-cells	count**	1.06 (0.92-1-22)	0.445	1.06 (0.91-1.23)	0.462	1.08 (0.94-1.24)	0.254	1.08 (0.94-1.24)	0.260
	% B	0.95 (0.85-1.05)	0.300	0.92 (0.83-1.03)	0.174	0.94 (0.81-1.08)	0.359	0.92 (0.78-1.08)	0.317
Transitional Zone B-cells	count**	1.05 (0.88-1.26)	0.580	1.06 (0.87-1.29)	0.539	1.07 (0.89-1.28)	0.459	1.07 (0.89-1.29)	0.446
	% B	1.00 (0.91-1.11)	0.945	0.99 (0.99-1.1)	0.916	0.94 (0.78-1.11)	0.448	0.94 (0.79-1.12)	0.456
Naive B-cells	count*	1.08 (0.91-1.28)	0.372	1.11 (0.90-1.39)	0.333	1.11 (0.94-1.30)	0.217	1.11 (0.94-1.30)	0.218
	% B***	0.92 (0.65-1.30)	0.639	0.92 (0.64-1.32)	0.662	0.89 (0.60-1.32)	0.569	0.90 (0.60-1.35)	0.625
Switched memory B-cells	count**	1.08 (0.96-1.23)	0.210	1.14 (0.96-1.35)	0.139	1.08 (0.97-1.20)	0.161	1.07 (0.97-1-19)	0.177
	% B***	1.27 (0.65-2.49)	0.489	1.21 (0.60-2.45)	0.600	1.07 (0.50-2.30)	0.849	1.03 (0.46-2.31)	0.942
$CD24^{+}CD38^{++}$ Regulatory B-cells	count	1.72 (0.42-7.13)	0.453	1.50 (0.34-6.72)	0.596	0.43 (0.05-3.41)	0.422	0.37 (0.04-3.26)	0.370
	% B	NA		NA		NA		NA	
Plasmablasts	count**	1.23 (0.87-1.73)	0.245	1.41 (0.89-2.24)	0.146	1.26 (0.90-1.76)	0.174	1.29 (0.91-1.85)	0.157
	% B	1.18 (0.94-1.48)	0.148	1.20 (0.94-1.52)	0.148	0.96 80.76-1.21)	0.731	0.96 (0.76-1.23)	0.756

3.2.2 Change of B-cell counts and frequency during ICI-treatment

A second blood draw was obtained from n=27 (69.2%) patients at the timepoint of first response evaluation, 8-12 weeks after initiation if ICI therapy. Twelve patients dropped out of the study before a second blood draw could be performed due to disease progression, death, unfitness for further treatment or patients did not have their first response evaluation or treatment for at least 8 weeks for any other reason.

Evaluation of absolute cell counts after 8-12 weeks of ICI therapy revealed a numerical increase of lymphocytes in n=11 (41%) patients and of total B-cells in n=8 (30%) patients. Furthermore, n=10 (37%) patients had an increase in $CD21^-$ B-cell counts and n=7 (26%) patients showed an increase in unswitched memory B-cells. Naïve B-cell counts increased in n=10 (37%) patients, while n=13 (48%) individuals had numerical increases in transitional zone B-cells counts and n=9 (33%) and n=15 (56%) patients had increases in switched memory B-cells and plasmablasts, respectively. Increases in $CD24^+CD38^{++}$ regulatory B-cell counts could not be observed in any patient.

A significant decrease of absolute counts of total B-cells (median change -789 cells/µl, p=0.0422), unswitched memory B-cells (median change -76 cells/µl, p=0.0237) and switched memory B-cells (median change -140 cells/µl, p=0.0463) upon 8-12 weeks of ICI treatment was found, whereas overall changes in lymphocyte counts (p=0.3997), $CD21^-$ B-cells (p=0.0815), naïve B-cells (p=0.1775), transitional zone B-cells (p=0.9859), $CD24^+CD38^{++}$ regulatory B-cells (0.6406) and plasmablasts (p=0.8919) were not statistically significant (**Figure *5B***).

Next; changes in the frequency of B-cell subtypes were evaluated. Increases of cell frequencies were observed in total B-cells (n=10, 37%), $CD21^-$ B-cells (n=12, 44%), unswitched memory B-cells (n=12, 52%), naïve B-cells (n=12, 52%), switched memory B-cells (n=12, 52%), transitional zone B-cells (n=18, 67%) and plasmablasts (n=18, 67%). Again, no changes in the frequency of CD24+CD38++ regulatory B-cells in peripheral blood could be observed in any patient.

However, overall changes in B-cell frequencies were non-significant in all analyzed cell populations (p>0.05 for all cell types, **Figure *5A***).

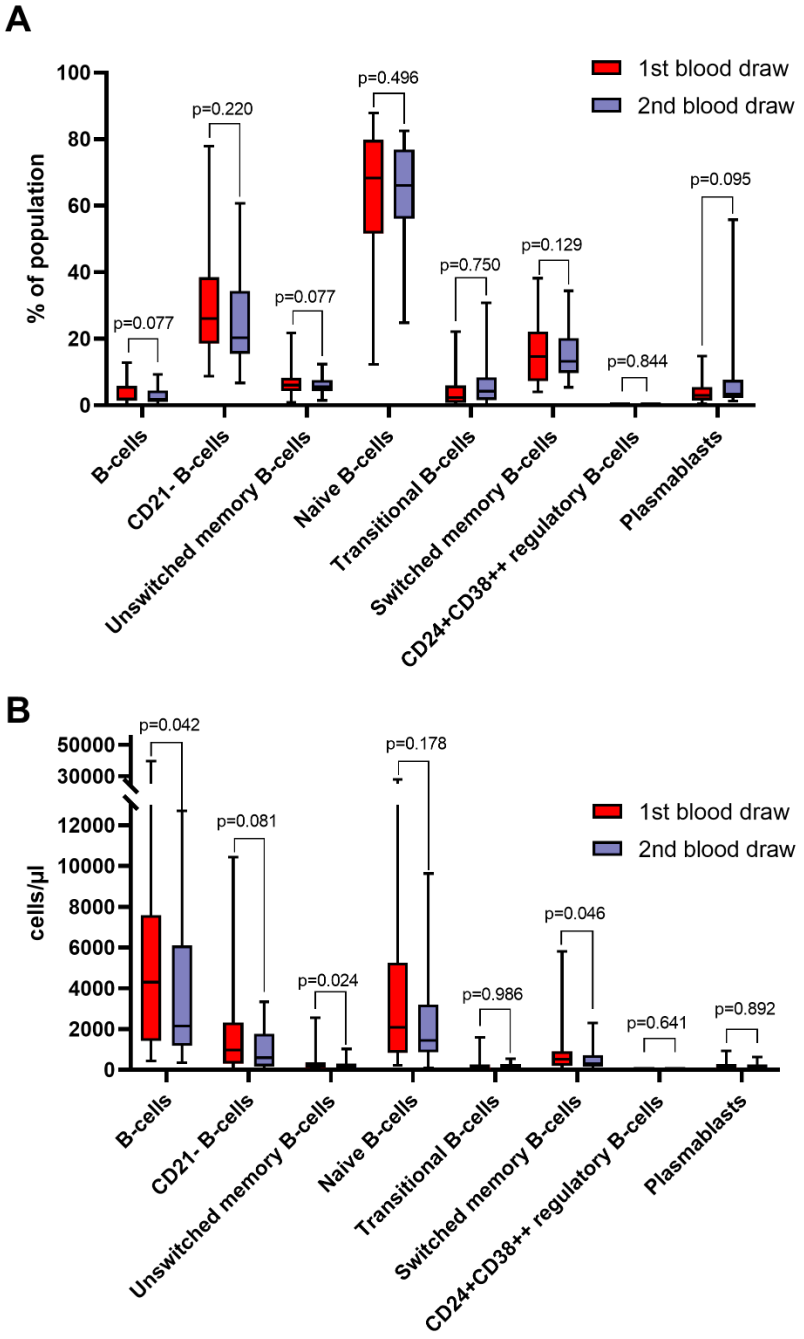

Figure 5: Changes in B-cell subtypes after 8-12 weeks of ICI treatment (as published in (174)**).**

A) Frequencies of different B-cell subtypes at baseline (1st blood draw) and after 8-12 weeks (2nd blood draw) of immune checkpoint inhibitor (ICI) treatment (n=27). Values are in percentages of total lymphocytes (for B-cells), and percentages of total B-cells (for B-cell subsets). B) Absolute counts of different B-cell subtypes at baseline (1st blood draw) and after 8-12 weeks (2nd blood draw) of ICI treatment (n=27). All groups compared by Mann-Whitney-U-test.

3.2.1 Association of changes in peripheral blood B-cell subtypes after 8-12 weeks of ICI therapy with treatment response

To evaluate the association of changes of B-cell subtypes between the 1st and 2nd blood draw with patient's response to ICI treatment, uni- and multivariable logistic regression models adjusted for tumor type were implemented and changes in absolute counts and frequencies of B-cells were analyzed 1) as dichotomous variables (increase vs decrease or no change), 2) absolute changes and 3) relative changes. Due to the hypothesis generating character of the study, p-values were not adjusted for multiple testing.

In the analysis, we neither observed associations of absolute counts nor frequencies of total B-cells, unswitched memory B-cells, transitional B-cells or plasmablasts with odds of response as indicated by both, DCR and ORR (**Table *12***, **Table 13** and **Table 14**).

However, as for the frequency of naïve B-cells an increase was associated with higher odds for DCR in both uni- (OR=7.00, 95%CI=1.10-44.61, p=0.039) and multivariable (OR=12.31, 95%CI=1.13-134.22, p=0.039) logistic regression (**Table *12***). In line with these results, patients with higher absolute changes in the frequency of naïve B-cells had significantly greater odds of response indicated by DCR per 10-unit increase: OR=1.94, 95%CI=1.05-3.59, p=0.035) and ORR (per 10-unit increase: OR=2.15, 95%CI=1.07-4.34, p=0.033) in the multivariable analysis adjusted for tumor type (**Table 13**). A significant association of relative changes in naïve B-cell counts and frequencies could not be observed (**Table 14**).

As opposed to naïve B-cells, an increase in the frequency of switched memory B-cells was linked to a decreased likelihood of experiencing response (CR, PR or SD) upon ICI therapy in the uni- (OR=0.06, 95%CI=0.01-0.62, p=0.018) and multivariable (OR=0.06, 95%CI=0.01-0.70, p=0.025) logistic regression analysis. Results for ORR were going in the same direction, while only a numerical and not statistically significant difference in the odds of response between patients with increased and decreased or non-changed switched memory B-cell frequencies was observed (**Table *12***). Across the analysis, there was no association of absolute counts as well as absolute and relative changes in frequencies of peripheral blood switched memory B-cells with response to ICI treatment (**Table *12***, **Table 13** and **Table 14**).

Table 12: Increase vs. Decrease or no change (reference) after 8-12 weeks of ICI treatment (as published in (174)**).**
lymph – percent of total lymphocytes; % B – percent of total B-cells; NA – not applicable, significant values are highlighted in bold.

		Disease Control Rate				**Objective Response Rate**			
Variable		**Univariable Analysis**		**Multivariable Analysis**		**Univariable Analysis**		**Multivariable Analysis**	
		OR (95%CI)	***p*-value**	**OR (95%CI)**	***p*-value**	**OR (95%CI)**	***p*-value**	**OR (95%CI)**	***p*-value**
Lymphocytes	count	1.60 (0.30-8.50)	0.581	1.64 (0.22-12.30)	0.628	0.95 (0.19-4.68)	0.952	1.29 (0.21-7.84)	0.785
B-cells – total	count	1.75 (0.28-11.15)	0.554	1.06 (0.14-7.85)	0.951	4.67 (0.80-27.10)	0.086	5.07 (0.75-34.20)	0.095
	% lymph	0.63 (0.12-3.22)	0.574	0.28 (0.04-2.13)	0.220	1.22 (0.25-6.11)	0.807	0.91 (0.15-5.33)	0.913
CD21- B-cells	count	0.63 (0.12-3.22)	0.574	0.23 (0.28-1.89)	0.170	1.22 (0.25-6.11)	0.807	0.88 (0.15-5.24)	0.891
	% B	0.11 (0.02-0.72)	**0.021**	0.05 (0.00-0.67)	**0.024**	0.18 (0.03-1.09)	0.061	0.09 (0.01-0.96)	**0.046**
Unswitched memory B-cells	count	1.35 (0.21-8.82)	0.757	0.74 (0.09-5.76)	0.771	3.11 (0.53-18.38)	0.210	3.04 (0.45-20.37)	0.253
	% B	0.40 (0.08-2.12)	0.282	0.30 (0.04-2.04)	0.217	0.47 (0.10-2.29)	0.348	0.39 (0.07-2.10)	0.271
Transitional Zone B-cells	count	1.25 (0.25-6.24)	0.785	1.15 (0.16-8.11)	0.886	4.28 (0.80-22.93)	0.090	3.88 (0.61-24.74)	0.151
	% B	2.08 (0.39-11.06)	0.390	2.52 (0.39-16.29)	0.330	2.80 (0.45-17.38)	0.269	3.10 (0.45-21.21)	0.248
Naive B-cells	count	2.80 (0.45-17.38)	0.269	2.86 (0.40-20.79)	0.298	4.88 (0.90-26.42)	0.066	5.63 (0.93-34.05)	0.060
	% B	7.00 (1.10-44.61)	**0.039**	12.31 (1.13-134.22)	**0.039**	3.33 (0.63-17.57)	0.156	4.41 (0.70-27.74)	0.113
Switched memory B-cells	count	1.00 (0.18-5.46)	1.000	0.54 (0.08-3.87)	0.541	1.60 (0.31-8.25)	0.574	1.20 (0.20-7.16)	0.845
	% B	0.06 (0.01-0.62)	**0.018**	0.06 (0.01-0.70)	**0.025**	0.23 (0.04-1.25)	0.090	0.18 (0.03-1.18)	0.073
$CD24^{+}CD38^{++}$ Regulatory B-cells	count	NA		NA		NA		NA	
	% B	NA		NA		NA		NA	
Plasmablasts	count	1.00 (0.20-5.00)	1.000	0.34 (0.03-4.15)	0.395	1.33 (0.27-6.50)	0.722	0.86 (0.12-6.19)	0.878
	% B	1.00 (0.18-5.46)	1.000	0.92 (0.12-7.03)	0.938	1.27 (0.24-6.82)	0.778	0.99 (0.16-6.07)	0.989

Table 13: Absolute changes of B-cells after 8-12 weeks of ICI treatment and associations with disease control rate (DCR) and objective response rate (ORR) (as published in (174)**).**

% B – percent of total B-cells, *per 1000-unit, **per 100-unit, *** per 10-unit increase; NA – not applicable, significant values are highlighted in bold.

		Disease Control Rate				**Objective Response Rate**			
Variable		**Univariable Analysis**		**Multivariable Analysis**		**Univariable Analysis**		**Multivariable Analysis**	
		OR (95%CI)	***p*-value**	**OR (95%CI)**	***p*-value**	**OR (95%CI)**	***p*-value**	**OR (95%CI)**	***p*-value**
Lymphocytes	count*	1.00 (0.99-1.01)	0.630	1.00 (0.99-1.01)	0.755	1.00 (0.99-1.01)	0.748	1.00 (0.99-1.01)	0.635
B-cells – total	count*	0.99 (0.86-1.14)	0.907	0.96 (0.78-1.19)	0.724	0.98 (0.86-1.12)	0.776	0.96 (0.84-1.11)	0.609
	% B	1.09 (0.81-1.45)	0.577	0.98 (0.69-1.40)	0.931	1.13 (0.84-1.53)	0.416	1.07 (0.77-1.49)	0.691
CD21- B-cells	count**	0.99 (0.95-1.04)	0.778	0.98 (0.92-1.04)	0.514	0.99 (0.94-1.03)	0.470	0.98 (0.94-1.02)	0.340
	% B***	0.32 (0.11-0.95)	**0.040**	0.19 (0.04-0.81)	**0.025**	0.49 (0.21-1.19)	0.116	0.34 (0.12-0.93)	**0.036**
Unswitched memory B-cells	count**	0.96 (0.78-1.20)	0.730	0.94 (0.71-1.25)	0.663	1.00 (0.83-1.21)	0.999	0.98 (0.81-1.19)	0.822
	% B	0.95 (0.75-1-20)	0.655	0.91 (0.70-1.19)	0.494	0.99 (0.79-1.23)	0.898	0.96 (0.76-1.21)	0.709
Transitional Zone B-cells	count**	1.08 (0.86-1.35)	0.525	1.05 (0.80-1.37)	0.742	1.12 (0.85-1.47)	0.427	1.11 (0.83-1.50)	0.484
	% B***	2.47 (0.58-10.48)	0.220	2.27 (0.54-9.61)	0.265	3.95 (0.76-20.53)	0.102	4.38 (0.88-21.80)	0.071
Naive B-cells	count*	1.01 (0.84-1.21)	0.931	1.00 (0.80-1.26)	0.976	0.99 (0.82-1.18)	0.879	0.97 (0.80-1.17)	0.738
	% B***	1.54 (0.85-2.79)	0.156	1.94 (1.05-3.59)	**0.035**	1.67 (0.87-3.20)	0.122	2.15 (1.07-4.34)	**0.033**
Switched memory B-cells	count**	0.97 (0.87-1.09)	0.619	0.96 (0.83-1.10)	0.528	1.00 (0.91-1.10)	0.970	0.99 (0.90-1.09)	0.886
	% B***	0.30 (0.07-1.32)	0.110	0.26 (0.05-1.38)	0.113	0.81 (0.30-2.23)	0.688	0.64 (0.21-1.93)	0.426
$CD24^{+}CD38^{++}$ Regulatory B-cells	count	NA		NA		NA		NA	
	% B	NA		NA		NA		NA	
Plasmablasts	count**	1.21 (0.81-1.82)	0.349	1.08 (0.63-1.87)	0.780	1.05 (0.70-1.56)	0.827	0.94 (0.57-1.54)	0.795
	% B	1.09 (0.88-1.34)	0.446	1.09 (0.84-1.41)	0.514	0.98 (0.88-1-08)	0.622	0.97 (0.87-1.08)	0.590

Table 14: Relative Changes per 100% increase of B-cells after 8-12 weeks of ICI treatment and associations with disease control rate (DCR) and objective response rate (ORR) (as published in (174)**).**

% lymph – percent of total lymphocytes; % B – percent of total B-cells; NA – not applicable

		Disease Control Rate				Objective Response Rate			
Variable		**Univariable Analysis**		**Multivariable Analysis**		**Univariable Analysis**		**Multivariable Analysis**	
		OR (95%CI)	***p*-value**	**OR (95%CI)**	***p*-value**	**OR (95%CI)**	***p*-value**	**OR (95%CI)**	***p*-value**
Lymphocytes	count	2.23 (0.54-9.31)	0.269	2.1 (0.43-10.34)	0.362	1.24 (0.65-2.34)	0.512	1.32 (0.68-2.59)	0.414
B-cells – total	count	1.23 (0.60-2.49)	0.274	1.12 (0.59-2.12)	0.729	1.36 (0.74-2.50)	0.351	1.42 (0.76-2.67)	0.274
	% lymph	0.74 (0.24-2.25)	0.589	0.47 (0.12-1.75)	0.257	1.20 (0.40-3.57)	0.748	1.01 (0.30-3-38)	0.992
CD21- B-cells	count	0.93 (0.49-1-76)	0.824	0.82 (0.41-1.64)	0.576	1.19 (0.63-2.24)	0.589	1.18 (0.60-2.32)	0.633
	% B	0.32 (0.07-1.51)	0.150	0.19 (0.03-1.33)	0.094	0.09 (0.01-1.25)	0.073	0.21 (0.00-0.71.9	**0.032**
Unswitched memory B-cells	count	1.13 (0.66-1.94)	0.661	1.05 (0.63-1.75)	0.854	1.24 (0.77-2.02)	0.374	1.26 (0.77-2.05)	0.335
	% B	0.63 (0.14-2.77)	0.537	0.45 (0.09-2.28)	0.332	0.31 (0.05-2.07)	0.228	0.21 (0.02-2.00)	0.175
Transitional Zone B-cells	count	1.12 (0.89-1.40)	0.326	1.10 (0.89-1.35)	0.377	1.10 (0.97-1.24)	0.154	1.11 (0.98-1.25)	0.112
	% B	1.11 (0.87-1.41)	0.393	1.11 (0.87-1.43)	0.395	1.20 (0.95-1.52)	0.132	1.20 (0.95-1.51)	0.123
Naive B-cells	count	1.38 (0.61-3.09)	0.437	1.31 (0.60-2.82)	0.498	1.41 (0.77-2.61)	0.261	1.51 (0.79-2.89)	0.209
	% B	9.25 (0.22-392.46)	0.245	15.04 (0.28-813.41)	0.183	3.47 (0.17-70.93)	0.418	7.38 (0.36-151.26)	0.195
Switched memory B-cells	count	0.96 (0.56-1.66)	0.890	0.90 (0.50-1.60)	0.710	1.27 (0.72-2.22)	0.412	1.27 (0.71-2.30)	0.421
	% B	0.28 (0.07-1.21)	0.089	0.28 (0.06-1.32)	0.108	0.29 (0.05-1.63)	0.162	0.16 (0.02-1.53)	0.112
CD24+CD38++ Regulatory B-cells	count	NA		NA		NA		NA	
	% B	NA		NA		NA		NA	
Plasmablasts	count	1.09 (0.87-1.35)	0.469	1.06 (0.86-1.29)	0.600	1.08 (0.92-1.25)	0.357	1.08 (0.92-1.27)	0.328
	% B	1.01 (0.81-1.26)	0.953	0.96 (0.76-1.21)	0.726	0.89 (0.65-1.21)	0.448	0.86 (0.59-1.23)	0.397

As for CD21$^-$ B-cells, an increase of the frequency 8-12 weeks after ICI treatment was associated with reduced odds of treatment response as indicated by DCR in the uni- (OR=0.11, 95%CI=0.02-0.72, p=0.021) and multivariable analysis (OR=0.05, 95%CI=0.00-0.67, p=0.024). Corroborating these results, multivariable adjustment for tumor type also revealed a significant negative connection with ORR (OR=0.09, 95%CI=0.01-0.96, p=0.046) (**Table *12***).

*Likewise, patients with a higher change of CD21$^-$ B-cell frequencies were less likely to responde to ICI treatment as indicated by DCR (per 10-unit increase: multivariable OR=0.19, 95%CI=0.04-0.81, p=0.025) and ORR (per 10-unit increase: multivariable OR=0.34, 95%CI=0.12-0.93, p=0.036) (**Table 13**). In addition, patients who responded to ICI therapy had significantly lower CD21$^-$ frequencies at the 2nd blood draw after weeks of ICI treatment (*
Figure *6*).

Interestingly, relative change of CD21$^-$ B-cell frequency was numerically but not statistically significantly associated with DCR, while ORR was significantly lower in patients with a high relative change of CD21$^-$ B-cell frequencies (multivariable OR=0.21, 95%CI=0.00-0.71.9, p=0.032) (**Table 14**).

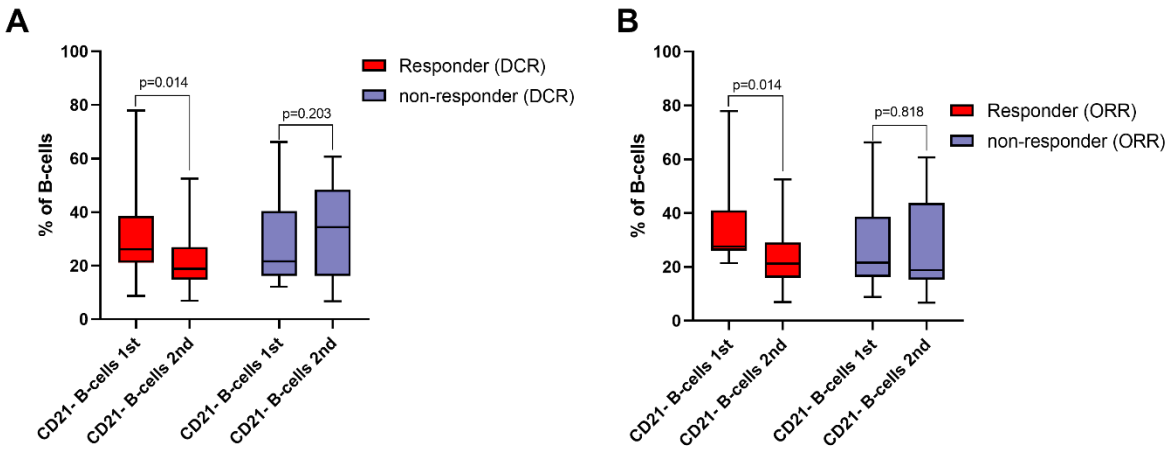

Figure 6: Distribution of CD21$^-$ B-cell frequencies in responders and non-responders to ICI therapy (as published in (174)**)**

A) Frequencies of CD21- B-cells at baseline (1st blood draw) and after 8-12 weeks (2nd blood draw) of immune checkpoint inhibitor (ICI) treatment in responders and non-responders for disease control rate (DCR). Values are in percentages of total B-cells. B) frequencies of CD21- B-cells at baseline (1st blood draw) and after 8-12 weeks (2nd blood draw) of ICI treatment in responders and non-responders for objective response rate (ORR). Values are in percentages of total B-cells. All groups compared by paired Sign-Rank-Test.

Discussion

Within this dissertation project, starting from the only poorly illuminated role of B-cells in ICI cancer treatment, a prospective longitudinal pan-cancer study was implemented in order to 1) evaluate the role of autoantibodies as predictors of treatment success and irAEs and 2) uncover associations of B-cell subtypes in the peripheral blood of cancer patients undergoing ICI therapy. Although no connections of autoantibodies with clinical endpoints could be drawn according to the results of this thesis, the abundance and change of B-cell subtypes during ICI therapy could be linked to patients' response to ICI treatment.

In this section, the findings of the studies are discussed separately at first, while final conclusions of this thesis are jointly summarized.

4.1 Study 1 – Autoantibodies

Parts of this section were reproduced from or similarly published in Barth et al. (173).

Several previous studies have aimed to elucidate the role of different autoantibodies as predictors of treatment response, patient's survival and the development of irAEs in cancer patients undergoing ICI treatment (181–186). However, evidence was inconclusive and prospective evaluations of autoantibodies in cancer immunotherapy were missing. As such, this is the first prospective longitudinal study on this matter, including a pan-cancer cohort of 44 cancer patients receiving mono- or combination ICI treatment and a wide panel of different autoantibodies including ANA ENA, hepatopathy-, rheumatoid arthritis- and myositis autoantibodies.

In the study, no significant association of the presence of autoantibodies at baseline with clinical endpoints, neither with response rates (DCR and ORR) nor PFS, could be observed. Furthermore, after 8-12 weeks of ICI therapy, autoantibodies did not predict for DCR, ORR or PFS. Subgroup analysis stratified by the three most frequent tumor types of the study cohort did not show any different results, except for NCSLC, where DCR was significantly higher in patients with NSCLC who had positive autoantibody measurements after 8-12 week of immunotherapy. Subgroup analysis for ANA did not reveal any statistically significant association with DCR, ORR or PFS, neither at baseline nor after 8-12 weeks of ICI treatment.

In addition, there was no connection between the presence of autoantibodies or ANA titers with the development of higher grade irAEs during the follow-up period.

As discussed earlier in the introduction section of this thesis (***see 1.3.3.2 Antibody production and antibody-mediated effects***) antibodies against neoantigens, tumor-associated antigens as well as anti-self-antibodies (or autoantibodies) secreted from B-cells and plasma cells involving antigen recognition, somatic hypermutation and affinity maturation, class switching and clonal expansion (140), may play an important role in the adaptive anti-cancer immune response. Interestingly, most tumor-associated antibodies are directed against wildtype cytoplasmatic or nuclear proteins but not against mutant proteins (97). Since mechanisms of antibodies and B-cells have already been illuminated in the introduction section, the discussion mainly concentrates on the comparison of clinical evidence on the presence of autoantibodies in cancer immunotherapy and the discussion of the results of the present study in this context.

Given the autoimmune nature of ICI-mediated adverse events, improved survival rates and treatment response in patients experiencing irAEs upon cancer immunotherapy (187), and the role of anti-tumor autoantibody responses and antibody-mediated direct and indirect cytotoxicity autoantibodies (97) have come into focus of clinical biomarker research for their potential as predictors of treatment response, survival as well as the development of irAEs.

Most studies investigating the impact of a variety of autoantibodies as predictors of safety and treatment efficacy of ICIs were performed in patients with NSCLC (181,183–186,188). However, the autoantibodies investigated as well as utilized cut-offs vary, making it difficult to reliably compare between studies.

Toi et al. (184) conducted a retrospective analysis of 137 individual patients with advanced or metastatic NSCLC who were treated either with pembrolizumab or nivolumab monotherapy and monitored several preexisting autoantibodies including rheumatoid factor, ANA, antithyroglobulin and antithyroid peroxidase antibodies. The authors reported that patients with any preexisting autoantibody had higher odds of response to ICI therapy and longer PFS. Response rates did not vary for the analysis of induvial autoantibodies, while patients with positive rheumatoid factor had significantly longer PFS (184). Corroborating these results, yet using a different set of autoantibodies, Giannicola at al. (181) retrospectively observed that positivity of at least one autoantibody (ANA, ENA or anti-smooth cell antigens) within the first 30 days of treatment with nivolumab was associated with higher PFS and OS in a cohort of 92 patients with metastatic NSCLC. These studies contrast with the results of the present study

where no observation between the presence of autoantibodies at baseline, nor after several weeks of ICI treatment were associated with clinical endpoints. Despite the comparably larger sample size of the studies by Toi et al. (184) and Giannicola at al. (181), the retrospective nature of the studies should be noted as a limitation. Furthermore, our study investigated autoantibodies in a pan-cancer cohort thus, evaluated autoantibodies as a site-agnostic biomarker during ICI treatment, whereas other studies focused on NSCLC only, which could further explain the controversial results. Lastly, differences in the autoantibody panel used, severely impede the comparability of the studies.

While available studies including a wide range of different autoantibodies for response and outcome prediction are limited, several studies only focused on ANA in their analysis. Antinuclear antibodies are a class of autoantibodies which are targeted against cellular components in the nucleus of a cell and, despite being not very specific, are widely associated with several autoimmune diseases including lupus erythematous, Sjögren's syndrome, and other connective tissue diseases (188).

A large retrospective study including 191 patients with solid organ malignancies independent of tumor type reported numerically but not significantly worse ICI efficacy in patients with preexisting ANA as indicated by ORR (12.5% vs. 26.5%, p=0.38) and DCR (37.5% vs 67.5%, p=0.08) (182). Since only 9 patients were defined as ANA positive (ANA ≥ 1:160) and cut-offs for ANA positivity are not fully established, analyses where also conducted for ANA ≥ 1:40 and ANA ≥ 1:80 with similar results (182). Supporting the direction of pretreatment ANA being linked to decreased treatment efficacy, Yoneshima et al. (185) observed significantly shorter PFS (2.9 vs. 3.8 months (p=0.03) and OS (11.6 vs 15.8 months, p=0.03) in NSCLC patients with ANA titers ≥ 1:160. Corroborating these results, another study in 77 consecutive NSCLC patients reported significantly shorter OS in patients with elevated ANA titers (183). However, this could not be validated in our present study where neither elevated ANA titers at baseline, nor high or increased ANA measurements after 8-12 weeks of ICI treatment were linked to treatment response. Furthermore, neither in the previously discussed study by Toi. et al (184) nor in another large-scaled study of 266 patients with NSCLC (186) a significant association of ANA levels at baseline with ICI efficacy or patient's survival could be found.

Notably, as already mentioned earlier, varying panels of analyzed autoantibodies and different cut-offs for biomarker dichotomization in the case of ANA make it difficult to compare between studies. Cut-offs for ANA in the literature ranging between ≥ 1:40 and ≥ 1:160, thus with a

chosen cutoff of ≥ 1:80 for ANA positivity, the present study lies well between previously published studies on this matter (182–186).

Apart from their potential predictive and prognostic utility, autoantibodies have been suggested as biomarkers for the development of irAEs, which is of major clinical importance considering severe and potentially life-threatening adverse effects of ICI treatment and the lack of respective established biomarkers in clinical routine (55,57).

The previously discussed study by Toi et al. (184) additionally evaluated autoantibodies in the context of irAEs and reported a more than 3-fold increase of the risk of developing irAEs of any-grade in patients with any elevated autoantibody titer at baseline and a significantly higher rate of thyroid dysfunction in patients with preexisting antithyroid antibodies. The relationship between thyroid dysfunction and related anti-thyroid autoantibodies is supported by several other studies in cancer patients undergoing ICI therapy (189–191), thus collectively representing robust evidence of the utility of antithyroglobulin and antithyroid peroxidase antibodies for risk prediction of thyroid-related irAEs. However, evidence is not as clear and quite controversial when it comes to other types of autoantibodies. For instance, ANA were reported to be increased in patients who developed colitis upon ICI therapy, yet there was no overall correlation of ANA positivity with the risk of developing any-grade irAEs (182), which is in line with the results of this thesis as well as another study conducted in NSCLC patients (186). In contrast, Morimoto et al. (183) observed a higher discontinuation rate of treatment due to severe adverse events in the ANA positive group, indicating more severe side effects of ICI treatment in this group. Autoantibodies were further suggested for the prediction of individual adverse events such as myositis (anti-acetylcholine receptor antibodies), hypophysitis (anti-GNAL, anti-ITM28) (192), pneumonitis (anti-CD74) (192), or skin reactions (anti-BP180) (193), but further studies are warranted to verify these results.

Les et al. (194) retrospectively evaluated an antibody battery of ANA, ANCA, rheumatoid factor and anti-thyroid antibodies (anti-thyroid-peroxidase and anti-thyroglobulin) in 26 cancer patients, which was tested positive more frequently in patients who developed irAEs and was able to predict the occurrence of irAEs with a diagnostic accuracy of 80%. In contrast, despite using a comparably or even more extensive panel of autoantibodies in a bigger sample size, the present prospective study found no relation between positive antibody screening and higher grade irAEs.

Finally, some limitations of the study should be discussed. First, selection bias may not be entirely excluded due to the single-center study design. Second, due to the limited sample size, subgroup analysis stratified for tumor types must be interpreted with caution. Third considering the pan-cancer study design, follow-up protocols and ICI treatment dosing schemes may vary depending on cancer type. Fourth, since PD-L1 status is not routinely assessed in all cancer types included in the study, we could not adjust our analysis for PD-L1 expression. Fifth, we did not monitor lower-grade irAEs in our study, thus conclusions regarding the impact of autoantibodies on lower- or any-grade irAEs cannot be drawn.
Lower-grade irAEs were not monitored in our study, thus no conclusion on the impact of autoantibodies on lower- and any-grade irAEs can be drawn.

4.2 Study 2 – peripheral blood B-cell subtypes

Parts of this section were reproduced or similarly published in Barth et al. (174).

As previously discussed in the introduction section of this thesis, B-cells and TIL-Bs were previously linked to the survival of cancer patients (130). This longitudinal cohort study prospectively evaluated the predictive potential and changes over time of different circulating B-cell subtypes in the blood of cancer patients undergoing ICI therapy in the form of a hypothesis-generating study. The aim of this project was to uncover novel B-cell-based biomarkers in ICI treatment and generate input from a clinical cohort and a clinical perspective for further research illuminating the roles of B-cells in tumor immunology and particularly ICI treatment. Within the study, no associations of different B-cell subtypes with response were observed at baseline, yet an increase of $CD21^-$ B-cells after 8-12 weeks of ICI mono- or combination therapy was consistently connected to reduced odds for treatment response. Likewise, we found signals for an increase of switched memory B-cells being associated with decreased likelihood of disease control and treatment response. Lastly, an increase in naïve B-cells between the 1^{st} and 2^{nd} blood draw was linked to improved odds of response.
Research in cancer immunity has primarily focused on the functions of T-cells since the discovery and further implementation of ICIs in cancer treatment (195,196), nonetheless the role of B-cells in anti-cancer immune responses has gradually gained more research interest, since fundamental functions of B-cells, such as antibody production, antigen presentation and

activation of T-cells may be key for the initiation and maintenance of effective anti-neoplastic responses of the immune system as a whole (93,197,198).

The importance of a deeper understanding of B-cell mediated immune responses, especially in the context of immune checkpoint blockade, is underlined by several reports, which highlight the relevance of B-cells in the light of ICI treatment response (86,90,138). In esophagogastric cancer it was shown that TIL-Bs were decreased in patients with PD-L1 expression indicating B-cells as a part of cancer immune evasions and potential target of ICI therapy (122).

As already broadly described in the introduction section of this thesis (***see 1.3.2 Tertiary lymphoid structures***), TIL-Bs mainly reside in TLS in the TME, where they may form specialized and demarked B-cell follicles (97). A recent study, which further investigated the role of TLS in the context of ICI treatment, emphasized the importance of TIL-Bs in TLS in terms of treatment response (199). The authors analyzed immune profiles of the TME in patients with soft tissue sarcoma from publicly available data bases such as TCGA and Gene Expression Omnibus (GEO) by analyzing characteristic gene signatures for various immune cells, vascularization and immune checkpoint molecules. Subsequentially, they defined five different immune profiles and found that patients with high abundance of T-cells, $CD8^+$ T-cells, natural killer cells and cytotoxic lymphocytes had significantly better survival outcomes if additional high expression of B-cell signatures was present. Patients who had high immune cell infiltration as well as high expression of immune checkpoint molecules did not show survival benefits in the absence of B-cell infiltration. In addition, this was also predictive for treatment response to pembrolizumab in a retrospective evaluation of the prospective phase-II study SARC028, with an ORR of 50%. Even more, CXCL13, which was considered as a marker indicating the presence of TLS, was highly expressed in patients with high T- and B-cell infiltration (199), corroborating the results of previous studies (125). Since sarcomas are generally considered immunologically ‘cold’ tumors, underlined by the fact that to date ICI treatment has not yet been established in routine treatment regimens (200), the astonishing reinstitution of immunogenicity by the mere presence of B-cells in the TME in the study by Petitprez et al. (199) , underlines the fundamental role of B-cells in the formation of anti-cancer immune responses, leading to the definition of a potential immunogenic and ICI-responsive subgroup of soft tissue sarcomas.

In another study, Wu et al. (201) defined a subset of TIL-Bs ($CD20^+CD22^+ADAM28^+$) with a memory B-cell phenotype in TLS which significantly correlated with survival outcomes in

melanoma, glioblastoma, NSCLC, RCC and head and neck squamous cell carcinoma in patients receiving ICI therapy. Moreover, the authors found that the abundance of these B-cells was predictive for ICI treatment response in NSCLC patients. Additional experiments in a murine-melanoma model with mice being depleted of $CD20^+CD22^+ADAM28^+$ B-cells and treated with ICI therapy, showed a resistance towards ICI treatment in depleted mice (201). ADAM Metallopeptidase Domain 28 (ADAM28) is mainly expressed on peripheral blood B-cells and plays a role in lymphocyte migration. Even more, it may promote the differentiation into marginal zone B-cells via the Notch2-RBP-Jκ pathway (201,202).

Corroborating the findings of Wu et al. (201), recently published data within the phase II NIVOREN GETUG-AFU 26 study suggest a significant association of unswitched memory B-cells (=marginal zone B-cells) with the survival of 44 metastatic RCC patients treated with nivolumab (203), thereby corroborating the findings of Wu et al. (201). In detail, higher baseline levels of unswitched memory B-cells ($CD19^+CD27^+IgD^+ IgM^+$) were significantly associated with longer OS (12-months OS 100% vs. 66.3%, p=0.002) and PFS (6.8 vs. 1.8 months, p=0.048) (203). In addition, unswitched memory B-cells significantly correlated with follicular T-helper cells and the abundance of TLS in the TME of tumor samples, which by themselves were associated with patient's survival outcomes (203). In line with the results of Carril-Ajuria et al. (203), high levels of IgM^+ memory B-cells at treatment start of anti-PD-(L)1 monotherapy (=unswitched memory B-cells) in the blood of advanced NSCLC patients were significantly associated with both, higher response rates and prolonged survival in a retrospective Chinese cohort study including two cohorts of 50 and 70 individuals, respectively (172). However, in a performed subgroup analysis, response prediction could only be achieved in patients undergoing PD-1 and not PD-L1 targeted therapy (172).

Unlike switched memory B-cells, unswitched memory B-cells have not undergone class switching to IgG^+, IgA^+ or IgE^+ and may enter germinal centers upon antigen rechallenge, thereby differentiating into germinal center B-cells undergoing class switching and affinity maturation (204–206). Thus, given their lower antigen affinity, unswitched memory B-cells may adapt upon antigen rechallenge and enable a faster IgM-mediated immune response (205). Furthermore, unswitched memory B-cells have a longer life span than switched memory B-cells and are therefore hypothesized to behave as a "peripheral imprint" of TLS and the TME (203). In the present study, a significant decrease of unswitched memory B-cells was reported, which may implicate that unswitched memory B-cells may be drawn to TLS in the TME, where

they may undergo class switching and eventually differentiate into mature memory B-cells or plasma cells. Although survival analysis was not performed in our study, at least in our present analysis, no signals of peripheral blood unswitched memory B-cells being connected to treatment response, neither at baseline nor after 8-12 weeks of ICI treatment, were observed. This may be explained by the smaller sample size or the heterogenous tumor types of our study cohort, yet conclusions should be drawn with caution, since the prognostic impact of a given biomarker may not automatically implicate the ability of reliable response prediction. Thus, further research and prospective studies are warranted to clarify this question.

While we did not observe associations of unswitched memory B-cells with response, we detected signals of switched memory B-cells as predictors of response after the initiation of ICI therapy. In detail, an increase of the frequency of switched memory B-cells after 8-12 weeks of ICI treatment was linked to decreased odds of treatment response.

In cancer, abundance of switched memory B-cells is higher in the TME as compared to peripheral blood of tumor patients (130,207). An interesting study by Helmink et al. (85) investigated B-cell in TLS in small individual cohorts of patients with melanoma and RCC as well as a TCGA RCC cohort. Besides demonstrating that a higher density of B-cells in TLS is linked to response to immune checkpoint blockade, intra-tumoral $CXCR3^{+}$ switched memory B-cells were increased in responders as compared to non-responders (85). In our study, we reported a decrease of the frequency of switched memory B-cells in the blood of responders and moreover found a significant decrease of absolute counts of switched memory B-cells after 8-12 weeks of ICI therapy. Thus, it may be hypothesized that switched memory B-cells could be drawn to TLSs in the TME of responders, resulting in a decrease in peripheral blood, where they might eventually differentiate into plasma cells inducing a cancer-specific immune response (85,208). Supporting this hypothesis, in the study by Helmink et al. (85) plasma cells were also enriched in the TME of responders. Furthermore, a study monitoring circulating memory B-cells of melanoma patients for IgG antibodies targeted against the tumors found an increase of antibody responses in melanoma patients as compared to healthy controls as well as a decrease in advanced disease stages. In addition, the authors were able to isolate antibodies from patient-derived B-cell cultures that effectively targeted and killed melanoma cells *in vitro* indicating an important role of B-cell mediated anti-tumor response through antibody production (209). Yet further research with larger sample sizes is still needed.

Besides memory B-cells, Helmink et al. (85) also reported that naïve B-cells were enriched in the blood and TME of patients who did not respond to ICI therapy. This is in contrast to observations of another study, which linked higher levels of naïve B-cells in the TME before treatment start with ICI treatment response (161). In line with these results, the present study found an increase of naïve B-cells after 8-12 weeks of ICI therapy to be significantly associated with higher rates of treatment response. However, in a murine model of malignant pleural effusion, activated naïve PD-L1^{+} B-cells were shown to inhibit the expansion of effector T-cells, such as Th-17 cells, via the PD-1/PL-L1 pathway, thereby promoting tumor growth (210). Collectively, the role of naïve B-cells in tumor immunity and especially in the context of checkpoint molecule blockade remains unclear and may vary by tumor site or even by targeted checkpoint molecules.

Lastly, the present study is the first to report a consistent connection between a CD21^{-} B-cell population and efficacy of ICI therapy.

Previously, CD21^{-}/low B-cells were linked to autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus or Sjogren's syndrome (211), where they were generally considered to represent an anergic B-cell population in the context of B-cell exhaustion (211–215). In cancer, in a study by Bruno et al. (86), the presence of so-called exhausted TIL-Bs (CD69^{+}HLA^{-}DR^{+}CD27^{-}CD21^{-}) correlated with a regulatory phenotype of CD4^{+} TIL-Ts in NSCLC. T_{regs} are frequently associated with cancer immune evasion and reduced ICI treatment efficacy (216,217). On the one hand, Bruno et al. (86) suggest that TIL-Bs may promote immunological antitumor response by antigen presentation to CD4^{+} lymphocytes in the TME. On the other hand, TIL-Bs could suppress antitumor immune response by expression of an exhausted B-cell phenotype (CD19^{+}CD20^{+}CD69^{+}CD27^{-}CD21^{-}) thereby exerting an immune-regulatory effect through their association with CD4^{+} TIL-Ts. The authors hypothesize that the purpose of these exhausted TIL-Bs might be the prevention of host damage in the light of chronic inflammation and suggest exhausted TIL-Bs as potential therapeutic targets in order to complement ICI therapy and potentially increase response rates in NSCLC (86). Although generally tumor tissues were tending towards enrichment with activated B-cells, patients with an exhausted TIL-B phenotype either faced recurrent disease or cancer-related death in their study (86). Notably, considering the comparably small sample size of 10 patients, these results should be interpreted with caution. However, these data are consistent with the results of this present study, where longitudinal measurements of B-cell subpopulations during ICI treatment

revealed a consistent association of an increase of peripheral blood $CD21^-$ cells with reduced ICI efficacy. Considering the results of Bruno et al. (86), an increase of $CD21^-$ B-cells during ICI therapy may resemble B-cell exhaustion upon ICI treatment and could thus be linked to treatment failure. Although no signals of $CD21^-$ B-cells being linked to ICI efficacy were present at baseline, interestingly, $CD21^-$ B-cells were significantly decreased after 8-12 weeks of ICI treatment in patients who showed response to ICI therapy, thereby supporting this hypothesis.

Interestingly, in patients with germline mutations in CTLA4 who developed severe immune dysregulation a progressive loss of circulating B-cells as well as an increase in $CD21^{lo}$ B-cells was observed (218). As a matter of fact, circulating $CD21^{lo}$ B-cells were also previously linked to the occurrence of irAEs. Das et al. (219) analyzed circulating B-cell subtypes at baseline and after the first administration of ICI therapy in melanoma patients and found individuals with a decrease in $CD21^{lo}$ B-cells at an increased risk of developing higher grade irAEs. In the analysis, no evaluation of treatment efficacy or survival outcomes considering $CD21^{lo}$ B-cells was performed. However, since the development irAEs upon ICI treatment is generally a marker for enhanced treatment response (220,221), the results may implicate and thus support our findings of a decrease in $CD21^-$ B-cells being a novel potential biomarker for ICI treatment efficacy. As such, the higher rate of irAEs in these patients may accompany the increase in activated B-cells with the initiation of an effective B-cell mediated anti-cancer immune response and the concomitant decline of an exhausted and anergic $CD21^-$ B-cell population upon blockade of immune checkpoint molecules. Yet further studies are warranted to verify this hypothesis.

Circulating B-cells at baseline were higher in responders as compared to non-responders in patients undergoing ICI therapy as reported in two recent studies in NSCLC patients undergoing treatment with the PD-1 inhibitor pembrolizumab (172) as well as in a pan-cancer cohort of 75 cancer patients (171). In this present study, these findings could not be validated, however, the retrospective nature of both mentioned studies should be noted as a noteworthy limitation. At least the latter study did not implement logistic regression models and did not further account for different B-cell subtypes in the analysis (171).

Some limitations of this study should be noted. First, due to the limited sample size especially concerning the second blood draw the study may be underpowered to detect small differences in the distribution of B-cell subtypes and their association with treatment response. Yet,

significant signals of three different subpopulations, namely naïve, switched and CD^- B-cells could be observed. Second, due to the single center design, selection bias cannot be entirely excluded. Third, considering the pan-cancer study design, follow-up protocols and ICI treatment dosing schemes may vary depending on cancer entity and cancer-specific changes in B-cell populations may not be considered. Fourth, a large proportion of the patients included in the study had undergone chemotherapy before inclusion in the study. Chemotherapy was previously shown to directly impact and alter B-cell frequencies during active treatment (222). Although long-term effects after termination of chemotherapy treatment on B-cells are unknown, this fact should at least be considered when interpreting the results. Fifth, PD-L1 expression was not available for most patients, since routine assessment is not performed in all cancer types. Sixth, single plasmablasts might have been lost due to conservative CD19 gating.

4.3 Conclusions

The prospective nature as well as longitudinal measurements of autoantibodies and B-cells should be noted as important strengths of this study.

Based on the results of this dissertation thesis, the presence of autoantibodies determined by an extensive panel of autoantibody measurements at treatment initiation and after 8-12 weeks of ICI therapy is neither associated with treatment efficacy of ICIs as indicated by DCR, ORR and PFS nor linked to an increased risk for higher-grade irAEs.

Considering B-cell measurements and longitudinal changes in circulating B-cell subtypes upon ICI therapy, changes in naïve, switched and $CD21^-$ B-cells may represent novel predictors of ICI treatment efficacy. Furthermore, this is the first study to report a link between an anergic and exhausted $CD21^-$ B-cell population with response to ICI therapy, yet further larger-scaled as well as functional studies are warranted to verify these results.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021;71(3):209–49.

2. Dagenais GR, Leong DP, Rangarajan S, Lanas F, Lopez-Jaramillo P, Gupta R, et al. Variations in common diseases, hospital admissions, and deaths in middle-aged adults in 21 countries from five continents (PURE): a prospective cohort study. The Lancet. 2020 Mar 7;395(10226):785–94.

3. Song M. Cancer overtakes vascular disease as leading cause of excess death associated with diabetes. Lancet Diabetes Endocrinol. 2021 Mar 1;9(3):131–3.

4. Krebserkrankungen [Internet]. STATISTIK AUSTRIA. [cited 2023 Feb 26]. Available from: https://www.statistik.at/statistiken/bevoelkerung-und-soziales/gesundheit/krebserkrankungen

5. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. CA Cancer J Clin. 2023 Jan;73(1):17–48.

6. Zhou F, Qiao M, Zhou C. The cutting-edge progress of immune-checkpoint blockade in lung cancer. Cell Mol Immunol. 2021 Feb;18(2):279–93.

7. Sabbatino F, Liguori L, Pepe S, Ferrone S. Immune checkpoint inhibitors for the treatment of melanoma. Expert Opin Biol Ther. 2022 May;22(5):563–76.

8. Parikh M, Bajwa P. Immune Checkpoint Inhibitors in the Treatment of Renal Cell Carcinoma. Semin Nephrol. 2020 Jan;40(1):76–85.

9. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science. 1996 Mar 22;271(5256):1734–6.

10. Kwon ED, Hurwitz AA, Foster BA, Madias C, Feldhaus AL, Greenberg NM, et al. Manipulation of T cell costimulatory and inhibitory signals for immunotherapy of prostate cancer. Proc Natl Acad Sci U S A. 1997 Jul 22;94(15):8099–103.

11. Hodi FS, Mihm MC, Soiffer RJ, Haluska FG, Butler M, Seiden MV, et al. Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. Proc Natl Acad Sci U S A. 2003 Apr 15;100(8):4712–7.

12. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J. 1992 Nov;11(11):3887–95.

13. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity. 1999 Aug;11(2):141–51.

14. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med. 2000 Oct 2;192(7):1027–34.

15. Iwai Y, Terawaki S, Honjo T. PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. Int Immunol. 2005 Feb;17(2):133–44.

16. ESMO. The Nobel Prize in Medicine 2018 Awarded for Discovery of CTLA-4 and PD-1 [Internet]. [cited 2023 Feb 26]. Available from: https://www.esmo.org/oncology-news/archive/the-nobel-prize-in-medicine-2018-awarded-for-discovery-of-ctla-4-and-pd-1

17. Powles T, Tomczak P, Park SH, Venugopal B, Ferguson T, Symeonides SN, et al. Pembrolizumab versus placebo as post-nephrectomy adjuvant therapy for clear cell renal cell carcinoma (KEYNOTE-564): 30-month follow-up analysis of a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol. 2022 Sep;23(9):1133–44.

18. Eggermont AMM, Blank CU, Mandalà M, Long GV, Atkinson VG, Dalle S, et al. Adjuvant pembrolizumab versus placebo in resected stage III melanoma (EORTC 1325-MG/KEYNOTE-054): distant metastasis-free survival results from a double-blind, randomised, controlled, phase 3 trial. Lancet Oncol. 2021 May;22(5):643–54.

19. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med. 2013;369(2):122–33.

20. Zhang Y, Zheng J. Functions of Immune Checkpoint Molecules Beyond Immune Evasion. Adv Exp Med Biol. 2020;1248:201–26.

21. Carlino MS, Larkin J, Long GV. Immune checkpoint inhibitors in melanoma. The Lancet. 2021 Sep 11;398(10304):1002–14.

22. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–74.

23. Bagchi S, Yuan R, Engleman EG. Immune Checkpoint Inhibitors for the Treatment of Cancer: Clinical Impact and Mechanisms of Response and Resistance. Annu Rev Pathol Mech Dis. 2021;16(1):223–49.

24. Shiravand Y, Khodadadi F, Kashani SMA, Hosseini-Fard SR, Hosseini S, Sadeghirad H, et al. Immune Checkpoint Inhibitors in Cancer Therapy. Curr Oncol Tor Ont. 2022 Apr 24;29(5):3044–60.

25. Valk E, Leung R, Kang H, Kaneko K, Rudd CE, Schneider H. T cell receptor-interacting molecule acts as a chaperone to modulate surface expression of the CTLA-4 coreceptor. Immunity. 2006 Nov;25(5):807–21.

26. Pentcheva-Hoang T, Egen JG, Wojnoonski K, Allison JP. B7-1 and B7-2 selectively recruit CTLA-4 and CD28 to the immunological synapse. Immunity. 2004 Sep;21(3):401–13.

27. Olsson C, Riesbeck K, Dohlsten M, Michaëlsson E. CTLA-4 ligation suppresses CD28-induced NF-kappaB and AP-1 activity in mouse T cell blasts. J Biol Chem. 1999 May 14;274(20):14400–5.

28. Fraser JH, Rincón M, McCoy KD, Le Gros G. CTLA4 ligation attenuates AP-1, NFAT and NF-kappaB activity in activated T cells. Eur J Immunol. 1999 Mar;29(3):838–44.

29. Sasidharan Nair V, Elkord E. Immune checkpoint inhibitors in cancer therapy: a focus on T-regulatory cells. Immunol Cell Biol. 2018;96(1):21–33.

30. Selby MJ, Engelhardt JJ, Quigley M, Henning KA, Chen T, Srinivasan M, et al. Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells. Cancer Immunol Res. 2013 Jul;1(1):32–42.

31. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. J Exp Med. 2013 Aug 26;210(9):1695–710.

32. Cha JH, Chan LC, Li CW, Hsu JL, Hung MC. Mechanisms controlling PD-L1 expression in cancer. Mol Cell. 2019 Nov 7;76(3):359–70.

33. Han Y, Liu D, Li L. PD-1/PD-L1 pathway: current researches in cancer. Am J Cancer Res. 2020;10(3):727–42.

34. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved Survival with Ipilimumab in Patients with Metastatic Melanoma. N Engl J Med. 2010 Aug 19;363(8):711–23.

35. Robert C, Ribas A, Wolchok JD, Hodi FS, Hamid O, Kefford R, et al. Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. Lancet Lond Engl. 2014 Sep 20;384(9948):1109–17.

36. Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus Ipilimumab in Advanced Melanoma. N Engl J Med. 2015 Jun 25;372(26):2521–32.

37. Hodi FS, Chiarion-Sileni V, Gonzalez R, Grob JJ, Rutkowski P, Cowey CL, et al. Nivolumab plus ipilimumab or nivolumab alone versus ipilimumab alone in advanced melanoma (CheckMate 067): 4-year outcomes of a multicentre, randomised, phase 3 trial. Lancet Oncol. 2018 Nov;19(11):1480–92.

38. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. N Engl J Med. 2015 Jul 2;373(1):23–34.

39. Motzer RJ, Tannir NM, McDermott DF, Aren Frontera O, Melichar B, Choueiri TK, et al. Nivolumab plus Ipilimumab versus Sunitinib in Advanced Renal-Cell Carcinoma. N Engl J Med. 2018;378(14):1277–90.

40. Motzer RJ, Powles T, Burotto M, Escudier B, Bourlon MT, Shah AY, et al. Nivolumab plus cabozantinib versus sunitinib in first-line treatment for advanced renal cell carcinoma (CheckMate 9ER): long-term follow-up results from an open-label, randomised, phase 3 trial. Lancet Oncol. 2022 Jul;23(7):888–98.

41. Motzer RJ, Penkov K, Haanen J, Rini B, Albiges L, Campbell MT, et al. Avelumab plus Axitinib versus Sunitinib for Advanced Renal-Cell Carcinoma. N Engl J Med. 2019 Mar 21;380(12):1103–15.

42. Rini BI, Plimack ER, Stus V, Gafanov R, Hawkins R, Nosov D, et al. Pembrolizumab plus Axitinib versus Sunitinib for Advanced Renal-Cell Carcinoma. N Engl J Med. 2019;380(12):1116–27.

43. Motzer RJ, Porta C, Eto M, Powles T, Grünwald V, Hutson TE, et al. Phase 3 trial of lenvatinib (LEN) plus pembrolizumab (PEMBRO) or everolimus (EVE) versus sunitinib (SUN) monotherapy as a first-line treatment for patients (pts) with advanced renal cell carcinoma (RCC) (CLEAR study). J Clin Oncol. 2021 Feb 20;39(6_suppl):269–269.

44. Riveros C, Ranganathan S, Xu J, Chang C, Kaushik D, Morgan M, et al. Comparative real-world survival outcomes of metastatic papillary and clear cell renal cell carcinoma treated with immunotherapy, targeted therapy, and combination therapy. Urol Oncol. 2023 Jan 5;S1078-1439(22)00479-3.

45. Waterhouse D, Lam J, Betts KA, Yin L, Gao S, Yuan Y, et al. Real-world outcomes of immunotherapy-based regimens in first-line advanced non-small cell lung cancer. Lung Cancer Amst Neth. 2021 Jun;156:41–9.

46. Högner A, Moehler M. Immunotherapy in Gastric Cancer. Curr Oncol Tor Ont. 2022 Mar 2;29(3):1559–74.

47. Chen K, Wang X, Yang L, Chen Z. The Anti-PD-1/PD-L1 Immunotherapy for Gastric Esophageal Cancer: A Systematic Review and Meta-Analysis and Literature Review. Cancer Control J Moffitt Cancer Cent. 2021;28:1073274821997430.

48. Shalhout SZ, Emerick KS, Kaufman HL, Miller DM. Immunotherapy for Non-melanoma Skin Cancer. Curr Oncol Rep. 2021 Aug 27;23(11):125.

49. Heeke AL, Tan AR. Checkpoint inhibitor therapy for metastatic triple-negative breast cancer. Cancer Metastasis Rev. 2021 Jun;40(2):537–47.

50. Horn L, Mansfield AS, Szczęsna A, Havel L, Krzakowski M, Hochmair MJ, et al. First-Line Atezolizumab plus Chemotherapy in Extensive-Stage Small-Cell Lung Cancer. N Engl J Med. 2018 Dec 6;379(23):2220–9.

51. Felip E, Altorki N, Zhou C, Csőszi T, Vynnychenko I, Goloborodko O, et al. Adjuvant atezolizumab after adjuvant chemotherapy in resected stage IB-IIIA non-small-cell lung cancer (IMpower010): a randomised, multicentre, open-label, phase 3 trial. Lancet Lond Engl. 2021 Oct 9;398(10308):1344–57.

52. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity. 1995 Nov;3(5):541–7.

53. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science. 1995 Nov 10;270(5238):985–8.

54. Xu C, Chen YP, Du XJ, Liu JQ, Huang CL, Chen L, et al. Comparative safety of immune checkpoint inhibitors in cancer: systematic review and network meta-analysis. The BMJ. 2018 Nov 8;363:k4226.

55. Ramos-Casals M, Brahmer JR, Callahan MK, Flores-Chávez A, Keegan N, Khamashta MA, et al. Immune-related adverse events of checkpoint inhibitors. Nat Rev Dis Primer. 2020;6(1):38.

56. Schneider BJ, Naidoo J, Santomasso BD, Lacchetti C, Adkins S, Anadkat M, et al. Management of Immune-Related Adverse Events in Patients Treated With Immune Checkpoint Inhibitor Therapy: ASCO Guideline Update. J Clin Oncol. 2021 Dec 20;39(36):4073–126.

57. Taylor J, Gandhi A, Gray E, Zaenker P. Checkpoint inhibitor immune-related adverse events: A focused review on autoantibodies and B cells as biomarkers, advancements and future possibilities. Front Immunol. 2022;13:991433.

58. Morad G, Helmink BA, Sharma P, Wargo JA. Hallmarks of response, resistance, and toxicity to immune checkpoint blockade. Cell. 2021 Oct 14;184(21):5309–37.

59. Pichler M, Steyrer J. Cost-effectiveness analysis of the use of immunotherapy in metastatic solid tumours in Austria by applying the ESMO-Magnitude of Clinical Benefit Scale (ESMO-MCBS) version 1.1. ESMO Open. 2021 Aug;6(4):100198.

60. Herbst RS, Soria JC, Kowanetz M, Fine GD, Hamid O, Gordon MS, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature. 2014 Nov 27;515(7528):563–7.

61. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med. 2012 Jun 28;366(26):2443–54.

62. Kulangara K, Zhang N, Corigliano E, Guerrero L, Waldroup S, Jaiswal D, et al. Clinical Utility of the Combined Positive Score for Programmed Death Ligand-1 Expression and the Approval of Pembrolizumab for Treatment of Gastric Cancer. Arch Pathol Lab Med. 2019 Mar;143(3):330–7.

63. Doroshow DB, Bhalla S, Beasley MB, Sholl LM, Kerr KM, Gnjatic S, et al. PD-L1 as a biomarker of response to immune-checkpoint inhibitors. Nat Rev Clin Oncol. 2021 Jun;18(6):345–62.

64. Janjigian YY, Shitara K, Moehler M, Garrido M, Salman P, Shen L, et al. First-line nivolumab plus chemotherapy versus chemotherapy alone for advanced gastric, gastro-oesophageal junction, and oesophageal adenocarcinoma (CheckMate 649): a randomised, open-label, phase 3 trial. Lancet Lond Engl. 2021 Jul 3;398(10294):27–40.

65. Sun JM, Shen L, Shah MA, Enzinger P, Adenis A, Doi T, et al. Pembrolizumab plus chemotherapy versus chemotherapy alone for first-line treatment of advanced oesophageal cancer (KEYNOTE-590): a randomised, placebo-controlled, phase 3 study. Lancet Lond Engl. 2021 Aug 28;398(10302):759–71.

66. Hendriks LE, Kerr KM, Menis J, Mok TS, Nestle U, Passaro A, et al. Non-oncogene-addicted metastatic non-small-cell lung cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. Ann Oncol Off J Eur Soc Med Oncol. 2023 Jan 17;S0923-7534(22)04785-8.

67. Powles T. Recent eUpdate to the ESMO Clinical Practice Guidelines on renal cell carcinoma on cabozantinib and nivolumab for first-line clear cell renal cancer: Renal cell carcinoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up1. Ann Oncol. 2021 Mar 1;32(3):422–3.

68. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med. 2014 Dec 4;371(23):2189–99.

69. Jardim DL, Goodman A, de Melo Gagliato D, Kurzrock R. The Challenges of Tumor Mutational Burden as an Immunotherapy Biomarker. Cancer Cell. 2021 Feb 8;39(2):154–73.

70. Yarchoan M, Hopkins A, Jaffee EM. Tumor Mutational Burden and Response Rate to PD-1 Inhibition. N Engl J Med. 2017 Dec 21;377(25):2500–1.

71. Ganesh K, Stadler ZK, Cercek A, Mendelsohn RB, Shia J, Segal NH, et al. Immunotherapy in colorectal cancer: rationale, challenges and potential. Nat Rev Gastroenterol Hepatol. 2019 Jun;16(6):361–75.

72. Overman MJ, McDermott R, Leach JL, Lonardi S, Lenz HJ, Morse MA, et al. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study. Lancet Oncol. 2017 Sep;18(9):1182–91.

73. Research C for DE and. FDA grants accelerated approval to pembrolizumab for first tissue/site agnostic indication. FDA [Internet]. 2019 Sep 2 [cited 2023 Mar 2]; Available from: https://www.fda.gov/drugs/resources-information-approved-drugs/fda-grants-accelerated-approval-pembrolizumab-first-tissuesite-agnostic-indication

74. Wouters MCA, Nelson BH. Prognostic Significance of Tumor-Infiltrating B Cells and Plasma Cells in Human Cancer. Clin Cancer Res. 2018;24(24):6125.

75. Kuroda H, Jamiyan T, Yamaguchi R, Kakumoto A, Abe A, Harada O, et al. Prognostic value of tumor-infiltrating B lymphocytes and plasma cells in triple-negative breast cancer. Breast Cancer Tokyo Jpn. 2021 Jul;28(4):904–14.

76. Yeong J, Lim JCT, Lee B, Li H, Chia N, Ong CCH, et al. High Densities of Tumor-Associated Plasma Cells Predict Improved Prognosis in Triple Negative Breast Cancer. Front Immunol. 2018;9:1209.

77. Garaud S, Buisseret L, Solinas C, Gu-Trantien C, de Wind A, Van den Eynden G, et al. Tumor infiltrating B-cells signal functional humoral immune responses in breast cancer. JCI Insight. 2019 Aug 13;5(18):e129641, 129641.

78. Qin Y, Peng F, Ai L, Mu S, Li Y, Yang C, et al. Tumor-infiltrating B cells as a favorable prognostic biomarker in breast cancer: a systematic review and meta-analysis. Cancer Cell Int. 2021 Jun 12;21(1):310.

79. Yang Z, Yin L, Zeng Y, Li Y, Chen H, Yin S, et al. Diagnostic and prognostic value of tumor-infiltrating B cells in lymph node metastases of papillary thyroid carcinoma. Virchows Arch Int J Pathol. 2021 Nov;479(5):947–59.

80. Brase JC, Walter RFH, Savchenko A, Gusenleitner D, Garrett J, Schimming T, et al. Role of Tumor-Infiltrating B Cells in Clinical Outcome of Patients with Melanoma Treated With Dabrafenib Plus Trametinib. Clin Cancer Res Off J Am Assoc Cancer Res. 2021 Aug 15;27(16):4500–10.

81. Martinez-Rodriguez M, Thompson AK, Monteagudo C. A significant percentage of CD20-positive TILs correlates with poor prognosis in patients with primary cutaneous malignant melanoma. Histopathology. 2014 Nov;65(5):726–8.

82. Rodgers CB, Mustard CJ, McLean RT, Hutchison S, Pritchard AL. A B-cell or a key player? The different roles of B-cells and antibodies in melanoma. Pigment Cell Melanoma Res. 2022 May;35(3):303–19.

83. Garg K, Maurer M, Griss J, Brüggen MC, Wolf IH, Wagner C, et al. Tumor-associated B cells in cutaneous primary melanoma and improved clinical outcome. Hum Pathol. 2016 Aug;54:157–64.

84. Erdag G, Schaefer JT, Smolkin ME, Deacon DH, Shea SM, Dengel LT, et al. Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. Cancer Res. 2012 Mar 1;72(5):1070–80.

85. Helmink BA, Reddy SM, Gao J, Zhang S, Basar R, Thakur R, et al. B cells and tertiary lymphoid structures promote immunotherapy response. Nature. 2020;577(7791):549–55.

86. Bruno TC, Ebner PJ, Moore BL, Squalls OG, Waugh KA, Eruslanov EB, et al. Antigen-Presenting Intratumoral B Cells Affect CD4+ TIL Phenotypes in Non-Small Cell Lung Cancer Patients. Cancer Immunol Res. 2017 Oct;5(10):898–907.

87. Liang J, Bi G, Shan G, Jin X, Bian Y, Wang Q. Tumor-Associated Regulatory T Cells in Non-Small-Cell Lung Cancer: Current Advances and Future Perspectives. J Immunol Res. 2022;2022:4355386.

88. Schneider T, Kimpfler S, Warth A, Schnabel PA, Dienemann H, Schadendorf D, et al. Foxp3(+) regulatory T cells and natural killer cells distinctly infiltrate primary tumors and draining lymph nodes in pulmonary adenocarcinoma. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2011 Mar;6(3):432–8.

89. Erfani N, Mehrabadi SM, Ghayumi MA, Haghshenas MR, Mojtahedi Z, Ghaderi A, et al. Increase of regulatory T cells in metastatic stage and CTLA-4 over expression in lymphocytes of patients with non-small cell lung cancer (NSCLC). Lung Cancer Amst Neth. 2012 Aug;77(2):306–11.

90. Sakimura C, Tanaka H, Okuno T, Hiramatsu S, Muguruma K, Hirakawa K, et al. B cells in tertiary lymphoid structures are associated with favorable prognosis in gastric cancer. J Surg Res. 2017 Jul;215:74–82.

91. Murakami Y, Saito H, Shimizu S, Kono Y, Shishido Y, Miyatani K, et al. Increased regulatory B cells are involved in immune evasion in patients with gastric cancer. Sci Rep. 2019 Sep 11;9(1):13083.

92. Zheng M, Li YM, Liu ZY, Zhang X, Zhou Y, Jiang JL, et al. Prognostic Landscape of Tumor-Infiltrating T and B Cells in Human Cancer. Front Immunol. 2021;12:731329.

93. Sharonov GV, Serebrovskaya EO, Yuzhakova DV, Britanova OV, Chudakov DM. B cells, plasma cells and antibody repertoires in the tumour microenvironment. Nat Rev Immunol. 2020;20(5):294–307.

94. Schumacher TN, Thommen DS. Tertiary lymphoid structures in cancer. Science. 2022 Jan 7;375(6576):eabf9419.

95. Wang B, Liu J, Han Y, Deng Y, Li J, Jiang Y. The Presence of Tertiary Lymphoid Structures Provides New Insight Into the Clinicopathological Features and Prognosis of Patients With Breast Cancer. Front Immunol. 2022;13:868155.

96. Pipi E, Nayar S, Gardner DH, Colafrancesco S, Smith C, Barone F. Tertiary Lymphoid Structures: Autoimmunity Goes Local. Front Immunol. 2018 Sep 12;9:1952.

97. Laumont CM, Banville AC, Gilardi M, Hollern DP, Nelson BH. Tumour-infiltrating B cells: immunological mechanisms, clinical impact and therapeutic opportunities. Nat Rev Cancer. 2022 Apr 7;

98. Rodriguez AB, Engelhard VH. Insights into tumor-associated tertiary lymphoid structures: novel targets for antitumor immunity and cancer immunotherapy. Cancer Immunol Res. 2020 Nov;8(11):1338–45.

99. Sautès-Fridman C, Petitprez F, Calderaro J, Fridman WH. Tertiary lymphoid structures in the era of cancer immunotherapy. Nat Rev Cancer. 2019 Jun;19(6):307–25.

100. Rodriguez AB, Peske JD, Woods AN, Leick KM, Mauldin IS, Meneveau MO, et al. Immune mechanisms orchestrate tertiary lymphoid structures in tumors via cancer-associated fibroblasts. Cell Rep. 2021 Jul 20;36(3):109422.

101. Engelhard VH, Rodriguez AB, Mauldin IS, Woods AN, Peske JD, Slingluff CL. Immune Cell Infiltration and Tertiary Lymphoid Structures as Determinants of Antitumor Immunity. J Immunol Baltim Md 1950. 2018 Jan 15;200(2):432–42.

102. Ruddle NH. Lymphatic vessels and tertiary lymphoid organs. J Clin Invest. 2014 Mar;124(3):953–9.

103. Gonzalez-Figueroa P, Roco JA, Papa I, Núñez Villacís L, Stanley M, Linterman MA, et al. Follicular regulatory T cells produce neuritin to regulate B cells. Cell. 2021 Apr 1;184(7):1775-1789.e19.

104. Stebegg M, Kumar SD, Silva-Cayetano A, Fonseca VR, Linterman MA, Graca L. Regulation of the Germinal Center Response. Front Immunol. 2018;9:2469.

105. Cyster JG, Allen CDC. B Cell Responses: Cell Interaction Dynamics and Decisions. Cell. 2019 Apr 18;177(3):524–40.

106. Peske JD, Thompson ED, Gemta L, Baylis RA, Fu YX, Engelhard VH. Effector lymphocyte-induced lymph node-like vasculature enables naive T-cell entry into tumours and enhanced anti-tumour immunity. Nat Commun. 2015 May 13;6:7114.

107. Carrega P, Loiacono F, Di Carlo E, Scaramuccia A, Mora M, Conte R, et al. NCR(+)ILC3 concentrate in human lung cancer and associate with intratumoral lymphoid structures. Nat Commun. 2015 Sep 23;6:8280.

108. Chaurio RA, Anadon CM, Lee Costich T, Payne KK, Biswas S, Harro CM, et al. TGF-β-mediated silencing of genomic organizer SATB1 promotes Tfh cell differentiation and formation of intra-tumoral tertiary lymphoid structures. Immunity. 2022 Jan 11;55(1):115-128.e9.

109. Lochner M, Ohnmacht C, Presley L, Bruhns P, Si-Tahar M, Sawa S, et al. Microbiota-induced tertiary lymphoid tissues aggravate inflammatory disease in the absence of RORgamma t and LTi cells. J Exp Med. 2011 Jan 17;208(1):125–34.

110. Peters A, Pitcher LA, Sullivan JM, Mitsdoerffer M, Acton SE, Franz B, et al. Th17 cells induce ectopic lymphoid follicles in central nervous system tissue inflammation. Immunity. 2011 Dec 23;35(6):986–96.

111. Ukita M, Hamanishi J, Yoshitomi H, Yamanoi K, Takamatsu S, Ueda A, et al. CXCL13-producing CD4+ T cells accumulate in the early phase of tertiary lymphoid structures in ovarian cancer. JCI Insight. 2022 Jun 22;7(12):e157215.

112. Li JP, Wu CY, Chen MY, Liu SX, Yan SM, Kang YF, et al. PD-1+CXCR5-CD4+ Th-CXCL13 cell subset drives B cells into tertiary lymphoid structures of nasopharyngeal carcinoma. J Immunother Cancer. 2021 Jul;9(7):e002101.

113. Barone F, Gardner DH, Nayar S, Steinthal N, Buckley CD, Luther SA. Stromal Fibroblasts in Tertiary Lymphoid Structures: A Novel Target in Chronic Inflammation. Front Immunol. 2016;7:477.

114. Qin Y, Lu F, Lyu K, Chang AE, Li Q. Emerging concepts regarding pro- and anti tumor properties of B cells in tumor immunity. Front Immunol. 2022 Jul 28;13:881427.

115. Vella G, Guelfi S, Bergers G. High Endothelial Venules: A Vascular Perspective on Tertiary Lymphoid Structures in Cancer. Front Immunol. 2021 Aug 17;12:736670.

116. Colbeck EJ, Jones E, Hindley JP, Smart K, Schulz R, Browne M, et al. Treg Depletion Licenses T Cell-Driven HEV Neogenesis and Promotes Tumor Destruction. Cancer Immunol Res. 2017 Nov;5(11):1005–15.

117. Hindley JP, Jones E, Smart K, Bridgeman H, Lauder SN, Ondondo B, et al. T-cell trafficking facilitated by high endothelial venules is required for tumor control after regulatory T-cell depletion. Cancer Res. 2012 Nov 1;72(21):5473–82.

118. Kroeger DR, Milne K, Nelson BH. Tumor-Infiltrating Plasma Cells Are Associated with Tertiary Lymphoid Structures, Cytolytic T-Cell Responses, and Superior Prognosis in Ovarian Cancer. Clin Cancer Res Off J Am Assoc Cancer Res. 2016 Jun 15;22(12):3005–15.

119. Zhu W, Germain C, Liu Z, Sebastian Y, Devi P, Knockaert S, et al. A high density of tertiary lymphoid structure B cells in lung tumors is associated with increased CD4+ T cell receptor repertoire clonality. Oncoimmunology. 2015 Dec;4(12):e1051922.

120. Cipponi A, Mercier M, Seremet T, Baurain JF, Théate I, van den Oord J, et al. Neogenesis of lymphoid structures and antibody responses occur in human melanoma metastases. Cancer Res. 2012 Aug 15;72(16):3997–4007.

121. Montfort A, Pearce O, Maniati E, Vincent BG, Bixby L, Böhm S, et al. A Strong B-cell Response Is Part of the Immune Landscape in Human High-Grade Serous Ovarian Metastases. Clin Cancer Res Off J Am Assoc Cancer Res. 2017 Jan 1;23(1):250–62.

122. Schlößer HA, Thelen M, Lechner A, Wennhold K, Garcia-Marquez MA, Rothschild SI, et al. B cells in esophago-gastric adenocarcinoma are highly differentiated, organize in tertiary lymphoid structures and produce tumor-specific antibodies. Oncoimmunology. 2019;8(1):e1512458.

123. Groeneveld CS, Fontugne J, Cabel L, Bernard-Pierrot I, Radvanyi F, Allory Y, et al. Tertiary lymphoid structures marker CXCL13 is associated with better survival for patients with advanced-stage bladder cancer treated with immunotherapy. Eur J Cancer Oxf Engl 1990. 2021 May;148:181–9.

124. Zhou L, Xu B, Liu Y, Wang Z. Tertiary lymphoid structure signatures are associated with survival and immunotherapy response in muscle-invasive bladder cancer. Oncoimmunology. 2021 May 18;10(1):1915574.

125. Yang M, Lu J, Zhang G, Wang Y, He M, Xu Q, et al. CXCL13 shapes immunoactive tumor microenvironment and enhances the efficacy of PD-1 checkpoint blockade in high-grade serous ovarian cancer. J Immunother Cancer. 2021 Jan 15;9(1):e001136.

126. Liu Z, Meng X, Tang X, Zou W, He Y. Intratumoral tertiary lymphoid structures promote patient survival and immunotherapy response in head neck squamous cell carcinoma. Cancer Immunol Immunother CII. 2022 Dec 8;

127. Deguchi S, Tanaka H, Suzuki S, Natsuki S, Mori T, Miki Y, et al. Clinical relevance of tertiary lymphoid structures in esophageal squamous cell carcinoma. BMC Cancer. 2022 Jun 24;22(1):699.

128. Johansson-Percival A, He B, Li ZJ, Kjellén A, Russell K, Li J, et al. De novo induction of intratumoral lymphoid structures and vessel normalization enhances immunotherapy in resistant tumors. Nat Immunol. 2017 Nov;18(11):1207–17.

129. Skeate JG, Otsmaa ME, Prins R, Fernandez DJ, Da Silva DM, Kast WM. TNFSF14: LIGHTing the Way for Effective Cancer Immunotherapy. Front Immunol. 2020;11:922.

130. Downs-Canner SM, Meier J, Vincent BG, Serody JS. B Cell Function in the Tumor Microenvironment. Annu Rev Immunol. 2022;40(1):169–93.

131. Ghosh D, Jiang W, Mukhopadhyay D, Mellins ED. New insights into B cells as antigen presenting cells. Curr Opin Immunol. 2021 Jun;70:129–37.

132. Wennhold K, Thelen M, Lehmann J, Schran S, Preugszat E, Garcia-Marquez M, et al. CD86+ Antigen-Presenting B Cells Are Increased in Cancer, Localize in Tertiary Lymphoid Structures, and Induce Specific T-cell Responses. Cancer Immunol Res. 2021 Sep;9(9):1098–108.

133. Zhang X, Zhang Y, Xu J, Wang H, Zheng X, Lou Y, et al. Antigen presentation of the Oct4 and Sox2 peptides by CD154-activated B lymphocytes enhances the killing effect of cytotoxic T lymphocytes on tumor stem-like cells derived from cisplatin-resistant lung cancer cells. J Cancer. 2018;9(2):367–74.

134. Cui C, Wang J, Fagerberg E, Chen PM, Connolly KA, Damo M, et al. Neoantigen driven B cell and CD4 T follicular helper cell collaboration promotes anti-tumor CD8 T cell responses. Cell. 2021 Dec 9;184(25):6101-6118.e13.

135. Liu K, Hoover AR, Krawic JR, DeVette CI, Sun XH, Hildebrand WH, et al. Antigen presentation and interferon signatures in B cells driven by localized ablative cancer immunotherapy correlate with extended survival. Theranostics. 2022;12(2):639–56.

136. Small M, Treilleux I, Couillault C, Pissaloux D, Picard G, Paindavoine S, et al. Genetic alterations and tumor immune attack in Yo paraneoplastic cerebellar degeneration. Acta Neuropathol (Berl). 2018 Apr;135(4):569–79.

137. Joseph CG, Darrah E, Shah AA, Skora AD, Casciola-Rosen LA, Wigley FM, et al. Association of the autoimmune disease scleroderma with an immunologic response to cancer. Science. 2014 Jan 10;343(6167):152–7.

138. Lo JA, Kawakubo M, Juneja VR, Su MY, Erlich TH, LaFleur MW, et al. Epitope spreading toward wild type melanocyte-lineage antigens rescues suboptimal immune checkpoint blockade responses. Sci Transl Med. 2021 Feb 17;13(581):eabd8636.

139. Shen P, Fillatreau S. Antibody-independent functions of B cells: a focus on cytokines. Nat Rev Immunol. 2015 Jul;15(7):441–51.

140. Zhang Y, Garcia-Ibanez L, Toellner K. Regulation of germinal center B-cell differentiation. Immunol Rev. 2016 Mar;270(1):8–19.

141. Gerstl B, Eng LF, Bigbee JW. Tumor-associated immunoglobulins in pulmonary carcinoma. Cancer Res. 1977 Dec;37(12):4449–55.

142. Kumar S, Mohan A, Guleria R. Prognostic implications of circulating anti-p53 antibodies in lung cancer – a review. Eur J Cancer Care (Engl). 2009;18(3):248–54.

143. Aziz M, Das TK, Rattan A. Role of circulating immune complexes in prognostic evaluation and management of genitourinary cancer patients. Indian J Cancer. 1997 Sep;34(3):111–20.

144. Fremd C, Stefanovic S, Beckhove P, Pritsch M, Lim H, Wallwiener M, et al. Mucin 1-specific B cell immune responses and their impact on overall survival in breast cancer patients. Oncoimmunology. 2015 Jun 5;5(1):e1057387.

145. Hamanaka Y, Suehiro Y, Fukui M, Shikichi K, Imai K, Hinoda Y. Circulating anti-MUC1 IgG antibodies as a favorable prognostic factor for pancreatic cancer. Int J Cancer. 2003 Jan 1;103(1):97–100.

146. Sobhani N, Roviello G, D'Angelo A, Roudi R, Neeli PK, Generali D. p53 Antibodies as a Diagnostic Marker for Cancer: A Meta-Analysis. Molecules. 2021 Oct 14;26(20):6215.

147. Li Y, Song R, Li X, Xu F. Expression and immunogenicity of NY-ESO-1 in colorectal cancer. Exp Ther Med. 2017 Jun;13(6):3581–5.

148. Fujiwara S, Wada H, Kawada J, Kawabata R, Takahashi T, Fujita J, et al. NY-ESO-1 antibody as a novel tumour marker of gastric cancer. Br J Cancer. 2013 Mar 19;108(5):1119–25.

149. Oshima Y, Shimada H, Yajima S, Nanami T, Matsushita K, Nomura F, et al. NY-ESO-1 autoantibody as a tumor-specific biomarker for esophageal cancer: screening in 1969 patients with various cancers. J Gastroenterol. 2016 Jan;51(1):30–4.

150. Endo M, de Graaff MA, Ingram DR, Lim S, Lev DC, Briaire-de Bruijn IH, et al. NY-ESO-1 (CTAG1B) expression in mesenchymal tumors. Mod Pathol Off J U S Can Acad Pathol Inc. 2015 Apr;28(4):587–95.

151. Garaud S, Zayakin P, Buisseret L, Rulle U, Silina K, de Wind A, et al. Antigen Specificity and Clinical Significance of IgG and IgA Autoantibodies Produced in situ by Tumor-Infiltrating B Cells in Breast Cancer. Front Immunol. 2018;9:2660.

152. Nielsen JS, Sahota RA, Milne K, Kost SE, Nesslinger NJ, Watson PH, et al. CD20+ tumor-infiltrating lymphocytes have an atypical CD27- memory phenotype and together with CD8+ T cells promote favorable prognosis in ovarian cancer. Clin Cancer Res Off J Am Assoc Cancer Res. 2012 Jun 15;18(12):3281–92.

153. Wieland A, Patel MR, Cardenas MA, Eberhardt CS, Hudson WH, Obeng RC, et al. Defining HPV-specific B cell responses in head and neck cancer patients. Nature. 2021 Sep;597(7875):274–8.

154. Kim SS, Sumner WA, Miyauchi S, Cohen EEW, Califano JA, Sharabi AB. Role of B Cells in Responses to Checkpoint Blockade Immunotherapy and Overall Survival of Cancer Patients. Clin Cancer Res Off J Am Assoc Cancer Res. 2021 Nov 15;27(22):6075–82.

155. Isaeva OI, Sharonov GV, Serebrovskaya EO, Turchaninova MA, Zaretsky AR, Shugay M, et al. Intratumoral immunoglobulin isotypes predict survival in lung adenocarcinoma subtypes. J Immunother Cancer. 2019 Oct 29;7(1):279.

156. Karagiannis P, Gilbert AE, Josephs DH, Ali N, Dodev T, Saul L, et al. IgG4 subclass antibodies impair antitumor immunity in melanoma. J Clin Invest. 2013 Apr;123(4):1457–74.

157. Hollern DP, Xu N, Thennavan A, Glodowski C, Recio SG, Mott KR, et al. B cells and T follicular helper cells mediate response to checkpoint inhibitors in high mutation burden mouse models of breast cancer. Cell. 2019 Nov 14;179(5):1191-1206.e21.

158. Janjic BM, Kulkarni A, Ferris RL, Vujanovic L, Vujanovic NL. Human B Cells Mediate Innate Anti-Cancer Cytotoxicity Through Concurrent Engagement of Multiple TNF Superfamily Ligands. Front Immunol. 2022 Mar 22;13:837842.

159. Hu HT, Ai X, Lu M, Song Z, Li H. Characterization of intratumoral and circulating IL-10-producing B cells in gastric cancer. Exp Cell Res. 2019 Nov 15;384(2):111652.

160. Lu L, Weng C, Mao H, Fang X, Liu X, Wu Y, et al. IL-17A promotes migration and tumor killing capability of B cells in esophageal squamous cell carcinoma. Oncotarget. 2016 Apr 19;7(16):21853–64.

161. Griss J, Bauer W, Wagner C, Simon M, Chen M, Grabmeier-Pfistershammer K, et al. B cells sustain inflammation and predict response to immune checkpoint blockade in human melanoma. Nat Commun. 2019;10(1):4186.

162. Ammirante M, Luo JL, Grivennikov S, Nedospasov S, Karin M. B-cell-derived lymphotoxin promotes castration-resistant prostate cancer. Nature. 2010 Mar 11;464(7286):302–5.

163. Roumenina LT, Daugan MV, Petitprez F, Sautès-Fridman C, Fridman WH. Context-dependent roles of complement in cancer. Nat Rev Cancer. 2019 Dec;19(12):698–715.

164. Dunkelberger JR, Song WC. Complement and its role in innate and adaptive immune responses. Cell Res. 2010 Jan;20(1):34–50.

165. Morgan BP. The membrane attack complex as an inflammatory trigger. Immunobiology. 2016 Jun;221(6):747–51.

166. Defendi F, Thielens NM, Clavarino G, Cesbron JY, Dumestre-Pérard C. The Immunopathology of Complement Proteins and Innate Immunity in Autoimmune Disease. Clin Rev Allergy Immunol. 2020 Apr;58(2):229–51.

167. Afshar-Kharghan V. The role of the complement system in cancer. J Clin Invest. 127(3):780–9.

168. Carroll MC, Isenman DE. Regulation of humoral immunity by complement. Immunity. 2012 Aug 24;37(2):199–207.

169. Rossbacher J, Shlomchik MJ. The B Cell Receptor Itself Can Activate Complement to Provide the Complement Receptor 1/2 Ligand Required to Enhance B Cell Immune Responses In Vivo. J Exp Med. 2003 Aug 18;198(4):591–602.

170. Jenkins RW, Barbie DA, Flaherty KT. Mechanisms of resistance to immune checkpoint inhibitors. Br J Cancer. 2018;118(1):9–16.

171. Yuan S, Liu Y, Till B, Song Y, Wang Z. Pretreatment Peripheral B Cells Are Associated With Tumor Response to Anti-PD-1-Based Immunotherapy. Front Immunol. 2020;11:563653.

172. Xia L, Guo L, Kang J, Yang Y, Yao Y, Xia W, et al. Predictable Roles of Peripheral IgM Memory B Cells for the Responses to Anti-PD-1 Monotherapy Against Advanced Non-Small Cell Lung Cancer. Front Immunol. 2021;12:759217.

173. Barth DA, Stanzer S, Spiegelberg J, Bauernhofer T, Absenger G, Posch F, et al. Evaluation of autoantibodies as predictors of treatment response and immune-related adverse events during the treatment with immune checkpoint inhibitors: A prospective longitudinal pan-cancer study. Cancer Med. 2022 Mar 16;

174. Barth DA, Stanzer S, Spiegelberg JA, Bauernhofer T, Absenger G, Szkandera J, et al. Patterns of Peripheral Blood B-Cell Subtypes Are Associated With Treatment Response in Patients Treated With Immune Checkpoint Inhibitors: A Prospective Longitudinal Pan-Cancer Study. Front Immunol. 2022;13:840207.

175. Chan EKL, Damoiseaux J, Carballo OG, Conrad K, de Melo Cruvinel W, Francescantonio PLC, et al. Report of the First International Consensus on Standardized Nomenclature of Antinuclear Antibody HEp-2 Cell Patterns 2014-2015. Front Immunol. 2015;6:412.

176. Rupp J, Dreo B, Gütl K, Fessler J, Moser A, Haditsch B, et al. T Cell Phenotyping in Individuals Hospitalized with COVID-19. J Immunol. 2021;206(7):1478.

177. Schulz E, Hodl I, Forstner P, Hatzl S, Sareban N, Moritz M, et al. CD19+IgD+CD27- Naïve B Cells as Predictors of Humoral Response to COVID 19 mRNA Vaccination in Immunocompromised Patients. Front Immunol. 2021;12:803742.

178. Wehr C, Kivioja T, Schmitt C, Ferry B, Witte T, Eren E, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. Blood. 2008 Jan 1;111(1):77–85.

179. Das A, Ellis G, Pallant C, Lopes AR, Khanna P, Peppa D, et al. IL-10-producing regulatory B cells in the pathogenesis of chronic hepatitis B virus infection. J Immunol Baltim Md 1950. 2012 Oct 15;189(8):3925–35.

180. Blair PA, Noreña LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. Immunity. 2010 Jan 29;32(1):129–40.

181. Giannicola R, D'Arrigo G, Botta C, Agostino R, Del Medico P, Falzea AC, et al. Early blood rise in auto-antibodies to nuclear and smooth muscle antigens is predictive of prolonged survival and autoimmunity in metastatic-non-small cell lung cancer patients treated with PD-1 immune-check point blockade by nivolumab. Mol Clin Oncol. 2019;11(1):81–90.

182. Sakakida T, Ishikawa T, Chihara Y, Harita S, Uchino J, Tabuchi Y, et al. Safety and efficacy of PD-1/PD-L1 blockade in patients with preexisting antinuclear antibodies. Clin Transl Oncol. 2020;22(6):919–27.

183. Morimoto K, Yamada T, Nakamura R, Katayama Y, Tanaka S, Takumi C, et al. Impact of preexisting antinuclear antibodies on combined immunotherapy and chemotherapy in advanced non-small cell lung cancer patients. Med Oncol. 2020;37(12):111.

184. Toi Y, Sugawara S, Sugisaka J, Ono H, Kawashima Y, Aiba T, et al. Profiling Preexisting Antibodies in Patients Treated With Anti-PD-1 Therapy for Advanced Non-Small Cell Lung Cancer. JAMA Oncol. 2019;5(3):376–83.

185. Yoneshima Y, Tanaka K, Shiraishi Y, Hata K, Watanabe H, Harada T, et al. Safety and efficacy of PD-1 inhibitors in non–small cell lung cancer patients positive for antinuclear antibodies. Lung Cancer. 2019;130:5–9.

186. Mouri A, Kaira K, Yamaguchi O, Hashimoto K, Miura Y, Shiono A, et al. Efficacy and Feasibility of Programmed Death-1/Programmed Death Ligand-1 Blockade Therapy in Non-Small Cell Lung Cancer Patients With High Antinuclear Antibody Titers. Front Oncol. 2021;11:610952.

187. Das S, Johnson DB. Immune-related adverse events and anti-tumor efficacy of immune checkpoint inhibitors. J Immunother Cancer. 2019 Nov 15;7(1):306.

188. Vlagea A, Falagan S, Gutiérrez-Gutiérrez G, Moreno-Rubio J, Merino M, Zambrana F, et al. Antinuclear antibodies and cancer: A literature review. Crit Rev Oncol Hematol. 2018 Jul;127:42–9.

189. Kobayashi T, Iwama S, Yasuda Y, Okada N, Tsunekawa T, Onoue T, et al. Patients With Antithyroid Antibodies Are Prone To Develop Destructive Thyroiditis by Nivolumab: A Prospective Study. J Endocr Soc. 2018;2(3):241–51.

190. MAEKURA T, NAITO M, TAHARA M, IKEGAMI N, KIMURA Y, SONOBE S, et al. Predictive Factors of Nivolumab-induced Hypothyroidism in Patients with Non-small Cell Lung Cancer. In Vivo. 2017 Sep 3;31(5):1035–9.

191. Kimbara S, Fujiwara Y, Iwama S, Ohashi K, Kuchiba A, Arima H, et al. Association of antithyroglobulin antibodies with the development of thyroid dysfunction induced by nivolumab. Cancer Sci. 2018 Nov;109(11):3583–90.

192. Tahir SA, Gao J, Miura Y, Blando J, Tidwell RSS, Zhao H, et al. Autoimmune antibodies correlate with immune checkpoint therapy-induced toxicities. Proc Natl Acad Sci U S A. 2019;116(44):22246–51.

193. Hasan Ali O, Bomze D, Ring SS, Berner F, Fässler M, Diem S, et al. BP180-specific IgG is associated with skin adverse events, therapy response, and overall survival in non-small cell lung cancer patients treated with checkpoint inhibitors. J Am Acad Dermatol. 2020;82(4):854–61.

194. Les I, Martínez M, Narro A, Pérez I, Sánchez C, Puntí L, et al. Association of immune-related adverse events induced by nivolumab with a battery of autoantibodies. Ann Med. 53(1):762–9.

195. Waldman AD, Fritz JM, Lenardo MJ. A guide to cancer immunotherapy: from T cell basic science to clinical practice. Nat Rev. 2020;20(11):651–68.

196. Paijens ST, Vledder A, de Bruyn M, Nijman HW. Tumor-infiltrating lymphocytes in the immunotherapy era. Cell Mol Immunol. 2021;18(4):842–59.

197. Largeot A, Pagano G, Gonder S, Moussay E, Paggetti J. The B-side of Cancer Immunity: The Underrated Tune. Cells. 2019;8(5):449.

198. Kinker GS, Vitiello GAF, Ferreira WAS, Chaves AS, Cordeiro de Lima VC, Medina T da S. B Cell Orchestration of Anti-tumor Immune Responses: A Matter of Cell Localization and Communication. Front Cell Dev Biol. 2021;9:678127.

199. Petitprez F, de Reyniès A, Keung EZ, Chen TWW, Sun CM, Calderaro J, et al. B cells are associated with survival and immunotherapy response in sarcoma. Nature. 2020 Jan;577(7791):556–60.

200. Dajsakdipon T, Siripoon T, Ngamphaiboon N, Ativitavas T, Dejthevaporn T. Immunotherapy and Biomarkers in Sarcoma. Curr Treat Options Oncol. 2022 Mar;23(3):415–38.

201. Wu Z, Zhou J, Xiao Y, Ming J, Zhou J, Dong F, et al. CD20+CD22+ADAM28+ B Cells in Tertiary Lymphoid Structures Promote Immunotherapy Response. Front Immunol. 2022 May 11;13:865596.

202. Zhang Y, Zhu G, Xiao H, Liu X, Han G, Chen G, et al. CD19 regulates ADAM28-mediated Notch2 cleavage to control the differentiation of marginal zone precursors to MZ B cells. J Cell Mol Med. 2017 Dec;21(12):3658–69.

203. Carril-Ajuria L, Desnoyer A, Meylan M, Dalban C, Naigeon M, Cassard L, et al. Baseline circulating unswitched memory B cells and B-cell related soluble factors are associated with overall survival in patients with clear cell renal cell carcinoma treated with nivolumab within the NIVOREN GETUG-AFU 26 study. J Immunother Cancer. 2022 May 1;10(5):e004885.

204. Hendricks J, Bos NA, Kroese FGM. Heterogeneity of Memory Marginal Zone B Cells. Crit Rev Immunol. 2018;38(2):145–58.

205. Cerutti A, Cols M, Puga I. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. Nat Rev Immunol. 2013 Feb;13(2):118–32.

206. Pape KA, Taylor JJ, Maul RW, Gearhart PJ, Jenkins MK. Different B Cell Populations Mediate Early and Late Memory During an Endogenous Immune Response. Science. 2011 Mar 4;331(6021):1203–7.

207. Liu C, Richard K, Wiggins M, Zhu X, Conrad DH, Song W. CD23 can negatively regulate B-cell receptor signaling. Sci Rep. 2016 May 16;6(1):25629.

208. Dogan I, Bertocci B, Vilmont V, Delbos F, Mégret J, Storck S, et al. Multiple layers of B cell memory with different effector functions. Nat Immunol. 2009;10(12):1292–9.

209. Gilbert AE, Karagiannis P, Dodev T, Koers A, Lacy K, Josephs DH, et al. Monitoring the systemic human memory B cell compartment of melanoma patients for anti-tumor IgG antibodies. PloS One. 2011 Apr 29;6(4):e19330.

210. Wu XZ, Shi XY, Zhai K, Yi FS, Wang Z, Wang W, et al. Activated naïve B cells promote development of malignant pleural effusion by differential regulation of TH1 and TH17 response. Am J Physiol-Lung Cell Mol Physiol. 2018 Sep;315(3):L443–55.

211. Thorarinsdottir K, Camponeschi A, Gjertsson I, Mårtensson IL. CD21−/low B cells: A Snapshot of a Unique B Cell Subset in Health and Disease. Scand J Immunol. 2015;82(3):254–61.

212. Isnardi I, Ng YS, Menard L, Meyers G, Saadoun D, Srdanovic I, et al. Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. Blood. 2010;115(24):5026–36.

213. Charles ED, Brunetti C, Marukian S, Ritola KD, Talal AH, Marks K, et al. Clonal B cells in patients with hepatitis C virus-associated mixed cryoglobulinemia contain an expanded anergic CD21low B-cell subset. Blood. 2011;117(20):5425–37.

214. Visentini M, Cagliuso M, Conti V, Carbonari M, Casato M, Fiorilli M. The VH1-69–expressing marginal zone B cells expanded in HCV-associated mixed cryoglobulinemia display proliferative anergy irrespective of CD21low phenotype. Blood. 2011;118(12):3440–1.

215. Rakhmanov M, Keller B, Gutenberger S, Foerster C, Hoenig M, Driessen G, et al. Circulating CD21low B cells in common variable immunodeficiency resemble tissue homing, innate-like B cells. Proc Natl Acad Sci U S A. 2009;106(32):13451–6.

216. Ohue Y, Nishikawa H. Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target? Cancer Sci. 2019 Jul;110(7):2080–9.

217. Togashi Y, Shitara K, Nishikawa H. Regulatory T cells in cancer immunosuppression - implications for anticancer therapy. Nat Rev Clin Oncol. 2019 Jun;16(6):356–71.

218. Kuehn HS, Ouyang W, Lo B, Deenick EK, Niemela JE, Avery DT, et al. Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. Science. 2014 Sep 26;345(6204):1623–7.

219. Das R, Bar N, Ferreira M, Newman AM, Zhang L, Bailur JK, et al. Early B cell changes predict autoimmunity following combination immune checkpoint blockade. J Clin Invest. 2018 Feb 1;128(2):715–20.

220. Shen Y, Chen Y, Wang D, Zhu Z. Treatment-related adverse events as surrogate to response rate to immune checkpoint blockade. Medicine (Baltimore). 2020 Sep 11;99(37):e22153.

221. Li Y, Wang Z, Guo T, Liu S, Feng C. Treatment-related adverse events and response rate to immune checkpoint inhibition. J Int Med Res. 2020 Mar;48(3):300060519886454.

222. Waidhauser J, Schuh A, Trepel M, Schmälter AK, Rank A. Chemotherapy markedly reduces B cells but not T cells and NK cells in patients with cancer. Cancer Immunol Immunother CII. 2020 Jan;69(1):147–57.