Significance of B cells in PD-1 checkpoint immunotherapy

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List of Abbreviations

A	
A	Area
Ab	Antibody
ADCC	Antibody dependent cellular cytotoxicity
Ag	Antigen
Akt	Protein kinase B
ALK	Anaplastic lymphoma kinase
ANOVA	One-way analysis of variance
APC	Allophycocyanin
Apo1	Apoptosis antigen 1
ASC	Antibody secreting cell
Asn	Asparagine
A.	Arthrobacter
B	
BCR	B cell receptor
BRAF	rapidly accelerated fibrosarcoma B-type
Breg	Regulatory B cell
BWH	Brigham & Women's Hospital
C	
CD	Cluster of differentiation
CDKN2A	Cyclin dependent kinase inhibitor 2A
CDK4	Cyclin dependent kinase 4
CDR	Complementarity determining regions
CD40L	CD40 ligand
CIS	Carcinoma in situ
ConA	Concanavalin A
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
C1qR	Complement component 1q receptor
CRC	Colorectal carcinoma
c-KIT	V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene
D	
DC	Dendritic cell
dMMR	Defective mismatch repair
DMSO	Dimethylsulfoxide
DN Mem	Double negative memory
DNA	Deoxyribonucleic acid
Dol-P	Dolicholphosphate
DPAGT1	Dolichylphosphate *N*-acetylglucosamine-phosphotransferase 1
DPBS	Dulbecco's phosphate-buffered saline
E	
ECA	Erythrina Cristagalli Lectin
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
ER	Endoplasmatic reticulum
ERK	Extracellular signal-regulated kinase
F	
FAS	FS-7-associated surface antigen
FASL	FAS Receptor/ CD95/ Apo1 ligand
FBS	Fetal bovine serum
FcR	Immunoglobulin crystallizable fragment receptor
FITC	Fluorescein isothiocyanate
FDA	Food and Drug Administration
FMO	Fluorescence minus one
FSC	Forward scatter
fT_H	Follicular T helper cell
G	
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
GC	Germinal center
GDP-Man	Guanosine diphosphate mannose
Glc	Glucose
GlcNAc	*N*-acetylglucosamine
GlcNAcT-I	*N*-acetylglucosaminyl-transferase-1
H	
H	Height
HCC	Hepatocellular carcinoma
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNSCC	Head and neck squamous cell carcinoma

I	
I	Isotype
ICC	Investigator´s choice chemotherapy
ICOS	Inducible T cell co-stimulator
IFN	Interferon
Ig	Immunoglobulin
IgC	Constant region of immunoglobulins
IgC_H	Constant region of heavy chain
IgH	Heavy chain of immunoglobulin
IgSF	Immunoglobulin-like super family
IgV	Variable region of Immunoglobulin
IgV_H	Variable region of heavy chain
IL	Interleukin
irAE	Immune related adverse effect
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITSM	Immunoreceptor tyrosine-based switch motif
L	
LacNAc	*N*-acetyllactosamine
M	
MAA	Maackia Amurensis Lectin
Man	Mannose
MAPK	Mitogen-activated protein kinase
Mem	Memory
MFI	Mean fluorescence intensity
MGAT	*N*-acetylglucosaminyltransferase
MHC	Major histocompatibility complex
MSI-H	Microsatellite instability high
N	
N	Nivolumab
NCT	National clinical trial
Neu5Ac	*N*-acetylneuraminic acid
NF1	Nuclear factor 1
NK	Natural killer
NRAS	Neuroblastoma rat sarcoma
NS Mem	Non-switched memory
NSCLC	Non-small cell lung cancer
O	
ORR	Objective response rate
OS	Overall survival
OST	Oligosaccharlytransferase
P	
P	Pembrolizumab
PB	Plasma blast
PBMC	Peripheral blood mononuclear cell
PC	Plasma cell
PD-L1/2	Programmed cell death 1 ligand-1/2
PD-1	Programmed cell death receptor-1
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PerCP-Cy5.5	PerCP-complex-cyanine 5.5
PFA	Paraformaldehyde
PFS	Progression free survival
PHA-L	Phytohemagglutinin-L
PI3K	Phosphoinositide 3 kinase
PKB	Protein kinase B
PMBCL	primary mediastinal large B cell lymphoma
PTEN	Phosphatase tensin homologue
R	
Ras	Rat sarcoma
RCC	Renal cell carcinoma
RPMI	Roswell Park Memorial Institute
RR	Risk ratio
S	
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SEM	Standard error of the means
Ser	Serine
Sia	Sialic acid
Siglec	Sialic acid-binding immunoglobulin-type lectin
SHM	Somatic hypermutations
SHP-2	SH2-containing protein tyrosine phosphatase-2
SH2	Src-homology 2
SNA	Sambucus Nigra Lectin
SSC	Side scatter
ST6Gal1	ST6 β-galactoside α2,6-sialyl-transferase 1
Swi Mem	Switched Memory

T

TAB	Tumor-associated B cell
TCR	T cell receptor
TD	Thymus-dependent
TGF-β	Transforming growth factor-β
Thr	Threonine
TI	Thymus-independent
TIL	Tumor-infiltrating lymphocyte
TLR	Toll-like receptor
TLS	Tertiary lymphoid structure
TME	Tumor microenvironment
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
Treg	Regulatory T cell

U

US	United States
UV	Ultraviolet

V

V.	Vibrio

5-FU	5-Fluorouracil

Introduction

1. Melanoma

Melanoma is a malignant tumor that develops from the pigment-producing cells known as melanocytes (Ali et al. 2013). Melanocytes are predominantly located in the skin, but can also be found in the eyes, inner ears, mucous membranes, and leptomeninges. Malignant melanoma can thus be classified into three subcategories: cutaneous melanoma (the most common, accounting for 90% of cases) arises from melanocytes in the epidermis, uveal melanoma from melanocytes in the ocular stroma, and mucosal melanoma from melanocytes in the mucous membranes (Ali et al. 2013, Tsao et al. 2012). Diagnosis of melanoma at early stages is highly curable, being successfully treated with surgery alone (Hartman & Lin 2019). However, approximately 3.6% of melanomas are diagnosed in an advanced stage (Schoffer et al. 2016), characterized by widespread metastatic lesions and a very poor prognosis (median survival of less than one year) (Bhatia et al. 2009). Metastatic melanoma has traditionally been resistant to most current modalities of tumor therapy (Bhatia et al. 2009). However, over the past decade, advances in cancer immunotherapies and targeted treatment modalities have revolutionized the clinical management of metastatic disease and dramatically improved melanoma prognosis (Domingues et al. 2018, Hartman & Lin 2019, Rodriguez-Cerdeira et al. 2017).

In this MD thesis, we will focus on metastatic cutaneous melanoma, highlighting its epidemiology, pathogenesis, prognosis, and current treatment options.

1.1. Epidemiology

Cutaneous melanoma is the deadliest form of skin cancer, being a major public health and economic burden on society. Worldwide incidence of cutaneous melanoma has been increasing annually by approximately 3-7%, thus growing at a faster rate than most other cancers (Leiter et al. 2014, Matthews et al. 2017). Today, cutaneous melanoma is the fifth most common cancer in male and female in the United States (US) and it is estimated that in 2024, 100,640 adults will be diagnosed with cutaneous melanoma (American Cancer Society 2024). In Germany, cutaneous melanoma is ranked as the fifth and fourth most common cancer among men and women, respectively (Ferlay et al. 2020). Although cutaneous melanoma is generally considered a disease of older, because its risk considerably increases

with age (Matthews et al. 2017), it is not uncommon for patients younger than 30 to be diagnosed with the disease. In fact, cutaneous melanoma is one of the most common cancer types within young adults (American Cancer Society 2024) and its annual incidence has constantly increased >8-fold among young women and >4-fold among young men (Reed et al. 2012). Interestingly, incidence rates, clinicopathological features, and prognosis of cutaneous melanoma vary greatly with race and ethnicity. In fact, there have been studies reporting that whites have a higher risk and poorer outcome, compared to multiple other ethnicities (Matthews et al. 2017, Wang et al. 2016).

Despite dramatic improvements in prognosis and treatment of metastatic disease over the past decade, melanoma continues to be the sixth most common fatal cancer type in the US and it remains by far the most fatal skin cancer, causing six out of seven skin cancer related deaths (Ferlay et al. 2020).

1.2. Pathogenesis

The development of cutaneous melanoma is strongly influenced by environmental and genetic factors. Exposure to ultraviolet (UV) light radiation from sunlight is the main environmental risk factor implicated in melanoma formation. Multiple studies have linked periodic and intermittent sun exposure during childhood and adolescence to increased risk of melanoma development (Leonardi et al. 2018). Consistently, Crombie and colleagues have reported that melanoma rates are higher in low latitude regions within North America, further supporting a key role of UV radiation in melanomagenesis (Crombie 1979). Exposure to UV radiation leads to the generation of pyrimidine dimers that impair correct amplification of deoxyribonucleic acid (DNA) (Palmieri et al. 2009, Riker et al. 2010). The acquisition and accumulation of UV-induced mutations in critical genes is the driving force behind the initiation of melanomagenesis, promoting cell proliferation, survival and apoptosis inhibition (Hanahan & Weinberg 2011, Palmieri et al. 2009, Riker et al. 2010). Indeed, somatic mutations in the rapidly accelerated fibrosarcoma B-type (BRAF) proto-oncogene are the most common oncogenic drivers of melanomagenesis (Ascierto et al. 2012). The BRAF-oncogene encodes for a serine (Ser)/ threonine (Thr) kinase and activates the pro-proliferative mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK) pathway, leading to cell activation and proliferation (Alqathama 2020). Approximately 50% of all advanced melanomas harbor BRAF mutations, most of which consist of a substitution from valine to glutamic acid at position 600 (BRAF V600E) (Ascierto et al. 2012). Mutated

BRAF proteins display an aberrant kinase activity and are responsible for abnormal activation of the MAPK/ERK pathway, leading to enhanced cell growth, cell cycle dysregulation and apoptosis inhibition (Leonardi et al. 2018, McCubrey et al. 2012).

Interestingly, BRAF mutations can also be detected in non-malignant nevi (Kumar et al. 2004, Pollock et al. 2003) and germline mutations of BRAF are not associated with an increased risk for malignant melanoma (Niihori et al. 2006, Rodriguez-Viciana et al. 2006). This suggests that BRAF mutation alone is not sufficient for melanoma development and that additional mutations are required for oncogenic transformation. In support, cutaneous melanoma frequently harbors additional mutations in pro-proliferative genes, such as those encoding for cyclin dependent kinase inhibitor 2A (CDKN2A), cyclin dependent kinase 4 (CDK4), and phosphatase and tensin homologue (PTEN) (Palmieri et al. 2009, Tsao et al. 2012).

1.3. Current treatment modalities for metastatic cutaneous melanoma

Metastatic melanoma is among the most aggressive and treatment-resistant cancers. Prior to 2011, chemotherapy was the standard treatment for metastatic melanoma, yielding modest clinical response and no significant improvement in progression free survival (PFS) or overall survival (OS) (Bhatia et al. 2009). The chemotherapeutic agents used included dacarbazine and temozolomide (Bhatia et al. 2009). Since 2011, breakthroughs in immune- and targeted therapy have significantly improved the treatment and prognosis of advanced-stage melanoma, and chemotherapy was replaced over time by these innovative therapeutic strategies (Domingues et al. 2018, Hartman & Lin 2019). Despite this, chemotherapy is still used as a palliative treatment for refractory, progressive, and relapsed melanomas (Domingues et al. 2018).

1.3.1. Targeted therapy

Most patients with cutaneous melanoma (70%) harbor somatic mutations, that are responsible for tumor growth and progression (Domingues et al. 2018). Consequently, highly selective small molecules that inhibit mutated proteins and thereby re-establish physiologic signaling offer a promising approach for melanoma treatment. In 2011, the US Food and Drug Administration (FDA) approved the first BRAF inhibitor, Vemurafenib (Zelboraf, Roche/ Plexxikon), for unresectable or metastatic melanomas harboring BRAF V600E mutations (Kim et al. 2014). Since then, two other BRAF inhibitors, Dabrafenib (Tafinlar,

Novartis) and Encorafenib (Braftovi, Pierre Fabre), were approved by the FDA (Ballantyne & Garnock-Jones 2013, Shirley 2018). Vemurafenib treatment results in a higher response rate (approximately 50%) and a more rapid onset, compared to dacarbazine treatment (Chapman et al. 2011). However, treatment with BRAF inhibitors is associated with early relapse and disease progression that occurs within months to years (Tsao et al. 2012). Interestingly, combination of BRAF and MEK inhibitors diminishes frequency of early relapse and improves OS (22.3 months versus 17.4 months) (Ascierto et al. 2016).

Currently, multiple research groups began evaluating the therapeutic efficacy of targeted agents against other melanoma driver mutations, including mutations at the neuroblastoma rat sarcoma (NRAS), nuclear factor 1 (NF1), and V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene (c-KIT) (Domingues et al. 2018, Shtivelman et al. 2014). While the importance of targeted therapy for the treatment of advanced cutaneous melanoma is undeniable, major drawbacks, including resistance and early relapse, still need to be addressed.

1.3.2. Immune checkpoint therapy

Cutaneous melanoma is one of the most immunogenic tumors, which harbors high levels of tumor-infiltrating lymphocytes (TILs). Several TIL-specific and cancer cell-intrinsic immunoregulatory mechanisms have been described to interfere with efficient tumor immune recognition and thus lead to suppression of antimelanoma immunity (Mahmoud et al. 2017, Passarelli et al. 2017). Accordingly, the molecular and cellular interplay between melanoma and immune cells within the tumor microenvironment (TME) plays a central role in melanoma progression (Mahmoud et al. 2017).

The destruction of tumor cells by the immune system is accomplished by a strict balance between immune-stimulatory and -inhibitory pathways: For instance, during activation of tumor-reactive T cells, the T cell receptor (TCR) initially engages a cognate peptide-major histocompatibility complex (MHC) molecule complex displayed on the cell surface of antigen- (Ag-) presenting cells in an Ag-specific manner. Subsequent co-stimulatory and co-inhibitory signals are required for complete activation versus anergy or unresponsiveness, apoptosis, and functional exhaustion of lymphocytes. The balance between co-stimulatory and co-inhibitory interactions of T cells and Ag-presenting cells thus determines the fate of T cell responses against tumor cells (Pardoll 2012). The goal of immunotherapy is to target

co-inhibitory pathways or immune checkpoints, which refer to a plethora of immune-inhibitory mechanisms that maintain self-tolerance and also modulate the duration and amplitude of antitumor immune responses (Pardoll 2012, Wei et al. 2018). Blockade of immune checkpoints removes inhibitory signals and enables tumor-reactive lymphocytes to mount an effective antitumor response (Pardoll 2012, Sahni et al. 2018, Wei et al. 2018).

The programmed cell death receptor-1 (PD-1) and the cytotoxic T lymphocyte-associated protein 4 (CTLA-4) are the two predominant immune checkpoint receptors that have been extensively studied in the context of melanoma immunotherapy (Baumeister et al. 2016, Sahni et al. 2018). Blockade of PD-1 and/or CTLA-4 has shown remarkable efficacy in restoring tumor-reactive immune cell functions capable of inhibiting melanoma growth and progression (Domingues et al. 2018, Linck et al. 2017). Thus, blocking antibodies (Abs) targeting PD-1 or CTLA-4 have been widely established in the treatment of advanced cutaneous melanoma. In this MD-thesis, we will focus on PD-1 therapy as a treatment option for advanced cutaneous melanoma.

2. Harnessing the PD-1 checkpoint pathway to treat advanced melanoma

PD-1 is a co-inhibitory receptor that dampens adaptive immune responses (Riley 2009). Stimulation of PD-1 by its ligands negatively regulates immune cell survival, proliferation, activation, and effector function (Francisco et al. 2010, Keir et al. 2008, Sharpe & Pauken 2017). PD-1 expression has mainly been studied in T cells, but can also be induced on B cells, natural killer (NK) cells, monocytes, and some dendritic cell (DC) subsets upon activation (Francisco et al. 2010, Patsoukis et al. 2020, Qin et al. 2019). More recently, PD-1 expression has also been described on multiple tumor cell types, including melanoma cells (Clark et al. 2016, Kleffel et al. 2015, Wang et al. 2020), hepatocellular carcinoma (HCC) cells (Li et al. 2017), and lung cancer cells (Du et al. 2018, Wang et al. 2020). However, compared to T cell-specific PD-1 immunobiology, much less is known regarding the regulation of the PD-1 receptor on other immune, non-immune or cancer cells.

PD-1 plays a central role in establishing peripheral tolerance and homeostasis (Okazaki & Honjo 2006, Riella et al. 2012). Yet, its expression can also limit protective immunity (Sharpe & Pauken 2017). Furthermore, PD-1 expression is involved in thwarting antitumor immunity, thereby promoting tumor progression (Francisco et al. 2010, Keir et al. 2008, Sharpe & Pauken 2017). PD-1 blockade via monoclonal Abs has revolutionized cancer treatment, exhibiting higher response rates and typically less severe adverse effects, compared to chemotherapy (Linck et al. 2017, Sahni et al. 2018). However, only a subset of patients with advanced melanoma responds to PD-1 therapy (Parakh et al. 2017, Zhao et al. 2019) and, therefore, a better understanding of how PD-1 attenuates protective antitumor immunity is required to improve upon predictive biomarkers of clinical response and optimize treatment efficacy.

2.1. PD-1 structure and signaling

PD-1 is a member of the immunoglobulin-like super family (IgSF) and is made of a variable region- (IgV-) like domain, a stalk region, a transmembrane region, and a cytoplasmatic tail with an immunoreceptor tyrosine-based switch motif (ITSM) and an immunoreceptor tyrosine-based inhibition motif (ITIM) (Riley 2009). PD-1 signaling was initially investigated in T cells, where PD-1 stimulation typically leads to its co-localization with the TCR and subsequent phosphorylation of ITSMs and ITIMs (Keir et al. 2008). The phosphorylated tyrosine residue Y248 within the ITSM then serves as Src-homology 2 (SH2)

binding domain, recruiting SH2-containing protein tyrosine phosphatase-2 (SHP-2) (Chemnitz et al. 2004, Riley 2009). SHP-2 binding to the PD-1/TCR complex then results in the inhibition of the phosphoinositide-3-kinase (PI3K)/ protein kinase B (PKB, also known as Akt) and/or the rat sarcoma (Ras)/ MEK/ Erk pathways (Parry et al. 2005, Patsoukis et al. 2012). Ultimately, stimulation of PD-1 culminates in attenuated T cell proliferation (Boussiotis et al. 2014), impairment of TCR/ Cluster of differentiation (CD) 28-dependent immune functions (Hui et al. 2017), and the establishment of immunosuppressive regulatory T cells (Tregs) (Francisco et al. 2009). Together, these PD-1 receptor functions down-regulate immunity to establish peripheral tolerance.

PD-1 has two ligands, programmed cell death 1 ligand-1 (PD-L1) and programmed cell death 1 ligand-2 (PD-L2) (Sharpe & Pauken 2017). PD-L1 is constitutively expressed on most hematopoietic cells and on multiple non-hematopoietic cells, including keratinocytes, vascular endothelium, fibroblastic reticular cells, mesenchymal cells, pancreatic islets, astrocytes, and neurons (Bardhan et al. 2016, Francisco et al. 2010). In contrast, expression of PD-L2 is limited to activated DCs, macrophages, B cells, and bone marrow derived mast cells (Latchman et al. 2001), although recent reports suggest that PD-L2 might also be inducibly expressed by some epithelial cancer cells (Derks et al. 2015, Wang et al. 2017). Additionally, significant levels of PD-L1 are detected on multiple cancer cell types, including non-small cell lung cancer (NSCLC) (Yamane et al. 2015), breast cancer (Mazel et al. 2015), and melanoma (Gadiot et al. 2011, Kaunitz et al. 2017). Although PD-L1 is the predominant PD-1 ligand in physiologic settings, PD-L2 exhibits 2-6-fold higher binding affinity to PD-1 than PD-L1 (Cheng et al. 2013, Youngnak et al. 2003). However, to date there is no evidence indicating differential patterns of PD-1/PD-L1 versus PD-1/PD-L2 intracellular signaling activity.

2.2. PD-1 blockade as a tool to restore immune cell function in melanoma

The majority of TILs express PD-1 on their surface (Ahmadzadeh et al. 2009, Gros et al. 2014, Krönig et al. 2012). Interaction of TIL-expressed PD-1 with its ligands impairs antitumor immunity and creates an immunosuppressive milieu within the melanoma microenvironment (Ahmadzadeh et al. 2009). Consequently, PD-1 blockade restores immune cell function by increasing cytotoxic T lymphocyte (CTL) frequency and function, interferon- (IFN-) γ secretion by Ag-specific effector cells and, ultimately, augmented

melanoma cell lysis (Wong et al. 2007). Thus, blockade of the PD-1 pathway results in an enhancement of local antitumor immunity.

There are two anti-PD-1 blocking Abs approved as first-line therapy for advanced and metastatic cutaneous melanoma. In September 2014, Pembrolizumab (Keytruda, Merck & Co., Inc.) was the first anti-PD-1 blocking Ab approved in the US (Raedler 2015a). Three months later, the FDA granted approval to Nivolumab (Opdivo, Bristol-Myers Squibb™) for the treatment of metastatic and unresectable melanoma (Raedler 2015b). Both PD-1 inhibitors block the interaction of PD-1 with its ligands in two distinct ways: (1) The binding epitope regions of both Abs overlap with the PD-Ls binding site and (2) binding of both PD-1 inhibitors induces small conformational changes within the PD-Ls binding interface, which are incompatible with PD-Ls binding to PD-1 (Fessas et al. 2017, Lee et al. 2016). Thus, PD-1 blockade via Nivolumab or Pembrolizumab results in inhibition of PD-1/PD-Ls interaction via direct occupation and/ or steric blockade of the receptor binding epitope.

The constant region of the heavy chain (IgC_H) of both, Nivolumab and Pembrolizumab, is a heavy chain (IgH) γ_4-chain, thus defining the isotype of both inhibitors as human immunoglobulin (Ig) G4 (Lee et al. 2016). Accordingly, both Abs exhibit low affinity to the complement component 1q receptor (C1qR) and the Ig-crystallizable fragment receptor (FcR) and are therefore unable to efficiently mount Ab-dependent effector functions including Ab-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (Lee et al. 2019b, Wang et al. 2014). Consistently, treatment with anti-PD-1 blocking Abs results in fewer severe (grade 3 or 4) immune related adverse effect (irAE, 10-22%), compared to treatment with the anti-CTLA-4 Ab Ipilimumab (Yervoy, Bristol-Myers Squibb™, IgG1 isotype, 10-42%) (Sosa et al. 2018).

Two randomized, open label/ double blinded studies (Checkmate-037 for Nivolumab, KEYNOTE-002 for Pembrolizumab) were conducted to compare efficiency of anti-PD-1 blocking agents versus investigator's choice chemotherapy (ICC) in patients with advanced melanoma. Median OS was 16 months for the Nivolumab group versus 14 months for ICC (Larkin et al. 2018) and 13.4 months for the Pembrolizumab group versus 11 months for ICC (Hamid et al. 2017). Moreover, two-year survival rate was improved by PD-1 blockade versus ICC (38.7% versus 33.9% for Nivolumab (Larkin et al. 2018); 36% versus 30% for Pembrolizumab (Hamid et al. 2017)). Additionally, objective response rate (ORR) and

median duration of response were substantially higher for Nivolumab/ Pembrolizumab versus ICC (Hamid et al. 2017, Larkin et al. 2018). Comparison of immune checkpoint inhibitors targeting distinct receptors (i.e. PD-1 and CTLA-4) further suggests that patients are more likely to respond to anti-PD-1 than to anti-CTLA-4 Abs (pooled risk ratios (RR) for ORR 2.51) (Hao et al. 2017). Patients treated with Nivolumab or Pembrolizumab showed improved PFS and 1-year OS, compared to Ipilimumab (anti-CTLA-4, pooled RR for PFS 0.58; pooled RR for 1-year OS 1.37) (Hao et al. 2017). However, approximately four out of five patients with advanced melanoma still do not benefit from PD-1 therapy (Topalian et al. 2012). Therefore, the establishment of optimized biomarkers for predicting response to PD-1 blockade and of improved combinatorial strategies is urgently needed.

Interestingly, there is emerging evidence indicating that biomarkers may exhibit different predictive value regarding treatment outcome with Nivolumab versus Pembrolizumab. For example, PD-L1 expression by squamous-cell NSCLC correlates with response to Pembrolizumab, but not to Nivolumab (Rossi et al. 2020b). This suggests at least partial differences in the mechanism of action of Nivolumab versus Pembrolizumab.

2.3. Comparison of the PD-1 inhibitors, Nivolumab and Pembrolizumab

Nivolumab and Pembrolizumab are both anti-PD-1 blocking Abs of the IgG4 isotype with similar receptor-mediated mechanisms of action (Lee et al. 2016). However, there has been emerging evidence suggesting that Nivolumab and Pembrolizumab might at least partially differ in their ability to restore antitumor activity.

2.3.1. Differences in Nivolumab versus Pembrolizumab clinical activity

Nivolumab and Pembrolizumab have several FDA approved indications for the treatment of multiple cancer types (**Table 1+2 in supplements**) (Stewart 2021a, 2021b). While some approvals overlap, others are discordant. Pembrolizumab has been approved as first-line therapy of NSCLC with strong PD-L1 expression, whereas Nivolumab monotherapy failed to show improved PFS or OS, compared to ICC in NSCLC patients, including those with PD-L1^{high} tumors (CheckMate-026) (Reck et al. 2016, Socinski et al. 2016). Moreover, Pembrolizumab has uniquely been approved as second-line for multiple advanced cancer entities, e.g. cervical (Frenel et al. 2017), and endometrial carcinoma (Ott et al. 2018), whereas respective studies for Nivolumab have not yet been completed (cervical carcinoma: NCT02257528 (phase II), endometrial carcinoma: NCT03367741 (phase II)).

Besides the aforementioned differences in FDA approval, variations in the frequency and nature of irAEs have also been observed between Nivolumab- and Pembrolizumab-treated patients: Chu and colleagues reported that Nivolumab has lower incidence of grade ≥3 adverse effects, compared to Pembrolizumab (Chu 2017). Common serious grade 3 and 4 adverse reactions of Nivolumab are diarrhea, hyponatremia, abdominal pain, elevated aspartate transaminase levels, and increased lipase levels (Raedler 2015b). In contrast, Pembrolizumab-treated patients tend to exhibit high rates (≥2%) of renal failure, dyspnea, coughing, and pneumonia (Raedler 2015a).

2.3.2. Distinct molecular and structural aspects of Nivolumab and Pembrolizumab immunobiology

While Nivolumab is a fully human anti-PD-1 Ab, Pembrolizumab is of non-human origin (Lee et al. 2016). Thus, Pembrolizumab contains murine sequence-derived complementarity determining regions (CDRs), is classified as a humanized murine Ab (Harding et al. 2010, Lu et al. 2020) and, as such, is a foreign protein in the human host potentially provoking an alloimmune reaction (Harding et al. 2010). However, its constant region of Igs (IgC) and its variable framework has been replaced by human sequences during the process of humanization (Lu et al. 2020). Since most human antiglobulin responses are directed against the IgC of foreign Abs, humanization of Pembrolizumab results in a significantly less immunogenic product and improves *in vivo* tolerability (Harding et al. 2010). Accordingly, the human or humanized Abs, Nivolumab and Pembrolizumab, respectively, carry only a small risk of inducing immunogenicity (Agrawal et al. 2017, van Vugt et al. 2019).

Besides structural differences, Nivolumab and Pembrolizumab are reported to at least partially differ in their molecular interactions with PD-1. Although both Abs directly occupy the PD-L1 binding interface of PD-1, there is only partial overlap between the binding epitopes of Nivolumab versus Pembrolizumab (Fessas et al. 2017, Lee et al. 2016): While Nivolumab mainly interacts with the N-terminal extension, the FG loop and the BC loops, of PD-1, Pembrolizumab binding strongly depends on the highly flexible CD′ loop and the CC′ strands of PD-1 (Lee et al. 2016, Na et al. 2016, Tan et al. 2017, Zak et al. 2015). These molecular and structural differences of the anti-PD-1 Abs, Nivolumab and Pembrolizumab, might contribute to differential binding affinities to human PD-1. Indeed, compared to Pembrolizumab, Nivolumab is reported to bind PD-1 with a significantly lower affinity (Fessas et al. 2017).

Notably, the differences between both Abs could potentially translate into differential blocking efficiency and resultant variations in patient response. Yet, there appears to be some controversy about Ab-dependent differences in the clinical activity. Some report no significant drug-dependent difference in clinical activity, e.g. in real-world comparison of patients with HCC (Chen et al. 2023). However, others claim drug-specific response rates, including a significantly higher ORR to Pembrolizumab in patients with advanced NSCLC, compared to Nivolumab (Cui et al. 2020). In support, Lepir and colleagues reported that a patient with V600E mutated advanced melanoma pretreated with BRAF/MEK inhibitors and Ipilimumab experienced progression upon Nivolumab treatment. However, after switching the anti-PD-1 blocking agent to Pembrolizumab the patient showed a robust clinical response (Lepir et al. 2019).

In summary, comparison of molecular structure and clinical characteristics reveals several differences between the PD-1 blocking agents, Nivolumab or Pembrolizumab. However, extensive studies directly evaluating differences in lymphocyte function upon blockade with either inhibitor are still lacking. Moreover, because several cell types are known to express PD-1, cell type-dependent inhibitor effects and their significance in PD-1 therapy require elucidation. Since melanoma-infiltrating B cells have recently been reported to sustain inflammatory processes within the tumor and seem to be associated with response to anti-PD-1 Abs (Cabrita et al. 2020, Griss et al. 2019, Helmink et al. 2020), this MD thesis aims to compare the effects of both PD-1 Abs on restoring B cell function and their overall capacity to boost antitumor immunity.

3. B cell immunoregulatory functions in antitumor immunity

B cells are key players of the humoral immune response. By secreting Abs, B cells can directly neutralize Ags and mark pathogens for destruction by phagocytes (opsonization), cytotoxic cells (ADCC) and activation of the complement system (Forthal 2014). Besides functioning as Ab-secreting cells (ASCs), B cells can modulate both innate and adaptive immunity in several additional ways: (1) They can act as professional Ag-presenting cells, presenting Ags via the MHC II to $CD4^+$ T cells, thereby enhancing T cell-mediated immunity (Chalasani & Rothstein 2014). (2) They can also promote the formation of tertiary lymphoid structures (TLS) and the migration and polarization of innate and adaptive immune cells via the secretion of pro-inflammatory cytokines and chemokines (Lund 2008), and (3) function as regulatory B cells (Bregs) to dampen immune responses via the secretion of interleukin-(IL-) 10, IL-35, and transforming growth factor-β (TGF-β) (Rosser & Mauri 2015). (4) Finally, B cells can contribute to activation-induced cell death and cellular cytotoxicity mechanisms via the expression of death-inducing molecules, including IFN-*γ*, FS-7-associated surface antigen (FAS) receptor/ CD95/ apoptosis antigen 1 (Apo1) ligand (FASL), tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and granzyme B (Jahrsdorfer et al. 2006, Lundy 2009).

3.1. Maturation and activation of B cells

The process of B cell maturation and activation occurs in multiple stages: First, hematopoietic precursors differentiate into transitional/ immature B lymphocytes within the bone marrow (Perez-Andres et al. 2010). In the periphery, transitional B cells then develop into fully mature, naïve B cells that express unique Ag-specific IgD and IgM molecules on their surface (Carsetti et al. 2004, Perez-Andres et al. 2010, Tobón et al. 2013). After migration into a secondary lymphatic organ, naïve B cells get exposed to a diverse pool of Ags (Berkowska et al. 2011). Upon encountering their cognate Ag, naïve B cells become activated. B cell activation occurs in a thymus-dependent (TD) or thymus-independent (TI) manner (Berkowska et al. 2011, Liao et al. 2017). TD Ags are immunogens that stimulate both Ag-specific B cells and $CD4^+$ follicular T helper cells (fT_H), leading to the formation of germinal center (GC) reactions (De Silva & Klein 2015). During GC reactions, extensive clonal expansion and somatic hypermutations (SHM) lead to affinity maturation and Ig class switching (Mesin et al. 2016). Follicular dendritic cells (fDCs) and fT_H strictly regulate this process via co-stimulatory signals, including CD40 ligand (CD40L), inducible T cell co-

stimulator (ICOS), and secretion of IL-4 and IL-21 (Tangye et al. 2002, Vu et al. 2008). Consequently, the GC reaction gives rise to two types of B cells: (1) high-affinity ASCs, including plasma blasts (PBs) and $CD138^+$ plasma cells (PCs), (Kaminski et al. 2012) and (2) memory (Mem) B cells which ensure sustained immune protection (Carsetti et al. 2004, Seifert & Küppers 2016).

In contrast, the diverse group of TI Ags usually exhibits highly repetitive epitopes or contains Toll-like receptor (TLR) ligands which cannot be recognized by T cells (Carsetti et al. 2004). Yet, strong B cell receptor (BCR) signaling bypasses the necessity of T cell help and triggers B cell proliferation, formation of ASCs, early IgM production, and gives rise to IgM^+ Mem B cells (Berkowska et al. 2011, Carsetti et al. 2004). TI responses coincide with high titers of low affinity IgM Abs and contribute to rapid immunity against numerous pathogens (Fagarasan & Honjo 2000, Weller et al. 2004).

3.2. Memory B cells` functions in adaptive immunity

Most B cells within human peripheral blood and lymph nodes are Ag-experienced cells. They can be distinguished from Ag-inexperienced IgD^+ $CD27^-$ naïve B cells by an increased lifespan and a faster and stronger response upon stimulation (Berkowska et al. 2011). These Ag-experienced B cells can be classified into multiple functional subsets: (1) In tonsils, B cells undergoing a GC reaction are reported to upregulate CD38 (De Silva & Klein 2015). Upon isotype switching and affinity maturation in secondary lymphatic organs, $CD38^+$ $IgD^–$ GC B cells then differentiate further (Kaminski et al. 2012, Seifert & Küppers 2016). (2) Most B cells derived from TD responses have experienced SHMs and class switching (IgA^+ or IgG^+) during GC reactions and express the memory marker CD27 on their surface (Berkowska et al. 2011). These switched memory (Swi Mem) B cells express a high affinity BCR and significantly contribute to rapid responses against subsequent encounter with their cognate Ag (Kaminski et al. 2012). (3) A small subset of peripheral B cells is characterized as IgD^+, $CD27^+$ non-switched memory (NS Mem) B cells (Seifert & Küppers 2016). There is evidence that they develop independently from the GC reaction and may undergo SHMs without T cell interaction (Berkowska et al. 2011, Kaminski et al. 2012). NS Mem B cells resemble splenic marginal zone B1b cells in that they are linked to rapid initial immunity and contribute to carbohydrate-reactive Ab responses to encapsulate bacteria (Kaminski et al. 2012). (4) Approximately 5% of peripheral $CD19^+$ B cells can be classified as IgD^- $CD27^-$ Mem B cells, also known as double negative memory (DN Mem) B cells (Wei et al. 2007).

This B cell subset exhibits high levels of surface IgA or IgG3 and fewer SHMs within its variable region (Berkowska et al. 2011, Seifert & Küppers 2016). DN Mem B cells are thus reported to arise from failed GC reactions (Wu et al. 2011) or develop independently from GC (Berkowska et al. 2011), and mainly contribute to primary immune responses (Wu et al. 2011). Interestingly, they are associated with either immunosuppressive phenotypes and/or autoimmune diseases, such as systemic lupus erythematosus (Wei et al. 2007). (5) While ASCs within the bone marrow are mainly classified as $CD138^+$ long-lasting PCs, approximately 50% of peripheral ASCs transiently proliferate and contribute to a rapid boost of Abs against cognate Ags, yet do not participate in the secretion of long-lasting Abs (Kaminski et al. 2012).

3.3. Relevance of B cells in antitumor immunity

While the importance of T cells in antitumor immunity is well established, little is known about B cell functions within the TME. However, the emerging role of B cells in modulating tumor-specific immune responses is being increasingly appreciated. Due to the heterogeneous functions of the diverse B cell pool, tumor-associated B cells (TABs) are reported to contribute to both tumor-promoting and -inhibitory immune responses **(Figure 1)** (Chiaruttini et al. 2017).

IL-10 secreting-TABs are associated with the promotion of tumorigenesis and cancer progression (Chiaruttini et al. 2017). They are reported to downregulate IFN-γ production by CTLs and NK cells, impair $CD8^+$ T cell-dependent cancer cytotoxicity, induce myeloid-derives suppressor cells (MDSCs), and support the conversion of $CD4^+$ T cells into tolerogenic Tregs (Sarvaria et al. 2017, Schwartz et al. 2016). However, B cells also prevent tumorigenesis and contribute to antitumor immunity in several ways: (1) TABs can produce high affinity and isotype class switched Igs (Cipponi et al. 2012, Quan et al. 2016, Simsa et al. 2005). Specific antitumor Igs may boost opsonization, ADCC and activation of the complement system, thereby initiating humoral antitumor immunity (Gilbert et al. 2011, Hazenbos et al. 1998, Schwartz et al. 2016). (2) TABs are reported to also co-localize with $CD8^+$ T cells within TME and promote T cell-dependent antitumor immunity through their role as professional Ag-presenting cells (DiLillo et al. 2010, Tsou et al. 2016). (3) B cells further secrete effector cytokines, including IFN-γ, that promote tumor-specific immunity (Castro et al. 2018). (4) Finally, B cells can directly kill tumor cells through TRAIL/Apo-2L-dependent mechanisms, FASL expression and/or granzyme B secretion, thus participating in

direct cancer cytotoxicity (Jahrsdorfer et al. 2006, Kemp et al. 2004). Consistently, the presence of TABs correlates with favorable outcome in multiple cancers, including breast cancer (Iglesia et al. 2014, Mahmoud et al. 2012, Schmidt et al. 2008), NSCLC (Al-Shibli et al. 2008, Lohr et al. 2013), ovarian cancer (Iglesia et al. 2014, Nielsen et al. 2012), and cervical squamous cell carcinoma (Nedergaard et al. 2008). Collectively, TABs may exert both pro-tumorigenic and tumor-inhibitory effects. Therefore, the composition of B cell subsets in the TME determines their overall effect on tumor development and progression.

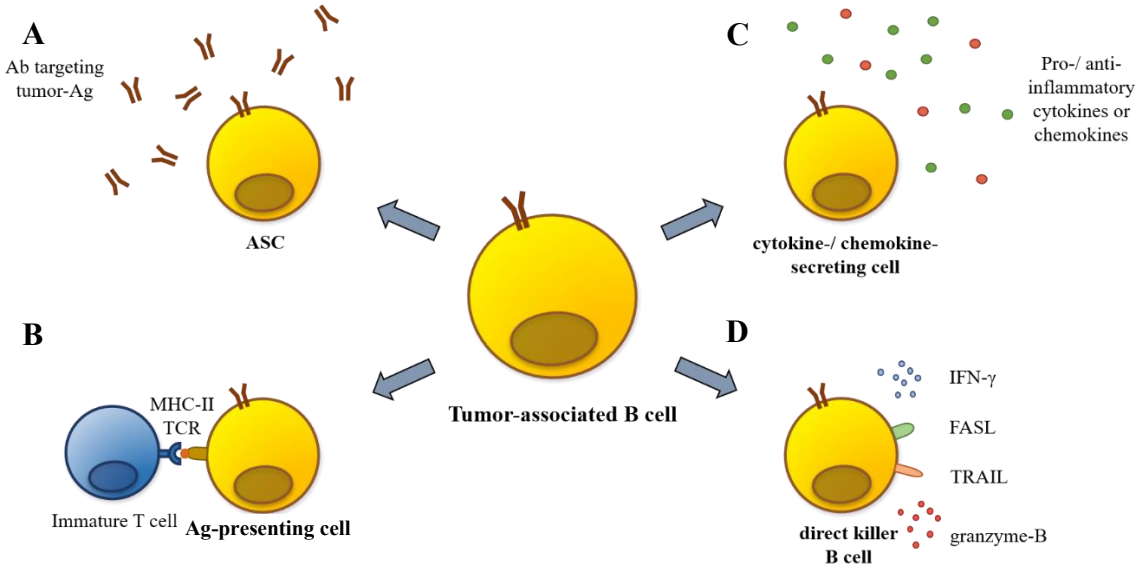

Figure 1: Function of TABs
TABs contribute to antitumor immunity via **(A)** the secretion of Ab targeting tumor Ag, **(B)** presenting tumor Ag to immature T cells thus activating them, **(C)** the secretion of pro-/ anti-inflammatory cytokines and/ or chemokines, and **(D)** direct killing of tumor cells. *Adapted from:* B cells and the humoral response in melanoma: The overlooked players of the tumor microenvironment. Chiaruttini, G. et al. OncoImmunology. 2017

In metastatic melanoma, TABs represent up to 33% of all infiltrating immune cells (Erdag et al. 2012). Yet, their significance in cutaneous melanoma has only recently gained some attention, and evidence for the potential prognostic and predictive value of B cells in this malignancy is rapidly growing, but still limited. Phenotypical analyses reveal that melanoma patients exhibit higher levels of Ig subclass switched B cells and clonal TAB expansion, compared to B cells in healthy skin, suggesting the presence and potential importance of B cell-dependent antitumor immunity in melanoma (Saul et al. 2016). Indeed, Iglesia and colleagues reported that B cell immune signatures are associated with improved OS in cutaneous melanoma. Moreover, BCR abundance and diversity of the BCR variable region is linked to favorable melanoma prognosis (Iglesia et al. 2016).

More recently, there has been an emerging body of literature shedding light into the mechanisms through which B cells shape antimelanoma immunity: (1) Patients with metastatic cutaneous melanoma develop Abs against cancer Ags, thereby boosting humoral antitumor immunity (Selitsky et al. 2019). Consistently, Griess and colleagues reported that *ex vivo* exposure of TABs to melanoma Ags induces PB-like B cells. Their presence within melanoma is reported to be associated with increased inflammation and longer OS in patients (Griss et al. 2019). (2) Stimulation of TABs with melanoma Ags leads to a release of pro-inflammatory and/or immunosuppressive cytokines, such as IL-1b, IL-6 and IL-35, and upregulation of BCR signaling pathways can thus directly sustain antitumor immunity (Griss et al. 2019). (3) There is also evidence that activation of TABs not only boosts B cell-derived antitumor immunity, but also enhances T cell-dependent antimelanoma responses: Cabrita and colleagues reported that B cell-rich melanomas also harbor higher frequencies of naïve and/ or memory T cells. Notably, the co-occurrence of melanoma-associated $CD8^+$ T cells and B cells contributes to the formation of TLSs and the induction of highly functional T cells and is thus associated with enhanced cellular immunity and improved survival in melanoma patients (Cabrita et al. 2020).

3.4. Role of PD-1 in regulating B cell immunity

Since B cells shape immune responses in multiple ways and substantially influence the efficiency and outcome of antitumor immunity, there is a pressing need to understand regulatory mechanisms determining TAB function and fate. For example, the immune checkpoint inhibitor, PD-1, is a key regulator of B cells within the TME (Thibult et al. 2012). Stimulation of B cell-PD-1 results in inhibitory signals, similar to the ones observed in T cells: Phosphorylation of ITSM/ITIM recruits SHP-2 to B cell-PD-1, followed by dephosphorylation of key BCR signaling mediators, resulting in dampened BCR activity (Okazaki et al. 2001). Thus, PD-1/PD-Ls interactions negatively regulate B cell functions. Consequently, PD-1 deficiency or pathway blockade is associated with enhanced B cell proliferation, expression of the co-stimulatory ligands, CD80 and CD86, and secretion of the pro-inflammatory cytokine, IL-6 (Nishimura et al. 1998, Thibult et al. 2012).

Because PD-1 shows varying expression between distinct B cell subsets, its influence on B cell activation and proliferation differs. PD-1 negatively regulates major functions essential for GC reactions. In fact, the downregulation of PD-1 is a crucial step in initiating an efficient GC reaction (Thibult et al. 2012). In contrast, Mem B cells exhibit higher levels

of PD-1 on their surface. NS Mem B cells, in particular, which develop in a GC-independent manner and determine TI B cell responses, express the highest levels of PD-1 among all established B cell subtypes (Thibult et al. 2012). Consistently, in PD-$1^{-/-}$ knockout mice, stimulation with TI Ags leads to significantly higher levels of B cell activation and increased IgG3 secretion, compared to PD-1 wildtype mice (Nishimura et al. 1998). Moreover, PD-1/PD-Ls interactions in human B cells were found to suppress Ag-specific B1b cell expansion (Haas 2011). Consistently, PD-1 blockade results in increased Ig production against TI-2 Ags boosting TI immunity.

Of note, PD-1^{+} B cells are prevalent in the TME of melanoma patient lesions (Tirosh et al. 2016). Thus, B cell-directed PD-1 blockade potentially contributes to the efficiency of immune checkpoint therapy. In support, the presence of TABs correlates with anti-PD-1 therapeutic outcome in patients with cutaneous melanoma and treatment with anti-PD-1 blocking Abs also leads to increased B cell frequency within TME (Amaria et al. 2018, Cabrita et al. 2020, Griss et al. 2019, Helmink et al. 2020). More recently, Helmink and colleagues reported that tumors from responders to PD-1 immune checkpoint inhibition exhibit significantly higher frequencies of Mem B cells, whereas lesions from non-responders display elevated numbers of naïve B cells (Helmink et al. 2020). However, the precise mechanisms of how anti-PD-1 blocking Abs influence B cell activation and function within the melanoma microenvironment remain uncertain. Therefore, the role of PD-1^{+} TABs during immune checkpoint therapy requires further investigation. Insights generated as part of such studies might improve ORRs and help optimize biomarkers for predicting and monitoring patient benefit.

4. Protein glycosylation

Glycosylation of proteins is one of the most common and complex forms of post-translational protein modification, which regulates many biological processes and contributes to the pathobiology of several diseases (Ohtsubo & Marth 2006). Glycoproteins can be classified into two types based on the nature and linkage of the glycan to the polypeptide backbone: *O*-Glycans are covalently bound to an oxygen-atom of a Thr or Ser residue, whereas *N*-Glycans are attached via a covalent nitrogen bond to an asparagine (Asn) in the sequence context Asn-X-Ser/Thr (Ohtsubo & Marth 2006, Reis et al. 2010). Glycosylation significantly modulates structure and biophysical properties of scaffold proteins, affecting folding and rigidity (Larkin & Imperiali 2011, Varki 2016). Moreover, post-translational addition of carbohydrates to either Asn (*N*-linked) or Ser/Thr residues (*O*-linked) can modify traditional receptor-ligand interactions, thereby determining a variety of biological processes, including cell signaling, differentiation, trafficking, and regulation of immune responses (Larkin & Imperiali 2011, Marth & Grewal 2008). Indeed, there has been emerging evidence that *N*-glycans can shape the efficiency of various immunotherapeutic regimens. Accordingly, *N*-glycoengineering strategies might be exploited to boost the effectiveness of current immunotherapeutics (Buettner et al. 2018, Solá & Griebenow 2010). Interestingly, the immune checkpoint receptor, PD-1, holds multiple *N*-glycosylation sites, including in its IgV-like domain or PD-Ls binding domain, and is heavily *N*-glycosylated *in vivo* (Agata et al. 1996, Ishida et al. 1992). Thus, the affinity to and effectiveness of anti-PD-1 blocking Abs might be influenced by the *N*-glycans decorating the PD-1 surface. In this MD thesis, we aim to assess the effects of surface *N*-glycosylation of B cells on B cell-specific PD-1 blockade.

4.1. *N*-Glycosylation and its role in immunity

N-Glycosylation requires the pre-assembly of *N*-glycans within the endoplasmic reticulum (ER) before these glycans get transferred en bloc from a lipid donor (dolicholphosphate, Dol-P) to nascent proteins (Helenius et al. 2001, Helenius & Aebi 2004). The construction of such oligosaccharide precursors is initiated by the transfer of a *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P) to Dol-P resulting in Dol-P-P-GlcNAc (Helenius & Aebi 2004, Stanley et al. 2015-2017). This initial step is catalyzed by dolichylphosphate *N*-acetylglucosamine-phosphotransferase 1 (DPAGT1), a GlcNAc-1-phosphotransferase that can be inhibited by tunicamycin *in vitro* to completely abolish intracellular *N*-glycosylation (Stanley et al. 2015-2017). Subsequent addition of GlcNAc and guanosine

diphosphate mannose (GDP-Man) generates $Man_5GlcNAc_2$-P-P-Dol (Helenius & Aebi 2002, Larkin & Imperiali 2011). Upon flipping to the luminal side of the ER, the glycan precursor is elongated via the addition of mannose (Man) and glucose (Glc) residues, originating the mature *N*-glycan precursor $Glc_3Man_9GlcNAc_2$-P-P-Dol (Helenius & Aebi 2002, Stanley et al. 2015-2017). The membrane-associated enzyme complex, oligosaccharyltransferase (OST), then catalyzes the transfer of this mature precursor to an Asn residue of a nascent protein (Helenius et al. 2001, Stanley et al. 2015-2017). Next, the glycan structure is trimmed by glucosidases and mannosidases within the ER (Larkin & Imperiali 2011). Upon translocation to the *cis*-Golgi, further removal of Glc and Man residues results in a uniform $Man_5GlcNAc_2$Asn *N*-glycan structure (Stanley et al. 2015-2017). Notably, the metabolic inhibitor, kifunensine, specifically suppresses the activity of the ER-associated mannosidases *in vitro*, thus preventing the consecutive modifications of *N*-glycan structures (Helenius & Aebi 2004). During the *cis-trans* Golgi movement of glycoproteins, the synthesis of hybrid structures is initiated via the addition of a GlcNAc residue to the C-2 of the α1,3-Man in the $Man_5GlcNAc_2$ structure (Helenius et al. 2001, Stanley et al. 2015-2017). This process is catalyzed by the *N*-acetylglucosaminyltransferase-1 (GlcNAcT-I/MGAT1) (Schachter 1991, Stanley et al. 2015-2017). Subsequently, the resulting structures are further elongated and give rise to hybrid *N*-glycans. However, the $GlcNAcMan_5GlcNAc_2$ structure may also lose the terminal α1,3-Man and α1,6-Man residues via activation of α-mannosidase II (Stanley et al. 2015-2017). *In vitro* inhibition of this Golgi α-mannosidase II via swainsonine prevents the initiation of complex *N*-Glycans synthesis (Elbein et al. 1981). Subsequent transfer of GlcNAc onto the free terminal Man is catalyzed by α1,6-mannosyl-glycoprotein 2-β-*N*-acetylglucosaminyltransferase (MGAT2), initiating the formation of complex *N*-glycans (Stanley et al. 2015-2017).

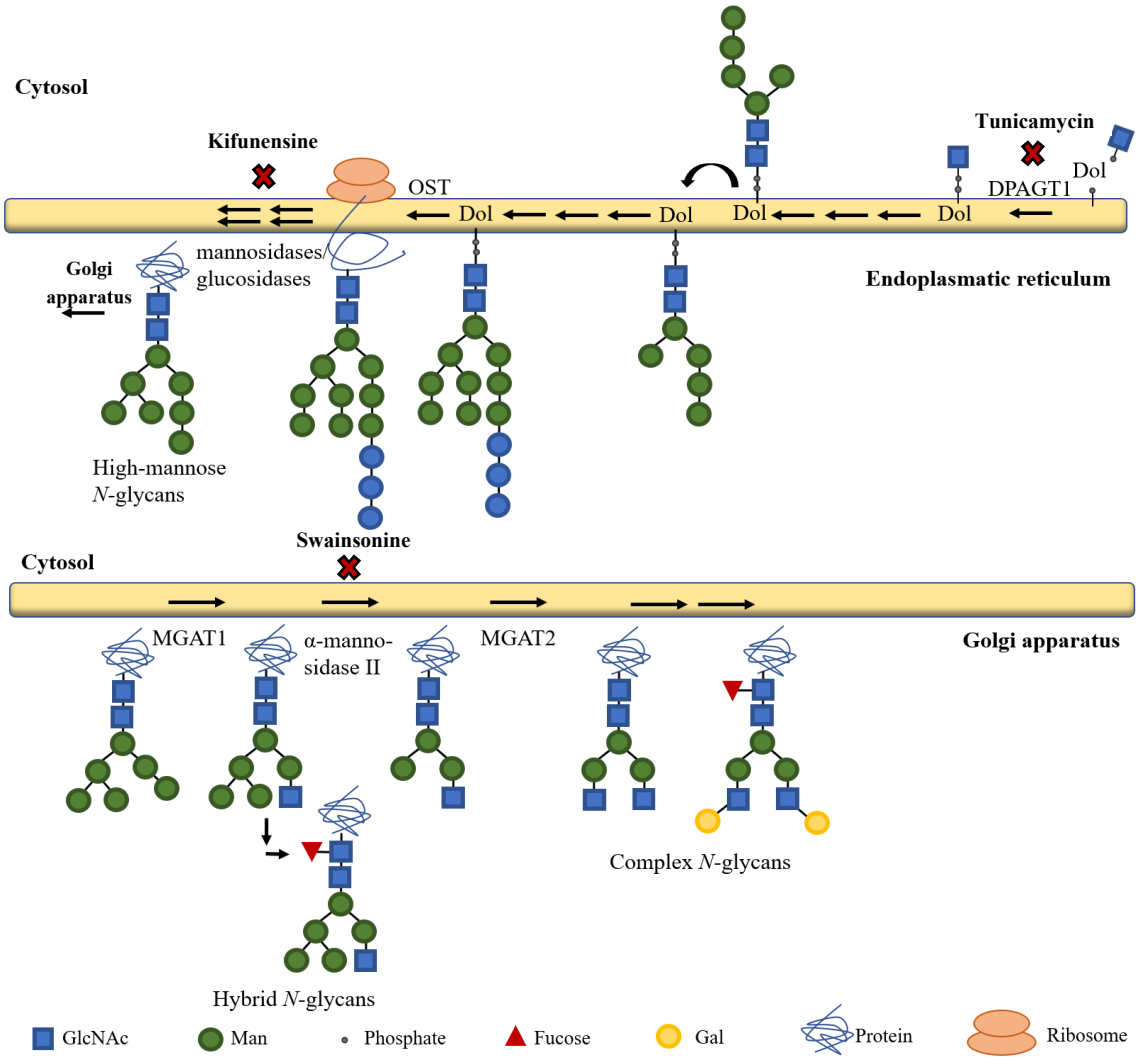

Figure 2: *N*-linked glycoprotein synthesis pathway
The synthesis of *N*-glycoproteins is initiated via the transfer of a core *N*-glycan structure from the Dol-P precursor to an Asn residue of a nascent protein. Subsequent modification of the glycan structures is carried out within the ER and the Golgi apparatus. The addition of GlcNAc to Man present on the α1,3-arm or the α1,6-arm of the core structure gives rise to hybrid or complex *N*-glycans. *Adapted from:* Stanley P, Taniguchi N, Aebi M. N-Glycans. 2017. In: Varki A, Cummings RD, Esko JD, et al., editors. Essentials of Glycobiology [Internet]. 3rd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2015-2017. Chapter 9. Figure 9.3. and 9.4. Available from: https://www.ncbi.nlm.nih.gov/books/NBK453020/ doi: 10.1101/glycobiology.3e.009

4.2. *N*-Glycosylation of PD-1

PD-1 is a glycoprotein containing four Asn residues in its IgV-like domain (N47, N58, N74, and N116), which serve as potential *N*-glycosylation sites (Ishida et al. 1992). In fact, several studies have confirmed the presence of *N*-Glycans on the PD-1 protein backbone (Agata et al. 1996, Chen et al. 2019, Okada et al. 2017). Interestingly, structural analyses revealed that *N*-glycosylation sites are located within the following PD-1 protein structures: BC loop

(N58), CC′ loop (N74), EF loop (N116), and the beginning of the B strand (N49). Whether any of these *N*-glycosylation sites overlap with PD-Ls binding epitopes is the subject of ongoing debate (Fessas et al. 2017, Lee et al. 2016, Liu et al. 2019). While the requirement of PD-L1 glycosylation for efficient binding has been well established (Lee et al. 2019a, Verdura et al. 2020), Liu et al. suggest that ligand binding structures within the PD-1 receptor may not harbor *N*-glycosylation sites (Liu et al. 2019), while others propose that N58 (Sun et al. 2020) and/or N74 may fall within the PD-L1 binding epitope (Fessas et al. 2017). Similarly, there is ongoing debate as to whether or not glycosylation might affect PD-1 inhibitor affinity (Fessas et al. 2017, Lee et al. 2016, Tan et al. 2017). A study by Lee and colleagues suggests that there may not be any glycosylation sites within the binding epitopes for neither Nivolumab nor Pembrolizumab (Lee et al. 2016). On the other hand, Tan and colleagues propose that N58 lies within the Nivolumab binding site (Tan et al. 2017). Similarly, Fessas and colleagues show that N74 is located in close proximity to the Pembrolizumab binding epitope (Fessas et al. 2017). Clearly, there appears to be some controversy as to what structures on the PD-1 receptor are actually bound by the two blocking agents. However, the mere fact that several *N*-glycan structures are present on the PD-1 protein backbone raises the possibility that glycosylation might affect ligand and/or inhibitor recognition and thus therapeutic efficacy. While Tan and colleagues did not observe differences in Nivolumab binding to the naked PD-1 peptide upon N58-specific de-glycosylation (Tan et al. 2017), it remains to be determined whether glycosylation of appropriately folded PD-1 expressed by native tissues modulates inhibitor recognition.

4.3. Glycosylation in humoral immunity

B cell immunity is highly dependent on the proper glycosylation of B cell surface receptors and Igs. Changes in B cell glycosylation modify biophysical properties of their protein scaffolds and/or alter binding determinants for a vast array of endogenous glycan binding proteins (lectins) with pivotal functions in immune regulation.

4.3.1. N-Glycosylation of B cells

Multiple cell surface receptors expressed by B cells are highly decorated with complex *N*-glycan structures that exhibit high levels of type 2 *N*-acetyllactosamine (LacNAc) chains of 2-4 units, also called poly-*N*-acetyllactosamine (poly-LacNAc) (Giovannone et al. 2018). *N*-glycans terminated with poly-LacNAc structures exhibit strong binding affinity to galectins,

also called S-type lectins, which are broadly expressed in immune and stromal tissues and participate in multiple immunoregulatory functions (Stanley & Cummings 2015-2017). Binding of galectins to poly-LacNAcs on B cells largely promotes development, activation, differentiation, and effector function via the enhancement of calcium flux, upregulation of activation markers, CD69 and CD86, and induction of B cell proliferation (Tsai et al. 2014). However, galectin binding is also reported to potentially downregulate B cell activity by inhibiting Akt signaling and promoting cell death (Cao et al. 2018, Giovannone et al. 2018).

4.3.2. N-Glycosylation of immunoglobulins

IgGs contain one *N*-glycosylation site within each IgC_H (Reily et al. 2019). Moreover, approximately 20% of IgGs exhibit an additional *N*-glycosylation motif within the variable region of the heavy chain (IgV_H) (Reily et al. 2019). Typically, both sites are decorated with complex bi-antennary glycans and core fucosylation – an addition of a fucose residue to the innermost GlcNAc (Higel et al. 2016, Reily et al. 2019). These *N*-glycans can affect structure and effector function of Abs in multiple ways, thus significantly influencing humoral immunity. Specifically, *N*-glycans of the IgCs can control ADCC and CDC by determining the binding to FcR and C1qR. While Igs with small *N*-glycans and low levels of fucose residues exhibit a high affinity to FcR and thus boost ADCC, an increase in terminal galactose (Gal) content enhances Ig binding to C1qR and promotes CDC (Higel et al. 2016, Reily et al. 2019). Moreover, *N*-glycans decorating the IgV may promote humoral immunity by adding an additional layer of Ab diversity, increasing Ab stability, or extending *in vivo* Ab half-life. (Koers et al. 2019, van de Bovenkamp et al. 2018).

Strikingly, glycosylation of Igs might lead to differential clinical outcomes and/or adverse events from immune checkpoint inhibition or other forms of cancer therapy. For instance, the presence of a α1,3-Gal sugar within the glycan of the Ag binding fragment of Cetuximab (Erbitux) is associated with severe anaphylactic reactions to the Ab, Cetuximab lacking this sugar is much less immunogenic (Chung et al. 2008).

4.3.3. Sialyation in humoral immunity

Sialic acids (Sias) are a diverse group of nine-carbon backbone terminal sugar moieties that are typically found attached to the outermost end of *N*-Glycans, *O*-Glycans and glycosphingolipids of all cell types (Varki et al. 2015-2017). The enzymes responsible for the addition of the terminal Sias to the acceptor glycan are called sialyltransferases. They transfer Sias from a donor substrate, *N*-acetylneuraminic cytidine monophosphate, to the terminal positions of acceptor glycans (Bhide & Colley 2017). Depending on their nature, sialyltransferases form multiple α-linkages between the C-2 of Sias and the underlying sugars (Harduin-Lepers et al. 2005). Most commonly, they utilize the C-3/C-6 positions of Gal residues or the C-6 position of GalNAc of the underlying sugar, forming α2,3- and/or α2,6-linkages (Varki et al. 2015-2017). Moreover, there are multiple enzymes that cleave Sias from the glycan scaffold, so called sialidases. Depending on the nature of the linkage they cleave, sialidases are referred to as α2,3-/α2,6-/α2,8-sialidases (Varki et al. 2015-2017).

Sias affect biophysical features by masking underlying structures and preventing cell-cell interaction via the repulsive effects of their negative charge (Varki & Gagneux 2012). Additionally, these acid sugars serve as receptors for specific lectins, including selectins and Sia-binding immunoglobulin-type lectins (Siglecs), and are reported to modulate multifaceted roles in immunity, such as cell migration, pathogen recognition, Ig function, and tumor immune evasion (Varki 2008, Varki & Gagneux 2012). Interestingly, B cells can express high levels of the ST6 β-galactoside α2,6-sialyltransferase 1 (ST6Gal1), an enzyme catalyzing the attachment of α2,6-linked Sias to complex *N*-Glycans. Consequently, mature B cells are predominantly decorated with α2,6-linked Sias that influence B cell immunity in two prominent ways (Irons & Lau 2018): (1) ST6Gal1-dependent sialyation of IgGs leads to an increased binding affinity to the inhibitory FcR, FcγRIIb. Thus, it significantly downregulates inflammation (Ohmi et al. 2016, Pfeifle et al. 2017, Samuelsson et al. 2001). (2) The membrane-bound glycoprotein, CD22, one of 16 members of the Siglec family that recognizes α2,6-linked Sias, is a potent regulator of B cell activation. When forming a complex with the BCR, its intracellular ITSM is phosphorylated, followed by SHP-1 recruitment. Consequently, signaling via the CD22 Siglec dampens BCR signaling and is thus essential for self-tolerance (Crocker et al. 2007, Varki & Angata 2005).

Understanding how *N*-glycosylation influences immunity is crucial to elucidate the mechanisms underlying protective immunity. Additionally, it may provide key insights for

the development of novel immunotherapeutic approaches. Yet, studies assessing the influence of global cell surface glycosylation on binding of PD-1 to PD-Ls and of PD-1 to anti-PD-1 Abs are still lacking. Thus, there is pressing need to evaluate surface *N*-glycosylation on PD-1 expressing cells and to investigate its influence on PD-1 binding to its ligands and/or PD-1 blocking agents.

Rationale and Objectives

The PD-1 receptor is an inhibitory immune checkpoint expressed on various lymphocyte subsets, as well as myeloid and cancer cells. Engagement of PD-1 by its ligands inhibits immune effector functions to maintain peripheral tolerance. In the context of cancer, the PD-1 pathway compromises antitumor immunity. Not surprisingly, the anti-PD-1 blocking Abs, Pembrolizumab and Nivolumab, have produced unprecedent clinical efficacy in patients with cancers of various etiology and have been approved as first-line therapy for the treatment of advanced melanoma. However, most patients still do not benefit from PD-1 checkpoint inhibition and predictive biomarkers of response require further optimization. Although both anti-PD-1 inhibitors block PD-Ls binding and bind to overlapping epitopes, there is emerging evidence supporting drug-dependent differences in therapeutic response to either inhibitor. Indeed, Nivolumab and Pembrolizumab have overlapping, yet discordant FDA approvals. For instance, in phase III clinical trials, patients with advanced NSCLC showed clinical benefit to Pembrolizumab monotherapy as first line treatment, but not to Nivolumab monotherapy, compared to ICC. There has also been a recent case report of a patient who experienced melanoma progression during Nivolumab treatment but produced a durable response to subsequent Pembrolizumab therapy.

Thus far, structural and mechanistic studies aimed at defining PD-1 pathway functions in tumor progression and therapy have mainly focused on T cell-PD-1. However, various other cell types present in the TME also express PD-1 and might therefore be targeted by PD-1 blocking agents. Recent studies have highlighted B cells as promising biomarkers of response to PD-1 checkpoint blockade. Yet, little is known about PD-1 inhibitor effects on B effector functions. Here, we hypothesize that blockade of B cell-PD-1 might contribute to the anticancer efficacy of immune checkpoint regimens in melanoma patients. Based on our preliminary findings of variations in tumoral B cell frequency in responders to Nivolumab versus Pembrolizumab, we further posit that B cells might contribute to differences in clinical activity between said PD-1 blocking agents. Thus, we designed the following leading questions of this MD thesis project:

Aim 1 - To define the binding affinity of anti-PD-1 Abs to B cells: To characterize the significance of B cell-specific PD-1 blockade, we aim to define the binding of Nivolumab and Pembrolizumab to B cell-expressed PD-1. Moreover, we aim to assess whether Nivolumab and/or Pembrolizumab target specific B cell subsets or B cells harboring distinct glycomic features.

Aim 2 - To assess functional consequences of B cell-directed PD-1 blockade: To investigate the effects of B cell-specific PD-1 blockade on antitumor immunity, we aim to compare the ability of Nivolumab versus Pembrolizumab to block B cell ligation and/or effector functions, including B cell proliferation, B cell maturation status, and secretion of cytokines and Abs.

Material and Methods

1. Peripheral blood mononuclear cells (PBMCs), tonsillar mononuclear cells, and clinical melanoma biopsies

Collection of mononuclear cells and clinical melanoma biopsies was performed under protocols approved by the Institutional Review Boards of Brigham & Women's Hospital (BWH), Boston Children's Hospital and University of Zurich, in agreement with the Declaration of Helsinki.

1.1. Isolation of human PBMCs

Ficoll-plaque density gradient centrifugation (GE Healthcare Life Sciences, Marlborough, MA, USA) was used as instructed to isolate human PBMCs from peripheral blood samples. Briefly, defibrinated peripheral blood from healthy donors, obtained from the Blood Donor Center of Boston Children's Hospital, was transferred into a 50 ml polystyrene centrifuge tube (Falcon^{TM}, Fisher Scientific, Bradford, MA, USA) and diluted 1:1 (v/v) with Dulbecco's phosphate-buffered saline (DPBS, gibco, Waltham, MA, USA) supplemented with 2% (v/v) heat inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, Bradford, MA, USA). Ficoll-plaque was added to a fresh Falcon tube. Next, diluted blood suspension was carefully overlaid on the Ficoll phase and centrifuged at 2200 rpm for 20 min at room temperature with the break-mechanism turned off. During high-speed centrifugation, cells were separated into multiple layers according to their density and capability to sediment in the presence of Ficoll-plaque. PBMCs were carefully collected, transferred to a new Falcon tube, and washed twice. To lyse residual erythrocytes, cells were resuspended in 10 ml of Red blood cell lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2-Ethylenediaminetetraacetic acid (EDTA, gibco)) at 4 °C for 10 min while shaking. Cells were washed in DPBS supplemented with 2% (v/v) heat inactivated FBS. The purity was routinely checked by flow cytometry and was typically >90%.

1.2. Isolation of circulatory B cells

Circulatory B cells were isolated from PBMCs by negative selection using the B Cell Isolation Kit II, human (MACS Miltenyi Biotech, Inc., Somerville, MA, USA), following the manufacturer's instructions. Briefly, freshly isolated PBMCs were resuspended in sterile ice-cold MACS buffer (DPBS with 0.5% (v/v) bovine serum albumin (Sigma-Aldrich, Inc.,

St Louise, MO, USA) and 2 mM EDTA, pH 7.2) and incubated at 4 °C for 5 min with biotinylated Abs recognizing CD2, CD14, CD16, CD36, CD43, CD235a. Subsequently, anti-biotin microbeads suspension was added to the cell solution and incubated at 4 °C for 10 min. Cells were washed once and resuspended in MACS buffer. Next, cell suspension was passed through a magnetic LS column and column was washed twice with MACS buffer. While biotinylated fraction was retained within the column, unlabeled B cells pass through the column and were collected in the flow-through. The purity of isolated B cells was routinely assessed by flow cytometry staining and was typically >90%.

1.3. Tonsillar samples

Previously isolated human tonsillar B cells were kindly provided by Associate Professor Charles J. Dimitroff, PhD, Department of Dermatology BWH, Harvard Medical School.

1.4. Digestion of melanoma samples

Frozen biopsies of human metastatic melanoma were kindly provided by the division of dermatology at the University Hospital of Zurich. Biopsies were quickly thawed in a 37 °C water bath for 20 sec and placed on a petri dish. Prewarmed Roswell Park Memorial Institute (RPMI) Media (gibco) was added until sample was completely thawed. Melanoma biopsies were then covered with RPMI media containing 2.4 U/ml dispase II protease (Sigma-Aldrich, Inc.) and mechanically dissociation using single-edged razor blades. Samples were incubated at 37 °C and 5% CO_2 for 2 hrs with shaking. Next, predigested melanoma biopsies were washed once and resuspended in collagenase IV solution (0.05 mg/ml, Worthington Biochem Corp., Lakewood, NJ, USA), containing 5 mM of $CaCl_2$. Samples were incubated at 37 °C and 5% CO_2 for 45 min with shaking. Subsequently, cell suspension was washed twice and filtered through a 70 µm nylon mesh cell strainer (Thermo Fisher Scientific). Cells were collected and used for flow cytometric analysis.

1.5. Determination of cell number

Improved Neubauer hemocytometer (Reichert Bright-Line, Hausser Scientific Company, Horsham, PA, USA) and the trypan blue exclusion assay were used to determine the number of viable cells. Briefly, cell suspension was diluted 1:10 (v/v) with 0.4% trypan blue solution (gibco) and loaded into the hemocytometer chamber. Viable cells remained unstained, whereas dead cells got stained. A light microscope (ECLIPSE T*i*-S, Nikon Instruments Inc.,

Melville, NY, USA) was used to manually count viable cells. Cell numbers were calculated as followed:

$$Total\ cell\ No. = \frac{No.\ of\ cells\ counted}{No.\ of\ quarters\ counted} \times Sample\ Volume\ \times Dilution\ Factor \times 10^4$$

1.6. Cryopreservation of cells

For cryopreservation, cells were collected after process of isolation, washed twice, and counted. Next, cells were resuspended in RPMI media supplemented with 20% FBS (v/v) and 10% (v/v) of the cryoprotective agent dimethylsulfoxide (DMSO, Fisher Scientific) at a density of approximately 10×10^6 cells/ml. Cells were aliquoted into cryogenic vials (Fisher Scientific) and stored in isopropanol containing freezing containers at -80 °C overnight. Subsequently, frozen cells were removed from freezing containers and transferred to liquid nitrogen tanks for long-term storage. To recover cryopreserved cells, vials were quickly thawed in a 37 °C water bath. To remove DMSO, cells were washed in 30 ml of DPBS supplemented with heat inactivated FBS and transferred to a Falcon tube.

2. Flow cytometry

Multiparameter flow cytometry was performed via BD FACSCanto™ (BD Bioscience, Franklin Lakes, NJ, USA) using a panel of anti-human Abs conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), PerCP-complex-cyanine 5.5 (PerCP-Cy5.5), allophycocyanin (APC), APC-Cy7 or PE-Cy7, as follows. BD Pharmingen™ (BD Bioscience): anti-PD-1 (MIH4, 10 µg/ml); abcam (Cambridge, MA, USA): anti-human IgG4 Ab (HP6023, 1:50 dilution); BioLegend® (San Diego, CA, USA): anti-CD3 (HIT3a, 1:20 dilution), anti-CD19 (HIB19, 1:20 dilution), anti-CD27 (M-T271, 1:20 dilution), anti-CD38 (HIT2, 1:20 dilution), anti-CD80 (2D10, 1:20 dilution), anti-CD86 (BU63, 1:20 dilution), anti-Ki67 (Ki-67, 1:20 dilution), anti-IgD (IA6-2, 1:20 dilution).

2.1. Extracellular staining

Flow cytometric analysis was performed following a series of sequential steps, including assessment of cell viability, FcR blockade, anti-PD-1 Ab indirect staining, direct cell surface marker detection and intracellular staining. To exclude dead cells, cells were counted,

resuspended in DPBS only at a density of approximately $1x10^6$ cells/ml and stained with Zombie NIR or Zombie Green fixable Viability Dye (BioLegend®, 1.2 µg/ml) at room temperature for 10 min protected from light. To minimize non-specific FcR binding, cells were washed twice and stained with human TruStain FcX (BioLegend®, 1:10 dilution) at 4 °C for 10 min protected from light. Cells were washed twice with 200 µl FACS buffer (DPBS supplemented with 2% (v/v) of FBS). Subsequent staining for all extracellular Ags was performed in FACS buffer.

If PD-1 staining was performed using the clinical anti-PD-1 Abs (Nivolumab (Opdivo, kindly provided by Dana Farber Cancer Institute, Boston, MA, USA) or Pembrolizumab (Keytruda, kindly provided by Dana Farber Cancer Institute), cells were incubated with 100 µg/ml of the clinical Abs or the respective isotype control human IgG4 (BioLegend®) at 4 °C for 45 min protected from light. After washing, cells were stained with a FITC-conjugated anti-human IgG4 Ab (1:50 dilution, abcam) for 30 min. Next, cells were washed two times and stained for surface Ags by direct staining for 30 min at 4 °C.

To fix Ab-binding after staining procedure, cells were resuspended in DPBS supplemented with 4% (v/v) paraformaldehyde (PFA, Electron Microscopy Science, Hatfield, PA, USA) and stored at room temperature for 10 min protected from light. Cells were then placed at 4 °C protected from light until fluorescence emission was acquired. Isotype control and/ or fluorescence minus one (FMO) staining was included in each experiment to allow for proper gating and to control for non-specific binding. For multiparameter flow cytometry, spectral overlap was corrected using compensation controls for each of the fluorophore-conjugated Abs. Data were analyzed using the FlowJo™ 10.0.6, LLC software (BD Life Science, Ashland, OR, USA).

2.2. Intracellular staining

For staining of the intracellular Ag Ki-67, cells were resuspended in DPBS supplemented with 4% (v/v) PFA to fixate prior Ab binding. Next, cells were resuspended in 200 µl BD Cytofix/Cytoperm (BioLegend®) and stored at room temperature for 20 min protected from light. Subsequently, cells were washed three times and resuspended in 200 µl 1X Perm/Wash buffer (BioLegend®). PE-conjugated anti-Ki67 (BioLegend®) monoclonal Ab or the respective isotype control was added, and cells were placed at 4 °C for 30 min protected from light. Next, cells were washed three times to remove excess Abs, resuspended in 200 µl of

DPBS, and placed at 4 °C protected from light until fluorescence emission was acquired. For multiparameter flow cytometry, spectral overlap was corrected using compensation controls for each of the fluorophore-conjugated Abs. Data were analyzed using the FlowJo™ 10.0.6, LLC software.

3. B cell activation, subset differentiation, PD-1/PD-L1 blocking assays and alteration of surface glycans

Isolated circulatory B cells were cultured in RPMI advanced 1640 media (gibco) supplemented with 10% (v/v) heat inactivated FBS, 1% (v/v) GlutaMAX supplements (gibco), 1% (v/v) Penicillin/ Streptomycin (gibco), 1% (v/v) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (gibco), and human IL-2 (10 ng/ml, BioLegend®) at 37 °C and 5% CO_2 in a humidified incubator using aseptic techniques.

3.1. B cell activation

For activation, freshly isolated circulatory B cells were resuspended in complete B cell culture media and plated in 24-well cell culture plates (Thermo Fisher) at a density of approximately $1x10^6$ cells/ml. Cells were treated with one of the two activation cocktails at 37 °C and 5% CO_2 for 72 hrs: a TD activation cocktail consisting of human IL-4 (20 ng/ml, BioLegend®), human IL-10 (10 ng/ml, BioLegend®), human IL-21 (50 ng/ml, BioLegend®), F(ab')2 Fragment goat anti-human IgA + IgG + IgM (H+L, 10 μg/ml, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and MEGACD40L (0.5 μg/ml, Enzo Life Science, Inc., Farmingdale, NY, USA), or a TI activation cocktail consisting of human IL-4 (20 ng/ml), human IL-10 (10 ng/ml) and ODN2006 (250 nM/ml, InvivoGen US, San Diego, CA, USA)

3.2. B cell subset differentiation

Circulatory and tonsillar B cell subsets were characterized by multiparameter flow cytometry analyses using the following monoclonal Abs: PE- or APC-conjugated anti-human IgD, APC- or PE- conjugated anti-human CD38, PE-Cy7-conjugated anti-human CD27, and FITC- or PerCP-Cy5.5 conjugated anti-human CD19. For characterization of B cell subsets, lymphocytes were electronically gated according to their forward scatter (FSC) and side scatter (SSC) properties. Doublets were discriminated using the FSC-Area (A) / FSC-Height (H) parameters. Cells were negatively gated to exclude viability dye^+ cells, and positively

gated for B cells ($CD19^+$). Next, circulatory subsets were characterized as follows: IgD^+ $CD27^-$ cells (Naive); IgD^- $CD27^+$ (Swi Mem); IgD^+ $CD27^+$ (NS Mem); IgD^- $CD27^-$ (DN Mem, **Figure 3**). Tonsillar subsets were characterized as follows: IgD^+ $CD27^-$ cells (Naive); IgD^+ $CD27^+$ (NS Mem); IgD^- $CD27^-$ (DN Mem); IgD^- $CD38^{low/mid}$ $CD27^+$ (Swi Mem); IgD^- $CD38^{high}$ $CD27^{+/-}$ (Swi Mem); IgD^- $CD38^{high}$ $CD27^+$ (PB, **Figure 4**). The CD27 gate was set based on a PE-Cy7 FMO gating control.

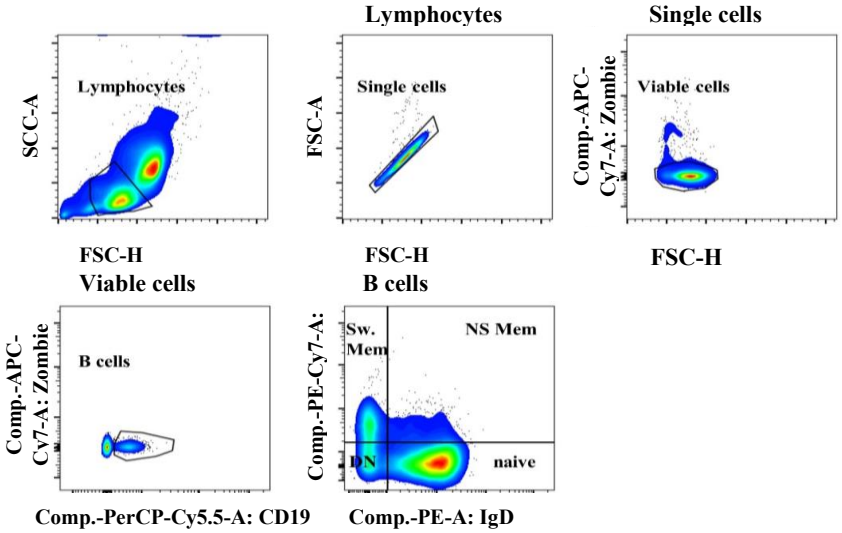

Figure 3: Gating strategy for circulatory B cell subset classification
Representative flow cytometry dot-plots showing the gating strategy for circulatory B cell subset classification. Gating on lymphocytes according their FSC and SSC. Negative gating to exclude doubles and dead cells. Positive gating to select $CD19^+$ B cells. Subset gating as followed: IgD^+ $CD27^-$ cells (Naive); IgD^- $CD27^+$ (Swi Mem), IgD^+ $CD27^+$ (NS Mem); IgD^- $CD27^-$ (DN Mem).

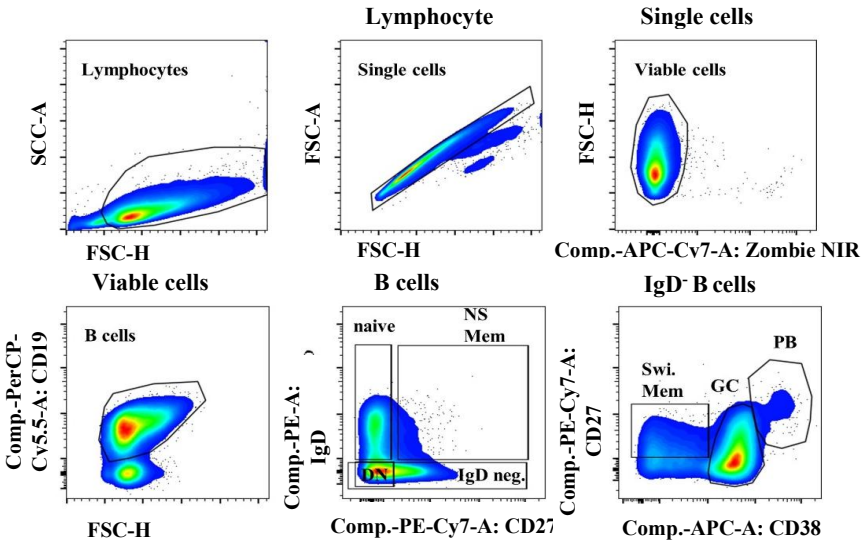

Figure 4: Gating strategy for tonsillar B cell subset classification
Representative flow cytometry dot plots showing the gating strategy for tonsillar B cell subset characterization. Gating on lymphocytes according their FSC and SSC. Negative gating to exclude doubles and dead cells. Positive gating to select $CD19^+$ B cells. Subset gating as followed: IgD^+ $CD27^-$ cells (Naive); IgD^+ $CD27^+$ (NS Mem); IgD^- $CD27^-$ (DN Mem), IgD^- $CD38^{low/mid}$ $CD27^+$ (Swi Mem), IgD^- $CD38^{high}$ $CD27^{+/-}$ (Swi Mem), IgD^- $CD38^{high}$ $CD27^+$ (PB)

3.3. PD-1/PD-L1 blocking assays

For PD-1/PD-L1 blockade assays, freshly isolated circulatory B cells were activated with one of the two activation cocktails, as described previously. To stimulate the PD-1/PD-Ls pathway, B cells were incubated with 10 µg/ml of human PD-L1 Fc chimera (R&D Systems, Inc., Minneapolis, MN, USA) or with control human IgG1 Fc (R&D Systems, Inc.). To block PD-1/PD-L1 interactions, 100 µg/ml of the clinical anti-PD-1 Abs (Nivolumab or Pembrolizumab) were added to the culture. As control, B cells were also treated with 100 µg/ml of human IgG4 isotype control or the vehicle control. After a 72 hrs activation period, B cells were harvested for flow cytometric analysis and supernatants were collected and stored at -20 °C for subsequent ELISA assays.

3.4. Alteration of surface glycan structures

3.4.1. Sialidase treatment

Freshly isolated PBMCs were counted, resuspended in RPMI without FBS at a density of approximately 10×10^6 cells/ml and transferred into three Eppendorf tubes. Media containing 0.1 U/ml sialidase obtained from Vibrio (V.) cholerae (Sigma-Aldrich, Inc.), 0.1 U/ml sialidase obtained from Arthrobacter (A.) ureafaciens (Sigma-Aldrich, Inc.), or the vehicle control was added, and tubes were placed at 37 °C. After a 30 min treatment period, cells were washed three times before proceeding with flow cytometric analyses. Efficiency and specificity of Sias removal was assessed by flow cytometric lectin staining with the following lectins: FITC-conjugated Erythrina Cristagalli Lectin (ECA, recognizing un-sialylated type 2 lactosamines, 1:500 dilution, Vector Laboratories, Inc., Burlingame, CA, USA), FITC-conjugated Maackia Amurensis Lectin (MAA, recognizing α2,3-linked Sias, 1:500 dilution, Vector Laboratories, Inc.) and FITC-conjugated Sambucus Nigra Lectin (SNA, recognizing α2,6-linked Sias, 1:500 dilution, Vector Laboratories, Inc.)

3.4.2. Treatment with glyco-metabolic inhibitors

Freshly isolated circulatory B cells were resuspended in complete TD B cell activation media and plated in 6-well cell culture plates (Thermo Fisher) at a density of approximately $1x10^6$ cells/ml. 1 µg/ml of swainsonine (MGAT2 inhibitor, Tocris Bioscience, Minneapolis, MN, USA), kifunensine (mannosidase inhibitor, Tocris Bioscience), tunicamycin (DPAGT1 inhibitor, Sigma-Alderich, Inc.) or the vehicle control was added, and cells were placed at 37 °C and 5% CO_2 in a humidified incubator. After a 48 hrs treatment period, cells were

washed three times, counted, and harvested for flow cytometric analyses. Efficiency and specificity of the glyco-alterations was assessed by flow cytometric lectin staining with the following lectins: biotinylated Concanavalin A (ConA, recognizing hybrid and high mannose *N*-glycans, 1:500 dilution, Vector Laboratories, Inc.) plus FITC-conjugated streptavidin (1:2000 dilution, BioLegend®), FITC-conjugated ECA (recognizing complex and hybrid *N*-glycans, 1:500 dilution), and FITC-conjugated Phytohemagglutinin-L (PHA-L, recognizing complex *N*-glycans, 1:500 dilution, Vector Laboratories, Inc.).

4. Enzyme Linked Immunosorbent Assay (ELISA)

Cytokine and Ig secretion was measured using ELISA kits, per the manufacturer's instructions. Human IL-6, IL-10, IgG, and IgM uncoated ELISA kits were purchased from eBioscience™ (Thermo Fisher Scientific). Cytokine and Ig concentrations were calculated using the specific standard curves.

5. Statistical Analysis

Data are expressed as the mean ± standard error of the means (SEM) if not mentioned otherwise. For test with two paired groups, differences were determined using a paired student´s t-test. One-way analysis of variance (ANOVA) was used for tests comparing three or more groups, with multiple comparisons performed by Turkey test. Test were considered significant when $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Results

1. Aim 1 – To define B cell-PD-1 glycosylation and inhibitor reactivity

There has been accumulating evidence that the PD-1 receptor is heavily *N*-gycosylated and that glycosylation is critical for PD-Ls binding (Sun et al. 2020). Because clinical PD-1 inhibitors recognize epitopes located within the PD-1 binding pocket, disparities in PD-1 glycostructures might alter clinical Ab reactivity. While PD-1 has mainly been studied in T cells (Baumeister et al. 2016), recent studies suggest that B cells play a crucial role in anti-PD-1 immunotherapy. Therefore, our studies aimed to investigate the significance of B cell-specific PD-1 clinical Ab binding affinities and glycosylation and their significance in PD-1 checkpoint immunotherapy.

1.1. PD-1 is expressed by B cells and differentially recognized by clinical Abs

First, the expression levels of PD-1 on circulatory and tonsillar B cells from healthy donors were screened via multiparameter flow cytometry analyses, using a PerCP-Cy5.5-conjugated mouse anti-human PD-1 Ab (clone MIH4). Our findings revealed that PD-1 is expressed by small subpopulations of unactivated circulatory or tonsillar B lymphocytes (*n*=7/7). Specifically, the percentage of PD-1$^+$ cells among circulatory B cells averaged 1.2% ± 0.2% (Mean ± SEM, *n*=4, **Figures 5A+C**). B cells obtained from tonsils exhibit a higher frequency of PD-1$^+$ B cells averaging 5.2% ± 0.6% (Mean ± SEM, *n*=3, **Figures 5B+C**). These findings were used as reference for PD-1 expression on B cells for the assessment of Nivolumab versus Pembrolizumab affinity.

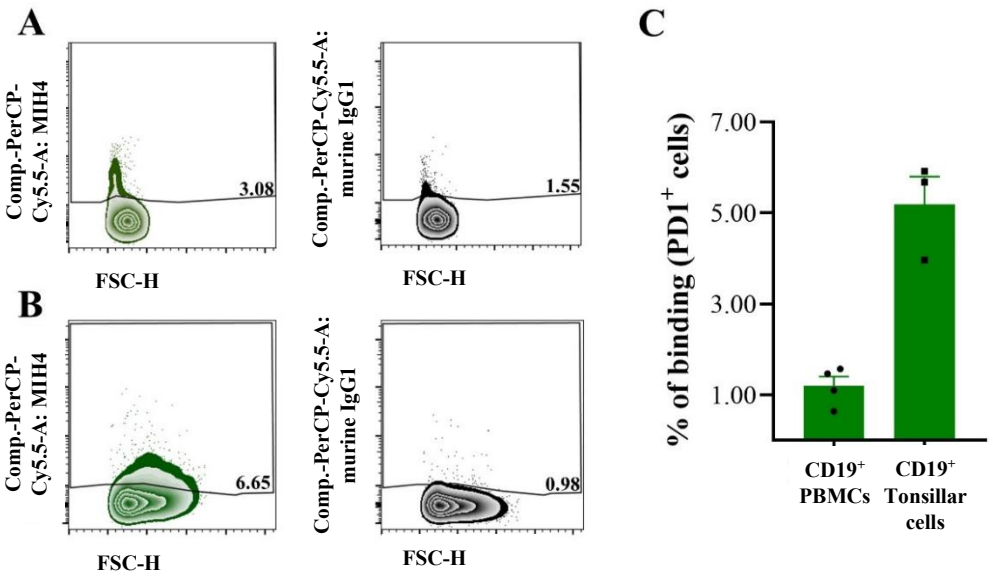

Figure 5: Human B cells obtained from healthy donors express surface PD-1.
(A+B) Representative flow cytometry dot plots of MIH4 binding to the PD-1 surface receptor of **(A)** Circulatory B cells (*n*=4*)* and **(B)** tonsillar B cells (*n=3)* from healthy donors. **(C)** Percentages (Mean ± SEM) of MIH4 binding to PD-1 surface receptor of circulatory $CD19^+$ B cells and tonsillar $CD19^+$ B cells from healthy donors.

To characterize the binding affinity of Nivolumab and Pembrolizumab to B cell-PD-1, we performed flow cytometry staining with either PD-1 Ab. Consistent with the modest reactivity to MIH4 (1.2% ± 0.2%, Mean ± SEM, *n*=4 donors, **Figure 5**), Pembrolizumab recognized PD-1 expressed by a small subset of circulatory B cells (1.3% ± 0.5%, Mean ± SEM, *n*=5, **Figure 6**). However, we only detected marginal (0.3%, Mean, *n*=2/5) to no (0.0%, *n*=3/5) Nivolumab binding. Thus, Pembrolizumab bound B cell-PD-1 significantly more efficiently than Nivolumab across all independent B cell samples *tested* (1.3% ± 0.5% versus 0.1% ± 0.1%, Mean ± SEM, *n*=5, *p*=0.0466*,* **Figures 6A+C**). Next, we performed flow cytometric affinity studies for Nivolumab and Pembrolizumab to PD-1 expressed by tonsillar B cells. Consistent with the previous results, tonsillar B cell-PD-1 showed a substantially higher reactivity to Pembrolizumab compared to Nivolumab (2.0% ± 0.8 % versus 0.2 % ± 0.1%, Mean ± SEM, *n*=6, *p*=0.0559), respectively **(Figures 6B+D)**.

Moreover, in all independent samples *tested* (*n*=11/11), we identified a small subset of B cells that exhibited a PD-1^{high} phenotype. These PD-1^{high} B cells were exclusively recognized by Pembrolizumab (circulatory B cells: 0.4% ± 0.1%, Mean ± SEM, *n*=5, **Figures 6A+E**; tonsillar B cells: 0.5% ± 0.1%, Mean ± SEM, *n*=6, **Figures 6B+F**), but not Nivolumab (circulatory PD-1^{high} B cells: 0.0% ± 0.0%, Mean ± SEM, *n*=5, **Figures 6A+E**; tonsillar B cells: 0.0% ± 0.0%, Mean ± SEM, *n*=6, **Figures 6B+F**). Consistent with our results pertaining to inhibitor reactivities to B cell-PD-1, side by side comparative analyses across

identical donors revealed that Pembrolizumab also exhibits significantly higher binding to PD-1^{high} B cells, compared to Nivolumab (circulatory B cells: n=5, p=0.0112, tonsillar B cells: n=6, p=0.0031, **Figure 6)**.

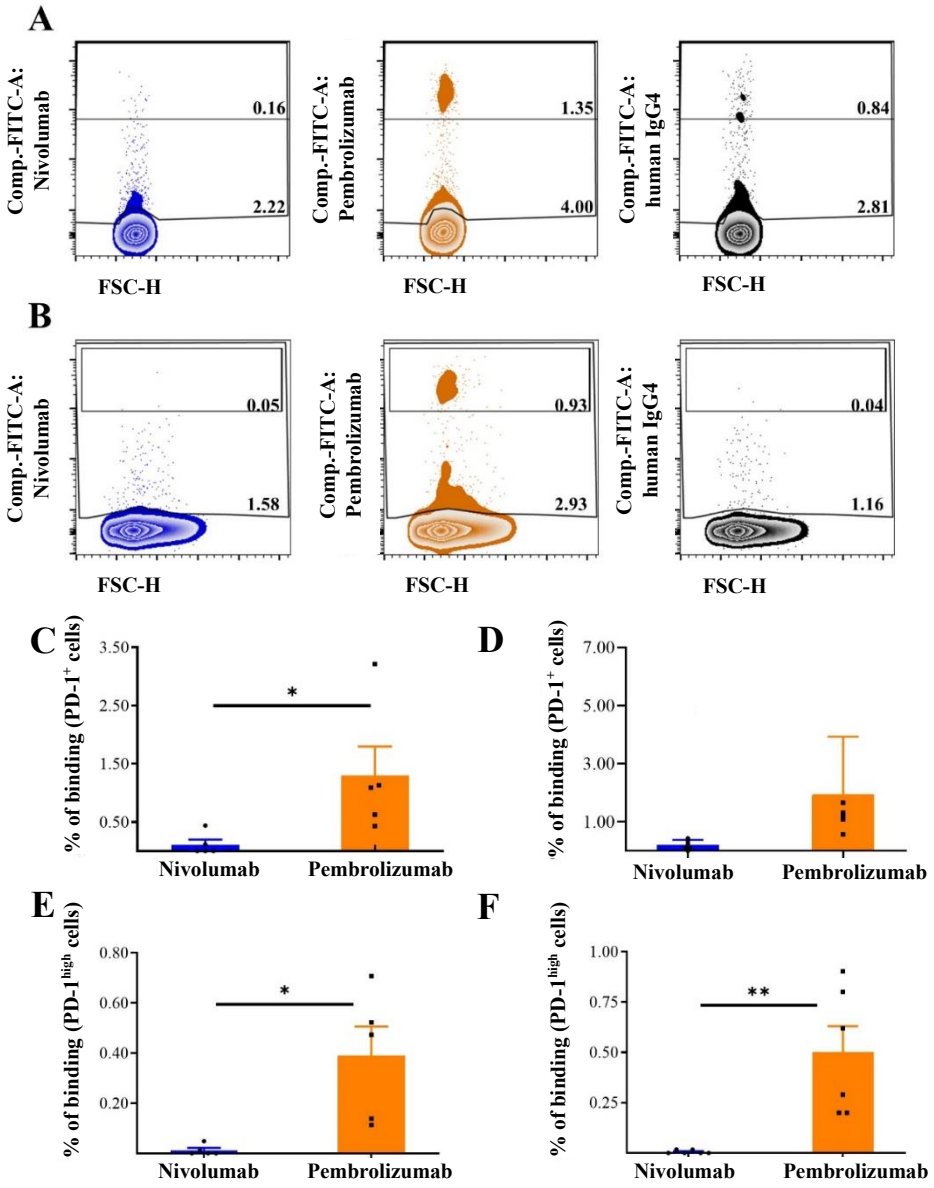

Figure 6: Human B cells obtained from healthy donors display a higher binding affinity to Pembrolizumab versus Nivolumab.
(A+B) Representative flow cytometry dot plots of Nivolumab and Pembrolizumab binding to PD-1 surface receptor of **(A)** circulatory B cells (n=5) and **(B)** tonsillar B cells (n=6) from healthy donors. **(C+D)** Percentages (Mean ± SEM) of Nivolumab and Pembrolizumab binding to the PD-1 surface receptor of **(C)** circulatory B cells (n=5) and **(D)** tonsillar B cells (n=6) from healthy donors. **(E+F)** Percentages (Mean ± SEM) of Nivolumab and Pembrolizumab binding to PD-1^{high} **(E)** circulatory B cells(n=5) and **(F)** tonsillar B cells (n=6) from healthy donors.

1.2. Pembrolizumab exhibits a higher binding affinity to PD-1 expressed by melanoma-infiltrating B cells, compared to Nivolumab

To investigate the binding affinity of Nivolumab and Pembrolizumab to melanoma-infiltrating B cells, we first characterized the frequency of B cells within the TME of patient melanoma tumor biospecimens. As shown in **Figures 7A+C**, the majority of TILs were $CD3^+$ T cells (42.0% ± 17.6%, Mean ± SEM, *n*=5). However, robust numbers of B cells were detected in 3 of 5 independent melanoma samples. The percentage of melanoma-infiltrating B cells ranged from 3.8% to 31.9% of all TILs (**Figures 7A+C**). Next, we assessed Nivolumab versus Pembrolizumab immunoreactivity to melanoma-infiltrating B cells by multiparameter flow cytometry. As shown in **Figures 7B+D**, Pembrolizumab bound to a subset (0.7% ± 0.6%, Mean ± SEM, *n*=3) of melanoma-infiltrating B cells, while Nivolumab showed no significant reactivity.

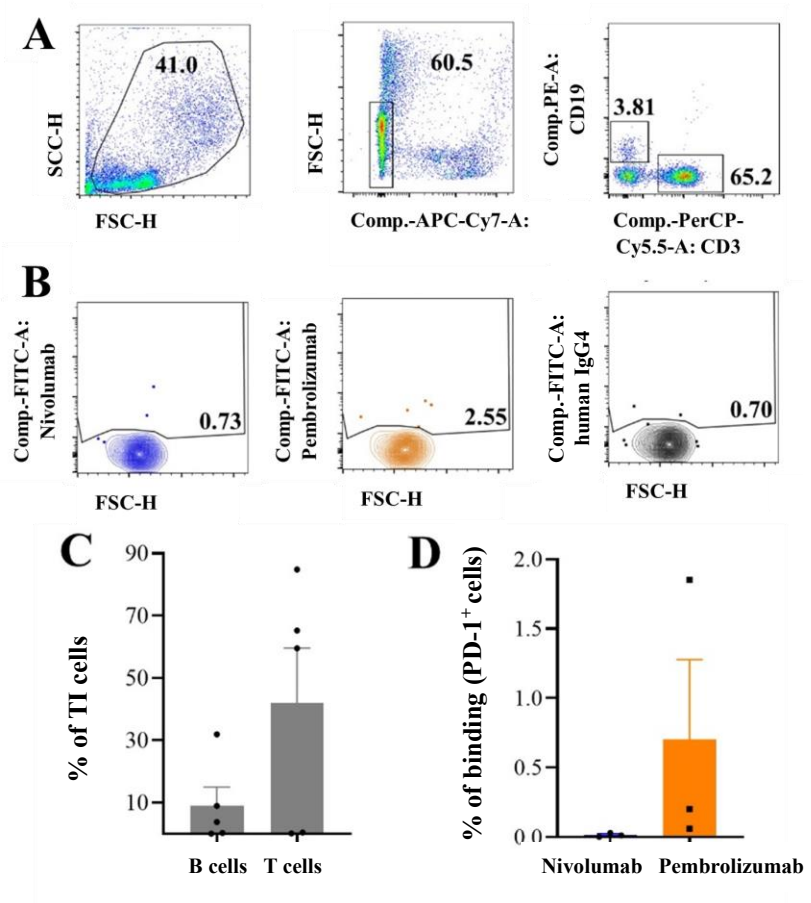

Figure 7: Melanoma-infiltrating B cells bind to Pembrolizumab, but not Nivolumab.
(A) Dead cells were discriminated from the scatter plot via exclusion of cell debris and Zombie positive cells. Lymphocytes were characterized according their expression of CD3 and CD19. **(B)** Representative flow cytometry dot plots and **(C)** percentage (Mean ± SEM) of melanoma infiltrating $CD19^+$ cells in *n*=5 melanoma lesions. **(D)** Percentages (Mean ± SEM) of Nivolumab and Pembrolizumab binding to PD-1 surface receptor of melanoma infiltrating B cells (*n*=3).

1.3. B cell subset- and glycosylation-dependence of PD-1 inhibitor binding

Next, we investigated whether Nivolumab and/or Pembrolizumab recognize PD-1 expressed by specific B cell subsets, and whether these clinical inhibitors bind to B cell-PD-1 in a glycan-dependent manner.

1.3.1. Pembrolizumab preferentially targets PD-1 expressed by B cells of the memory compartment

To investigate the phenotypic variation of PD-1$^+$ cells among human circulatory and tonsillar B cells, we analyzed the expression of IgD, CD27 and CD38 markers, delineating circulatory and tonsillar B cells into four or six distinct subsets, respectively, including naïve B cells (IgD$^+$ CD27$^-$), Swi Mem B cells (IgD$^-$ CD27$^+$), NS Mem B cells (IgD$^+$ CD27$^+$), DN Mem B cells (IgD$^-$ CD27$^-$), GC cells (IgD$^-$ CD38$^+$) and PBs (IgD$^-$ CD27^{high} CD38^{high}) (**Figures 3 and 4)**. As shown in **Figure 8**, flow cytometric analyses revealed modest MIH4 reactivity to a small subpopulation in all samples *tested* (*n*=7/7). The percentage of MIH4$^+$ circulatory cells averaged 0.3% ± 0.2% (Mean ± SEM, *n*=3) among naïve B cells, 0.6% ± 0.2% (Mean ± SEM, *n*=3) among DN Mem B cells, 4.0% ± 0.7% (Mean ± SEM, *n*=3) among NS Mem B cells, and 2.1% ± 0.1% (Mean ± SEM, *n*=3) among Swi Mem B cells **(Figure 8A)**. Tonsillar B cells showed a higher reactivity to MIH4. The frequency of MIH4$^+$ B cells averaged 2.2% ± 1.3% (Mean ± SEM, *n*=4) among naïve B cells, 3.2% ± 0.8% (Mean ± SEM, *n*=4) among DN Mem B cells, 15.1% ± 6.0% (Mean ± SEM, *n*=4) among NS Mem B cells, 14.0% ± 0.8% (Mean ± SEM, *n*=4) among Swi Mem B cells, 5.0% ± 1.1% (Mean ± SEM, *n*=4) among GC cells, and 7.3% ± 5.5% (Mean ± SEM, *n*=4) among PBs **(Figure 8B)**.

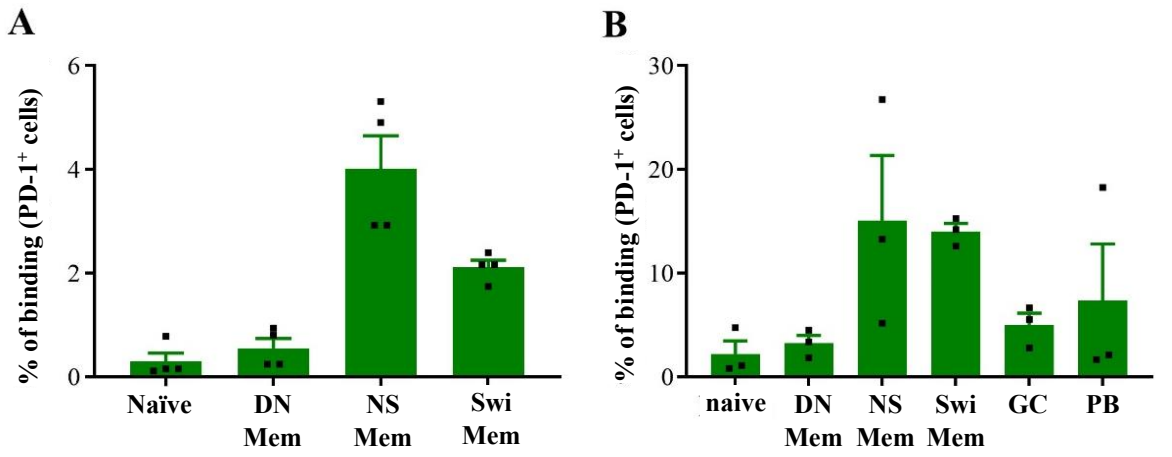

Figure 8: PD-1 is predominantly expressed by the memory compartment of circulatory and tonsillar B cells.
(A) Percentage (Mean ± SEM) of MIH4 binding to PD-1 surface receptor of subsets of circulatory B cells and tonsils from healthy donors (*n*=4). **(B)** Percentage (Mean ± SEM) of MIH4 binding to PD-1 surface receptor of subsets of tonsillar B cells from healthy donors (*n*=3*)*.

Next, we performed similar flow cytometric analyses with Nivolumab and Pembrolizumab to determine respective Ab reactivities among these B cell subsets. Consistent with our previous results using the PD-1 FACS Ab, MIH4, both PD-1 inhibitors predominantly bound to B cells of the memory compartment (**Figures 9+10**). Compared to Nivolumab, Pembrolizumab exhibited higher binding to all circulatory B cell subsets *examined*, including naïve B cells (0.5% ± 0.3% versus 0.1% ± 0.1%, Mean ± SEM, *n*=5, *p*=0.9789), DN Mem B cells (1.7% ± 0.6% versus 0.0% ± 0.0%, Mean ± SEM, *n*=5, *p*=0.2406), Swi Mem B cells (1.4% ± 0.5% versus 0.3% ± 0.2%, Mean ± SEM, *n*=5, *p*=0.5998) and NS Mem B cells (7.0% ± 1.6% versus 0.4% ± 0.2%, Mean ± SEM, *n*=5, *p*<0.0001, **Figures 9A+B**). Relative differences in Pembrolizumab versus Nivolumab affinity were highest among NS Mem B cells. Consistently, we detected higher binding of Pembrolizumab, compared to Nivolumab, to all tonsillar B cell subsets *tested*, including naïve B cells (0.8% ± 0.3% versus 0.1% ± 0.1%, Mean ± SEM, *n*=6, *p*=0.9708), DN Mem B cells (0.5% ± 0.2% versus 0.1% ± 0.1 %, Mean ± SEM, *n*=6, *p*=0.9973), GC B cells (0.3% ± 0.0% versus 0.3% ± 0.1%, Mean ± SEM, *n*=6, *p*>0.9999), PBs (2.1% ± 0.7% versus 0.6% ± 0.2%, Mean ± SEM, *n*=6, *p*=0.4683), Swi Mem B cells (3.8% ± 0.7% versus 0.7% 0.2%, Mean ± SEM, *n*=6, *p*=0.0063), and NS Mem B cells (12.4% ± 2.2% versus 1.5% ± 0.6%, Mean ± SEM, *n*=6, *p*<0.0001), with highest Pembrolizumab reactivity detected for NS Mem B cells **(Figures 10A+B)**.

Next, we focused on the PD-1^{high} B cell subpopulation of circulatory and tonsillar B cells and defined their phenotype. As shown in **Figures 9C** and **10C**, our data indicated that the vast majority of PD-1^{high} Pembrolizumab^{+} B cells was CD27^{+} IgD^{+}, characterized as NS Mem B cells. Consistent with our previous results, these PD-1^{high} NS Mem B cells exhibited the greatest divergence in clinical inhibitor binding (circulatory B cells: 5.2% ± 1.7% versus 0.2% ± 0.2%, Mean ± SEM, n=5, p<0.0001; tonsillar B cells: 11.3% ± 2.2% versus 0.1% ± 0.1%, Mean ± SEM, n=6, p<0.0001) and were almost exclusively recognized by Pembrolizumab **(Figures 9C+10C)**.

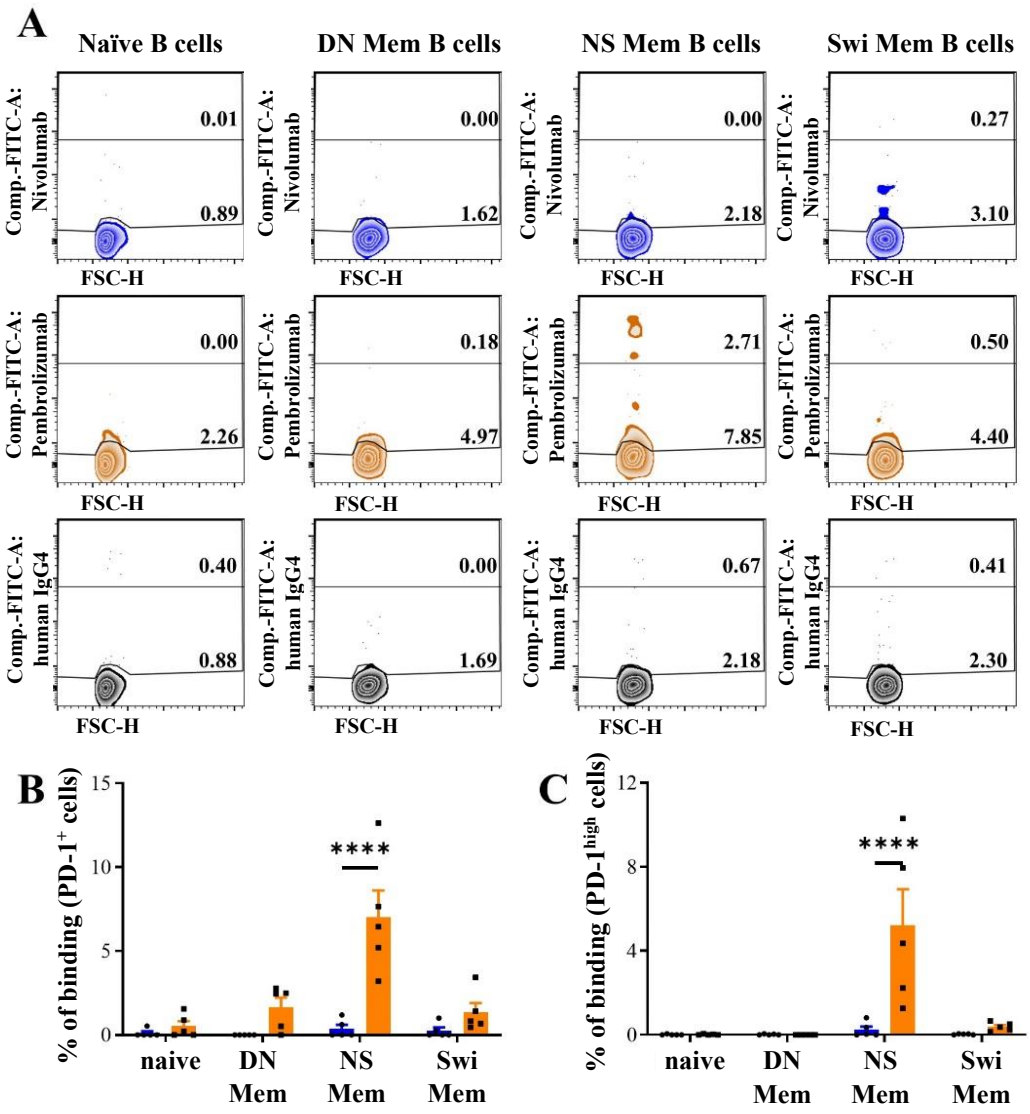

Figure 9: Pembrolizumab preferentially binds to PD-1 expressed by NS Mem circulatory B cells. **(A)** Representative flow cytometry dot plots and **(B+C)** percentages (Mean ± SEM) of Nivolumab and Pembrolizumab binding to PD-1 surface receptor of circulatory B cell subsets (n=5).

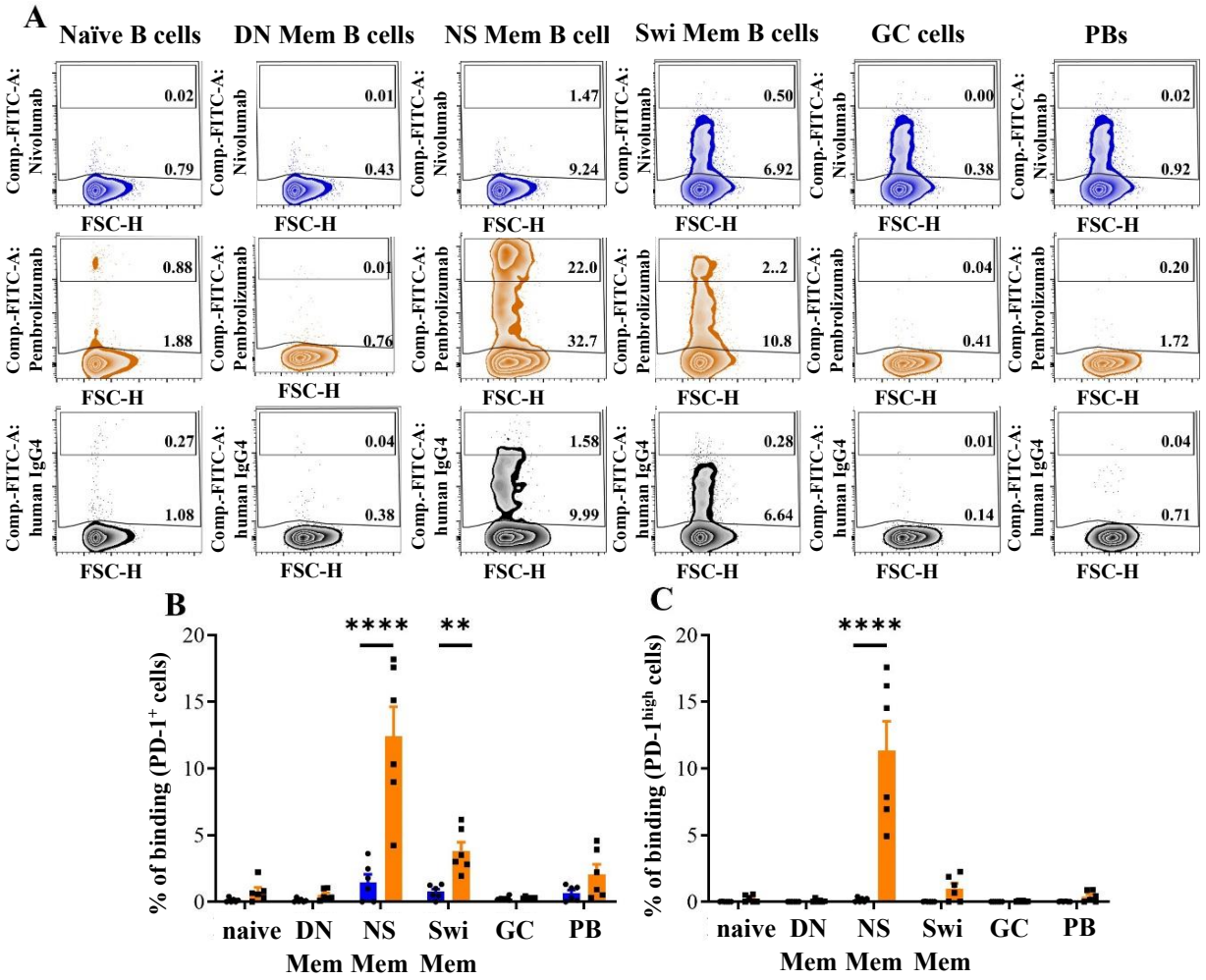

Figure 10: Pembrolizumab preferentially targets PD-1 expressed by Swi Mem and NS Mem tonsillar B cells.
(A) Representative flow cytometry dot plots and **(B+C)** percentages (Mean ± SEM) of Nivolumab and Pembrolizumab binding to PD-1 surface receptor of tonsillar B cell subsets (*n*=6).

1.3.2. PD-1 receptor recognition by its clinical inhibitors depends on PD-1 glycosylation patterns

1.3.2.1. De-sialylation of B cells reduces binding of Pembrolizumab to PD-1

Sias regulate multiple immunological processes, including immune cell activation and cell signaling, as described in the introduction. In this MD thesis, we assessed whether exogenous de-sialylation, via V. cholerae sialidase (cleaving α2,3-linked Sias) or A. ureafaciens sialidase (cleaving α2,6-linked Sias) treatment, affects Nivolumab or Pembrolizumab binding affinity to B cell-PD-1.

To confirm the efficiency of sialidase treatment, we first performed lectin staining with FITC-labeled ECA (recognizing un-sialylated type 2 lactosamines), MAA (recognizing α2,3-

linked Sias) and SNA (recognizing α2,6-linked Sias). As shown in **Figures 11A+B**, treatment with both sialidases increased ECA binding, indicating effective de-sialylation. Moreover, treatment with V. cholerae sialidase substantially reduced MAA binding, indicating efficient removal of Sias in α2,3-linkages. Yet, it only slightly reduced SNA binding, indicating limited α2,6-linked Sias cleavage **(Figure 11A)**. Treatment with A. ureafaciens sialidase decreased SNA binding, suggesting efficient removal of α2,6-linked Sias. Additionally, it also resulted in a substantial decrease of MAA binding, representing both α2,3-linked and α2,6-linked Sias cleavage **(Figure 11B)**.

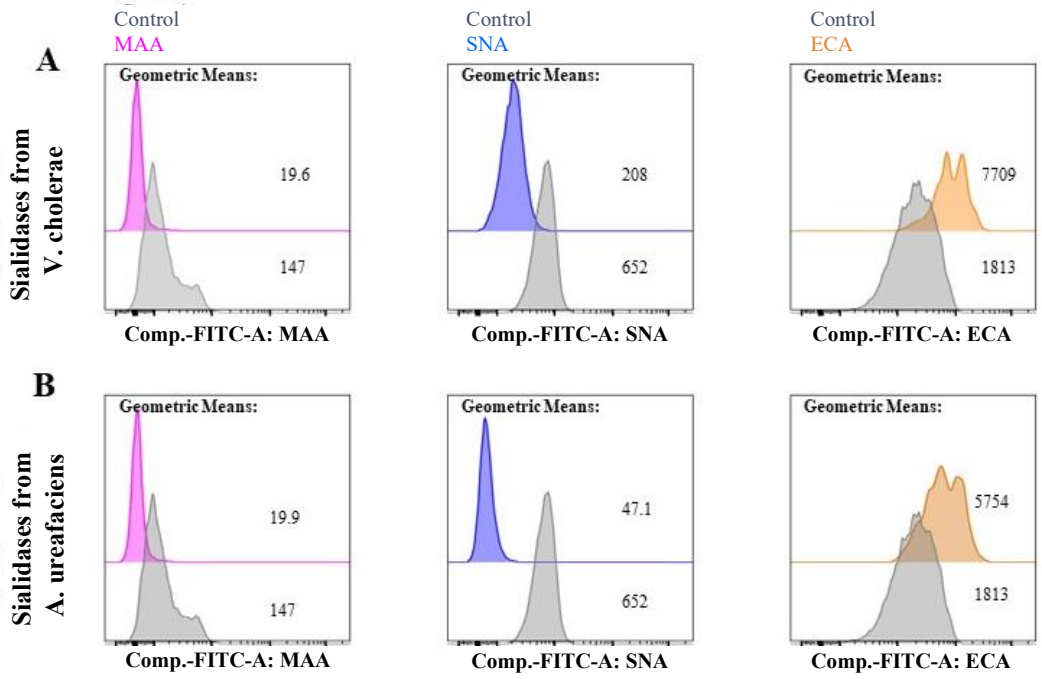

Figure 11: Effectiveness and specificity of sialidase treatment regimens
Representative histogram of MAA (left), SNA (middle), ECA (right) lectin binding by flow cytometry to circulatory B cells treated with sialidases obtained from **(A)** V. cholerae or **(B)** A. ureafaciens for 30 min (*n*=2). Gray histograms represent untreated control samples.

Next, we next assessed Nivolumab and Pembrolizumab binding to sialidase-treated or untreated B cells by flow cytometry. As shown in **Figure 12A**, Nivolumab did not show any reactivity. In contrast, Pembrolizumab bound PD-1 expressed by both sialidase-treated and untreated B cells. However, cleavage of α2,3-linked and α2,6-linked Sias tended to decrease Pembrolizumab binding to PD-1$^+$ B cells, compared to untreated cells (0.2% ± 0.2% and 0.7% ± 0.7% versus 1.1% ± 0.6%, *n*=2, *p*=0.4479 and *p*=0.7993, **Figure 12B**). However, differences failed to reach statistical significance and experimental cohort size needs to be increased for further analyses.

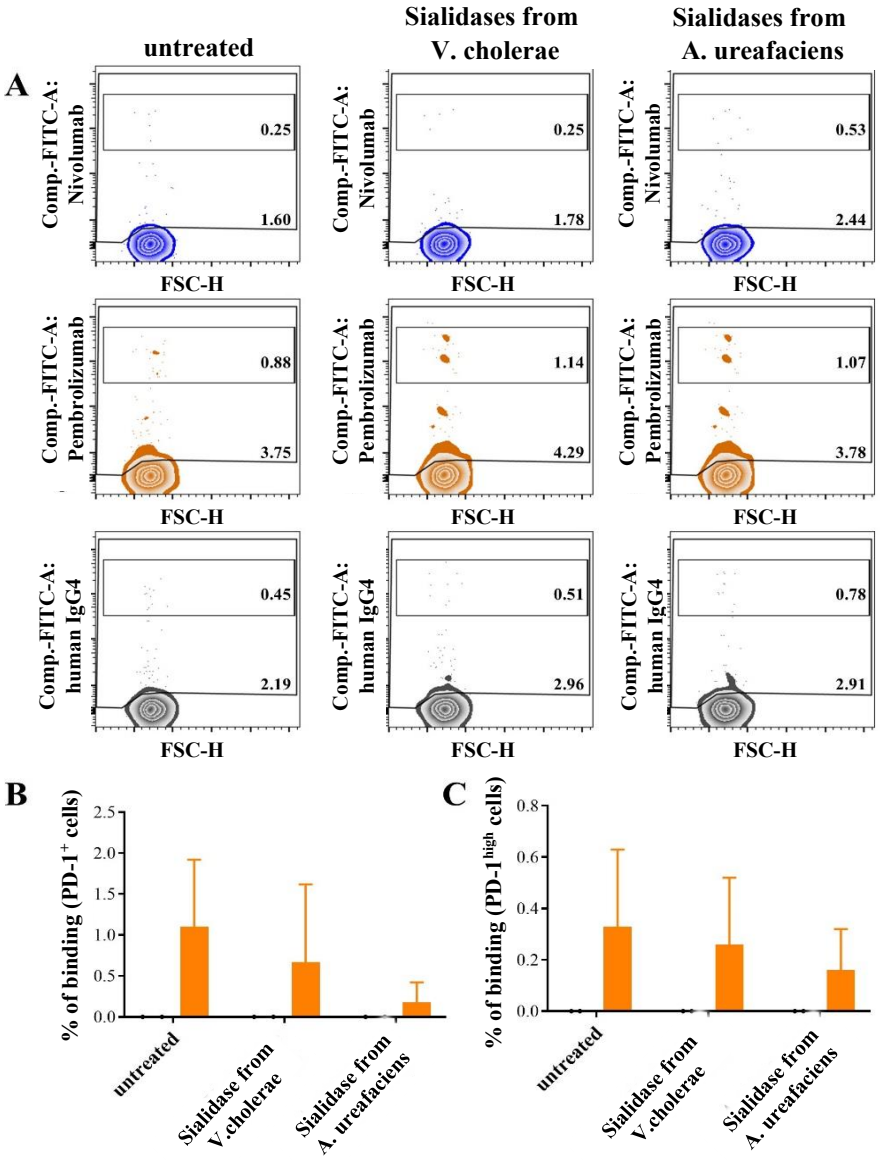

Figure 12: Sia removal reduces Pembrolizumab affinity to B cell-PD-1.
(A) Representative flow cytometry dot plots and **(B+C)** percentages (Mean ± SEM) of Nivolumab and Pembrolizumab binding to PD-1 surface receptor of circulatory B cells that were exogenously de-sialylated with sialidase obtained from V. cholerae or A. ureafaciens or left untreated for 30 min (n=2).

1.3.2.2. Nivolumab binding to B cell-PD-1 is diminished upon modification of the *N*-glycan composition

The PD-1 receptor is a transmembrane glycoprotein that contains four heavily *N*-glycosylated Asn-residues within its IgV-like domain. To investigate the impact of these *N*-glycans on Nivolumab and Pembrolizumab binding, we isolated circulatory B cells, followed by activation in a TD manner and treatment with metabolic inhibitors to modify the relative proportions of high-mannose, hybrid, or complex *N*-glycans. We subsequently performed flow cytometric staining for Nivolumab or Pembrolizumab.

The efficiency of the *N*-glyco-alteration regimens was confirmed by lectin staining with biotinylated ConA (recognizing hybrid and high mannose *N*-glycans) plus FITC-conjugated streptavidin, FITC-conjugated ECA (recognizing complex and hybrid *N*-glycans), or FITC-conjugated PHA-L (recognizing complex *N*-glycans). Compared to untreated samples, treatment with swainsonine decreased binding of ECA and PHA-L, resulting from an inhibition of complex *N*-glycans synthesis. Yet, binding of ConA increased due to a relative increase of high mannose and hybrid *N*-glycans (**Figure 13A**). Treatment with kifunensine largely suppressed the synthesis of hybrid and complex *N*-glycans, as demonstrated by a strong reduction in ECA binding and PHA-L binding, and a slight reduction in ConA binding (**Figure 13B**). As shown in **Figure 13C**, treatment with tunicamycin decreased binding of ECA and PHA-L, resulting from an effective abolishment of overall *N*-glycan synthesis. However, tunicamycin treatment increased binding of ConA, indicating incomplete abolishment of *N*-glycan synthesis.

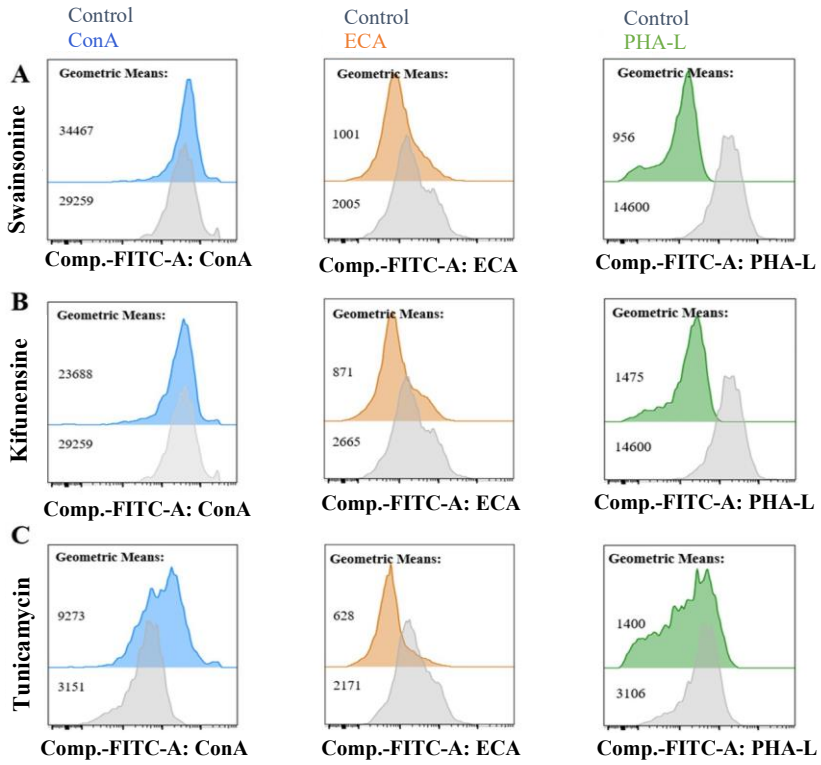

Figure 13: Effectiveness and specificity of swainsonine, kifunensine and tunicamycin glycometabolic inhibitor treatment
Representative histogram of ConA (left), ECA (middle), PHA-L (right) lectin binding by flow cytometry to circulatory B cells treated with of **(A)** swainsonine**, (B)** kifunensine, **(C)** tunicamycin for 48 hrs (*n*=3). Gray histograms represent untreated control samples.

Next, we assessed the binding of Nivolumab and Pembrolizumab to PD-1 expressed by B cells that were predominantly decorated with either high mannose, hybrid or complex *N*-

glycans. As shown in **Figure 14**, we detected binding of Nivolumab and Pembrolizumab to B cells subjected to either glycometabolic inhibitor regimen or no treatment. Enrichment of hybrid *N*-glycan synthesis via swainsonine treatment resulted in a 0.763-fold ± 0.060-fold (Mean ± SEM, n=5, p=0.6096) decrease in Nivolumab binding, compared to untreated control samples (**Figures 14A+B**). Likewise, enrichment of high mannose *N*-glycans via kifunensine suppressed binding of Nivolumab (0.558 ± 0.065-fold, Mean ± SEM, n=5, p=0.1667). The complete abolishment of *N*-glycosylation via tunicamycin treatment resulted in a 0.687 ± 0.385-fold (Mean ± SEM, n=4, p=0.4487) decrease in Nivolumab binding to B cell-PD-1. In contrast, changes in *N*-glycan diversity via swainsonine or kifunensine treatment did not substantially influence Pembrolizumab binding to PD-1 (swainsonine: 1.008 ± 0.196-fold, p>0.9999, kifunensine: 1.066 ± 0.263-fold, p=0.9911, Mean ± SEM, n=5), compared to untreated samples **(Figures 14A+C)**. Only the complete abolishment of *N*-glycan synthesis via tunicamycin treatment substantially suppressed Pembrolizumab binding (0.506 ± 0.264-fold, Mean ± SEM, n=4, p=0.2735), compared to binding to the untreated control samples. Together, these findings suggest that *N*-glycan composition might modulate PD-1 inhibitor binding, particularly in the case of Nivolumab. However, results did not reach statistical significance and further experiments with larger cohort sizes are needed to validate.

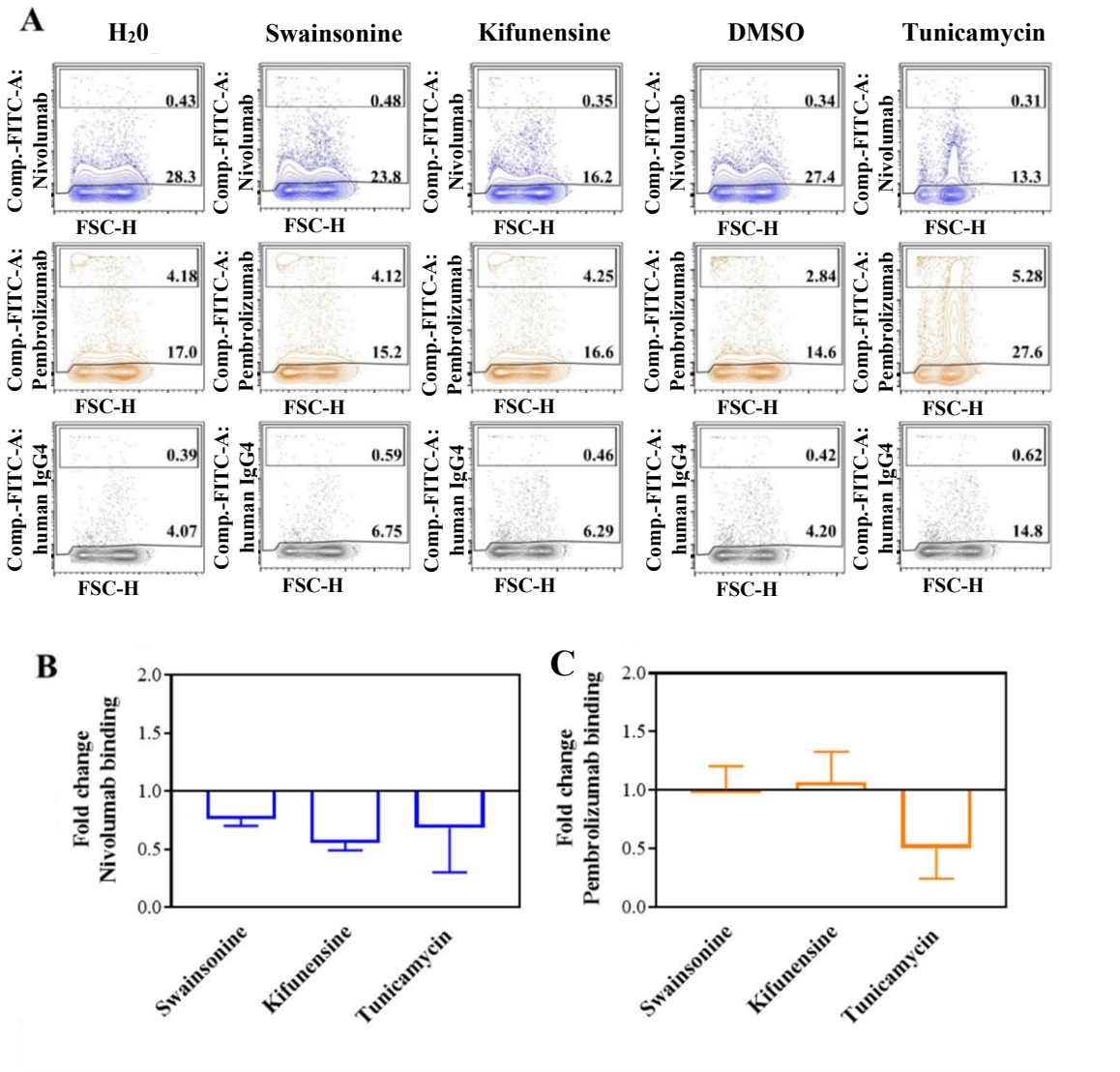

Figure 14: Modification of N-Glycan complexity modulates Nivolumab binding to B cell-PD-1. **(A)** Representative flow cytometry dot plots of Nivolumab and Pembrolizumab binding to PD-1 surface receptor expressed by circulatory B cells upon treatment with of swainsonine, kifunensine, or tunicamycin for 48 hrs. **(B+C)** Fold change (Mean ± SEM) of **(B)** Nivolumab and **(C)** Pembrolizumab binding to PD-1 expressed by circulatory B cells treated with of swainsonine (*n*=5), kifunensine (*n*=5), or tunicamycin (*n*=4) for 48 hrs, compared to PD-1 expressed by untreated circulatory B cells.

2. Aim 2 – To characterize effects of Nivolumab- versus Pembrolizumab-mediated PD-1 blockade on B cell effector functions

To determine whether the observed differences between Nivolumab versus Pembrolizumab affinity to B cell-PD-1 translate into distinct B effector functions, we isolated circulatory B cells, activated them in a TD or TI manner, and subsequently treated them with PD-L1 Fc chimera or human control IgG1, in the presence or absence of Nivolumab, Pembrolizumab, or human IgG4 control. Subsequently, we assessed the level of bound clinical anti-PD-1 Abs, reactivity of the alternative PD-1 blocking Ab, MIH4, B cell proliferation, maturation, cytokine secretion, and Ig secretion.

2.1. Pembrolizumab binds B cell-PD-1 with greater efficacy than Nivolumab

To define the capacity of Nivolumab and Pembrolizumab to block B cell-expressed PD-1 during B cell activation, we first treated cells as described above and assessed MIH4 binding. Because the MIH4 binding epitope overlaps with those of both Nivolumab and Pembrolizumab, MIH4 reactivity to B cell-PD-1 decreases as a result of PD-1 ligation by either inhibitor. Accordingly, MIH4 reactivity inversely correlates with Nivolumab or Pembrolizumab binding to B cell-PD-1. As shown in **Figures 15A+B**, B cells treated with Pembrolizumab during TD activation exhibited a significant decrease in MIH4 binding ($MIH4^+$ cells: 29.9% ± 2.4% versus 37.6% ± 3.5%, Mean ± SEM, n=6, p=0.0223, mean fluorescence intensity (MFI) MIH4: 284.9 ± 61.5 versus 341.5 ± 64.6, Mean ± SEM, n=6, p=0.0817), compared to isotype control treatment. However, MIH4 binding did not significantly decrease following treatment with Nivolumab ($MIH4^+$ cells: 34.5% ± 3.8% versus 37.6% ± 3.5%, Mean ± SEM, n=6, p=0.6089, MFI MIH4: 325.9 ± 68.4 versus 341.5 ± 64.6, Mean ± SEM, n=6, p=0.9200, **Figures 15A+B**). Moreover, the direct comparison between MIH4 reactivity following treatment with either clinical Abs revealed substantially reduced binding to Pembrolizumab- versus Nivolumab-treated cells. However, the results did not reach statistical significance ($MIH4^+$ cells: p=0.2788; MFI MIH4: p=0.3641, **Figures 15A+B**). Consistent with our findings in B cells activated in a TD manner, Nivolumab did not display significantly reduced MIH4 binding, compared to isotype control treatment, following TI B cell activation ($MIH4^+$ cells: 5.4% ± 1.0% versus 5.4% ± 0.6%, n=3, p>0.9999, MFI MIH4: 125.1 ± 5.4 versus 124.7 ± 12.7, Mean ± SEM, n=3, p=0.9999, **Figures 15C+D**). However, treatment with Pembrolizumab substantially reduced MIH4 reactivity, compared to Nivolumab treatment ($MIH4^+$ cells: 4.5% ± 0.9% versus 5.4% ±

1.0%, Mean ± SEM, *n*=3, *p*=0.2760, MFI MIH4: 117.7 ± 3.4 versus 125.1 ± 5.4, Mean ± SEM, *n*=3, *p*=0.6300, **Figures 15C+D**) and control treatment (MIH4$^+$ cells: 4.5% ± 0.9% versus 5.4% ± 0.6%, Mean ± SEM, *n*=3, *p*=0.2760, MFI MIH4: 117.7 ± 3.4 versus 124.7 ± 12.7, Mean ± SEM, *n*=3, *p*=0.6674, **Figures 15C+D**), although results did not reach significance.

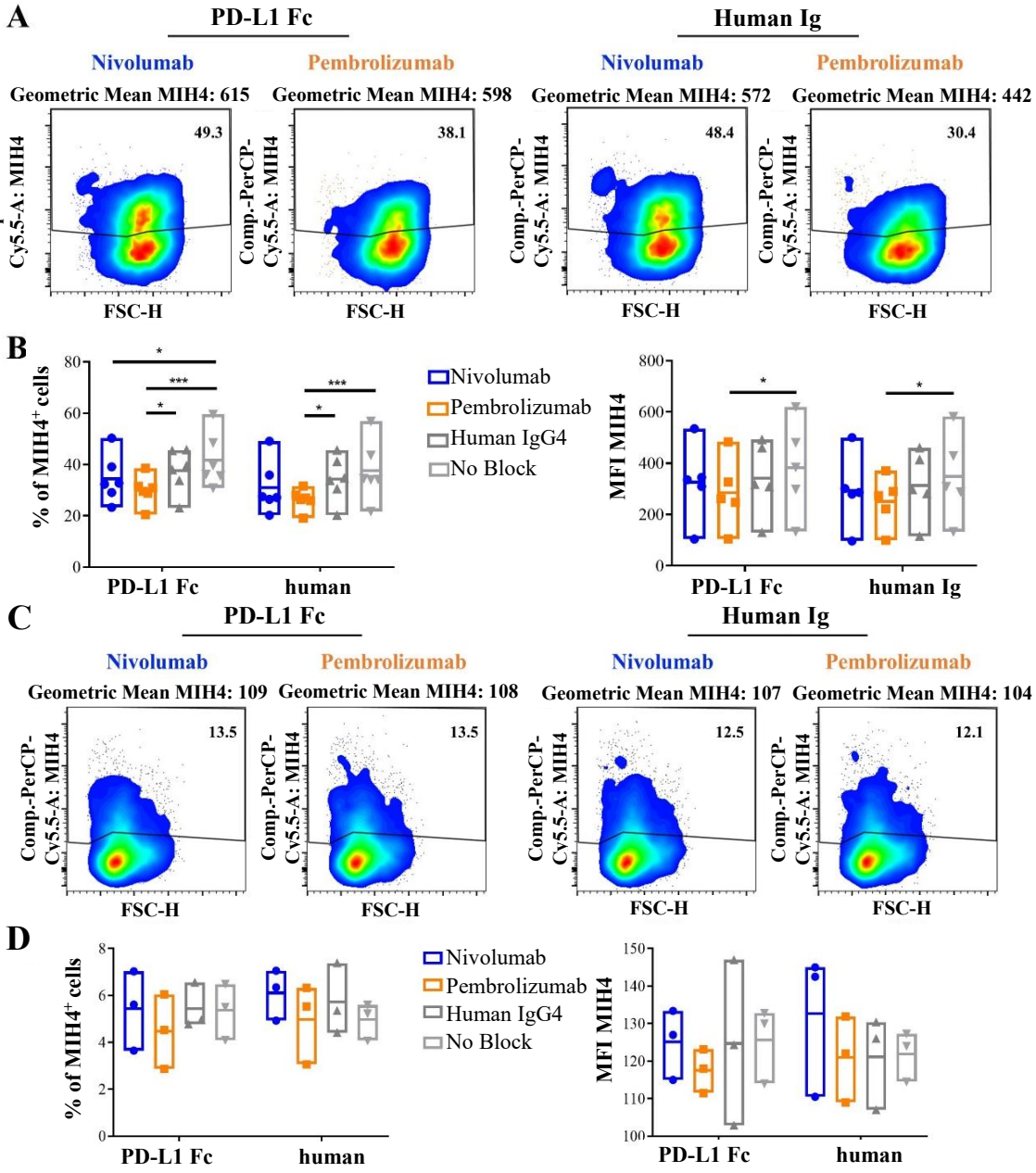

Figure 15: PD-1 blockade with Pembrolizumab, but not with Nivolumab, decreases MIH4 binding affinity to B cell-PD-1.

(A) Representative flow cytometry dot plots, **(B)** percentages (Mean ± SEM) and MFI (Mean ± SEM) of MIH4 binding to PD-1 expressed by B cells activated in a TD manner and stimulated with PD-L1 Fc or respective isotype control for 72 hrs (*n*=6). PD-1/ PD-L1 interaction was simultaneously blocked with Nivolumab, Pembrolizumab, hIgG4, or no blocking agent. **(C)** Representative flow cytometry dot plots, **(D)** percentages (Mean ± SEM) and MFI (Mean ± SEM) of MIH4 binding to PD-1 expressed by B cells activated in a TI manner and stimulated with PD-L1 Fc or respective isotype control for 72 hrs (*n*=3). PD-1/ PD-L1 interaction was simultaneously blocked via Nivolumab, Pembrolizumab, hIgG4, or no blocking agent.

2.2. PD-1 blockade with Pembrolizumab, but not Nivolumab enhances B cell proliferation

To investigate the effect of Nivolumab and Pembrolizumab treatment on B cell proliferation, we treated circulatory B cells, as described, and quantified the frequency of proliferating B cells via flow cytometric intracellular staining for the proliferation marker Ki67. Upon TD B cell activation, we detected a substantially higher frequency of $Ki67^+$ B cells treated with Pembrolizumab, compared to isotype control-treated B cells ($Ki67^+$ cells: 65.6% ± 6.6% versus 57.6% ± 7.8%, Mean ± SEM, n=6, p=0.0550*;* MFI Ki67: 1781.6 ± 428.4 versus 1526.4 ± 295.6, Mean ± SEM, n=6, p=0.6460, **Figures 16A+B**). However, treatment with Nivolumab did not result in increase of $Ki67^+$ proliferating cells, compared to isotype control ($Ki67^+$ cells: 60.2% ± 7.6%, versus 57.6% ± 7.8%, Mean ± SEM, n=6, p=0.8220, MFI Ki67: 1591.5 ± 320.6 versus 1526.4 ± 295.6, Mean ± SEM, n=6, p=0.9904, **Figures 16A+B**). Similar findings were observed when B cells were activated in a TI manner. As shown in **Figures 16C+D**, treatment with Pembrolizumab ($Ki67^+$ cells: 40.4% ± 4.1% versus 33.6% ± 3.5%, Mean ± SEM, n=5, p=0.0484; MFI Ki67: 539.8 ± 96.8 versus 498.6 ± 78.1, Mean ± SEM, n=5, p=0.1431), but not Nivolumab ($Ki67^+$ cells: 38.2% ± 3.2% versus 33.6% ± 3.5%, Mean ± SEM, n=5, p=0.2692; MFI Ki67: 528.6 ± 83.3 versus 498.6 ± 78.1, Mean ± SEM, n=5, p=0.3838), resulted in a markedly higher frequency of $Ki67^+$ B cells, compared to isotype control. Together, these findings suggest that Pembrolizumab might induce B cell proliferation more efficiently than Nivolumab, although experiments involving additional patients that possibly focus on B cell subsets as opposed to bulk populations, as reported herein, are required to further solidify our findings with statistical significance.

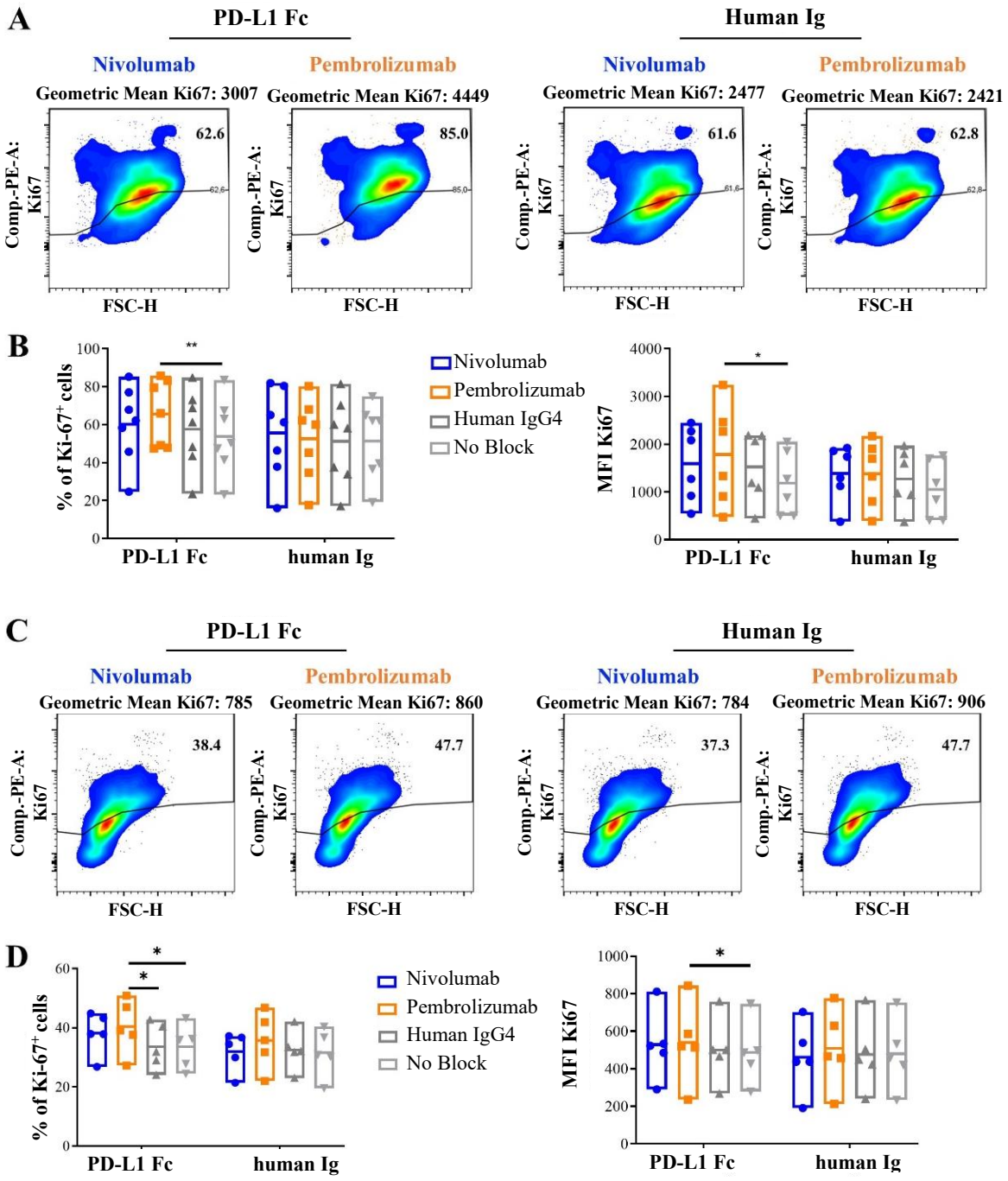

Figure 16: PD-1 blockade with Pembrolizumab, but not with Nivolumab enhances B cell proliferation, compared to controls.
(A) Representative flow cytometry dot plots and **(B)** percentages (Mean ± SEM) and MFI (Mean ± SEM) of intracellular Ki67 staining to B cells activated in a TD manner and stimulated with PD-L1 Fc or respective isotype control for 72 hrs (*n*=6). PD-1/ PD-L1 interaction was simultaneously blocked with Nivolumab, Pembrolizumab, human IgG4, or no blocking agent. **(C)** Representative flow cytometry dot plots and **(D)** percentages (Mean ± SEM) and MFI (Mean ± SEM) of intracellular Ki67 staining B cells activated in a TI manner and stimulated with PD-L1 Fc or respective isotype control for 72 hrs (*n*=5). PD-1/ PD-L1 interaction was simultaneously blocked with Nivolumab, Pembrolizumab, human IgG4, or no blocking agent.

2.3. Neither Nivolumab- nor Pembrolizumab-based PD-1 blockade modulates B cell maturation

The engagement of PD-1 by PD-Ls induces inhibitory signals leading to impaired B cell activation and maturation (Okazaki et al. 2001). To investigate whether Nivolumab or Pembrolizumab treatment can abrogate these inhibitory signals to reinvigorate B cell maturation, we assessed the expression of the B cell activation markers, CD80 and CD86, and the plasma cell marker, CD38, by flow cytometry. As shown in **Figure 17A**, CD86 was expressed by the vast majority of B cells activated in a TD manner (96.7% ± 0.5%, Mean ± SEM, *n*=5). However, we did not detect any difference in CD86 expression between B cells treated with Nivolumab, Pembrolizumab or isotype control (Nivolumab (N) 95.9% ± 1.9%, Pembrolizumab (P) 96.4% ± 1.6%, isotype (I) 96.8% ± 1.3%, Mean ± SEM, *n*=5, **Figure 17A**). Similarly, most B cells activated in a TD manner expressed CD80 (88.9% ± 1.5%, Mean ± SEM, *n*=4, **Figure 17A**) and/or CD38 (77.8% ± 1.3%, Mean ± SEM, *n*=4, **Figure 17A**). However, we did not detect any difference in CD80 or CD38 expression between B cells treated with Nivolumab, Pembrolizumab or isotype control (CD80: N 87.7% ± 5.0%, P 88.6% ± 5.1%, I 88.7% ± 5.2%, Mean ± SEM, *n*=4; CD38: N 77.3% ± 4.6%, P 77.7% ± 3.8%, I 77.8% ± 4.4%, Mean ± SEM, *n*=4, **Figure 17A**). As shown in **Figure 17B**, 81.8% ± 0.6% (Mean ± SEM, *n*=3) of the B cells activated in a TI manner expressed CD86 and 71.7% ± 0.2% (Mean ± SEM, *n*=1) of the B cells expressed CD38 on their surface, while CD80 expression was more restricted (27.8% ± 2.0%, Mean ± SEM, *n*=3). No difference in CD86, CD80 or CD38 expression was observed between B cells treated with Nivolumab, Pembrolizumab or isotype control (CD86: N 81.8% ± 1.7%; P 82.4% ± 1.9%, I 81.8% ± 1.7%, Mean ± SEM, *n*=3; CD80: N 27.8% ± 6.9%, P 25.8% ± 7.7%, I 28.7% ± 6.2%, Mean ± SEM, *n*=3; CD38: N 72.4%, P 72.9%, I 70.9%, *n*=1, **Figure 17B**). Together, these results indicate that the two clinical PD-1 inhibitors, Pembrolizumab and Nivolumab, do not affect B cell maturation.

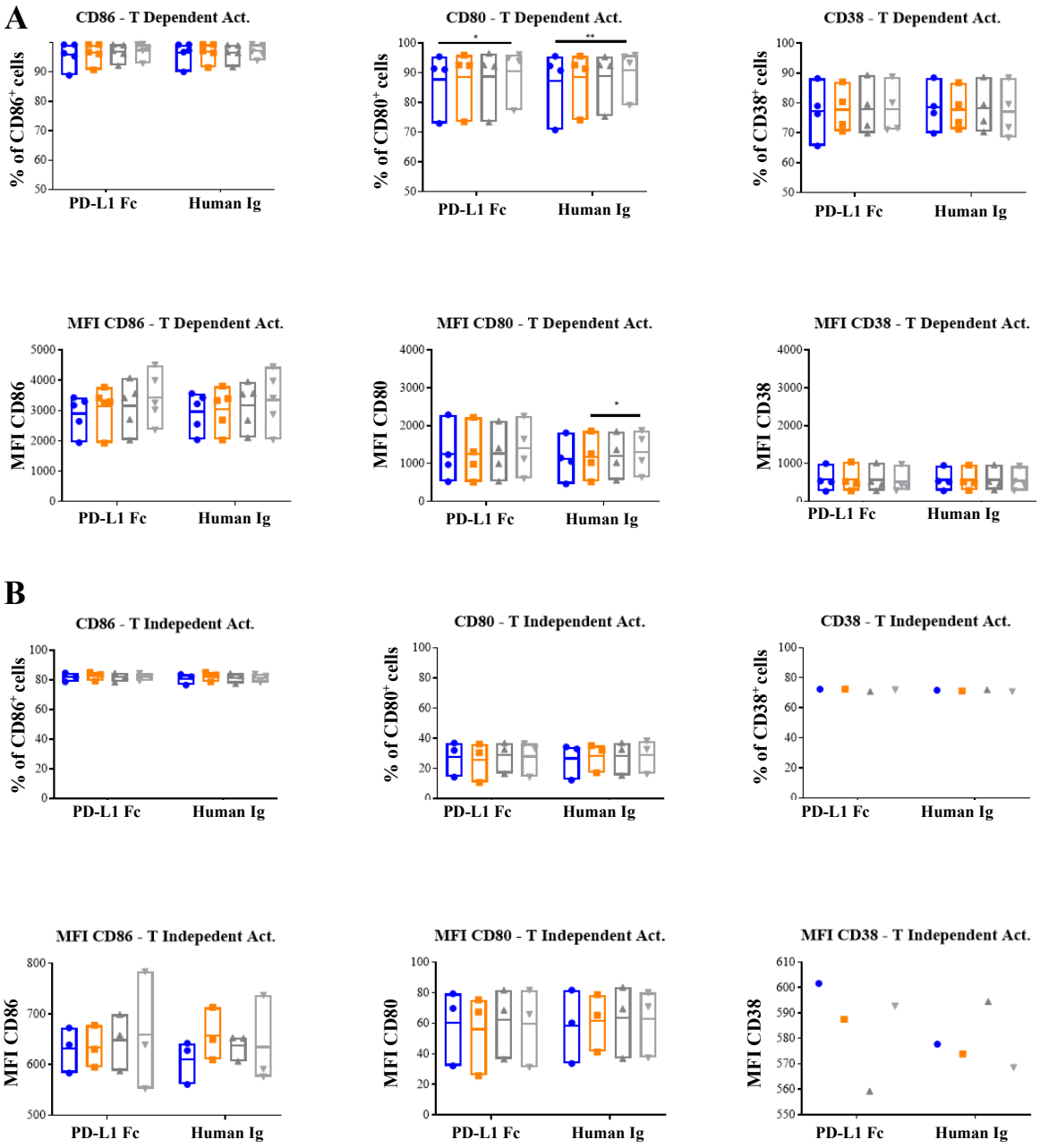

Figure 17: PD-1 blockade with Nivolumab or Pembrolizumab does not affect B cell maturation. **(A)** Percentage (Mean ± SEM) and MFI (Mean ± SEM) of CD86 *(n=5)*, CD80 *(n=4)*, CD38 *(n=4)* expression of B cells activated in a TD manner, stimulated with PD-L1 Fc chimera or respective isotype, and blocked with Nivolumab, Pembrolizumab or isotype control for 72 hrs. **(B)** Percentage (Mean ± SEM) and MFI (Mean ± SEM) of CD86 *(n=3)*, CD80 *(n=3)*, CD38 *(n=1)* expression of B cells activated in a TI manner, stimulated with PD-L1 Fc chimera or respective isotype, and blocked with Nivolumab, Pembrolizumab or isotype control for 72 hrs.

2.4. PD-1 blockade with Nivolumab or Pembrolizumab does not differentially impact cytokine secretion by B cells

IL-6 is a pro-inflammatory cytokine produced by various lymphocytes, including B cells, which critically influences B cell proliferation and isotype switching. IL-10 is produced by a small subpopulation of B cells that can act as either pro- or anti-inflammatory cytokine orchestrating multiple processes involved in adaptive immunity (Vazquez et al. 2015). Here, we activated circulatory B cells, as described above, and evaluated the effect of Nivolumab versus Pembrolizumab treatment on B cell secretion of IL-6 and IL-10 using ELISAs. As shown in **Figure 18A**, high levels of IL-6 and IL-10 were detected in all samples *tested.* Upon TD B cell activation and stimulation with PD-L1 Fc chimera, we did not detect any significant differences in IL-6 and IL-10 production by B cells treated with Nivolumab, Pembrolizumab, or isotype control (IL-6: N 5734.4 ± 578.3 pg/ml, P 5573.3 ± 451.2 pg/ml, I 6065.9 ± 510.1 pg/ml, Mean ± SEM, *n*=5; IL-10: N 1882.0 ± 409.8 pg/ml, P 2153.7 ± 488.9 pg/ml, I 2178.7 ± 533.6 pg/ml, Mean ± SEM, *n*=6, **Figure 18A**). Following TI B cell activation, IL-6 and IL-10 levels were lower compared to supernatants obtained during TD B cell activation (**Figure 18**). Similar to our previous findings, however, no significant differences in cytokine production were detected between Nivolumab, Pembrolizumab, or isotype control treatment groups (IL-6: N 460.7 ± 147.1 pg/ml, P 501.2 ± 121.8 pg/ml, I 443.8 ± 118.6 pg/ml, Mean ± SEM, *n*=6; IL-10: N 1805.1 ± 268.5 pg/ml, P 1656.0 ± 313.3 pg/ml, I 1615.6 ± 342.9 pg/ml, Mean ± SEM, *n*=6, **Figure 18B**). These findings show that PD-1 blockade with the clinical inhibitors, Nivolumab or Pembrolizumab, does not impact production of the B cell cytokines, IL-6 and IL-10.

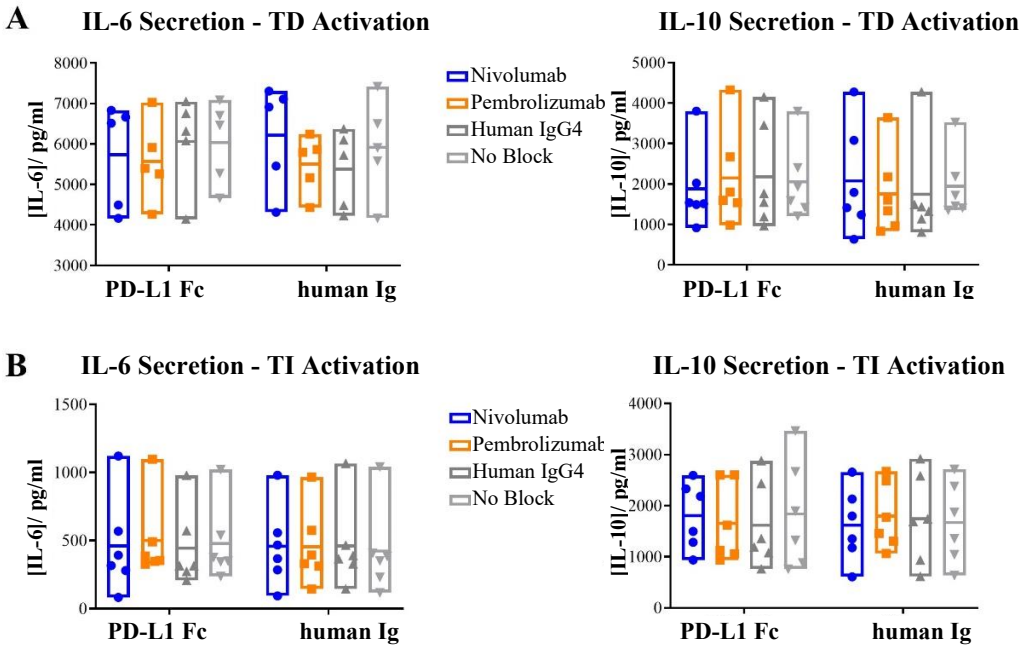

Figure 18: PD-1 blockade with Nivolumab or Pembrolizumab does not significantly influence secretion of IL-6 or IL-10.
(A) Concentration (Mean ± SEM) of IL-6 (left, *n*=5) and IL-10 (right, *n*=6) in culture supernatant upon culturing B cells activated in a TD manner, stimulating with PD-L1 Fc chimera or respective isotype, and blocking with Nivolumab, Pembrolizumab or the isotype control. **(B)** Concentration (Mean ± SEM) of IL-6 (left, *n*=6) and IL-10 (right, *n*=6) in culture supernatant upon culturing B cells activated in a TI manner, stimulating with PD-L1 Fc chimera or respective Ig control, and following blockade with Nivolumab, Pembrolizumab or isotype control treatment.

2.5. PD-1 blockade with Nivolumab or Pembrolizumab does not appear to influence IgM, but might possibly differentially affect IgG production by B cells

B cells can release an extensive repertoire of Ab isotypes to directly neutralize Ags or boost cellular immunity (Chiaruttini et al. 2017). To investigate whether Nivolumab- or Pembrolizumab-based B cell-PD-1 blockade affects Ab secretion, we performed ELISAs to assess the concentration of IgG and IgM in culture supernatants. Our findings are summarized in **Figure 19**. Upon TD B cell activation, high levels of IgGs were detected in all culture supernatants (3309.9 ± 526.3 ng/ml, Mean ± SEM, *n*=5, **Figure 19A**). However, we did not detect any significant difference between IgG secretion of Nivolumab, Pembrolizumab or isotype control-treated cells (N 2470.4 ± 820.0 ng/ml, P 3621.0 ± 1461.8 ng/ml, I 3582.6 ± 1567.5 ng/ml; Mean ± SEM, *n*=5, **Figure 19A**), although IgG levels in the Pembrolizumab treatment group tended to be higher. As shown in **Figure 19A**, we did not detect secreted IgMs upon TD B cell activation, regardless of whether B cells were treated

with Nivolumab, Pembrolizumab or isotype control. Upon TI B cell activation, we detected a robust amount of secreted IgMs and IgGs (IgM: 306.1 ± 37.5 ng/ml, Mean ± SEM, *n*=5; IgG: 3233.1 ± 538.1 ng/ml, Mean ± SEM, *n*=5, **Figure 19B**). However, our data revealed no significant difference in IgM secretion between B cells treated with Nivolumab, Pembrolizumab, or isotype control (N 312.7 ± 107.4 ng/ml, P 293.4 ± 87.0 ng/ml, I 270.6 ± 105.6 ng/ml, Mean ± SEM, *n*=5, **Figure 19B**). While our results did not reach significance for IgG, we found higher IgG contents in the supernatants of Pembrolizumab versus Nivolumab treated B cell cultures (3648.5 ± 1474.1 ng/ ml versus 2276.9 ± 782.1 ng/ml, Mean ± SEM, *n*=5, *p*=0.1258, **Figure 19B**). Together, these findings suggest that there might possibly be PD-1 inhibitor-specific differences in IgG production following PD-1 blockade in B cells.

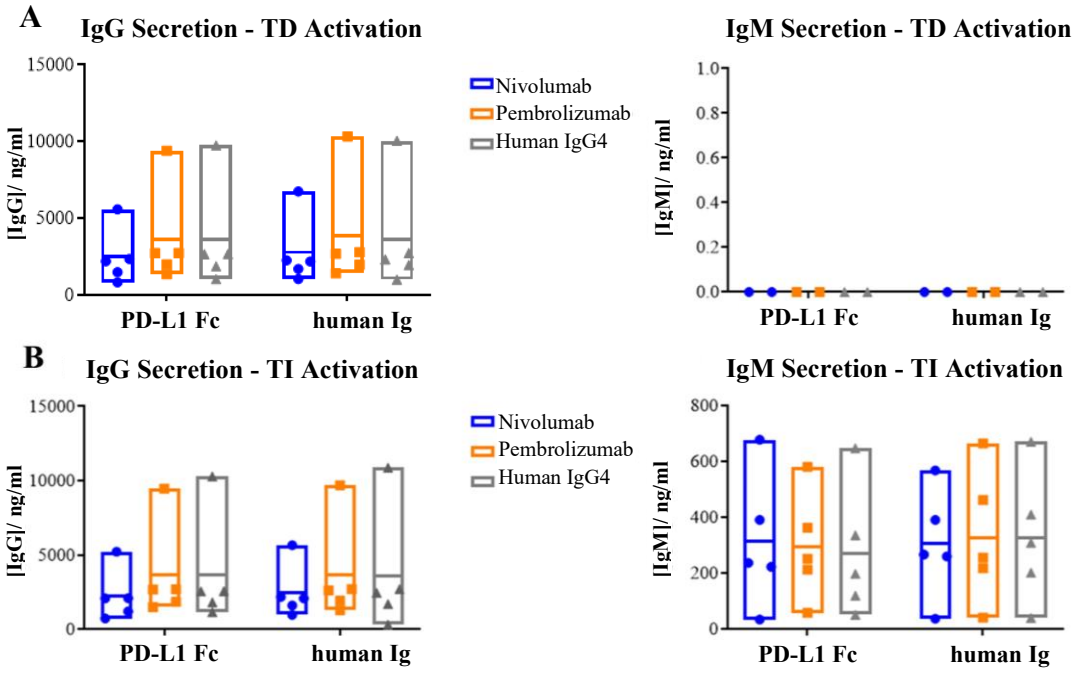

Figure 19: PD-1 blockade with Nivolumab or Pembrolizumab does not appear to influence IgM, but might possibly differentially affect IgG production by B cells.
(A) Concentration (Mean ± SEM) of IgG (*n*=5) and IgM (*n*=2) in culture supernatant upon culturing B cells activated in a TD manner, stimulating with PD-L1 Fc chimera or respective isotype, and blocking with Nivolumab, Pembrolizumab or the isotype control. **(B)** Concentration (Mean ± SEM) of IgM (*n*=5) and IgG (*n*=5) in culture supernatant upon culturing B cells activated in a TI manner, stimulating with PD-L1 Fc chimera or respective isotype, and blocking with Nivolumab, Pembrolizumab or the isotype control.

Discussion

TABs predict response to PD-1 blockade in melanoma patients

Melanoma growth and progression is often sustained by an immunosuppressive microenvironment characterized by dampened cytotoxic T cell activity and tumor immune recognition. Increased understanding of the tumor immune ecosystem has led to the development of cancer immunotherapies that successfully target mechanisms of tumor immune evasion and have revolutionized melanoma treatment. Particularly, therapies targeting immune checkpoint molecules, such as the PD-1, rescue tumor-associated T cell immunity and yield significant clinical benefit for many patients with advanced stage cancers of various etiology, including melanoma. While it is clear that T cells play a critical role in mediating anti-PD-1 therapeutic efficacy, other immune cell types, including B cells, might also contribute to clinical benefit (Gooden et al. 2011). Nevertheless, the possible contribution of tumor-infiltrating B cells to PD-1 inhibitor-induced antimelanoma immunity is not well understood. Recent data revealed that high levels of melanoma-infiltrating B cells and the presence of melanoma-specific Abs in the serum are associated with improved response to PD-1 blockade, suggesting that B cells are potential predictors of and might contribute to patient response to PD-1 therapy (Amaria et al. 2018, Fässler et al. 2019, Helmink et al. 2020).

As outlined in the introduction, TABs may engender multiple functions during antitumor immunity, including tumor-reactive Ab and pro-inflammatory cytokine secretion, tumoral Ag presentation, and direct tumor killing activity (Abate-Daga et al. 2013, Cabrita et al. 2020). Recently, Griess and colleagues reported that TABs comprise multiple B cell subsets enriched for co-stimulatory, pro-inflammatory, and B cell exhaustion markers, that significantly contribute the orchestration and maintenance of melanoma-directed immunity (Griss et al. 2019).

Given the emerging data suggesting that TABs may play a critical role in promoting response to PD-1 therapy, we investigated whether the two main clinical anti-PD-1 Abs, Nivolumab and Pembrolizumab, bind to B cell-expressed PD-1 and performed phenotypic and functional analyses of B cells upon anti-PD-1 Ab treatment. We also investigated how the B cell glycome may affects PD-1 inhibitor recognition.

Human B cells display higher affinity to Pembrolizumab versus Nivolumab

Consistent with the published literature, our preliminary results revealed that human circulatory, tonsillar and melanoma-infiltrating B cells express PD-1 **(Figure 5+7)** (Okazaki et al. 2001, Thibult et al. 2012, Tirosh et al. 2016). Interestingly, while Pembrolizumab efficiently recognizes PD-1 expressed by B cells, Nivolumab displays low B cell-PD-1 reactivity in comparison **(Figure 6+7)**. Additionally, pretreatment of activated B cells with Pembrolizumab, but not with Nivolumab decreased B cell-PD-1 binding by the alternative anti-PD-1 Ab clone MIH4 **(Figure 15**), further suggesting enhanced Pembrolizumab mediated PD-1 recognition and possibly also blocking activity, compared to Nivolumab.

Given the divergent B cell reactivity between the two clinical anti-PD-1 Abs, Pembrolizumab and Nivolumab, we investigated whether these inhibitors recognize specific B cell populations. Our data indicated that Pembrolizumab, and to a lesser extent Nivolumab, mostly target B cells of the memory compartment **(Figure 9+10)**. Furthermore, we found that Pembrolizumab, but not Nivolumab, recognizes a PD-1^{high} B cell population that exclusively contains NS Mem B cells **(Figure 9+10)**. NS Mem B cells are a functionally diverse subpopulation of unswitched B cells that contribute to fast immunological memory producing low affinity IgM- and IgD-Abs (Pauli et al. 2011). Interestingly, NS Mem B cell frequency is significantly reduced in advanced and metastatic melanoma, compared to healthy donors (Crescioli et al. 2023). Thus, their presence might be associated with favorable disease outcome. To our knowledge, these data are the first to reveal divergent binding affinities to B cell-PD-1, and especially to PD-1^{high} NS Mem subsets, between two FDA-approved anti-PD-1 Abs. Accordingly, our findings further suggest that Pembrolizumab, but not Nivolumab, might be involved in the restoration of rapidly activated IgM-derived memory B cell function.

It should be noted that because we utilized circulatory B cells for many of our analyses, our results not fully reflect divergent inhibitor effects on PD-1^{high} NS Mem B cells within the melanoma microenvironment. To fully elucidate the role of B cells in mediating PD-1 inhibitor responses, more research into the function(s) of NS Mem B cells during antitumor immunity is needed. Moreover, further experiments are needed to characterize the unique role of B cells exclusively recognized by Pembrolizumab, but not Nivolumab, in tumor-specific immunity.

Nevertheless, our data indicates that B cells of the NS Mem compartment exhibit a significantly higher binding affinity to Pembrolizumab, compared to Nivolumab. Accordingly, the frequency of IgD^+ $CD27^+$ B cells within the TME might serve as a unique tool to predict and/or monitor response to Pembrolizumab. Due to their unique binding profile to PD-1 inhibitors, these cells could play a critical role in inhibitor-specific clinical outcomes during PD-1 immune checkpoint inhibition.

Pembrolizumab elicits greater B cell proliferation, compared to Nivolumab-based PD-1 inhibition

While it is well known that PD-1 blockade results in increased cytotoxic $CD8^+$ T cell proliferation and secretion of effector cytokines (Waldman et al. 2020, Wang et al. 2014), the role of PD-1 in tumor-infiltrating B cells remains unclear. Accordingly, we sought to investigate B cell proliferation, maturation, cytokine and Ig secretion upon PD-1 blockade with Nivolumab versus Pembrolizumab. Notably, B cells treated with Pembrolizumab displayed higher proliferation when compared to B cells treated with Nivolumab or human IgG4 isotype control **(Figure 16)**. On the other hand, treatment of B cells, with either Nivolumab or Pembrolizumab did not differentially affect B cell maturation (as assessed by CD86, CD80 and CD38 expression **(Figure 17)**), cytokine **(Figure 18)** or overall Ig secretion **(Figure 19)**. Thus, our findings suggest that B cell-specific PD-1 blockade with Pembrolizumab, but not Nivolumab results in enhanced B cell proliferation, and blockade with either Ab does not majorly affect B cell maturation, cytokine or Ig secretion. Consistently, recently published data indicates that anti-PD-1 therapy leads to an increase in TAB numbers (Griss et al. 2019), thereby supporting the notion that B cell-specific PD-1 blockade results in enhanced B cell proliferative activity. Moreover, a recent report describing the case of a melanoma patient who achieved durable response to Pembrolizumab after treatment failure with Nivolumab, further highlights the possibility of at least partially distinct mechanisms of action between these two PD-1 blocking agents (Lepir et al. 2019). Our data suggests that B cells might contribute to the observed variation in PD-1 inhibitor-induced clinical activity and might light into the cellular mechanism underlying Pembrolizumab response and/or lack of benefit from Nivolumab treatment in this patient.

While multiple recent reports have linked the presence of melanoma-associated B cells to improved clinical outcome following PD-1 inhibition, our results might provide some

mechanistic insights in this regard. Treatment with Pembrolizumab induces greater B cell proliferation and may thus result in higher B cell frequencies within the TME, compared to Nivolumab-based therapy. On the other hand, treatment with either PD-1 inhibitor does not appear to majorly affect (1) B cell maturation and differentiation, (2) Ig secretion, or (3) production of the pro- and anti-inflammatory cytokines *tested*, which included IL-6 and IL-10. The precise molecular mechanisms underlying proliferative activity and possibly other processes induced by Pembrolizumab, but not Nivolumab, in B cells, requires further study. Nevertheless, our results represent a first important step towards appreciating PD-1 inhibitor-specific differences in B cell affinity and function that could possibly be exploited for therapy and therapeutic monitoring.

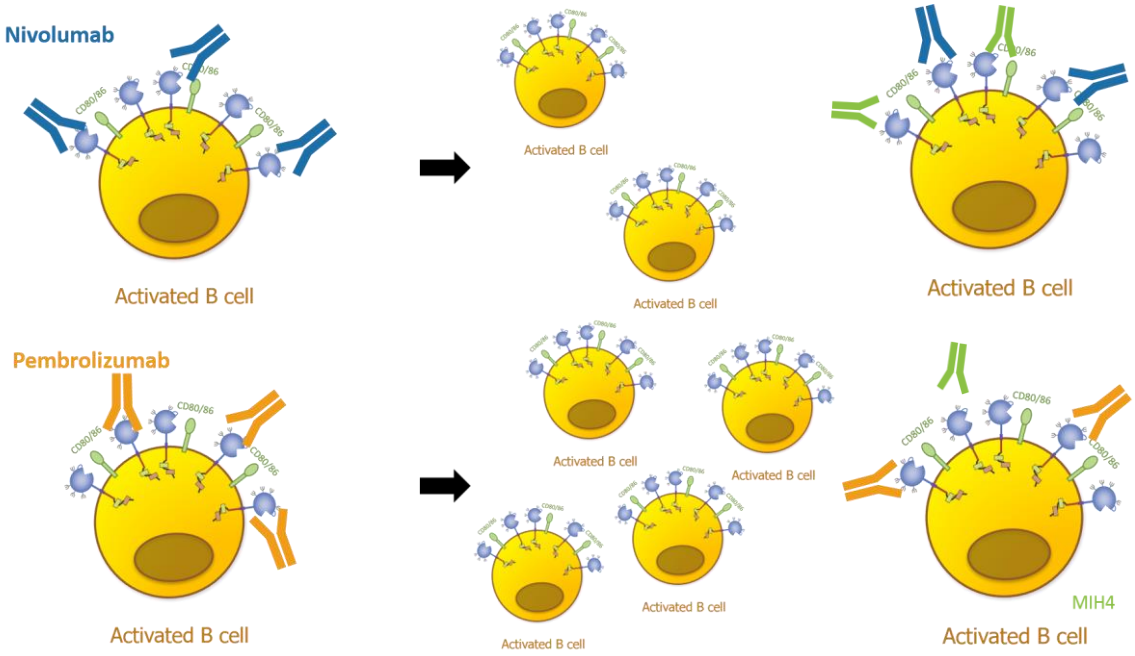

Figure 20: Differential effects of PD-1 blockade with Nivolumab versus Pembrolizumab
B cell-specific PD-1 blockade with Pembrolizumab leads to increased proliferation of B cells, compared to blockade with Nivolumab. Moreover, blockade with Pembrolizumab sufficiently blocks the receptor and reduces binding of MIH4 to B cell-specific PD-1, whereas blockade with Nivolumab does not.

B cell glycome affects binding of PD-1 inhibitors to B cell-PD-1

While PD-1 checkpoint inhibitors have become the standard of care for many malignancies including melanoma, only 30-40% of patient respond to this novel form of therapeutics (Rossi et al. 2020a). Poor therapeutic efficacy in some patients might result from limited Ab binding to target cell-PD-1 (Fessas et al. 2017). Accordingly, understanding the mechanisms underlying anti-PD-1 Ab affinity might help to improve clinical response to PD-1 inhibitors.

As described in the Introduction Chapter 4.3.3, terminal Sia residues shape biophysical properties of the cell surface by adding negative charges, masking underlying protein or glycan structures, or acting as direct ligands for Siglec immune inhibitor receptors (Varki & Gagneux 2012). Notably, the PD-1 receptor is heavily *N*-gycosylated (Chen et al. 2019, Sun et al. 2020). However, the PD-1 glycoform distribution and its significance in clinical Ab binding remains unknown. We observed that enrichment of high mannose and hybrid *N*-glycan structures via kifunensine and swainsonine treatment, respectively, decreased Nivolumab binding to B cells, but did not significantly affect Pembrolizumab binding (**Figure 14**). Complete abrogation of *N*-glycans achieved via tunicamycin treatment prevented Nivolumab and Pembrolizumab binding to B cells (**Figure 14**), and removal of cell surface Sia from B cells reduced Pembrolizumab binding (**Figure 12**). Altogether, our data suggests that both Nivolumab and Pembrolizumab binding to B cells is affected by their glycosylation profile. However, given that our findings are based on a limited number of donors, they should thus be treated with caution and must be further verified.

The published data regarding the impact of PD-1-specific *N*-glycosylation on the blocking Ab affinity are somewhat controversial. In line with our findings, some reports state that Nivolumab only binds glycosylated PD-1, but not non-glycosylated PD-1 (Weis & Putman 2014). On the other hand, others report that Nivolumab binding is independent of PD-1 glycosylation (Tan et al. 2017). However, most of these studies focus on anti-PD-1 Ab binding to either T cells or the naked PD-1 peptide, while we assessed binding to B cell-PD-1. The cellular glycome is highly diverse, and it vastly differs depending on the cell type (e.g. T cell versus B cell versus cancer cell), differentiation and/or activation status (Marth & Grewal 2008, Reily et al. 2019, Taniguchi & Kizuka 2015). Additionally, while Tan and colleagues utilized a mammalian cell expressed PD-1 protein with a specific mutation within the *N*-glycosylation site of PD-1 to assess the effect of *N*-glycan alterations directly within

the PD-1 receptor (Tan et al. 2017), we investigated the effect of the total cell surface glycome on PD-1 binding to clinical Abs.

Our observations provide an exciting translational rationale for exploring glycoengineering and/or the development of glycan-specific anti-PD-1 Abs as potential therapeutic strategies to improve anti-PD-1 binding affinity. Moreover, based on our data one ought to assess the utility of TAB glycan structures as predictors of response to PD-1 therapy.

Collectively, our data demonstrates that the affinities of Nivolumab and Pembrolizumab vary depending on sialyation and composition of *N*-glycan residues decorating the B cell surface. These insights could ultimately offer unique tools to enhance the binding affinity of anti-PD-1 Abs and improve therapeutic outcomes.

Conclusion

In this MD thesis, we showed for the first time that B cells isolated from different sources (circulatory, tonsillar, and melanoma-infiltrating B cells) display a higher binding affinity to Pembrolizumab, compared to Nivolumab. While both PD-1 inhibitors preferentially target PD-1^{+} B cells of the memory compartment, Pembrolizumab exclusively recognizes a PD-1^{high} B cell population that mostly consists of NS Mem B cells. Treatment with Pembrolizumab blocks PD-1 more efficiently and elicits greater B cell proliferation, compared to Nivolumab treatment. However, Pembrolizumab does not differentially affect B cell maturation, cytokine secretion or overall Ig secretion, compared to Nivolumab. Interestingly, the complexity of the B cell glycome might influence binding of both PD-1 inhibitors to B cell-expressed PD-1. Thus, our findings suggest that *N*-glycan and/or Sia modification might offer unique tools to improve response to PD-1 checkpoint blockade.

Although we acknowledge that our study has some limitations (mainly utilizing circulatory B cells to model effects within the TME, limited number of independent samples), our result could significantly contribute to a greater understanding of mechanisms underlying PD-1 immune checkpoint therapy in cutaneous advanced melanoma. Moreover, our data highlights the potential significance of B cell-specific PD-1 blockade for antitumor immunity and indicate the need to comprehensively investigate inhibitor-specific effects on all PD-1^{+} lymphocytes and non-immune cells in the TME to improve our understanding of the mechanisms underlying clinical benefit. Collectively, the results of the present MD thesis may offer novel perspectives on potential inhibitor-specific differences in PD-1 checkpoint inhibition and overcoming poor ORRs.

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Suppl. Table 1: List of FDA Approvals of Nivolumab (January 2025)

Modified from: Drugs.com: Opdivo FDA Approval History. (https://www.drugs.com/history/opdivo.html) [Accessed 02.01.2025]

Melanoma	22/12/14	Phase 3 CheckMate -037	Nivolumab	for treatment of patients with	unresectable or metastatic melanoma		
	10/01/15	CheckMate -067	Nivolumab plus Ipilimumab	for first line treatment of patients with	BRAF V600 wildtype unresectable or metastatic melanoma		
	23/01/16	Phase 3 CheckMate -067	Nivolumab plus Ipilimumab	for first line treatment of patients with	BRAF status		
	20/12/17	Phase 3 CheckMate -238	Nivolumab	for adjuvant treatment of patients with	melanoma with involvment of lymph nodes or metastatic disease	who have undergone complete resection	
		Phase 3 CheckMate -76K	Nivolumab	for adjuvant treatment of patients with	melanoma		
HNSCC	10/11/16	Phase 3 CheckMate -141	Nivolumab	for second treatment of patients with	recurrent or metastatic HNSCC	with disease progression on or after plantinum-based therapy	
NSCLC	04/03/15	Phase 3 CheckMate -017	Nivolumab	for second line treatment of patients with	advanced metastatic squamous NCSLC	with progression on or after platium-based therapy	
	09/10/15	Phase 3 CheckMate -057	Nivolumab	for second line treatment of patients with	advanced or metastatic NCSLC	with progression on or after platium-based therapy	
	15/05/20	CheckMate -227	Nivolumab plus Ipilimumab	for first line treatment of patients with	metastatic NSCLC	whose tumors express PD-L1 >1%, with no EGFR or ALK genomic tumor aberrations	
	26/05/20	CheckMate -9LA	Nivolumab plus Ipilumumab given with two cychus of platinum-doublet	for first line treatment of patients with	metastatic or recurrent NSCLC	with no EGFR or ALK regradless of the PD-L1 staus	
	07/03/22	CheckMate -816	Nivolumab plus platinum-doublet	for neoadjuvant treatment of patients with	resectable NSCLC	regardless of the PD-L1 status	
	03/10/23	Phase 3 CheckMate -77T	Nivolumab plus platinum-doublet/ Nivolumab mono as adjuvant	for perioperative treatment of patients with	resectable NSCLC	with no EGFR or ALK regradless of the PD-L1 staus	
SCLC	17/08/18	Phase 1/2 CheckMate -032	Nivolumab	for third line treatment of patients with	metastatic SCLC	whose cancer has progressed after platinum-based therapy and at least one other line of therapy	
Withdrawal	29/12/20	CheckMate -032					
RCC	23/11/15		Nivolumab	for second line treatment of patients with	advanced metastatic RCC	who have received a prior anti-angiogenic therapy	
	16/04/18	Phase 3 CheckMate -214	Nivolumab plus Ipilimumab	for first line treatment of patients with	intermediate and poor risk advanced RCC		
	22/01/21	Phase 3 CheckMate -9ER	Nivolumab plus Cabozantinib	for first line treatment of patients with	advanced RCC		
classical Hodgkin lymphoma	17/05/16	Phase 2 CheckMate -205	Nivolumab	for third line treatment of patients with	classical Hodgkin lymphoma	who have relapsed or progressed after autologous hematopoetic stem cell therapy and post transplantation	
Urothelia carcinoma	02/02/17	Checkmate -275	Nivolumab	for second line treatment of patients with	locally advanced or metastatic urothelial carcinoma	containing therapy or disease progression within 12 month or neoadjuvant treatment with plantium-based therapy	
	20/08/21	Checkmate -274	Nivolumab	for adjuvant treatment of patients with	carcinoma	for recurrence	
	07/03/24	Phase 3 Checkmate - 901	Nivolumab plus cisplatin and gemcitabin	for first line treatment of patients with	unresectable or metastatic urothelial carcinoma (UC)		
Metastatic CRC	01/08/17	Phase 2 CheckMate -142	Nivolumab	for second line treatment of patients with	MSI-H or dMMR metastatic CRC	that have progressed following treatment with 5-FU, Oxaliplatin and Irinotecan	
	11/07/18	Phase 2 CheckMate -142	Nivolumab plus Ipilimumab	for second line treatment of patients with	MSI-H or dMMR metastatic CRC	that have progressed following treatment with 5-FU, Oxaliplatin and Irinotecan	
esophageal Cancer/ Esophageal Junction Adenocarcinoma	16/04/21	Phase 3 CheckMate -649	Nivolumab plus 5-FU, leucovorin, and oxaliplatin or capecitabine and oxaliplatin	for first line treatment of patients with	locally advanced or metastatic gastric or gastroesophageal junction or esophageal adenocarcinoma	regardless of PD-L1 status	

Suppl. Table 1 (continued): List of FDA Approvals of Nivolumab (January 2025)

Esophageal squamous cell carcinoma	10/06/20	Phase 3 ATTRACTION 3	Nivolumab	for second line treatment of patients with	metastatic esophageal squamous cell carcinoma	after prior 5-FU- and platinum-based therapy
	20/05/21	Phase 3 CheckMate -577	Nivolumab	for adjuvant treatment of patients with	gastroesophageal junction cancer with residual pathologic disease	in patients who have received neoadjuvant chemoradiotherapy
	30/03/22	Phase 3 CheckMate -648	Nivolumab + 5-FU and platinum-based therapy	for first line treatment of patients with	unresectable or metastatic esophageal squamous cell carcinoma	regardless of PD-L1 status
HCC	22/09/17	CheckMate -040	Nivolumab	for second line treatment of patients with	HCC	who have previously been treated with sorafenib
	11/03/20	Phase 1/2 CheckMate -40	Nivolumab plus Ipilimumab	for second line treatment of patients with	HCC	who have previously been treated with sorafenib
Pleural mesothelioma	02/10/20	CheckMate -743	Nivolumab plus Ipilimumab	for first line treatment of patients with	unresectable malignant pleural mesothelioma	

Suppl. Table 2: List of FDA Approval of Pembrolizumab (January 2025)

Modified from: Drugs.com: Keytruda FDA Approval History. (https://www.drugs.com/history/keytruda.html) [Accessed 02.01.2025]

Melanoma	04/09/14	P001	Pembrolizumab	for treatment of patients with	advanced or unrecetable melanoma	who no longer responding to other drugs
	18/12(15	PN006	Pembrolizumab	for first line treatment of patients with	advanced or unrecetable melanoma	regradless of the BRAF status
	19/02/19	FORTC1325/ KEYNOTE-054	Pembrolizumab	for adjuvant treatment of patients with	melanoma with involvment of lymph nodes (IIIA - C)	who have undergone complete resection
HNSCC	05/08/16	KEYNOTE-012	Pembrolizumab	for second line treatment of patients with	recurrent or metastatic HNSCC	containing therapy
	11/06/19	Phase 3 KEYNOTE-048	Pembrolizumab	for first line treatment of patients with	metastatic or unresectable recurrent metastatic or unresectable recurrent HNSCC	whose tumors express PD-1 (combined positive scale ≥
	11/06/19	Phase 3 KEYNOTE-048	Pembrolizumab plus platinum and 5-FU	for first line treatment of patients with		regardless of PD-L1 status
Cutanous SCC	24/06/20	KEYNOTE-629	Pembrolizumab	for first line treatment of patients with	metastatic or recurrent cutanous SCC	that is not curable by surgery or radiation
	06/07/21	KEYNOTE-629	Pembrolizumab	for first line treatment of patients with	locally advanced cutanous SCC	that is not curable by surgery or radiation
Merkel cell carcinoma	19/12/18	KEYNOTE-017/ CITN-09	Pembrolizumab	for first line treatment of patients with	recurrent locally advanced or metastatic Merkel cell carcinoma	who had not received prior systemic therapy for advanced disease
NSCLC	02/10/15	KEYNOTE-20	Pembrolizumab	for treatment of patients with	advanced (metastatic) NSCLC	whose disease has progressed after other treatments and with tumors that express PD-L1
	24/10/15	KEYNOTE-20	Pembrolizumab	for first line treatment of patients with	metastatic NSCLC	proportion score of 50% or more), with no EGRF or ALK genomic aberrations
	10/05/17	KEYNOTE-021	Pembrolizumab plus pemetrexed and carboplatin	for first line treatment of patients with	metastatic nonsquamous NSCLC	irrespective of PD-L1 expression
	20/08/18	KEYNOTE-189	Pembrolizumab plus pemetrexed and carboplatin	for first line treatment of patients with	metastatic nonsquamous NSCLC	with no EGRF or ALK genomic aberrations regardless of the PD-L1 status
	30/10/18	KEYNOTE-407	Pembrolizumab plus carboplatin and (nab-) paclitaxel	for first line treatment of patients with	metastatic squamous NSCLC	irrespective of PD-L1 expression
	11/04/19	KEYNOTE-042	Pembrolizumab	for first line treatment of patients with	with stage III NSCLC	who are not candidates for surgical resection or definitive chemoradiation and whose tumors express PD-L1 (tumor proportion score ≥ 1 with no EGFR or ALK genomic aberrations)
	11/04/19	KEYNOTE-042	Pembrolizumab	for first line treatment of patients with	metastatic NSCLC	whose tumors express PD-L1 (tumor proportion score ≥ 1 with no EGFR or ALK genomic aberrations)
	27/01/23	KEYNOTE-091	Pembrolizumab	for adjuvant treatment of patients with	with stage IB, II, or IIIA NSCLC	following surgical resection and platinum-based chemotherapy
	16/10/23	KEYNOTE-671	Pembrolizumab + platin-based therapy (for adjuvant without chemotherapy)	for (neo)adjuvant treatment of patients with	resectable NSCLC	
SCLC voluntary withdrawal	18/06/19	G); KEYNOTE-028 (cohort C1)	Pembrolizumab	for third line treatment of patients with	metastatic SCLC	with disease progression on or after platinum-based therapy and at least one other prior line of therapy
	01/03/21	Phase 3 KEYNOTE -604				
RCC	22/04/19	KEYNOTE-426	Pembrolizumab plus axitinib	for first line treatment of patients with	advanced RCC	
	18/11/21	KEYNOTE-564	Pembrolizumab	for adjuvant treatment of patients with	RCC	following nephrectomy, or following nephrectomy and resection of metastatic lesions
classical Hodgkin lymphoma	14/03/17	KEYNOTE-087	Pembrolizumab	for forth line treatment of patients with	refractory cassical Hodgkin lymphoma	who have relapsed after three or more prior lines of therapy

Suppl. Table 2 (continued): List of FDA Approval of Pembrolizumab (January 2025)

PMBCL	13/06/18	KEYNOTE-170	Pembrolizumab	for third line treatment of patients with	refractory PMBCL	who have relapsed after two or more prior lines of therapy
Urothelia carcinoma	18/05/17	KEYNOTE-052	Pembrolizumab	for first line treatment of patients with	carcinoma	who are ineligible for cisplatin-containing therapy
	18/05/17	KEYNOTE-045	Pembrolizumab	for second line treatment of patients with	locally advanced or metastatic urothelial carcinoma	who have disease progression during or following platinum-containing therapy or within 12 months of neoadjuvant or adjuvant platinum-containing therapy
	08/01/20	KEYNOTE-057	Pembrolizumab	for second line treatment of patients with	BSG unresponsive, high-risk, non-muscle invasive bladder cancer with CIS with or without papillary tumors	who are ineligible for or have elected not to undergo cystectomy
	03/04/23	KEYNOTE-869	Pembrolizumab + enfortumab vedotin	for treatment of patients with	locally advanced or metastatic urothelial carcinoma	who are ineligible for cisplatin-containing treatment
	15/12/23	Phase 3 EV-302 clinical trial	Pembrolizumab + enfortumab vedotin	for treatment of patients with	locally advanced or metastatic urothelial carcinoma	
MSI-H/ dMMR tumors	23/05/17	KEYNOTE-012, 028, 016, 158, 164	Pembrolizumab	for treatment of patients with	unresectable or metastatic solid tumors referred to as MSI-H or dMMR	that have progressed following prior treatment and who have no satisfactory alternative treatment options
	29/06/20	KEYNOTE-177	Pembrolizumab	for first line treatment of patients with	metastatic CRC	
	21/03/22	KEYNOTE-158	Pembrolizumab	for second line treatment of patients with	advanced endometrial carcinoma that is MSI-H or dMMR	therapy in any setting and are not candidates for curative surgery or radiation
	29/03/23	Phase 2 KEYNOTE-158, -164 KEYNOTE-051	Pembrolizumab	for treatment of patients with	unresectable or metastatic MSI-H or dMMR solid tumors	that have progressed following prior treatment and who have no satisfactory alternative treatment options
Gastric cancer voluntary withdrawal	22/09/17 01/07/21	KEYNOTE-059	Pembrolizumab	for third line treatment of patients with	recurrent locally advanced or metastatic gastric or gastroesophageal adenocarcinoma	whose tumor express PD-L1 (combined positive score ≥1) with disease progression on or after two or more prior lines of therapy (5-FU- or platinum-containing or HER2-targeted therapy)
	05/05/21	KEYNOTE-811	Pembrolizumab plus Trastuzumab, 5-FU- and platinum-based therapy	for first line treatment of patients with	locally advanced unresectable or metastatic gastric or gastroesophageal junction adenocarcinoma	whose tumor express HER2
	16/11/23	Phase 3 KEYNOTE-859	Pembrolizumab plus 5-FU- and platinum-based therapy	for first line treatment of patients with	gastric or gastroesophageal junction adenocarcinoma	whose tumor express HER2
Esophageal squamous cell carcinoma	31/07/19	KEYNOTE-181	Pembrolizumab	for second line treatment of patients with	recurrent locally advanced or metastatic esophageal squamous cell carcinoma	whose tumor express PD-L1 (combined positive score ≥1) with disease progression on or after one or more prior lines of systemic therapy
	23/03/21	KEYNOTE-590	Pembrolizumab plus plantinum- or 5-FU-based therapy	for first line treatment of patients with	locally advanced or metastatic esophageal or gastroesophageal junction carcinoma	that is not amenable to surgical resection or definitive chemoradiation in combination with platinum- and 5-FU-based therapy
HCC	09/11/18	KEYNOTE-224	Pembrolizumab	for second line treatment of patients with	HCC	who have previously been treated with sorafenib
Cervix cancer	12/06/18	KEYNOTE-158	Pembrolizumab	for second line treatment of patients with	recurrent or metastatic cervival cancer	whose tumors express PD-L1 (combined positive score ≥ 1)
	13/10/21	KEYNOTE-826	chemotherapy +/- Bevacizumab	for treatment of patients with	with persistent, recurrent or metastatic cervical cancer	whose tumor express PD-L1 (combined positive score ≥ 1)
	12/01/24	Phase 3 KEYNOTE-A18 trial	Pembrolizumab + chemoradiotherapy	for treatment of patients with	FIGO 2014 Stage III-IVA cervical cancer	

Suppl. Table 2 (continued): List of FDA Approval of Pembrolizumab (January 2025)

Endometrial carcinoma	17/09/19	KEYNOTE-146/ Study 111	Pembrolizumab plus Lenvima (lenvatinib)	for treatment of patients with	advanced endometrial carcinoma	that is not MSI-H or dMMR who have disease progression following prior systemic therapy and are not candidates for curative surgery or rediation
	22/07/21	KEYNOTE-775/ Study 309	Pembrolizumab plus Lenvima (lenvatinib)	for treatment of patients with	advanced endometrial carcinoma	progression following prior systemic therapy in any settings and are not canddates for curative surgery or rediation
	17/06/24	KEYNOTE -868	Pembrolizumab plus Carboplatin/ Paclitaxel, followed by Pembrolizumab mono	for treatment of patients with	primary advanced or recurrent endometrial carcinoma	
Breast Cancer	13/11/20	KEYNOTE-355	Pembrolizumab plus chemotherapy	for treatment of patients with	locally recurrent unresectable or metastatic triple-negative breast cancer	whose tumors express PD-L1 (combined positive score ≥1)
	27/07/21	KEYNOTE-522	Pembrolizumab plus chemotherapy as neoadjuvant followed by Pembrolizumab monotherapy as adjuvant therapy	for treatment of patients with	high-risk early-stage triple-negative breast cancer	
Biliary tract cancer	01/11/23	Phase 3 KEYNOTE-966	Pembrolizumab plus cisplatin and gemcitabine	for treatment of patients with	locally advanced or metastatic biliary tract cancer	
Malignant pleura mesothelioma	28/09/24	Phase 2/3 KEYNOTE - 483	Pembrolizumab plus Pemitrexed and platinum chemotherapy	for first line treatment of patients with	unresectable advanced or metastatic malignant pleura mesothelioma	

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To my dear parents for all the constant encouragement, support and love.

Affidavit according to § 5 (1) for Data Retention

I hereby declare that the original data forming the basis of this doctoral thesis are stored
(Hiermit erkläre ich, dass die dieser Dissertation zu Grunde liegenden Originaldaten

- by me personally (bei mir),

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(hinterlegt sind).

Affidavit according to § 5 (1) § 11 (3) 12 of the doctoral studies regulations

I, **Anna Meurer,** hereby declare on oath that I independently collected and prepared the following results portrayed in the dissertation "**Significance of B cells in PD-1 checkpoint immunotherapy**". I had the following assistance with completing the dissertation. These are listed in the acknowledgments.

Hiermit erkläre ich, **Anna Meurer,** an Eides statt, dass ich folgende in der von mir selbstständig erstellten Dissertation „**Bedeutung von B Zellen in der PD-1 Checkpoint-Immuntherapie**" dargestellten Ergebnisse erhoben habe.
Bei der Durchführung der Arbeit hatte ich folgende Hilfestellungen, die in der Danksagung angegeben sind.

	A. Meurer	M. Silva, PhD	T. Schatton, PhD, PharmD	Dr. med. I. Appelmann	PD Dr. med. F. Beier	Summe (%)
Studienüberwachung (study supervision)		40	50	5	5	100
Konzeption (conception)	10	40	50			100
Datenauswertung (data evaluation)	100					100
Durchführung Experimente (performing experiments) Fig. 5-14, 18+19	100					100
Statistische Auswertung (statistical evaluation)	80	20				100
Bereitstellung von Materialien (delivery of matrials)			100			100
Interpretation der Datenauswertung (interpretation of the data evaluation)	60	20	20			100
Durchführung Experimente (performing experiments) Fig 15-17	50	50				100

_____
Unterschrift der Doktorandin/des Doktoranden

Als Betreuer der obigen Dissertation bestätige ich die Angaben von Anna Meurer

_____
Unterschrift des Doktorvaters

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Clinical Skills and Work Experience

10/2022 – Present	Charité Universitätsmedizin Berlin, Berlin, Germany **Clinician Scientist** – Department of Hematology, Oncology and Cancer Immunology
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Education

2018 – Present	RWTH Aachen University, Aachen Germany Harvard Medical School, Boston USA **MD Research Student** „Significance of B cells in PD-1 checkpoint immunotherapy“
2014 – 2021	RWTH Aachen University, Aachen, Germany **Studies of human medicine**, Class of 2021
2006 – 2014	St. Bernhard Gymnasium, Willich, Germany High School Diploma (Abitur), Class of 2014

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