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Investigation of the potential regulation of the PD-1-PD-1 ligand immune checkpoint axis by inhibition of the B cell receptor signaling pathway

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Lea Reemann
aus Wesel

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Dekan: Universitätsprofessor Dr. med. G. R. Fink
1. Gutachter: Universitätsprofessor Dr. med. M. Hallek
2. Gutachter: Universitätsprofessor Dr. rer. nat. H. Kashkar

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For my parents

Table of contents

Abbreviations

°C	Degree Celsius
µg	Microgramm
µl	Microliter
ALL	Acute lymphoblastic leukemia
APC	Antigen-presenting cell
ATM	Ataxia telangiectasia mutated
BCR	B cell receptor
BCL	B cell lymphoma
BLNK	B cell linker protein
BSA	Bovine serum albumin
BTK	Bruton's tyrosine kinase
C	Constant region
CAR	Chimeric antigen receptor
CARD11	Caspase recruitment domain-containing protein 11
CD	Cluster of differentiation
CD40L	CD40 ligand
cf.	Confer
CLL	Chronic lymphocytic leukemia
cm	Centimeter
CML	Chronic myelogenous leukemia
CR	Complete remission
CRISPR	Clustered regularly interspaced short palindrome repeats
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
del	Deletion
dl	Deciliter
DLBCL	Diffuse large B cell lymphoma
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid

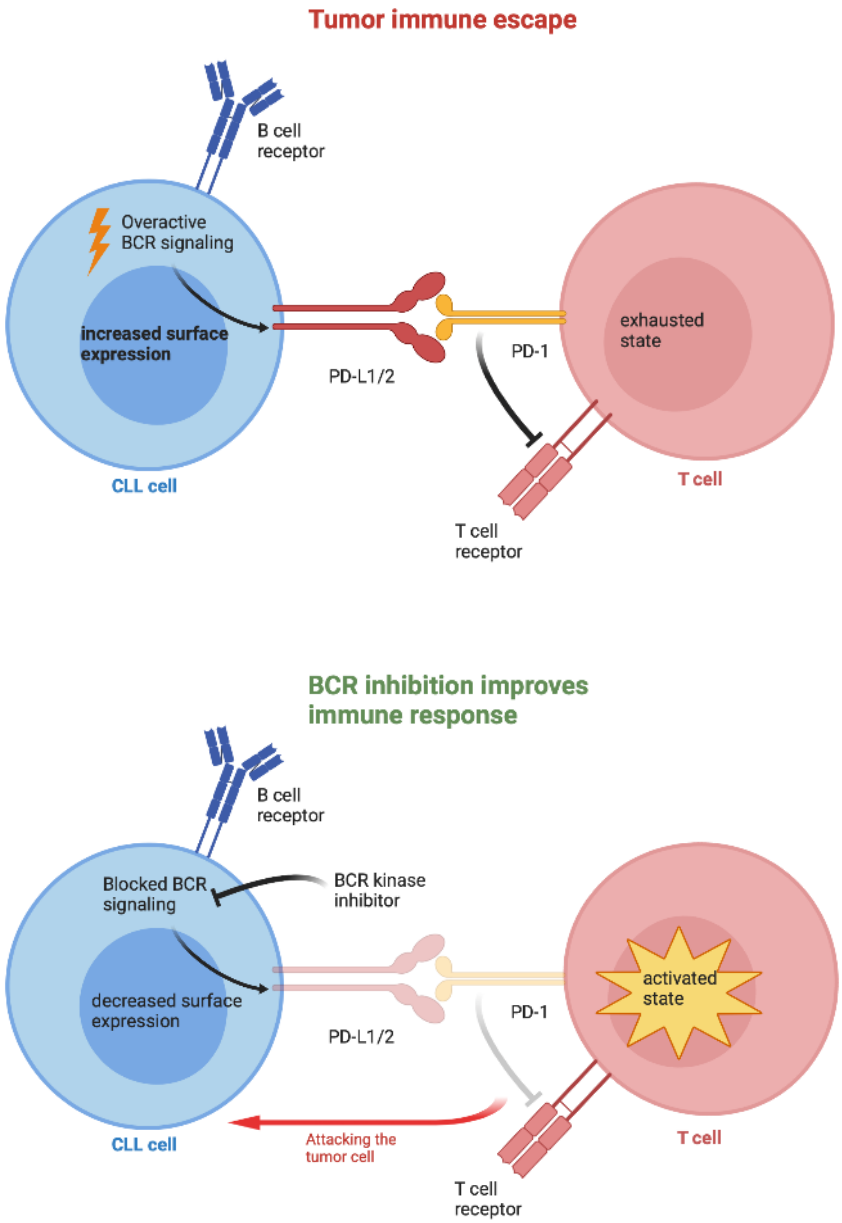
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular-signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FCR	Fludarabine + chlorambucil + rituximab
FISH	Fluorescence In Situ Hybridization
g	Gramm
GAB1	GRB2-associated binding protein 1
h	Hour
Hb	Hemoglobin
ICB	Immune checkpoint blockade
IFN	Interferon
Ig	Immunoglobulin
IgHV	Immunoglobulin heavy chain variable region
IgLV	Immunoglobulin light chain variable region
IL	Interleukin
IPI	International Prognostic Index
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITSM	Immunoreceptor tyrosine-based switch motif
JAK2	Janus kinase 2
kDa	Kilo Daltons
l	Liter
LAG3	Lymphocyte activation gene 3
LMP1	Epstein-Barr virus latent membrane protein 1
LPS	Lipopolysaccharide
LYN	Lck/Yes novel tyrosine
M	Molar
MACS	Magnetic activated cell sorting
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
mg	Milligramm
MHC	Major histocompatibility complex

min	Minute
ml	Milliliter
mM	Millimolar
MSI-h	Microsatellite instability high
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of the rapamycin
NF-κB	Nuclear factor kappa light polypeptide gene enhancer in B cell
NFATC1	Nuclear factor of activated T cells
NHL	Non-Hodgkin lymphoma
NK	Natural killer cell
nM	Nanomolar
ns	not significant
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed Death 1
PD-L1	Programmed Death Ligand 1
PD-L2	Programmed Death Ligand 2
PFS	Progress-free survival
PI3K	Phosphoinositide 3 kinase
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKC β	Protein kinase C β
PLCγ2	Phospholipase C γ 2
PMA	4 beta-phorbol 12-myristate 13-acetate
PR	Partial remission
qRT-PCR	Quantitative real time polymerase chain reaction
RAF	Rat fiber sarcoma
RAS	Rat sarcoma
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	Richter's Transformation
STAT3	Signal transducer and activator of transcription 3
SD	Standard deviation
SHIP	SH2 domain-containing inositol 5'-phosphatase
SHP	SH2 domain-containing protein tyrosine phosphatase

sgRNA	single guide RNA
SLO	Secondary lymphoid organ
SYK	Spleen tyrosine kinase
t	Time
TCR	T cell receptor
TGF-β	Transforming growth factor β
TKI	Tyrosine kinase inhibitor
TME	Tumor microenvironment
TP53	Tumor protein p53
US	United States
VDJ	Variable diversity joining
w&w	Watch and wait
ZAP-70	70 kDa zeta associated protein

1. Summary

1.1. Graphical Abstract



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1.2. Abstract

Chronic lymphocytic leukemia (CLL) is characterized by abnormal proliferation of monoclonal B lymphocytes. A hallmark of CLL is the strong dependency of the malignant B cells on the interactions with the tumor microenvironment. CLL cells have established various strategies to escape the endogenous anti-tumor immune response.

One key mechanism that is known to regulate T cell-mediated immune responses is the PD-1-PD-L1/2 immune checkpoint axis. The PD-1 receptor is located on the surface of effector T cells, and upon binding to its cognate ligands PD-L1 or PD-L2, activation of the T cell is suppressed.

It has been shown that PD-1 and PD-L1 might be upregulated on T cells and CLL cells, respectively, in this malignancy as a way of escaping the immune system.

The B cell receptor (BCR) signaling pathway is crucial for B cell survival and function. In aberrant cells such as CLL, it contributes importantly to anti-apoptotic, pro-survival signaling. Some recent evidence could show that the BCR signaling pathway might regulate PD-L1 expression, for example, in diffuse large B cell lymphoma. Preliminary data from our group suggested that BCR-inhibition could lead to a decrease in PD-L1 surface levels.

Based on these findings, this study aims to investigate the potential regulation of the PD-1-PD-L1/2 immune checkpoint axis by the BCR signaling pathway, focusing on CLL cells.

The central element of this work is to analyze the effects of various BCR kinase inhibitors on a wide range of B cell lines as well as primary CLL cells. Flow cytometry was used as the main readout assay for PD-L1 expression. In those experiments, a reduction of PD-L1 surface levels could often be observed, but with considerable variation depending on the cell line, inhibitor, and treatment timepoint. Effects of BCR inhibition on PD-L1 varied even in the different biological replicates, suggesting very dynamic regulation of PD-L1 expression and instead seems to contradict a clear, one-dimensional way of regulation by the BCR pathway.

However, we found that the SRC-kinase inhibitor dasatinib exerted a consistent and significant downregulation of PD-L1 expression.

Furthermore, it was examined whether a stimulation of the BCR-pathway with Immunoglobulin M (IgM) could increase PD-L1 surface levels. Also in this experimental set-up, targeting the BCR-pathway had no clear impact on PD-L1.

Taken together, a clear mechanism that links BCR inhibition to a reduction of PD-L1 surface levels could not be found, but we identified dasatinib as a substance which could possibly have synergistic effects with PD-1 inhibitors. This effect is probably independent

from the BCR-pathway and related to other targets of this kinase inhibitor. The results of this work thus led to a research project further investigating the influence of dasatinib on PD-L1 expression.

Furthermore, additional data using patient-derived primary CLL cells suggest that PD-L1 surface levels, in contrast to previous publications, might not be significantly higher than on healthy B cells.

1.3. Zusammenfassung

Die chronische lymphatische Leukämie (CLL) ist charakterisiert durch eine abnormale Proliferation monoklonaler B-Lymphozyten. Diese maligne Erkrankung ist, wie viele andere, in hohem Maße abhängig von ihrem Tumormikromilieu. Normalerweise attackiert und zerstört das Immunsystem körperfremde, bösartige Zellen. CLL-Zellen sind jedoch in der Lage, die Immunantwort auf vielfältige Art und Weise zu unterdrücken.

Ein Schlüsselmechanismus, der die Immunantwort im Allgemeinen kontrolliert, ist die PD-1-PD-L1/2-Immuncheckpoint-Achse. Der PD-1-Rezeptor befindet sich auf T-Zellen und durch die Bindung einer seiner Liganden PD-L1 oder PD-L2 wird die Aktivierung der T-Zelle unterdrückt.

Die Rolle dieser Immuncheckpoint-Achse bei CLL ist noch wenig verstanden. Es konnte bereits gezeigt werden, dass PD-1 und PD-L1 hochreguliert sein könnten und so auch in dieser Erkrankung einen Mechanismus darstellen, um sich einem Angriff durch das Immunsystem zu entziehen.

Der B-Zell-Rezeptor (BZR) Signalweg ist essenziell für das Überleben und die Funktionstüchtigkeit der B-Zelle. In aberranten Zellen gibt er Signale ab, die die Apoptose unterdrücken und das Überleben der Zelle fördern.

In neuen Publikationen konnten Hinweise darauf gefunden werden, dass der BZR-Signalweg die PD-L1 Expression regulieren könnte, z.B. bei dem diffus-großzelligen B-Zell-Lymphom. In unserer Gruppe haben einige vorläufige Daten darauf hingedeutet, dass BZR-Inhibition zu einer Reduktion der PD-L1 Oberflächenexpression führen könnte.

Basierend auf diesen Daten wurde die zentrale Hypothese dieser Arbeit generiert. Das Ziel der Studie ist die potenzielle Regulierung der PD-1-PD-L1/2 Immuncheckpoint-Achse durch den BZR-Signalweg zu untersuchen, mit Fokus auf die CLL als Krankheitsbild. Das zentrale Element dieser Arbeit ist es, den Effekt diverser BZR-Inhibitoren auf eine Reihe verschiedener B-Zelllinien sowie CLL-Patientenzellen zu analysieren. Zur Messung der PD-L1 Expression wurde hauptsächlich die Durchflusszytometrie verwendet. In diesen Experimenten konnte oft eine Reduktion der PD-L1 Expression beobachtet werden,

jedoch mit einer sehr großen Variation abhängig von der Zelllinie, spezifischem Inhibitor und Zeitpunkt. Selbst in den verschiedenen biologischen Replikaten konnte der Effekt nicht zuverlässig reproduziert werden, was eine sehr dynamische Regulation der PD-L1 Expression vermuten lässt. Eine klare Regulierung hauptsächlich durch den BZR-Signalweg erscheint hierdurch eher unwahrscheinlich.

Wir konnten jedoch zeigen, dass der SRC-Kinase-Inhibitor Dasatinib zu einem konsistenten und signifikanten Abfall der PD-L1 Expression führte.

Außerdem wurde untersucht, ob eine Stimulation des BZR-Signalwegs durch Immunglobulin M (IgM) zu einer Erhöhung der PD-L1 Expression führt. Auch in diesem Experiment konnte kein direkter Einfluss des BZR-Signalwegs auf PD-L1 beobachtet werden.

Insgesamt konnte kein eindeutiger Mechanismus gefunden werden, der die BZR-Inhibition mit einer Reduktion der PD-L1 Expression verbindet. Wir konnten aber Dasatinib als Substanz identifizieren, die möglicherweise eine Synergie mit PD-1-Inhibitoren aufweisen könnte. Dieser Effekt ist wahrscheinlich unabhängig vom BZR-Signalweg, sondern auf andere Angriffspunkte dieses Kinase-Inhibitoren zurückzuführen. Die Ergebnisse dieser Arbeit führten zu einem nachfolgenden Projekt, in dem der Einfluss von Dasatinib auf die Expression von PD-L1 weiter untersucht wurde.

Zudem geben Durchflusszytometrie-Analysen von CLL-Patientenzellen einen Hinweis darauf, dass PD-L1 Level hier oft nicht signifikant höher sind als auf gesunden B-Zellen, anders als dies in den bisherigen Publikationen angenommen wurde.

2. Introduction

2.1. Chronic lymphocytic leukemia (CLL)

2.1.1. Definition

CLL is characterized by excessive clonal proliferation of mature $CD19^{+}CD5^{+}$ B cells. These cells usually accumulate in the blood, lymph nodes, bone marrow, and spleen. [1]

2.1.2. Epidemiology, Symptoms & Classification

CLL is the most common leukemia in western countries and usually occurs in elderly patients with a median age of 67-72 years. In the U.S., there is an age-adjusted incidence of 4.1/100 000 inhabitants per year. [2] Given the current demographic changes, a worldwide increase in morbidity and mortality can be expected in the upcoming years.

At clinical manifestation, CLL patients can present with B-symptoms which comprise fever, night sweat, and weight loss. Furthermore, swelling of lymph nodes, enlargement of the spleen and liver, and frequent infections due to impaired immune function can occur. [1]

A severe complication of CLL with a very negative impact on survival is the Richter's Transformation (RT). This describes the transformation of the disease into a highly malignant Non-Hodgkin lymphoma (NHL) and can be observed in $\leq$ 5% of the patients. [3] For the classification of CLL, the two staging systems of Rai [4] and Binet [5] are commonly used (Table 1, Table 2). [1] According to the modified Rai staging system, a low-risk disease is defined as a lymphocytosis with more than 30% lymphoid leukemic cells in the blood and/or bone marrow. Patients who additionally show lymphadenopathy, splenomegaly, and/or hepatomegaly are classified as intermediate risk. If disease-related anemia (Hemoglobin <11 g/dl) or thrombocytopenia (<100 x 10^9/l) occur, the patient is considered as high risk. The Binet staging system includes the hemoglobin (Hb) level, thrombocyte count, and the number of areas with enlarged lymph nodes or organs. In particular, head and neck, axillae, groins, spleen as well as the liver represent the five different areas affected most. Binet A is defined as Hb $\geq$10 g/dl, platelets $\geq$100 x 10^9/l, and up to two areas affected. In contrast, Binet B signifies 3 or more areas of enlargement, and stage C implies a Hb <10 g/dl or platelets <100 x 10^9/l.

Table 1: Rai Staging System

Low Risk	Lymphocytosis > 30% of lymphoid leukemic cells in the blood and/or bone marrow

Intermediate Risk	Additional enlargement of lymph nodes/spleen/liver
High Risk	Hb < 11 g/dL or platelets < 100 x 10^9/L

Table 2: Binet Staging System

A	≤ 2 lymph node areas/organ systems enlarged
B	> 2 lymph node areas/organ systems enlarged
C	Hb < 10 g/dL or platelets < 100 x 10^9/L

Due to the scientific advances in CLL research, specific genetic and biological prognostic factors have complemented the two-staging systems. This has led to the establishment of the CLL International Prognostic Index (CLL-IPI, Table 3), which comprises the presence of TP53 deletions or mutations, the immunoglobulin heavy chain variable region (IGHV) mutational status, the serum level of β_2-microglobulin, the clinical status, as well as the age of the patient. [6] By using a weighted grading of all these factors, four risk groups can be distinguished, which show a significant difference in overall survival and require differential treatment regimes.

Table 3: CLL-IPI

Risk group	Treatment recommended
Low risk	No treatment
Intermediate risk	No treatment, except for highly symptomatic patients
High risk	Treatment, except for asymptomatic patients
Very high risk	Treatment, if possible with novel agents or enrollment in new clinical trials

2.1.3. Pathogenesis

Leukemogenic transformation of B cells in CLL is associated with recurrent chromosomal aberrations such as deletion 13q, deletion 11q, deletion 17p, or trisomy 12. [7] The most frequent alteration, *del(13q14)*, can be detected in approximately 55% of CLL patients. It is usually associated with a mild course of the disease. Moreover, *del(11q)* is found in 25% of chemotherapy-naïve patients with advanced and 10% with early-stage disease. This

region carries the gene locus for the DNA damage response kinase ATM, while the loss of this area leads to a less effective repair of DNA mutations. These patients usually show a high-risk form of the disease. Trisomy 12 can be detected in 10-20 % of the cases, but the underlying mechanisms of this aberration are not well-understood so far. The least favorable genomic alteration, occurring in 5-8% of all CLL patients, is *del(17p)*. Often, band 13 is affected, where *TP53* is located. The loss of this tumor suppressor gene leads to severe defects in cell cycle arrest, DNA-damage response, and the induction of apoptosis and is associated with poor prognosis. Furthermore, an unmutated IgHV gene, corresponding to the status found in naïve B cells, correlates with a more severe disease.[1]

Besides, additional mutations can largely determine the clinical disease course. Particularly, genes affecting RNA procession and export, MYC activity, MAPK signaling, and DNA damage response and repair are involved. [7]

Aside from tumor cell-intrinsic factors, the tumor microenvironment plays a crucial role in CLL progression. [8] A variety of different bystander cell types support the proliferation and survival of CLL cells, including, for example, mononuclear cells that differentiate into “nurse-like” cells. [9] Furthermore, an immunosuppressive milieu prevents immune cells such as T cells from attacking the leukemic cells. In addition, the direct interaction with leukemic cells induces changes in gene expression in both CD4 and CD8 positive T cells. This leads to alterations in pathways involved in cytotoxicity, vesicle trafficking, and cytoskeleton formation. [10] Additionally, soluble factors produced by CLL cells, such as IL-10, inhibit T cell activation, expansion, and function as well as antigen response. [11]

2.1.4. Diagnostics

The peripheral blood count is a central diagnostic tool in CLL. Over 5000 B lymphocytes/µl for more than 3 months are a criterion for diagnosis. Furthermore, flow cytometry is used to confirm the clonality of the cells. Other alterations which can potentially be observed in the peripheral blood are anemia, thrombocytopenia, and granulocytopenia due to bone marrow failure. [12]

In blood smear analysis, much higher numbers of mature and small lymphocytes can be found compared to healthy individuals. In addition, the so-called Gumprecht nuclear shadows can be frequently observed, which derive from fragile CLL cells that have been destroyed during sample preparation. [12]

Immunophenotyping is also routinely performed. Typically, the co-expression of the T cell antigen CD5 and the B cell antigens CD19, CD20, and CD23 can be found on the surface of the malignant CLL cells. Furthermore, each CLL cell clone is restricted to express either kappa or lambda light chains. [12]

Further diagnostic tools include FISH-analysis for chromosomal aberrations and the molecular analysis of the IGHV mutation status. Bone marrow cytology and histology are usually not required for diagnosis. [13]

2.1.5. Therapy

As the CLL-IPI score (Table 3) indicates, patients at early stages or with asymptomatic disease should not receive any treatment and follow a "Watch and Wait" procedure. Requirement for treatment is indicated by the first occurrence or rapid progression of symptoms, such as bone marrow failure or an increase in lymphocytosis of more than 50% over 2 months. [6]

Nowadays, a wide range of pharmaceutics is used in CLL treatment. First, there are the classic cytostatic agents, including alkylating chemotherapeutics such as chlorambucil, purine analogs, and bendamustine. [14] Furthermore, monoclonal antibodies play an important role in treatment regimens, especially anti-CD20 antibodies like rituximab which is often combined with chemotherapy. [15]

Another emerging drug class targets the B cell receptor signaling pathway, which is one of the most active pathways in CLL cells. [16] Different effector kinases of this complex signaling network can be inhibited, thus reducing pro-survival and anti-apoptotic signaling of the CLL cells. Important agents are, for example, idelalisib targeting the phosphatidylinositol 3-kinases (PI3Ks) or ibrutinib inhibiting Bruton's tyrosine kinase (BTK). [17] Besides the BCR inhibitors, the BCL-2 inhibitor venetoclax is used to induce apoptosis of CLL cells. [18]

Immunomodulatory drugs like lenalidomide represent another option for CLL patients in consolidation therapy but are currently only administered within clinical trials [19].

The immune checkpoint blocking antibody pembrolizumab, targeting the programmed death 1 (PD-1) receptor, has also been investigated to treat CLL patients. However, in a clinical trial by Ding et al., none of the 16 enrolled CLL patients with relapsed disease responded to treatment. In contrast, 4 out of 9 patients with Richter's transformation (RT, 44%) showed an objective response. Higher PD-L1 levels and a tendency towards increased PD-1 expression were observed in those responders before treatment. [20]

Recently, also chimeric antigen receptor (CAR) T cells have been tested in CLL. [21, 22] Efficacy of the treatment varied immensely between the different clinical trials, with a rate of complete remissions (CR) ranging from 0 to 60% in studies with CAR T cells targeting CD19. Compared to other B cell malignancies, the response rates were rather low. [23] Interestingly, a concurrent administration of ibrutinib with CAR T cells showed promising results in two clinical trials. [24, 25]

As a general principle in CLL therapy, combining the different agents mentioned above is vital to make treatment more effective. In patients with del(17p) or p53 mutation, ibrutinib or venetoclax + obinutuzumab or idelalisib + rituximab are the current first-line therapies. Patients without those genetic alterations receive different regimes depending on their fitness. Fludarabine + cyclophosphamide + rituximab (FCR) or ibrutinib monotherapy are preferred for fit patients. Unfit patients should be treated with venetoclax + obinutuzumab or chlorambucil + obinutuzumab or ibrutinib monotherapy. In relapsed patients, other therapeutic regimens, including allogeneic stem cell transplantation, should be considered. [1]

All in all, to further improve treatment in CLL and overcome the acquisition of resistance mechanisms, further preclinical and clinical studies remain imperative. [1]

2.2. The B cell receptor (BCR) signaling pathway

2.2.1. Physiological role & function

The BCR is a transmembrane protein complex that is crucial for the development and function of each B lymphocyte. [26] It consists of a pair of heavy- and light-chain immunoglobulins, forming a unique antigen-binding site (epitope). [27] During the transition from the pro- to the pre-B cell stage, the immunoglobulin heavy chain variable, diversity and joining genes are rearranged. This is called VDJ recombination. After splicing, this creates a functional immunoglobulin μ heavy chain which is coupled with the surrogate light chain λ5 and VpreB. Thus, the pre-BCR is generated and its signaling leads to the arrangement of immunoglobulin light chain V and J sequences with κ or λ constant regions (Cκ, Cλ). This forms the IgM on immature B cells. Additionally, IgD is generated by splicing the heavy chain VDJ locus to the *∂* constant region (C*∂*) and is then co-expressed with IgM. The further development of the B cell moves from the bone marrow to the secondary lymphoid organs (SLOs). Here, somatic hypermutation occurs. This process adds mutations to the immunoglobulin heavy and light chain V region (IGHV, IGLV) and implies a further diversification of the immunoglobulin repertoire. To create IgA, IgG or IgE, the μ constant region needs to be replaced by the respective constant region specific for each immunoglobulin, which is called class switch. B cells can furthermore develop into memory B cells or plasma cells, which are able to secrete specific antibodies. [26]

Upon antigen binding to the BCR, a cascade of downstream kinases and phosphatases is activated (Figure 1). [28] First, the tyrosine kinase LYN phosphorylates the immunoreceptor tyrosine-based activation motif (ITAM) of the CD79α CD79β heterodimer, allowing the spleen tyrosine kinase (SYK) to bind. Together with BTK, it activates B cell linker protein (BLNK), which connects this cascade to more distal signaling effectors. Above that, the

activation of BTK is dependent on the phosphorylation of PI3K via SYK. PI3K can also be activated directly by LYN, but in this case the response is weaker. Downstream pathways then mobilize calcium and activate RAS and MAPK, which play an important role in regulating cell proliferation and survival. [29] Furthermore, phospholipase C γ 2 (PLCγ2), protein kinase C β (PKCβ) and caspase recruitment domain-containing protein 11 (CARD11) activate the Nuclear factor-κB (NF-κB) pathway. This family of transcription factors also promotes cell proliferation and survival and coordinates differentiation as well as inflammatory and immune responses. [30, 26,31]

In addition, negative feedback loops are also vital to regulate BCR signaling. Here, LYN plays a dual role in positive and negative signaling events downstream of the BCR-pathway by phosphorylating both effector kinases and phosphatases. [32]

Furthermore, a variety of co-stimulating and co-inhibitory factors contributes to the complexity of the BCR signaling network. For example, CD19 takes part in the activation of the PI3K/AKT pathway and thus promotes B cell survival. Via PI3K*∂*, phosphatidylinositol-3,4,5-triphosphate (PIP3) is generated as a second messenger and further recruits BTK and PLCγ2. This results in a downstream activation of AKT and mTOR which again facilitate cell survival, proliferation, and growth. [33] In contrast, the co-receptors CD5 and CD22, together with phosphatases like SH2 domain-containing inositol 5'-phophatase 1 and 2 (SHIP1 and SHIP2) inhibit the transcriptional changes in the nucleus caused by BCR-activation. This results for instance in an upregulation of BCL6, which serves as a repressor of transcription.

Of note, the processes described above also vary depending on maturation status of the B cell. This includes the class switch from IgM/IgD to IgG, which for example leads to a more intensified early antigen response. [34]

In addition to this antigen-dependent activation, a tonic low-level BCR-signaling also exists. [26] This could first be shown in a mouse model with ablation of the surface immunoglobulin, which resulted in rapid apoptosis of the B lymphocytes. [35]

All in all, the BCR is of vital importance in the maturation process of B cells, their differentiation to plasma cells or memory B cells, and the specific antibody response to foreign antigens. It promotes B cell activation, proliferation and survival and thus aberrations in this pathway play a key role in the pathogenesis of B cell lymphomas. [31]

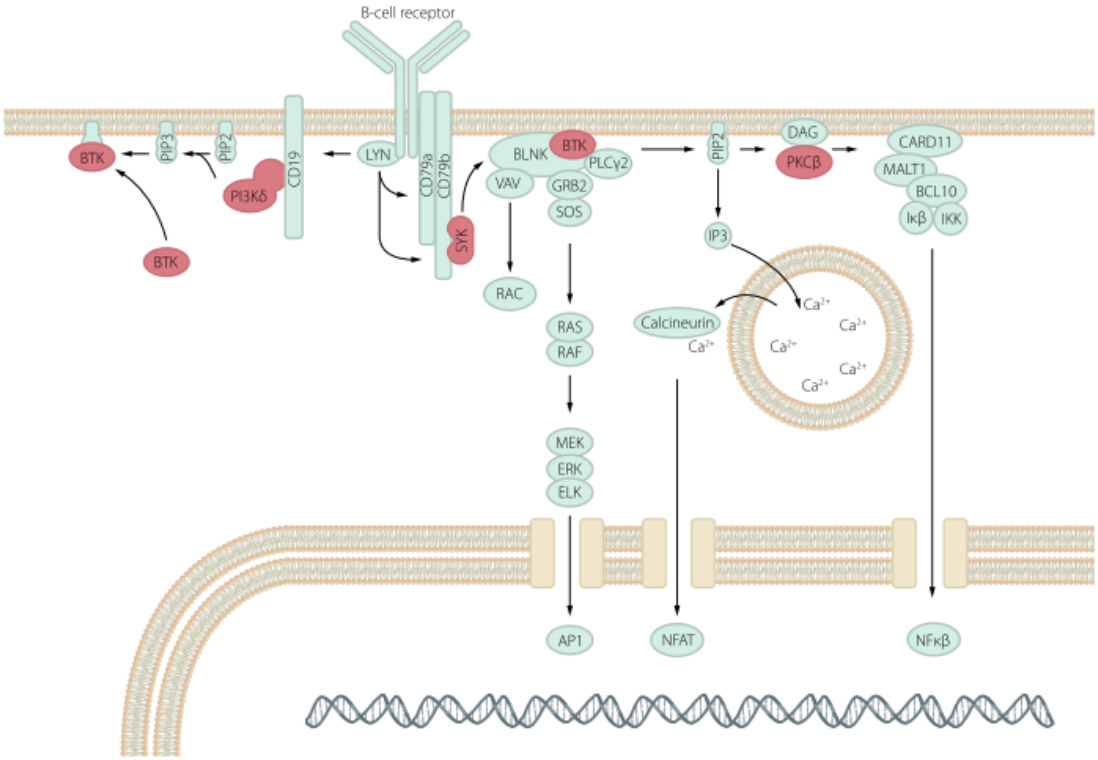

Figure 1: The B cell receptor signaling pathway: The complex downstream network following BCR-activation is depicted. Kinases which represent current therapeutic targets are highlighted in red. Adopted from [28].

2.2.2. Role of the BCR in B cell malignancies

Due to frequent DNA modifications in the germinal center, such as somatic hypermutation, B cells are prone to develop not only favorable DNA mutations but also those that might eventually lead to malignant transformation. Moreover, aberrant cells tend to maintain IgM expression instead of IgG. [36] Subsequently, pro-survival signaling cascades downstream of CD79α and CD79β lead to Nf-kB activation. [31]

In CLL, the Ig shows an unusual structure, while predominantly IgM and IgD are expressed. Furthermore, the IGHV mutation status is a central prognostic marker. [31,37] Surprisingly, when comparing different CLL patients, very similar BCR Igs have been found, hinting at common antigens or specific features of these BCRs that might play a role in the CLL pathogenesis. [38,39] However, mutations in BCR-associated proteins are rarely found. [40,41] In contrast, it was shown that the tyrosine kinase LYN is upregulated in many patients, which leads to increased inhibition of apoptotic signals. [42] Another key contributor to activating the BCR pathway and avoiding apoptosis is PI3K, mediated through adaptor proteins such as GRB2-associated binding protein 1 (GAB1). [31] Furthermore, expression of zeta-chain (TCR)-associated protein kinase 70 kDa (ZAP-70), which is a protein normally found in T cells and not in healthy B cells, is associated with unmutated IGHV and has a negative prognostic impact for CLL patients. [43,44]

Taken together, inhibiting essential molecules of the BCR signaling network is an effective therapeutic approach for the treatment of various lymphoma entities including CLL.

2.2.3. Small-molecule inhibitors of BCR-kinases

The development of small-molecule inhibitors targeting essential kinases of the BCR-pathway has revolutionized the treatment of B cell malignancies. The following section will give a short overview of the function, and clinical use of the BCR-inhibitors studied in this work.

An inhibitor commonly used in the clinic is ibrutinib, which blocks the kinase BTK. This leads to decreased B cell survival and reduces signaling of multiple cell surface receptors. [45,46] The first clinical studies in which the efficacy of ibrutinib could be shown were published in 2013 by Byrd et al. [47] and Advani et al. [48]. After the RESONATE-2 trial [49], ibrutinib became an essential agent in CLL first-line treatment and is often given as monotherapy. [1]

Another irreversible BTK inhibitor is represented by acalabrutinib, which is supposed to have higher selectivity than ibrutinib. A first study published in 2016 showed promising results in patients with relapsed CLL. [1,50] In the phase III ELEVATE-TN trial (published in 2019), improved progression-free survival (PFS) could be observed for acalabrutinib monotherapy and in combination with obinutuzumab compared to chlorambucil + obinutuzumab in treatment-naïve CLL patients. [51] Also, the phase III ASCEND trial published in 2020 showed significantly longer PFS with acalabrutinib than idelalisib + rituximab or bendamustine + rituximab in patients with relapsed or refractory CLL. [52]

Other BTK-inhibitors studied in this work are spebrutinib and tirabrutinib, which are currently evaluated in clinical trials. [53] A phase Ib study comparing tirabrutinib vs tirabrutinib + entospletinib vs tirabrutinib + idelalisib was published in 2020. [54] The different treatments were tolerated well, but for the 53 patients enrolled in this study, no superiority of the combination therapy could be observed.

Another important small-molecule inhibitor is idelalisib, which inhibits PI3K∂ and thus reduces survival signals, chemotaxis, and migration. Furthermore, secretion of chemokines is decreased. [55] In 2014, a first phase I trial showed a good response in relapsed and refractory CLL patients. [56] Today, in combination with rituximab, idelalisib is a first-line therapy option for patients with del(17p) or p53 mutation if there are contraindications for ibrutinib. Moreover, it is a second-line option in refractory disease or early progression. [1]

Another important molecule of the BCR-signaling cascade is SYK. The first agent which has been developed for targeting SYK was fostamatinib. Furthermore, fostamatinib was

also the first BCR-inhibitor ever investigated in a clinical trial. [57] Although it showed promising results, it was later overshadowed by the success of the BTK and PI3K∂-inhibitors. [26] In contrast, entospletinib, a more selective inhibitor for SYK, has shown remarkable clinical efficacy in a phase II trial with relapsed or refractory CLL patients. [58] Another trial studied the combination of entospletinib with idelalisib, where an objective response could be observed in 60% of CLL patients, but the trial had to be stopped due to severe cases of pneumonitis. [59]

Dasatinib is an unspecific inhibitor of multiple kinases and was initially developed to block ABL. Thus, it is primarily used in chronic myelogenous leukemia (CML) since an overactive fusion BCR-ABL kinase is the central driver of this disease. Furthermore, it is a therapeutic option in Philadelphia chromosome-positive acute lymphoblastic leukemia (ALL). [60,61]

Taken together, none of the small-molecule inhibitors mentioned above is entirely specific for only one kinase, while the functional activity of their primary targets is not limited to the BCR-pathway. This implies complex and multifaceted functional mechanisms for each of the approved BCR-inhibitors, accompanied by interference with multiple off-target molecules that can lead to undesirable toxicity. [26] Thus, a second generation of more specific inhibitors is needed, which is currently under clinical investigation.

2.3. The PD-1-PD-L1/2 immune checkpoint axis

2.3.1. Physiological role

The interaction between programmed death protein 1 (PD-1; CD279) and its cognate ligands PD-1 ligand 1 (PD-L1; CD274; B7-H1) and 2 (PD-L2; CD273; B7-DC) represents a critical checkpoint axis to balance T cell mediated immune responses. [62] Upon ligand binding, the PD-1 receptor, predominantly expressed on activated T cells, initiates inhibitory signaling events that reduce T cell activity and proliferation while promoting self-tolerance and homeostasis (Figure 2). [63,64] In addition, PD-1 can also be found on the surface of natural killer (NK) T cells, B cells, activated monocytes, and dendritic cells (DCs). [62] In contrast, PD-L1 is expressed on diverse hematopoietic and nonhematopoietic cells, [62] while the expression of PD-L2 is more restricted and can be induced, for example, on DCs or macrophages. [65]

Usually, T cells become activated after specific binding of the T cell receptor (TCR) to peptide-major histocompatibility complexes (MHC) on the surface of antigen-presenting cells (APCs) or malignant cells. In addition, T cell responses are tightly controlled by various co-stimulatory or co-inhibitory signals. Regarding T cell inhibition by PD-1, PD-L1

seems to be the dominant ligand compared to PD-L2 since PD-L1 antibodies are clinically as effective as PD-1 antibodies. [66]

PD-1, PD-L1, and PD-L2 are type 1 transmembrane proteins that belong to the immunoglobulin superfamily. [63,67] After PD-1 binds to its ligands, the cytoplasmic domain undergoes conformational changes, allowing the recruitment of SRC-family kinases that phosphorylate the intracellular tyrosine-based inhibitory motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM) of the receptor. Afterward, the cytoplasmic protein tyrosine phosphatases SHP-2 and SHP-1 can bind the ITIM and ITSM motifs, leading to their activation. Subsequently, SHP-1 and SHP-2 counteract the TCR-signaling cascade by dephosphorylating essential downstream kinases. This affects various T cell effector functions, including proliferation, cytokine production, and survival. [66]

Besides, there is accumulating evidence for reversed signaling through PD-L1, which, for example, has been shown to provide pro-survival signals to tumor cells overexpressing PD-L1 on their surface. [66,68]

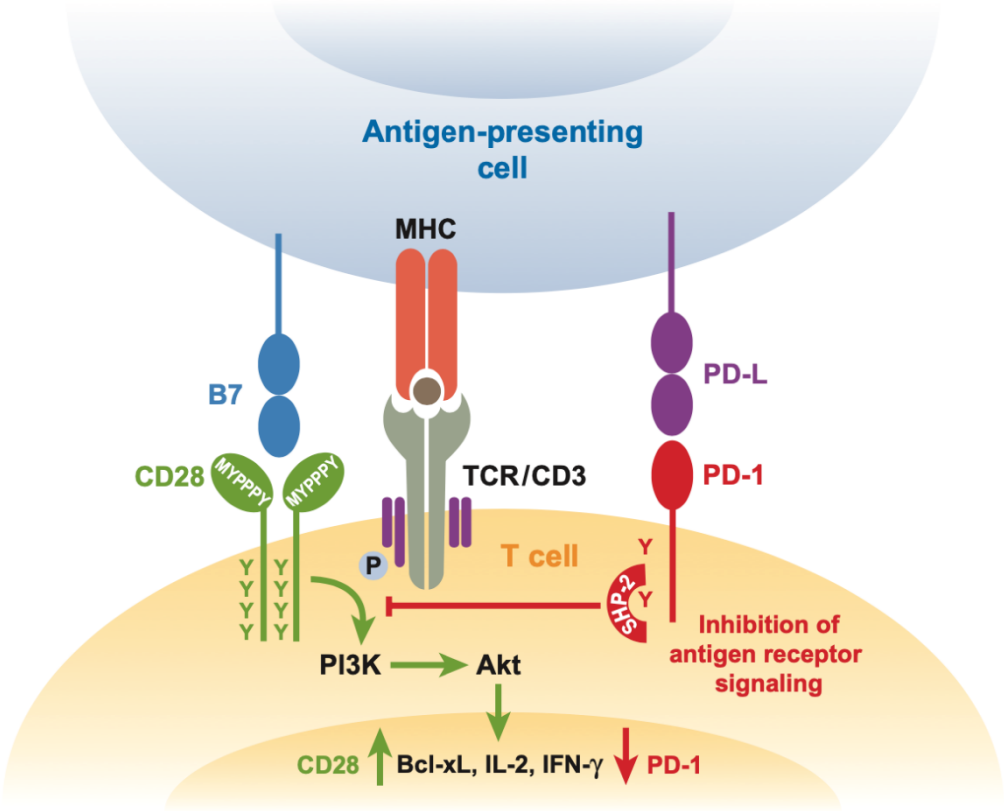

Figure 2: The PD-1-PD-L1/2 immune checkpoint axis: After antigen presentation via the MHC and binding to the TCR, the T cell is activated. Ligation of CD28 to CD80 or CD86 (B7) provides essential co-stimulatory signals, whereas interaction of PD-1 and PD-L1/PD-L2 leads to inhibitory signal transduction reducing T cell activity. Adopted from [62].

2.3.2. Role of the PD-1 axis in the pathology of CLL

Cancer is often associated with the constant presence of tumor cell-derived neoantigens. Moreover, chronic antigen exposure can put effector T cells into a so-called exhausted state, characterized by progressive functional defects that can also be frequently observed in CLL. [69,70] Exhausted T cells show high expression of PD-1, which contributes to maintaining the dysfunctional phenotype. [71] Besides constant TCR-signaling, cytokines such as IL-10 [72] and TGF-β [73] can further induce the expression of PD-1.

In addition to T cells, the expression of PD-1 on the surface of other immune cells has been shown to play a functional role in cancer pathology. For example, it has been observed that tumor cell phagocytosis can be increased by blocking PD-1 on the surface of macrophages. [74]

In CLL patients, Brusa et al. have shown a significant increase of PD-1 on T cells compared to healthy donor controls, as well as an upregulation of PD-L1. Notably, some CLL cells also expressed PD-1, whereas healthy B lymphocytes were mostly PD-1 negative. Furthermore, *in vitro* activation of CLL cells with CpG + IL-2 led to a substantial increase in surface PD-L1 levels. [70]

In contrast, Grzywnowicz et al. observed no significant differences in PD-L1 levels of healthy donors compared to CLL patients. However, they found PD-1 surface expression to be higher in CLL cells. Above that, they quantified the mRNA expression of PD-1 via qRT-PCR and found an increased level of the full-length transcript in CLL cells. In contrast, the level of the full-length PD-L1 transcript was similar to the healthy donors, while a splicing variant lacking exon 2 was significantly higher in CLL. [75]

Additionally, Ramsay et al. could show that CLL cells use, among others, PD-L1 to impair actin synapse formation in T cells. This immunosuppressive mechanism can also be found in other lymphomas and solid cancers. Furthermore, they could show an upregulation of immune-inhibitory ligands, including PD-L1 on B cells and PD-1 on T cells, in peripheral blood and lymph node samples of CLL patients. Significantly increased expression was associated with poor prognosis. [76]

All in all, the PD-1-PD-L1/2 axis is supposed to be active in CLL, but more studies need to be conducted in order to unravel the functional role in the pathogenesis and pathophysiology of this disease.

2.3.3. Immune checkpoint blockade

In recent years, therapeutic antibodies blocking the interaction between PD-1 and its ligands have achieved unprecedented success in treating various cancer entities because of the long-lasting effect in the responsive subgroup. In 2010, a first phase I clinical trial

reported the antitumoral activity of single-agent anti-PD-1 therapy in melanoma, colorectal cancer, and renal cell carcinoma. [77] Since then, many more studies have followed, leading to approvals of different anti-PD1 and PD-L1 blocking antibodies to treat various cancer entities, including Hodgkin's lymphoma as the first hematological malignancy. [78] However, often only a subgroup of patients shows a significant response, and there are also many tumor types where the therapeutic activity is very limited. This led to comprehensive investigations on how to predict the response to anti-PD-1 checkpoint blockade of a given tumor. In addition to PD-L1 expression, many factors have been identified to contribute to the success of immune checkpoint blockade (ICB) therapy. According to Sun et al., 2018, those include "(1) the "foreignness" of the tumor, (2) the immune status of the patient, (3) the presence and activity of the intratumoral T cell infiltrate, (4) the presence of other inhibitory processes within the tumor, and (5) the sensitivity of tumor cells to tumor-specific T cells at that site" [66]. Here "foreignness" of the tumor refers to the mutational load leading to the presentation of neoantigens. In this regard, it has been observed that microsatellite instable-high (MSI-H) solid tumors, for instance, frequently respond well to PD-1/PD-L1 inhibition. [66,79,80] This type of tumor displays a difference in allele length of short, repetitive DNA sequences compared to normal tissue. Together, the parameters mentioned above determine whether ICB can restore effective anti-tumor immune responses.

Expression of PD-L1 on tumor cells as well as in the tumor microenvironment (TME) has been shown to have a predictive value in many, [81-85] but not all studies, [86,87] which have been conducted so far. Interestingly, there are PD-L1 positive cancers that do not respond to immune-checkpoint blockade, as well as PD-L1 negative cancers which do. [66] The highly dynamic expression pattern of the PD-L1 protein might be a possible explanation for these contradicting observations.

In CLL, data on treatment with PD-1 or PD-L1 antibodies are limited. In a phase II clinical trial, [20] pembrolizumab monotherapy was administered to 16 patients with relapsed disease and 9 patients with Richter's Transformation. There was no response in the CLL group, while there was 1 patient with complete remission (CR) and 3 patients with partial remission (PR) in the RT group. Consistently, in another phase II trial investigating the combination of ibrutinib with the PD-L1 antibody nivolumab, the response rates in CLL were similar to ibrutinib monotherapy, while for patients with RT there was a significant benefit from the combination treatment. [88] Consequently, PD-1 blockade has been considered a treatment option in RT but does not seem to have any clinical activity in CLL. However, the underlying reason for the lack of response in CLL patients is still unclear.

Interestingly, there are some studies pointing to a possible improvement of ICB-therapy in CLL by combination with other agents.

It has already been shown that the CD40-stimulation of CLL cells in co-culture with CD40L expressing feeder cells stimulates the anergic T cells, which illustrates the immunogenic potential of CLL cells. [89]

In an adoptive transfer mouse model of CLL, the combination of anti-PD-1 and anti-LAG3 immune checkpoint blockade has been shown to be superior to PD-1 monotherapy. [90]

Furthermore, in 2021 Ioannou et al. published a preclinical study demonstrating how the immune-modulatory drug avadomide reinforces the efficacy of anti-PD-L1/PD-1 treatment in CLL by inducing IFN signaling in T cells. [91]

Also in clinical trials, numerous combination therapies have already been tested or are currently under investigation. This includes the BCR-inhibitors ibrutinib, acalabrutinib and idelalisib as well as chemotherapy, radiotherapy, ipilimumab (anti-CTLA-4), rituximab (anti-CD20), brentuximab-vedotin (anti-CD30 antibody-drug-conjugate), MEDI-551 (anti-CD19), blinatumomab (bispecific anti-CD19/CD3 T engager), lenalidomide, AFM13 (bispecific anti-CD30/CD16A), varlilumab (anti-CD27) and many more. [92]

Taken together, those studies emphasize the potential of ICB in CLL despite poor response rates in the first trials. Further investigations in this field are of vital importance to discover new treatment options for CLL patients in the future.

2.4. Possible link between the BCR and the PD-1 pathway

The understanding of PD-L1 expression and regulation in B cell lymphoma is still limited. Recently there have been studies suggesting an expressional regulation of PD-L1 by the BCR pathway. One study, focusing on DLBCL, proposed a model in which the BCR signaling cascade activates the transcription factor NFATc1. This in turn induces IL-10 secretion which activates JAK2/STAT3 signaling, and STAT3 finally increases the expression of PD-L1 in the lymphoma cells. Consistently, they observed a decrease in PD-L1 expression after BTK inhibition. [93] In another publication by Kondo et al., 2018, CLL patient samples were analyzed for the expression of PD-L1 after ibrutinib treatment. Interestingly, they observed a significant downregulation of PD-L1 on the surface of $CD19^+$ B cells 3 months after initiation of treatment and identified STAT3 as an important mediator of this effect. In addition, the IL-10 production was also reduced. Taken together, the authors concluded that ibrutinib acts not only by direct BCR inhibition but also by modifying the TME as an immune modulator. [94]

In summary, these studies provide new insights into the underlying mechanisms of immune evasion in CLL and could hint at the possible clinical efficacy of combining PD-1/PD-L1 antibodies with BCR-inhibitors.

2.5. Study aims

The regulation of PD-1 ligand expression is still poorly understood in CLL. Although it has been shown that PD-1 and PD-L1 are frequently upregulated in this malignancy and thus contribute to immune evasion, clinical trials investigating the use of PD-1 and PD-L1 antibodies did not show promising results. A more profound knowledge about this pathway can help identifying novel strategies to improve the response to ICB therapy in CLL. This study aims to investigate the potential link between the BCR pathway and the expression regulation of PD-L1 and PD-L2. Based on previous findings, we hypothesize that the overactivated BCR signaling in CLL cells leads to increased surface expression of PD-1 ligands, which reduces T cell response and thus promotes immune escape by the tumor cells. To confirm our hypothesis, we want to address the following questions:

1. **How does inhibition of the BCR pathway affect PD-L1 expression on CLL cell lines?**

2. **What are the underlying mechanisms of this regulation?**

3. Materials & Methods

3.1. Materials

3.1.1. Technical devices

Name	Description	Manufacturer
5415 R	Centrifuge	Eppendorf, Hamburg, Germany
5810 R	Centrifuge	Eppendorf, Hamburg, Germany
Accu-jet pro	Pipet filler	Brand, Wertheim, Germany
C200	CO_2 Incubator	Labortech, Göttingen, Germany
CASY	Cell counter	OMNI
HI3220	pH/ORP Meter	Hanna Instruments, Woonsocket, RI, USA
JB Aqua 12 Plus	Water bath	Grant Instruments, Shepreth, UK
Lab Dancer	Test tube shaker	IKA, Staufen, Germany
Macs Quant VYB	Flow cytometer	Miltenyi Biotech, Bergisch Gladbach, Germany
Macs Quant X	Flow cytometer	Miltenyi Biotech, Bergisch Gladbach, Germany
Mars Safety Class 2	Laminar Flow Hood	Labogene, Vassingerod, Denmark
Mastercycler epgradient S	Thermocycler	Eppendorf, Hamburg, Germany
Mili-Q	Water purification system	Milipore, Eschwege, Germany
MR 3001	Heater/Magnet stirrer	Heidolph, Schwabach, Germany
Mr Frosty	Freezing Container	Nalgene, Neerijse, Belgium

Pipetus	Pipet filler	Hirschmann, Eberstadt, Germany
Research®	Pipettes (P10-1000)	Eppendorf, Hamburg, Germany
Scout II	Analytical Balance	Ohaus, Pine Brook, NJ, USA
SRT9	Tube roller	Stuart, Staffordshire, U.K.
Telaval 31	Microscope	Zeiss, Oberkochen, Germany
Vibrax VXR	Shaker	IKA, Staufen, Germany

3.1.2. Disposable materials

Item	**Manufacturer**
Cell scraper 25 cm	Sarstedt, Nümbrecht, Germany
Cell strainer 25 µm	BD, Franklin Lakes, NJ, USA
Experimental gloves	Paul Hartmann, Heidenheim, Germany
Experimental tubes 5, 15 and 50 ml	Sarstedt, Nümbrecht, Germany
Falcon™ tissue culture flasks T25 and T75	VWR International, Radnor, PA, USA
Filtered pipet tips	Sarstedt, Nümbrecht, Germany
Multiwell plates 6-,12-,24-,96-well	PAA, Pasching, Austria
Parafilm	Pechiney Plastic Packaging, IL, USA
Petri dishes 10 cm	Corning, NY, USA
Pipet tips 10, 100 and 1000 µl	Sarstedt, Nümbrecht, Germany
Safe-Lock Tubes “Eppi” 0.5, 1.5 and 2 ml	Eppendorf, Hamburg, Germany
Serological pipettes 5, 10, 25 and 50 ml	Sarstedt, Nümbrecht, Germany
Tissue culture dishes 10 cm	PAA, Pasching, Austria

3.1.3. Chemicals and reagents

Reagent	**Manufacturer**
Bovine serum albumin (BSA)	PAA, Pasching, Austria
Dimethylsulphoxide (DMSO)	Carl Roth, Karlsruhe, Germany
Ethanol	Carl Roth, Karlsruhe, Germany

Flow Cleanse Solution	Beckman Coulter, Brea, CA, USA
Flow Sheath Fluid	Beckman Coulter, Brea, CA, USA

3.1.4. Buffer, media, and solutions

Solution	**Composition**
ACK lysis buffer	150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA
Cell culture growth medium supplements	10% FBS, 1% Penicillin/Streptomycin
Freezing medium	50% RPMI 1640, 40% FBS, 10% DMSO
GibcoTM DPBS (Dulbecco's phosphate-buffered saline)	Thermo Fisher Scientific, Waltham, MA, USA (14190)
MACsQuant running buffer (1x)	PBS, EDTA, stabilizer, 0.09% sodium azide
MacsQuant washing solution (1x)	Detergent and stabilizer
PBS (phosphate buffered saline)	137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4

3.1.5. Cell culture media

Name	**Manufacturer**	**Catalog no.**
GibcoTM DMEM (Dulbecco's Modified Eagle Medium)	Thermo Fisher Scientific, Waltham, MA, USA	41965
GibcoTM RPMI 1640 (Roswell Park Medium Institute)	Thermo Fisher Scientific, Waltham, MA, USA	21875

3.1.6. Kits

Name	**Manufacturer**
Annexin V Apoptosis Detection Kit	BD, Franklin Lakes, NJ, USA

3.1.7. Cell lines

Name	**Description**

DB	Human B lymphoblast cell line established from ascites of a 45-year-old male with large cell lymphoma
DOHH-2	Human B cell lymphoma cell line established from pleural effusion of a 60-year-old male with refractory immunoblastic B cell lymphoma
HEK293	Human epithelial cell line established from fetal embryonic kidney
HT	Human B lymphoblast cell line established from ascites of a 70-year-old male with diffuse mixed lymphoma
JVM3	Human EBV-transformed lymphoblast cell line established from peripheral blood of a male in his late 60's with B-prolymphocytic leukemia
Karpas 422	Human B cell non-Hodgkin lymphoma cell line established from pleural effusion of a 73-year-old female with DLBCL
L-540	Human Hodgkin lymphoma cell line established from the bone marrow of a 20-year-old female with Hodgkin lymphoma
Mec1	Human chronic B cell leukemia cell line established from peripheral blood of a 61-year-old female with chronic B cell leukemia
OSU-CLL	Human EBV-transformed chronic B cell leukemia cell line established from peripheral blood of a male of unknown age with CLL
P30-OHKUBO	Human B cell precursor acute lymphoblastic leukemia cell line established from the bone-marrow of an 11-year-old female with acute lymphoblastic leukemia (ALL)

THP-1	Human monocyte cell line established from pleural effusion of 37-year-old male with histiocytic lymphoma
WSU-DLCL2	Human B cell lymphoma cell line established from pleural effusion of a 41-year-old male with B cell non-Hodgkin lymphoma (B-NHL)

3.1.8. Software

Name	**Manufacturer**
GraphPad Prism 9	GraphPad Software, San Diego, CA, USA
Kaluza	Flow Analysis Beckman Coulter, Brea, CA, USA
Microsoft Office	Microsoft, Redmond, WA, USA

3.2. Methods

3.2.1. Cell culture experiments

3.2.1.1. Cell lines

All cell lines described above – except for HEK293 – were cultured in a T25 or T75 tissue culture flask with Gibco™ RPMI 1640 medium (life technologies, 11875093) supplemented with 10% heat-inactivated Gibco™ fetal bovine serum (FBS; life technologies, 10500056) and 1% Gibco™ Penicillin-Streptomycin (pen-strep; life technologies, 15140122) and kept at 37°C and 5% CO_2. Cells were split every 48-72 hours, transferring $1*10^6$ cells to a new flask with fresh medium. The adherent HEK293 cells were cultured in a $10cm^2$ dish with Gibco™ DMEM Medium (life technologies, 11965092) supplemented with 10% heat-inactivated FBS and 1% pen-strep. For splitting, cells were detached by incubating with 0.5% trypsin/EDTA (Thermo Fisher Scientific) for 5 minutes at 37 °C. Trypsin was inactivated by adding culture medium. To count absolute cell numbers, the Macs Quant VYB or X flow cytometers (Miltenyi Biotech) were used according to the manufacturer's instructions.

3.2.1.2. In vitro stimulation of THP-1 monocytes

To prolong the ex vivo survival of primary human CLL cells, a co-culture system with THP-1 feeder cells has been established in which the expression of PD-L1 and PD-L2 has been monitored by flow cytometry over a period of up to ten days. Before co-culture, $3*10^6$ THP-1 cells were stimulated with 1 ng/ml phorbol-12-myristate-13-acetate (PMA; Sigma, #P8139) on a 10 cm^2 dish for 48-72 hours to induce the differentiation into macrophages. Afterwards, the cells were washed twice with PBS to remove dead cells. Subsequently, the adherent cells were detached with trypsin/EDTA as described earlier (c.f. 3.2.1.1). Afterwards $1*10^5$ cells/well were distributed on a 24-well plate in 500 µl RPMI 1640 medium supplemented with 10% FBS and 1% pen-strep and incubated overnight to attach to the bottom of the well. The next day, primary patient derived CLL cells were added to each well in a 1:5 ratio.

3.2.1.3. Primary CLL cells

Primary CLL cells were isolated from peripheral blood samples of CLL patients treated in the University Clinic of Cologne.

For primary human B cell isolation, whole blood was distributed into 50 ml tubes with a maximum volume of 15 ml per tube. Afterward, 50 µl RosetteSep® Human B Cell Enrichment cocktail per ml blood were added and incubated at room temperature for 20 minutes. Subsequently, the samples were mixed with an equal volume of washing buffer consisting of DPBS supplemented with 2% FBS. Next, the samples were transferred to a new 50 ml tube containing a minimum of 10 ml of Lymphoprep™ without mixing the fluid layers. Then, the samples were centrifuged for 20 minutes at 1200 g without brake. Afterwards, the blood plasma was removed, and the enriched B cells were transferred to a separate 50 ml tube. Washing buffer was added, and the tube was centrifuged for 10 minutes at 300 g with brake. The supernatant was discarded, and erythrolysis was performed with ammonium chloride solution. Then, the enriched B cells were washed again with washing buffer and centrifuged for 10 minutes at 300 g. After discarding the supernatant, the cell pellet was resuspended in culture medium. Here RPMI 1640 Medium GlutaMAX™ + 10% FBS + 1% Pen/Strep was used. Finally, cell concentration and viability were determined by flow cytometry using Annexin V staining as a marker for apoptotic cells.

For storage, the samples were kept at -150 °C. For thawing, cells were put into a 37 °C water bath, washed twice with PBS, and suspended in RPMI 1640 medium supplemented with 10% FBS and 1% pen-strep. All patient samples utilized in this work have been

provided by the local bio bank of the german CLL study group. The following table (Table 4) provides an overview of the samples used in this work.

Table 4: Characterization of CLL patient samples: The CLL patient samples analyzed in this work are characterized. Sex, Age, Binet stage, Genetics and previous therapeutic interventions are depicted. In general, the patient samples analyzed show a low to intermediate risk and are mostly untreated.

Patient ID	Sex	Age	Binet stage	Genetics	Therapy
25	M	76	A	IgVH mutated, del13q	w&w
78	F	46	A	IgVH mutated, del13q	w&w
100	M	68	A	del13q	w&w
122	F	61	B	ZAP-70 positive	w&w
182	M	62	A	IgVH mutated	w&w
200	M	74	B	IgVH unmutated, del11q	w&w
262	M	69	A	ZAP-70 positive	w&w
332	M	53	A	IgVH unmutated	6x Bendamustine + Rituximab (Binet C at time of diagnosis)
358	F	50	A	IgVH mutated	w&w
398	M	64	A	Complex aberrant karyotype	w&w

3.2.1.4. BCR inhibitor treatment

To evaluate the impact of BCR-pathway inhibition on PD-L1 and PD-L2 surface expression, different small molecule inhibitors were used to block various kinases of the BCR-signaling cascade (Table 5).

Table 5: BCR inhibitors used and their target kinases

Inhibitor	Target	Manufacturer/Cat. number
Acalabrutinib	BTK	Provided by Gilead Sciences
Dasatinib	BCR-ABL, SRC	Santa Cruz, #302962-49-8
Entospletinib	SYK	Selleckchem, S7523
Ibrutinib	BTK	Selleckchem, S2680

Idelalisib	PI3K	Provided by Gilead Sciences
Tirabrutinib	BTK	Provided by Gilead Sciences
Spebrutinib	BTK	Provided by Gilead Sciences

The potential effect on the surface expression of the PD-1 ligands has been evaluated for each of these inhibitors in diverse human B cell lines, including JVM3, OSU-CLL, DB, DOHH2, HT, Karpas 422, L-540, Mec1, P30-OHKUBO, and WSU-DLCL2. For JVM3 and OSU-CLL, $5x10^5$ cells/ well were suspended in 500 µl of cell culture medium and distributed on a 24-well-plate. Subsequently, the cells were incubated with 1 or 5 µM of one of the mentioned inhibitors (Table 5). For the treatment of the other B cell lines,$1.25x10^5$ cells were seeded on a 96-well-plate. Here, only the inhibitors dasatinib, entospletinib, ibrutinib, and idelalisib were used at a concentration of 1 µM each. Primary CLL cells were obtained from peripheral blood as described in 3.2.1.3. Primary CLL cells. The frozen cells were thawed, and $1x10^6$ cells were suspended in 500 µl of cell culture medium. Afterwards, they were distributed on top of a layer of THP-1 feeder cells seeded in a 24-well-plate as described in 3.2.1.2. In vitro stimulation of THP-1 monocytes. Finally, PD-L1 and PD-L2 expression was determined by flow cytometry at 24 and 48 hours after treatment (cf. 3.2.2. Cell analysis).

3.2.1.5. Stimulation of primary CLL cells

Samples of three different CLL patients were treated with cell stimulating agents and with IgM, which directly activates the BCR. $2x10^5$ patient cells diluted in 150 µl of RPMI were seeded on a layer of THP-1 feeder cells (c.f. 3.2.1.2. In vitro stimulation of THP-1 monocytes) in a 96-well plate. Then samples were treated with either 25 ng/ml of IFNγ (Miltenyi, #130-094-048), 5 µg/ml of IgM, 1 µg/ml of PMA (Sigma, #P8139), 1 µg/ml of CD40L, 1 µg/ml of LPS (Sigma, #L4391) or 0,25 nM of CpG. PD-L1 expression was determined by flow cytometry at 6, 24 and 48 hours after treatment with PMA, IFNγ or IgM and 6, 24 and 72 hours after treatment with CpG, CD40L or LPS.

3.2.2. Cell analysis

To determine the expression levels of PD-L1 and PD-L2 on the surface of human B cells, we used flow cytometry. Different sample volumes ranging from 50 – 250 µl (depending

on the cell concentration) were used for staining. To prevent unspecific antibody binding, the cells were incubated at room temperature for 10 minutes with 5 µl of FcR-blocking reagent human (Miltenyi, #130-059-901). Afterwards, the cells were centrifuged at 300 g for 5 minutes, the supernatant was discarded, and the cells were resuspended in 200 µl of 1x Annexin V binding buffer. Subsequently, 0.5 µl of each antibody (Table 6) were added. Annexin V staining was used as a marker for apoptotic cells. In addition, for each cell line, one separate sample was stained with the respective isotype control for PD-L1 and PD-L2. Subsequently, the samples were incubated for 20 minutes at room temperature in the dark. Afterwards, the samples were washed twice with PBS by centrifugation for 5 minutes at 300 g, and the cells were finally resuspended in 200 µl PBS. The measurement was performed either using the MACSQuant X or the MACSQuant VYB flow cytometer (Miltenyi).

To determine the surface expression levels of PD-L1 and PD-L2, only Annexin V negative cells have been considered. For both PD-L1 and PD-L2, MFI values have been subtracted by the respective isotype control and normalized to the untreated control.

Table 6: Reagents used for PD-L1 and PD-L2 staining after inhibitor treatment

Name	Fluorochrome	Manufacturer	Cat. number
Annexin V	FITC	Biolegend	640906
Anti-human PD-L1	PE	Biolegend	329706
Anti-human PD-L2	APC	Biolegend	329708
Mouse IgG2b, κ isotype	PE	Biolegend	400314
Mouse IgG2a, κ isotype	APC	Biolegend	400246

3.2.3. Data analysis, illustration & storage

For flow cytometry, data analysis was performed using the Kaluza Analysis software (Beckmann Coulter).

Line and column diagrams show the mean values for each group. Error bars represent the standard deviation (SD). All diagrams were generated with the Prism 9 software (GraphPad).

All data presented in this thesis are stored on the Scale-out File-Server (SoFS) of the University of Cologne and can be accessed under the following server address: \\sofs2.uni-koeln.de\AGHallek\AG Hallek\SeLe

3.2.4. Statistical analysis

Statistical analysis of flow cytometry data was performed with the GraphPad Prism 9 software using an unpaired t-test. Statistical significance was assumed at P values < 0.05.

4. Results

4.1. PD-1 ligand expression on the surface of human B cell lines after *in vitro* treatment with clinically relevant BCR-inhibitors

4.1.1. CLL cell lines JVM3 & OSU-CLL

At first, we investigated the effect of six clinically relevant BCR-inhibitors (dasatinib, entospletinib, ibrutinib, idelalisib, acalabrutinib, and spebrutinib) on the surface expression of PD-L1 and PD-L2 on the human CLL cell lines JVM3 and OSU-CLL (Figure 3, Figure 4, Figure 5, Figure 6). We used two different concentrations (1 and 5 µM) for each inhibitor, while the expression levels of PD-L1 and PD-L2 were determined 24 and 48 hours after treatment initiation using flow cytometry.

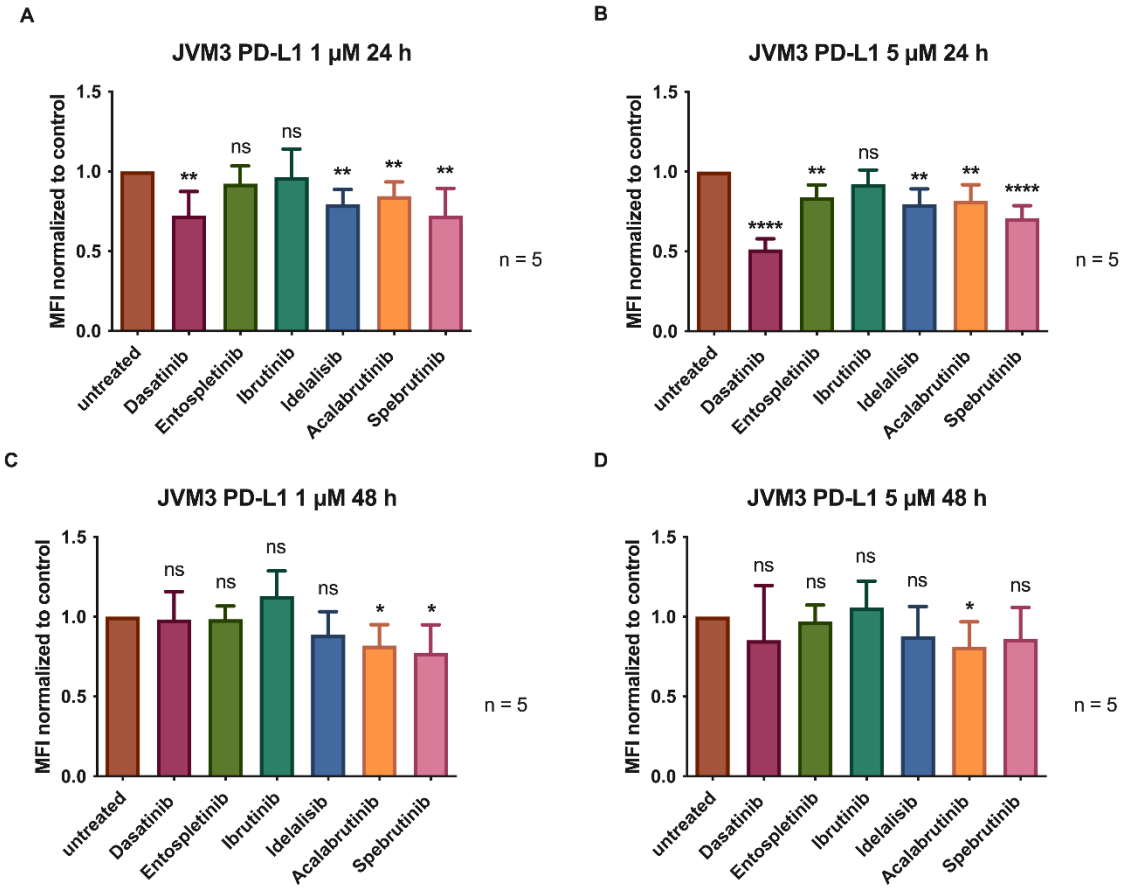

Figure 3: PD-L1 surface expression after BCR-inhibitor treatment of JVM3 cells: A-B) At 24 hours, treatment with most BCR-inhibitors led to a significant decrease in the PD-L1 surface level. The most significant decline was observed with 5 µM dasatinib 24 hours after treatment. **C-D)** At 48 hours, the reduction in PD-L1 surface level disappeared comparable to the untreated control, except for cells treated with acalabrutinib and spebrutinib. Surprisingly, ibrutinib did not affect the surface expression of PD-L1.

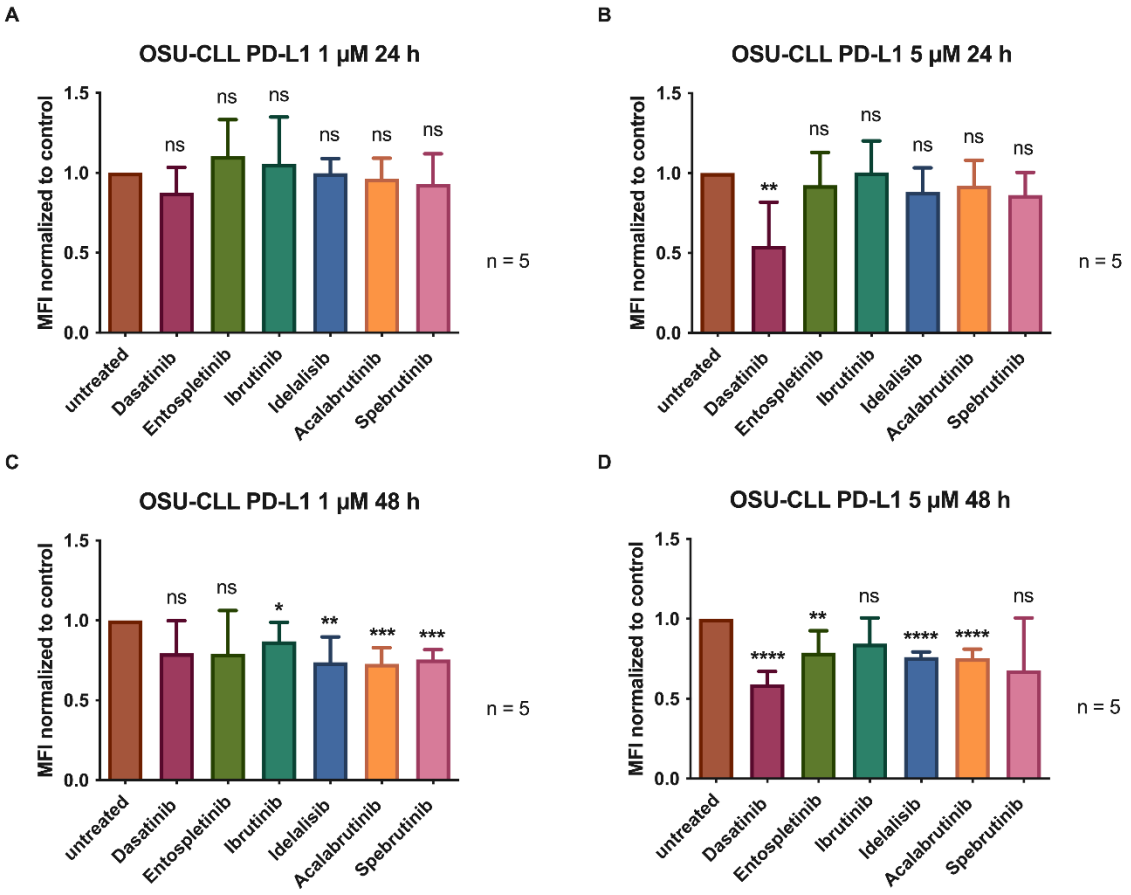

Figure 4: PD-L1 surface expression after BCR-inhibitor treatment of OSU-CLL cells: A-B) At 24 hours, a reduction of PD-L1 surface expression could only be observed with 5 µM of dasatinib. **C-D)** However, at 48 hours, there was a significant concentration-dependent decrease in PD-L1 for each BCR-inhibitor.

Most of the BCR-inhibitors we tested seem to have a time- and dose-dependent capacity to significantly alter the expression of PD-L1 on the surface of JVM3 and OSU-CLL cells (Figure 3, Figure 4). However, the reduction of surface PD-L1 was not durable and varied strongly between the different inhibitors. Moreover, we observed a high variation between biological replicates, suggesting a very dynamic expression of PD-L1.

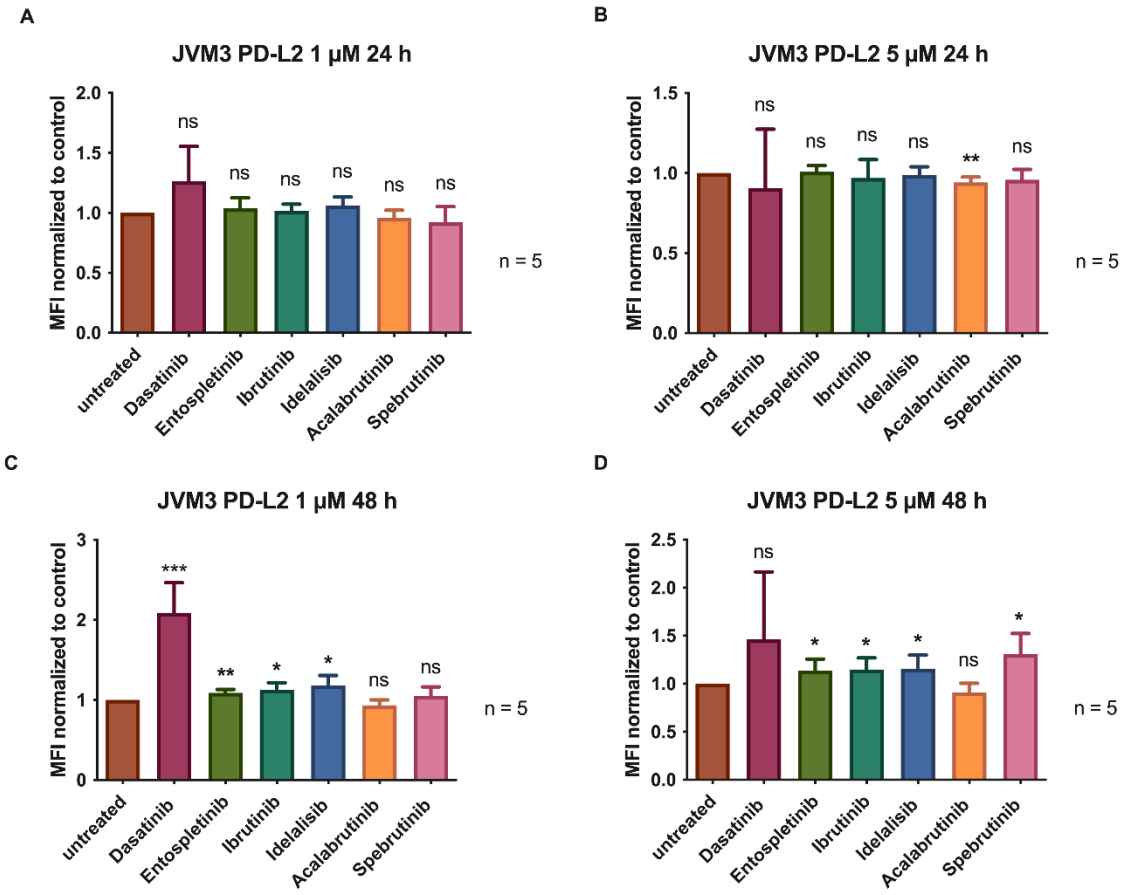

Figure 5: PD-L2 surface expression after BCR-inhibitor treatment of JVM3: In contrast to PD-L1, the impact of BCR-inhibition on the surface expression of PD-L2 was much less pronounced and led to a mild increase. **A-B)** 24 hours after treatment initiation, a slight but significant increase in PD-L2 expression could be observed for 5 µM of acalabrutinib. **C-D)** After 48 hours, a mild upregulation of surface PD-L2 could be observed for most inhibitors, while after treatment with 1 µM of dasatinib, there was even a 2-fold increase.

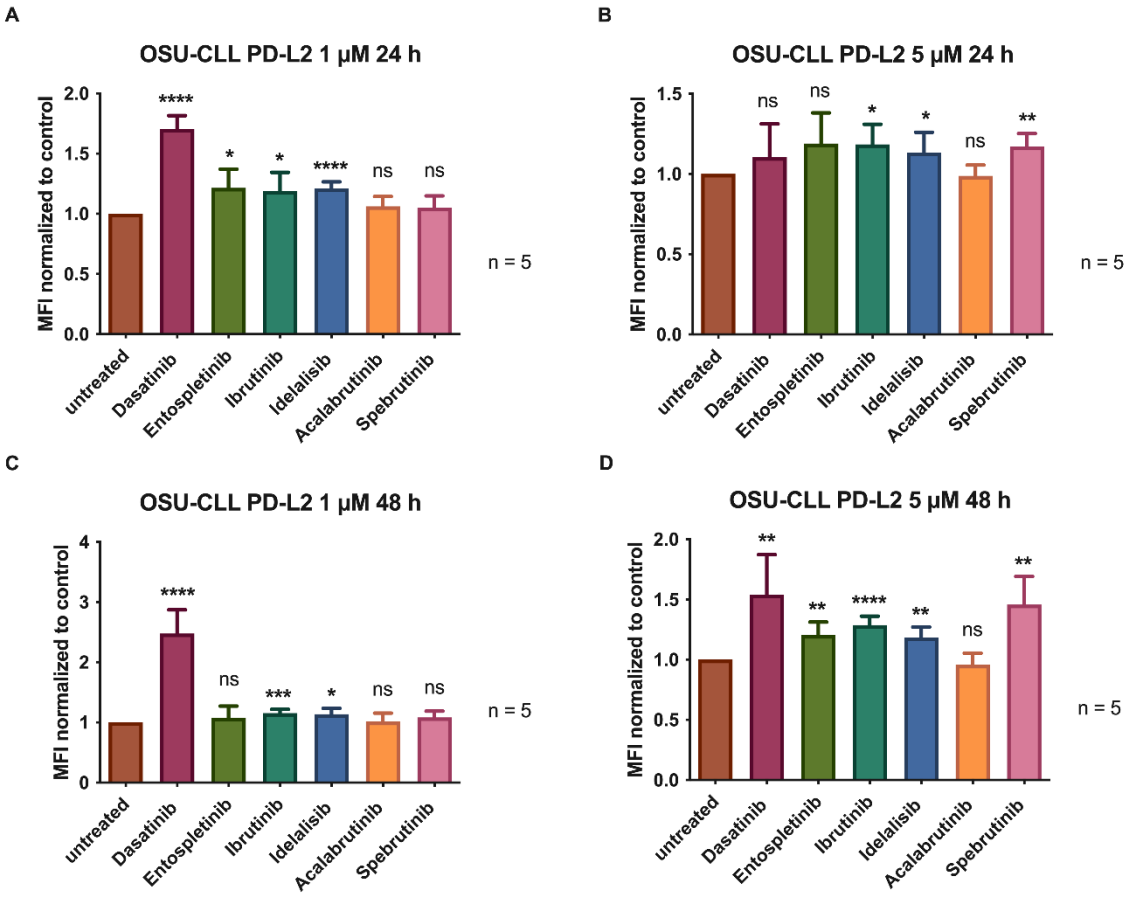

Figure 6: PD-L2 surface expression after BCR-inhibitor treatment of OSU-CLL: In general, BCR-inhibition increased the PD-L2 surface levels on OSU-CLL cells. Compared to JVM3 cells, this effect was even more pronounced. The only inhibitor that did not affect the expression of PD-L2 was acalabrutinib.

Interestingly, in contrast to PD-L1, there was a tendency for PD-L2 surface levels to increase after BCR-inhibition (Figure 5, Figure 6). Similar to our observations for PD-L1, this effect was highly time- and dose-dependent and varied considerably between the different BCR-inhibitors.

4.1.2. BCR-inhibitor combination treatment of JVM3 & OSU-CLL

As single-agent treatment with BCR-inhibitors indicated a potential regulatory effect on the PD-L1 and PD-L2 surface expression on JVM3 and OSU-CLL cells, we wanted to investigate the benefit of combinatorial treatment with two different BCR-inhibitors (Figure 7, Figure 8, Figure 10, Figure 11). Simultaneous blockage of two separate effector kinases of the same signaling pathway was supposed to induce a more robust and durable response. Thus, we examined the combination of tirabrutinib and entospletinib as well as tirabrutinib and idelalisib, as these combinations reflect therapeutic regimes currently under clinical investigation.

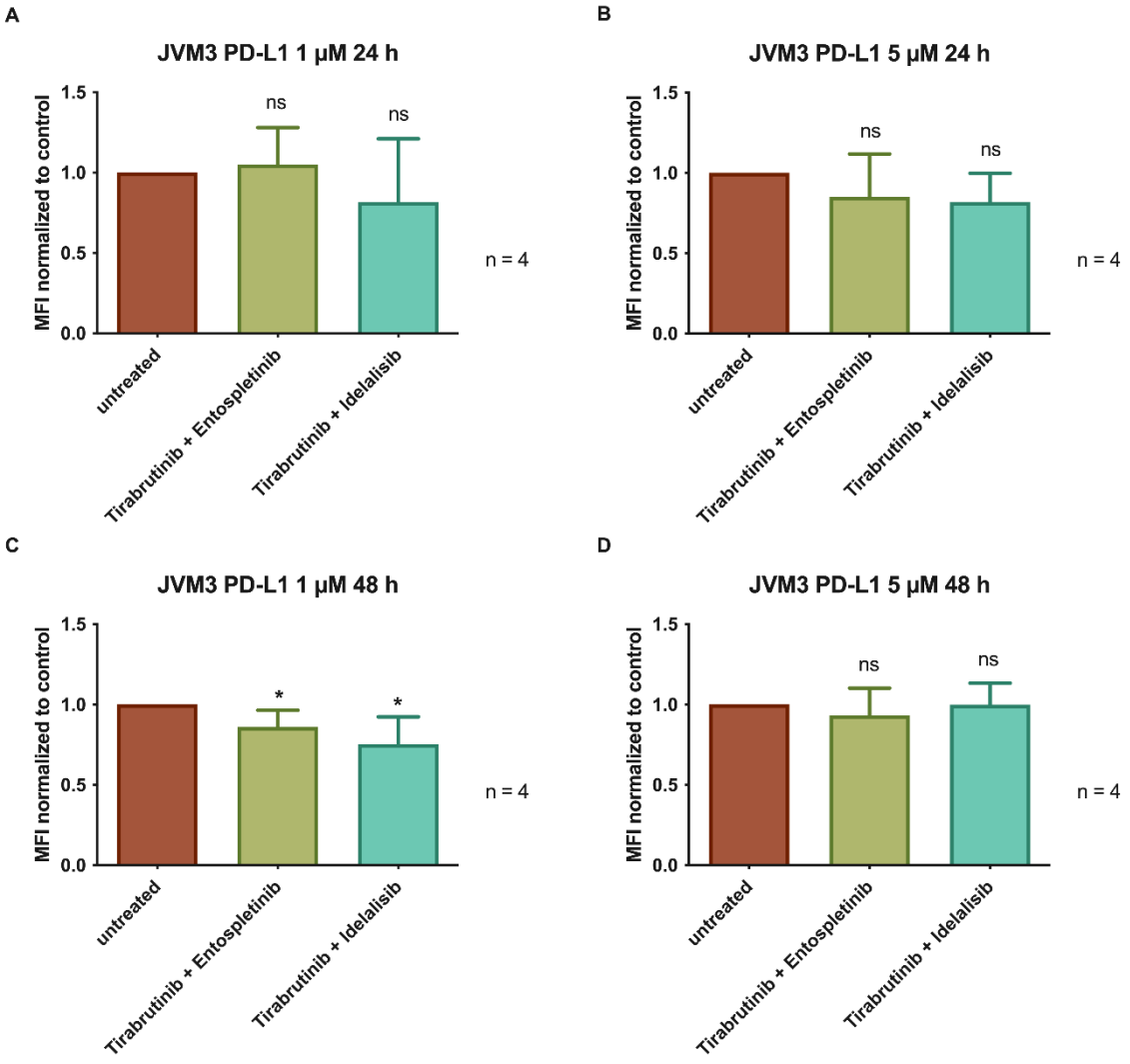

Figure 7: PD-L1 surface expression after BCR-inhibitor combination treatment of JVM3 cells: A-B) 24 hours after combination treatment, there was no significant difference in the surface expression of PD-L1 compared to the untreated control. **C)** 48 hours after treatment with 1 µM, a significant but mild decrease of PD-L1 surface levels was observed for both combinations. **D)** Surprisingly, using a concentration of 5µM per inhibitor did not affect the PD-L1 surface level compared to the untreated control after 48 hours of treatment.

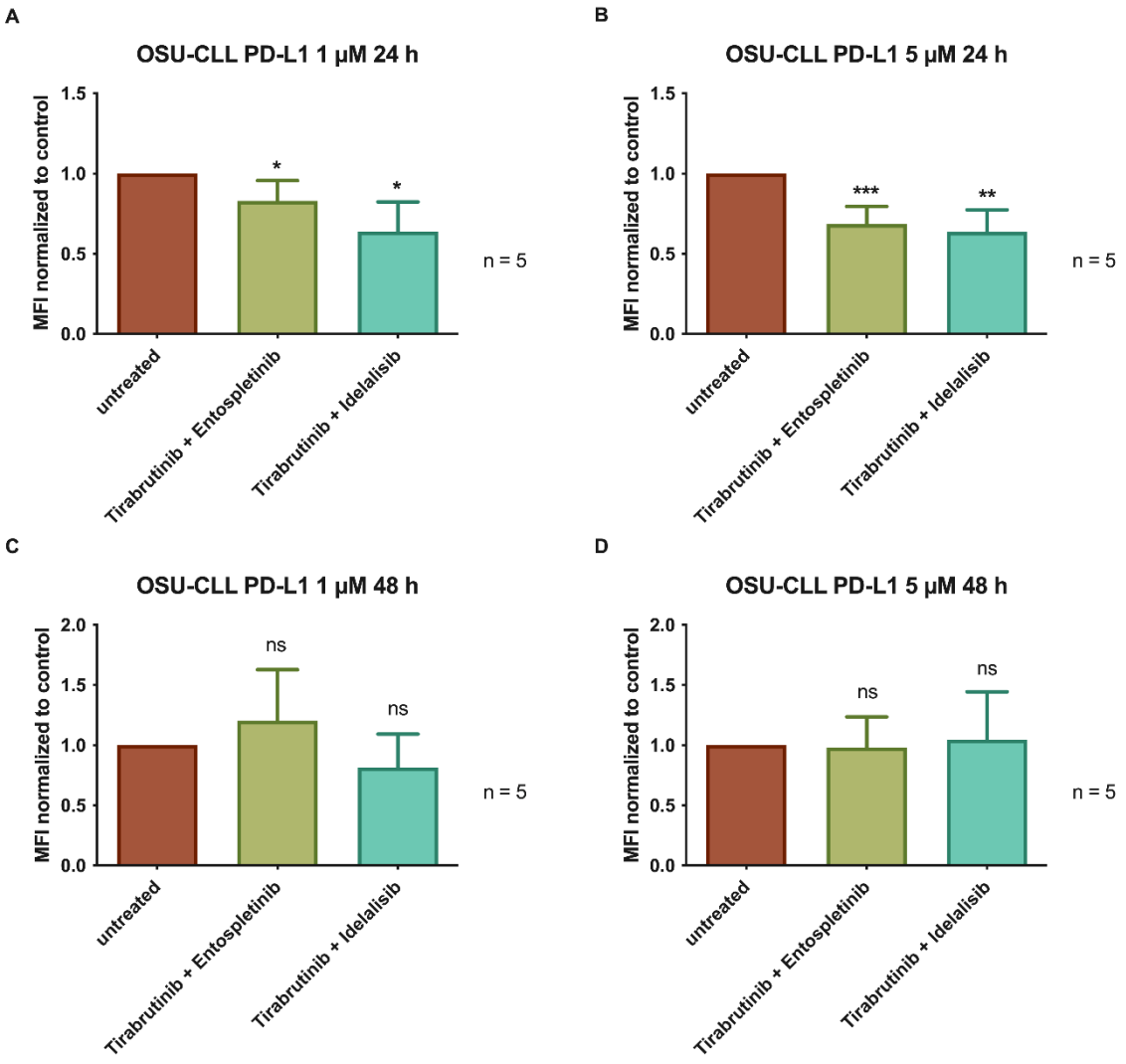

Figure 8: PD-L1 surface expression after BCR-inhibitor combination treatment of OSU-CLL cells: A-B) After 24 hours of treatment, PD-L1 surface levels were significantly decreased for both inhibitor combinations at both concentrations. **C-D)** Intriguingly, the reduction of PD-L1 was not maintained until 48h after treatment initiation.

A significant reduction of PD-L1 surface expression could only be observed in OSU-CLL after the 24-hour time point, while for the JVM3 cells, only at a concentration of 1 µM per inhibitor, there was a significant downregulation of PD-L1 after 48 hours of treatment (Figure 8).

Strikingly, the overall effect of the combination treatment with two different BCR-inhibitors (tirabrutinib + entospletinib, tirabrutinib + Idelalisib) on the surface expression of PD-L1 on JVM3 and OSU-CLL cells was less pronounced in comparison to the respective single-agent treatment (Figure 9). This contradicts an additive or synergistic effect when combining two different BCR-inhibitors.

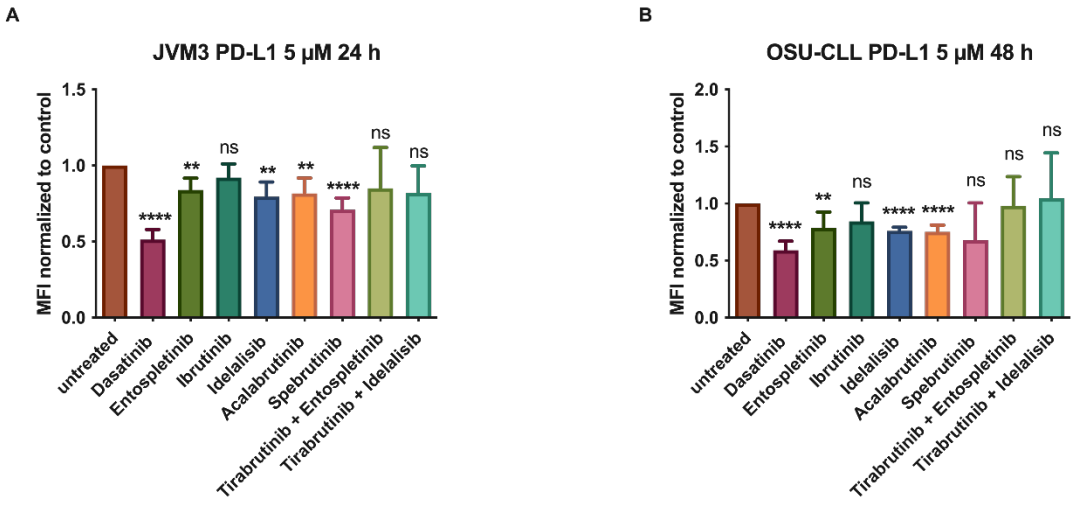

Figure 9: Direct comparison of PD-L1 expression after single agent- vs. combination treatment: The graph directly compares PD-L1 expression after treatment with 5 µM of each inhibitor, at t = 24h for JVM3 and t = 48h for OSU-CLL. Although single agent-treatment often resulted in a reduction of PD-L1 surface levels at the concentration and time point selected for this graph, the combination treatment showed no significant difference. For both cell lines, dasatinib led to the strongest decrease in PD-L1 expression.

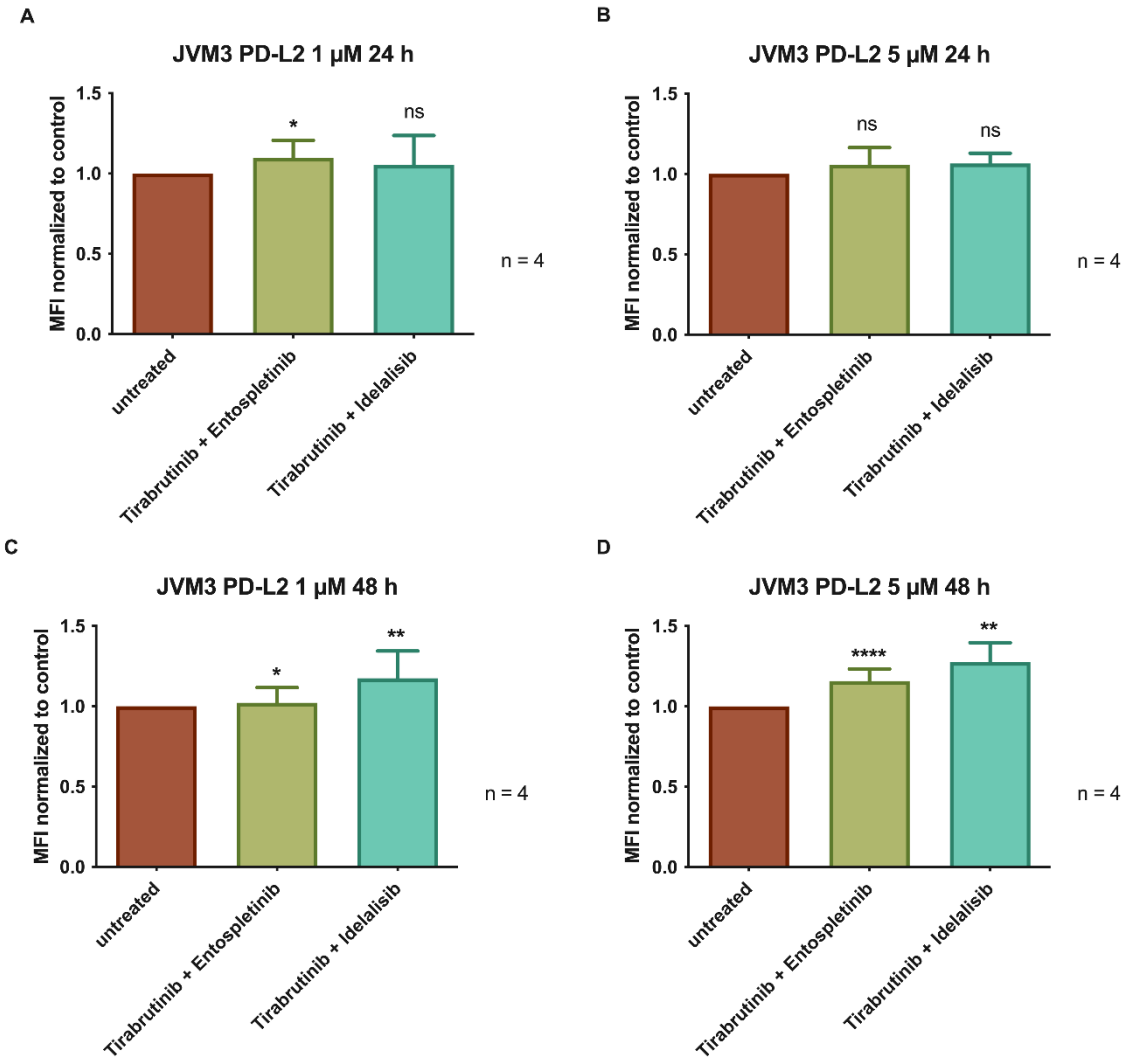

Figure 10: PD-L2 surface expression after BCR-inhibitor combination treatment of JVM3 cells: Similar to the single-agent treatment, the combination treatment with two BCR-inhibitors increased the PD-L2 surface levels. **A-B)** After 24 hours, this was firstly visible for the combination of 1 µM tirabrutinib + 1µM entospletinib. **C-D)** After 48 hours, upregulation of PD-L2 was observed for both inhibitor combinations at both concentrations.

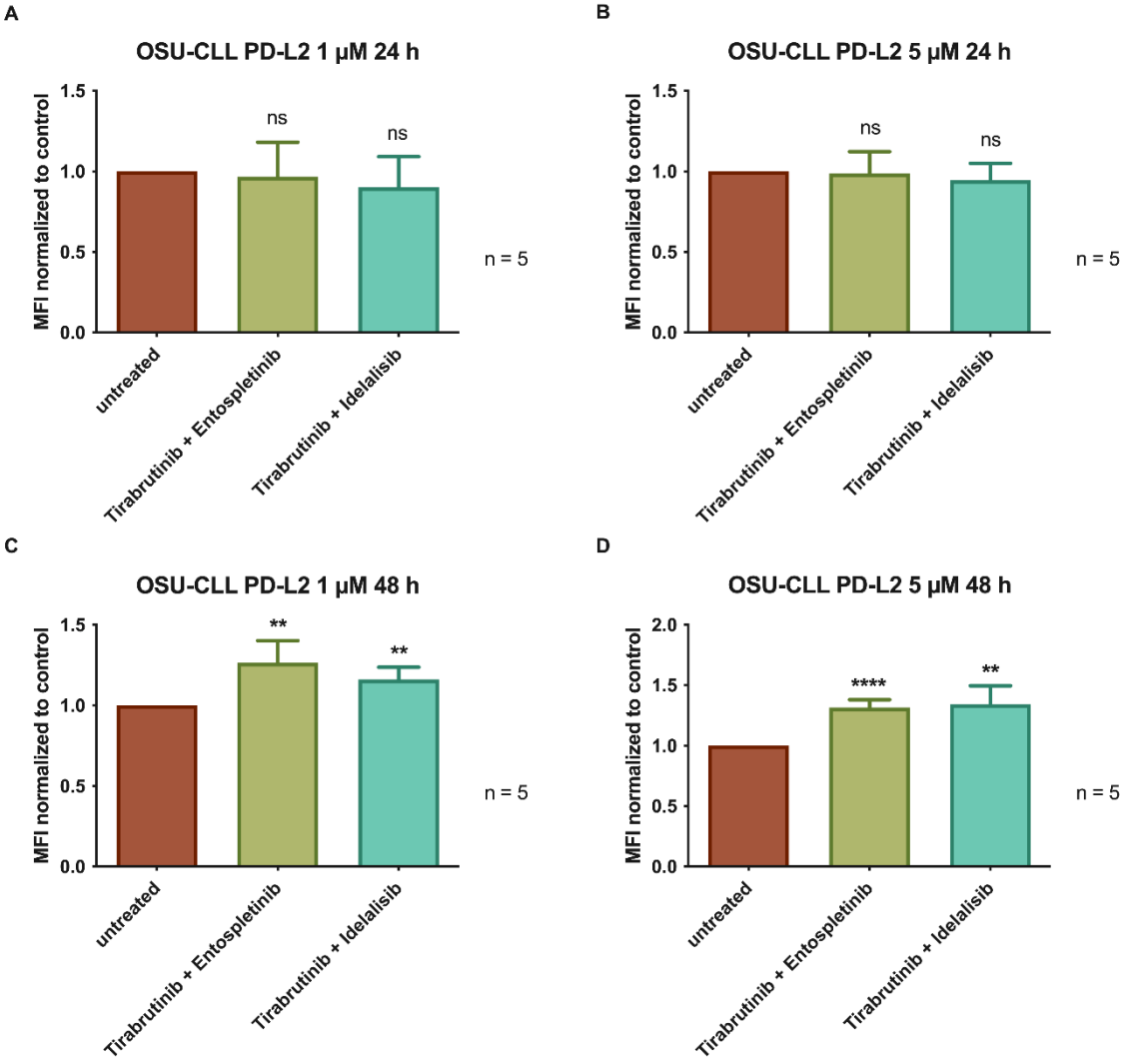

Figure 11: PD-L2 surface expression after BCR-inhibitor combination treatment of OSU-CLL cells: A-B) After 24 hours, the combination treatment had no significant effect on the PD-L2 surface expression compared to the untreated control. **C-D)** In contrast, there was a significant increase in the PD-L2 surface expression after 48 hours of treatment with both inhibitor combinations at both concentrations.

Similar to the single-agent treatment, the combination treatment significantly increased the PD-L2 protein level on the surface of JVM3 and OSU-CLL cells (Figure 10, Figure 11). However, the effect was slightly delayed and detected mainly after 48h, while the relative increase of PD-L2 was also less pronounced.

4.1.3. BCR-inhibitor treatment of multiple human B cell line systems

To verify a general regulatory role of the BCR-signaling pathway for the surface expression of PD-1 ligands, we expanded our previous experiment using various human B cell lines of diverse lymphoma origins. JVM3 and OSU-CLL cells served as control CLL models. Surprisingly, the treatment of JVM3 and OSU-CLL cells with dasatinib did not lead to a reduced PD-L1 expression as previously observed (Figure 3, Figure 4). Only for the

DOHH-2 and L540 cell lines, dasatinib treatment led to a progressive reduction of surface PD-L1 (Figure 12), which was consistent with our previous findings. However, none of the other BCR-inhibitors significantly affected the PD-L1 surface expression on most of the examined B cell lines.

Together, these findings show that inhibition of the BCR-signaling cascade does not significantly influence the surface expression of PD-L1 in lymphoma cell lines. We also measured PD-L2 surface levels, but there were several cell lines in which we could not detect any PD-L2 expression. Hence studying PD-L2 in this experimental set-up was not feasible.

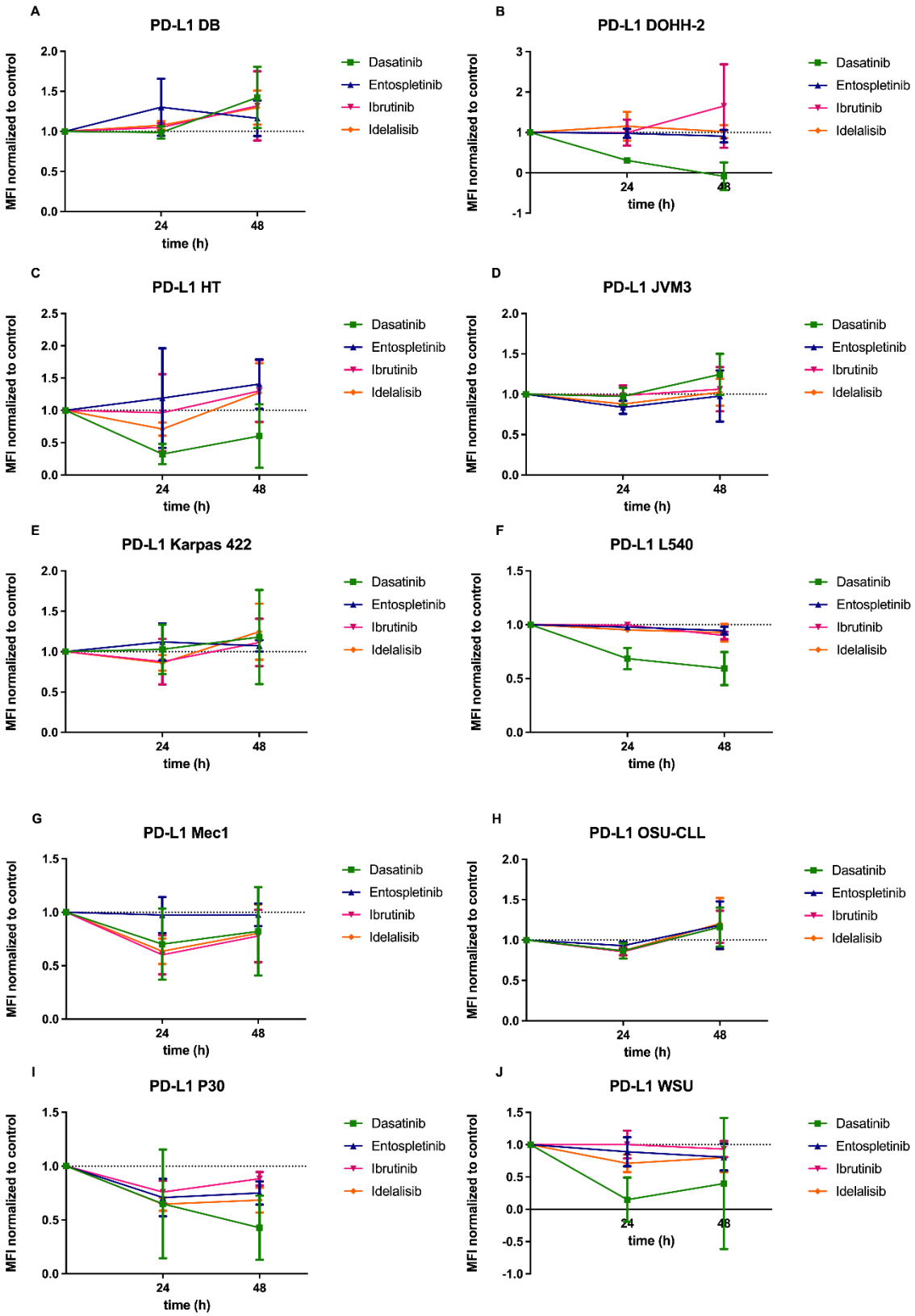

Figure 12: PD-L1 surface expression after BCR-inhibitor treatment of various B cell lines: In this experimental set-up, no significant differences could be observed in most treatment cases compared to the untreated control. **B, F)** Only for the DOHH-2 and L540 cell lines dasatinib treatment led to a progressive reduction of the PD-L1 surface level. **D, H)** Strikingly, the downregulation of PD-L1 on the surface of JVM3 and OSU-CLL, which has previously been observed, could not be reproduced in this experimental set-up.

4.2. In vitro treatment of primary CLL cells

4.2.1. With BCR inhibitors

To overcome the artificial and immortal characteristics of cell line systems, we also investigated the effect of BCR-inhibition on the surface expression of the PD-1 ligands in primary patient-derived CLL cells (Figure 13, Figure 14). For this, we established a co-culture system using THP-1 feeder cells, which allowed us to extend the CLL cell viability *ex vivo* for up to 10 days. We only used ibrutinib to treat the CLL cells as this inhibitor represents the most frequently used BCR inhibitor for treating CLL patients. Moreover, it has been shown that the PD-L1 expression is reduced on the surface of $CD19^+$ cells in peripheral blood of CLL patients after three months of Ibrutinib monotherapy. [95]

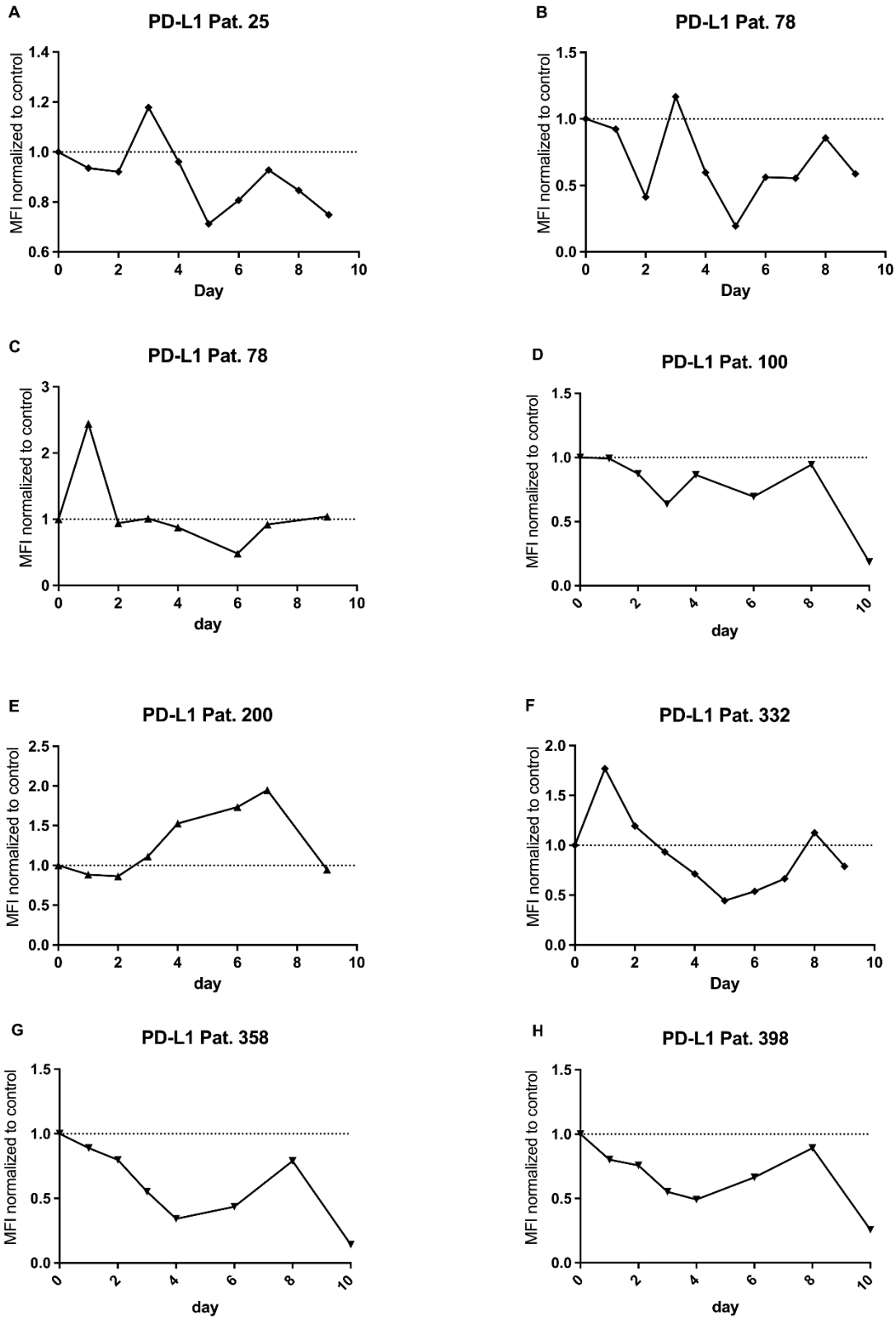

Figure 13: PD-L1 surface expression on primary CLL cells after ibrutinib treatment: Each graph represents the cells of a single CLL patient treated with ibrutinib in vitro, normalized to untreated controls. Most graphs show high variability of PD-L1 expression, with values being both lower and higher than the control during the observed time course. **B-C)** shows the cells of the same patient evaluated in two independent experiment rounds. However, no consistent impact on the PD-L1 surface expression can be observed.

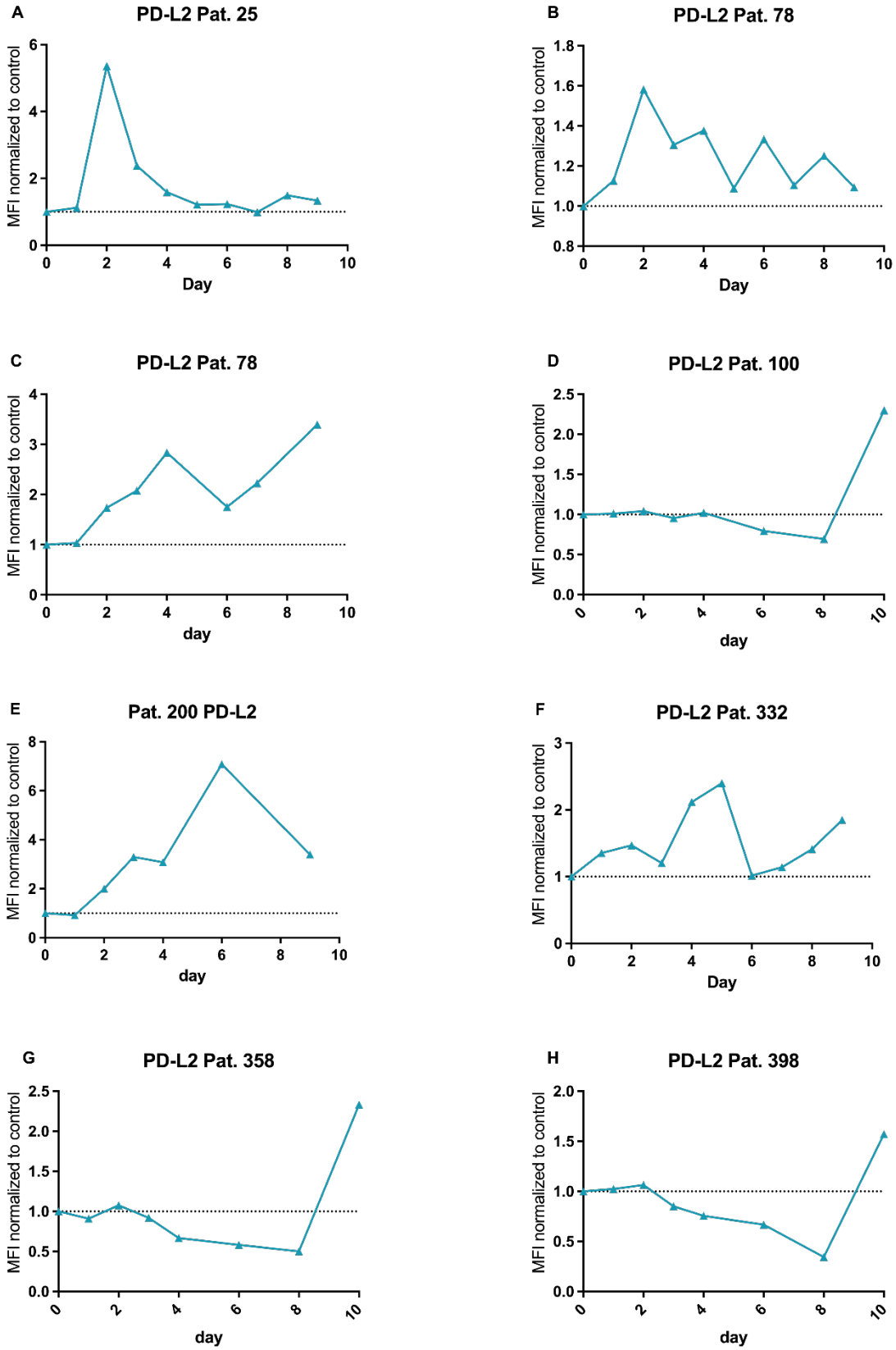

Figure 14: PD-L2 surface expression on primary CLL cells after ibrutinib treatment: Similar to the cell lines, most patient-derived CLL cells showed an increase in PD-L2 surface expression after BCR-inhibition. However, the PD-L2 levels showed high variability and dynamics. B-C) show in vitro treatments of the same patient from two independent experiment rounds. Again, BCR-inhibition of the same patient cells led to inconsistent effects on the PD-L2 surface expression level.

The graphs above indicate highly variable PD-L1 and PD-L2 expression levels on the CLL cell surface in each individual patient (Figure 13, Figure 14). Moreover, the expression is also highly dynamic throughout the observed time course. These findings contradict an apparent and consistent effect of ibrutinib on the surface expression of PD-1 ligands, at least for the period observed in this experiment.

Notably, the PD-L1 expression on the primary CLL cells was much lower than in the human B cell lines, while PD-L2 was completely absent in some samples (Figure 15).

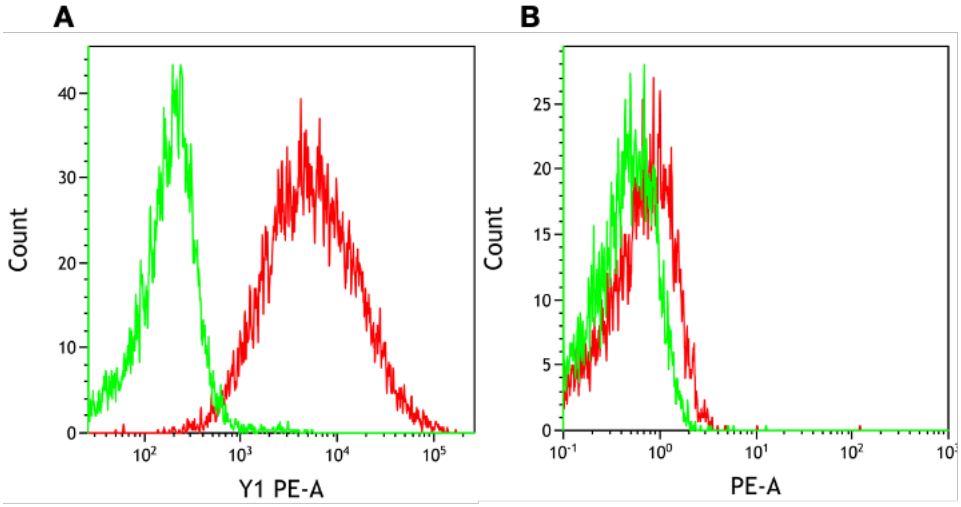

Figure 15: PD-L1 surface expression on JVM3 vs. primary CLL cells: Representative histogram of the PD-L1 surface expression (red) compared to the respective isotype control (green) determined by flow cytometry. **A)** Shows the MFI signal for PD-L1 on the surface of JVM3 cells, which was significantly higher than the isotype control. **B)** In contrast, primary CLL cells showed only a slightly higher MFI than the isotype control, indicating minimal PD-L1 surface expression.

4.2.2. Induction of PD-L1 surface expression using diverse BCR stimulating agents

Next, we investigated the effect of BCR stimulation on PD-1 ligand expression on the surface of primary CLL cells. We hypothesized that stimulation with IgM induces the surface expression of PD-L1 via BCR-mediated signaling. Additionally, we evaluated the effectiveness of other stimulating agents such as IFNγ and PMA in increasing the low baseline expression of PD-L1 on primary human CLL cells (Figure 16).

PMA is a phorbol ester which activates PKC and subsequently Ras, Raf-1, ERK and NF-κB, [96] thus regulating gene transcription, cell growth, differentiation, and survival.

IFNγ regulates the transcription of various genes linked to the immune system. It stimulates macrophages, induces antitumor and antimicrobial responses, and enhances antigen presentation. Furthermore, it affects cell growth, maturation and differentiation. [97]

PD-L1 expression was measured on three different patient samples at 6, 24, and 48 hours after treatment.

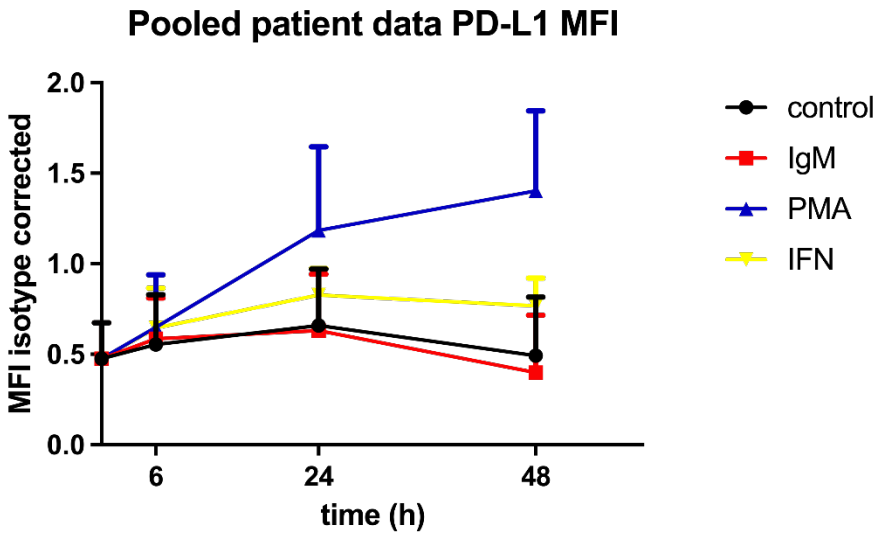

Figure 16: PD-L1 surface expression after in vitro stimulation of primary CLL cells with PMA, IFNγ, and IgM: The surface expression of PD-L1 was determined by flow cytometry on two consecutive days after in vitro stimulation. The absolute MFI values of the untreated control and the stimulated samples corrected with the isotype control are depicted. The graphs show data from three independent patient samples. PMA was the only agent that could significantly upregulate PD-L1 on the CLL cell surface in all three patient samples tested. In contrast, IFNγ only mildly affected the PD-L1 surface expression, while IgM showed no effect on the surface expression of PD-L1 on primary human CLL cells.

Baseline PD-L1 expression was low in all three patients compared to the expression levels usually found in cell lines. This has already been observed previously in the inhibitor treatment experiments (Figure 15). Only PMA could significantly upregulate PD-L1 expression, while IFNγ just led to a slight increase in PD-L1 surface levels. BCR-stimulation with IgM did not have any effect on PD-L1 compared to the untreated control (Figure 16).

In a second repetition, more stimulating agents were investigated to see whether we could find other molecules that upregulate PD-L1 on primary CLL cells. Samples were analyzed over a longer time period, after 6, 24 and 72 hours (Figure 17).

CD40 ligand is found on activated T cells and upon binding to its receptor CD40 on the B cell surface, it promotes growth and differentiation. In combination with cytokines, it also stimulates immunoglobulin secretion. [98]

CpGs are oligodeoxynucleotides with immunomodulatory properties. They stimulate Toll-like receptor 9 (TLR9) on plasmacytoid dendritic cells and B cells, which leads to the production of pro-inflammatory cytokines. [99] Furthermore, they promote cellular polarization and migration in CLL. [100]

LPS can be found in the cell wall of gram-negative bacteria and thus strongly activates immune cells. It induces cytokine production and proliferation as well as differentiation of B cells into antibody secreting cells. This effect is mediated by PI3K. [101]

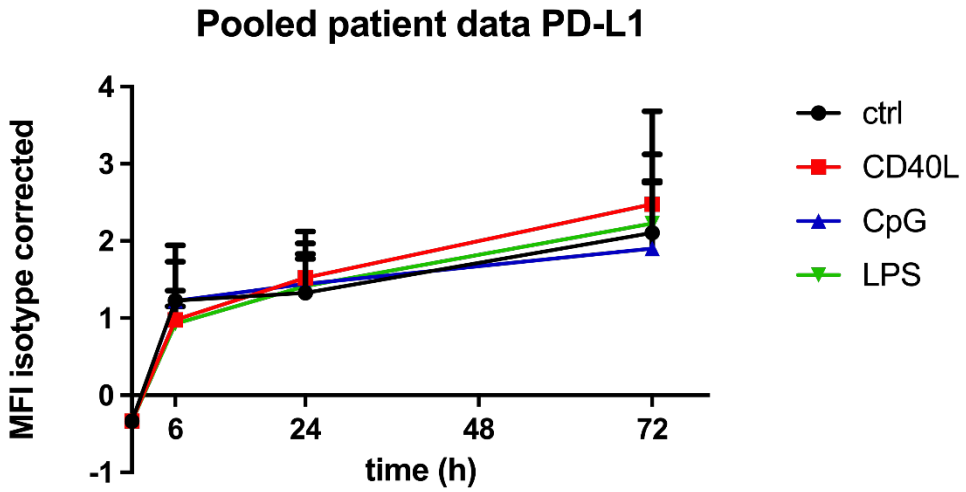

Figure 17: PD-L1 surface expression after in vitro stimulation of primary CLL cells with CD40L, CpG and LPS: The surface expression of PD-L1 has been determined by flow cytometry 6, 24. and 72 hours after in vitro stimulation. The absolute MFI values of the untreated control and the stimulated samples corrected with the isotype control are depicted. The graphs show data from three independent patient samples which are the same ones used in the first experimental run (Figure *16*), Here, the first measurement at t = 0 showed an absence of PD-L1 on the surface of all three patient samples. At the other time points, PD-L1 could be detected again, but no significant differences could be observed between the untreated control and the stimulated samples.

Strikingly, although using cells from the same patients as in the first run, during the initial measurement before treatment no PD-L1 could be detected on the cell surface. During the further course of the experiment, PD-L1 was expressed again in all three patient samples, but very weakly. None of the stimulants used was able to significantly upregulate PD-L1 (Figure 17).

Taken together, in these experiments PMA was the only agent that could increase the low baseline expression of PD-L1 in the patient samples investigated. Since BCR-stimulation with IgM could not achieve an increase in PD-L1 surface expression, we concluded that the regulation of PD-L1 by the BCR pathway is very unlikely.

5. Discussion

The PD-1-PD-L1/2 immune checkpoint axis has become an important target for ICB therapy in many tumor entities. However, this therapeutic approach has not been very successful in CLL, except for a subset of patients with RT, for which the response could be further improved by combining ICB with a tyrosine kinase inhibitor (TKI). [20,88] This is somewhat surprising since the PD-1-PD-L1/2 pathway has been shown to be active in CLL. [92] Moreover, upregulation of PD-1 and PD-L1 has been observed in the peripheral blood and lymph node samples of CLL patients. [70,75,102,103] This discrepancy indicates the urgent need to investigate further the role of the PD-1/PD-L1/PD-L2 immune checkpoint axis in CLL. Recent publications suggested a potential regulation of the PD-1-PD-L1/2 immune checkpoint axis by the BCR-signaling pathway. Li et al. have shown that BCR-mediated NFATc1 activation induces the expression of PD-L1 via an IL-10/STAT3 signaling axis in DLBCL. [93] Moreover, they found that BTK-inhibition reduces the protein level of PD-L1 in a DLBCL and a Hodgkin lymphoma cell line. In agreement with this, Kondo et al. observed a significant downregulation of PD-1 on T cells and PD-L1 on CLL cells at 3 and 6 months after initiating ibrutinib monotherapy. [94]

In this study, we wanted to evaluate the regulatory potential of the BCR-signaling pathway for the surface expression of PD-L1 and PD-L2 on CLL cells *in vitro*. Therefore, we conducted a series of *in vitro* experiments where we stimulated or inhibited the BCR-pathway using both primary human CLL cells and multiple human B cell lines.

5.1. The effects of BCR-inhibition on PD-1 ligand expression in cell lines

First, we focused on the CLL-like cell lines JVM3 and OSU-CLL. We used six different BCR-inhibitors (dasatinib, entospletinib, ibrutinib, idelalisib, acalabrutinib and spebrutinib) in order to be certain that the observed effects are dependent on the BCR and not on other off-targets of those TKIs. Furthermore, we measured two different concentrations and timepoints to assess whether the effects are time- and/or dose-dependent. Here we observed a tendency of PD-L1 downregulation and PD-L2 upregulation, but the effects were highly inconsistent. Since we only analyzed surface expression and not protein levels of the PD-1 ligands, possible intracellular changes in mRNA and protein levels could have remained undetected. Furthermore, JVM3 and OSU-CLL are cell lines immortalized with Epstein-Barr virus (EBV). It has been shown that LMP1, which is a latent gene product of EBV, increases PD-L1 expression. [104] Especially JVM3 cells display large amounts of

LMP1. [105] This could be an explanation for the high basal expression of PD-L1 in this cell line.

Next, we investigated the synergistic or additive potential of different BCR-inhibitor combinations. Here we chose inhibitor combinations that are being investigated in clinical trials, namely tirabrutinib + entospletinib and tirabrutinib + idelalisib. By targeting two different kinases in the BCR-pathway, both the BTK and ATK pathways are blocked and thus compensatory mechanisms cannot take effect. We assumed this might result in a more pronounced change in PD-1 ligand expression. However, the combination of two different inhibitors led to weaker effects than the respective single-agent treatment. This somewhat contradicts a regulation by the BCR-pathway.

Then we extended our BCR-inhibitor treatment to other B cell lines (DB, DOHH-2, HT, Karpas 422, L-540, Mec1, P3-OHKUBO and WSU-DLCL2) to evaluate the impact of BCR-inhibition on cell lines representing other types of lymphomas and thus to broaden our analysis to malignant B cells in general. This should help us exclude that we were missing a possibly more prominent effect BCR-inhibition might have in other cell lines. Furthermore, the publication by Li et al. [93] supports our initial hypothesis in DLBCL cell lines, and thus we wanted to investigate whether we could obtain similar results in this lymphoma entity. However, in those experiments we still included JVM3 and OSU-CLL to evaluate the reproducibility of the effects observed in the previous experiments and to have a direct comparison to the PD-1 ligand expression in the other cell lines. Surprisingly, we could not reproduce the results obtained in the first experiments with JVM3 and OSU-CLL. A possible reason for this is the different experimental set-up using a 96-well plate and thus smaller volumes. Still, this supported our notion that the changes we observed in PD-1 ligand expression did not correspond to a robust, reproducible effect. Also in the other cell lines, inhibitor treatment did not result in a different PD-1 ligand expression compared to the untreated control. PD-L2 surface expression, being lower than PD-L1 levels in general, could not even be detected in some cell lines.

Taken together, our findings in the cell line experiments showed that BCR inhibition did not consistently influence the expression of the PD-1 ligands in vitro. PD-1 ligand regulation appears to be very dynamic in these cell lines and above that highly dependent on other environmental factors. Clearly, a robust, one-dimensional regulation of PD-1 ligand expression by the BCR seems unlikely.

5.2. The effect of BCR-inhibition on PD-1 ligand expression in primary CLL cells

In CLL patients, PD-L1 and PD-1 have been shown to be upregulated, resulting in impaired T cell function. [70,76] The observation that ibrutinib therapy could reduce PD-L1 expression in CLL patients [94] hinted at a possible modulation of this immunosuppressive pathway by the BCR signaling cascade.

In our in vitro setting, ibrutinib treatment had very different effects on each individual patient, and the PD-L1 surface expression showed high variability. Even in one patient whose cells were analyzed twice, the development of PD-L1 expression over time was quite different in the two analyses.

For PD-L2, the results looked similar, and we could observe very dynamic levels of this protein. Like the cell lines, the PD-L2 expression on the surface of primary CLL cells was even lower than that of PD-L1.

One problem in working with primary CLL cells is their rapid apoptosis *ex vivo*, which might negatively impact experimental readout. We could establish a co-culture system with THP-1 feeder cells which significantly improved survival in cell culture, but inevitably the viability deteriorated over time.

Furthermore, the results of the previous publication on the effect of ibrutinib treatment on PD-1 and PD-L1 expression in CLL patients have been obtained after 6 months of continuous *in vivo* therapy with this agent. [94] Various systemic factors might have influenced the readout, which is not reproduceable in an *in vitro* system.

Surprisingly, in most of our primary CLL patient samples, we could not observe the high PD-L1 expression levels that have been described for CLL patients in the literature. This limits the potential regulatory effect of BCR-inhibition that can be observed in the *in vitro* experiments. Recently, a co-culture system with HS5 cells has been established in our laboratory increasing the low baseline expression of PD-L1 on primary CLL cells up to threefold. Subsequently treating the cells with dasatinib led to a decrease in PD-L1 surface levels (data by Greta Steinmetz, c.f. Figure 18, Figure 19). This needs to be investigated further, but since dasatinib has multiple off-targets and other BCR-inhibitors, including ibrutinib (Figure 13), did not have this impact, this seems to be a BCR-independent effect. Additionally, dasatinib is known for its considerable toxicity, which needs to be taken into account when evaluating this data.

5.3. Induction of PD-L1 expression by BCR stimulation

To investigate whether we could enhance PD-L1 expression in primary CLL cells, we tested different cell stimulating agents in our THP-1 co-culture system. We were especially interested in the effect of BCR-specific stimulation with IgM on PD-L1 surface levels. With this reverse approach we wanted to elicit further whether those two pathways are interlinked.

However, stimulation with IgM did not affect PD-L1 expression. Only PMA could significantly increase PD-L1 surface levels. This revealed a possible way to enhance PD-L1 expression on primary CLL cells. Above that, further experiments were conducted in our laboratory establishing a successful stimulation of PD-L1 with IFNγ or using a coculture system with HS5 cells.

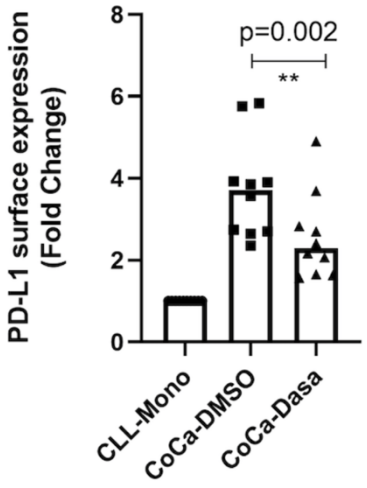

Figure 18: PD-L1 surface levels on CLL patient cells after coculture with HS5 cells and dasatinib treatment: Coculture of primary CLL cells with HS5 cells increased PD-L1 expression compared to monoculture. Subsequent treatment with dasatinib led to a significant reduction of PD-L1, measured via flow cytometry (data by Greta Steinmetz).

Interestingly, Li et al. observed an upregulation of PD-L1 protein levels upon IgM stimulation, determined by Western Blot analysis [93]. Similar to the inhibitor treatments, this could reveal a regulatory effect of the BCR pathway on protein level which does not affect PD-L1 surface expression, at least not during the observed time period.

Taken together, our experiments showed no upregulation of PD-L1 after BCR-specific stimulation and no consistent downregulation after treatment with BCR-TKIs. Only dasatinib repeatedly led to a stronger reduction of PD-L1 surface levels. Since no other kinase inhibitor showed a similar effect and dasatinib is not very specific to the BCR-pathway, we concluded that the underlying mechanisms are BCR-independent. This

mechanism still needs to be investigated further. This observation led to further research projects subsequently conducted in our laboratory.

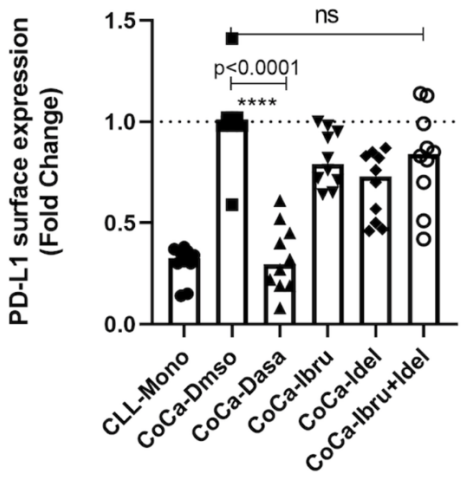

Figure 19: PD-L1 surface levels on CLL patient cells after coculture with HS5 cells and treatment with different BCR-inhibitors: As depicted in figure 18, coculture with HS5 cells increased the low baseline PD-L1 expression on primary CLL cells. Treatment with dasatinib led to a significant reduction of PD-L1, while ibrutinib and idelalisib as well as a combination of both did not have a significant impact. This data by Greta Steinmetz reinforces the conclusion that the effect of dasatinib on PD-L1 expression is not mediated by the BCR-pathway.

PD-L2 surface expression increased after treatment with some of the inhibitors, but the results were hardly reproduceable and many of the B cell lines used in this work did not express any PD-L2. Also, in primary CLL cells, PD-L2 expression was even lower than that of PD-L1. Considering the limited literature on the role of PD-L2 in B cell malignancies, this molecule seems to be of minor importance in the pathogenesis of CLL.

All in all, our data led us to the conclusion that PD-L1 and PD-L2 surface expressions are highly variable and do not underlie a clear regulation by the BCR pathway.

6. Outlook

6.1 Quantification of total PD-1 ligand expression and mRNA levels upon BCR inhibition and stimulation

Since only PD-1 ligand surface expression was investigated with flow cytometry in this work, a possible regulatory effect of the BCR signaling pathway on intracellular protein or mRNA levels remains to be examined. Thus, a further step would be to use Western Blot as an alternative readout to flow cytometry in order to determine the effect of BCR inhibition and stimulation on PD-L1 protein levels. Here, Li et al. could already show an effect of three different BTK-inhibitors as well as after stimulation with IgM in DLBCL cell lines [93].

In addition to protein quantification, measuring mRNA levels after BCR inhibition and stimulation with qRT-PCR would provide further insight into possible regulatory impacts of the BCR-pathway on PD-1 ligand transcription.

For those experiments, working again with various cell lines representing different B cell malignancies as well as primary CLL cells would show most comprehensively whether the observed effects apply on B cells in general and whether cell lines and patient cells are systems which are well-comparable.

Furthermore, it could be of interest to investigate PD-1 ligand expression on the cells surface as well as on protein and mRNA levels in patient samples after *in vivo* treatment with TKIs. This could help to elucidate the effects of BCR-inhibition not just on the malignant cells directly but also on the TME and the function of the host's immune system.

6.2 Identification of the regulatory potential of individual dasatinib off-targets for the expression of PD-1 ligands on the surface of malignant B cells

Since dasatinib showed the strongest effect on PD-L1 expression in our experiments, further investigating the off-targets of this kinase inhibitor would be of particular interest. In the cell lines used in our experiments as well as in primary CLL cells (data by Greta Steinmetz, see figure 18 and 19), after stimulating PD-L1 surface expression, dasatinib led to a decrease in PD-L1 levels. Since other BCR-inhibitors did not consistently show this effect, we concluded that dasatinib treatment targets other molecules and pathways influencing PD-L1 expression. Dasatinib is known to have various off-targets, and to determine which one impacts PD-L1, a CRISPR-Cas9 knock-out screen would be a

suitable tool. This method elucidates the link between genotype and phenotype by a genome-wide analysis of gene expression and subsequent phenotypic alterations. To achieve this, libraries of single guide RNAs (sgRNAs) are used to knock out genes on a genome-wide scale. Thus, loss-of-function mutations can be introduced into various targets of dasatinib. Subsequently, the phenotypic alterations in PD-L1 expression can be investigated. This way, still unknown regulatory pathways of PD-L1 in malignant B cells can be identified.

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8. Appendices

8.1. List of figures

8.2. List of tables