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Regulation of PD-L1 surface expression on CLL cells by small molecule inhibitors and the functional consequences on T cell mediated cytotoxicity

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Primäre humane CLL-Proben wurden aus dem peripheren Blut von CLL-Patienten an der Universitätsklinik Köln nach schriftlicher und informierter Zustimmung gemäß der Deklaration von Helsinki und mit Genehmigung des Institutional Review Board der Universität zu Köln Nr. 21-1317 und Nr. 19-1438_1 gesammelt.

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For my dad

who keeps asking the question until I find my answer.

Table of contents

Index of abbreviations

°C	degree Celsius
ALL	acute lymphocytic leukaemia
APC	antigen presenting cell
ATP	adenosine triphosphate
BCR	B cell receptor
Bcr-abl	Breakpoint cluster region-Abelson murine leukemia (gene)
Ca^{2+}	Calcium
Cas9	CRISPR-associated protein 9
CD	Cluster of differentiation
aCD3	anti-CD3 (Figure 14,15)
CFSE	Carboxyfluorescein succinimidyl ester
CLL	chronic lymphocytic leukaemia
CML	chronic myelocytic leukaemia
CO_2	carbon dioxide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CT	computed tomography
DC	dendritic cell
Del	deletion
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmatic reticulum
FcR	fragment crystallizable receptor
Fig.	Figure
HIF	hypoxia induced factor
HL	Hodgkin lymphoma
i.e.	id est (this is)
Ig	immunoglobulin
IGHV (IgVh)	immunoglobulin heavy-chain variable (gene region)
IL	interleukin
INFγ	interferon gamma
IPI	international prognostic index (score)
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif

ITSM	immunoreceptor tyrosine-based switch motif
LDH	lactate dehydrogenase
MHC	major histocompatibility complex
MRD	minimal residual disease
Mut	mutated
NHL	Non-Hodgkin-lymphoma
NK	natural killer cells
NSCLC	non-small-cell lung cancer
PE	phycoerythrin (Facs channel)
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cell
PD-1/ PD-L1	programmed cell death protein/ ligand 1
PMA	Phorbol-12-Myristat-13-Acetat
PS	phosphatidylserine
RNA	ribonucleic acid
Rpm	revolutions per minute
RT	room temperature
SEM	standard error (of the mean)
TGFβ	transforming growth factor beta
T_h	T-helper cell
TME	tumor microenvironment
TP53	tumor (suppressor) protein (transcription factor)
Unk	unknown
Unmut	unmutated
VEGF	vascular endothelial growth factor
VpreB	variable pre B cell
WW	watch and wait
X-ray	roentgen radiation
Z-Vad-fmk	carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (pan caspase inhibitor)

1. Zusammenfassung

PD-L1 und sein Rezeptor PD-1 sind Oberflächenproteine auf vielen verschiedenen Zellen, die an der Immunantwort im menschlichen Körper beteiligt sind. Die Bindung der Liganden an ihren Rezeptor hemmt die Immunantwort, was physiologischerweise eine überschießende Immunantwort an den sogenannten Immun-Checkpoints verhindert.

PD-L1 wird jedoch auch auf Tumorzellen exprimiert, z. B. auf CLL-Zellen, und so können leukämische Zellen der Immunabwehr des Tumors entgegenwirken. Die PD-L1/PD-1-Achse wird in der Onkologie als therapeutisches Ziel genutzt, um die Immunabwehr gegen Tumorzellen zu verbessern. Zu diesem Zweck werden sogenannte Checkpoint-Inhibitoren eingesetzt, die die Interaktion zwischen dem Liganden und dem Rezeptor blockieren.

In dieser Arbeit haben wir uns mit der Regulation der PD-L1-Expression auf primären CLL-Zellen beschäftigt. Zum einen kann die PD-L1-Expression auf der Zelloberfläche durch verschiedene Methoden stimuliert werden, zum anderen wird eine Reduktion von PD-L1 auf primären CLL-Zellen durch verschiedene Checkpoint-Inhibitoren erreicht. Wir haben uns besonders auf den Inhibitor Dasatinib konzentriert, da er die deutlichste Wirkung bei der Reduzierung der PD-L1-Expression zeigte. In verschiedenen Experimenten untersuchten wir die Rolle des B-Zell-Rezeptor-Signalwegs und den Einfluss seiner einzelnen Kinasen auf die Regulation der PD-L1-Expression. In diesen Experimenten erwies sich der B-Zell-Rezeptor-Signalweg nicht als kritischer Regulator.

Da Dasatinib zelltoxisch ist und bekanntermaßen viele klinische Nebenwirkungen verursacht, wurden in den Experimenten ausschließlich lebensfähige CLL-Zellen berücksichtigt, um eine beeindruckende Verringerung von PD-L1 auf CLL-Zellen allein durch eine toxische Wirkung auszuschließen.

Eine signifikante Verringerung von PD-L1 konnte durch die Behandlung mit Dasatinib auch auf Hochrisiko-CLL-Zellen erreicht werden. Dies weist darauf hin, dass Dasatinib eine potenzielle Therapie für CLL-Patienten mit hohem Risiko darstellt.

Außerdem wurde die Wirkung von PD-L1 exprimierenden CLL-Zellen auf T-Zellen untersucht. Zu diesem Zweck wurden verschiedene Bedingungen für B-T-Zell-Kokulturen mit hoher oder niedriger PD-L1-Expression auf CLL-Zellen und aktivierten oder inaktivierten T-Zellen angewendet. Erste Ergebnisse bestätigten die Hypothese, dass das Überleben von CLL-Zellen durch aktivierte T-Zellen stärker reduziert wird und dass die T-Zell-Antwort durch hoch exprimiertes PD-L1 gehemmt wird.
Diese Ergebnisse bedürfen jedoch einer weiteren Validierung. Unsere Idee ist es, die Interaktion zwischen B- und T-Zellen durch Live-Bildgebung sichtbar zu machen. Ein künftiges Ziel ist die Etablierung von Dasatinib plus einem PD-L1-Antikörper als Kombinationstherapie für CLL. Dies könnte die therapeutischen Möglichkeiten für CLL Hochrisikopatienten, die auf eine Immuntherapie ansprechen, erweitern.

2. Introduction

2.1 B lymphocytes

2.1.1 B cell development and function

B lymphocytes belong to the white blood leukocytes and play an important role in the specific humoral immune system to fight off infections. They form antibodies in response to an antigenic stimulus and, together with the T lymphocytes, make up the crucial component of the adaptive immune system.

B cells were identified long time ago and since then our understanding of development, maturation and function has progressed enormously (Balfour et al., 1965). B cell development is a complex process, which proceeds in two stages and takes place at distinct anatomical areas.

The first, antigen-independent stage involves the formation and verification of functional B cells, derived from multipotent hematopoietic stem cells. In mammals, it takes place in the fetal liver and later in the bone marrow. The immature B cells undergo negative selection due to the specificity of their receptor (Arneth, 2018). Cells expressing an autoreactive B cell receptor are rendered harmless. Only about 10% of the 10^7 immature B cells generated daily reach the peripheral lymphatic system in mice after negative selection.

The second section describes the differentiation steps triggered by activation of B cells in the periphery by binding of foreign antigens. If a mature B cell binds to a suitable antigen via its B cell receptors, it is activated via intracellular signaling cascades and stimulated to proliferate and further differentiate. The B cell now functions as an antigen-presenting cell (APC) to produce specific antibodies at the end. For this purpose, B cells express the major histocompatibility complex (MHC class I and II). After internalizing and processing the antigen, an oligopeptide of this antigen is presented by the MHC on the surface (**Figure 1**). A T helper cell binds to this complex and, after verification of the antigen as foreign, activates CD4+ and CD8+ effector T cells (Reichardt et al., 2007).

Depending on the form of the antigen, the B cell now differentiates directly into an antibody-secreting plasma cell, or it requires interaction with T lymphocytes for this step (Cambier et al., 2007); (Wang et al., 2020).

The crucial element in both sections is the mentioned B cell receptor. Its functionality, in combination with the right microenvironment and the right growth factors, is a prerequisite for reaching the next developmental stage in each case. This complex process naturally offers many different possibilities and points of attack where errors occur, a cell develops incorrectly or even a disease develops (LeBien & Tedder, 2008); (Mauri & Ehrenstein, 2008).

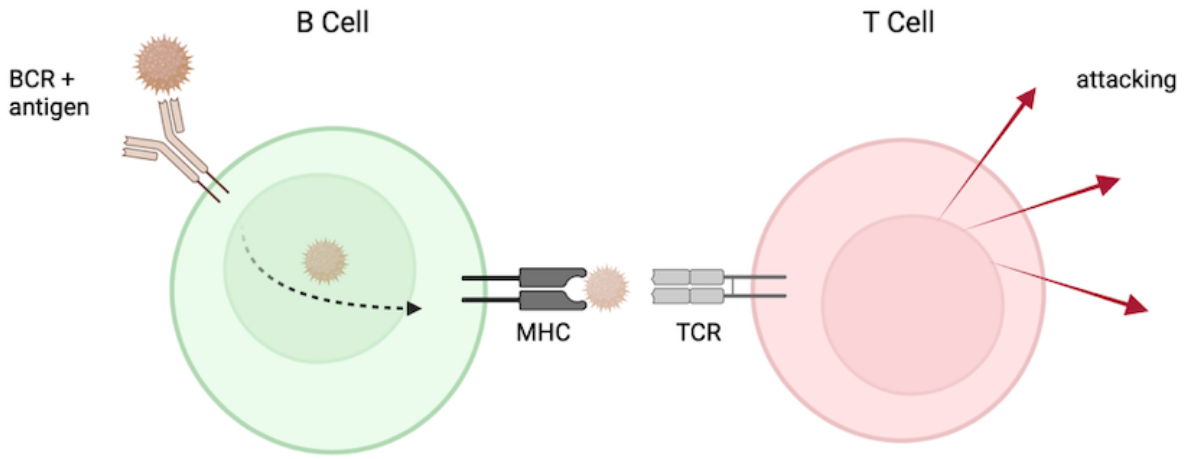


Figure 1. Antigen presenting. Figure created with BioRender. *Schematic view of a B cell presenting an antigen to a T cell. Binding of an antigen to the BCR leads to internalizing and processing the antigen. Finally, the antigen is presented via MHC to the T cell. Binding of the antigen to the TCR leads to activation of the T cell.*

2.1.2 B cell receptor signaling pathway

As mentioned, the B cell receptor (BCR) is not only fundamentally important for B cell development and maturation, but also for the binding and uptake of antigens. Binding of an antigen to the receptor transmits signals inside the cell, triggering an entire signaling cascade that activates the B cell (**Figure 2**). BCR activation is a multi-step process that is self-regulating, in terms of reinforcing and also inhibiting.

B cells have several hundred thousand copies of BCRs on their plasma membrane. The distribution of receptors on the cell surface is not random, but is shaped by the cell's cytoskeleton. The receptors cluster as oligomers in microdomains (Yang & Reth, 2010a). Multivalent binding of an antigen to the receptor leads to cross-linking and dissociation of the BCRs and thus stimulation of the quiescent B cell (Woodruff et al., 1967); (Yang & Reth, 2010b). In summary, and to indicate the direction of my topic,

the BCR signaling pathway, as a central regulatory site in B cell development, offers many targets to influence this development and thus becomes one focus of research.

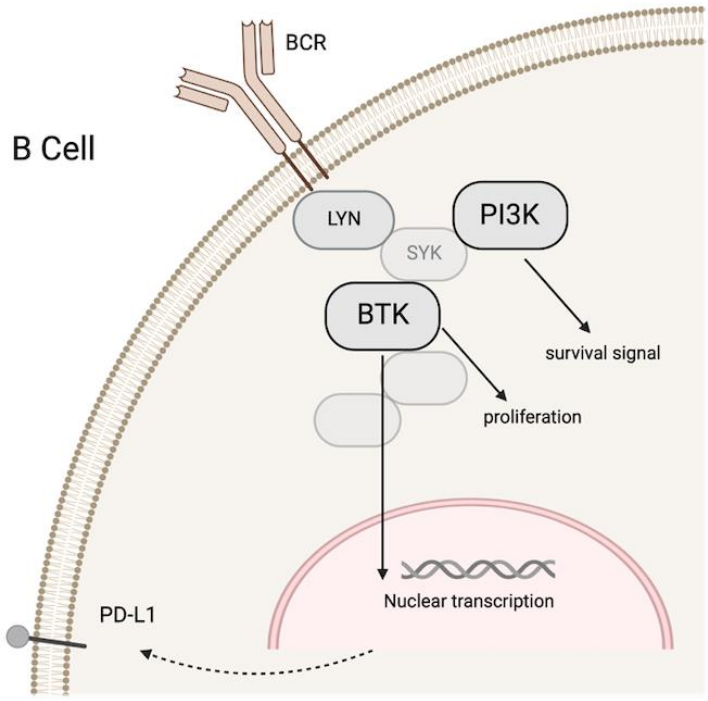


Figure 2. The B cell receptor signaling pathway. ***Figure created with BioRender.*** *Schematic view of the BRC-signaling pathway. Antigen biding to the BCR induces signalosome formation at the cytosolic plasma membrane. Influenced by stimulatory an inhibitory co-receptor signaling this triggers a signal cascade and leads to the transduction of survival, apoptosis, proliferation or migration signals, as well as the transcription of proteins, e.g. PD-L1.*

2.2 B cell malignancies

When cells grow and divide in an uncontrolled manner, tumors develop. Such degeneration is based on genetic alterations caused by cancer-causing substances (carcinogens) and/or heredity, or may also be associated with infections. In detail, cellular homeostasis like proliferation, differentiation or survival is defective, caused by oncogenic mutations in regulatory genes, chromosomal translocations, amplification and deletions. Tumors of the lymphatic system are called lymphomas because these diseases originate from the lymphatic organs such as lymph nodes and spleen or from the lymphatic cells (T and B cells). Thus, in the case of lymphoma, lymphocytes proliferate uncontrollably, which can be very slow (low-malignant, indolent) but also rapidly proliferating and aggressive (high-malignant) and has a wide clinical spectrum.

Within malignant lymphomas, two major groups are distinguished, Hodgkin lymphomas (Morbus Hodgkin) and non-Hodgkin lymphomas (NHL). The non-Hodgkin lymphomas are further subdivided into very many different types that must be distinguished for treatment and estimation of prognosis. About 85% of NHLs are B-cell lymphomas, which are further divided into additional groups. Basically, lymphomas are classified according to two criteria, cell type and malignancy. For example, a typical low-malignant B-cell lymphoma is chronic lymphocytic leukemia. The term "leukemia" generally refers to a degeneration and malignant proliferation of leukocytes (white blood cells). Lymphatic leukemias are diseases in which a certain type of leukocyte, namely lymphocytes, is found to be increased in the blood.

B-cell diseases are of immense medical importance, as they can affect anyone and can have far-reaching consequences due to massive deficiencies in the immune defense system. A constant threat, challenging genomic integrity in B lymphocytes is the rearrangement of immunoglobulin genes in early development. Here chromosomal translocations can occur replacing the promoter region of a gene with heterologous regulatory elements which results in inappropriate expression patterns (Shaffer et al., 2002).
Compared to solid tumors lymphomas and leukemias occur rather rarely. Together they account for about 10% of all cancers in Germany, but often young patients are affected and if therapy is performed in time, patients can be well helped and even cured.

Common symptoms of lymphoma include fever, night sweats and weight loss (B-symptomatology), and persistent or growing, painless lymph nodes. There may also appear splenomegaly and displacement of bone marrow due to increasingly degenerated cells. In this case, anemia (displacement of erythrocytes), hemorrhage (displacement of platelets), or clustered infections (displacement of leukocytes) occur. Malignant lymphomas are diagnosed by blood tests and fine tissue examinations (histology) of lymph nodes and other tumor-infiltrated tissues, which can be used to identify the various types of malignant lymphoma and assign them to one of the types. Imaging in the form of sonography, X-ray or CT can provide information about the exact localization and spread in the body.

For therapy selection, a basic distinction is made between low-malignant and high-malignant NHLs. In general, the "watch-and-wait" strategy, radiotherapy or various systemic therapies are available. Depending on the case, stem cell transplantation may also be considered as ultima ratio. Whether the disease should be classified as curative or palliative, depends on whether the finding is localized or spread systemically. In general, highly malignant NHLs are curatively treatable, as they respond better to therapeutic agents due to their high cell division rate. The International Prognostic Index (IPI) is used to estimate prognosis of NHL, considering age, stage, extra nodal involvement, LDH concentration and general condition of the patient. Therapeutic advances have been made over the past decades and options continue to expand through good research.

2.2.1 Chronic lymphocytic leukemia (CLL)

Chronic lymphocytic leukemia belongs to the low-malignant B cell lymphomas with lymphocytic leukocytosis in the blood count. The small B lymphocytes accumulate in the blood, lymph nodes, and lymphoid organs and immunophenotypically exhibit aberrant expression of CD5, normal levels of CD19, CD20, and CD23, as well as less surface immunoglobulin and deficient CD22 (Jaffe, 2009). It has been shown that the BCR signaling pathway is highly active in CLL cells, which is mediated by overactivation of kinases such as LYN, SYK, and BTK. This leads to the expansion and persistence of neoplastic cell clones and plays a key role in the pathogenesis of the disease (Davids & Brown, 2012).

The highly variable clinical manifestation occurs due to biological genetic heterogeneity. In CLL, hypermutations in the immunoglobulin heavy chain gene (IGHV), specific genomic aberrations such as deletions in chromosome 13 (del13q14) and recurrent mutations in oncogenes and tumor suppressors are known. These and many other alterations are becoming more common and have a high impact on prognosis (Hallek, 2013). For example, there are high-risk aberrations such as del(17p) with loss of the tumor suppressor gene TP53, which can lead to a more aggressive course and worse outcome of the disease. Furthermore, possible overexpression of the anti-apoptotic protein B-cell lymphoma 2 (BCL-2) and hypomethylation of its promoter leads to a disbalance in the regulation of programmed cell death (Yosifov, Wolf, Stilgenbauer, &

Mertens, 2019). Moreover, the tumor microenvironment (TME), including T cells, nurse-like cells and macrophages, plays a critical role for CLL support and survival. In particular, the overactivated tyrosine kinase LYN and its substrate BTK in macrophages promote leukemic progression (Nguyen et al., 2016). Taken together, the genetic heterogeneity is another criterion to determine the prognosis of CLL patients and therefore is part of the CLL-IPI Score. Compared to the standard NHL-IPI it considers also specific mutations (IGHV-, TP53 status) next to patients age and clinical stage of each individual CLL patient.

CLL is the most common form of leukemia in western industrialized countries (about 5600 new cases annually in Germany) with an increasing incidence with age (median: 67-72 years) and occurs more frequently in men. It is usually asymptomatic for a long time and is often diagnosed late or by chance in blood smears and by immunophenotyping. Displacement of hematopoietic bone marrow and functional B lymphocytes with subsequent antibody deficiency syndrome and impaired immune defense is the most common cause of death. Therapy is basically based on the Binet staging classification and thus on the general condition of the patient. Although significant progress has been made in recent years, CLL is still not curable and allogeneic stem cell transplantation remains the only known curative option, albeit reserved for young, fit patients (Yosifov et al., 2019).

Standard chemoimmunotherapy is offered for CLL. One of the best-known agents is the anti-CD20 antibody rituximab, but also the purine analogue fludarabine, and the alkylants cyclophosphamide, chlorambucil and bendamustine. Combinations of these agents are expected to achieve steady remission of the disease and a state of minimal residual disease (MRD). New research findings in CLL pathogenesis yielded additional targeted agents, most notably the BTK inhibitor ibrutinib, the PI3Kδ inhibitor idelalisib (**Figure 3**) and the apoptosis regulator BCL-2 antagonist venetoclax (Burger & O'Brien, 2018). These represent a particular advantage for high-risk patients, who show a higher frequency of mutations. Such alterations can be, for example, a p53 mutation, 17p deletion or complex altered karyotypes. The gene p53 plays a crucial role in the regulation of the cell cycle and acts as a tumor suppressor (Vieler & Sanyal, 2018) in various tumors. P53 physiologically suppresses degenerated cell division and thus the development of cancer. In CLL patients with a mutation in the p53 gene, this

mechanism is defective and the risk of tumor progression is increased. In addition, such patients are more difficult to treat, the course of the disease is more often aggressive, the response to therapy is poorer, and the tumor is more likely to be refractory (Buccheri et al., 2018). Such mutations are becoming more common and thus represent an increasing problem for patient care (Dohner et al., 1995), (Cordone et al., 1998). This group of patients is thus all the more in need of improved therapy and new options to combat the ever-evolving resistance. Of course, not many substances can be considered for this, only highly effective agents that are usually also very aggressive.

In addition, new anti-CD20 antibodies, obinutuzumab and ofatumumab, were introduced, enabling a low-intensity regiment even for elderly fragile patients. Last but not least, immune checkpoint inhibitors have become more important and have sparked interest in many other research approaches. Immune checkpoints regulate the balance between immune defense and tolerance, here an important example, also in CLL, is the PD-1/ PD-L1 axis. While the understanding of these molecular mechanisms continues to grow, the PD-1 inhibitor nivolumab, for example, has already shown promising results in early trials in refractory B-cell non-Hodgkin lymphoma (Xu-Monette et al., 2018). Numerous new discoveries have been made, but CLL also continues to struggle with new mutations and resistances. The success story must continue.

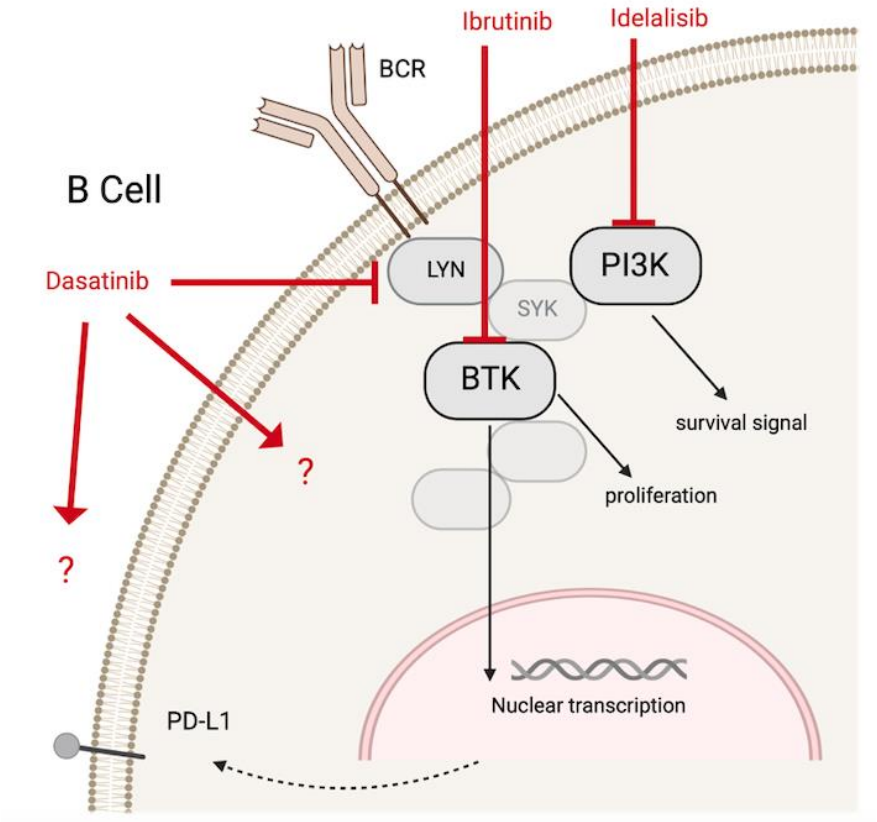


Figure 3. Possible targets of the BCR-signaling pathway. Figure created with BioRender. *Schematic view of the BCR-signaling pathway with single kinases that can be targeted, thus inhibited by BCR inhibitors. Ibrutinib inhibits the kinase BTK, idelalisib is a PI3K-inhibitor, whereas dasatinib is a pan inhibitor. The molecular targets of the inhibitors are not yet fully investigated.*

2.3 Targeted therapy

2.3.1 Small molecule BCR-kinase inhibitors

For a long time, chemotherapy was the standard treatment for cancer as well as CLL with aggressive progression. Alkylating agents, such as chlorambucil, generally attack rapidly proliferating and dividing cells and were long considered the first-line therapy in CLL. They were soon joined by nucleoside analogues, such as fludarabine and cladribine, which were designed to prevent first-line treatment failure and increase response rates. They act mainly by apoptosis induction through inhibition of DNA synthesis (Robak & Kasznicki, 2002). However, since nucleoside analogues are more suitable for young fit patients due to a high side effect profile and refractory CLL became an increasing concern, the need for new efficient options continued to grow. For example, the alkylator bendamustine was developed, which also induces cell death

and is considered more manageable and tolerable than fludarabine (Chang & Kahl, 2012). An equally promising hot topic has been the combination of chemoimmunotherapy. Immunotherapeutics specifically promote the immune system, including the defense against cancer cells, without causing as broad damage as chemotherapeutics. The monoclonal antibody rituximab plays a particularly important role here. Rituximab specifically targets the transmembrane phosphoprotein CD20 on developing B cells, which is not present on mature or progenitor B cells, thus reducing side effects (Pierpont et al., 2018); in combination with fludarabine and cyclophosphamide, progression-free as well as overall survival of CLL patients was increased (Hallek et al., 2010). With this successful outcome, not only the era of targeted therapy in oncology began, but also further research on immune regulation in cancer.

The increasing understanding about CLL and its biology has also exposed numerous targets for therapeutics. CLL is known to be highly dependent on survival and growth factors as well as interaction with cells of the tumor microenvironment. Nurse-like cells, stromal cells and also T cells secrete chemokines and other factors that support CLL survival by activating specific signaling pathways and enzymes (Kipps & Choi, 2019). Targeted drugs can act precisely at these sites. Kinase inhibitors cover a large area, such as the BCR inhibitors ibrutinib, acalabrutinib, and idelalisib. Such kinases catalyze the B cell signaling pathway cascade, leading to B cell stimulation and CLL cell growth and survival (as described in 1.1.3). The small molecule ibrutinib (and acalabrutinib as second-generation of ibrutinib) covalently irreversibly binds and inhibits the ATP-binding domain of the kinase BTK, a key enzyme in the B cell signaling pathway. Ibrutinib has shown improvements in overall survival, especially in high-risk patients (with del (17p) and/or TP53 mutations) and could even be recommended as initial single-agent therapy for CLL patients (Davids, 2014), (Woyach et al., 2018), (Wierda et al., 2019). Idelalisib, as a small molecule inhibitor of the BCR kinase PI3K, was also able to achieve significant reductions in lymph node growth and lymphocytosis, but was not further supported due to increased toxicity (Kipps, 2019). Despite the success, especially of ibrutinib, BCR-associated kinase inhibitors failed to achieve complete remission even when combined with other drugs, necessitating further therapy.

2.3.2 Immune Checkpoint blockade

The human immune system is tightly regulated and controlled. It requires a fine-tuned balance between immune defense and immune tolerance. More precisely, foreign particles and pathogens, such as viral antigens or cancer cells, should be fought without immune response overshooting and attacking own tissue. In fact, this can lead from an acute or chronic inflammatory reaction to an immunopathology or autoimmunity. In addition to regulatory T cells, certain macrophages and regulatory cytokines such as IL-10 and TGF-ß, immune checkpoints are responsible for controlling this balance. Immune checkpoints are molecularly composed of surface receptors and their ligands (**Figure 4**). An important example is the receptor PD-1, which is among others expressed on activated T cells, with its ligand PD-L1. Ligation of PD-1 with PD-L1 leads physiologically to suppression of activated T cells in the effector phase in order to prevent tissue damage and cell death. Such immune checkpoints are often elevated in chronic infections and in many cancers, for example PD1/PD-L1 has been shown to be increased in CLL patients (Ramsay et al., 2012), (Brusa et al., 2013). Tumors use this mechanism with the consequence of T cell exhaustion to evade immune system attacks, which is called immune evasion (**Figure 5**). Consequently, these checkpoints have become important targets for cancer therapy (Curry & Lim, 2015),(Dyck & Mills, 2017).

Checkpoint inhibitors activate tumor defense by disrupting inhibitory interactions between antigen-presenting cells and T lymphocytes at the so-called checkpoints. Pharmacologically, these are monoclonal antibodies.
Checkpoint inhibitors have already been approved for several tumors and are in clinical use. In 2014, for example, the PD-1 inhibitor pembrolizumab was approved for the first time for advanced melanoma, as well as for non-small cell lung cancer, first in the USA and a short time later also in Europe. Since then, knowledge of molecular mechanisms has grown and further approvals have been added (Alexander, 2016), (Darvin et al., 2018). PD-L1 inhibitors showed in particular in a CLL mouse model that the tumor burden could be reduced and the immune effector functions could be reactivated (McClanahan et al., 2015). Also advantageous is the relatively low side effect profile of PD-1/PD-L1 blocking antibodies. The relatively mild phenotypes of PD-1/ PD-L1 mice may be a reason for rather less collateral immune toxicity (Pardoll, 2012).

Furthermore, clinical trials demonstrated that the PD-1 inhibitor pembrolizumab achieved benefits for CLL patients with Richter transformation (Ding et al., 2017). However, similar to conventional chemotherapy, the effect of these inhibitors is often not durable, as tumor cells develop genetic variants through mutation and selection that bypass the therapeutic effect of checkpoint inhibitors through novel pathways of immune evasion. A huge amount of patients fails to respond to PD-1/PD-L1axis inhibitors completely. Since expression of PD-L1 on tumor cells has been associated with enhanced response rates towards PD-1/PD-L1 inhibition, an improved understanding about the regulation of PD-L1 expression has become more important (Sun et al., 2018). It is anticipated that PD-L1 may serve as a prognostic marker regarding the therapy response and outcome rates in tumor patients, and this not only for several tumor entities, but also in microbial infections (Herbst et al., 2014), (Aguiar et al., 2017).

The role of PD-1/PD-L1 inhibitors, specifically for CLL, has not yet been conclusively evaluated, but initial successful results and especially the potential relevance for high-risk CLL patients, make further research desirable.

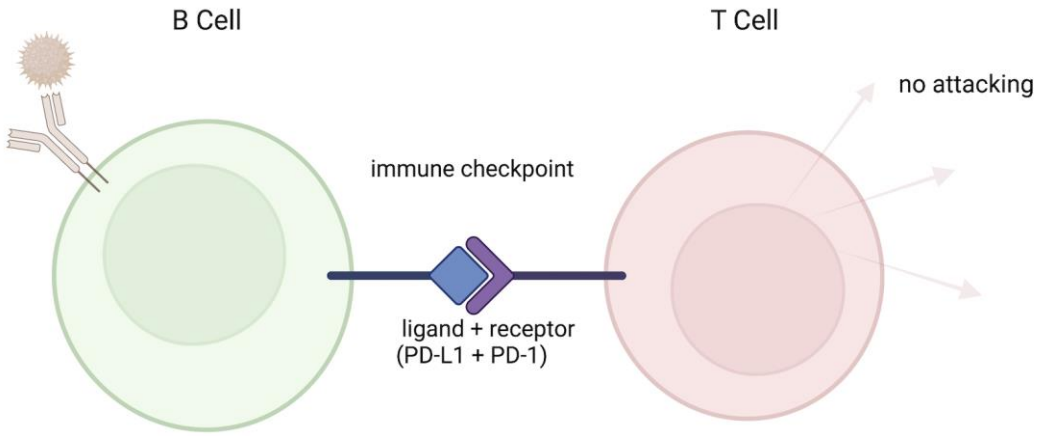


Figure 4. Immune checkpoint. Figure created with BioRender. *Schematic view of a immune checkpoint, consisting of a receptor and a ligand. Binding of the ligand to its receptor leads to suppression of T cell activation and therefore to suppression of immune defense.*

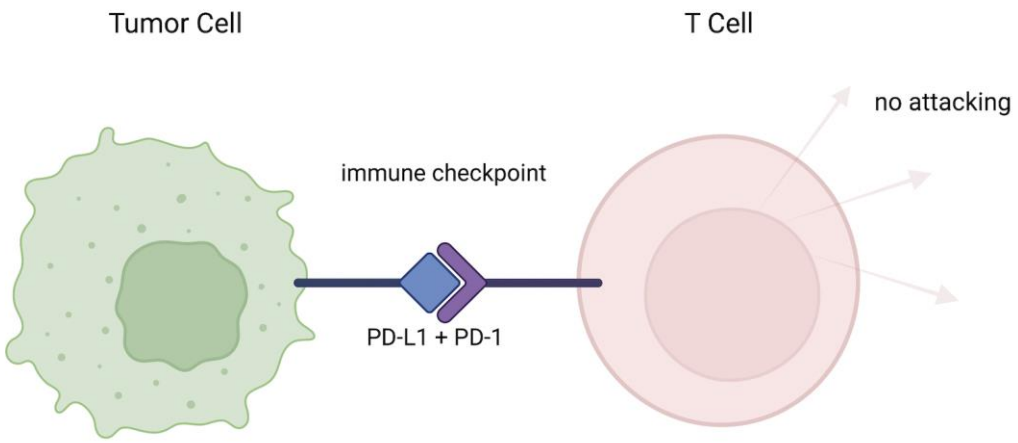


Figure 5. PD-1/ PD-L1 axis. Figure created with BioRender. *Schematic view of the PD-1/ PD-L1 immune checkpoint. Tumor cells express PD-L1 to inhibit immune answer towards themselves. Binding of PD-L1 to its receptor PD-1, expressed on T cells, leads to suppression of T cell activation.*

2.4 PD-L1

Programmed-cell death ligand 1, also known as CD274 or B7-H1, is a broadly expressed glycosylated surface protein involved in immune system inhibition as described above. It occurs physiologically on immune cells, such as T cells, B cells, dendritic cells and macrophages, as well as on parenchymal tissue cells, such as mesenchymal stem cells, epithelial, endothelial cells and brown adipocytes. It was found to be elevated on tumor cells, leading to tumor immune evasion (Iwai et al., 2002).

PD-L1 was co-discovered and described by Gordan Freeman in 2000. The protein is encoded on chromosome 9 at gene locus 9p24.1. The gene contains 7 exons. The PD-L1 protein consists of a total of 290 amino acids and has a molecular weight of approximately 33 kilodaltons (kDa) (Dong et al., 1999). It is composed of 3 domains, the largest IgV- and IgC-like domain in the extracellular region, the hydrophobic transmembrane domain and the short, charged intracellular domain (Freeman et al., 2000).

PD-L1 and its major receptor PD-1 (CD279), which is also mainly expressed on activated T cells, belong to the B7/CD28 family. The B7 family includes several

molecules that, in addition to MHC antigen presentation at the T cell receptor, regulate the T cell response through activating and also inhibitory signals. Of these molecules, CD80 (B7-1), CD86 (B7-2), PD-L1 (B7-H1 or CD274), PD-L2 (B7-DC or CD273), ICOSL (B7-H2), CD276 (B7-H3), and B7S1 (B7-H4) are known to date (Zou & Chen, 2008).

PD-L1 is thought to be the dominant inhibitory ligand of PD-1 (Sun et al. 2018), but can also interact with the costimulatory surface protein CD80 (B7-1) and bind to the T cell receptor CD28. The engagement of CD28 is required for T cell activation (Shen et al., 2019). In turn, binding of PD-L1 to the CD28 receptor leads to inhibition of T cell proliferation and cytokine production. Thus, together with the PD-1/PD-L1 axis, PD-L1 has a dual strategy to block the T cell response. This makes PD-L1 antibodies even more relevant (Butte et al., 2007).
The related ligand PD-L2 is genetically located at the same site as PD-L1, but has different expression patterns. PD-L2 is mainly present on activated dendritic cells and macrophages and is also involved in T cell inhibition (Yearley et al., 2017).

The interaction of PD-L1 and PD-1 has been thoroughly explored at the molecular level. PD-L1 provides three hotspots on its surface composed of different amino acids to bind PD-1 (Zak et al., 2015). Binding results in a conformational change in PD-1 with subsequent phosphorylation of the cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM) by Src family kinases. This in turn recruits the tyrosine phosphatases SHP-1 and-2, which ultimately prevent T cell activation. More specifically, T cell proliferation and survival, cytokine production and other effector functions are inhibited (Sun et al. 2018). In CLL patients, dysfunction of Th2 cells, a subset of T helper cells responsible for primary B cell activation and isotype switch, has been demonstrated. Reduced secretion of INFγ and IL-4 ultimately lead to decreased B and T cell differentiation, as well as impaired regulation of inflammatory processes and bacterial defense (Brusa et al., 2013).

Basically, when PD-1 senses a fragmented antigen, which is presented by MHC molecules, the T cell produces interferons. This in turn induces PD-L1 expression (Zak et al., 2015). As previously described, PD-L1 inhibits this communication between T cell and antigen-presenting immune cell itself, a negative feedback mechanism.

Genetic alterations of chromosome 9p24.1 affect PD-L1 expression and vary between cancer types. Thus, in some cancer types a gain of copy numbers has been found in this chromosome, while in others, e.g. melanoma, NSCLC and also diffuse large B cell lymphoma, PD-L1 gene deletions are typical. Such gene alterations may be responsible for increased PD-L1 expression (Georgiou et al., 2016). More specifically, other somatic mutations and genetic mechanisms have been identified as structural variants for PD-L1 overexpression, such as a disruption of the PD-L1 3′-untranslated region (Kataoka et al., 2016). In general, PD-L1 regulation can be divided into 2 distinct patterns: inducible or constitutive. In addition to gene alterations, as described above, there are many other mechanisms of constitutive PD-L1 expression. These include furthermore epigenetic regulation by DNA methylation, histone modification, and microRNAs, which belong to a group of small noncoding RNAs. Global DNA hypomethylation has been shown to lead to increasing PD-L1 expression in melanoma cells (Chatterjee et al., 2018), while inhibitors of histone deacetylases have been presented as positive or even negative regulators in several studies (Woods et al., 2015), (Booth et al., 2017). Likewise, microRNAs can negatively affect PD-L1 level, mostly via binding of the PD-L1 3′-untranslated region (Xie et al., 2018) or also upregulate it (Tang et al., 2018). Furthermore, constitutive PD-L1 regulation is also intrinsically controlled by oncogenic signals, such as the transcription factors MYC, STAT3, and c-Jun or receptor kinases of the MEK-ERK and PI3K-AKT pathways. In addition, mutations associated with loss of tumor suppressors, such as TP53 or PTEN, also play a role. Inducible regulation of PD-L1 is mainly controlled by extrinsic factors. These include viral infections, inflammatory signals such as INFγ and TNFα but also the anti-inflammatory cytokine TGF-β, hypoxia with thereby activated hypoxia-induced factors (HIFs), the angiogenesis factor VEGF or also previous treatment with radiotherapy or chemotherapy. Last but not least, post-translational modifications such as phosphorylation, ubiquitination, glycosylation or lipidation at the protein level also play a role in PD-L1 regulation. The complicated, multi-layered regulation of PD-L1 at different levels thus offers many potential targets for targeted therapy and also options to improve therapy response rates and counteract resistance (Shen et al., 2019).

In summary, PD-L1 plays an important role in the regulation of the immune system in the human body. Physiologically, it prevents excessive immune response and tissue damage as well as autoimmune reactions by inhibiting the T cell response. However,

tumors also exploit this mechanism to evade anti-tumor targeting of T cells, ensure prolonged tumor survival and therefore increasingly express PD-L1. Thus, the PD1/PD-L1 axis has become an important target for therapeutic approaches. Especially the immune checkpoint antibodies have gained a high clinical relevance, but due to a progressing understanding of PD-L1 regulation also other compounds are coming into the focus of current research.

2.4.1 The role of PD-L1 in CLL

Each tumor has its own characteristics. CLL is known as a compartmentalized disease in which cell proliferation is mainly localized in the lymphoid organs. The fine tuning of cell proliferation, in terms of cell survival, growth and also resistance development is strongly dependent on environmental factors and requires a high interaction of CLL cells with bystander cells. An increase in PD-1 and PD-L1 has been demonstrated in CLL and leads overall to prolonged CLL survival linked to poor patient prognosis (Ramsay et al., 2012), (Brusa et al., 2013).

Increased PD-L1 correlates with increased PD-1 on T cells and macrophages and leads to T cell exhaustion. It has been demonstrated that CLL T cells express increased exhaustion markers, such as PD-1, and exhibit decreased proliferation function. This was due to a reduction in the proportion of divisible CD8+ T cells and a prolonged division time. Furthermore, due to impaired granzyme-mediated cell lysis, cytotoxicity is reduced (Riches et al., 2013).

Nevertheless, proteins like PD-1, CD69 and granzyme B can be physiologically seen as activation markers of T cells.

CD 69 is a human transmembrane lectin protein that is expressed on T cells, among others. It is one of the first proteins to be induced during lymphoid activation and is therefore considered an early activation marker. It is also implicated in T cell differentiation as well as lymphocyte retention in lymphoid organs (Lopez-Cabrera et al., 1993).

Granzyme B, on the other hand, is a specific serine protease and is secreted by cytotoxic T cells to fight off pathogens and infections. It induces apoptosis in infected cells via caspase 3 activation and thus serves as a killing marker (Cullen et al., 2010).

Activation of T cells occurs physiologically via recognition and contact of a T cell from

a pathogen. In vitro, T cells can be stimulated and activated by CD3 antibodies (Li & Kurlander, 2010).

Nevertheless, T cells from CLL patients are still partially capable of producing cytokines, here INFγ and TNFα, thus favoring the Th1-differentiated cell fraction. This could also strongly contribute to create an immunosuppressive environment (Riches et al., 2013), (Grzywnowicz et al., 2015). However, this hypothesis is in contrast to other research findings (see above, Brusa et al. 2013) and highlights the still lacking understanding of the impact of PD-L1 in CLL.

For example, the reasons for increased PD-L1 expression on CLL cells has not yet been elucidated in detail. However, as also shown by Grzywnowicz et al, it is independent of clinical parameters and prognostic markers in CLL, such as age, LDH levels, leukocyte count, Binet stage, IGHV status or other specific mutations.

This made numerous studies all the more important, using different classes of drugs to try to influence PD-L1 regulation, thereby elucidating further mechanisms and ultimately restoring T cell function through PD-L1 blockade (**Figure 6**).

Lenalidomide, an immunomodulatory drug used in CLL therapy, increases the production of proinflammatory cytokines (Luptakova et al., 2013). In patients with multiple myeloma, PD-L1 downregulation has even been specifically demonstrated after lenalidomide therapy (Benson et al., 2010). In contrast, thalidomide, a related agent, could not show relevant changes regarding PD-L1 expression in CLL patients (Grzywnowicz et al., 2015).

PD-L1 blockade in general results in more active T cells in non-Hodgkin lymphomas, which is characterized by increased cytokine production and thus leads to an improved anti-tumor response (Andorsky et al., 2011). Consequently, BCR inhibitors also came to the fore, especially ibrutinib, that represents a successful new therapy for CLL (Davids & Brown, 2014), (Hallek, 2013),(Kim, 2019), (Younes et al., 2019) (Hanna et al., 2020). It has been investigated whether ibrutinib causes PD-L1 reduction and thus whether the B cell signaling pathway is involved in PD-L1 regulation. Although not all studies found a direct effect of ibrutinib on PD-L1 expression, there are also results showing ibrutinib to be a PD-1 and PD-L1 blocker via inhibition of the STAT3 pathway (Kondo et al., 2018), (Fraietta et al., 2016). Thus, the detailed effect of ibrutinib and the impact of other BCR inhibitors on PD-L1 expression is not yet fully understood and requires additional elucidation.

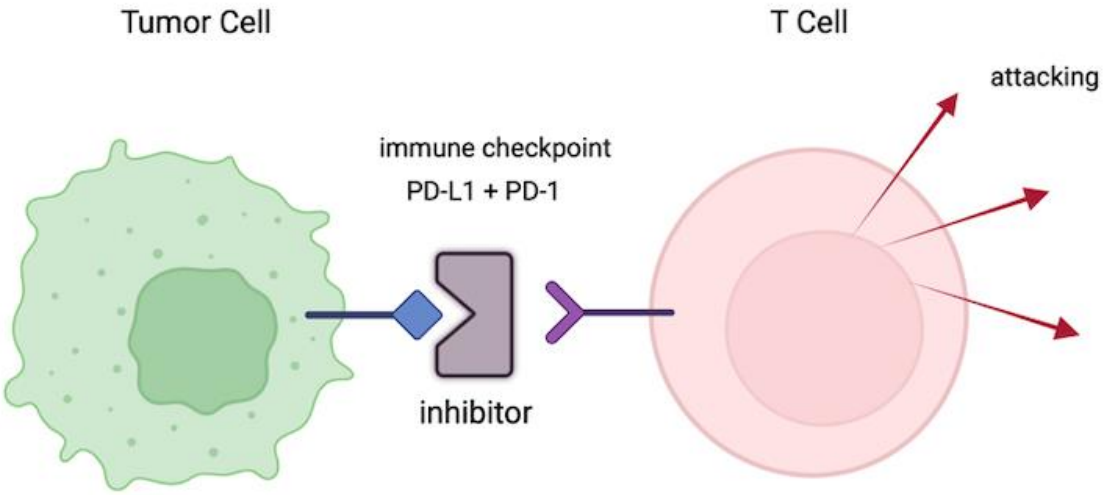


Figure 6. Inhibition of PD-1/ PD-L1 checkpoint. Figure created with BioRender. *Schematic view of an inhibitor binding PD-L1. The inhibitory checkpoint is blocked, thus T cell activation is not inhibited anymore and T cell attacking towards tumor cells is possible.*

2.5 Aims of research

Extensive research and numerous evidence have shown the important role of the PD-1/PD-L1 axis in regulating human immune response by various immune cells and factors including B- and T cells. Consistently, PD-L1 was implicated in essential cellular processes such as proliferation, differentiation and survival. Its main function is to control overshooting immune reaction by inhibiting T cell response in order to prevent tissue damage and autoimmune disorders. Since PD-L1 is also known to be expressed on tumor cells and therefore used by the tumor to reduce T cell defense against itself, PD-L1 has become an important target for cancer therapy. The understanding of PD-L1 regulation has already made huge strides, but is still lacking in some points, including the role of PD-L1 in CLL. To our knowledge the role of the BCR-signaling pathway for PD-L1 regulation is not fully understood so far. Given the striking success of BCR-inhibition in CLL and some previous promising studies about ibrutinib´s effect on PD-L1 expression, we postulated that the BCR signaling pathway and its downstream kinases might be important for PD-L1 regulation.

The first aim of this project was to decipher the role of the BCR-signaling pathway by treating primary CLL patient cells with different BCR-inhibitors, such as ibrutinib, idelalisib and dasatinib, regarding the influence on PD-L1 surface expression. This

assumed high levels of PD-L1 on tumor cell´s surface and therefore stimulation experiments and also included investigation on regulating mechanisms on protein level via Flow Cytometry.

Since both cell types, B- and T-cells, express PD-L1 and are influenced by PD-L1 interactions, we wondered if there might be detectable changes in cell phenotypes or behavior in a setting with low compared to high PD-L1 expression on B cell surface.

A second aim was to examine how (possibly BCR-mediated) alterations in PD-L1 expression affect T cell activity in a B-/T-cell coculture system (**Figure 7**). To find out more about the interaction between these cell types and to characterize underlying mechanisms in cell alterations possibly mediated by PD-L1, first we wanted to establish a functioning system of B-/T cell Coculture.

PD-L1 is an inhibitory protein, which suppresses immune reaction of T cells. T cells no longer attack antigens, as well as tumor cells, which is used by several tumors themselves e.g. CLL and called tumor evasion (Brusa et al., 2013).

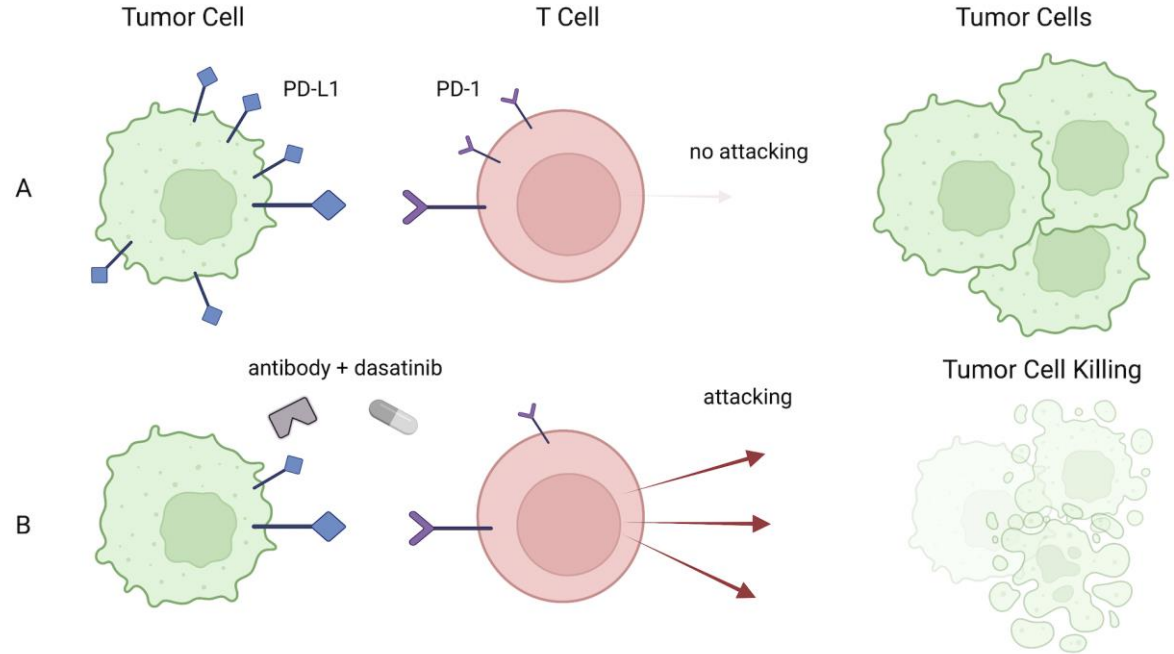


Figure 7. How does the PD-L1 expression on CLL cells affect T cell activation (towards tumor cells)?. Figure created with BioRender*. Schematic view of T cell activity in a B-T-cell Coculture. Tumor cells with high PD-L1 expression lead to lower T cell answer, thus less tumor cell killing. Inhibition and reduced number of PD-L1 allows T cell attacking towards tumor cells.*

There are several publications about T cell alterations in CLL patients (Gorgun et al., 2005). With this set of experiments, we aimed to explore

1) if higher PD-L1 expression on CLL cells would inhibit T cell response against tumor cells, for example by a higher CLL survival;
2) would combination of dasatinib and the available PD-1/PD-L1 antibodies be possible to enhance T cell responses. Our hypothesis was that this combination could improve the efficacy of immune checkpoint blockage for high-risk CLL patients.

Further approaches were to investigate and characterize T cell activation status by quantifying different activation markers and the overall idea was to lay the foundation for a possible new treatment combination in cancer therapy; the combination of a BCR-inhibitor plus a PD-1/ PD-L1 antibody. We postulated that this combination might have synergistic effects on PD-L1 regulation and therefore the overall CLL survival.

3. Material and methods

3.1 Material

3.1.1 Consumables

item	manufacturer
CryoPure container 1.8ml	Sarstedt, Nümbrecht, Germany
Dermagrip gloves	WRP, Amsterdam, Netherlands
FACS Analyse Tubes	Sarstedt, Nümbrecht, Germany
Falcon: 15ml, 50ml	Sarstedt, Nümbrecht, Germany
Filter Filtropur S 0.2µm	Sarstedt, Nümbrecht, Germany
Cannula	BD, Drogheda, Co.Louth, Ireland
Multiwell Plates 12-, 24-, 48-, 96- well F, U bottom	Falcon^{R,} Corning Incorporated NY, USA
Pipets 2, 5, 200, 1000µl	Eppendorf, Hamburg, Germany
Serological pipets: 5, 10, 25, 50 ml	Sarstedt, Nümbrecht, Germany
Pipet tips: 10, 100, 1000µl	Sarstedt, Nümbrecht, Germany
Reaction container: 0.5, 1.5, 2 ml	Sarstedt, Nümbrecht, Germany
Syringe: 1ml, 5ml	BD S.A., Madrid, Spain
Cellculture flasks: 25, 75, 175 cm^2	Falcon^{R,} Corning Incorporated NY, USA

3.1.2 Devices

name	description	manufacturer
ZOETM Fluorescent Cell Imager	Microscope	Bio-Rad, Singapore
CASY Model TT 150	Cell Counter	Roche, Mannheim, Germany
C200	CO2 Incubator	Labotect, Göttingen, Germany
FLUOstar OPTIMA	Microplate Reader	BMG Labtech, Ortenberg, Germany
JB Aqua 12 Plus	Water Bath	Grant Instruments, Shepreth, UK
Mixer Uzusio VTX-3000L	Vortex	Tanaka Bldg, Tokyo, Japan
Macs Quant VYB	Flow Cytometer	Miltenyi Biotech
Macs Quant X	Flow Cytometer	Miltenyi Biotech
Mars Safety Class 2	Laminar Flow Hood	Labogene, Vassingerod, Danmark
Milli-Q	Water filter system	Millipore, Eschwege, Germany
Pipetus	Pipette Filler	Hirschmann, Eberstadt, DE
SnowWhite	Freezer -80°C	SANYO Electric, Japan
SRT9	Roller, Mixer	Stuart, Staffordshire, UK
Telaval 31	Microscope	Zeiss, Oberkochen, Germany
ViCellTM XR	Cell Counter/ Viability Analyzer	Beckman Coulter, Brea, CA
5810 R	Centrifuge	Eppendorf, Hamburg, Germany
5430 R	Centrifuge	Eppendorf, Hamburg, Germany

3.1.3 Chemicals and reagents

reagent	manufacturer
AnnexinV Pacific Blue, APC	BioLegend, SanDiego, CA
AnnexinV FITC	Immuno Tools, Friesoythe, Germany
Dimethylsulphoxide (DMSO)	Carl Roth, Karlsruhe, DE
Ethanol	Carl Roth, Karlsruhe, DE
FcR Blocker	BD Pharmingen, Franklin Lakes, NJ, USA
Isopropanol	Carl Roth, Karlsruhe, DE
ViCell Reagent Pak, (Trypanblau, Cleaning Agent, Buffer Solution, Disinfectant)	Beckman Coulter, Brea, CA, USA
0.5% Trypsin/EDTA	Life Technologies, Paisley, UK, (Thermo Scientific; 15400-054)
7AAD	Miltenyi Biotec, Bergisch Gladbach, Germany

3.1.4 Inhibitors und stimuli

name	manufacturer
Acalabrutinib	Selleckchem, Houston, Texas, USA
Dasatinib	Selleckchem, Houston, Texas, USA
Ibrutinib	Selleckchem, Houston, Texas, USA
Idelalisib	Selleckchem, Houston, Texas, USA
aCD3 (clone UCHT1, HIT3)	BioLegend, SanDiego, CA
Brefeldin A	BioLegend, SanDiego, CA
INFɣ	Life Technologies, Paisley, UK
PMA	Sigma, Saint Lousi, Missouri, USA

3.1.5 Buffers and media

solution	composition	manufacturer
Annexin V Binding Buffer		BD Pharmingen, Franklin Lakes, NJ, USA; 51-66121E
FACS Buffer (1x)	5 g/l BSA, 2 mM EDTA, 0,09 % Na-Azid, PBS	Made by lab
Freezing Medium	50 % RPMI 1640, 40 % FBS, 10 % DMSO	Made by lab
MACS Buffer Running Buffer	PBS, EDTA, Na-Azide, Stabilizer	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS Buffer Storage Solution	0.09% Azide	Miltenyi Biotec, Bergisch Gladbach, Germany
MACSQuant Washing Solution	Detergent, stabilizer	Miltenyi Biotec, Bergisch Gladbach, Germany
PBS	Phosphate Buffered Saline	Life Technologies, Paisley, UK; 14190144)
RPMI 1640 Culture media	10 % FBS (life technologies; 10500056), 1 % Penicillin/Streptomycin (life technologies; 15140122)	GibcoTM (Life Technologies, Paisley, UK; 11875093)

3.1.6 Antibodies and cytokines

antibody	isotype	#number	manufacturer
CD3-FITC	/	130-113-128	Miltenyi Biotec, Bergisch Gladbach, Germany
CD4-FITC	/	130-114-531	Miltenyi Biotec, Bergisch Gladbach, Germany
CD4-VioBlue	/	130-113-781	Miltenyi Biotec, Bergisch Gladbach, Germany
CD5-VioBlue	/	130-110-995	Miltenyi Biotec, Bergisch Gladbach, Germany
CD8-Brilliant Violet	Mouse IgG1,k	344731	BioLegend, SanDiego, CA
CD19-VioGreen	/	130-113-174	Miltenyi Biotec, Bergisch Gladbach, Germany
CD19-PE	/	130-113-169	Miltenyi Biotec, Bergisch Gladbach, Germany
CD69-FITC	/	310903	BioLegend, SanDiego, CA
CD274-PDL1	Mouse IgG2b,k	329706	BioLegend, SanDiego, CA
CD279-PD1	Mouse IgG1,k	329905	BioLegend, SanDiego, CA
IL-2	/	500305	BioLegend, SanDiego, CA
GranzymeB-Pacific Blue	Mouse IgG1,k	515407	BioLegend, SanDiego, CA

3.1.7 Cell lines

name	description
HS5	Human fibroblast established from bone marrow stroma of 30-year-old Caucasian male

3.1.8 Primary cells and PBMCs

ID	age	sex	first diagnosis	Binet	IgHV	p53/ del17p	del	other	therapy
1	68	male	Jan 07	A	mut	no	no		ww
2	75	male	Nov 07	B	unmut	no	13q, 11q		1x FC, 5x FCR (CR) -> Ritux mono -> Ibrutinib
3	74	male	Aug 11	B	unmut	no	13q, 11q		ww
4	53	male	Aug 13	C	unmut	no	no	SF3B1, DDX3X, POT1	6x BR
5	50	fem	Aug 16	A	mut	no	no		ww
6	63	male	Jan 02	A	unk	no	no	com. aberr.KT	ww
7	65	fem	Jan 14	C	unmut	no	13q, 11q		Acalabrutinib
8	61	fem	Dec 11	A	unk	no	no	ZAP70+	ww
9	76	male	May 07	A	mut	no	13q		ww
10	46	fem	Aug 11	A	mut	no	13q		ww

11	67	male	Jan 09	A	unmut	no	13q, 11q		ww
12	56	male	Sep 11	B	mut	mut	13q		ww, i.n. allo. SCT
13	54	male	Apr 16	C	unmut	no	no		ww
14	86	fem	Oct 16	C	unmut	no	13q, 11q		Ibrutinib → Venetoclax mono
15	53	fem	Aug 12	C	mut	no	13q	t(8:13)	ww
16	67	male	Jan 01	C	mut	no	13q	com. aberr.KT	Bend/Rit (13/14)
17	57	male	Sep 16	C	unk	mut	13q		ww
18	80	male	Sep 11	A	mut	mut	no	com. aberr.KT	Bend/Rit (2013), Rit/Chlor (2016), Ibrutinib (2017)
19	70	male	May 05	A	unmut	mut	6q21 , 13q		5x FCR (termination: drug exanthema)
20	63	male	Feb 16	A	mut	del	13q		ww
21	66	fem	Jul 04	B	mut	no	13q		Bend/Rit (2011)
22	56	male	Jan 13	A	unk	unk		ZAP70+, CD38+	ww
23	81	male	Jan 13	unk	unk	unk			unk
24	56	fem	Feb 14	A	mut	no	13q	ZAP70+	ww
25	63	fem	Feb 16	C	unk	no	13q	Trisomie 21	ww
26	65	fem	2008	A	unk	unk			unk
27	71	male	Jan 02	C	unmut	del	13q, 11q	com. aberr.KT	Venetoclax (ABT-199 Studie)
28	71	male	Apr 09	C	unmut	del	13q		Bendamustin, Obinutuzumab + Idelalisib
29	53	male	Nov 14	C	unmut	no	13q		2x Benda 4x Obinutuzumab Erhaltung, Stopp auf Pat Wunsch
30	75	male	May 17	A		no	no		ww
31	54	fem	Mar 18	A	unmut	no	13q, 11q, 6q		ww
32	62	male	Jan 02	A	mut	no	no	BIRC3 mutation	ww

name	**description**
PBMC	Healthy donor cells from buffy coats (containing lymphocytes and monocytes)

Primary human CLL samples were collected from the peripheral blood of CLL patients at the University Hospital of Cologne after written and informed consent according to the Declaration of Helsinki and with Institutional Review Board approval at the University of Cologne no. 21-1317 and no. 19-1438_1.

3.1.9 Software

name	manufacturer
Microsoft Office	Microsoft, Redmond, WA, USA
GraphPad Prism	GraphPad Software, San Diego, CA, USA
FlowJo	FlowJo Software, Ashland, OR, USA

3.2 Methods

3.2.1 Cell culture with primary CLL cells

Viably frozen primary patient derived CLL cells were thawed at 37°C in the water bath before each experiment. Afterwards the cells were washed with 10ml PBS by centrifugation at 300 rpm for 5 min. and resuspended in 10ml supplemented Gibco™ RPMI 1640 medium. The cell number was determined using the Vi-Cell Cell counter system (Beckmann Coulter) or the Macs Quant X flow cytometer (Miltenyi). Finally, the cells were diluted to the required concentration (1x 10^6 cells/ml) in cell culture medium and seeded onto cell culture plates.

3.2.2 Cell culture with HS5 cell line

Human HS5 cells were cultured in Gibco™ RPMI 1640 medium supplemented with 10% Gibco™ fetal calf serum and 1% Gibco™ penicillin-streptomycin at 37°C and 5% CO2 in nontreated tissue culture flasks. Every 48 to 72 hours, cells were split 1:10 or 1:100 with fresh cell culture medium. The adherent cells were detached for splitting by incubating with 4ml 0.5% Trypsin/EDTA for 2-3 minutes. Trypsin was inactivated by adding 4ml Gibco™ RPMI 1640 medium, the cells were washed by centrifugation at 300 rpm for 5min at room temperature (RT), resuspended in fresh medium and split accordingly. Absolute cell numbers were determined using the Casy cell counter or Vi-Cell according to the manufacturer's instructions.

3.2.3 Induction of PD-L1 surface expression

To increase the surface expression of PDL1 on primary CLL cells as well as cell lines (JVM3), the cells were stimulated with phorbol-12-myristate-13-acetate (PMA) or interferon gamma (INFγ). Depending on the experimental setup, different concentrations of PMA (1ng/ml) and INFγ (2.5, 10, 25, 50ng/ml) were added directly to the wells and incubated for 24-72 hours at 37°C and 5% CO2. As vehicle control, 1µl 1:100 solution of dimethyl sulfoxide (DMSO) with Gibco™ RPMI 1640 medium was used for all experiments.

As an alternative induction of PD-L1 surface expression, primary CLL cells were cocultured with HS5 stromal cells. For this, 0.5 x 10^5 HS5 cells were seeded in 0.5 ml of the supplemented Gibco™ RPMI 1640 medium (1 x 10^5/ml) onto a 24-well cell culture plate and incubated overnight at 37°C and 5% CO2 to allow them to attach to the bottom. The next day, 5 x 10^5 CLL cells in 0.5 ml culture medium (1 x 10^6/ml) were then added to the HS5 feeder layer. After 48-72 hours of incubation at 37°C and 5% CO2 the surface levels of PD-L1 were examined with flow cytometry and the cells were used for further experiments.

To separate the CLL cells from the HS5 cells for sample collection or subsequent analysis, the plate was gently agitated and the suspension CLL cells were gently mixed by pipetting. Afterward 100 µl samples were collected or (depending on the experimental setup) the CLL cells were transferred completely to another cell culture plate. During this, the stromal cells remained attached to the bottom of the plate.

3.2.4 Small molecule inhibitor treatment

After the induction of PD-L1 surface expression, the CLL cells were treated with various small molecule inhibitors targeting the B cell receptor signaling pathway. Ibrutinib (1µM), idelalisib (1µM) or dasatinib (100nM) were added to the coculture and incubated for 24 hours at 37°C and 5% CO2.

3.2.4.1 Pretreatment of HS5 cells with Dasatinib

HS5 cells were plated in medium as described above (in 2.2.2), pretreated with 100nM Dasatinib and incubated overnight. The medium was then carefully pipetted off, the HS5 cells adhering to the bottom were washed twice with fresh medium in the plate. Finally, fresh medium with CLL cells was added.

3.2.5 Surface molecule staining for flow cytometry

For surface marker staining, 100 µl samples of approximately 1x 10^5 CLL cells were transferred to 5ml flow cytometry tubes (Sarstedt). Afterwards the cells were incubated for 15 min at RT with 5µl FcR Blocking Reagent human (Miltenyi) diluted in 10µl/sample of 1x Macs Quant Running Buffer (Miltenyi) to prevent unspecific antibody binding. For Annexin V stainings, the cells were incubated for 15 min at RT with 5µl FcR Blocking Reagent human (Miltenyi) diluted in 200µl/sample of 1x Annexin V Binding Buffer (BD Pharmingen). Subsequently, surface marker were stained by adding 0.5µl of the respective antibody/sample and incubating for 20-30 minutes at RT in the dark. For each staining, necessary isotype controls conjugated to the same fluorochrome were included in a separate sample tube. Before measuring the stained cells with the Macs Quant X flow cytometer, samples were washed with 2ml 1x Macs Quant Running Buffer by centrifugation at 300rpm for 5min. The supernatant was discarded and the cells were resuspended in the residual fluid. In addition, 100µl of 1x Macs Quant Running Buffer were added to increase the final volume of the sample to approximately 200-300µl.

3.2.6 T cell activation in healthy donor PBMCs

PBMCs from various healthy blood donors were thawed before each experiment, washed in PBS and resuspended in normal cell culture medium (supplemented Gibco™ RPMI 1640 medium). Afterwards, the cells were counted using the Vi-Cell cell counter, diluted to 2x 10^6 cells/ml and transferred in 0.5 ml to a 48-well cell culture plate. Then the cells were allowed to rest in the incubator (37°C, 5% CO2) for at least 1 hour before 5µg/ml of soluble anti-CD3 antibody (clone UCHT1 or HIT3) was added. In another attempt to stimulate the T cells, the CD3 antibody was immobilized on the bottom of the 96-well culture plate by pre-incubation of 50µl of 10µg/ml antibody

solution diluted in sterile PBS at 4°C overnight. The next day antibody solution was decanted from the plate, washed 3 times with sterile PBS and then 200µl CLL cell suspension diluted to 1 x 10^6/ml in Gibco™ RPMI 1640 medium was aliquoted. As an alternative stimulus, 25ng/ml PMA was added. Subsequently, the cells were incubated at 37°C and 5% CO2 for 72 hours.
To validate successful T cell activation after 72 hours, various activation markers such as CD69, PD-1 and Granzyme B were monitored by flow cytometry as described in 2.2.5 and 2.2.6.1.

3.2.6.1 Intracellular protein staining for flow cytometry

To be able to stain intracellular proteins for flow cytometry the IntraPrep Permeabilization Reagent Kit (Beckman Coulter) was used according to the manufacturer's instruction. Before intracellular staining, surface markers were stained as described in 2.2.5.
To determine elevated levels of Granzyme B indicating T cell activation, the cells were incubated with 5µM Brefeldin A, a protein transport inhibitor that prevents the release of intracellular proteins, 4 hours before performing the staining protocol.

3.2.7 Co-culture of primary human CLL and T cells

First, PDL1 surface expression on primary CLL cells was induced by coculture with HS5 cells for 72 hour as described in 2.2.3. Simultaneously, PBMC's of healthy blood donors were incubated with CD3 antibody for 72 hours at 37°C and 5% CO2, to activate the T cells (c.f. 2.2.6). Afterwards, enhanced PDL1 expression on the CLL cells, as well as the activation status of T cells was validated by flow cytometry.
After successful validation, the CLL cells were labeled with CFSE to be distinguishable from the PMBCs in the co-culture. CFSE is a fluorescent cell staining dye for in vitro or in vivo labeling of cells. It covalently attaches to surface amino acids or to the interior of the cell and holds its signal for several days. It is considered non-toxic to cells, easy to use and is detectable by a flow cytometer. For the labeling, 1x 10^6 CLL cells/ml were diluted in 10ml PBS and incubated with 1µM CFSE for 30min at 37°C and 5% CO2. Then cells were washed 3x with 10ml PBS by centrifugation at 300rpm for 5 min.

Afterwards, the cells were resuspended in normal culture medium and the absolute cell number was determined.
Finally, PBMCs and CLL cells were combined in a 48-well plate. Here, a total number of 5 x 10^5 CLL cells and 1 x 10^6 PBMC's were combined in a total volume of 1 ml normal culture medium. With approximately 50% T cells in the PBMC's, this resulted in a ratio of 1:1 CLL-/T cells.
For each round of experiment, 4 different co-culture conditions were set up combining stimulated CLL cells or unstimulated CLL cells with activated or non-activated PBMC's. After 1, 48- and 72 hours of co-culture the CLL cell viability was determined by flow cytometry as described in 2.2.7.1.

3.2.7.1 Apoptosis quantification by flow cytometry

The survival rate of CLL cells in the different co-culture conditions was determined by Annexin V staining. The extracellular protein Annexin V can bind calcium-dependently with high affinity to negatively charged phospholipid surfaces (phosphatidylserine (PS)), marking the cell as apoptotic (Boersma et al., 2005). When conjugated to a fluorochrome, Annexin V can be detected as a surface marker in flow cytometry. Annexin V negative cells are thus defined as living cells.
For the Annexin V staining, 100µl samples from the co-culture were incubated with 200µl Annexin V Binding Buffer and 5µl FcR block for 15min in the dark at RT. Afterwards, 0.5µl/sample of Annexin V as well as 0.5µl/sample of the surface antibodies CD5 and CD19 were added to the cells and incubated for another 20min at RT in the dark. Finally, the samples were washed once with 2ml 1x MACs Quant Running Buffer by centrifugation at 300rpm for 5min. to remove the unbound antibodies and measured with the Macs Quant X flow cytometer.
The ratio of living cells, which were classified as Annexin V negative cells out of the CFSE positive population, to all measured cells in the sample represented the survival rate.

3.2.8 XTT Cell Proliferation Assay

To determine the viability of HS5 cells after treatment with dasatinib, the XTT assay was performed using the associated kit (Thermo Fisher) according to the

manufacturer's instruction. Here, 50µl cell proliferation suspension and 1µl activator from the kit was added per well of the 96-well plate with 100µl medium containing HS5 cells, incubated for 2 hours at 37°C and 5% CO2 and then measured with the FLUOstar Optima. The viability was defined by the absorbance (measured at 450 and 620 nM) of the water-soluble XTT reagent, which is reduced and converted to an orange-colored formazan product by actively respiring viable cells. Data was normalized according to kit instructions ($A450_{sample} - A450_{control} - A620_{sample}$).

3.2.9 Data illustration

Flow cytometry data were analyzed by using the FlowJo analysis software (BD). All raw data were transferred into Excel spreadsheets and normalized. All graphs were made by using the Prism 8 software (GraphPad).
The error bars in the graphs represent the standard errors of the mean (SEM). For all statistically significant differences, t-test was performed and the P-value was indicated.

3.2.10 Statistics

For statistical analysis, Student's t test or Mann-Whitney test was used. All statistical differences were calculated using Graphpad Prism 8 software.
Statistical significance was assumed at P values $\leqslant 0.05$ (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

4. Results

4.1 PD-L1 surface expression on peripheral blood CLL cells was increased after PMA or INFγ stimulation

PD-L1 or "programmed death-ligand 1" is a surface protein, which modulates immune response. It was found to be higher expressed on CLL cells in the lymph nodes rather than on healthy B cells (Ramsay et al., 2012). However, CLL cells from peripheral blood of most patients do not express PD-L1 on their surface, suggesting that PD-L1 expression might be induced by the tumor microenvironmental factors in the lymphoid organs whereas circulating CLL cells did not receive sufficient stimuli to express PD-L1 at a high levels.

For all experiments, an isotype was included, (consisting of a mixture of all sample conditions in this experiment). An isotype served as control for determination of non-specific antibody binding to prove a basal expression of the molecule of interest in the untreated, unstimulated cell. Our data proved that there is no PD-L1 expression on unstimulated CLL cells (**Figure 8a**). In order to analyze the regulation of PD-L1 surface expression on primary patient CLL cells, we investigated the potential to increase the PD-L1 surface expression on these cells. For this purpose, cells from different CLL patients were thawed, cultured in Gibco^{TM} RPMI 1640 medium (1x 10^6/ml) and PD-L1 surface level was measured via flow cytometry after stimulation with either 1ng/ml PMA or 10ng/ml INFγ for 48 hours. PMA is known to activate B cells resulting in increased cell proliferation, RNA synthesis and IgM secretion and this can be further enhanced through additional INFγ. INFγ is a physiological stimulus of PD-L1 as proven in previous studies (Tangye et al., 1995), (Tangye et al., 1997), (Galbraith et al., 2020). The Gating strategy of how the data was analyzed is shown below (**Figure 8b**). All data shown in this thesis is gated on only viable cells (AnnexinV negative cells) and provably B cells (CD19 positive/ CD5 positive cells).

Our studies proved as well that PD-L1 surface level was increased significantly after stimulation with PMA or INFγ compared to untreated cells or DMSO control. PMA shows a higher effect than INFγ, but also a higher standard deviation (**Figure 8c**).

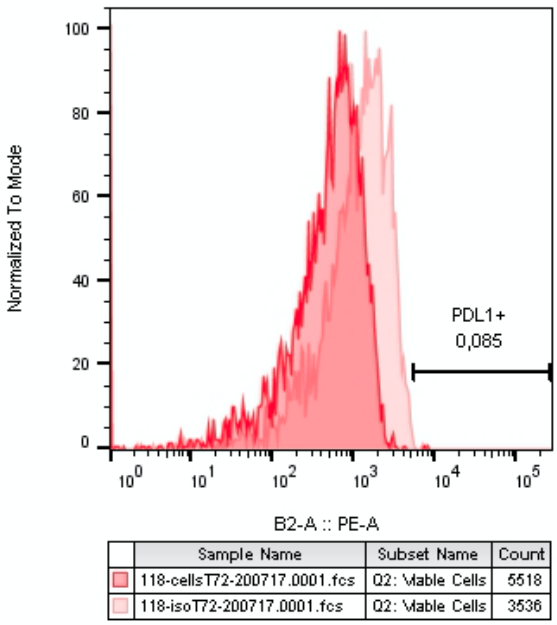


Figure 8a. PDL1 surface expression on primary CLL cells, isotype vs. Mono. *Primary CLL cells of one patient were cultured in Gibco™ RPMI 1640 medium supplemented with 10% Gibco™ fetal calf serum and 1% Gibco™ penicillin-streptomycin for 72 hours (T72). PDL1 surface expression is quantified via Flow cytometry. Single cell data is analyzed with FlowJo software. The isotype peak shows a right shift compared to the Mono-cell peak (118-cells = cells mono, 118-iso = isotype control).*

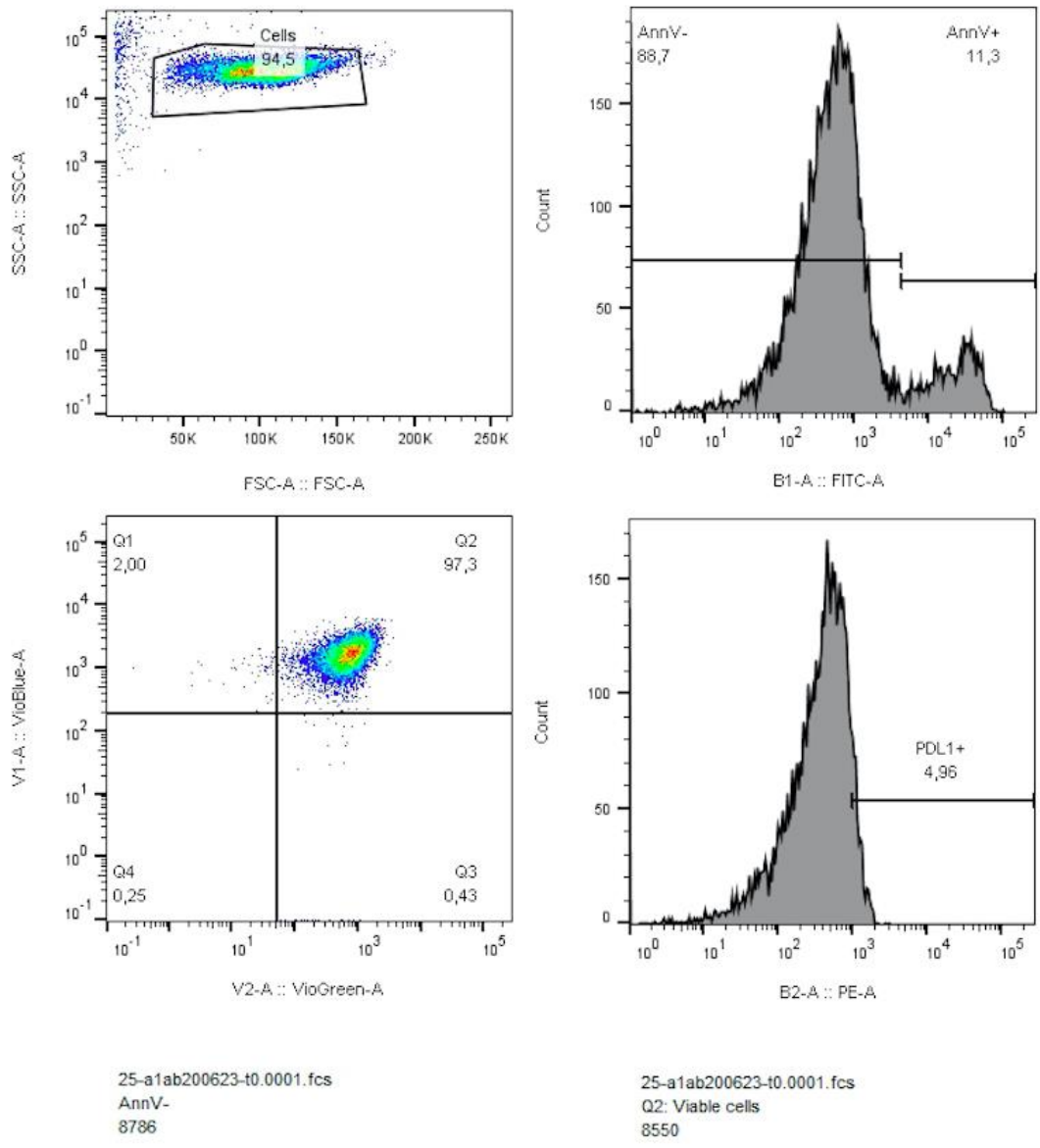


Figure 8b. Gating strategy. *Values depicted in all graphs in this thesis are gated on Cells (FSC-A/SSC-A), AnnexinV negative cells (FITC-A), CD19 positive (VioGreen-A)/ CD5 positive (VioBlue-A), PD-L1 positive cells (PE-A). In this example we gated on PD-L1 positive cells. The data was further isotype corrected and normalized to DMSO control.*

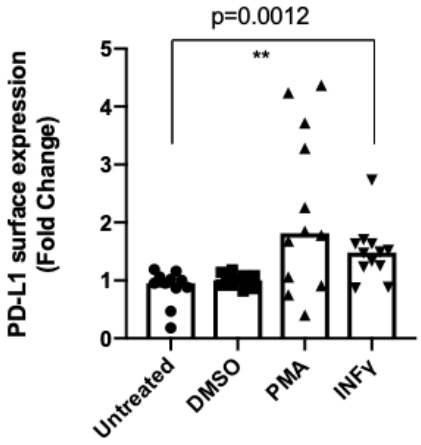


Figure 8c. PDL1 surface expression on primary CLL cells after stimulation with PMA or INFγ. *Primary CLL cells from 6 different patients (biological replicates) were stimulated for 48 hours with either 1ng/ml PMA or 10ng/ml INFγ compared to DMSO control (1µM). PDL1 surface expression was quantified via Flow cytometry. Values depicted in the graph represent data from 2 independent experiments, gated on AnnexinV negative and CD5/CD19 positive cell population. 2 technical replicates of each patient are shown in the graph. Data was isotype corrected and normalized to DMSO control. Both stimuli, PMA and INFγ could induce an increase of PD-L1 surface expression, Median values: Untreated = 0.95, DMSO = 1, PMA = 1.82, INFγ = 1.48.*

4.2 BCR inhibitors downregulate PD-L1 surface expression on primary CLL cells

Based on previous data from the lab, showing that PD-L1 surface level was decreased by several BCR inhibitors in human cancer cell lines (JVM3 and OSU, data not shown) and already published data that BCR inhibition by ibrutinib downregulated PD-L1 on CLL cells (Kondo et al., 2018), we validated these findings on primary CLL cells with ibrutinib and also dasatinib. Thus, we checked the influence of different BCR inhibitors on PD-L1 surface expression.

CLL cells of one patient were stimulated with PMA or INFγ and then treated with 1µM ibrutinib or 100nM dasatinib for 24 hours. After successful stimulation with PMA or INFγ, both inhibitors decreased PD-L1 surface expression to a lower level than in the unstimulated sample. The combination of INFγ stimulation and dasatinib treatment had the most effective impact (**Figure 9**).

a) b)

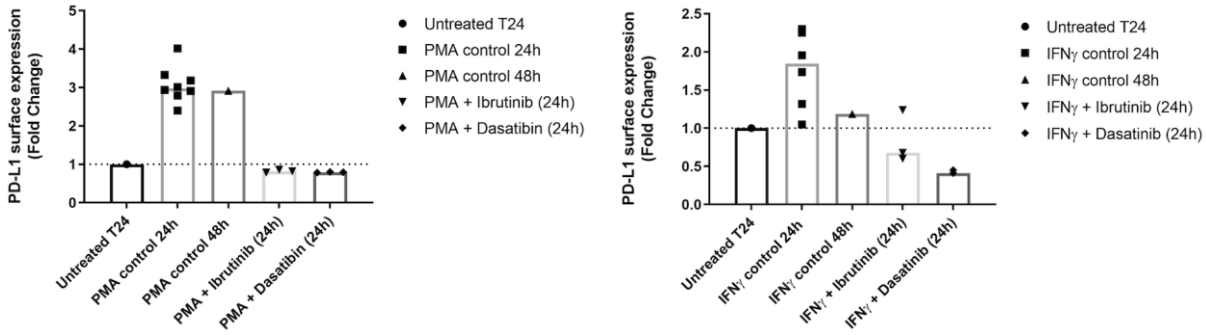


Figure 9. PDL1 surface expression on primary CLL cells of one patient after PMA or INFγ stimulation and BCR inhibitor treatment. *Primary CLL cells from one patient were stimulated with 1ng/ml PMA or 10ng/ml INFγ for 24 hours (48 hours) and then treated with 1µM ibrutinib or 100nM dasatinib for another 24 hours. PDL1 surface expression is quantified via Flow cytometry. Calculation of the Median (PE-A/ PDL1 channel) is gated on AnnexinV negative and CD5/CD19 positive cell population. Data is isotype corrected and normalized to Unstimulated control. (a) PMA increases PD-L1 surface expression after 24 and still after 48 hours, a clear reduction after treatment is shown, no relevant difference between ibrutinib and dasatinib, Median values: Unstimulated = 1, PMA24h = 2.97, PMA+ibrutinib = 0.82, PMA+dasatinib = 0.79. (b) After INFγ stimulation and inhibitor treatment PDL1 surface expression decreases, more efficient with dasatinib, Median values: Unstimulated = 1, IFNγ24h = 1.85, IFNγ+ibrutinib = 0.68, IFNγ+dasatinib = 0.41.*

These findings were validated with more patient samples. The pooled data of 6 or 10 different patient samples from 3 independent experiments showed neither an increase of PD-L1 surface expression after stimulation with PMA or INFγ nor an effect of inhibitor treatment in combination with PMA. Only after INFγ stimulation there was a significant downregulation of PD-L1 compared to DMSO control, especially by dasatinib (-0,74 fold) (**Figure 10a+b**).

a) b)

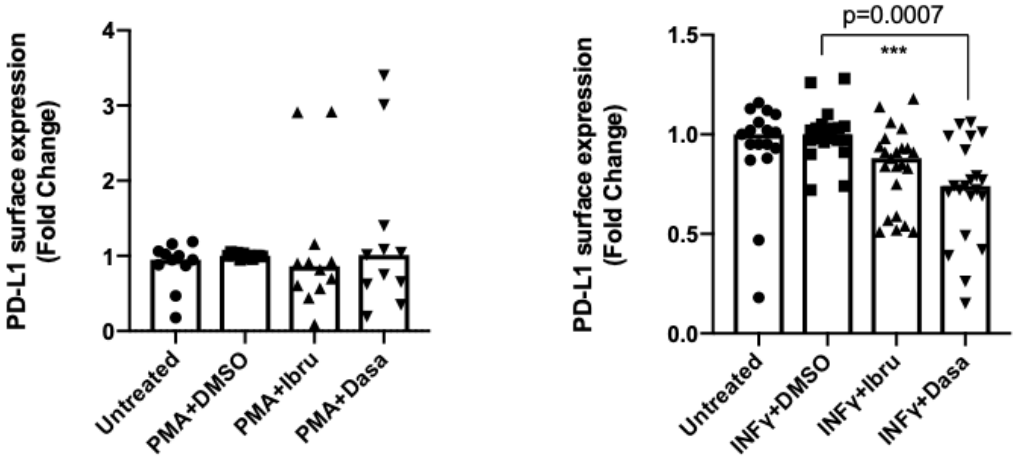


Figure 10a+b. PDL1 surface expression on primary CLL cells of several patients after PMA or INFγ stimulation and BCR inhibitor treatment. *Primary CLL cells of 6 (PMA) or 10 (INFγ) different patients were stimulated for 48 hours with 1ng/ml PMA or 10ng/ml INFγ and treated for 24 hours with 1µM ibrutinib or 100nM dasatinib. PDL1 surface expression is quantified via Flow cytometry. Calculation of the Mean (PE-A/ PDL1 channel) is gated on AnnexinV negative and CD5/CD19 positive cell population, duplicates of each patient are shown. Data is isotype corrected and normalized to DMSO control. (a) Neither PMA stimulation nor the BCR inhibitor treatment shows a relevant effect on PDL1 surface expression, Median values: Untreated = 0.95, PMA+DMSO = 1, PMA+ibru = 0.86, PMA+dasa = 1.01. (b) BCR inhibitor treatment reduces the PDL1 surface expression, dasatinib more than ibrutinib, Median values: Untreated = 1, INFγ+DMSO = 1, IFNγ+ibru = 0.88, IFNγ+dasa = 0.74. (ibru = ibrutinib, dasa = dasatinib).*

Previous studies had shown that dasatinib inhibited CLL cell survival in vitro, especially CLL with unmutated IgV_H genes (Veldurthy et al., 2008), (McCaig et al., 2011), (Giannopoulos et al., 2021). Concerns regarding the toxicity of dasatinib were discovered, for example in studies after long-term treatment with dasatinib in CML patients inducing hypoxic pulmonary vasoconstriction and pulmonary endothelial damage leading to increased susceptibility to pulmonary arterial hypertension (Guignabert et al., 2016). To clarify the effect of dasatinib in our experiments and exclude distortion of the results deriving from dasatinib-toxicity, the viability of CLL cells under inhibitor treatment was tested (**Figure 11**). The cells were incubated with Annexin V reagent and viability was quantified by Annexin V negative cell population via Flow Cytometry. There was no relevant reduction of CLL cell survival after INFγ stimulation in combination with dasatinib treatment, indicating that the used dasatinib dose did not induce significant CLL cell death after 24 hours. This was important to make sure that PD-L1 decrease was not because of dying cells after dasatinib treatment.

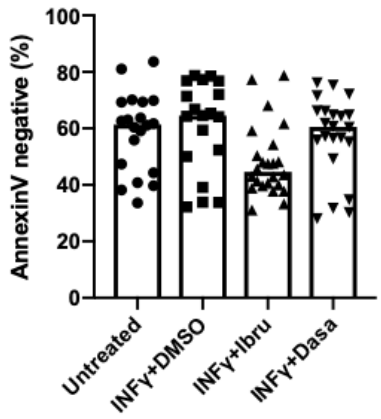


Figure 11. Viability of primary CLL cells after INFg stimulation and BCR inhibitor treatment*. Primary CLL cells of 10 different patients (2 technical repliactes of each patient) were stimulated for 48 hours with 10ng/ml INFγ and treated for 24 hours with 1µM ibrutinib or 100nM dasatinib. AnnexinV negative cells are quantified via Flow cytometry, gated on CLL cell population. Dasatinib treatment shows no relevant reduction in CLL cell survival, Median values: Untreated = 61.4, INFγ+DMSO = 64.5,IFNγ+ibru = 44.6, IFNγ+dasa = 60.55.*

4.2.1 10ng/ml INFγ is the most efficient concentration stimulating PD-L1

INFγ is well described to be a physiological stimulator of PD-L1 (Chen et al., 2018) and according to our data it could efficiently increase the PD-L1 surface expression on primary CLL cells. Therefore we first titrated for the best concentration of INFγ for PD-L1 expression induction.

CLL cells from one patient were stimulated with 10, 25 or 50ng/ml INFγ for 24 hours and then with a second dose of the same concentration for another 24 hours. After 24 hours the PD-L1 surface expression with 10 and 50ng/ml INFγ stimulation is upregulated similarly, 25ng/ml has no effect in this patient. After 24 more hours with a second dose, PD-L1 was increased to slightly higher levels with all 3 concentrations, no relevant difference between the different conditions could be observed. The 50ng/ml concentration-samples showed the highest increase (1.27 fold after 24 hours, 1.77 after 48 hours), but not significant and since one dose of 50ng/ml was even more efficient than two doses, this could also be a random deviation (**Figure 12a+b**). This data allowed us to further use 10ng/ml INFγ as the standard dose to induce PD-L1 expression.

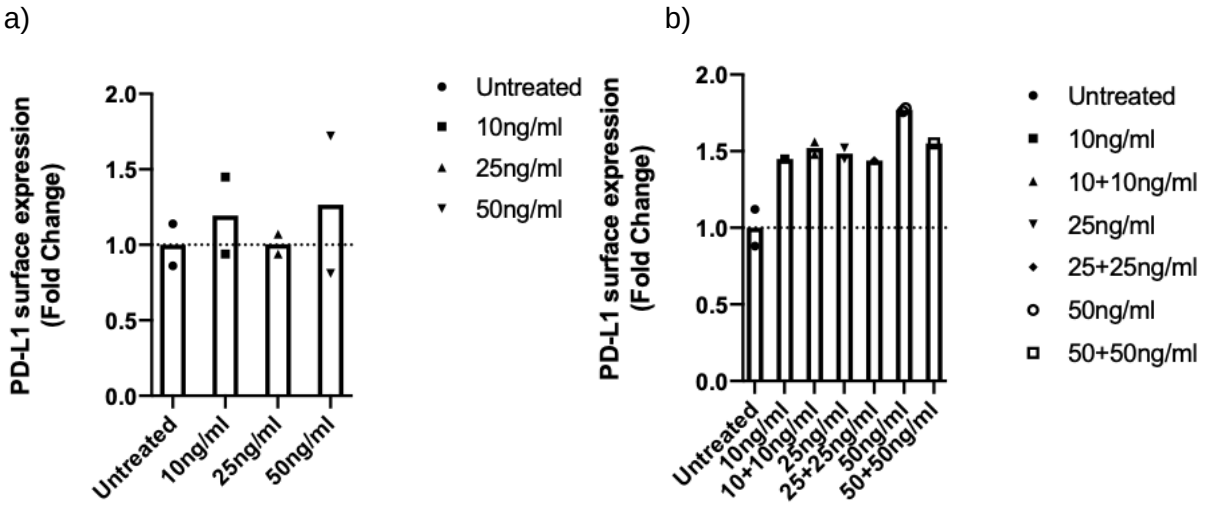


Figure 12a+b. PDL1 surface expression after different concentrations of INFγ stimulation. *Primary CLL cells from one patient were treated with 10, 25 or 50ng/ml INFγ for 24 hours, then a second dose of the same concentration was added to each condition and incubated for another 24 hours. PDL1 surface expression is quantified via Flow cytometry. Calculation of the Mean (PE-A/ PDL1 channel) is gated on AnnexinV negative and CD5/CD19 positive cell population, duplicates of each condition are shown. Data is isotype corrected and normalized to untreated control. (a) 10 and 50ng/ml INFγ stimulation for 24 hours show a similar effect on PDL1 surface upregulation, 25ng/ml has almost no effect in this patient, Median values: Untreated = 1, 10ng/ml = 1.2, 25ng/ml = 1.01, 50ng/ml = 1.27. (b) After 48 hours of stimulation and with the second dose all conditions show a similar increase in PDL1 surface expression, Median values: Untreated = 1, 10ng/ml = 1.45, 10 ng/ml +10ng/ml = 1.52, 25ng/ml = 1.49, 25 ng/ml +25ng/ml = 1.44, 50ng/ml = 1.77, 50 ng/ml +50ng/ml = 1.55.*

4.3 HS5 cells support CLL survival and upregulate PD-L1 surface expression

CLL cells are known to undergo rapid apoptosis ex vivo and in vitro without support of a feeder layer. It was found that a Coculture system with HS5 stromal cells maintains CLL cell survival for long time in vitro (Seiffert et al., 2007). In the attempt to ensure better conditions for experiments with CLL cells over several days in culture and to investigate the influence of stromal cell support on PD-L1 surface levels in primary CLL cells (Böttcher et al., 2020), we used a coculture consisting of primary patient-derived CLL cells and the human bone marrow-derived stromal cell line HS5.

The Coculture was prepared as described above. The Coculture was incubated for 48 or 72 hours (**Figure 13a+b**). PD-L1 surface levels on CLL cells were measured via Flow Cytometry before Coculture and after 72 hours of Coculture with HS5 cells. An increase of PD-L1 surface expression on CLL cells in presence of HS5 stromal cells is

shown in the FlowJo-Overlay by a clear right-shift of the CoCulture-sample-peak compared to the CLL Mono-peak (**Figure 14**).

a) b)

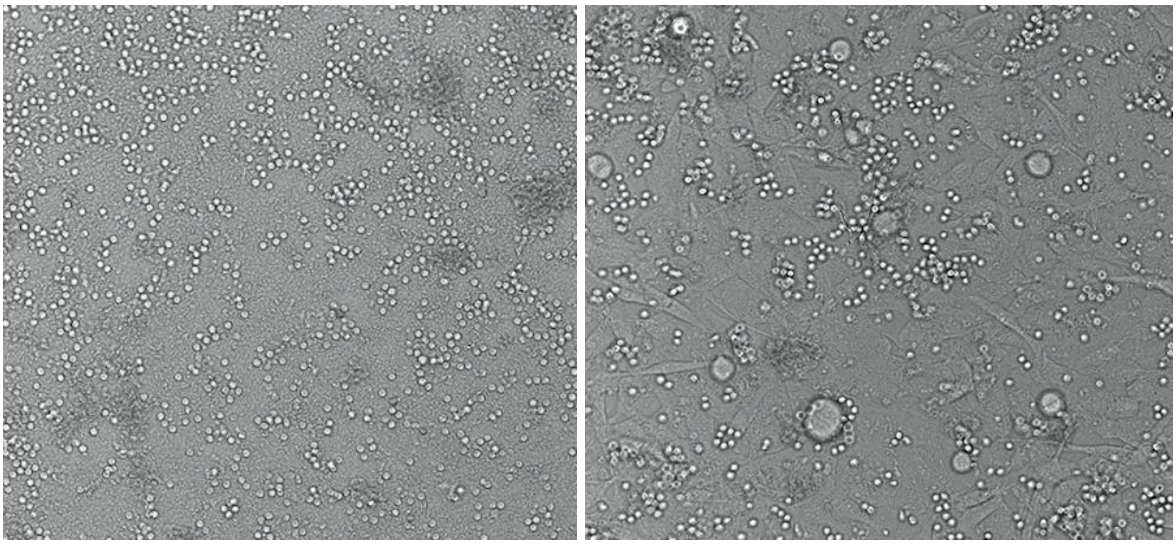
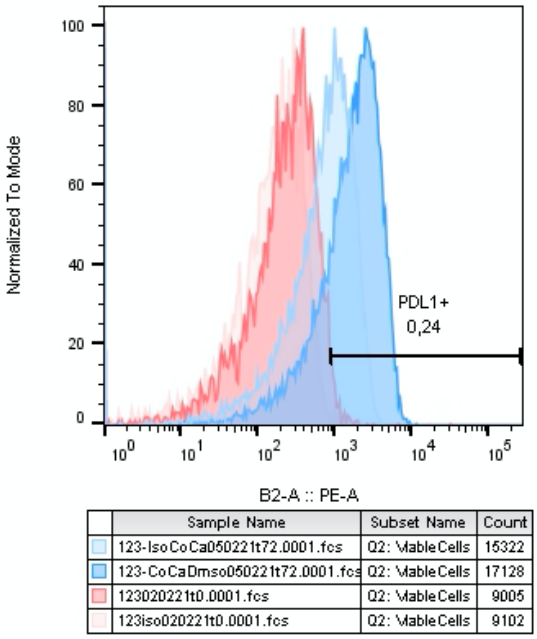


Figure 13a+b. Photo of primary CLL cells Mono and in Coculture with HS5 cells. *(a) Primary CLL cells (1*10^6/ml) of one patient were incubated in 1ml supplemented Gibco^{TM} RPMI 1640 medium on a 48-well plate for 48 hours. (b) Primary CLL cells (1*10^6/ml) of the same patient were cocultured with HS5 cells (1*10^5/ml) in 1ml supplemented Gibco^{TM} RPMI 1640 medium on a 48-well plate for 48 hours.*



	Sample Name	Subset Name	Count
	123-IsoCoCa050221t72.0001.fcs	Q2: \MableCells	15322
	123-CoCaDmso050221t72.0001.fcs	Q2: \MableCells	17128
	123020221t0.0001.fcs	Q2: \MableCells	9005
	123iso020221t0.0001.fcs	Q2: \MableCells	9102

Figure 14. PDL1 surface expression on primary CLL cells Mono and after 72 hours HS5 Coculture. *Primary CLL cells of one patient were cultured in supplemented Gibco™ RPMI 1640 medium and measured for PDL1 surface expression after thawing (t0) and 72 hours after Coculture with HS5 stroma cells (t72). Flow cytometry-Single cell raw data is analysed with FlowJo software, peak shifts to the right are shown between the sample of interest and its isotype as well as between CLL cells mono and Coculture of CLL and HS5 cells (123 = untreated Cells mono, 123iso = untreated cells isotype control, 123CoCa = Coculture of CLL and HS5 cells, 123IsoCoCa = Coculture isotype control).*

4.3.1 Dasatinib significantly reduced PD-L1 surface expression on primary CLL cells

In the next step, cells were treated with 1µM ibrutinib, 1µM idelalisib or 100nM dasatinib for 24 hours and again examined for PD-L1 surface expression after treatment. After Coculture with HS5 stromal cells PD-L1 surface expression was increased by 4-fold. Dasatinib reduced PD-L1 significantly whereas ibrutinib and idelalisib had no relevant effect (**Figure 15**).

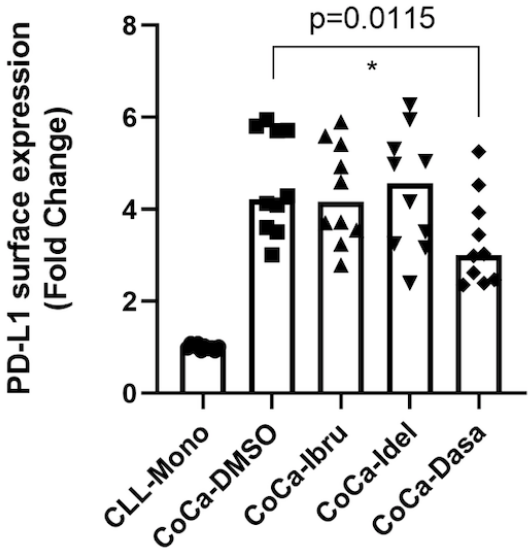


Figure 15. PDL1 surface expression on primary CLL cells after HS5 WT-Coculture and treatment with different BCR inhibitors*. Primary CLL cells of 5 different patients were cocultured with HS5 cells for 48 hours and then treated with different BCR inhibitors for another 24 hours. PDL1 surface expression is quantified via Flow cytometry. Calculation of the Mean (PE-A/ PDL1 channel) is gated on AnnexinV negative and CD5/CD19 positive cell population, duplicates of each condition are shown. Data is normalized to untreated Mono control. After treatment with 1µM ibrutinib, 1µM idelalisib or 100nM dasatinib, only dasatinib shows a significant reduction of PDL1 surface expression compared to DMSO control, Median values: CLL Mono = 1, CoCaDMSO = 4.22, CoCaIbru = 4.16, CoCaIdel = 4.57, CoCaDasa = 3.01. (CoCa ≙ Cuculture)*

These results clearly indicated that dasatinib was very potent to decrease PD-L1 on primary CLL cells compared to other inhibitors of the BCR signaling pathway. This finding was validated with more patient samples. Using more patient samples, the previous result in Figure 7 that dasatinib reduced PD-L1 surface expression on primary CLL cells after HS5 coculture could be confirmed (**Figure 16**).

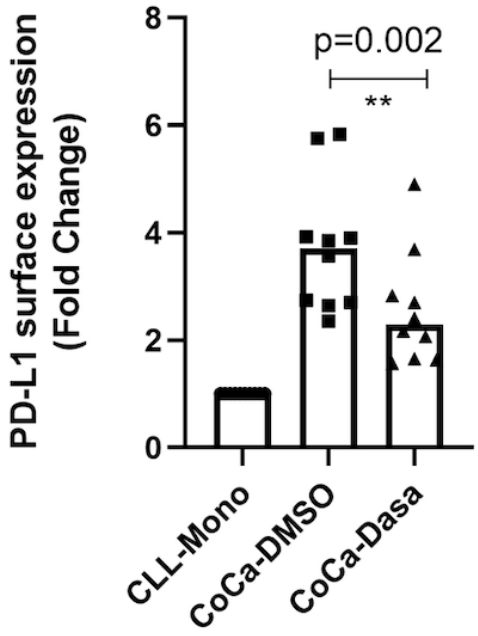


Figure 16. PDL1 surface expression on primary CLL cells after Coculture with HS5 WT cells and dasatinib treatment. *Primary CLL cells of 10 different patients were cocultured with HS5 cells for 48 hours and then treated with 100nM dasatinib for another 24 hours. PDL1 surface expression is quantified via Flow cytometry. Calculation of the Mean (PE-A/ PDL1 channel) is gated on AnnexinV negative and CD5/CD19 positive cell population, the mean of duplicates of each condition is shown. Data is normalized to untreated Mono control. Dasatinib shows a highly significant reduction of PDL1 surface expression after stimulation with HS5 cells, Median values: CLLMono = 1, CoCaDMSO = 3.71, CoCaDasa = 2.29. (CoCa ≙ Cuculture)*

4.3.2 The BCR signaling pathway is not a key player of PD-L1 regulation

In order to investigate whether Dasatinib regulated PD-L1 expression via inhibition of the BCR signaling pathway, we set up an experiment comparing the combination of ibrutinib and idelalisib treatment to dasatinib treatment. Ibrutinib is a covalent inhibitor of BTK (Davids & Brown, 2014) and idelalisib of PI3K (Greenwell et al., 2017), both kinases downstream of the B cell receptor. Combining both drugs, the whole BCR

signaling pathway should be blocked (compare Fig. 3 in Introduction). Instead, dasatinib is a pan-inhibitor, among others of the SRC-family kinases including LYN (Montero et al., 2011). With this experiment we aimed to find out if the BCR signaling pathway plays a role in downregulating PD-L1 and more specifically, if the BCR kinases are a target of dasatinib reducing PD-L1.

After Coculture of CLL cells and HS5 stromal cells to enhance PD-L1 surface expression, cells were treated with 100nM dasatinib, 1µM ibrutinib, 1µM idelalisib or a combination of 1µM ibrutinib + 1µM idelalisib for 24 hours. As shown before, dasatinib reduced PD-L1 level significantly, while neither ibrutinib nor idelalisib nor the combination of ibrutinib and idelalisib showed any relevant impact on PD-L1 expression (**Figure 17a**).

This led us to the conclusion, that inhibition of BCR kinases is not the mechanism behind dasatinib-related PD-L1 downregulation, but the decreased PD-L1 level was rather a consequence of other inhibited targets of dasatinib that needs further investigation.

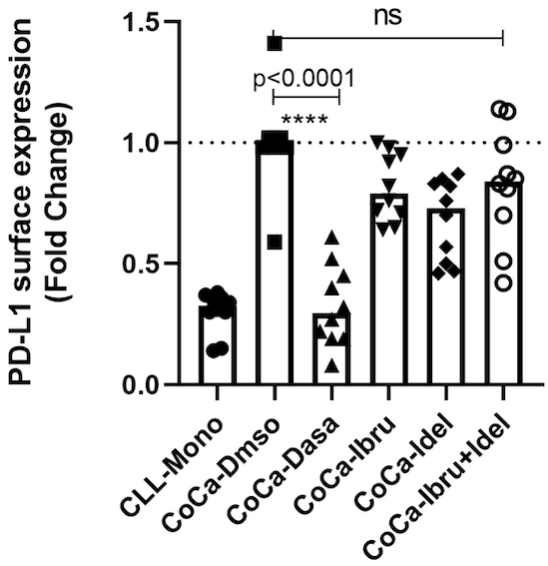


Figure 17a. PDL1 surface expression on primary CLL cells after HS5 Coculture and treatment with different BCR inhibitors/ combinations. *Primary CLL cells of 5 different patients were cocultured with HS5 cells for 48 hours and then treated with 100nM dasatinib, 1µM ibrutinib, idelalisib or the combination of both for another 24 hours. PDL1 surface expression is quantified via Flow cytometry. Calculation of the Mean (PE-A/ PDL1 channel) is gated on AnnexinV negative and CD5/CD19 positive*

cell population, duplicates of each condition are shown. Data is isotype corrected and normalized to Coculture DMSO control. Coculture with HS5 cells increases PDL1 surface expression on CLL cells, dasatinib reduces PDL1 significantly while ibrutinib, idelalisib or the combination of both don´t, Median values: Untreated-Mono = 0.33, CoCaDMSO = 1, CoCaDasa = 0.3, CoCaIbru = 0.79, CoCaIdel = 0.73, CoCaIbru+Idel = 0.84. (CoCa ≙ Cuculture)

To prove that dasatinib -induced cell death was not the major cause for reduced PD-L1 level, the viability of CLL cells during this experiment was measured as described above with Annexin V staining. Again, CLL cell viability was not reduced in the presence of inhibitors including dasatinib (**Figure 17b**). To exclude the concern about dasatinib toxicity further, the shown data of all experiments was gated on the Annexin V negative cell population and therefore all shown results in my thesis proceed only from viable cells.

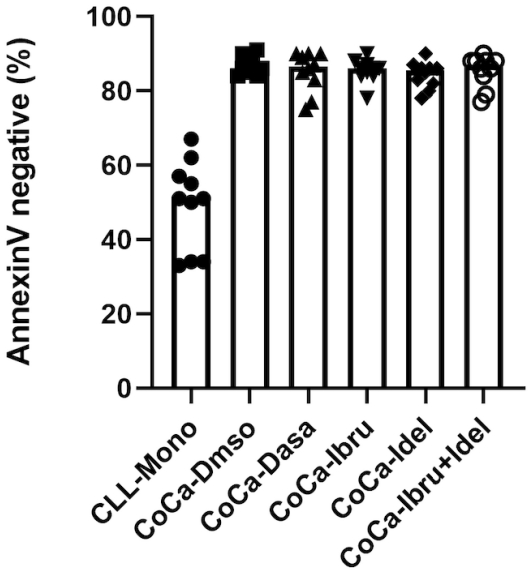


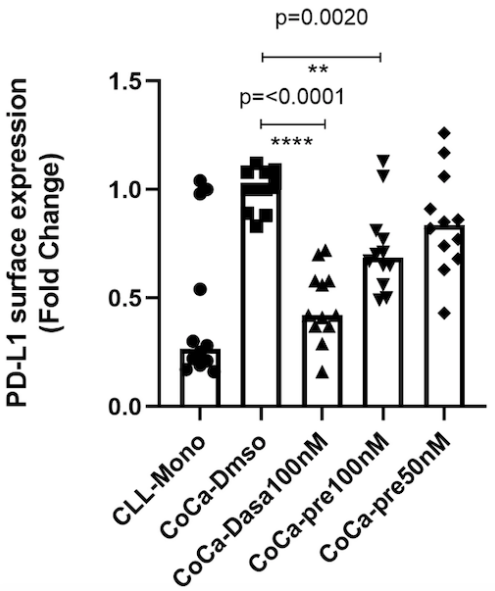
Figure ***17b***. Viability of CLL cells after HS5 Coculture and treatment with different BCR inhibitors/ combinations. Primary CLL cells of 5 different patients were cocultured with HS5 cells for 48 hours and then treated with 100nM dasatinib, 1µM ibrutinib, 1µM idelalisib or the combination of both for another 24 hours. AnnexinV negative cells are quantified via Flow cytometry, gated on CLL cell population, duplicates of each patient are shown. Coculture with HS5 cells supports the CLL cell survival, none of the inhibitors has a relevant effect on the viability, Median values: Untreated-Mono = 51, CoC*a*DMSO = 86, CoC*a*Dasa = 86.5, CoC*a*Ibru = 86, CoC*a*Idel = 85.5, CoC*a*Ibru+Idel = 87*. (CoCa ≙ Cuculture)*

4.4 Pretreatment of HS5 cells with dasatinib reduces PD-L1 levels on CLL cells, but also the stromal cell viability

To completely prevent toxic effects of dasatinib on CLL cells, we set up an experiment comparing CLL-HS5 Coculture with direct dasatinib treatment as usual (described above) to CLL-HS5 Coculture with dasatinib-pretreated HS5 cells. This setting excluded potential side effects on CLL cells by dasatinib toxicity, because CLL cells never had direct contact to dasatinib. HS5 cells were treated with 50 or 100nM dasatinib for 24 hours and then the dasatinib-containing medium was exchanged with fresh medium before CLL cells were added to pretreated HS5 cells, which adhered to the plate bottom. The CLL-HS5 Coculture was incubated for 48 hours and then the measured PD-L1 expression was compared to the CLL-HS5 coculture with direct dasatinib treatment.

Surprisingly, the pretreated conditions show a significant reduction of PD-L1 surface expression on CLL cells, the 100nM-pretreatment more than the 50nM-pretreatment, but not as much as direct treatment with 100nM dasatinib (**Figure 18a**). The viability of CLL cells in this setting was not influenced relevantly, neither with direct dasatinib nor with pretreatment (**Figure 18b**).

a)



b)

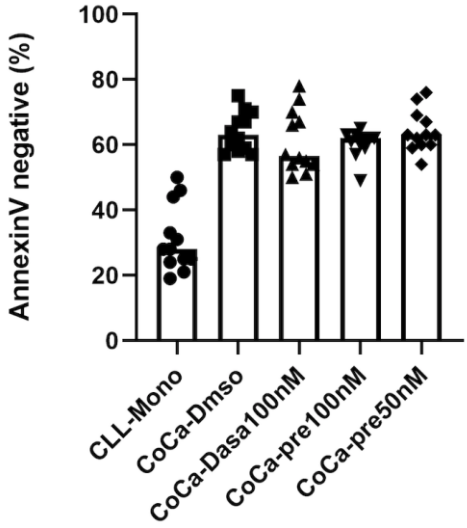


Figure 18a+b. PDL1 surface expression and CLL-viability after Coculture with HS5 cells and direct dasatinib treatment compared to dasatinib-pretreated HS5 cells in Coculture with CLL cells. *Primary CLL cells of 5 different patients were cocultured with HS5 cells for 48 hours and 24 hours direct dasatinib treatment (100nM) or with 24 hours-dasatinib-pretreated HS5 cells (50 or 100nM). PDL1 surface expression was quantified via Flow cytometry. Calculation of the Mean (PE-A/ PDL1 channel) is gated on AnnexinV negative and CD5/CD19 positive cell population, values depicted in the graph represent data from 3 independent experiments, duplicates or triplicates of each condition are shown. Data is isotype corrected and normalized to Coculture DMSO control. (a) Pretreatment of HS5 cells with dasatinib shows lower PDL1 surface levels compared to untreated DMSO Coculture control, but direct dasatinib treatment still has the highest effect, Median values: CLL Mono = 0.27, CoCaDMSO = 1, CoCaDasa100nM = 0.42, CoCapre100nM = 0.69, CoCapre50nM = 0.84. (b) Pretreatment or direct treatment with dasatinib shows no relevant reduction in CLL cell viability, Median values: CLL Mono = 28, CoCaDMSO = 63, CoCaDasa100nM = 56.5, CoCapre100nM = 62, CoCapre50nM = 63. (CoCa ≅ Cuculture)*

Considering the fact that PD-L1 was reduced in Coculture with HS5 cells also after dasatinib pretreatment, we checked the viability of HS5 cells during this experiment to exclude that the PD-L1 reduction was only because of dying HS5 cells and therefore missing feeder cell support. The viability of HS5 cells is measured via XTT assay and quantified by the absorbance of the reduced XTT reagent, which was added to HS5 cells at the end of the experiment. The HS-5 viability was not significantly reduced in the conditions with 50nM dasatinib pretreatment, but in presence of 100nM dasatinib while evaluating the viability in the FluoStar-reader, HS5-viability is decreased significantly (**Figure 18c**). This observation needs further investigation, because it gives the impression that prolonged treatment with dasatinib reduces HS5 viability and the higher amount of dead HS5 cells might be a reason for PD-L1 reduction on CLL cells.

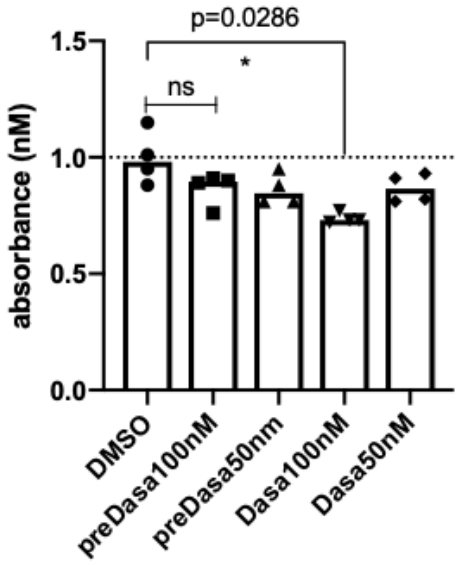


Figure 18c. Viability of HS5 cells after direct treatment or pretreatment with dasatinib. *A second 96-well plate with only HS5 cells was prepared and treated with exactly same conditions like in 10a,(b) just without CLL cells and also 50nM of direct dasatinib treatment. XTT assay was used to measure viability of HS5 cells with the FluoStar Optima-Reader, quantified by the absorbance of an orange colored formazan product in viable cells. Data is normalized according to kit instructions, quadruplicates of each condition are shown. Viability of HS5 cells in presence of (100nM) dasatinib is significantly reduced, but not in pretreated samples, Median values: DMSO = 0.98, preDasa100nM = 0.9, preDasa50nM = 0.85, Dasa100nM = 0.73, Dasa50nM = 0.87.*

4.5 Dasatinib directly reduces PD-L1 expression on CLL cells

The pretreatment-experiment raised the concern that dasatinib influence on HS5 cell viability may lead to a reduced supporter effect to CLL cells, thereby indirectly reduced PD-L1 level on CLL cells. To answer this question, we set up an experiment comparing the effect of dasatinib in the usual HS5-CLL cell Coculture system with transferred CLL cells only after Coculture with HS5 cells.

After 48 hours of Coculture, CLL cells were transferred to another well by carefully suspending the medium because the HS5 cells adhered to the plate bottom. This was verified by microscopy (**Figure 19a-d**). Then both conditions, Coculture and transferred CLL cells were treated with 100nM dasatinib for 24 hours and PD-L1 surface expression was measured. Dasatinib reduced PD-L1 surface levels on CLL cells in Coculture with HS5 cells as well as on transferred CLL cells (mono). Interestingly, the difference in PD-L1 expression between untreated and dasatinib-

treatment on the transferred cells results was smaller than the difference observed in Coculture and did not reach statistical significance (**Figure 20a**). This might indicate that dasatinib reduced PD-L1 expression both directly on CLL cells and indirectly via killing of the HS-5 feeder cells.

a) b)

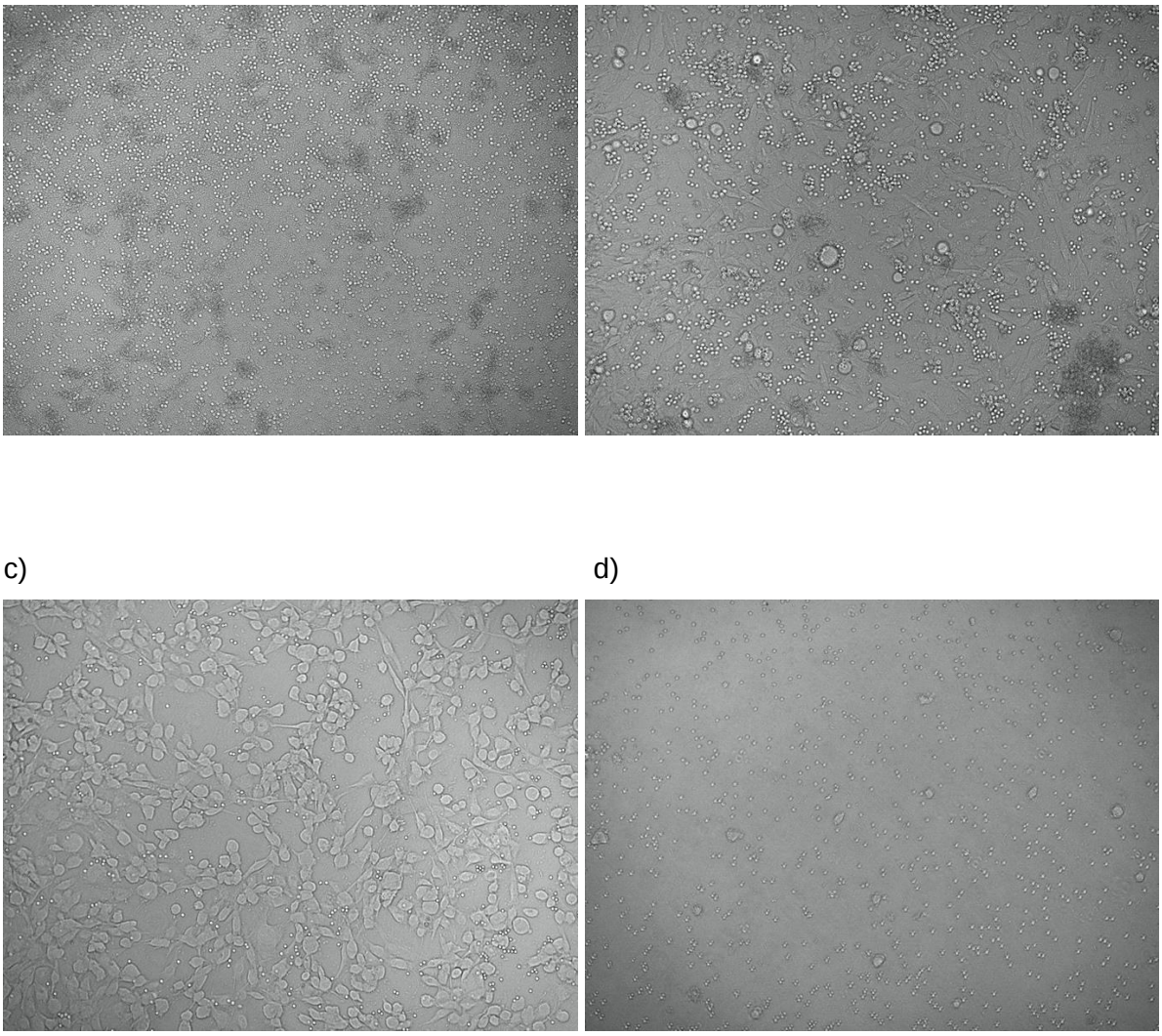


Figure 19a-d. microscopy of CLL cells mono or in Coculture with HS5 cells and after separation of CLL and HS5 cells. *CLL cells were cocultured with HS5 cells for 48 hours and then transferred to another well to be treated with dasatinib. The different conditions were checked by microscopy. (a) CLL cells mono after 48 hours (t48). (b) CLL-HS5 Coculture after 48 hours (t48). (c) HS5 cells after transfer of CLL cells to another well (t48), a few left CLL cells stick to HS5 cells. (d) CLL cells after transfer (t48), a few clotted dead cells are in the picture.*

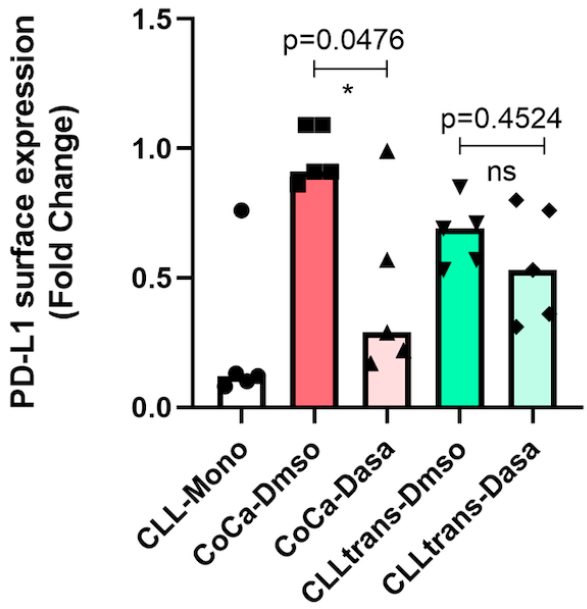


Figure 20a. PDL1 surface expression on primary CLL cells in HS5 Coculture compared to transferred CLL cells after HS5 Coculture with and without dasatinib treatment. *Primary CLL cells of 3 different patients were cocultured with HS5 cells for 48 hours and then treated with 100nM dasatinib for another 24 hours or CLL cells were separated from HS5 cells after 48 hours Coculture to another well and treated with 100nM dasatinib for 24 hours. PDL1 surface expression was quantified via Flow cytometry. Calculation of the Mean (PE-A/ PDL1 channel) is gated on AnnexinV negative and CD5/CD19 positive cell population, values depicted in the graph represent data from 2 independent experiments, duplicates of each condition are shown. Data is isotype corrected and normalized to T0 DMSO control. Dasatinib reduces PDL1 surface expression in HS5 Coculture as well as on transferred CLL cells, reduction after transfer is not significant, Median values: CLLMono = 0.12, CoCaDmso = 0.91, CoCaDasa = 0.29, CLLtransDmso = 0.69, CLLtransDasa = 0.53. (CoCa ≙ Cuculture)*

Again, the viability of CLL cells in all conditions was measured and compared to exclude relevant toxicity of dasatinib on CLL cells. The viability is quantified by Annexin V negative cell population (**Figure 20b**). As expected and in agreement with previous results, CLL viability is reduced without the feeder effect of stroma cells but there was no significant difference after dasatinib treatment compared to DMSO controls.

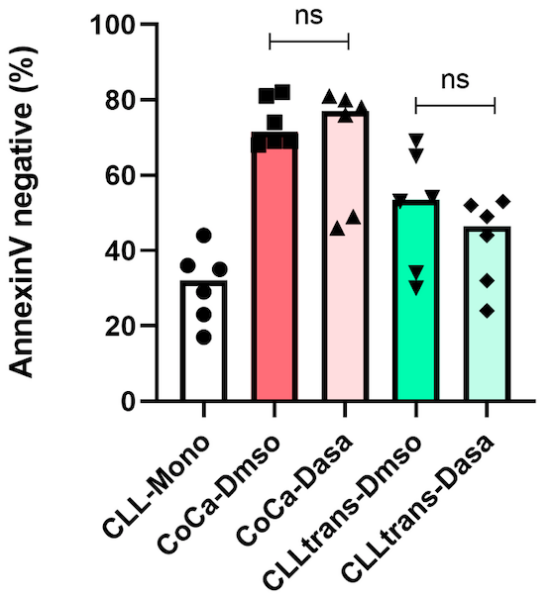


Figure 20b. Viability of CLL cells in HS5 Coculture compared to transferred CLL cells after HS5 Coculture with and without Dasatinib treatment. *Within the same experiments like in 12a, AnnexinV negative CLL cells were quantified via Flow cytometry, gated on CLL cell population. Duplicates of each patient are shown. Viability of CLL cells is reduced without the support of HS5 feeder cells but dasatinib treatment doesn´t reduce viability significantly, Median values: CLLMono = 32, CoCaDmso = 71.5, CoCaDasa = 77, CLLtransDmso = 53.5, CLLtransDasa = 46.5. (CoCa ≙ Cuculture)*

4.6 Dasatinib-induced reduction of PD-L1 surface expression was also observed in high-risk CLL samples

The findings of dasatinib reducing PD-L1 surface expression on primary CLL cells was impressive and convincing but we wanted to point out a higher relevance for the clinic. Since dasatinib is known to be a highly toxic drug with several side effects it is not used for standard CLL therapy regiments as first line, but rather ibrutinib. However ibrutinib-resistant cases are frequently associated with high-risk features and need new therapy (Cheng et al., 2015), (Parikh, 2018). Therapy for high-risk CLL patients still need reliable options and also the readiness from those patients to accept the risk of potential side effects is higher.

The same experiment (like in 3.3.1) was performed with primary CLL cells from high-risk patients to verify the effect of dasatinib. High-risk patients were defined according to the CLL-IPI Score, including age >65 years, clinical stage (Binet B-C), serum ß2 microglobulin >3.5 mg/L, unmutated IGHV and p53 status (deletion 17p and/or p53 mutated). The cells were stimulated in a 48 hours Coculture with HS5 cells and then

treated with 100nM dasatinib for another 24 hours, this was compared to DMSO control (**Figure 21**).

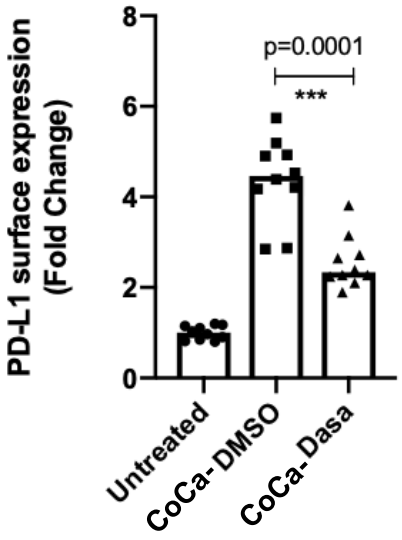


Figure 21. PDL1 surface expression on highrisk-patient CLL cells after HS5 Coculture and dasatinib treatment*. Primary CLL cells of 5 different patients were cocultured with HS5 cells for 48 hours and then treated with 100nM dasatinib for another 24 hours. PDL1 surface expression is quantified via Flow cytometry. Calculation of the Mean (PE-A/ PDL1 channel) is gated on AnnexinV negative and CD5/CD19 positive cell population. Data is normalized to Untreated control. Dasatinib shows a highly significant reduction of PDL1 surface expression after stimulation with HS5 cells, Median values: Untreated = 1, CoCaDMSO = 4.46, CoCaDasa = 2.34.*

The successful reduction of PD-L1 could also be confirmed in high-risk CLL patient samples, thus delivering a promising relevance of dasatinib for clinical purposes.

4.7 Interaction between CLL cells and T cells

4.7.1 T cells are activated after CD3 antibody stimulation

To activate T cells, we stimulated PBMC´s from healthy donors with CD3 antibody. The amount of T cells in the different healthy donor samples was already measured and provided by the group, we used samples with 50% to 60% of T cells. PBMC´s were incubated with 10µg/ml immobilized or soluble CD3 antibody for 48 hours (**Figure 22a+b**). Because soluble CD3 antibody activated T cells more efficiently, we focused on and improved the protocol with soluble anti-CD3. Activation status of T cells was

observed over 7 days by quantifying several activation markers via Flow Cytometry, results of PD-1- and CD69 expression on CD4+ or CD8+ T cells are shown (**Figure 23a-d**). PMA was used as positive control, since it is known to stimulate T cells.

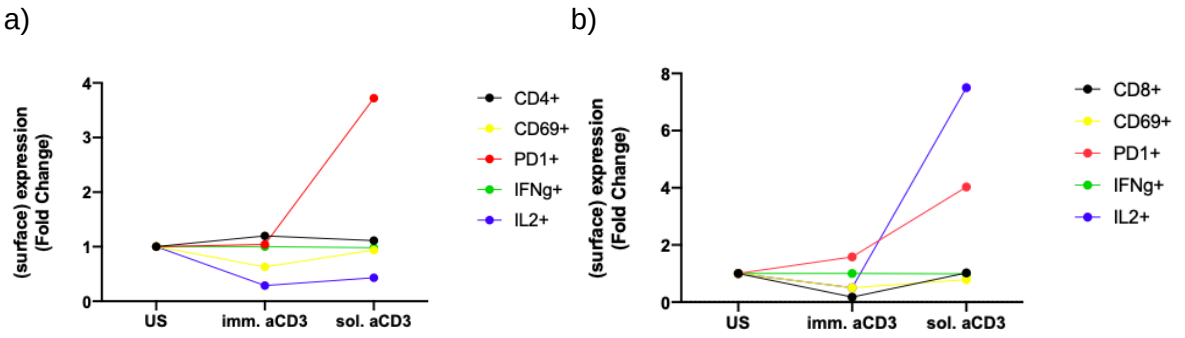


Figure 22a+b. expression of different activation markers on Tcells after stimulation with immobilized vs. soluble CD3 antibody*. PBMcs of a healthy Donor were activated with either 10µg/ml immobilized or soluble CD3 antibody (clone UCHT1). (surface) marker expression on T cells was measured via Flow Cytometry after 48 and 72 hours incubating (t72 data not shown). The calculated Mean of each marker is isotype corrected and normalized to Unstimulated control, Gating on AnnexinV negative and CD4 or CD8 positive cell population. (a) On CD4+ Tcells PD1 surface expression is increased after activation with soluble anti-CD3. (b) On CD8+ Tcells IL2 and PD1 expression is increased after soluble anti-CD3 activation. Immobilized anti-CD3 shows no effect neither on CD4+ nor CD8+ Tcells.*

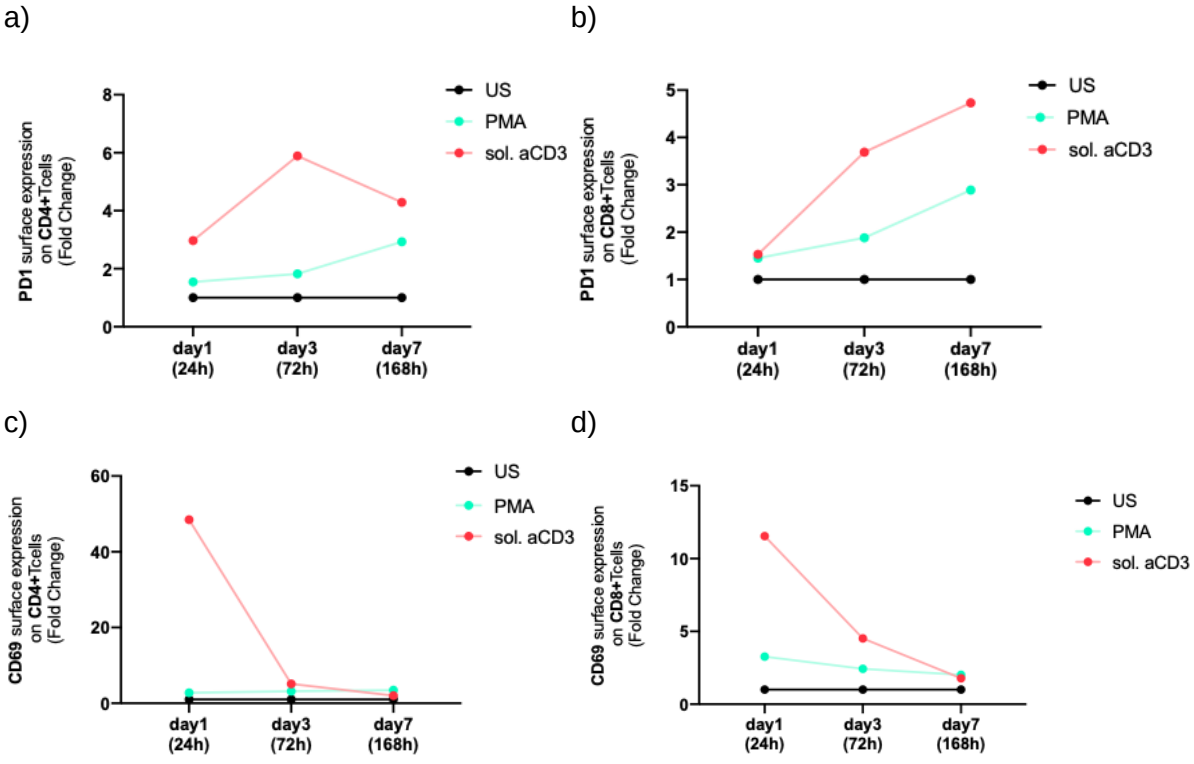


***Figure 23a-d. PD1 and CD69 surface expression on Tcells after soluble anti-CD3-activation**. PBMcs of a healthy Donor were activated with either 5µg/ml soluble CD3 antibody (clone UCHT1) or 25ng/ml PMA. Surface marker expression on T cells was measured via Flow Cytometry after 1, 3 and 7 days incubating. The calculated mean of each marker is isotype corrected and normalized to Unstimulated control, Gating on AnnexinV negative and CD4 or CD8 positive cell population. (a) On CD4+ Tcells PD1 surface expression is highly increased after soluble anti-CD3 activation at all timepoints, but decreases between day 3 and day7. (b) On CD8+ Tcells PD1 surface expression increases steadily over time after activation, best with soluble anti-CD3. (c) On CD4+ Tcells CD69 surface expression is highly increased after one day of activation with soluble anti-CD3, then it decreases to PMA and Unstimulated levels. (d) On CD8+ Tcells CD69 surface expression is also increased after one day of soluble anti-CD3 activation and then decreases with time.*

The soluble CD3 antibody showed better results in activating T cells. PD-1 receptor was strongly increased to a level comparable to PMA stimulation. CD69 marker was also increased after CD3 stimulation, most strongly after 24 hours and decreased over time. These results proved successful activation of T cells from healthy PBMC.

4.7.2 Activated T cells in Coculture with CLL cells

The next step was to coculture activated PBMC´s with HS-5-stimulated CLL cells and examine the activation status of T cells in the Coculture system. We compared unstimulated PBMC´s mono to activated PBMC´s after 72 (and more) hours stimulation with 5µg/ml soluble CD3 antibody and also Coculture of either activated or inactivated PBMC´s with stimulated CLL cells. CLL cells were stimulated as described before in Coculture with HS5 cells for 72 hours before transferring them to PBMC´s.

The conditions with activated PBMC´s, Mono or in Coculture, presented clearly higher levels of PD-1, CD69 and the intracellular Granzyme B (**Figure 24a-f**). Granzyme B is a specific serine protease in cytotoxic T cells and is important for defense of viral infections through apoptosis induction in infectious cells. PD-1 surface expression on T cells increases the longer the cells are stimulated, while CD69 and Granzyme B expression decreases with time.

These results were the repetitive successful activation of T cells, also in presence of CLL cells and therefore ready for the next step in our Coculture protocol establishment with different Coculture conditions of B- and T cells.

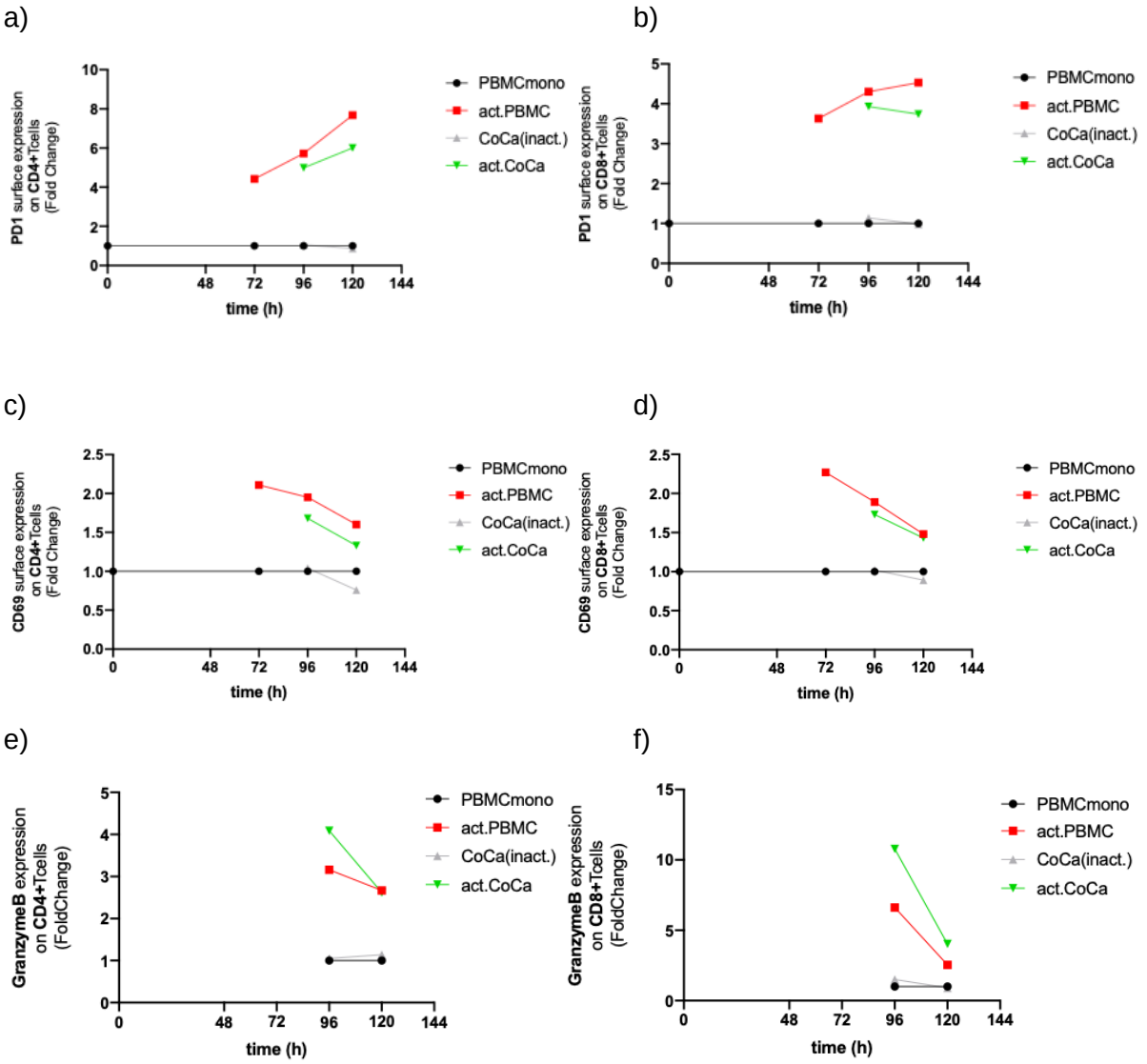


Figure 24a-f. PD1, CD69 and GranzymeB expression on Tcells of healthyDonor PBMCs after anti-CD3 stimulation and in presence of PDL1-stimulated CLL cells. *PBMCs of a healthy Donor were activated with 5µg/ml soluble anti-CD3 for 72 hours while primary CLL cells of one patient were also stimulated for PDL1 surface expression in HS5 Coculture, then the stimulated CLL cells were cocultured with activated PBMCs or nonactivated PBMCs. Activation marker expression was measured via Flow Cytometry after 72, 96 and 120 hours (1, 24 and 48 hours after starting CLL-PBMC Coculture). The calculated mean of each marker is isotype corrected and normalized to Unstimulated control, Gating on AnnexinV negative and CD4 or CD8 positive cell population. (a-f) All markers are clearly increased in Coculture with activated PBMCs and in activated PBMCs Mono compared to inactivated controls.*

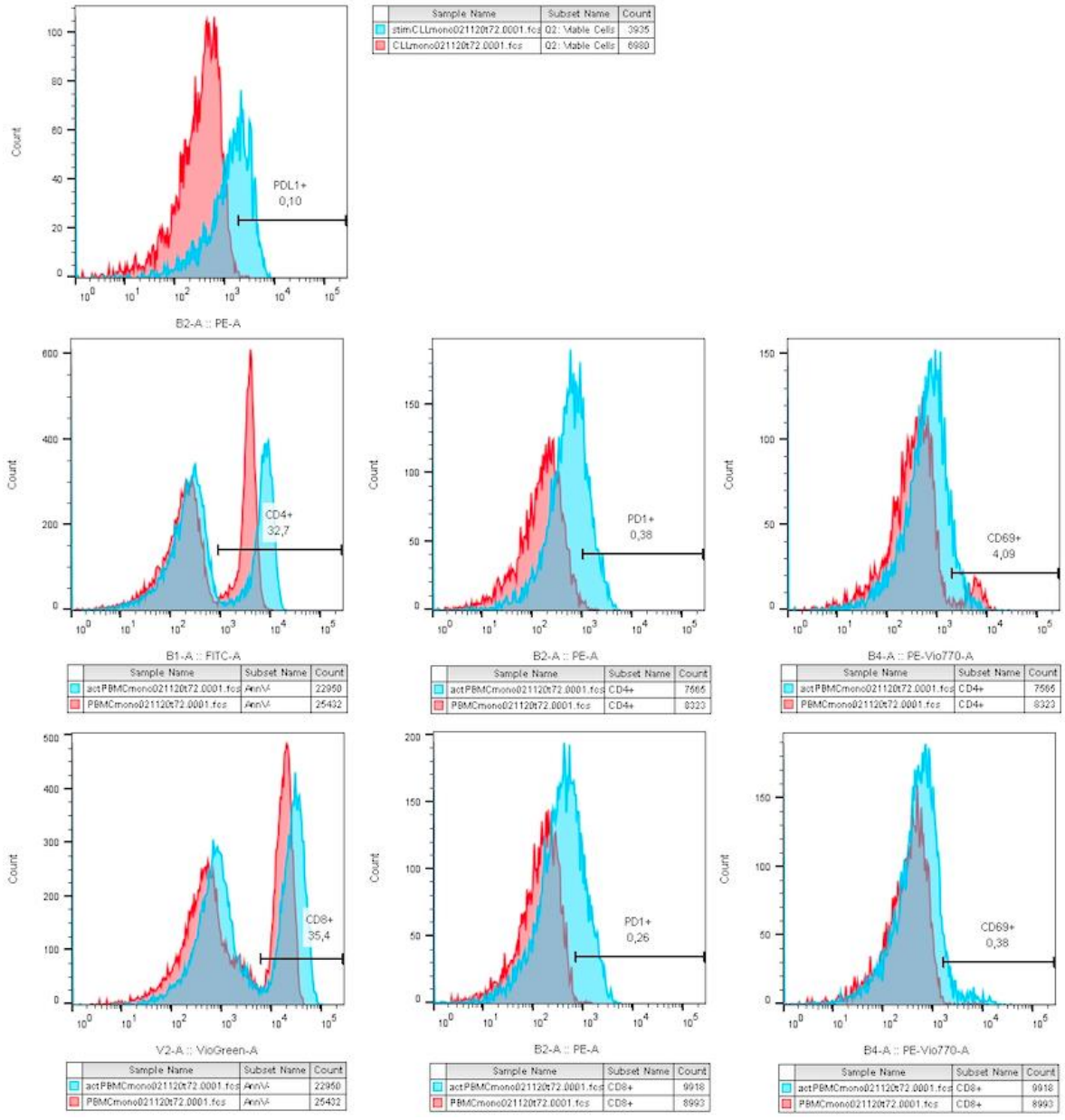
4.7.3 T cell activation in different Coculture conditions of B- and T cells

Now we were prepared to mix either stimulated or unstimulated CLL cells with either activated or inactivated PBMC´s. This resulted in 4 different Coculture conditions.

Before CLL cells and PBMC´s were mixed, the successful stimulation of CLL cells for PD-L1 and activation of T cells for PD-1, CD69 (and Granzyme B) was checked after 72 hours (**Figure 25**).

The increase of the proteins and markers of interest were first analyzed with FlowJo software and presented by a right shift in the histograms. PD-L1 is increased in stimulated CLL cells compared to unstimulated CLL cells mono and PD-1 as well as CD69 is increased on CD4 positive as well as CD8 positive T cells (**Figure 25a**). This data is also analyzed with Prism software and shown in the different graphs (**Figure 25b-e**).

a)



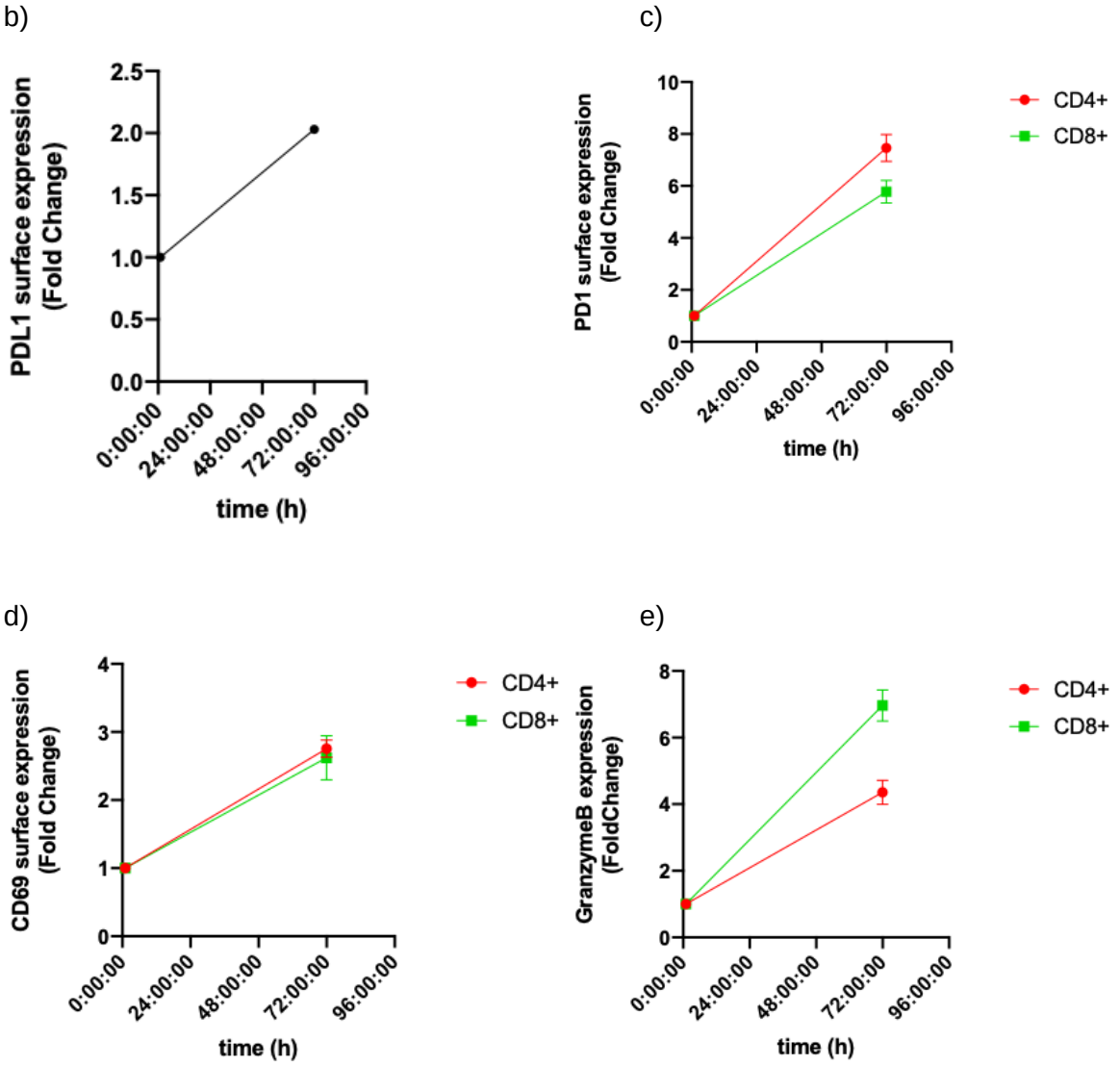


Figure 25a-e. check for successful PDL1 stimulation on CLL cells and activation of Tcells regarding PD1, CD69 and GranzymeB expression before putting them in Coculture. *PBMCs of a healthy Donor were activated with 5µg/ml soluble anti-CD3 for 72 hours while primary CLL cells of one patient were also stimulated for PDL1 surface expression in HS5 Coculture. Before CLL cells (unstimulated and stimulated) were cocultured with PBMCs (inactivated and activated), PDL1 surface expression on CLL cells and PD1/ CD69 expression on T cells was measured via Flow Cytometry. The calculated mean of each marker is isotype corrected and normalized to Unstimulated control, Gating on Annexin V negative cell population. (a) Single cell data is analyzed with FloJo software, the right shift of stimulated CLL cells for PDL1 surface expression or activated T cells for CD4/ CD8, PD1 and CD69 expression is clearly shown compared to their unstimulated controls. (b) PDL1 surface expression on CLL cells of one patient is also shown in PRISM software, PDL1 is increased after 72 hours stimulation with HS5 cells. (c-e) PD1, CD69 and Granzyme B expression on Tcells is also shown in PRISM software, all markers are increased after 72 hours incubation with soluble anti-CD3.*

4.7.4 Comparison of different PBMC-healthy donors for their activation status

A side experiment was to test the existing PBMC samples of different healthy donors for their ability to be activated with anti-CD3 antibody stimulation. This was to pick out the best samples for our experiments and also for the database of the lab group for future experiments.

PBMC´s from 5 different healthy donors were stimulated with 5µg/ml soluble CD3 antibody for 72 hours and then expression of PD-1, CD69 and Granzyme B was measured via Flow Cytometry (CD69 and Granzyme B data is not shown). PD-1 surface expression presents the highest increase on CD4- and CD8 positive T cells on donor II and IV (**Figure 26a+b**).

a) b)

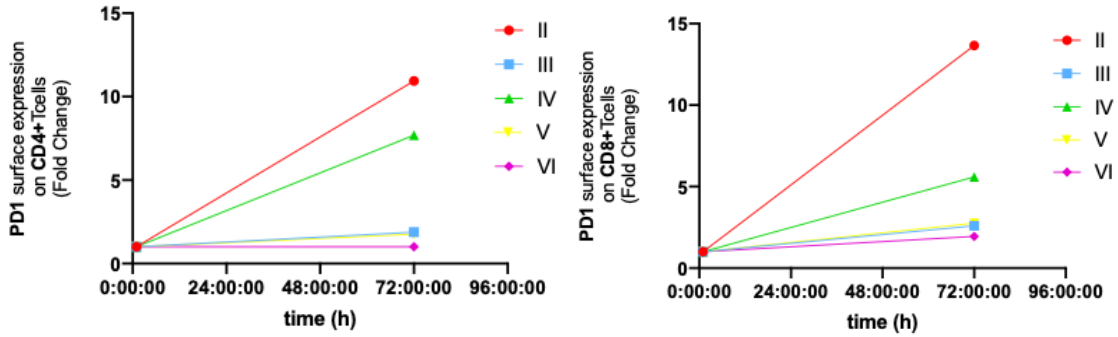


Figure 26a+b. comparison of activation marker expression on different healthy Donor PBMCs after anti-CD3 stimulation. *PBMCs from 5 different healthy donors were activated with 5µg/ml soluble CD3 antibody for 72 hours and compared for their expression of PD1 (CD69 and GranzymeB data not shown). Marker expression was measured via Flow Cytometry. The calculated mean of each marker is isotype corrected and normalized to Unstimulated control, Gating on Annexin V negative and CD4 or CD8 positive cell population. (a,b) On CD4+ and CD8+ T cells the PD1 stimulation works best in healthy donor II and IV.*

For our experiments we used healthy donor IV, because it showed good results for all tested markers and we had enough vials for the planned experiments. It was also interesting to see, that activation with CD3 antibody doesn´t work in all donors. This is not fully understood and needs further investigation.

4.7.5 CLL survival decreases in Coculture with activated T cells

After the check for successful stimulation of PD-L1 and activation of T cells, CLL cells and PBMC´s were mixed. $1*10^6$ CLL cells/ml diluted in Gibco^{TM} RPMI 1640 medium were cocultured with $2*10^6$ PBMC´s/ml, also diluted in Gibco^{TM} RPMI 1640 medium, to ensure a ratio of 1:1 CLL and T cells (this donor contained around 50% T cells). The mix of either unstimulated or stimulated CLL cells (after HS5 Coculture for 72 hours) with either inactivated or activated PBMC´s (after 72 hours anti-CD3 stimulation) added up to 4 different Coculture conditions. CLL cells were stained with CFSE before to distinguish them from PBMC´s. Furthermore, CLL cells were characterized by CD19/ CD5 surface expression.

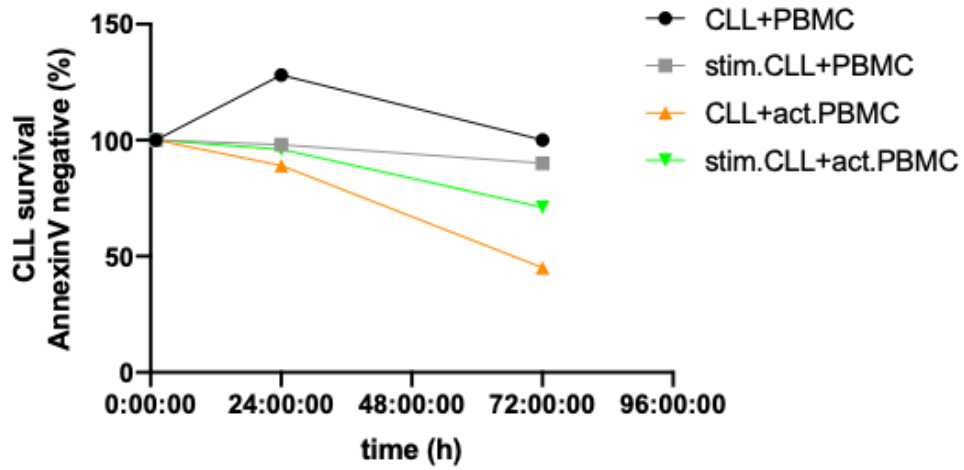
Our hypothesis was that more CLL cells are killed in presence of activated T cells rather than inactivated T cells. In addition, we suggested that high PD-L1 expression (stimulated CLL cells) inhibits T cell attacking towards tumor cells and therefore leads to higher CLL survival compared to the conditions with unstimulated CLL cells (low PD-L1 levels). Viable CLL cells were characterized as Annexin V negative cells, gated on the CFSE positive cell population to focus only on CLL cells and exclude PBMC´s. As shown below CLL viability of this patient decreases the most in Coculture with activated T cells, whereas the viability of CLL cells with high PD-L1 is a little better even in presence of activated T cells (**Figure 27a**).

This confirms our hypothesis, activated T cells attack tumor cells better than inactivated, but high PD-L1 expression inhibits T cell answer leading to a higher tumor cell survival.

In another graph we present the survival of CLL cells quantified only by the amount of CFSE positive cells (all CLL cells), assuming that dead cells lose their stability, burst and wouldn´t be recognized via Flow Cytometry as CFSE stained cells. Comparing the different conditions in this graph, similar results are shown. It has to be mentioned that in the beginning there was about the same amount of CLL cells in all conditions, at least the unstimulated CLL cells as well as the stimulated CLL cells come from the same cell pool and the same volume was added to each Coculture condition. Comparing the Cocultures with inactivated and activated PBMC´s, CLL survival is at

lower levels in presence of activated T cells (**Figure 27b**). Admittedly, it seems like the amount of CFSE positive cells in the same condition doesn´t change over time within the 3 measurements, which is against our expectations. Possibly CFSE positive gating is not a reliable read out for living cells, because it is a quite bright color even recognized in apoptotic cells.

a)



b)

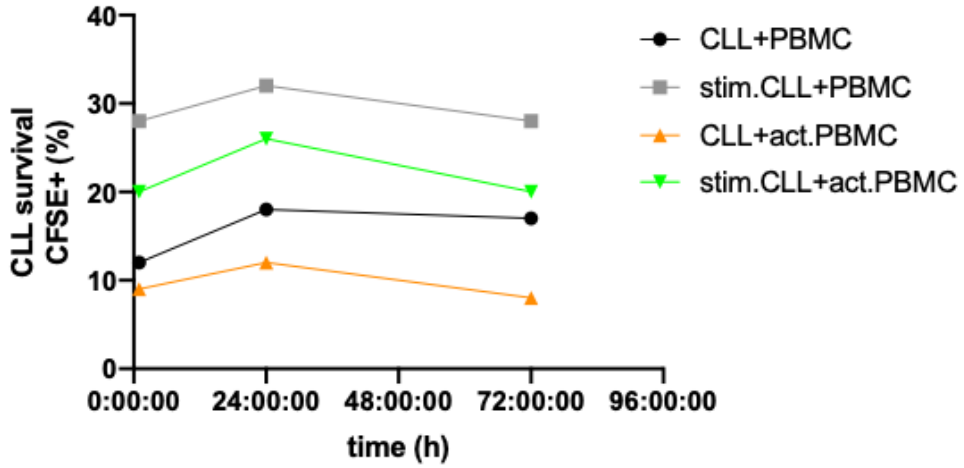


Figure 27a+b. CLL cell survival in different CLL-PBMC Coculture conditions. *PBMCs of a healthy Donor were activated with 5µg/ml soluble anti-CD3 for 72 hours while primary CLL cells of one patient were also stimulated for PDL1 surface expression in HS5 Coculture. CLL cells were labeled with CFSE, then the 4 different conditions of Cocultures were plated. CLL cell survival was measured via Flow Cytometry after 1 hour, 24 and 72 hours. (a) CLL cell survival is defined as Annexin V negative cell population, gated on CFSE+ labeled CLL cells, normalized to total cell count in this condition and to T1 (measurement after 1 hour). CLL cell survival decreases mostly in activated Coculture conditions, in coculture with PDL1-stimulated CLL cells less than with unstimulated CLL cells. (b) CLL cell survival is quantified by the Count of CFSE+ labeled CLL cells. The amount of CFSE+ (CLL) cells doesn´t seem to decrease over time.*

This is only the results from one patient and needs to be confirmed by more samples and also with different read outs and more detailed techniques, but this experiment series proves that a Coculture system with stimulated or unstimulated B- and T cells works successfully. Furthermore, these results support our previous hypothesis that high PD-L1 may have an influence on T cell behavior in this in-vitro setting and allows further consideration about possible therapy options to interfere PD-L1 regulation on CLL cells and enhance immune answer towards the tumor by T cells.

5. Outlook

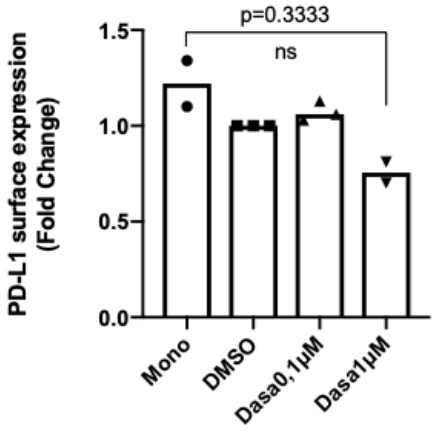
5.1 Reduction of PD-L1 surface expression by dasatinib on JVM3 Cas9 cells remains to be confirmed

In order to identify the targets of dasatinib involved in the regulation of PD-L1 surface expression on primary CLL cells, one possible idea is to create knock-outs of different kinases known to be inhibited by dasatinib in a CLL-like cell line. The CLL-like lymphoma JVM3 cell line expressing Cas9 ribonucleoprotein was already generated by our lab group. Cas9 is an endonuclease which binds to specific RNA-sequences and cuts out DNA. With this technique, known as CRISPR, it is possible to knock-out specific molecules, e.g. kinases.

The plan is to measure JVM3 cells with knock out of different dasatinib-kinases for PD-L1 surface expression without the need of direct dasatinib treatment. Firstly, this would avoid toxic side effects of direct dasatinib treatment and secondly, this could reveal specific kinases as key players in PD-L1 regulation and possible targets of dasatinib reducing PD-L1.

As the first step it was necessary to check the ability of dasatinib to reduce PD-L1 expression on those cells. JVM3 Cas9 cells were treated with 0.1 or 1µM dasatinib for 48 hours and measured for PD-L1 surface expression via Flow Cytometry. 1µM dasatinib showed a reduction of PD-L1 expression, but not significantly (**Figure 28a+b**). The viability of the cells in the same experiment quantified by the Annexin V negative cell amount also presented a decrease in the 1µM-dasatinib sample, again not significantly. So far, these results are not sufficient to claim a relevant role of dasatinib in reducing PD-L1 expression in these cells. Moreover, we could not exclude cell apoptosis caused by dasatinib as the reason for PD-L1 reduction.

a)



b)

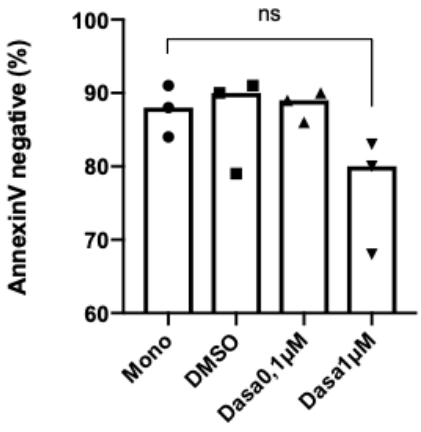


Figure 28a+b. PDL1 surface expression on JVM3 Cas9 cells after Dasatinib treatment*. JVM3 Cas9 cells were treated with 0.1 or 1µM dasatinib for 48 hours (data after 24 hours not shown). PDL1 surface expression is quantified via Flow Cytometry. (a) Calculation of the Mean (PE-A/ PDL1 channel) is gated on AnnexinV negative cell population. Data is isotype corrected and normalized to DMSO control, triplicates of each condition are shown in the graph. 1µM dasatinib reduces PDL1 surface expression significantly after 48h hours, Median values: Mono=, DMSO=, Dasa0.1µM=, Dasa1µM=. (b) Within the same experiment like in Xa, AnnexinV negative JVM3 Cas9 cells were quantified via Flow cytometry, gated on GFP positive cell population. Triplicates of each condition are shown. Viability of JVM3 Cas9 cells is reduced after treatment with 1µM dasatinib, but not significant, Median values: Mono=32, Dmso=71.5, Dasa0.1µM=77, Dasa1µM=53.5.*

5.2 New drug combinations for clinical future therapy lines in CLL treatment

The data shown in 3.8.5 allows the assumption that high PD-L1 on CLL cells influences T cell behavior towards tumor cells. T cell attacking towards CLL cells is inhibited and therefore CLL cell survival enhanced. This finding needs further investigation to understand the interaction between B- and T cells, especially considering the PD-L1 level, more precisely.

For an outlook our idea is to try out the Incucyte Real time visualization, a technique to monitor the dynamics of cell-cell interactions in real-time photos/videos. With this technique it would be possible to track and quantify T-cell induced apoptosis of CLL cells with high or low PD-L1 expression in the Coculture setting.

Finally, the overall aim is to test a new drug combination of dasatinib plus a PD-1 antibody to detect possible additional or synergistic effects in reducing the CLL tumor load (**Figure 29**).

In our data dasatinib showed promising results in reducing the PD-L1 surface expression on CLL cells while PD-1 antibodies, e.g. Nivolumab or Pembrolizumab block the corresponding receptor PD-1 on T cells (and also B cells as well as dendritic cells). So far, the immune checkpoint inhibition is not established in common CLL therapy strategies, but is an emerging field. There is no proofed response to monotherapy with checkpoint inhibitors despite CLL expressing high PDL1 level (Ntsethe et al., 2020), (Ding et al., 2017), (Brusa et al., 2013). Considering the concerns about toxicity of dasatinib, this drug could still play a crucial role for high-risk CLL and in combination with PD-1 antibodies may contribute to promising improvement of CLL therapy options regarding immune dysfunctions of T cells in CLL.

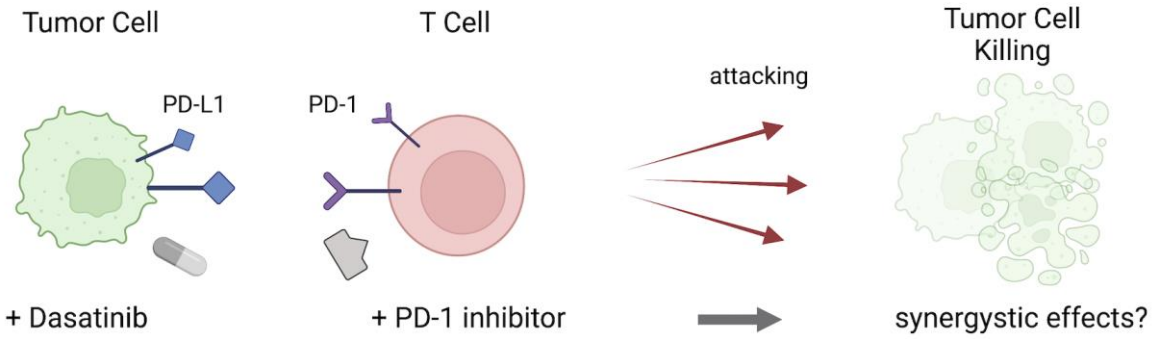


Figure 29. CLL-T-cell Coculture to test optional treatment combinations in order to reduce CLL viability. *CLL cells expressing PD-L1 are treated with checkpoint inhibitors, e.g. dasatinib and T cells are treated with PD-1 inhibitors. These cells are then cocultured to check for synergistic effects of this treatment combination regarding T cell behavior towards tumor cells and overall CLL cell survival.*

6. Discussion

6.1 Stimulation of PD-L1 expression in CLL cells.

In recent years, a few studies have already associated the immunomodulatory protein PD-L1 with progression of CLL ((Ramsay et al., 2012), (Brusa et al., 2013). The principle of regulation of this protein is therefore all the more significant to ultimately explore new targets for cancer therapy. In order to make precise studies with PD-L1, the surface expression on primary blood CLL cells had to be stimulated first. Ramsay et al. and Brusa et al. were able to detect increased PD-L1 expression on lymph-node CLL cells compared to healthy B cells, but nevertheless primary CLL cells in the periphery usually do not express detectable PD-L1.

In the first part of the thesis, we showed that PD-L1 expression could be significantly increased in flow cytometry measurements, on the one hand by the known cell stimulants PMA and INFγ, but also, as recently discovered, by Coculture with HS5 stromal cells (Trimarco et al., 2015).

Although PMA first showed a more pronounced increase in PD-L1 in our experiments, INFγ is more physiological than PMA, because it is produced and secreted by immune cells themselves (Salerno, Guislain, Cansever, & Wolkers, 2016), (Mackensen, Galanos, & Engelhardt, 1991). PMA acts as a cell stimulant by activating various kinases, especially protein kinase C signaling pathways also downstream of the BCR (Jiang & Fleet, 2012); (Tangye et al., 1995). INFγ stimulates cellular defense via activation of the Jak-Stat signaling pathway (Mimura et al., 2018). INFγ also yielded better results in our experiments related to BCR inhibitors, meaning that after INFγ stimulation the decrease of PD-L1 by dasatinib was significant, but not after PMA stimulation. Therefore, we have focused on INFγ to stimulate PD-L1 expression, eliciting the most effective concentration of 10ng/ml.

Since relatively recent studies found significant PD-L1 stimulation after co-culture with HS5 cells, a human stromal cell line (Bottcher et al., 2021), we also tested this method. Coculture of CLL cells with HS5 stromal cells for 48-72 hours confirmed these results and we were able to increase PD-L1 expression up to 4-fold. Support of CLL cells by fibroblasts has been widely studied, but the exact mechanism of PD-L1 stimulation by HS5 cells is still unclear. Involvement and activation of the Notch-signaling pathway was suggested, which led to rescue of CLL cells from apoptosis and may even promote

resistance to chemotherapy (Nwabo Kamdje et al., 2012). Böttcher et al. could previously proof a positive correlation between c-Myc and PD-L1 levels in CLL cells when cocultured with HS5 cells. They found that stromal cells activate the Notch-c-Myc axis via cell-to-cell contact by detecting upregulated Notch and c-Myc target genes in CLL cells together with stimulated PD-L1 expression.
The significantly increased PD-L1 levels provided us with a good basis and prerequisite to detect changes in surface expression by treatment with inhibitors.

6.2 The effect of BCR inhibitors on PD-L1 expression in CLL cells

Previous studies in our laboratory on PD-L1expression after BCR inhibitor treatment on human CLL cell lines (JVM3 and OSU) had shown a successful impact and lowered surface expression (data not shown). Previously published studies on ibrutinib also hypothesize that the BTK inhibitor has an impact on PD-L1 expression (Hong et al., 2019). While ibrutinib has long been a successful therapeutic agent in CLL, its effect via PD-L1 inhibition has not been described, and PD-1/PD-L1 antibodies have not yet been included in standard CLL therapy. Above mentioned evidence, that BCR inhibitors can decrease PD-L1 surface expression, requires further elucidation. Thus, we now wanted to investigate this effect on primary CLL cells. Comparison of ibrutinib, idelalisib and dasatinib, dasatinib consistently showed the most significant results in terms of PD-L1 inhibition, both after PMA/INFγ stimulation and in HS5 Coculture. These results also confirmed our previous data from the cell line experiments that dasatinib had the strongest effect (courtesy Lea Reemann). To our knowledge, this is the first time that dasatinib is specifically highlighted as a potential PD-L1 inhibitor. To further validate this hypothesis, several aspects still needed to be investigated and clarified, such as concerns about toxicity, as well as possible molecular targets of dasatinib. This work shows a beginning to uncover possible mechanisms and potential targets of dasatinib, thus further understanding the regulation of PD-L1 and possibly paving new therapeutic options for CLL.

6.2.1 The role of the BCR-signaling pathway for PD-L1 expression

A major goal of the project was to investigate the role of the B-cell signaling pathway

in relation to PD-L1 regulation. Triggered by the already positive data on various BCR inhibitors to decrease PD-L1, we hypothesized that blockade of individual B-cell kinases decreases (is involved in) PD-L1 expression on CLL cells. However, in our experiments, neither the BTK inhibitor ibrutinib nor the PI3K inhibitor idelalisib significantly reduced PD-L1 in CLL cells. BTK and PI3K each represent important kinases of the 2 main arms of the B cell signaling pathway, and thus the combination of ibrutinib plus idelalisib treatment is supposed to be comparable to dasatinib-induced blockade of the BCR pathway (See Fig. 3 in Introduction). Consistent with this, the treatment combination also showed no effect on PD-L1 surface expression. In contrast, the pan-inhibitor dasatinib again caused a significant reduction in the same experiment. From this, we concluded that the B cell signaling pathway does not play a significant role in PD-L1 regulation and thus unlikely to be a target at the molecular level for dasatinib to decrease PD-L1. Thus, the mechanism of action behind PD-L1 reduction by dasatinib remains to be investigated.

6.3 Toxicity of dasatinib

As a pan-kinase inhibitor, there has been a growing concern about the toxicity of dasatinib. Possible side effects include cardiac failure, myelosuppression, gastrointestinal bleeding, hypocalcemia, pneumonia, and bone metabolism (Al-Asmakh et al., 2021). Dasatinib is a pan-inhibitor that blocks key tyrosine kinases, mainly src-family kinases and bcr-abl, as well as many others. These proteins are involved in cell differentiation, proliferation and survival and thus play a critical role in cancer pathogenesis (Lindauer & Hochhaus, 2018), (Steinberg, 2007), (Horinkova et al., 2019). Our results show that dasatinib is also involved in PD-L1 regulation. Through which approach it decreases PD-L1 expression, or whether this occurs through an as yet unknown mechanism, remains open. However, it is known that dasatinib is toxic to cells and therefore is not used as the preferred standard therapy in the clinic, although it is approved as first-line therapy in CML. It has been established in the treatment of CML patients resistant to imatinib with good tolerance and outcome (Aguilera & Tsimberidou, 2009) or Philadelphia chromosome-positive acute lymphoblastic leukemia (ALL), but has not been recommended for CLL to date (Talpaz et al., 2006), (Amrein, 2011). The mostly indolent form of CLL can be treated well with the already

known agents and the use of such toxic agents is not necessary. Aggressive forms and resistance, however, are becoming more frequent and it is precisely in such cases that dasatinib has already shown promising results (O'Hare et al., 2005), (Shah et al., 2004). Since our goal was to investigate whether dasatinib is a potential therapeutic agent for aggressive CLL therapy, we wanted to exclude possible toxic side effects in our experiments of dasatinib killing the CLL cells. As our data show, dasatinib has no effect on CLL cell viability in our experiments, as the annexin V negative cell percentage remains constant at a high level, even in the presence of dasatinib. This is listed for all of our measurements to always demonstrate evidenced sample quality. This also allowed us to conclude that the PD-L1 level did not decrease because cells go under apoptosis and thus can no longer express PD-L1. This aspect is further supported by the fact that in all experiments the PD-L1 surface measurement was performed from the annexin V negative gate; thus, only viable cells were considered.

A crucial investigation was the direct effect of dasatinib on CLL cells. This was done with a series of experiments in which CLL cells were transferred to a new plate after co-culture with HS5 cells and verification of a thereby successfully stimulated PD-L1 expression and only then treated with dasatinib. This excluded any indirect influence of dasatinib on stromal cells and underlined the effect of dasatinib exclusively on CLL cells. For comparison, PD-L1 was also measured in the same experiment after dasatinib treatment in the presence of HS5 cells. In the results, we could show that dasatinib induced a reduction of PD-L1 expression in both conditions, but without significance in the transferred CLL cells. However, this might be due to the small sample size and this experiment should be repeated with more patient samples to confirm the result. The aim is to investigate whether dasatinib has a direct effect on CLL cells with respect to PD-L1 regulation. In parallel, the viability of the CLL cells was determined to exclude that a reduction of the PD-L1 level is solely due to increased cell mortality. Of course, the viability of CLL cells without the presence of HS5 cells was lower than in HS5 Coculture due to the lack of feeder support. Crucially, CLL cell viability is not significantly altered by dasatinib, neither with nor without HS5 cells. Thus, we can again emphasize that our experimental concentration of dasatinib did not induce significant CLL apoptosis before reducing PD-L1 level.

The potential toxic effect of dasatinib should also be tested for HS5 cells. For this purpose, we developed a protocol according to which HS5 cells should be pre-treated with dasatinib and only come into contact with CLL cells after exchange of the treated medium with fresh medium. Dasatinib binds reversibly, so it can be assumed that no dasatinib was left in the co-culture. This method was used firstly to investigate the effect of dasatinib specifically on HS5 cells and secondly to measure PD-L1 expression after stimulation with pre-treated HS5 cells compared with CLL-HS5 Coculture stimulation in the presence of dasatinib. Contrary to our assumption, the results of this experiment show that PD-L1 surface expression decreases even in the pretreated sample, although no inhibitor should be present anymore. This suggests that dasatinib has such an effect on HS5 cells that they can no longer stimulate PD-L1 expression as before. To find only one possible reason for this result, the viability of HS5 cells under dasatinib treatment, more precisely under exactly the conditions of this experiment, was determined by a XTT assay. Here, a reduction of viability after treatment with 100nM dasatinib was shown. Thus, a possible explanation for the reduced PD-L1 expression after stimulation with the pre-treated HS5 cells could be a reduced HS5 number and thus a lower feeder capacity. It has been thoroughly investigated that CLL cell survival is supported by various stimuli of cells from the microenvironment. This is achieved by crosstalk between malignant cells and mesenchymal stromal cells via cell-to-cell contact, communication via soluble chemokines or via extracellular vesicles. Malignant cells can even manipulate their environment into a supportive microenvironment better than healthy B cells (Dubois et al., 2020), (Panayiotidis et al., 1996). The exact impact of HS5 cells on PD-L1 expression on CLL cells needs further investigation. It is a fact that dasatinib also has a direct effect on CLL cells with respect to their PD-L1 expression and has also shown promising results in lowering PD-L1 independently of HS5 stimulation (see INFγ experiments).

6.4 Dasatinib effect on PD-L1 expression in high-risk CLL cells

To this extent, it is becoming increasingly clear that dasatinib has a significant effect on PD-L1 expression. As explained earlier, elevated PD-L1 on tumor cells contributes to tumor progression, including in CLL. Drugs that lower PD-L1 levels and thus interfere with CLL survival may therefore, in theory, be a promising gain for therapy

and patient outcome. In this regard, dasatinib is showing the best results in this project and is coming to the fore as a potential agent for CLL. Despite ample evidence that these impressive results are not solely due to excessive toxicity of dasatinib, we also performed the studies with high-risk patient cells. High-risk patients have a poorer prognosis from the outset because they have specific alterations in their genome, as described above (in the introduction). On top these patients are more difficult to treat and in this case, somewhat higher side effect profiles are also accepted. Dasatinib could therefore be considered for high-risk CLL patients. As our data also show, dasatinib also works specifically in high-risk patient samples and significantly lowers the PD-L1 level. This crystallizes another major goal of the work, namely to present dasatinib as a potential therapeutic for clinical use in high-risk patients.

6.5 The effect of PD-L1 levels on T cell behaviour

As already explained, the PD-1/PD-L1 axis primarily alters T cell behavior. In brief, the interaction of PD-1 with its ligand PD-L1 inhibits the T cell response against pathogens and tumor cells. T cells are thus inactivated. PD-L1 accordingly plays an important role in the interaction between B and T cells. Since PD-L1 is mainly expressed on B cells and PD-1 is present on T cells, it is obvious to use a combination of inhibitors of both proteins to achieve the interaction between both cell types, in fact to enhance it. Treatment combinations are often used clinically, because they cover a larger spectrum of activity and the doses of the individual components can be reduced in order to curb specific side effects. This aspect is very helpful for our further goal, because a combination therapy could possibly reduce toxic side effects of dasatinib, especially at higher doses. This must now be characterized as a very speculative hypothesis, but one idea is to combine dasatinib with a PD-1 antibody to achieve synergistic effects if necessary, to inhibit the PD-1/PD-L1 axis and thus reactivate T cell attacking towards tumor cells. At the same time, this could mitigate against concerns about the high side effect profile of dasatinib. Of course, drug interactions have to be considered in such combinations and much further work is needed to justify future studies and to convince of the potential success of this therapy for high-risk patients.

6.5.1 Activation of T cells

To characterize the activation state of T cells, we determined specific markers, including PD-1, CD69, and granzyme B.
PD-1 was considered to some extent as a control that it was actually present and stimulable on the T cells. With increased PD-1 on the T cells, it can be assumed that the cell is immunologically active.
Activation of T cells occurs physiologically via recognition and contact of a T cell from a pathogen. In vitro, T cells can be stimulated and activated by CD3 antibodies (Li & Kurlander, 2010). Incubation of PBMC sample with anti-CD3 was performed for 7 days and measured at different time points. After successfully activating T cells using soluble CD3 antibody, as measured by an increase in these activation markers, we could focus on the interaction between B and T cells, whether and to what extent they influence each other.

6.5.2 B-/T-cell Coculture

Here, B cells with low or high PD-L1 levels were cocultured with unstimulated or activated T cells after HS5 Coculture. Previously, the successful stimulation of PD-L1 on the B cells and the different activation markers on the T cells, respectively, were checked. We hypothesized that activated T cells would increasingly kill CLL cells, while increased PD-L1 would lead to decreased T cell activity. In flow cytometry, decreased T cell activity can be seen by again decreasing activation markers. An enhanced T cell response after activation by CD3 antibodies should be measurable by a decreased proportion of living CLL cells in Coculture. We focused on the latter in this work, measuring the four different Coculture conditions of stimulated or unstimulated CLL cells with either inactive or activated PBMC samples at different timepoints after incubation. For the time being, only the survival of CLL cells in the different conditions was compared, measured by Annexin V staining, here by counting Annexin V negative CLL cells. The result of the first measurement with one patient was quite promising and corresponded to our expectations. The percentage of Annexin V negative CLL cells, i.e. living CLL cells, logically decreased with time in Coculture with T cells. Moreover, it was clearly seen that the proportion is lower in the conditions with activated PBMC's than with inactive PBMC's. This suggests that activated T cells

increasingly attack and eliminate CLL cells. Encouragingly, it could nevertheless be seen that the proportion of living CLL cells in the condition with stimulated CLL cells, meaning with increased PD-L1 expression, was still higher than in unstimulated CLL cells with lower PD-L1. This suggests that higher PD-L1 expression inhibits T cells more strongly and thereby also inhibits tumor cell killing. These results need to be validated with more patient samples, but show already the successful establishment of a Coculture with B and T cells, a successful concept/readout to measure the interaction and behavior of the cells and also the confirmation of our hypothesis about the influence of PD-L1 on T cells.

To be able to distinguish CLL cells in Coculture from PBMC's after measurement, they were labeled with CFSE. Thus, a second readout was the percentage of CFSE positive cells and thus the percentage of CLL cells present in the culture at the time of measurement. This variant assumed that the detected CFSE via flow cytometry corresponded to the remaining living CLL cells and, accordingly, killed or apoptotic CLL cells no longer expressed CFSE. As described in the Results Part, CLL survival was lower in the activated PBMC conditions, but the overall amount of CFSE positive cells remained stable over the 3 days, so CLL cells would not die (neither with time nor by T cell attacking). This result is therefore not fully reliable and it remains to be clarified which readout is best suited here.

6.6 Combination treatment to reinforce the immune system against tumor cells

In this work, we could at least set a foundation suggesting the importance and consequences of PD-L1 changes for the functionality of T cell mediated cytotoxicity.

Provided that these data are reproducible and convincing, the next step in this setting would be to add a combination treatment of dasatinib and a PD-1 antibody, as already described in the Outlooks in 4.2. Dasatinib, as shown in our data, reduces PD-L1 levels and a PD-1 antibody reduces PD-1 levels. We will test if a combo of the two may act synergistically to throttle the PD1/PD-L1 axis in tumor cells to the point where the immune response is no longer limited. The T cells would no longer be inhibited by the PD-1/PD-L1 axis and could increase their targeting of tumor cells.

Treatment with novel targeted drugs, such as the Bruton tyrosine kinase (Btk) inhibitor ibrutinib or the Bcl-2 antagonist venetoclax significantly improved treatment outcomes

for CLL patients. Unfortunately, resistance to these therapies is increasingly observed (Pula et al., 2019), (Haselager et al., 2020). Similarly, mutations are occurring with increasing frequency, leading to high-risk variants and posing an increasing problem in oncology. Our combination could be a real value-add for high-risk patients who no longer respond to standard therapies and have developed resistance.
However, one problem with the idea is that single-agent immune checkpoint blockade with antibodies targeting PD-1 or PD-L1 were not effective in initial clinical trials with CLL patients (Xu-Monette et al., 2018), (Ntsethe et al., 2020). The reasons for this unexpected failure of the therapeutic approach are unclear and require further investigation and testing. It also remains to be seen whether PD-1/ PD-L1 antibodies in the right combination might not achieve success after all, also for CLL (high-risk) patients.

With this work, we have addressed an important topic from the overlapping field of immunology and oncology. The immune system plays an important role in fighting cancer. The human body has developed impressive mechanisms of its own to distinguish between foreign and own, to communicate, to regulate highly sensitively and to keep the balance. Medically, this regulation can be manipulated, mechanisms can be strengthened or even blocked and dysregulations can be prevented. An example of such an intersection and the subject of this work is the immune checkpoint PD-1/PD-L1. This checkpoint is physiologically designed to control immunological responses, inhibit T cell attacks and thereby prevent tissue damage and autoimmune responses. However, malignant cells can also exploit this mechanism to block attacks against themselves and ensure tumor progression. This is exactly the point where we can now intervene medically and inhibit the PD-1/PD-L1 axis on tumor cells. With this project, we were able to further investigate and confirm the importance of the protein for CLL and even, to our knowledge, gain new insights that give hope for future progress in understanding CLL pathogenesis as well as further development of therapy. In my opinion, the most important and a new finding of this work is that dasatinib decreases PD-L1 expression on primary CLL patient cells. One has to admit, and this is probably the biggest weakness of this project, that dasatinib is known to be a toxic drug. It has many side effects and therefore will be reluctant to be used in the clinic and will have difficulty gaining acceptance for CLL therapy. It is tempting to find the mechanism of dasatinib at the molecular level and thereby further clarify the regulation

of PD-L1. In any case, we were able to convincingly demonstrate the efficacy of dasatinib with regard to PD-L1 reduction in several independent trials and also in different systems and settings. Based on earlier data from the laboratory showing PD-L1 reduction by dasatinib in human cell lines (JVM3 and OSU), this project has emerged and we could confirm that dasatinib also reduces PD-L1 expression on primary CLL cells, consistently after different stimulations. Although in a few places the data need to be validated with more patient samples and the exact effect of dasatinib on stromal cell function and viability needs to be clarified, we could already exclude the toxic effects of dasatinib. Furthermore, we were able to exclude the B cell signaling pathway as a key player and target of dasatinib in PD-L1 regulation. The elucidation of where dasatinib acts in this regard requires further experiments and is in planning.

7. Summary

PD-L1 and its receptor PD-1 are surface proteins on many different cells that are involved in the immune response in the human body. Binding of the ligands to its receptor inhibits the immune response, which physiologically prevents an exuberant immune response at the so-called immune checkpoints.

However, PD-L1 is also expressed on tumor cells, for example on CLL cells, and thus leukemic cells can counter tumor immune evasion. The PD-L1/ PD-1 axis is used as a therapeutic target in oncology to enhance the immune defense against tumor cells. For this purpose, the so-called checkpoint inhibitors are used, which block the interaction between the ligand and the receptor.

In this work, we focused on the regulation of PD-L1 expression on primary CLL cells. On the one hand, PD-L1 expression on the cell surface can be stimulated by different methods, and on the other hand, a reduction of PD-L1 on primary CLL cells is achieved by different checkpoint inhibitors. We particularly focused on the inhibitor dasatinib, as it showed the clearest effect in reducing PD-L1 expression. In different experiments, we tested the role of the B-cell receptor signaling pathway and influence of its individual kinases on the regulation of PD-L1 expression. In these experiments, the B-cell receptor signaling pathway was not shown to be a critical regulator.

Since dasatinib is toxic to cells and is known to cause many clinical side effects, it were strictly considered only viable CLL cells in the experiments to exclude the impressive reduction of PD-L1 on CLL cells by a toxic effect alone.

A significant reduction of PD-L1 could also be achieved on high risk CLL cells by dasatinib treatment. This points out dasatinib as a potential therapy for high risk CLL patients.
Further, the effect of PD-L1 expressing CLL cells on T cells was investigated. For this purpose, different conditions of B-T cell co-cultures with high or low PD-L1 expression on CLL cells and activated or inactivated T cells were applied. Initial results confirmed the hypothesis that CLL cell survival is more markedly reduced by activated T cells and that the T cell response is inhibited by highly expressed PD-L1.

However, these results need further validation. Our idea is to visualize the interaction between B and T cells by live imaging. A future goal is to establish dasatinib plus a PD-L1 antibody as a combination therapy for CLL. This could expand the therapeutic options for high-risk CLL patients who respond to immunotherapy.

7.1 Graphical summary

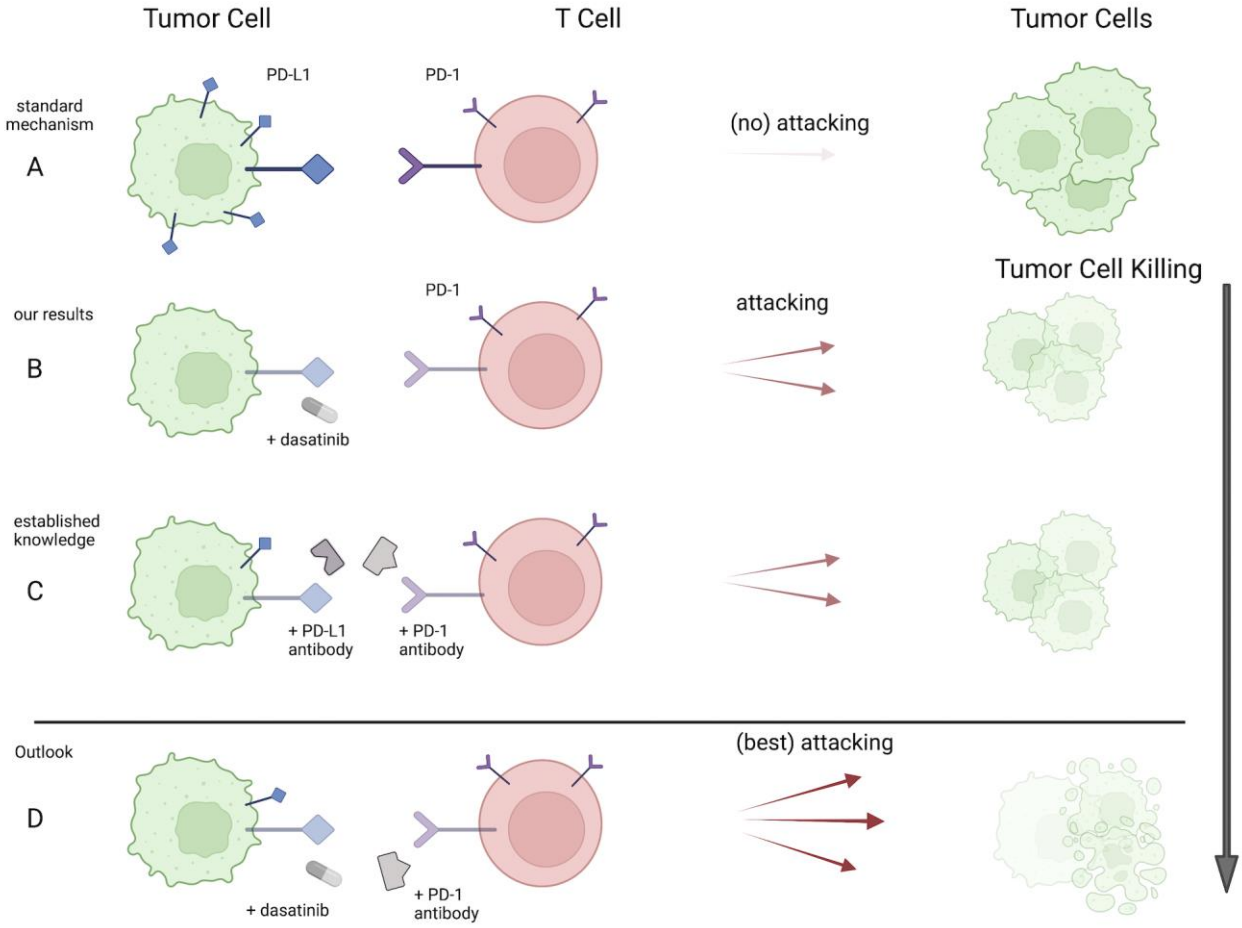


Figure 30. Graphical Summary. *Row A shows the standard mechanism of tumor cells expressing PD-L1 to suppress T Cell activation against themselves via binding PD-1. Row B shows our main results, depicting that dasatinib reduces PD-L1 expression on tumor cells and therefore promotes T cell attacking against tumor cells. Row C shows already established knowledge of blocking PD-L1 and PD-1 with antibodies which also leads to T cell attacking against tumor cells. Row D presents our outlook idea to investigate the combination of dasatinib (reducing PD-L1) with a PD-1 antibody to achieve the (best) attacking of T cells against tumor cells.*

8. References

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9. Appendices

9.1 List of Figures