Immunotherapeutic Approaches in Multiple Myeloma

Doctoral thesis

to obtain a doctorate (PhD)

from the Faculty of Medicine

of the University of Bonn

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from Hamburg, Germany

2023

Written with authorization of

the Faculty of Medicine of the University of Bonn

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Day of oral examination: 30.11.2023

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List of Abbreviations

°C	Degrees celsius
Ab	Antibody
ADC	Antibody drug conjugate
ADCC	Antibody dependent cellular cytotoxicity
ADCP	Antibody dependent cellular phagocytosis
AKT	Protein kinase B (PKB)
Allo-SCT	Allogenic stem cell transplantation
APC	Antigen presenting cell
ASCT	Autologous stem cell transplantation
BCA	Bicinchoninsäure-Assay
BCMA	B cell maturation antigen
BCR	B cell receptor
BiTes	Bispecific T cell engagers
BM	Bone marrow
BMSC	Bone marrow stem cells
bp	base pairs
BSA	Bovines Serum Albumin
BsAbs	Bispecific antibodies
CAR	Chimeric antigen receptor
CCL12	Chemokine (C-C motif) ligand (SDF-1α)
CD	Cluster of differentiation
cDNA	complementary DNA
CTLA-4	Cytotoxic T lymphocyte associated protein-4
CXCR4	C-X-C motif chemokine receptor 4
Da	Dalton
DAMPs	Damage associated molecular patterns

DC	Dendritic cell
Dex	Dexamethasone
DNA	Deoxyribonucleic acid
ECM	Extracellular Matrix
e.g.	*exempli gratia*
EMD	Extramedullary disease
EMT	Epithelial-mesenchymal transition
ER	Endoplasmatic reticulum
et al.	*et alii*
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
g	Gramm or force
GFP	Green fluorescent protein
GvHD	Graft versus host disease
h	Hour
HDAC	Histone deacetylase
HET	Haus für Experimentelle Therapie
HIF-1α	Hypoxia inducible factor-1α
HRP	Horseradish peroxidase
HSCT	Hematopoietic stem cell transplantation
ICD	Immunogenic cell death
i.e.	*id est*
IL	Interleukin
IMiDs	Immunomodulatory drugs
INFγ	Interferon γ
iNKT	Invariant Natural Killer T cell
i.p.	intraperitoneal

i.v.	intravenous
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
kB	kilo base
kDa	kilo Dalton
L	Liter
M	Molar
mAbs	Monoclonal antibodies
MDSC	Myeloid-derived suppressor cells
Mg	Milligramm
MGUS	Monoclonal Gammopathy of Undetermined Significance
MHC	Major histocompatibility complex
Min	Minute
miRNA	Micro RNA
mM	Millimolar
MM	Multiple Myeloma
MMP	Matrix metalloproteinase
MRD	Minimal residual disease
mRNA	messenger RNA
ms	Millisecond
mTor	Mammalian target of rapamycin
MW	Molecular Weight
n/a	Not available or not assessed
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NK	Natural Killer
NKT	Natural Killer T cell

PB	Pacific blue
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PD-L1	Programmed death-ligand 1
PD-1	Programmed death 1
PE	Phycoerythrin
pH	Potentium hydrogenium
PIs	Proteasome inhibitors
Ras	Rat sarcoma
RBC	Red blood cells
rcf	Relative centrifugational force
RNA	Ribonucleic acid
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute
RT-PCR	Rerverse transcriptase polymerase chain reaction
s	Second
SH2	Src homology 2
SHP2	Src homology region 2 domain containing phosphatase 2
SSC	Side scatter
Syk	Spleen tyrosine kinase
TAA	Tumor associated antigen
TAMs	Tumor associated macrophages
TCR	T cell receptor
T_{fH} cell	Follicular helper T cell
TGF-β	Tissue growth factor-β
TILs	Tumor infiltrating lymphocytes
TNF	Tumor necrosis factor
TP53	Tumor protein p53

wt	Wild type
Zeb1	Zinc finger E-box-binding homeobox 1

1. Introduction

1.1 Multiple Myeloma: Overview and aim of this work

Multiple Myeloma (MM) accounts for 13% of hematological malignancies representing the second most common hematological neoplasm behind non-Hodgkin lymphoma (NHL) and 1% of all cancer diseases. In general, it is diagnosed in the elderly at an average age of approximately 70 years. Roughly 37% of patients are under 65 years (Palumbo et al., 2011). The annual incidence of MM is 4-6 cases per 100.000 population. Despite the advances in therapy the disease still remains incurable (Harousseau and Moreau, 2009).

Checkpoint inhibition (CPI) has shown dramatic improvements in overall survival in many malignant diseases. However, in multiple myeloma the results were disappointing resulting in an early termination of clinical trials.

The first aim of this work was to seek the possibilities of CPI in MM and evaluate its efficiency and immunological mechanisms with focus on PD-1/PD-L1 blockade. The second aim was to investigate the MM pathomechanisms in relation to the PD-1/PD-L1 singnaling pathway. The role of PD-L1 expression on myeloma cells was analyzed by generating a PD-L1 KO of the 5T33 cell line using the CRISPR/Cas9 technology. *In vivo* analysis using KaLwRij mice that develop MM upon injection of 5T33 myeloma cells were performed to identify key players in tumor progression and possible targets for MM therapy. Bone marrow (BM), lymph nodes and spleen cells were analyzed by flow cytometry for the phenotype of immune cells to elaborate immune cell regulations and the crosstalk with tumor cells. Immunotherapeutic approaches with CPI and HDAC inhibitors were performed to identify possibly synergistic approaches to optimize CPI therapy in MM which might have an impact on the clinical efficiency.

1.1.1 Multiple Myeloma: clinical criteria of the disease

MM is a plasma cell disorder (Alexander et al., 2007). Plasma cells arise from the lymphocyte lineage of the immune system and develop from differentiated B cells that

can produce antibodies to protect the body from invading pathogens and other potentially harmful foreign bodies (Shapiro-Shelef and Calame, 2005). B and T cells build the adaptive immune system. Whereas T cells enable the cell mediated immune response, B cells mediate the antibody responses. MM is characterized by a clonal expansion of terminally differentiated plasma cells in the bone marrow (BM), the site of origin of hematopoiesis (Alexander et al., 2007). The dysfunctional plasma cells secrete monoclonal immunoglobulins, typically IgG or IgA, which have lost their function in an ongoing immune response as they are not specific for combating any current pathology and do not provide a diverse repertoire but instead are monoclonal. These antibodies now increasingly accumulate in other tissues. This leads in the end to organ damage and failure as well as an increased risk for infection. The immunoglobulins can be measured as paraprotein or monoclonal (M) protein in the serum or as urinary M protein (Bird et al., 2009). Besides the paraprotein level, the so called CRAB criteria (hypercalcemia, renal failure, anemia and bone lesions) are used as basis for the diagnosis. Clinical manifestations include an increased level of calcium release from the bone (hypercalcemia), renal failure due to the accumulation of immunoglobulins, anemia because of the replacement of hematopoietic cells by the tumor cells in the bone marrow as well as the development of lytic bone lesions (Durie and Salmon, 1975; Nakaya et al., 2017).

1.1.2 Plasma cell maturation and function

Plasma cells can have different life spans although long-lived plasma cells only occur in the bone marrow. The BM microenvironment supports plasma cells with growth and survival factors (Moser et al., 2006). Plasma cells can also be found in the red pulp of the spleen as well as the medullary cords of the lymph nodes, where they have both a shorter life span as well as less antibody secretory properties (Shapiro-Shelef and Calame, 2005). The critical receptor for bone marrow homing is CXCR4, which expression is also responsible to the initial transit from the germinal centers of the lymph nodes to the BM (Nagasawa et al., 1996). The importance of the CXCR4 receptor will be discussed later in **chapter 1.1.6.**

Plasma cells are terminally differentiated B cells. During hematopoiesis progenitor B cells arise in the BM. Following the first antigen contact, naïve B cells are activated and undergo further maturation in the germinal center of the lymph nodes (Pape et al., 2003). For the proliferation and survival of the B cells, it is most important that its B cell receptor (BCR) shows a high affinity to the antigens presented by T lymphocytes. During this selection most of the B cells die, when an inappropriate B cell receptor repertoire is presented (Rajewsky, 1996). A large repertoire of BCRs is achieved by genetic recombination of V (variable segment), D (diversity segment, only for the constant chain) and J (joining segment) regions of the immunoglobulin gene (Mckean et al., 1984). Immunoglobulins can either be transmembrane receptors, like the BCR, or secreted as antibodies. They are made up of a heavy and a light chain (two types, κ and λ) which are bound together via disulfide bridges. The antigen recognition part of the antibody is the variable part of the heavy and the light chain. They include three different complementary-determining regions (CDR) with specific amino acid sequence and hence detecting various epitopes of antigens. The initially produced antibody type has always a constant region (Fc fragment) usually composed of IgM and to a lesser extent IgD class isotypes. The ideotype of an immunoglobulin represents the characteristic variable regions of the antigen binding fragment (Fab) (Tonegawa et al., 1978). During VDJ gene rearrangement the basic repertoire of diverse immunoglobulins is produced (Weigert et al., 1978; Weigert et al. 1980). For the improvement of the adaptive immune response, the process of somatic hypermutation and isotope switching ensures higher antigen binding ability (di Noia and Neuberger, 2002). The enzyme activation-induced cytidine deaminase (AID) initiates point mutations in the variable genes for the diversification of the immunoglobulins (Longerich et al., 2006). This process is called affinity maturation. Because of all the mutational steps enabling genetic variability, B cell malignancies are much more frequent than T cell malignancies (Arakawa, 2002).

Activated B cells can either differentiate into memory B cells or into plasma cells. The B cell maturation depends on interaction with T follicular helper (T_{fH}) cells and the antigen presentation of the T helper cells. Here, only if the BCR recognizes the antigen and the CD40 receptor of the B cells is engaged with the T helper cell, the process will continue. In this context, CD278 (ICOS) is a hallmark of T_{fh} cells and serves as co-stimulatory receptor, whereby the programmed death receptor 1 (PD-1) serves as co-inhibitory

receptor in this maturation process (Kerfootet al., 2011). Depending on ICOS expression and the cytokine level secreted by the specific interaction of T_{fh} cells with plasmablasts (differentiation state between activated B cell and plasma cell), the isotype is determined (Mcadam et al., 2001). Besides IgM and IgD, there are also the isotypes IgA, IgE and IgG (Poljak, 1991). The latter comprises the most abundant isotypes in the human immune response and has four subtypes namely IgG1, IgG2a, IgG2b and IgG3 (Schroeder and Cavacini, 2010). Interestingly, the T_{fh} cell reflects with its cytokine signature the ongoing immune response of the T cells leading to the plasma cell maturation. For example, TGF-β induces the IgG2b and IgA isotype, whereas IL-4 induces IgG1 and IgE. Besides IL-4 and TGF-β, also IFN-γ regulates class switching, whereby all three cytokines inhibit the ongoing production of the initial isotype IgM (Odegard et al., 2008). The isotypes differ in their molecular mass, how many antigens they can bind and in their specific life span (Schur et al., 1988). IgG has the longest life span at approximately 20 days (Murphy, 2011).

1.1.3 Plasma cell differentiation and the link to immunomodulatory drugs

The fate of B cell differentiation is dependent on the transcription factors BCL6 and PAX5. These regulators inhibit differentiation into a plasma cell and therefore promote differentiation into a memory B cell (Nutt et al., 1997) (Lin et al., 2003). The key transcription factor for plasma cell differentiation is the interferon regulatory factor 4 (IRF-4), which in turn activates the transcription factor Blimp-1 leading to B cell proliferation, class switching and affinity maturation (Recaldin and Fear, 2016). Another important regulator of plasma cell function is cereblon. Cereblon is a multifunctional protein that can form an active E3 ubiquitin ligase complex (Liu et al., 2014). Cereblon is active in various tissues and cell compartments and its downstream effects vary in these different cell types. This ligase protein is the main reason for the anti-myeloma effects of immunomodulatory drugs (IMiDs) which serve as first line treatment strategy today against MM (Holstein and McCarthy, 2017). IMiDs have a multitude of anti-cancer effects including cytotoxic, anti-angiogenic and immunoregulatory. It was found that cereblon is the main target of thalidomide derivates like the second generation IMiD lenalidomide (Ito et al., 2010). Besides its neuronal and metabolic regulatory functions,

which also serve for the teratogenic effect of thalidomide, it has also important functions in cell proliferation and apoptosis. Cereblon therefore serves as a proto-oncogene and as a biomarker that can predict the IMiD sensitivity of MM patients (Y. X. Zhu et al., 2011). Additionally, in IMiD resistance relapsed/refractory multiple myeloma (RRMM) patients, cereblon expression is often decreased (Franssen et al., 2017). In plasma cells, the downstream signaling targets of cereblon are the B cell transcription factors Ikaros (IKZF1) and Aiolos (IKZF3). These transcription factors induce proliferation and development of plasma cells. Their degradation due to the cereblon associated E3 ubiquitin ligase complex following IMiD application is the main reason for their therapeutic effect (Krönke et al., 2014). Interestingly, the downstream target of IKZF1/3 is the previous mentioned transcription factor IRF-4, which is an essential survival factor for plasma cells. Besides these various effects, cereblon and its downstream substrates IKZF1/3 are also known to suppress CD4+ T cells and IL-2 induction. As IL-2 is an important T cell proliferation and activation cytokine, the immunomodulatory effects of lenalidomide are also at least partly explained by the increased IL-2 production and suppression of CD4+ T cells inhibition (Shi and Chen, 2017).

1.1.4 Plasma cell metabolism and the link to Proteasome Inhibitors

The proteasome is a multi-enzyme complex that degrades polyubiquitinated proteins (Driscoll and Goldberg, 1989). Therefore, the proteasome is essential to regulate a homeostatic protein turnover (proteostasis), clear the cell from unfolded and misfolded proteins and therefore ensures a homeostatic trafficking, supply with amino acids, cell cycle control, growth and differentiation (Goldberg, 2003). Since cancer cells are highly proliferating and often have mutational abnormalities leading to an accelerated protein turnover with a high amount of misfolded proteins, proteasome inhibitors (PIs) were developed as anti-cancer drugs. Additionally, the increased nutritional demand of cancer cells points to a high dependency of the proteasome degradation pathway to help supply that need (Ito, 2020). Indeed, the highest efficiency of PIs is in MM tumor cells compared to cells from other cancer types. This might be due to their production of massive

amounts of immunoglobulins (Cenci et al., 2012). To enable the secretory function, when B cells differentiate into plasma cells, the endoplasmatic reticulum (ER) and Golgi apparatus expand massively to ensure more space and a higher capacity for folding and secreting all the immunoglobulins (Kirk et al., 2010). Interestingly, the life span of plasma cells, which can range from only a few days to several years, also depends on their tolerance to produce and store those proteins. As a high level of immunoglobulin secretion is accompanied by high ER stress, this in general limits the survival of the plasma cell. The loading capacity of the cell is regulated by the UPR and proteasomal activity. Myeloma cells have been shown sensitive to PIs if they have a high workload of immunoglobulin production but a low proteasomal capacity (Auner et al., 2010) (Saavedra-García et al., 2020).

1.1.5 Pathophysiological characteristics of the disease

The initial genetic event of disease development occurs in the germinal center of the lymph nodes where the B cells mature. During isotype (class) switching and somatic hypermutation for high affinity maturation of the antibodies, mutations accumulate over time and form the malignant founder clone (Mlynarczyk et al., 2019). The main myeloma initiating events transforming a healthy long-lived plasma cell to a malignant one are trisomies and chromosomal translocations involving the 14q32 immunoglobulin heavy chain (IgH) locus. The DNA modification mechanisms that actually serve to generate the genetic diversity of B cells, in error result in oncogenes translocating near to the strong enhancer of the IgH locus leading to dysregulation and overexpression of those oncogenes (Prideaux et al., 2014). Most important genes involved and consequently aberrantly expressed are multiple myeloma SET domain containing protein (*MMSET*), fibroblast growth factor receptor 3 (*FGFR3*), cyclin D1 (*CCND1*), cyclin D3 (*CCND3*) and musculoaponeurotic fibrosarcoma oncogene homolog (*MAF* and *MAFB*) (Maura et al., 2019) (Chesi et al., 1997) (Chesi et al., 1996) (Shaughnessy et al., 2001) (Hanamura et al., 2001). Overexpression of these oncogenes is generally associated with poor prognosis, as for example MMSET is a histone methyltransferase regulating cell proliferation, adhesion and suppression of apoptosis while FGFR3 is a

receptor tyrosine kinase mediating cell growth and proliferation (Brito et al., 2009) (Tian et al., 2014).

The first asymptomatic stage of the disease is called monoclonal gammopathy of undetermined significance (MGUS). MGUS is characterized by abnormal proliferation of plasma cells but with the absence of the CRAB criteria and end-organ damage, serum M protein < 3 g/dL and bone marrow plasma cells (BMPC) < 10 % (Kyle and Rajkumar, 1999). Inevitably further mutations accumulate and lead to the high heterogeneity of this cancer type. This the disease progresses to the advanced but still asymptomatic smoldering MM. Smoldering MM (SMM) is mainly distinguished from MGUS by the high risk of disease progression: 10 % of patients with SMM progress to MM in the first 5 years after diagnosis compared to 1 % of the MGUS patients (Waxman et al., 2010). SMM is characterized by plasma cells present in the bone marrow ≥ 10-60 % and serum M protein level ≥ 3 g/dL or urine M protein ≥ 500 mg/day (Lionetti et al., 2021).

High risk groups can be identified via cytogenetic abnormalities and the so-called Mayo Clinic criteria. Patients with chromosomal translocation t(4;14) (encoding for MMSET and FGFR3), t(14;16), t(14;20) (encoding the MAF genes) and del(17;17p) (mainly P53) are considered as high-risk patients with poor prognosis. The cytogenetic classification is of importance for the patient specific treatment strategy especially in regards to immunomodulatory drugs. Furthermore, the risk factors published by the Mayo Clinic to distinguish between low, low-intermediate, high-intermediate and high risk patients include M protein > 1.5 g/dL, non-IgG isotype (IgA or IgM) and the κ and λ free light chain ratio (Sonneveld et al. 2016). Especially the monoclonal immunoglobulin free light chains in the serum serve as biomarker for the prediction of the disease progression from MGUS to MM (Rajkumar et al., 2004). In very rare cases, approximately 1 % of newly diagnosed patients, the initial driver for the disease is a single catastrophic genetic event called chromothripsis (Magrangeas et al., 2011). Chromothrispis is a mutational event whereby localized areas of one or two chromosomes are rearranged in a random order. This chromosome shattering leads to aggressive forms of tumors and correlates with poor prognosis (Luijten et al., 2018).

1.1.6 Role and mechanism of bone remodeling in MM

Almost 80-90 % of MM patients will have osteolytic bone lesions and pathological fractures which increases the risk of death by about 20 % (Melton et al., 2005) (Saad et al., 2007). Bone structure underlies a constant dynamic process of bone remodeling (Frost, 1969). Bone formation is facilitated by osteoblasts and bone resorption is enabled by osteoclasts (Eriksen, 1986). During the progression of myeloma, there is a strong shift to an increased activity of osteoclasts and an inhibition of osteoblasts (Garrett et al., 1987). Consequently, osteolytic bone lesions develop and leading to severe pain, fractures and increased morbidity and mortality (M. A. Dimopoulos et al., 2000). The BM niche includes but is not limited to osteoclasts, osteoblasts, osteocytes, bone marrow stromal cells as well as immune cells (Baccin et al., 2019) (Delgado-Calle et al., 2014). The BM microenvironment, the site of hematopoiesis, is highly dysregulated in myeloma patients (Taube et al., 1992). The malignant plasma cell clones infiltrate the BM and replace the normal hematopoietic cells while simultaneously inducing bone resorptions which further leads to the release of growth factors from the bone matrix leading to a MM cell growth feedback loop. Key signaling pathways in the bone metabolism include RANK/RANKL/OPG, notch, Wnt and NFκB signaling as well as many cytokines like IL-6 and TNF α/β influencing the disease progression (Moser-Katz et al., 2021). In the following section the importance and relevance for MM disease progression is described in more detail.

Progenitor cells of osteoclasts are monocytes and macrophages which differentiate into multinuclear osteoclasts (osteoclastogenesis) (Udagawa, N. et al., 1990). In contrast, osteoblasts and osteocytes are mononuclear cells derived from mesenchymal stem cells (osteoblastogenesis) (Dominici et al., 2004). The latter have a very long life span, do not undergo mitosis and typically die through senescence (Marotti, G. et al. 1990). Osteocytes, which constitute the most numerous cell type in the bone marrow, mainly regulate the activity of osteoclasts and osteoblasts in the bone matrix (Manolagas & Parfitt, 2010). Besides their secretory capabilities, osteocytes modulate the transport of bone material and waste through gap junctions of the bone matrix (Doty, S. B., 1981). The homeostasis of the bone remodeling process includes various complex interacting feedback loops which are highly aberrant in bone associated disorders (Redlich and

Smolen, 2012). The apoptosis of osteocytes for example leads to the secretion of apoptotic bodies which in turn attracts osteocytes to further resorb the bone tissue (Kogianni et al., 2008). Key research investigations in myeloma point to the bone marrow stromal cells (BMSC) as critical players. Osteoclasts express the receptor activator of NFκB (RANK) and are activated by binding of the receptor to its ligand RANKL (Kong, Y. et al., 1999). RANKL is expressed by osteoblasts, bone marrow stroma cells, immune cells and also cancer cells (Mizukami et al., 2002). Osteoprotegerin (OPG) is a decoy receptor for RANKL, that is also expressed on osteoblasts, and its binding to RANK prevents bone resorption (Martin and Sims, 2005). MM cells inhibit the availability of OPG, increase the expression of RANKL and inhibit the apoptosis of osteoclasts both through cell-cell contact as well as cytokine secretion promoting bone destruction and disease progression (Sezer et al., 2003). For example, MM cells highly express syndecan-1 (CD138) which binds OPG and accounts for its lysosomal degradation (Standal et al., 2002). An important regulator of the bone remodeling homeostasis is canonical Wnt signaling. Activation of Wnt signaling prevents β-catenin from degradation and enables its translocation to the nucleus and consequently binding to the transcription factor of the TCF/LEF family which enables β-catenin to facilitate the transcription of the Wnt target genes (Manolagas, 2014). Consequently, this activates osteoblastogenesis in the mesenchymal cell lineage. MM cells are known to secret dickkopf-1 (DKK1) which inhibits canonical Wnt signaling (Niehrs, 2006). Thus, the MM cell derived DKK1 inhibits OPG and enhances RANKL production in osteoblasts therefore attenuating the Wnt signaling and contributing to bone destruction (F. Zhou et al., 2013). DKK1 serum level in patients correlates with the level of bone lesions (Heider et al., 2009).

The successive bone resorption releases various growth factors from the bone matrix including TGF-β, calcium and extracellular matrix proteins which further stimulates MM cell survival and growth (Kolb and Bussard, 2019). The adhesion of MM cells to the BMSC is essential for their growth and survival. MM cells stimulate mesenchymal stromal cells to secrete Interleukin-6 (IL-6) which additionally promotes osteoclastogenesis. The enhanced level of IL-6 in the MM bone marrow microenvironment enables MM cell growth and development as well as the inhibition of apoptosis (Gunn et al., 2006). IL-6 is produced in the acute phase of infection or tissue injury and works as warning signal

and bridge between the innate and adaptive immune system. IL-6 positively regulates inflammation, immune response and hematopoiesis. Constant activation of IL-6 is accompanied by chronic inflammation which serves as basis for many diseases including cancer and autoimmunity (Tanaka et al., 2014).

Another important pathway for plasma cell development is the CXCR4/CXCL12 signaling pathway. The interaction of CXCR4 (C-X-C motif chemokine receptor 4) with its ligand CXCL12 (stromal cell derived factor 1α; SDF-1α) regulates adhesion, retention and homing to the BM as well as proliferation, migration, invasion, dissemination, metastasis and drug resistance in many solid cancer types including breast, pancreatic and lung cancer (Espinoza-Sánchez et al., 2018) (Salazar et al., 2014) (Weekes et al., 2012) (Y. C. Wu et al., 2016). In the context of MM, CXCL12 is secreted by osteoblasts, bone marrow stromal and endothelial cells, whereas CXCR4 is strongly expressed on MM cells. Therefore, CXCL12 serves as a chemokine for attracting MM cells to the BM or to different sides of bone marrow niches while promoting tumor progression and aggressiveness by its binding to CXCR4 (Alsayed et al., 2007).

CXCR4 is known for its pro-tumorigenic properties and high expression of CXCR4 on MM cells is associated with poor prognosis (Darash-Yahana, M. et al., 2004). Due to its interaction with integrins and adhesion molecules, it provides a protective BMSC microenvironment for the cancer cells and promotes relapse and a refractory phenotype (Ullah, 2019a). Additionally, vascular endothelial growth factor (VEGF) is secreted, which enables angiogenesis and also tumor growth (Marzo et al., 2016). This is of major importance since the BM microenvironment is a highly hypoxic niche and acts as a regulator of metastasis (Johnson et al., 2017). Both, the hypoxia inducible factor 1α (HIF-1α) and the CXCR4/CXCL12 signaling axis promote the epithelial-to-mesenchymal transition (EMT) phenotype and its reverse phenotype mesenchymal-to-epithelial transition (MET) of the cancer cell and initiates the metastatic cascade (Mathieu et al., 2011). Thereby, matrix metalloproteinases (MMPs) are secreted and degrade the extracellular matrix (ECM) to promote migratory capabilities (Kessenbrock et al., 2010). The EMT-MET formation is associated with increased gene expression of the pro-metastatic transcription factors zinc finger E-box-binding homeobox 1 (Zeb1) as well as the zinc finger protein transcription factors snail, slug and twist (Drake et al., 2009)

(Zheng et al., 2014). Interestingly, both HIF-1α and CXCR4 activate osteoclastogenesis and in turn HIF-1α has activating capacities for the CXCR4/CXCL12 signaling pathway (M. Guo et al., 2014) (Hulley et al., 2017). The dissemination of cancer cells due to the degradation of the ECM by MMPs allows mobilized cancer cells with the obtained mesenchymal phenotype not only to establish in other BM niches, but also to enter the blood stream to reach distant organs. This advanced and aggressive disease stage in MM with tumor cell manifestations outside the bone marrow is called extramedullary disease (EMD) (Azab et al., 2012).

1.1.7 Importance of the tumor immune microenvironment

In addition to the interaction of B cells and CD4+ T cells during plasma cell development, other T cell subpopulations also play an essential role during tumorigenesis. The homeostatic microenvironment of immune cell regulation is essential for the health of each organism (Noonan & Borrello, 2011). Highly regulated mechanisms are responsible for the balance of effective and specific immune responses, either against invading pathogens or aberrant host cells such as cancer cells. These control mechanisms prevent an overactive immune response to avoid autoimmunity or severe allergic reactions (Shalapour & Karin, 2015). CD4+ helper T cells and CD8+ effector T cells are the center of the adaptive cellular immune response. In contrast to B and plasma cells, which serve for the humoral response, T lymphocytes are the main actor of the cellular immune response. Depending on their differentiation, which is strongly dependent on their cytokine microenvironment and cell surface receptor interactions, they can develop either into an immunosuppressive or a pro-inflammatory phenotype (Vazquez et al., 2015). Pro-inflammatory cytokines include but are not limited to IL-1β, IL-2 and TNFα and promote effector cell proliferation, whereas IL-4, IL-10 and TGF-β promote differentiation into immunosuppressive cell types such as regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSC). A remarkable phenomenon is the shift from pro-inflammatory Th1 to immunosuppressive Th2 cells responses, M1 to M2 macrophage polarization and type 1 Natural Killer T cells (iNKT) to type 2 NKT cells (Lindau et al., 2013) (Mirlekar, 2022). It is well known that in many cancer types this homeostatic balance is highly dysregulated and shifted towards the immunosuppressive

pro-tumorigenic microenvironment. Due to genetic and environmental factors, a chronic inflammatory tissue with a shift towards immunosuppressive cells clearly severs a basis for the establishment of many diseases including cancer (Fisher et al., 2014). Especially in MM, where the cancer cell is an immune cell, the immune system of patients is highly dysregulated, in which an increasing immunosuppressive milieu correlates with advanced disease stage (Díaz-Tejedor et al., 2021). The reasons for this imbalance are as complex as the immune system with all its interactions. Therefore, the focus here is on the interaction of MM cells with T cells, NKT cells, dendritic cells (DCs) and the BMSC.

1.1.8 The process of cancer immunoediting

The intensively studied process of tumor cell interaction with immune cells to promote a pro-tumorigenic microenvironment is called cancer immunoediting. This escape of tumor cells from immune cell attack in an established tumor tolerant immunosuppressive microenvironment is clearly one of the hallmarks of cancer. In almost all diagnosed cancers such a loss of anti-tumor immunity can be observed. The process of cancer immunoediting can be divided into three steps: (i) elimination, (ii) equilibrium and (iii) escape. **Fig. 1.1** illustrates the process of cancer immunoediting (Dunn et al., 2002).

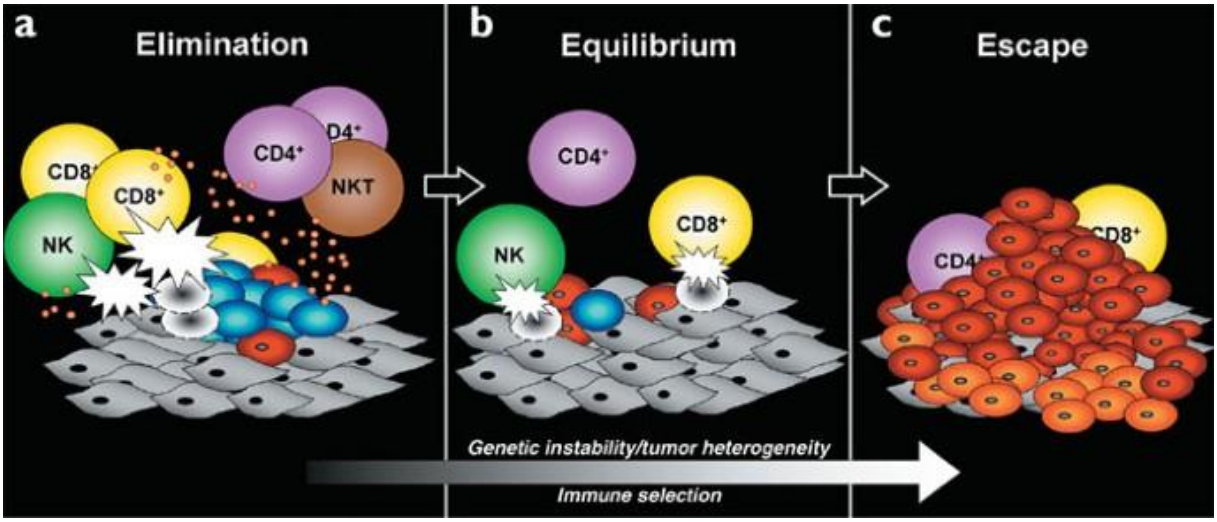


Figure 1.1: Cancer immunoediting. The three steps of cancer immunoediting comprise: **a)** the elimination phase, which is known as immunosurveillance, **b)** the equilibrium, which characterizes the balance of tumor cell clearance and tumor cell clone selection, and **c)**

demonstrates the escape phase, where the tumor cells overgrow the host cells in an uncontrolled manner (Dunn et al., 2002).

Decades ago the elimination phase was considered as tumor immunosurveillance (immune surveillance). During the homeostatic interplay of cells of the adaptive and innate immune system, cells constantly scan all tissues for malignant transformed cells and sufficiently eliminate these aberrant host cells by detecting specific surface antigens. New emerging tumor cells display antigens which are well recognized and presented by antigen presenting cells (APCs) such as M1 polarized macrophages and DCs (Smyth et al., 2001). Via the major histocompatibility complex I (MHC-I) antigens are effectively cross-presented by DCs to activate effector lymphocytes like CD8+ T cells and NK cells. These cells kill the aberrant host cells via granzymes, perforine and establish an anti-tumor microenvironment with cytotoxic cytokines (e.g. IFN type I). Furthermore, they recruit additional immune cells and stimulate their proliferation by bioactive cytokines (e.g. IL-1, IL-2) (Labani-Motlagh et al., 2020). In addition, DCs present lipid antigens via the unconventional MHC like receptor CD1d to NKT cells. NKT cells are a small subpopulation of T cells which also display NK cell functions (Joyce et al., 2011). They show rapid effector function by the secretion of perforin, granzymes and IFN-γ secretion, but have also highly regulatory functions. Their cytokine repertoire is extremely broad and so they can induce either an anti-tumorigenic or a tumor-tolerant microenvironment. NKT cells express both a TCRβ-CD3 complex as well as the typical NK cell receptors like CD16 and NKG2D. NKT cells bridge the adaptive and innate immune system by activating T cells, NK cells, macrophages and DCs (van Kaer et al., 2011). The interaction of NKT cells with DCs via CD40-CD40L activates DCs to secrete IL-12 which in turn increases IL-2 production and further activates iNKT cells (Nowak et al., 2013). Their fine tuning role is apparent by their capability to induce both Th1 and Th2 cell responses. Typically, iNKT cells display an antitumor immunity and induce Th1 cell phenotype development, whether type II NKT cells in general induce the shift to a tolerant Th2 phenotype. Therefore, NKT cells display a powerful part in the antitumor immunity in the elimination phase of tumor immunosurveillance (Krijgsman et al., 2018).

Following the continuous interaction of MM cells with immune cells, the phenotype of effector T cells shifts to a less functional and exhausted phenotype that is characterized

by extensive expression of different co-inhibitory receptors including PD-1 (Dong et al., 2002). The elimination phase now becomes an equilibrium between tumor cell elimination and tumor cell escape. The chronic activation of both co-stimulatory and co-inhibitory signals of the T cell, which serve as second signal upon TCR activation, which is necessary for their proliferation, lead to a tumor tolerant phenotype (Willimsky and Blankenstein, 2005). Besides the exhausted T cells and other effector cells, the number of senescent T cells also increases (Ramello et al., 2014). The tumor microenvironment starts to shift from an anti-tumor to a pro-tumorigenic microenvironment. While the tumor cells upregulate co-inhibitory receptors and secrete anti-inflammatory cytokines to suppress the antitumor responses and recruit additional immunosuppressive cells, the immune cells edit the tumor cell clones (Khong and Restifo, 2002). Comparable to the Darwinian evolution of the survival of the fittest, only those cancer clones which are best adapted to the tissue in which they grow and bear a phenotype which allows them to hide from the immune attack, will survive. These clones display a decreased immunogenicity that allows them to hide from the immune system. Here, in one mechanism for tumor dormancy, they survive in a state of dormancy which underlines why the equilibrium stage is also referred to tumor cell dormancy (Schreiber et al., 2011).

1.1.9 Role of co-inhibitory receptors in cancer immunoediting and immunotherapy

The intensity of an immune response is dependent on the overall input of co-stimulatory and co-inhibitory receptor signals upon the first stimulus- for T cells through the engagement of the TCR with an antigen. Only if the amount of co-stimulatory receptor signallings outweighs the signalling by co-inhibitory receptors and consequently the third activation signal of stimulating cytokines is induced, the effector cell is activated and reacts with an anti-tumor response. If not, the effector cell response is dampened and the cells die or differentiate into immunosuppressive phenotypes such as Tregs with a characteristic tumor tolerant phenotype (Chen and Flies, 2013). The constant activation of co-inhibitory receptors leads to an exhausted and anergic phenotype. This fine tuning mechanism of immune response control is very important for the overall homeostasis of

various tissues. Cancer cells use this phenomenon and upregulate co-inhibitory receptors to dampen the immune response and hide from the immune attack (Wherry and Kurachi, 2015). The knowledge of this phenomenon opened the door for immunotherapy. In terms of MM, the most important co-inhibitory receptors, also called checkpoints, are the programmed death ligand 1 (PD-L1, B7-H1, CD274) and the T cell immunoglobulin and ITIM domain (TIGIT) (Tamura et al., 2013) (Papadas and Asimakopoulos, 2018). Tumor cells of patients with advanced MM display highly upregulated PD-L1 expression on their cell surface in the BM and peripheral blood. On the other hand, TIGIT is expressed on the surface of T cells and competes with its counterpart DNAM for the binding to CD155 or CD112 on tumor cells and APCs (Yadav et al., 2016). This mechanism is similar to that of one of the cytotoxic T lymphocyte associated protein 4 (CTLA-4) competing with CD28 on T cells for the engagement to CD80 and CD86 on APCs (Ge et al., 2021). In contrast, PD-1 which is expressed on hematopoietic cells like T cells, binds either to PD-L1, which is typically highly expressed on MM cells or PD-L2, which displays tissue specific expression and is less common on MM cells (Ray et al., 2015). Following the shift to the overexpression of immune checkpoints, the tumor microenvironment becomes increasingly immunosuppressive. This includes both, the differentiation to immunosuppressive cell types like Tregs and the further recruitment of tumor tolerant immune cells like MDSC. These cells are attracted by chemokines such as IL-6, IL-10, TGF-β and VEGF and further promote the tumor cell growth (Lopes et al., 2021).

Immunosurveillance is facilitated by macrophages and DCs which constantly scan the body for foreign structures and engulf pathogenic invaders, infected or aberrant cells. Upon phagocytosis of these potentially dangerous cells, they process the proteins and present the peptide fragments to other immune cells to maintain a homeostatic environment and mount an effective immune response (Savina and Amigorena, 2007). In order to fulfill these tasks, an important mechanism for myeloid cells, and especially macrophages, is the CD47-SIRPα signaling pathway (Seiffert et al., 2001). The expression of the transmembrane receptor CD47 on various hematopoietic and non-hematopoietic cells serves for the discrimination between self and non-self-structures (Oldenborg et al., 2000). Interestingly, senescent cells display less CD47, which enables macrophages to migrate towards these cells and phagocytose them (Lv et al., 2015).

Hence, CD47 serves additionally as an immune checkpoint and provides a “don´t eat me” signal especially to macrophages. This ensures that healthy normal cells are protected from the host immune system (Logtenberg et al., 2020). Myeloid cells, including macrophages, express the signal regulatory protein α (SIRPα) which mediates phagocytosis and migration due to its C-terminal intracellular signaling domain. The latter comprises an ITIM motif which becomes phosphorylated upon receptor ligation and recruits the tyrosine phophatases SHP-1 and SHP-2. This interaction inhibits the capabilities of macrophages to migrate and phagocytose (Barclay and Brown, 2006). In contrast, if the signaling pathway is weakened due to the missing signal of CD47, macrophages are unleashed to phagocytose their target cell. Many tumors, including myeloma, exploit this immune checkpoint mechanism by overexpressing CD47 to evade the anti-tumor immune attack (Gu et al., 2018) (Kim et al., 2012).

At some point during disease progression, the effector immune cells are impaired to the extent, that it permits tumor escape. Here, the high heterogeneity of the tumor cells serves to allow the immune selection of those cancer clones which are most effective in hiding from the immune system. In parallel, there is also selection for the immune cells primarily select for those cancer clones with the minimal immunogenicity. Together this selects for increased aggressiveness and immune escape (Beatty and Gladney, 2015). In this context, interferons secreted by effector cells are an important driver for cancer immunoediting, since interferons are strongly cytotoxic and anti-proliferative cytokines. Hence, highly immunogenic cancer clones are eliminated whereas the most resistant clones are untouched with the potential for future outgrowth (von Locquenghien et al., 2021). These malignant clones adapt by downregulating antigens, MHC complexes and increasing signaling pathways for survival by preventing effective induction of apoptosis (Dhatchinamoorthy et al., 2021). This clonal expansion of the malignant plasma cell then overwhelms the immune cells and establishes as active MM which can be clinically observed. In the case of treated patients, the escape phase represents the stage of refractory and relapsed patients (RRMM) (Nakamura et al., 2020a). Based on this elaboration, it appears obvious, that the modulation of the immune system is a highly promising potential option for therapy of MM. Nonetheless, the more advanced and dysregulated the TME already is, the more difficult might it be to achieve patient cure.

The high complexity of cell-cell interactions and redundancy of key receptors and cytokines explains the failure of various therapeutic approaches. Therefore, it is of main importance to diagnose cancer types in early stages before they are clinically measurable and treat the patients as precise as possible to prevent on-target off-tumor effects and adverse events to enable a sufficient anti-tumor response. One example in this context of MM therapy are the highly immunoregulative IMiDs, which ensure various and at the same time precise immunomodulatory actions to restore the appropriate microenvironment (Dhodapkar, 2016) (McCachren et al., 2021).

1.1.10 Molecular mechanism: PD-1/PD-L1 signaling

PD-1 (CD279) is a 288 amino acid transmembrane protein responsible for the maintenance of peripheral tolerance of self-antigens and for the limitation of the intensity and duration of immune responses (Boussiotis, 2016). PD-1 is mainly expressed on effector immune cells such as T, B and myeloid cells (Buchbinder and Desai, 2016). Following the recognition of an antigen, PD-1 is presented by the MHC of APCs building the immunological synapse. The immunologic synapse is the nano-scaled gap between T cell and APC interaction including TCR and antigen, adherence molecules as well as co-stimulatory and co-inhibitory receptors that determine the immunological response (Pentcheva-Hoang et al., 2007). As the second signal after T cell induction, the ligation of the co-inhibitory receptors determine the differentiation fate of the immune cell by interfering with the TCR signaling pathway. In consequence, there is modulation of the resulting cytokine signature, which serves as a third activation signal. All three activation signals must be given for T cell activation, otherwise the cell becomes anergic or apoptotic (Kaech and Cui, 2012). Extracellular PD-1 has an immunoglobulin-like domain. The intracellular cytoplasmatic domain consists of an immunoreceptor tyrosine based inhibitory motif (ITIM) and an immunoreceptor tyrosine switch motif (ITSM). These tyrosine residues are phosphorylated upon ligation of the receptor to one of its ligands. As a consequence, the src-homology 1 (SH1) and src-homology 2 (SH2) domain containing phosphatases are recruited to the ITSM, whereby especially the SH2 domain containing phosphatase SHP-2 is responsible for the downstream signaling pathway attenuation (Chemnitz et al., 2004). Focusing on T cells, following the formation of the

immunological synapse by engagement of the TCR-CD3 with the antigen-MHC complex, two main signaling pathways are activated leading to cell cycle progression, proliferation and differentiation towards effector cell functions: (i) the PI3K/Akt/mTor and (ii) the Ras/MAPK/Erk signaling pathway (J. Chen et al., 2016) (Hwang et al., 2020). PI3K/Akt/mTor signaling is negatively regulated by the phosphatase and tensin homolog (PTEN). In the context of T cell activation, IL-2 serves in an autocrine manner to increase the proliferation and pro-inflammatory capabilities of the appropriate T cell. In a paracrine way, it recruits and activates adjacent T cells. If PD-1 is engaged following TCR activation, the recruited SHP-2 interferes with the downstream TCR signaling pathway, activates PTEN and therefore abolishes the activation of Akt. As a consequence, cell cycle arrest is induced and IL-2 production is inhibited (Buckler et al., 2008) (Long and Adler, 2006). The other main intracellular inhibited pathway following PD-1 engagement is that one of Ras/MAPK/Erk signaling. This abrogation results in a metabolic reprogramming by downregulation of glycolysis and promotion of the fatty acid oxidation. The change in metabolism in turn promotes differentiation into exhausted or regulatory T cells instead of effector or memory T cells (Patsoukis et al., 2015). **Figure 1.2** illustrates how PD-1 controls the effector phase of the immunological response following antigen contact.

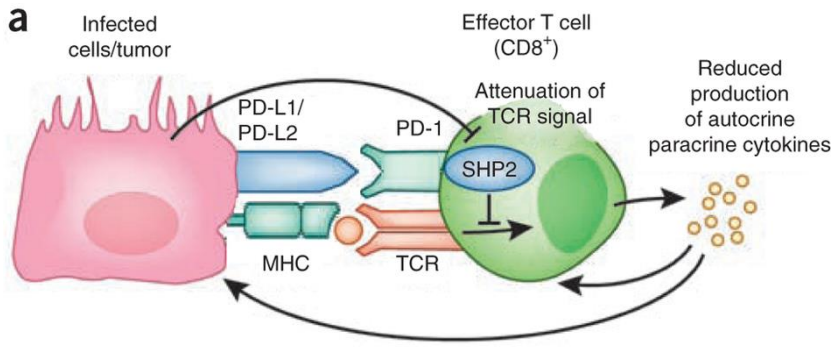


Figure 1.2: Mechanism of PD-1/PD-L1 mediated immunosuppression. PD-1 expressed on CD8 (and CD4) T cells regulates the effector function following the formation of the immunological synapsis. The binding of PD-1 to its ligand PD-L1 or PD-L2 attenuates the TCR signaling pathway and hence inhibits the cytokine expression which is necessary for the complete activation of the immune cell. Modified from Okazaki et al., 2013.

Programmed death ligand 2 (PD-L2, B7-DC) is typically inducible expressed tissue specific on e.g. activated macrophages and dendritic cells. In contrast, PD-L1 is constitutively expressed on hematopoietic cells and many non-hematopoietic cells and can be upregulated to higher levels upon immune cell activation (Butte et al., 2007). PD-L1 is a type I transmembrane glycoprotein of the immunoglobulin superfamily. Although there have been intense studies of the PD-1 receptor and downstream signaling, surprisingly less is known about the intracellular signaling of PD-L1. One reason for this lack of knowledge is the absence of an obvious cytoplasmatic signaling domain (Patsoukis et al., 2020) (Wen et al., 2021). Nevertheless, mass spectrometry studies have revealed that the intracellular domain of PD-L1 includes at least three potential motifs for signal transduction. Whether they serve through direct interaction, phosphorylation or the recruitment of adapter proteins remains to be identified (Gato-Cañas et al., 2017a). But several studies lead to the assumption of an intracellular, PD-1 independent, pro-survival function of PD-L1 enabling resistance and a protective shield against pro-apoptotic signals (Azuma et al., 2008) (Clark et al., 2016a) (Escors et al., 2018a).

1.1.11 Epigenetic changes are of crucial importance during MM disease progression

Besides the aforementioned molecular changes in the tumor microenvironment, the epigenome of MM patients displays multiple aberrations contributing to disease progression. Epigenetic processes describe the reversible genomic mechanism that modulates gene expression without altering the DNA sequence and hence builds the link between the genotype and the phenotype of a cell. Quite common in various cancer types, including MM, is an altered activity of chromatin modifying enzymes prone to lead to genomic instability, cell cycle progression and malignant transformation (de Smedt et al., 2018). In MM, especially patients with high risk genetics including the t(4;14) occurring in almost 15-20% of MM patients, is associated with increased expression of the epigenome modifying enzymes MMSET and EZH2 (Stec et al., 1998) (Walker et al., 2011). During MM disease progression, changes in the epigenome result in an overall hypoacetylation and hypermethylation of tumor suppressor genes. As a consequence, increased gene activity of oncogenes and inhibition of tumor suppressor genes are

crucial drivers of the disease (Agirre et al., 2015) (Heuck et al., 2013). The epigenetic regulation machinery is compost of counteracting chromatin modifying enzymes and various non-coding microRNAs. The main regulatory mechanisms are DNA methylation and the posttranslational modifications of histone complexes. The latter comprise reversible acetylation, methylation, ubiquitination, phosphorylation and SUMOylation of the N-terminal tail protruding from the nucleosome (Bannister and Kouzarides, 2011). DNA methylation has major impact on genome stability and gene expression patterns (Sriraman et al., 2020). Even though it often serves for the repression of genes, it is now known that both, the degree of methylation as well as the position of the amino acids methylated is crucial for the overall output. Furthermore, the different epigenetic modifications influence each other to regulate the chromatin structure (Barski et al., 2007). Similar to the histone modifications, the DNA methylation process is reversible, demonstrating the meaningful plasticity of the epigenome. In general, DNA methylation occurs on the cysteine residues of cysteine-phosphate-guanine (CpG) residues at promotor sides or at the beginning of exons. These methylation hot spots often cluster in so called CpG islands (Bestor, 2000). The DNA methyltransferases MMSET and EZH2, which are often overexpressed in MM patients in advanced disease, transfer methyl groups especially to tumor suppressor genes leading to their gene silencing (Martinez-Garcia et al., 2011) (Pawlyn et al., 2017). Promoter hypermethylation of cyclin dependent kinase inhibitors is seen in up to 40% of MM patients. Today, intensive research focuses on epigenetic modifiers as anti-cancer drug targets, with DNA methylation inhibitors and histone deacetylation inhibitors entering clinical applications (K. Dimopoulos et al., 2014). Histones are nucleic acid packaging proteins with 147 bp DNA wrapped around an octamer made up of a pair of the histones H2a, H2b, H3 and H4 building together the nucleosome (Davey et al., 2002). Histone acetylation is an essential regulatory mechanism to switch between the open euchromatin gene structure, allowing active gene transcription, and the dense packed heterochromatin structure, which is responsible for gene silencing (Eberharter and Becker, 2002). In MM and among other cancers, the alteration of histone acetyltransferases (HAT) and histone deacetylases (HDACs) increasingly shifts during disease progression. HATs transfer acetyl groups from Acetyl-CoA to the positively charged lysine residues of histones and thereby contributing to neutralization of charge, which results in a more open chromatin

structure. In contrast, HDACs remove the acetyl group from the histone, unleashing the positive charged lysine residue that binds tightly to the negative charged phosphate backbone of the DNA. The tight binding finally facilitates the inhibition of gene transcription (Harada et al., 2016). There are several HDACs consisting of 18 family members and four different classes described in man (Clocchiatti et al., 2011). Besides histones, HDACs and HATs also modulate other proteins such as chaperones, DNA repair proteins and transcription factors (Spange et al., 2009). Upregulation of HDACs in MM is correlated with decreased progression free and overall survival (Mithraprabhu et al., 2014). Therefore, HDAC inhibitors are in clinical use for relapsed and refractory patients who received at least two prior therapies including IMiDs. The pan-HDAC inhibitors panobinostat and vorinostat are FDA approved and have demonstrated tolerable safety profiles and improved therapeutic outcome (Afifi et al., 2015). Clinical studies showed the beneficial use of panobinostat, even though it is not sufficient as monotherapy for most of the patients (Laubach et al., 2015). It is worth mentioning that especially pan-HDAC inhibitors have additional modulating capacities as the broad blockade of the deacetylases also interferes with other proteins besides histones. In the context of MM, one side effect is the induction of the PD-L1 gene by interfering with the IFNγ pathway (Terranova-Barberio et al., 2016) (West et al., 2014).

In MM cells, cyclin D is often overexpressed. Cyclin D promotes cell cycle progression and thus tumor cell proliferation (Zhan et al., 2006). HDAC inhibitors promote cell cycle arrest in the transition from G1 to S phase inhibiting growth of the malignant cells. Involved in this regulatory process is induction of the tumor suppressor p21, a cyclin dependent kinase inhibitor (Bernhart et al., 2017). HDAC inhibitors work synergistically with other drugs approved for MM. One reason is the open gene structure which makes the cells more susceptible to other interfering drugs including the DNA alkylating agent cyclophosphamide (Cosenza and Pozzi, 2018). Additionally, HDAC inhibitors work synergistically with IMiDs as they both induce apoptosis in the malignant cells (Hideshima et al., 2015). But especially the combination of HDAC inhibitors with the proteasome inhibitor bortezomib, also in bortezomib refractory patients, has been shown to increase the therapeutic outcome (Ocio et al., 2010). HDAC6 mediates the aggresome protein degradation pathway via the lysosome. Ricolinostat is a selective HDAC6 inhibitor and showed promising results in phase II clinical trials (Imai et al.,

2019). MM plasma cells have a strongly increased protein turnover, not only because of increased proliferation, a hallmark for cancer in general, but additionally due to the massive production of immunoglobulins. Therefore, the survival of the cells is strongly dependent on the ubiquitin degradation pathway. Here, misfolded or unfolded proteins are ubiquitinated and consequently degraded either by the proteasome or by the aggresome. If this unfolded protein response (UPR) pathway is inhibited, for example by the proteasome inhibitor bortezomib, the cells become apoptotic. A salvage pathway is the protein degradation by the aggresome and lysosome, but this can be blocked via the HDAC inhibitors leading to consequent protein overload and tumor cell death (Catley et al., 2006).

2. Materials and Methods

2.1 Materials

2.1.1 Laboratory instruments

Instrument	Company
CO_2 incubators HeraCell 240i	Thermo Scientific, Waltham MA, USA
Centrifuge Allegra X-15R	Beckman Coulter, Krefeld, Germany
Centrifuge Heraeus Multifuge 3SR+	Thermo Scientific, Waltham MA, USA
Centrifuge 5417R	Eppendorf AG, Hamburg, Germany
Centrifuge 5427R	Eppendorf AG, Hamburg, Germany
Centrifuge PMC-060 Capsulefuge	Tomy, Woonsocket, US
Flow cytometer BD FACS Canto II	BD GmbH, Heidelberg, Germany
Flow cytometer Navios	Beckman Coulter, Krefeld, Germany
Neon™ Transfection System	Invitrogen, Carlsbad CA, USA
Fluorescence microscope CKX41	Olympus, Düsseldorf, Germany
Laminar flow hoods KSP18	Thermo Scientific,

	Waltham MA, USA
Vortexer Vortex-Genie 2	Thermo Scientific, Waltham MA, USA
Thermomixer comfort	Eppendorf AG, Hamburg, Germany
Waterbath GFL	LAUDA, Lauda-Königshofen, Germany
Shaker plate Titramax 100	Heidolph, Schwabach, Germany
Shaker plate BioMixer	Benchmark Scientific, Sayreville, USA
Power supplier PowerPac Basic	Bio-Rad Laboratories, München, Germany
SDS-PAGE chamber	Bio-Rad Laboratories, München, Germany
SDS-PAGE gel casting stand Mini Protean	Bio-Rad Laboratories, München, Germany Germany
Pure water supply Arium pro UV	Sartorius, Göttingen, Germany
Weighing scale BP 2100S	Sartorius, Göttingen, Germany
Spectrophotometer NanoDrop 2000	Thermo Scientific, Waltham MA, USA
Pipettes Research (10 / 20 / 100 / 200 / 1000 µL)	Eppendorf AG, Hamburg, Germany

Piped aid Pipetboy	Integra Biosciences GmbH Biebertal, Germany
QIAxcel	Qiagen, Hilden, Germany
Thermocycler Veriti	Applied Biosystems GmbH, Darmstadt, Germany

2.1.2 Ready to use kits, reagents and marker

Kit	Company
RNeasy Mini Kit™	Qiagen, Hilden, Germany
PureLink Genomic DNA Mini Kit	Thermo Scientific, Waltham MA, USA
Neon™ Transfection System Kit (10 / 100 µL)	Invitrogen, Carlsbad CA, USA
SuperScript III First-Strand	Thermo Scientific, Waltham MA, USA
PrimeScript 1st strand cDNA Synthesis Kit	Takara Bio, Kusatsu, Japan
Pierce BCA Protein Assay Kit	Thermo Scientific, Waltham MA, USA
WesternBright Sirius and WesternBright Peroxide	Advansta Inc, San Jose, USA
BD OptEIA™ Substrate Reagent A / B	BD Biosciences, San Diego CA, USA

Foxp3/Transcription Factor Staining Buffer Set	Thermo Scientific, Waltham MA, USA
DreamTaq Green PCR Master Mix	Fermentas, Waltham MA, USA
DNase I	Sigma-Aldrich, Steinheim, Germany
Collagenase (*Clostridium histolyticum*)	Sigma-Aldrich, Steinheim, Germany
Alt-R® S.p. Cas9 Nuclease V3	IDT Inc., San Diego CA, USA
Rainbow Molecular Weight Marker	GE Healthcare, Freiburg, Germany
PageRuler Prestained Protein Ladder	Fermentas, Waltham MA, USA
Zombie Aqua™ Fixable Viability Kit	BioLegend GmbH, Fell, Germany

2.1.3 Disposables

Item	Company
6 Well Cell Culture Plate	Greiner Bio-One GmbH, Frisckenhausen, Germany
12 Well Cell Culture Plate	Greiner Bio-One GmbH, Frisckenhausen, Germany

24 Well Cell Culture Plate	Corning Inc., NY, USA
24 Well Inserts 8 µm	Sarstedt AG, Nümbrecht, Germany
96 Well Cell Culture Plate	Greiner Bio-One GmbH, Frisckenhausen, Germany
Nitrocellulose Blotting Membrane 0.2 µm	GE Healthcare, Solingen, Germany
PCR Tubes 0.2 mL	Corning Inc., NY, USA
Reaction tubes 1.5 mL	Eppendorf AG, Hamburg, Germany
FACS tubes 5 mL	Sarstedt AG, Nümbrecht, Germany
FACS culture tubes Superclear	Labcon, North America, USA
Reaction tubes 15 mL	Sarstedt AG, Nümbrecht, Germany
Reaction tubes 50 mL	Sarstedt AG, Nümbrecht, Germany
Cryotubes 1.8 mL	Sarstedt AG, Nümbrecht, Germany

Cell culture flasks (T25 / T75 / T175)	Sarstedt AG, Nümbrecht, Germany
Filter Tips (10 / 100 / 200 / 1000 µL)	Nerbe Plus, Winsen, Germany
Pipettes Serological (5 / 10/ 25 / 50 mL)	Corning Inc., NY, USA
Cell Strainer 70 µm	Corning Inc., NY, USA
Canules Sterican 26G	B. Braun Melsungen AG, Melsungen, Germany
Canules Microlance 3 27G	BD GmbH, Heidelberg, Germany
Syringe Omnifix-F 1 mL	B. Braun Melsungen AG, Melsungen, Germany
Filter Millex-GV 0.22 µm	Merck Millipore Ltd, Darmstadt, Germany

2.1.4 Chemicals

Chemical	Company
Tween® 20	Sigma-Aldrich, Steinheim, Germany
Methanol ≥99 %	Carl Roth GmbH,

	Karlsruhe, Germany
Iso-Propanol 100 %	VWR International GmbH, Darmstadt, Germany
Ethanol absolute z. A.	Geyer GmbH, Renningen, Germany Karlsruhe, Germany
Acrylamide/ Bis Solution 37.5:1	Serva, Heidelberg, Germany
APS (Ammonium Peroxodisulfate)	Carl Roth GmbH, Karlsruhe, Germany
TEMED	Serva, Heidelberg, Germany
BSA Protein Standard	Thermo Scientific, Waltham MA, USA
Dimethylsulfoxide (DMSO)	Carl Roth GmbH, Karlsruhe, Germany
Bovine Serum Albumin (BSA)	AppliChem GmbH, Darmstadt, Germany
HEPES Pufferan® ≥ 99.5 %	Carl Roth GmbH, Karlsruhe, Germany
Paraformaldehyde	Carl Roth GmbH, Karlsruhe, Germany
SDS (Sodium Dodecyl sulfate) ≥ 99.5 %	Carl Roth GmbH, Karlsruhe, Germany

7-AAD staining solution	Beckman Coulter, Krefeld, Germany
β-mercaptoethanol	Gibco/ Life Technologies GmbH, Darmstadt, Germany

2.1.5 Cell Culture reagents and antibiotics

Reagent	Company
RPMI-1640 Medium with L-Glutamine	Gibco/ Life Technologies GmbH, Darmstadt, Germany
FCS (Fetal Calf Serum)	Invivogen, San Diego CA, USA
Penicillin-Streptomycin (10.000 U/ mL)	Invitrogen, Carlsbad CA, USA
X-VIVO™ 20 Medium	Lonza, Basel, CH
Dulbecco´s PBS (1x)	Gibco/ Life Technologies GmbH, Darmstadt, Germany
Trypan Blue Solution	Gibco/Life Technologies GmbH, Darmstadt, Germany

2.1.6 Buffers and solutions

Buffer/Solution	Company
Cell Lysis Solution	Promega, Madison WI, USA
10x Running Buffer	250 mM Tris 1.92 M Glycin
RIPA Lysis Buffer	PBS pH 7.0 – 7.3 2 mM EDTA 2 mg/mL Aprotinin 1 mM PMSF 1 mM Na-Orthovanadat 0.1 % (w/v) SDS 0.5 % (w/v) Na-Deoxycholat
3x SDS PAGE Sample Buffer	180 mM Tris HCl 6 % SDS 28 % Glycerol 0.001 % (w/v) Bromphenol blue
Running buffer SDS PAGE	1x Running buffer 0.1 % (w/v) SDS
Transfer Buffer	1x Running buffer

	0.05 % (w/v) SDS
	20 % (v/v) MeOH
10x TBS Buffer	500 mL Tris HCl pH 8.0
	420.75 g NaCl
	ad 5.000 mL dH_2O
Blocking Buffer	1x TBS Buffer
	5 % (w/v) BSA
	0.1 % (v/v) Tween 20
Wash Buffer TBS-T	1x TBS Buffer
	0.1 % (v/v) Tween 20
Fixation Buffer	2 % Paraformaldehyd
	pH 7.0 in dH_2O
Nuclease Free Duplex Buffer	IDT Inc.,
	San Diego CA, USA

2.1.7 Cell lines and Mouse model

Name	Company/Reference
5T33 murine myeloma cells	n/a
C57BL/6J	n/a
C57BL/KaLwRij	Amend et al., 2015
NOD.*scid*.*Il2Rγc*^{null}	Shultz et al., 1995

2.1.8 Drugs

Inhibitor	Company
Panobinostat (LBH589)	Selleckchem, Houston TX, USA
*InVivo*MAb anti-mouse PD-1 (CD279)	BioXCell, Lebanon NH, USA
*InVivo*MAb anti-mouse CD47 (IAP)	BioXCell, Lebanon NH, USA

2.1.9 Antibodies

Antibody	Company
BD™ CompBeads Anti-Rat and Anti-Hamster Igκ	BD Biosciences, San Diego CA, USA
Goat anti-rabbit IgG-HRP	Santa Cruz Biotech, Heidelberg, Germany
Anti-beta Actin Mouse IgG	Abcam plc., Cambridge, UK
Anti-PD-L1 Rabbit monoclonal	Abcam plc., Cambridge, UK
Human PD-L1 Synthetic Peptide	Thermo Scientific, San Diego CA, USA

Rabbit anti-ZEB1 Polyclonal	OriGene Technologies, Rockville MD, USA
TruStain FcX™ (anti-mouse CD16/32)	BioLegend GmbH, Fell, Germany
Anti-Mouse CD274 (B7-H1) PE, Clone MIH5	Thermo Scientific, San Diego CA, USA
Anti-Mouse CD273 (B7-DC) PE, Clone TY25	Thermo Scientific, San Diego CA, USA
Anti-Mouse CD279 (PD-1) PerCP-eFluor 710, Clone J43	Thermo Scientific, San Diego CA, USA
Anti-Mouse GPNMB eFluor 660, Clone CTSREVL	Thermo Scientific, San Diego CA, USA
PE anti-mouse CD154, Clone MR1	BioLegend GmbH, Fell, Germany
PE anti-mouse TCR β chain, Clone H57-597	BioLegend GmbH, Fell, Germany
PerCP/Cy5.5 anti-mouse CD279 (PD-1), Clone 29F.1A12	BioLegend GmbH, Fell, Germany
PerCP/Cy5.5 anti-mouse CD152, Clone UC10-4B9	BioLegend GmbH, Fell, Germany
PE/Cy7 anti-mouse CD3, Clone 17A2	BioLegend GmbH, Fell, Germany
APC/Cyanine7 anti-mouse CD47, Clone miap301	BioLegend GmbH,

	Fell, Germany
APC anti-mouse CD1d (CD1.1, Ly-38), Clone 1B1	BioLegend GmbH, Fell, Germany
APC anti-mouse CD366 (Tim-3), Clone B8.2C12	BioLegend GmbH, Fell, Germany
APC anti-mouse NK1.1, Clone PK136	BioLegend GmbH, Fell, Germany
APC anti-mouse αGalCer:CD1d complex, Clone L363	BioLegend GmbH, Fell, Germany
APC/Cy7 anti-mouse CD8α, Clone 53-6.7	BioLegend GmbH, Fell, Germany
APC/Cy7 anti-mouse CD62L, Clone MEL-14	BioLegend GmbH, Fell, Germany
Brilliant Violet 421 anti-mouse CD4, Clone GK1.5	BioLegend GmbH, Fell, Germany
Brilliant Violet 421 anti-mouse CD184 (CXCR4), Clone L276F12	BioLegend GmbH, Fell, Germany
Brilliant Violet 421 anti-mouse I-A/I-E, Clone M5/114.15.2	BioLegend GmbH, Fell, Germany
PE anti-mouse TIGIT (Vstm3), Clone 1G9	BioLegend GmbH, Fell, Germany
PE anti-mouse FoxP3, Clone MF-14	BioLegend GmbH,

	Fell, Germany
APC/Cy7 anti-human/mouse/rat CD278 (ICOS), Clone C398.4A	BioLegend GmbH, Fell, Germany
PE/Cy7 anti-mouse Ly-6G, Clone 1A8	BioLegend GmbH, Fell, Germany
APC anti-mouse CD223 (LAG-3), Clone C9B7W	BioLegend GmbH, Fell, Germany
APC/Cy7 anti-mouse Ly-6C, Clone HK1.4	BioLegend GmbH, Fell, Germany
APC anti-mouse CD73, Clone TY/11.8	BioLegend GmbH, Fell, Germany
APC anti-mouse CD184 (CXCR4), Clone L276F12	BioLegend GmbH, Fell, Germany
APC anti-mouse CD25, Clone PC61	BioLegend GmbH, Fell, Germany
APC anti-mouse CD274 (B7-H1, PD-L1), Clone 10F.9G2	BioLegend GmbH, Fell, Germany
PE anti-mouse/human CD11b, Clone M1/70	BioLegend GmbH, Fell, Germany
PE anti-mouse CD40, Clone 3/23	BioLegend GmbH, Fell, Germany
PE anti-mouse H-2Kb/H-2Db, Clone 28-8-6	BioLegend GmbH,

	Fell, Germany
PE anti-mouse Galectin-9, Clone 108A2	BioLegend GmbH, Fell, Germany
PE anti-mouse GITR Ligand, Clone YGL386	BioLegend GmbH, Fell, Germany
PerCP/Cy5.5 anti-mouse CD8α, Clone 53-6.7	BioLegend GmbH, Fell, Germany
PerCP/Cy5.5 anti-mouse CD80, Clone 16-10A1	BioLegend GmbH, Fell, Germany
PerCP/Cyanine5.5 anti-mouse CD357 (GITR), Clone DTA-1	BioLegend GmbH, Fell, Germany
PerCP/Cy5.5 anti-mouse CD226 (DNAM-1), Clone 10E5	BioLegend GmbH, Fell, Germany
Pacific Blue anti-mouse CD11c, Clone N418	BioLegend GmbH, Fell, Germany
Pacific Blue anti-mouse NK1.1, Clone PK136	BioLegend GmbH, Fell, Germany
Brilliant Violet 421 anti-mouse CD86, Clone GL-1	BioLegend GmbH, Fell, Germany
PE/Cy7 anti-mouse CD38, Clone 90	BioLegend GmbH, Fell, Germany

2.1.10 Oligonucleotides

Gene, Primer Sequence (5´→ 3´)	Company
Mouse β Actin_for ggg aat ggg tca gaa gga ct	Biomers.net GmbH, Ulm, Germany
Mouse β Actin_rev ttt gat gtc acg cac gat tt	Biomers.net GmbH, Ulm, Germany
Mouse PD-L1_for atg agg ata ttt gct ggc att at	Biomers.net GmbH, Ulm, Germany
Mouse PD-L1_rev tta cgt ctc ctc gaa ttg tgt	Biomers.net GmbH, Ulm, Germany
Alt-R® CRISPR-Cas9 crRNA PD-L1 Exon 3.1 rGrGrC rUrCrC rArArA rGrGrA rCrUrU rGrUrA rCrGrG rUrUrU rUrArG rArGrC rUrArU rGrCrU	IDT Inc., San Diego CA, USA
Alt-R® CRISPR-Cas9 crRNA PD-L1 Exon 3.2 rUrGrC rUrGrC rArUrA rArUrC rArGrC rUrArC rGrGrG rUrUrU rUrArG rArGrC rUrArU rGrCrU	IDT Inc., San Diego CA, USA

2.2 Methods

2.2.1 Cell biology

2.2.1.1 Cell culture

Cell culture procedures were carried out under aseptic conditions using laminar flow hoods. Murine 5T33 myeloma cells were cultivated in RPMI-1640 media containing L-Glutamine and were supplemented with 10 % fetal calf serum (FCS) and 2 % Penicillin/Streptomycin (referred to RPMI media). The cultivation took place in the

Heracell incubator at 37°C and 5 % CO_2. Cells were split every 2-4 days in a ratio of 1:2 to 1:10. 5T33 cells were already stable transduced with GFP which enables live dead discrimination and tracking of the tumor cells *in vivo*.

2.2.1.2 Freezing, thawing and counting of cell lines

Cells were frozen using FCS with 10 % DMSO and stored at -80°C or in liquid nitrogen for long term storage. For each vial $5x10^6$ cells were prepared. Thawing was performed by transferring the cells in 50 mL media and washed for 5 min at 1500 rpm. Cells were transferred in a T25 cell culture flask with prewarmed media.

Cells were counted using Neubauer chambers and dead cell exclusion was performed using trypan blue.

2.2.1.3 Treatment with panobinostat

Phenotyping was performed using $1x10^6$ 5T33 cells in each of a 6 well plate. RPMI media was supplemented with 10 nM panobinostat and cells cultured for 24 h at 37°C and 5 % CO_2. Analyses were compared with cells in DMSO control medium. Before staining the immunoregulatory cell surface receptors, cells were washed with PBS for 5 min at 800 rpm.

2.2.1.4 Cell proliferation assay

Comparison of the proliferation rate of wt, Mock and PD-L1 KO 5T33 cells was performed using the ^{3}H-Thymidine incorporation assay. For each cell line $1x10^4$, $5x10^4$ and $1x10^5$ cells were cultured for 24 h in a 96 well plate in RPMI media and for further 18 h supplemented with ^{3}H-Thymidine that incorporates into the DNA during cell growth. Cells including the tritium were counted using the MicroBeta TriLux.

2.2.1.5 Migration assay

Measurement of migratory capabilities of 5T33 Mock and PD-L1 KO cells was performed using 20 % FCS in RPMI media as attractant for cells plated in RPMI media without supplements on the top of the transwell membrane. RPMI media which is used as culture media (10 % FCS) served as control. Cells were incubated for 4 h at 37°C and 5 % CO_2. The pore size of the transwell membrane was 8 µm and cells which migrated to the bottom were counted using flow cytometry.

2.2.1.6 Generating 5T33 PD-L1 KO cells using CRISPR/Cas9 technology

The gene knock out of PD-L1 in murine 5T33 cells was generated by self-designed guideRNAs (gRNAs) targeting two separate parts of the exon 3 of the *PD-L1* gene (IDT DNA Technologies). The Neon™ Transfection System was used for the delivery of the CRISPR/Cas9 complex by electroporation. The transfection procedure was performed according to the manufacturer´s protocol (IDT DNA Technologies: Alt-R CRISPR-Cas9 System: Delivery of RNP into Jurkat cells (Neon Transfection System)) without the use of an enhancer. The concentration of the Alt-R CRISPR-Cas9 tracrRNA was 100 µM. Cells were transfected in a 96 well plate (U-bottom) using either $2x10^5$ cells 1200 V, 20 ms pulse width and 2 pulses or $5x10^5$ cells 1600 V, 10 ms pulse width and 3 pulses. The cells were cultured at 37°C and 5 % CO_2 for three days and then expanded to be analyzed by flow cytometry. Sorting of the PD-L1 negative 5T33 cells was performed using the FACS cell sorter (BD). Hoechst staining was used to discriminate between live and dead cells according to manufacturer instructions. $1x10^6$ 5T33 cells were sterile stained for PD-L1 cell surface receptor expression for 15 min at RT and washed with PBS. Cells were resuspended in 1 mL PBS and filtrated through a 70 µM membrane to ensure a single cell suspension. The cell sorter separated 5-10 PD-L1 negative, GFP positive and Hoechst negative cells into one well of the 96 well plate (U-bottom) with 100 µL prewarmed full RPMI media. The sorted PD-L1 negative 5T33 cells were cultured at 37°C and 5 % CO_2 to generate a pure PD-L1 negative cell line.

2.2.2 Flow Cytometry

2.2.2.1 Phenotyping and detection of apoptosis

Flow cytometry was used to phenotypically characterize the cells by analyzing the cell surface markers. First, cells were washed with PBS for 5 min at 800 rpm. Approximately $1x10^6$ cells were transferred into FACS tubes or 96 well plates and stained with the fluorescence dye labeled antibodies according to the manufacturer´s protocol. The incubation with the fluorescence dye labeled antibodies was performed for 15 min at room temperature (RT) or 30 min at 4°C in the dark. Cell lines were washed for 5 min at 800 rpm and resuspended 0.3 – 0.5 mL PBS for flow cytometry analysis. The first gate was set to exclude doublets and dead cells as well as cell fragments, which were positive stained for the zombie dye (BioLegend). Percentage and mean fluorescence intensity (MFI) were analyzed with the software FlowJo (Version 10, Ashland OR, USA).

Apoptosis rate was measured following incubation with panobinostat using the DNA fragmentation method (Nicoletti assay). Cells were prepared using a Triton-X containing propidium iodide (PI) buffer. Triton-X permeabilizes the cells and the PI incorporates into the DNA which enables the flow cytometric analysis of the nuclei.

2.2.3 Molecular biology

2.2.3.1 Isolation of nucleic acids

Genomic DNA was isolated using the PureLink Genomic DNA Mini Kit (Thermo Scientific, Waltham MA, USA) according to manufacturer instructions. The DNA was eluted using elution buffer and stored at -80°C. The DNA concentration was measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham MA, USA).

RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. RNase free water was used for the elution process and the RNA was stored at -80°C. The RNA concentration was measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham MA, USA).

2.2.3.2 cDNA synthesis

The transcription of genomic RNA into complementary DNA (cDNA) was performed using the PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Kusatsu, Japan) according to manufacturer instructions. Oligo-dT primer were used for the synthesis. For each reaction 1 µg of isolated RNA were used. The DNA concentration was measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham MA, USA) and stored at -20°C.

2.2.3.3 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was carried out using the Thermocycler Veriti and the DreamTaq PCR Master Mix (Applied Biosystems GmbH, Darmstadt, Germany). Primer were designed using the BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). For the strand elongation 0.5 µg cDNA was used as template. The PCR protocol for the amplification of the transcripts is shown in table 1:

Table 1: qRT-PCR reaction scheme

Step	Duration	Temperature	Cycles
Initial Denaturation	7 min	95°C	1
Denaturation	1 min	95°C	
Annealing	1 min	50-52°C*	x 35
Elongation	1 min	72°C	
Final Elongation	10 min	72°C	1
Stop	∞	4°C	

*depending on primer annealing temperature

The analysis of the PCR products was carried out using the QIAxcel (Qiagen) and BioCalculator® software (Hilden, Germany).

2.2.4 Protein biochemistry

2.2.4.1 Preparation of protein lysates

Frozen cells were thawn and resuspended in the appropriate amount of RIPA lysis buffer. Cells were incubated for 30 min on ice and vortexed regularly. The samples were centrifuges at 14.000 g for 1 h at 4°C. The supernatant was transferred into a new reaction tube and prepared for protein concentration measurement by BCA test or stored at -20°C.

2.2.4.2 Evaluation of the protein concentration

The protein concentration of the cell lysates was measured using the BCA Protein Assay Kit (Thermo Scientific, Waltham MA, USA) according to manufacturer instructions. BSA standard dilution series were prepared as reference to determine the quantification of the protein concentration. The absorption of the BCA reagent was measured at 560 nm using the Gen5-Software (Biotek, Bad Friedrichshall, Germany).

2.2.4.3 SDS-PAGE

The proteins within the cell lysates were separated regarding their molecular size using the Sodium Dodecyl Sulfate Polyacrylamide Gel-Electrophoresis (SDS-PAGE). The SDS enables a constant negative charge of the proteins correlating to their molecular mass. By applying a voltage 100 – 130 V, the denaturated proteins run through the gel matrix in direction of the Anode in dependence of their molecular size. 12 % separation gels were used for the separation of the proteins. Samples were denaturated at 95°C for 5 min and 10 – 20 µg protein were loaded to the gel.

2.2.4.4 Western Blot and immuno detection

Following the SDS-PAGE, the gel was removed to transfer the separated proteins on to a nitrocellulose membrane. A wet-transfer was applied by assembling the necessary

components in the following orientation: Cathode, sponge, Whatman paper, SDS-PAGE gel, nitrocellulose membrane, Whatman paper, sponge, Anode. The tank was filled with ice cold transfer buffer containing 10 % Methanol and the transfer was carried out by applying 250 mA at 4°C for 1 h.

The nitrocellulose membrane including the immobilized proteins was washed with TBS-T three times for 5 min and blocked with TBS-T + 5 % BSA for 1h at room temperature (RT) on a shaker. Membranes were stained with the primary antibodies with dilutions according to manufacturer instructions. The antibody solution was applied in TBS-T + 2.5 % BSA and incubated over night at 4°C on a shaker. Membranes were washed with TBS-T three times for 5 min on a shaker and the appropriate secondary HRP-conjugated antibodies were applied. Incubation with the secondary antibody in TBS-T + 2.5 % BSA was 1 h at RT on a shaker. Membranes were washed with TBS-T three times for 5 min on a shaker. The membrane was moistened with 1 mL Western Bright ECL solution. Chemiluminescence was detected digital using a blot imaging and developer system.

2.2.5 Animal experiments

2.2.5.1 Breeding and housing of laboratory mice

Mice were kept in the animal facility of the HET (Haus für Experimentelle Therapie) of the University Clinic Bonn according to the national regulations for animal experiments. Individually Ventilated Cages were placed under specific pathogen-free conditions as recommended by the GV-SOLAS. C57BL/KaLwRij mice were breed in the HET facility and NOD.*scid*.*Il2Rγc*^{null} (NSG) mice were originally obtained from the Jackson laboratory (Bar Harbor, ME, USA). Animal experiments were approved by the federal office for Nature, Environment and Consumer Protection, North Rhine Westphalia (Landesamt für Natur, Umwelt und Verbraucherschutz NRW; animal experiment protocol 81-02.04.2019.A194).

2.2.5.2 C57BL/KaLwRij mouse model

C57BL/KaLwRij mice have a genetically predisposition to develop MM following i.v. injection of syngenetic murine myeloma cells. Freshly harvested $3x10^5$ 5T33 wt, Mock or PD-L1 KO cells in their log growth phase were resuspended in PBS and injected i.v. on day zero. C57BL/KaLwRij mice mice develop benign idiopathic paraproteinemia (BIP) similar to human MGUS till day 14 post injection. Mice were observed daily from day 14 and analysis were carried out either in the early disease stage three weeks post inoculation or at the disease end stage, when mice develop signs of leg paraplegia or a tumor.

2.2.5.3 NOD.*scid*.*Il2Rγc*^{null} (NSG) mice strain

NSG mice are highly immunodeficient due to mutations in their genetic background rendering them B and T cell deficient. This immunodeficiency makes them eligible as xenograft model. The injection of the 5T33 myeloma cells was carried out in the same way as described above for the C57BL/KaLwRij mice.

2.2.5.4 Drug treatment

Panobinostat was solved according to manufacturer instructions for *in vivo* applications (Selleckchem, Houston TX, USA) and stored at -80°C. Per mouse, 0.4 mg panobinostat was injected i.p. every second day from d 19 post tumor cell inoculation. Anti-PD-1 *InVivo*MAb (BioXCell, Lebanon NH, USA) was solved in PBS and 0.25 mg was injected i.p. every 4 days from d 17 post inoculation. The *in vivo* monoclonal antibody anti-CD47 (BioXCell, Lebanon NH, USA) was solved in PBS and 0.2 mg was injected i.p. every second day from d 17 post inoculation.

2.2.5.5 Organ isolation and preparation

Mice were sacrifized by cervical dislocation to obtain the organs. To obtain the spleen, the abdomen was opened, the spleen was isolated and immediately transferred into a reaction tube with ice cold PBS. The spleen was meshed through a 70 µM cell strainer (Thermo Scientific, Waltham MA, USA). The cell suspension was washed with PBS at 1500 rpm for 5 min at 4°C. Erythrocytes were lysed by incubation with 5 – 10 mL RBC lysis buffer (Promega, Madison WI, USA) for 10 min at RT. The single cell suspension was washed with PBS at 1500 rpm for 5 min at 4°C and prepared by staining for flow cytometry analysis.

The bone marrow of a mouse was taken from both hind legs, the tibia and femur. Bones were carefully opened at one site and centrifuged at 10.000 rpm for 10 sec to flush the bone marrow suspension in a 1.5 mL reaction tube. The cell suspension was resuspended in ice cold PBS and meshed through a 70 µM cell strainer (Thermo Scientific, Waltham MA, USA). Erythrocytes were lysed by incubation of 1 mL RBC lysis buffer (Promega, Madison WI, USA) for 5 min at RT. Cells were washed with PBS at 1500 rpm for 5 min at 4°C and used for staining and flow cytometry analysis.

Lymph nodes were pooled from superficial cervicals, axillary, brachial, mediastinal, thymus and inguinal per mouse. Lymph nodes were transferred into full RPMI media with 1 mg/mL collagenase and 0.1 mg/mL DNase to carefully lyse the organs without damaging the cells. Lymph nodes were incubated 30 min at 37°C and 5 % CO_2. Lymph nodes were carefully meshed using a pipette and incubated again 20 min at 37°C and 5 % CO_2. The cell suspension was resuspended in PBS and flushed through a 70 µM cell strainer (Thermo Scientific, Waltham MA, USA). Erythrocytes were lysed by incubation of 1 mL RBC lysis buffer (Promega, Madison WI, USA) for 5 min at RT. Cells were washed with PBS at 1500 rpm for 5 min at 4°C and used for staining and flow cytometry analysis.

Spleens were meshed and the cell suspension was resuspended in PBS and flushed through a 70 µM cell strainer (Thermo Scientific, Waltham MA, USA) till further organ processing.

Staining for flow cytometry analysis was carried out as described for cell lines (**section 2.2.2.1** Phenotyping). TruStain FcX (BioLegend GmbH, Fell, Germany) was added to each antibody master mix according to manufacturer instructions to minimize unspecific bindings. Cells were fixed in 2 % paraformaldehyde for 30 min at RT and washed with PBS at 1500 rpm for 5 min at 4°C.

2.2.6 Statistical analysis

Statistical significance was determined by the unpaired two-tailed student *t* test as well as 2way ANOVA (GraphPad Prism, San Diego CA, USA). All results were presented as ± standard error of the deviation (SED) and significance was defined as p-value of <0.05. For *in vivo* survival analysis the Log rank test (Mantel-Cox analysis) was applied.

3. Results

3.1 Characterization of 5T33 cells and the generation of 5T33 PD-L1 KO cells

During the progression of MM, increased PD-L1 expression on tumor cells is an important factor that enables the establishment of a tumor tolerant immune microenvironment. As patients with more progressed MM show strong expression of PD-L1 in tumor and peripheral blood samples compared to healthy control groups, we sought to investigate whether the *knockout* of PD-L1 on myeloma cells would diminish their capacity to downregulate the anti-tumor response *in vivo* and thus limit tumor growth.

3.1.1 5T33 cells express PD-L1

In order to characterize the 5T33 myeloma cells, we first analyzed the surface expression of PD-L1 using flow cytometry. The 5T33 cell line we use was already stably transduced with GFP, which enables both tumor cell tracking *in vivo*/ *ex vivo* and characterizes living cells *in vitro*. As seen in **fig. 3.1**, at least 97 % of 5T33 cells highly express PD-L1.

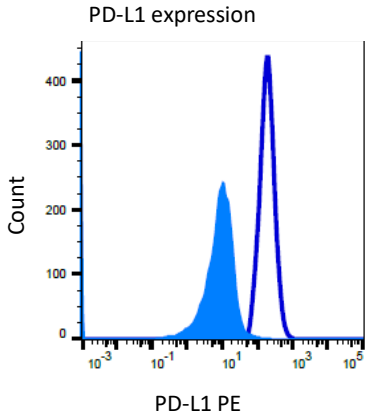


Fig. 3.1: 5T33 cell surface expression of PD-L1. 97,3 % of 5T33 cells express PD-L1 measured by flow cytometry. The left peak shows the IgG isotype control, the right peak shows PD-L1 positive cells. Result shown is representative for the experiments.

3.1.2 Generating the PD-L1 KO using CRISPR/Cas9 technology

There are several methods for generating a gene KO, one effective method is the very specific clustered regularly interspaced short palindromic repeats (CRISPR) system.

In order to deplete the target gene PD-L1, the CRISPR approach was chosen by designing a mix of guideRNAs (gRNAs) which were added together with the activated Cas9 as a complex to the cell. This way of delivery has been proven by us to be much more effective than introducing a whole plasmid encoding for the particular gRNA, Cas9 and eventually a marker peptide, as these plasmids generally reach a size which is not easily internalized by the target cell line. The gRNAs mix is made of three gRNAs targeting different exons in the PD-L1 gene, except the first and the last exon. There are also several methods for the delivery of the genetic material, but as 5T33 cells are suspension cells, we used the electroporation.

In addition to the generation of the PD-L1 KO 5T33 cell line, we applied the same approach without the gRNAs to simultaneously produce a “Mock” transfected cell line. This Mock cell line was treated exactly as the PD-L1 KO cell line, but without the gRNAs and therefore should be equivalent to the wt 5T33 cell line.

Following transduction, the cells were grown in standard media and could be analyzed first via flow cytometry. **Fig. 3.2** shows the transduced 5T33 population which includes cells without any surface expression of PD-L1, the newly generated PD-L1 KO cells.

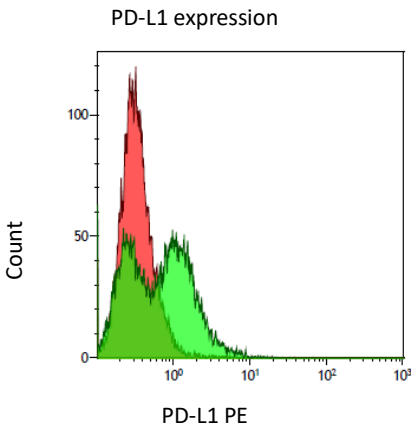


Fig. 3.2: Cell surface expression of PD-L1 of the gRNA/Cas9 transduced 5T33 cell line. 27,02 % of the cells do not show PD-L1 expression following transduction with gRNA/Cas9 complex targeting the murine PD-L1 gene. Measurement was performed using flow cytometry. Result shown is representative for the experiments.

In order to obtain a pure PD-L1 negative 5T33 cell population, these cells were stained for PD-L1 and separated from the PD-L1 positive cells using a cell sorter. The cells were stained with Hoechst to discriminate between live and dead cells. Only cells which were Hoechst negative, GFP positive and PD-L1 negative were collected (**fig. 3.3**).

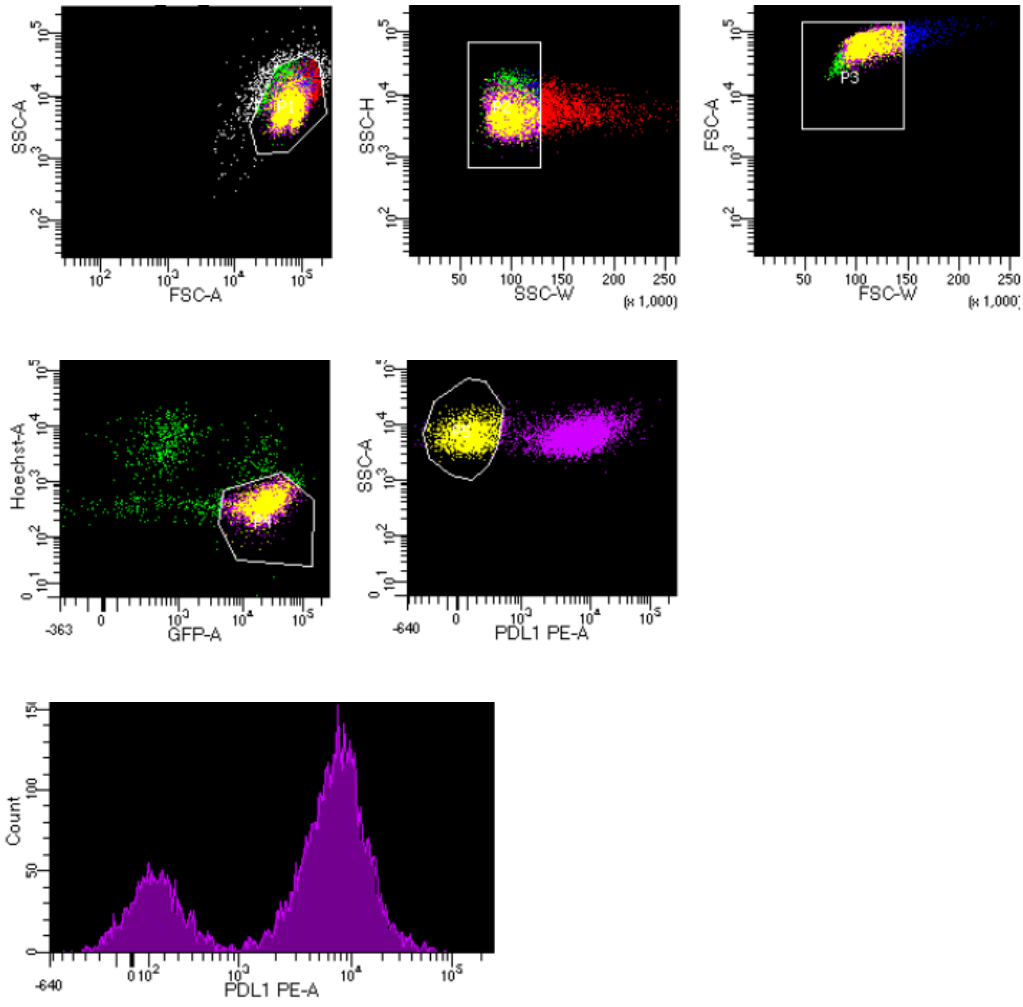


Fig. 3.3: Gating strategy for the sorting of PD-L1 negative 5T33 cells using the cell sorter. The first three gates show the exclusion of doublets (SSC-A vs FSC-A, SSC-H vs SSC-W and SSC-A vs SSC-W), the Hoechst-33342 staining discriminates between live (GFP+) and dead (Hoechst-33342+) cells and the last gate as well as the histogram show the clear separation of 5T33 cells expressing PD-L1 (PE+) and those who do not show any PD-L1 (PE-) expression on

the cell surface. 5T33 PD-L1 negative cells were separated and cultured in the appropriate cell culture media to generate a pure PD-L1 negative cell line.

3.1.3 Verification of the generated PD-L1 KO

Following cell sorting, the pure PD-L1 negative population was cultured and before performing further experiments the genetic KO of PD-L1 was verified by using flow cytometry analysis (**fig. 3.4**), Western Blot (**fig. 3.5**) and PCR (**fig. 3.6**).

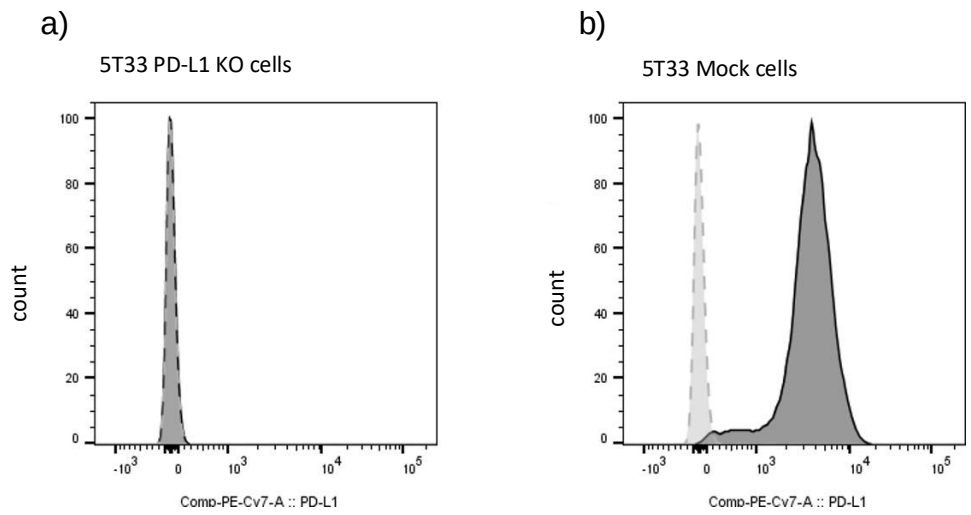


Fig. 3.4: Sorted PD-L1 KO 5T33 cells do not show PD-L1 cell surface expression. a) No PD-L1 cell surface expression of sorted 5T33 PD-L1 KO cells, **b)** 5T33 Mock transfected cells show 90,5 % PD-L1 expression measured by flow cytometry.

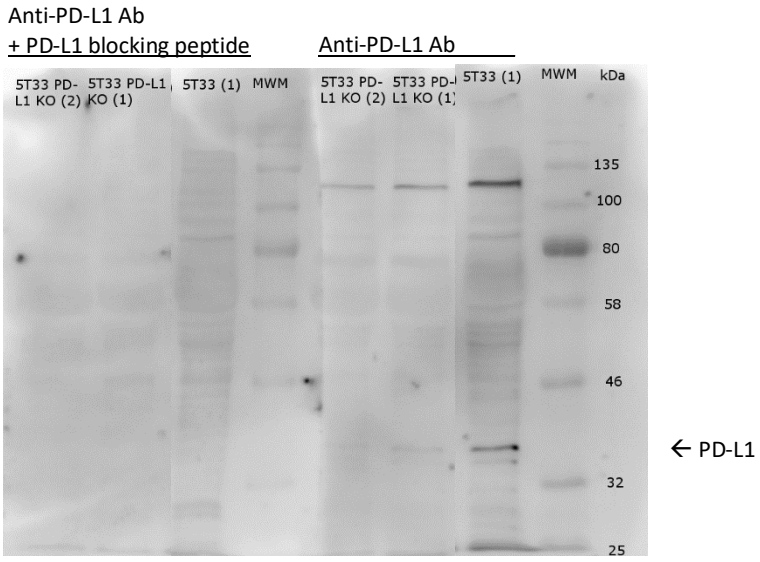


Fig. 3.5: Western Blot analysis of 5T33 PD-L1 KO and 5T33 Mock cells. The blot on the right shows the membrane treated with the PD-L1 antibody, whereas the left membrane shows the protein bands following treatment with PD-L1 antibody and the corresponding blocking peptide to verify the PD-L1 protein band. The expression of PD-L1 could only be observed in the cell lysates of the 5T33 Mock cells, not in that of the PD-L1 KO cells. The addition of the PD-L1 blocking peptide leads to the disappearance of the presumed PD-L1 band.

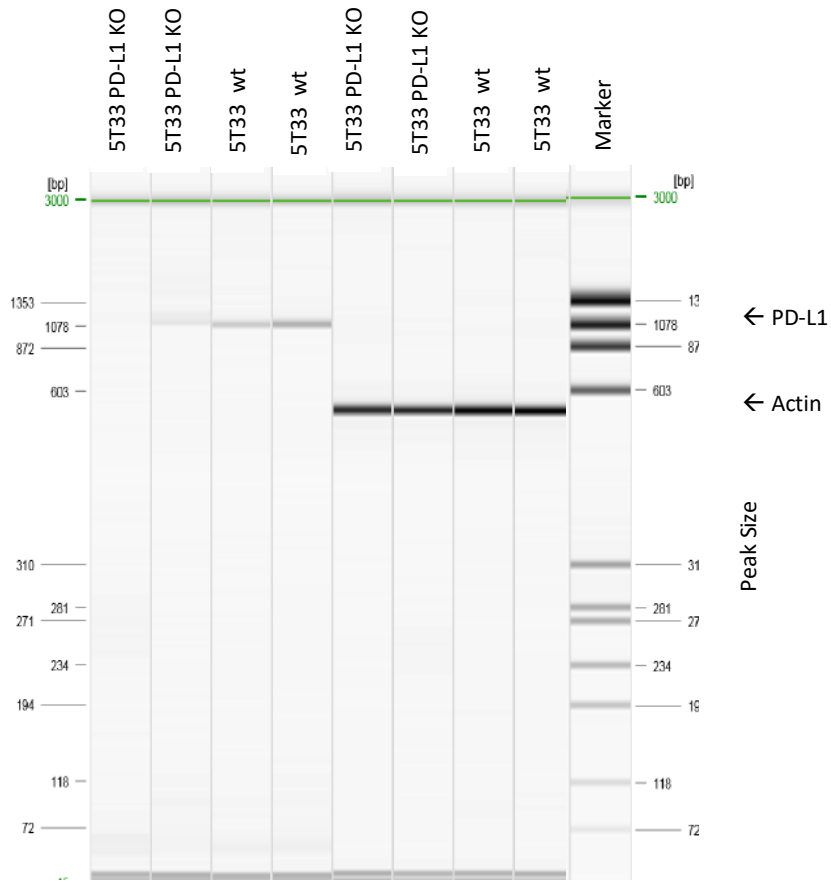


Fig. 3.6: PCR analysis of PD-L1 KO and wt 5T33 cells. The PD-L1 gene could be verified for 5T33 wt cells but not for the PD-L1 KO cells. Actin expression was used as control. Analysis was performed using the QIAxcel Advanced System.

3.1.4 Functional assays of 5T33 PD-L1 KO cells

We asked whether the 5T33 PD-L1 KO cells that lack the PD-L1 gene expression differ from the Mock transfected and wt cells in other cellular properties, testing proliferation and migration capabilities as well as expression of other important immunoregulatory cell surface receptors. The Thymidine incorporation assay (see **fig. 3.7**) shows that there is no difference in cell proliferation between the 5T33 PD-L1 KO and Mock

transfected cells. To test migratory capability, we performed a transwell assay using 8 µm pores and 20 % FCS cell culture media at the bottom as an attractant. The migration assay (see **fig. 3.8**) shows no difference in the migration of the cell lines. As we could not find any differences in the functional assays performed between the PD-L1 KO and Mock transfected as well as wt cells, we could assume that the genetic KO of PD-L1 was specific and the cells could be used for further *in vitro* and *in vivo* studies.

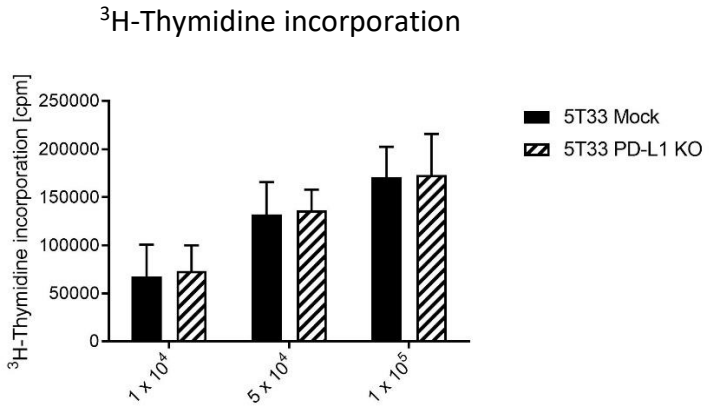


Fig. 3.7: The proliferation of 5T33 Mock and PD-L1 KO cells remains the same. The ^{3}H-Thymidine incorporation assay was performed with 1x10^4, 5x10^4 and 1x10^5 cells and shows that there is no difference in cell proliferation between 5T33 Mock and PD-L1 KO cells, (n=3). Cell count was measured using the MicroBeta TriLux.

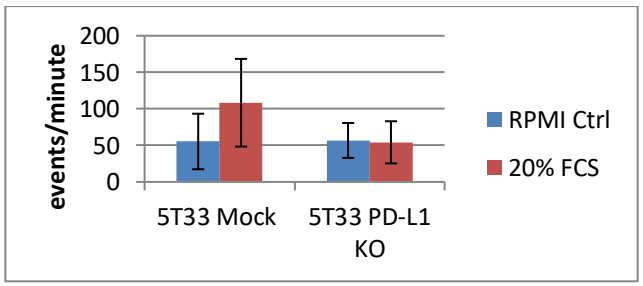


Fig. 3.8: Migratory capabilities of 5T33 Mock and PD-L1 KO cells are comparable. 20 % FCS in RPMI media was used as attractant for cells plated in RPMI media without supplements on the top of the transwell membrane. RPMI media which is usually used as culture media (10 % FCS) served as control. Cells were incubated for 4 h in 37°C and 5 % CO_2. The pore size of the transwell membrane was 8 µm and cells which were migrated to the bottom were counted using flow cytometry. No difference in migration was measured between the cell lines, (n=3).

3.1.5 Cell surface characterization of 5T33 Mock and 5T33 PD-L1 KO cells

For the phenotyping of the myeloma cells, we focused on immunoregulatory receptors and their sensitivity to the pan-HDAC inhibitor panobinostat. Panobinostat is in clinical use for RRMM patients and new combinatorial approaches are urgently needed as the development of drug resistance is a serious difficulty in MM therapy. By examining the regulation of immunoregulatory cell surface receptors of the tumor cell line we aim to investigate the potential interactions with other immune cells contributing to the tumor growth progression and establishment of a tumor tolerant microenvironment *in vivo*.

Drug sensitivity was tested with a panobinostat cytotoxicity assay (see **fig. 3.9**) to identify the optimal drug concentration for comprehensive cell characterization. Panobinostat was applied for 24 h and the nucleic fragmentation rate was determined. The 5T33 wt cells showed a similar sensitivity for panobinostat treatment as the Mock and PD-L1 KO cells. Cells were analyzed with panobinostat concentrations ranging from 5 to 10 nM to obtain a potential strong drug regulation one hand and enough live cells on the other hand for further experimentation. There were no significant differences measured in cell surface receptor regulation over a range of 5-10 nM panobinostat. Therefore, most analysis were performed at 10 nM panobinostat for 24 h compared to the DMSO control media.

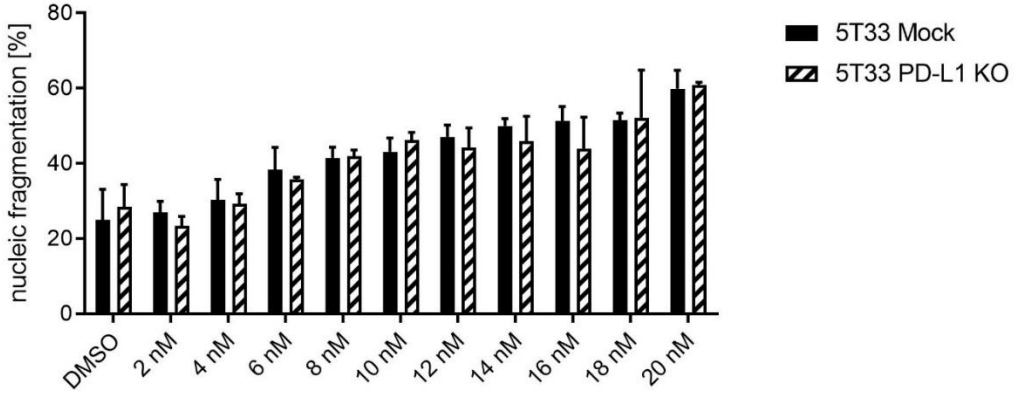


Fig. 3.9: Panobinostat cytotoxicity assay for 5T33 Mock and PD-L1 KO cells. The 5T33 Mock and PD-L1 KO cells show an apoptosis rate of 20 % in the DMSO control. To analyze the cells under cytotoxic stress we aimed for a drug concentration that increases apoptosis rate over 20 %. This drug concentration was given 8-10 nM panobinostat for 24 h. Propidium Iodide was used to distinguish between life and apoptotic cells in a flow cytometry analysis, (n=3).

3.1.6 Phenotyping of 5T33 Mock and PD-L1 KO cells

To investigate if the *knockout* of PD-L1 has impact on other immunoregulatory receptors which could influence our *in vivo* studies, we analyzed the expression of the cell surface receptors MHCI, MHCII, CD86, CD155 and GITR (**fig. 3.10a**). As no differences were found between the Mock and PD-L1 KO cells regarding these immunoregulatory receptors, we further analyzed if there are any differences in the regulation patterns of 5T33 PD-L1 KO cells compared to 5T33 Mock cells when treated with panobinostat. These analyses are important to suggest that potential differences to responses in vivo studies exclusively rely on the KO of PD-L1.

The expression of the following receptors, which are involved in immunoregulatory functions, were analyzed: PD-L1, CD86 (shown in **fig. 3.10b** representative for further analysis), MHC-I, Osteoactivin, CD155, CD62L, GITR, GITRL, CD44, CD25, ICOS, PD-1, PD-L1, MHCII as well as CXCR4 and CD47.

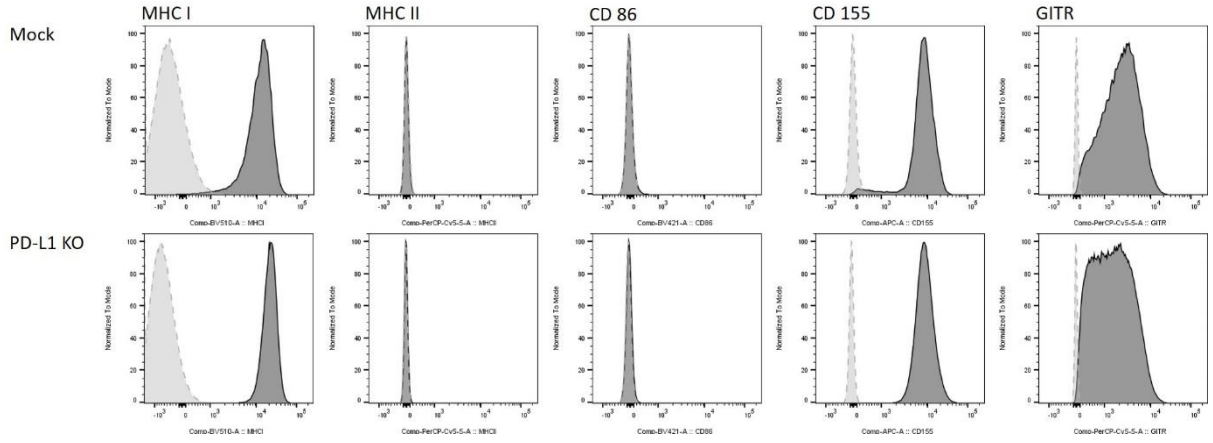


Fig. 3.10a: Expression of the immunoregulatory receptors MHCI, MHCII, CD86, CD155 and GITR of 5T33 Mock and PD-L1 KO cells are identical. 5T33 Mock and PD-L1 KO cells show the same expression of the immunoregulatory receptors MHCI, MHCII, CD86, CD155 and GITR. Analysis was performed using flow cytometry, (n=3). Statistics: one-way ANOVA.

a) b)

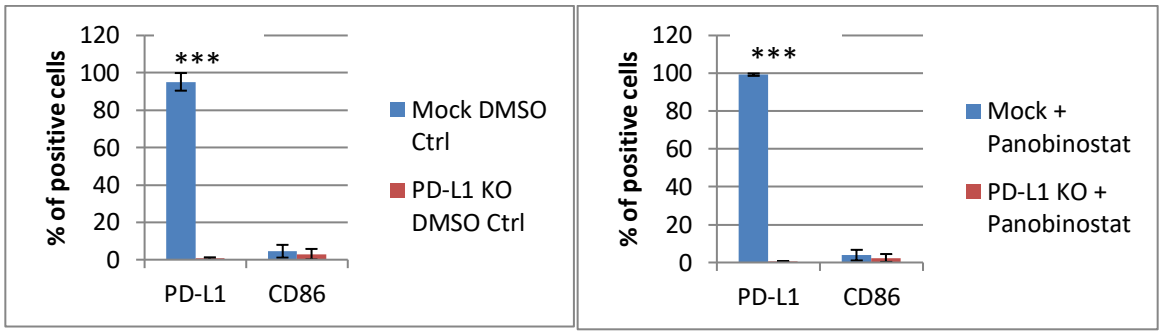


Fig. 3.10b: Expression of PD-L1 and immunoregulatory receptors such as CD86 of 5T33 Mock and PD-L1 KO cells are not regulated by panobinostat. 5T33 PD-L1 KO cells show no expression of PD-L1 and it is not induced by panobinostat ($p<0.001$). **a)** Number of 5T33 Mock and 5T33 PD-L1 KO cells expressing either PD-L1 or CD86 in control media. **b)** Number of 5T33 Mock and 5T33 PD-L1 KO cells expressing PD-L1 and CD86 in media supplemented with10 nM panobinostat. Panobinostat can not regulate PD-L1 or CD86 receptor expression on 5T33 Mock and PD-L1 KO cells. CD86 is shown representatively for all other analyzed immunoregulatory receptors of 5T33 Mock and PD-L1 KO cells when treated with 10 nM panobinostat for 24 h. Analysis was performed using flow cytometry, (n=3). Statistics: one-way ANOVA.

Our comprehensive analysis of immunoregulatory cell surface receptors reveal that only CD1d (**fig. 3.12**) is significantly altered after panobinostat treatment, but in both 5T33 Mock and PD-L1 KO myeloma cells. However, 5T33 PD-L1 KO cells show a 50 % decrease of CD1d expression compared to the Mock transfected cells (**fig. 3.11**). Therefore, we assume a link between the tumor cell intrinsic signaling of PD-L1 and the regulation of the CD1d expression. Overall, we verified that PD-L1 is not expressed on the 5T33 PD-L1 KO cells and PD-L1 is not induced by panobinostat.

a) b)

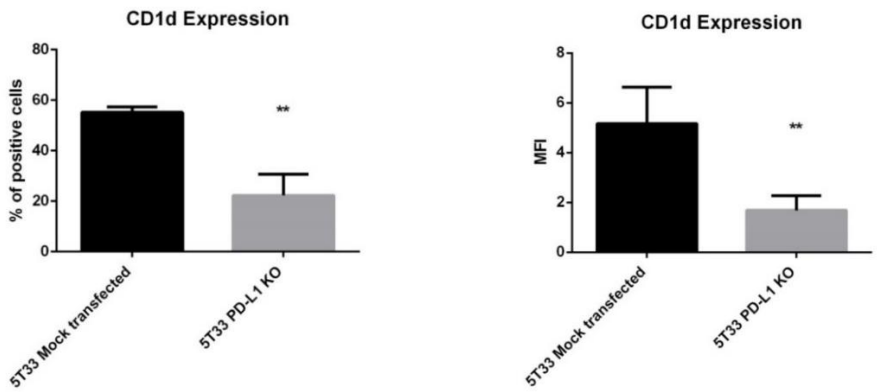


Fig. 3.11: CD1d expression by 5T33 Mock and PD-L1 KO cells. The expression of CD1d in 5T33 PD-L1 KO cells is significantly reduced compared to the Mock transfected cells. Both, **a)** the number of 5T33 PD-L1 KO cells expressing CD1d and **b)** the receptor expression level is significant reduced ($p<0.01$) by approximately 50 % of the 5T33 Mock cells. Analysis was performed using flow cytometry, (n=3). Statistics: unpaired t-test.

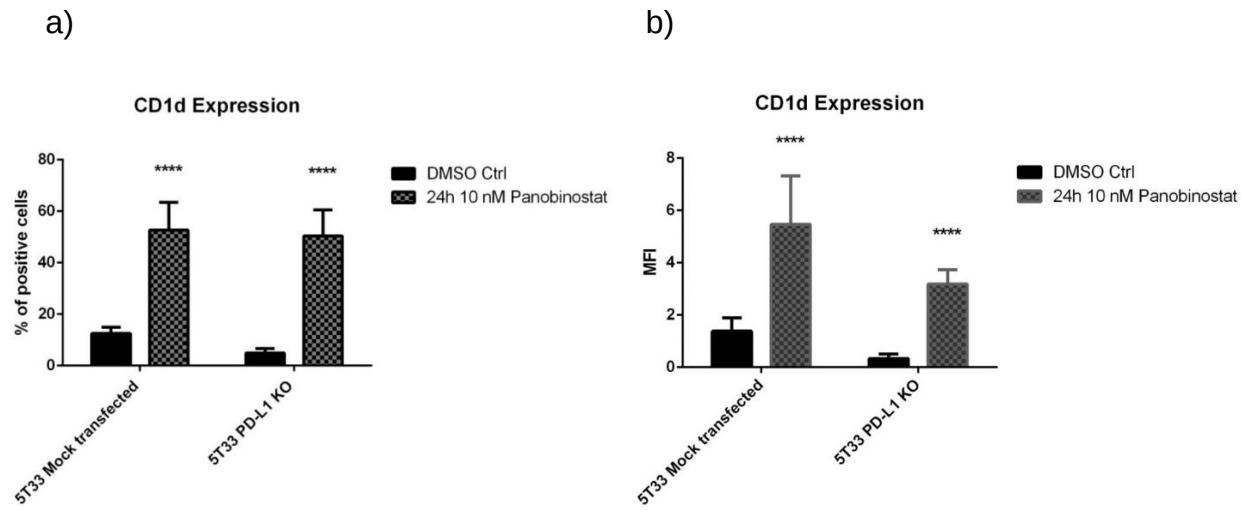


Fig. 3.12: Panobinostat increases CD1d expression on 5T33 Mock and PD-L1 KO cells. Significant induction of CD1d upon the treatment of 24 h 10 nM panobinostat for both cell lines 5T33 PD-L1 KO and 5T33 Mock in **a)** percentage of positive cells ($p<0.0001$) and **b)** MFI ($p<0.0001$). Analysis was performed using flow cytometry, (n=3). Statistics: two-way ANOVA.

In summary, we observed a potential regulatory mechanism between pan-HDAC inhibitors and NKT cell trafficking indicated by the ability of panobinostat to increase the CD1d expression on myeloma cells. This might have influence on the CD1d-dependent anti-tumor response as well as the modulation of the TME by NKT cells *in vivo.* These results are supported by the work of Tiper *et al.* who hypothesized that tumors use epigenetic mechanisms to dysregulate CD1d-mediated antigen presentation and therefore impairing NKT cells to recognize and kill lymphoma cells. The authors showed that HDAC inhibitors can enhance CD1d-dependent NKT cell responses to mantle cell lymphoma cells (Tiper and Webb, 2016). Except the expression of CD1d, other evaluated immunoregulatory receptors do not differ significantly between these cell lines. This finding enables the assumption that changes in tumor growth of *in vivo* studies might be mainly dependent on PD-L1 receptor expression. Furthermore, we found strong induction of CD1d on both Mock and PD-L1 KO cells by panobinostat, pointing to the

immunoregulatory function of the compound by CD1d suggesting its ability to modulate NKT cell activation and trafficking. Interestingly, the genetic presence of the PD-L1 gene seems to be a prerequisite for the CD1d expression, which further points to a relationship between MM and NKT cells in the context of the tumor immune editing.

3.1.7 Panobinostat reduces the expression of Zeb1 and TGF-β

Panobinostat has been shown to significantly inhibit tumor growth in our myeloma *in vivo* model which will be discussed later (**fig. 3.24**). Nonetheless, panobinostat has limited efficiency as monotherapy in RRMM patients, therefore we further aim to investigate the mechanism of action on the protein level (Wolf et al., 2012). Pan-HDAC inhibitors have multiple effects besides gene activation including the suppression of the transcription factor Zeb1 which is also known to induce PD-L1 (Meidhof et al., 2015) (Y. Guo et al., 2021a). Therefore, we examined the protein expression of this proto-oncogenic transcription factor following panobinostat treatment using Western Blot. Here, we found that 5T33 Mock and 5T33 PD-L1 KO cells show decreased expression of Zeb1 following panobinostat treatment (**fig. 3.13a**). Zeb1 is known to induce CXCR4. Hence, this might be an explanation why CXCR4 is decreased on different T cell populations and marks a distinct difference of diminished tumor burden.

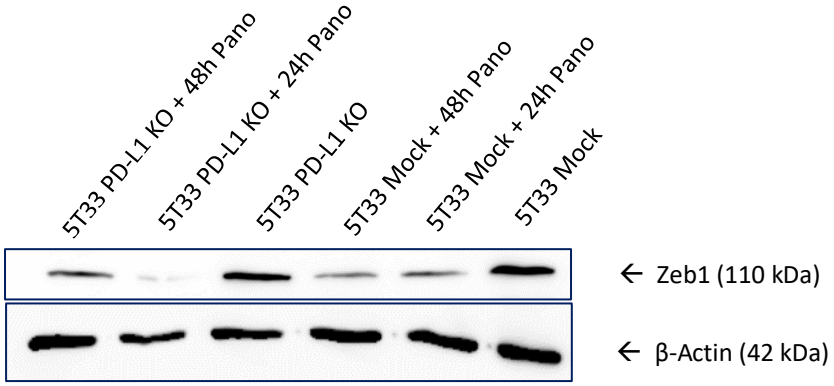


Fig. 3.13a: Zeb1 protein expression is reduced in 5T33 Mock and PD-L1 KO cells following panobinostat treatment. Western Blot analysis of 5T33 Mock vs 5T33 PD-L1 KO cells treated with 10 nM panobinostat for 24 h or 48 h compared to DMSO control. β-Actin was used as loading control (42 kDa). Protein expression of Zeb1 (110 kDa) is diminished upon panobinostat treatment in 5T33 Mock as well as PD-L1 KO cells.

To further investigate the mode of action of the pan-HDAC inhibitor *in vitro*, we performed an ELISA to measure the TGF-β secretion of 5T33 wt cells following a 24 h, 10 nM panobinostat treatment. As shown in **fig. 3.13b**, 5T33 wt cells secrete significant less pro-tumorigenic TGF-β ($p<0.05$) following panobinostat treatment.

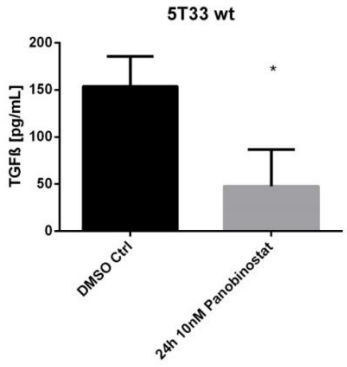


Fig. 3.13b: The treatment of panobinostat reduces the TGF-β secretion of 5T33 wt cells *in vitro*. 5T33 wt cell secret significant ($p<0.05$) less TGF-β following panobinostat treatment compared to the untreated control. 5T33 wt cells were treated with 10 nM panobinostat for 24 h and the TGF-β concentration of the conditioned media was measured using ELISA, (n=3). Statistics: unpaired t-test.

3.2 Comprehensive screening of immune cell populations in primary and secondary lymphoid organs of myeloma bearing mice and healthy controls

To get insights into the regulation of immune cells during the development of myeloma disease progression and to potentially identify new drug targets and biomarkers to monitor disease progression related to the establishment of the immunosuppressive tumor tolerant microenvironment, we analyzed different immune cell populations in the bone marrow and secondary lymphoid organs. Functional receptors of the immune cells and their co-inhibitory markers were measured in mice three and four weeks post 5T33 wt, Mock or PD-L1 KO challenge as well as at disease end stage using flow cytometry.

3.2.1 NKT cells are augmented in the bone marrow of myeloma bearing mice compared to healthy control

NKT cells function to fine tune the immune response and to bridge the adaptive and innate immune responses. NKT cells influence various immune cells including macrophages, DCs, NK and T cells through their cytokine expression. NKT cells have decisive regulatory influences on the responses against infections, grafts or tumors (L. Wu and van Kaer, 2011).

By analyzing different immune cell populations in the bone marrow of end stage disease mice, we found that NKT cells are tremendously augmented. Interestingly, this increase in the number of NKT cells in the BM is already established in early disease stage while other analyzed immune cells including T cells, Tregs and NK cells remain stable (**fig. 3.16**). The state of health of mice at four weeks post inoculation is comparable to the SMM in human and benign idiopathic proteinemia (BIP) stage in myeloma bearing mice. Here, we already found the increase in NKT cells, which further increases until the final myeloma disease end stage (**fig. 3.14**). Additionally, the NKT cell number in the bone marrow was associated with the type of disease manifestation. In contrast to mice developing the typical symptom of leg paraplegia, mice which developed a solid tumor did not show the increased NKT cell numbers (**fig. 3.15**). As generally most of the mice develop leg paraplegia, the analysis with solid tumor bearing mice was limited. The reasons for the formation of a solid tumor compared to tumor cell manifestation in the bone marrow is still an open question and remains to be elucidated. As CXCR4 is the bone marrow homing receptor which is associated with extramedullary disease (EMD) formation, we also analyzed the NKT cell populations in the BM for the expression of CXCR4. In addition to CXCR4 we further analyzed the NKT cells for the expression of different co-inhibitory receptors which are associated with the tumor tolerant immune setting. As shown in **fig. 3.14** the NKT cells increase gradually during MM disease progression, while the NKT CD4-/CD4+ ratio increases correspondingly.

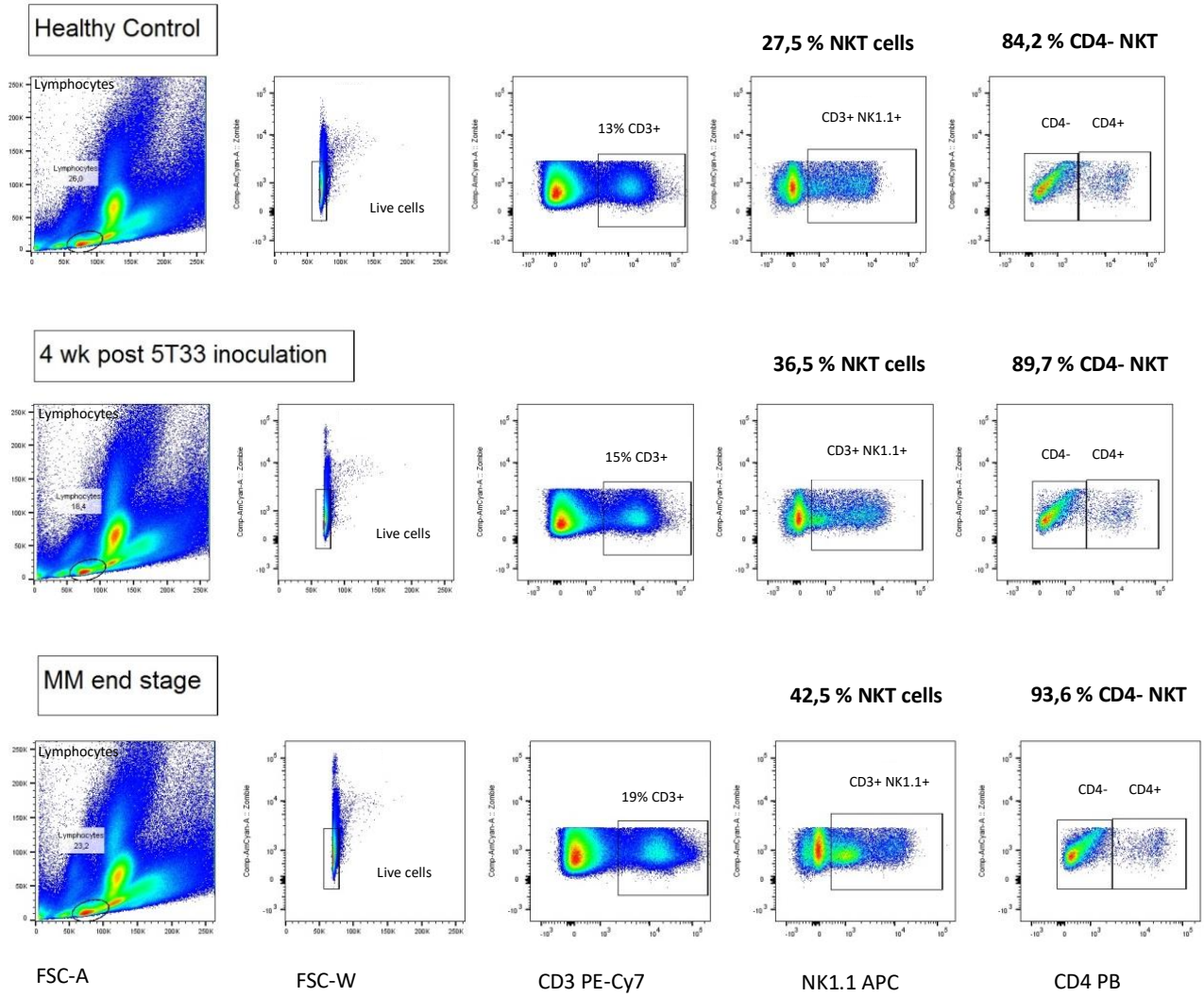
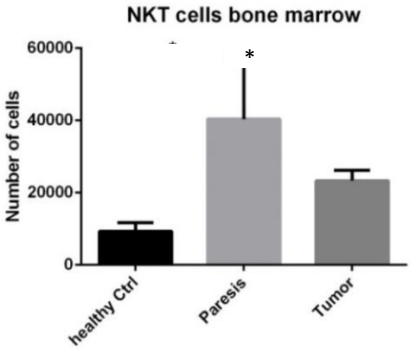


Fig. 3.14: Gating strategy for CD4+ and CD4- NKT cells in the bone marrow. Analysis was performed for healthy control mice, at four weeks post 5T33 wt inoculation and at MM disease end stage. NKT cells gradually increase during MM disease progression, while the ratio of CD4-/CD4+ NKT cells increases correspondingly. Results shown are representative for the experiments.

a)



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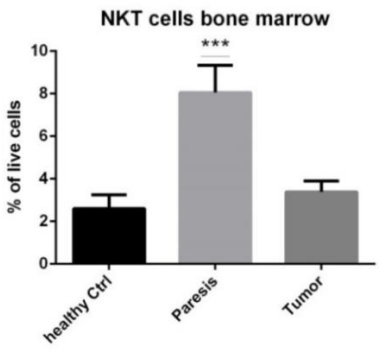

Fig. 3.15: NKT are increased in the bone marrow of MM bearing mice. Significant increase of **a)** the total number ($p<0.05$) and **b)** the percentage ($p<0.001$) of NKT cells in the BM of myeloma bearing mice at disease end stage developing symptoms of leg paraplegia compared to healthy control mice or solid tumor bearing mice ($p<0.01$). Analysis was performed using flow cytometry, (n=3). Statistics: one-way ANOVA.

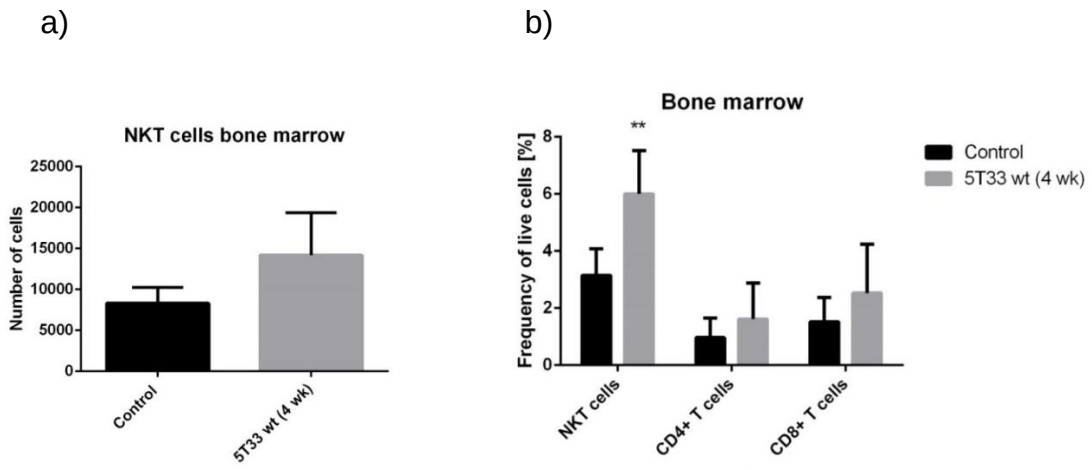

Fig. 3.16: Increase of NKT cells in the bone marrow of mice four weeks post inoculation with 5T33 wt cells. a) Total number of NKT cells in the BM of healthy control mice compared to mice four weeks post tumor cell injection, (n=3). Statistics: unpaired t-test. **b)** Significant increase ($p<0.01$) of NKT cells compared to CD4+ and CD8 + T cells in mice of early MM disease stage compared to the healthy control group, (n=5). Analysis was performed using flow cytometry. Statistics: two-way ANOVA.

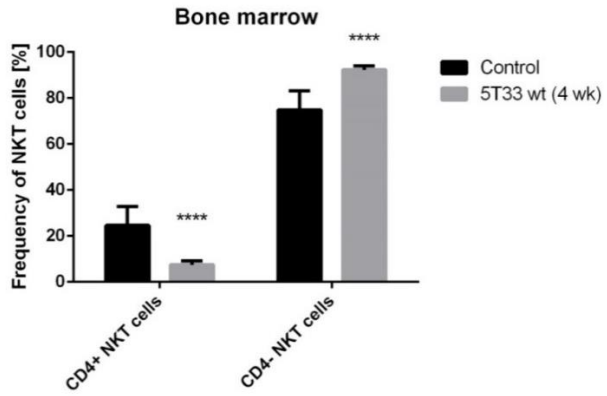

Fig. 3.17: The proportion of CD4- NKT cells increases during MM disease progression. Significant increase ($p<0.0001$) of the CD4-/CD4+ ratio of NKT cells in the bone marrow of mice four weeks post 5T33 wt injection compared to the healthy control. Analysis was performed using flow cytometry, (n=5). Statistics: one-way ANOVA.

As seen in **fig. 3.17** in our analysis of healthy control vs myeloma bearing mice we found that the CD4- NKT cell population increases in mice four weeks post tumor cell inoculation. This might be a profiling marker of disease progression, as the CD4- NKT cell ratio further increase till the end stage (fig. 3.14) but no significant increase is observed after three weeks in the bone marrow.

3.2.2 NKT cells in myeloma bearing mice show increased CXCR4 expression

This result showing increased expression of NKT cell CXCR4 leads to the conclusion of interaction with the tumor cell protecting bone marrow niche and promotion of disease progression.

Therefore, we stained NKT cells in the BM and spleen of healthy control mice, myeloma early disease stage as well as MM disease end stage mice for CXCR4 expression and the co-inhibitory receptors PD-1, PD-L1 and CD47. No differences were found 3 weeks post tumor cell inoculation, but already 4 weeks post tumor cell injection the NKT cells in the BM positive for CXCR4 and PD-L1 are phenotypically less diverse in relation to their PD-L1 and CXCR4 expression pattern and show strong upregulation of these markers (**fig. 3.18**). We did not find any differences in the NKT CXCR4 expression in the splenic cells from MM disease end stage mice compared to controls. In contrast, the BM showed a shift of NKT cells positive for the co-expression of PD-L1 and CXCR4 during MM disease progression as demonstrated in **fig. 3.19**. Interestingly, the $CXCR4^{high}$ PD-L1+ NKT cells increased proportional to the degree of leg paraplegia, whereas mice which developed a tumor did not show the same pattern of NKT cell alterations. Here, the appropriate NKT cell population primarily infiltrates the tumor instead of residing in the bone marrow. Remarkably and in line with the findings in **fig. 3.17**, the significant increase of CXCR4 expression on BM infiltrating NKT cells corresponding to MM disease progression is exclusively found on the CD4- NKT cell population (**fig. 3.20**).

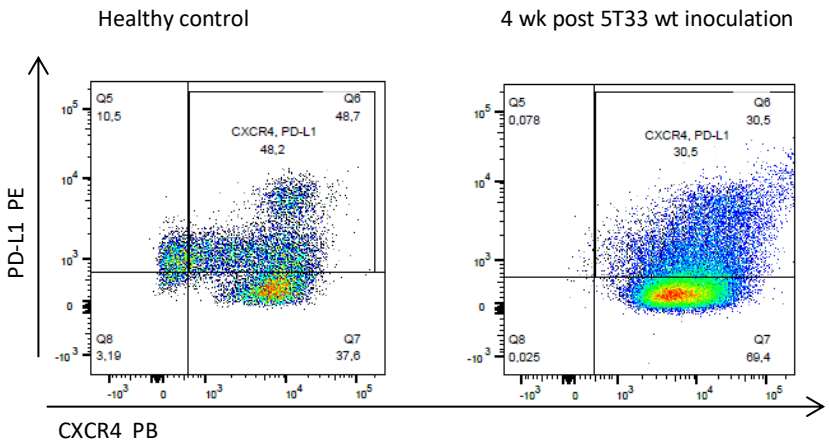

Fig. 3.18: Expression pattern of PD-L1+ CXCR4+ NKT cells in myeloma-bearing mice. FACS analysis show increased CXCR4 expression four weeks post 5T33 wt inoculation compared to healthy control. Cells from the BM of healthy control and mice 4 weeks post 5T33 injection were analyzed for CD3+ NK1.1+ cells and gated on the cell surface marker PD-L1 and CXCR4. Analysis was performed using flow cytometry. Results shown are representative for the experiments.

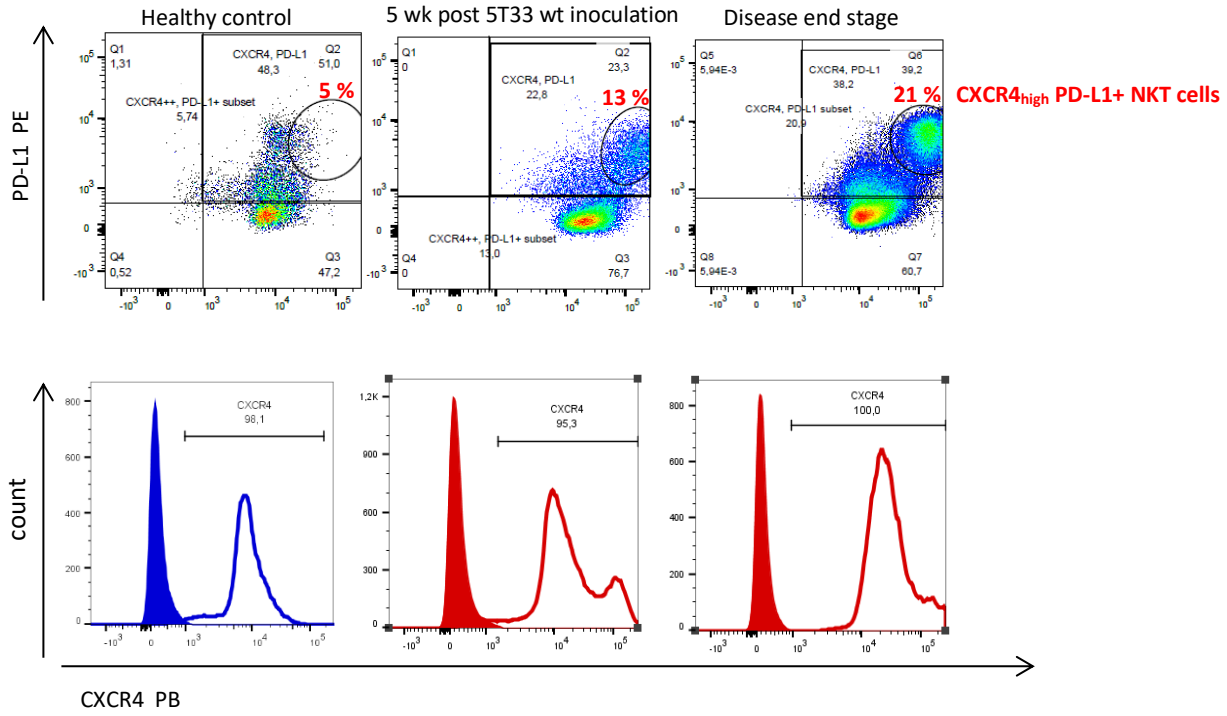

Fig. 3.19: The gradually increase of CXCR4$_{high}$ PD-L1+ NKT cells as biomarker to monitor MM disease progression. Depending on the grade of bone tumor cell infiltration characterizing the disease stage, the CXCR4 expression on PD-L1+ NKT cells increases correspondingly. Cells from the BM of healthy control, 5 weeks post tumor cell inoculation and at MM disease end

stage were analyzed for CD3+ NK1.1+ cells and gated on the cell surface marker PD-L1 and CXCR4. Analysis was performed using flow cytometry. Results shown are representative for the experiments.

a) b)

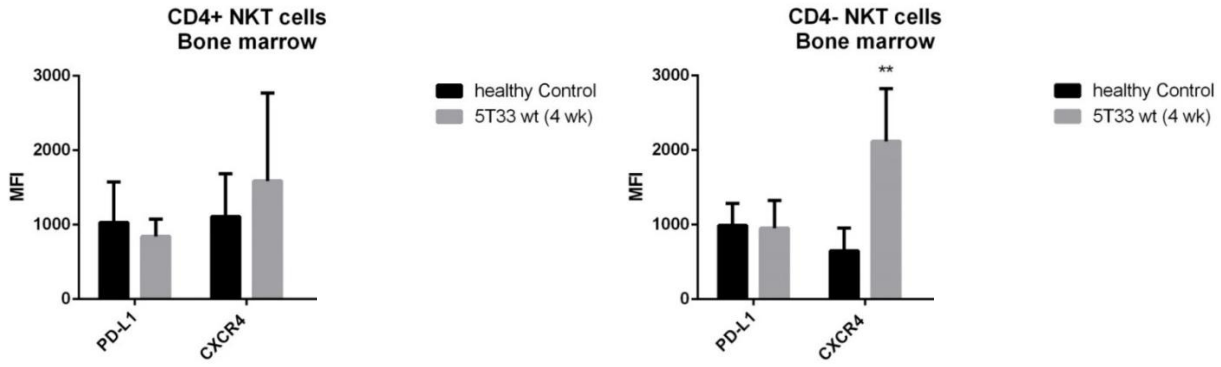

Fig. 3.20: Significant increase of CXCR4 expression of CD4- NKT cells in the bone marrow of myeloma-bearing mice. a) The receptor expression level of PD-L1 and CXCR4 of CD4+ NKT cells in the BM of healthy control and mice four weeks post 5T33 wt injection remain constant. **b)** The expression of CXCR4 is significant increased ($p<0.01$) of CD4- NKT cells in the BM of mice four weeks post 5T33 tumor cell injection compared to the control group. The percentage of the appropriate cells does not change. Cells from the BM of both groups were stained for CD3+ NK1.1+ and the cell surface marker PD-L1 and CXCR4 (n=5). Analysis was performed using flow cytometry. Statistics: two-way ANOVA.

In summary, these results indicate that especially the upregulation of CXCR4 on NKT cells and the increased CD4-/CD4+ NKT cell ratio might be a marker of myeloma disease progression and raise the hypothesis that it could be a driver of this progression. To ask whether these changes in NKT cells might also have an impact on therapy, we further investigated NKT cell regulation in mice models treated with panobinostat (**fig. 3.26**). In **chapter 3.3.1** we will discuss the effect of an increase in CD4+ CXCR4+ NKT cells associated with prolonged survival in the therapeutic groups. It is already known that CXCR4 on tumor cells modulates the tumor immune environment and enables the dissemination of MM cells to distant bone marrow niches. Here we show that NKT cells are associated with the establishment of the tumor tolerant microenvironment and MM disease progression and that their engagement relies on PD-L1 expression on the MM cells. The function of the increased number of NKT cells with aberrant immunoregulatory expression pattern still remains to be elucidated. For a deeper understanding of the role

of CXCR4 in MM disease progression we move to analysis of CD4+ and CD8+ T cells in the primary and secondary lymphoid organs.

3.2.3 CD4+ and CD8+ T cells in the bone marrow of myeloma-bearing mice show increased CXCR4 expression

The function of CXCR4 in the promotion of MM aggressiveness, metastasis and drug resistance makes CXCR4 a promising target in myeloma cancer therapy. It has been shown that the exclusion of CD8+ T cells from the tumor microenvironment can be prevented by a CXCL12 inhibitor. The increased CD8+ T cell effector functions synergized with additional anti-PD-1 immune checkpoint inhibition (ICI) (Zboralski et al., 2017). In lymphoma bearing mice CD8+ T cells in the BM express increased levels of CXCR4 enabling their migration along the CXCL12 gradient and demonstrate increased effector function and protection against the tumor (A. B. Khan et al., 2018). Therefore, we analyzed the expression of CXCR4 on CD4+ and CD8+ T cells as well as PD-1, PD-L1 and the NKT cell activating receptor CD1d in both BM and spleen. As shown in **fig. 3.21** there is a significant increase in the CXCR4 receptor expression level on CD4+ and CD8+ T cells in the bone marrow of mice four weeks post tumor cell challenge.

a) b)

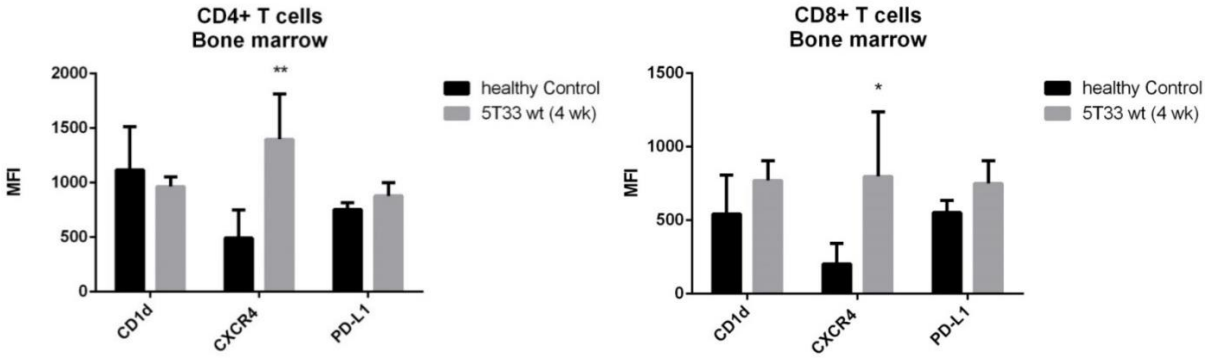

Fig. 3.21: CXCR4 receptor expression is increased on T cells in the bone marrow of myeloma bearing mice. While PD-L1 and CD1d remain constant, the receptor expression on **a)** CD4+ T cells ($p<0.01$) and **b)** CD8+ T cells ($p<0.05$) is significant increased in the BM of mice four weeks post 5T33 wt inoculation compared to the healthy control. Cells from the BM of both groups were stained for CD3+ CD4+ or CD8+ and the cell surface marker PD-L1, CXCR4 and CD1d. Analysis were performed using flow cytometry, (n=4). Statistics: two-way ANOVA.

In summary, the results of the analysis four weeks post 5T33 wt injection show early immunological changes in the primary tumor site, which is not found in the disease stage one week before or in the secondary lymphoid organs. We found that the number of NKT cells increases in the bone marrow depending on the type of disease manifestation, linking the NKT cell activity directly to tumor cell interactions in the BM. Comprehensive analysis of the NKT cells revealed that the ratio of CD4-/CD4+ NKT increases during MM disease progression (**fig. 3.14** and **3.17**) and that the CXCR4 expression level further increases on the CD4- NKT cells (fig. **3.20**). While the number of CD4+ and CD8+ T cells remains constant in the BM, they both increase in CXCR4 expression. As we found that the number of PD-L1+ CXCR4+ NKT cells seem to progressively increase during myeloma disease progression in the bone marrow, we assume that NKT cells play a major role in driving the fate of either a tumor supporting or anti-tumor immune microenvironment in the MM mouse model.

3.3 Immunotherapeutic approaches for the treatment of Multiple Myeloma

Immune checkpoint blockade has demonstrated promising outcomes for patients with many different cancers including myeloma, but their addition to MM standard therapy has been disappointing. With focus on the PD-1/PD-L1 signaling, we investigate the influence of different checkpoint inhibitors on immunological changes of the TME in the KaLwRij mouse model. These results give new insights into the complexity of action of ICI in the treatment of malignant diseases which might help to develop combinatorial approaches of checkpoint inhibitors in clinical trials.

3.3.1 Combining checkpoint inhibitors for MM therapy using the 5T33 KaLwRij mouse model

The immune checkpoint blockade of PD-1 has shown great benefit in patients with solid malignancies such as melanoma (Robert et al., 2015). However, as monotherapy in multiple myeloma, immune checkpoint blockade e.g. with nivolumab, has shown

disappointing results for the treatment of relapsed/refractory myeloma patients in several clinical studies (Costa et al., 2018). Nonetheless, PD-1 and PD-L1 have essential impact on the development of myeloma disease progression and new treatment combinations are urgently needed. Therefore, we seek to analyze the combinatorial checkpoint blockade of PD-1 and CD47. It was recently demonstrated that macrophages are suppressed in their antigen presentation capacity during tumor progression by the simultaneous increase of PD-1 and the “don´t eat me” signal receptor CD47 on their cell surface (Gordon et al., 2017). Hence, we tested the survival of KaLwRij mice injected with 5T33 wt cells when treating them with either anti-PD-1 or anti-CD47 alone or in combination. As shown in **fig. 3.22** there was no significant prolongation of the survival when mice were treated either with anti-PD-1 or anti-CD47 mAb or both compared to the untreated group. These results indicate that dual checkpoint blockade as well as checkpoint blockade as monotherapy are not sufficient to diminish myeloma tumor growth.

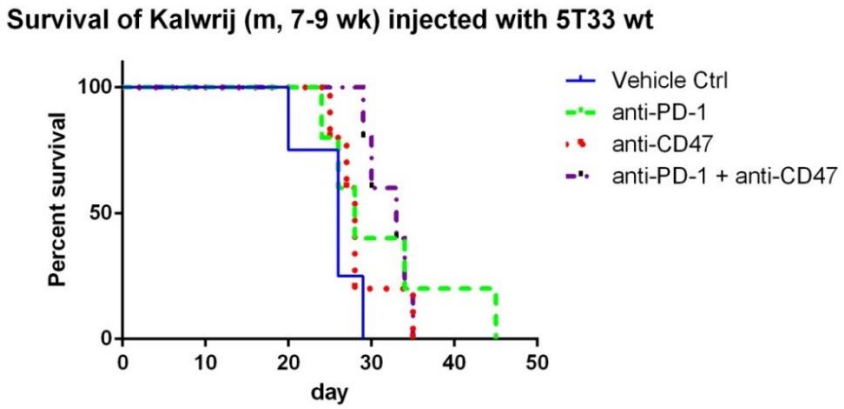

Fig. 3.22: Dual blockade of anti-PD-1 and anti-CD47 checkpoints has no effect on the survival of myeloma-bearing mice. Survival of KaLwRij mice injected with 5T33 wt cells and treated either with vehicle control, anti-PD-1 blocking antibody, anti-CD47 blocking antibody or double treated with anti-PD-1 and anti-CD47. No prolongation of the survival of mice treated with double checkpoint blockade anti-PD-1 and anti-CD47 compared to the vehicle control as well as the groups receiving the monotherapy. 5 mice per group each, treatment start d 17 post inoculation. Statistics: Log rank test (Mantel-Cox analysis): ns.

Since immune checkpoint therapy did not show promising therapeutic effects, other treatment options are needed. For the treatment of relapsed and refractory myeloma patients, the HDAC inhibitor panobinostat has shown promising results, although not as

monotherapy (Eleutherakis-Papaiakovou et al., 2020). Additionally, patients develop drug resistance following the treatment regimens, demonstrating the urgent need for new therapy modalities. The combination of HDAC inhibitors with immune checkpoint blockade can increase the immunotherapeutic efficiency *in vitro* and *in vivo* (Borcoman et al., 2022). By enhancing the anti-tumor immune response and increasing the immunogenicity, the combination of HDAC6i with anti-PD-1 mAbs has already demonstrated efficiency in decreasing the tumor burden in a melanoma mouse model (Knox et al., 2019).

Therefore, we questioned whether the combinatorial treatment of anti-PD-1 mAb with panobinostat would prolong the progression free survival of mice injected with 5T33 wt cells. As shown in **fig. 3.23**, the treatment of panobinostat monotherapy significantly increases the survival of myeloma bearing mice. Surprisingly, the application of the anti-PD-1 mAb inhibits the drug efficiency of the pan-HDAC inhibitor. This result indicates that PD-1 signaling blockade negatively interferes with the immunomodulatory mechanism of the panobinostat anti-myeloma effect.

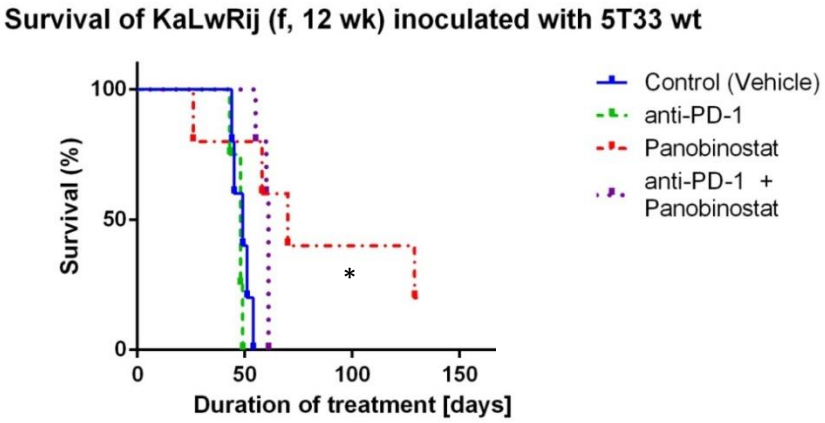

Fig. 3.23: Attenuation of the pan-HDAC inhibitors anti-myeloma effect by combination with anti-PD-1 checkpoint blockade. Survival of KaLwRij mice injected with 5T33 wt cells and treated either with vehicle control, anti-PD-1 blocking antibody, panobinostat or double treated with anti-PD-1 and panobinostat. 5 mice per group each, treatment start d 19 post inoculation. Statistics: Log rank test (Mantel-Cox analysis): $p<0.01$.

As clinical studies with the use of nivolumab have already been haltered due to serious ICI-specific immune related adverse events, the molecular mechanisms behind the diminishment of checkpoint blockade induced loss of survival benefit still has to be investigated (Hradska et al., 2021). To examine the immunological changes during ICI and HDAC therapy, we analyzed the tumor immune microenvironment at the end stage of the disease. Using flow cytometry we analyzed the regulation of CD4+, CD8+ and NKT cells in the BM and spleen of the different treatment groups. No changes were found in the BM regarding the analyzed immune cell populations and the receptor expression of PD-1, PD-L1, CXCR4 and CD1d. In contrast, immunological changes in the spleen were found (**fig. 3.24 - 3.27**) which might point to the negative effect of the PD-1 blockade on the survival of myeloma bearing mice, especially if combined with other immune modulatory drugs. Both, HDAC inhibitors and PD-1 blockade are known to up-regulate PD-L1 expression which might have a negative influence on the survival effect of the combinatorial approach. We could show that CD1d is strongly downregulated on CD4+ and CD8+ T cells in the animal group receiving the panobinostat monotherapy (**fig. 3.26**). Also, the CD4+ NKT CXCR4 expression pattern is differentially regulated following panobinostat monotherapy (**fig. 3.25**). The precise relation of HDAC inhibitors and NKT cells via regulation of CD1d and CXCR4 in this context still remains an open question.

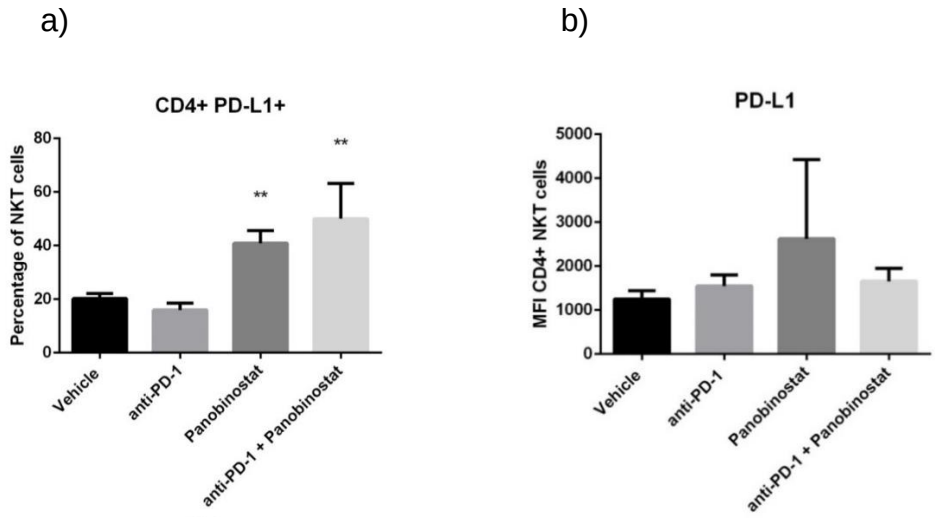

Fig. 3.24: CD4+ NKT cells in the spleen of mice treated with panobinostat monotherapy, anti-PD-1 monotherapy, combinatorial treatment and vehicle control. a) Significant increase ($p<0.01$) in the number of CD4+ PD-L1+ NKT cells of both, panobinostat treated and of panobinostat treated in combination with anti-PD-1 mAb compared to anti-PD-1 monotherapy

and vehicle control. **b)** There was no difference in the number of PD-L1 receptors expressed on CD4+ NKT cells. Flow cytometry analysis were performed at the disease end stage. 3 mice per group each were analyzed. Statistics: one-way ANOVA.

a) b)

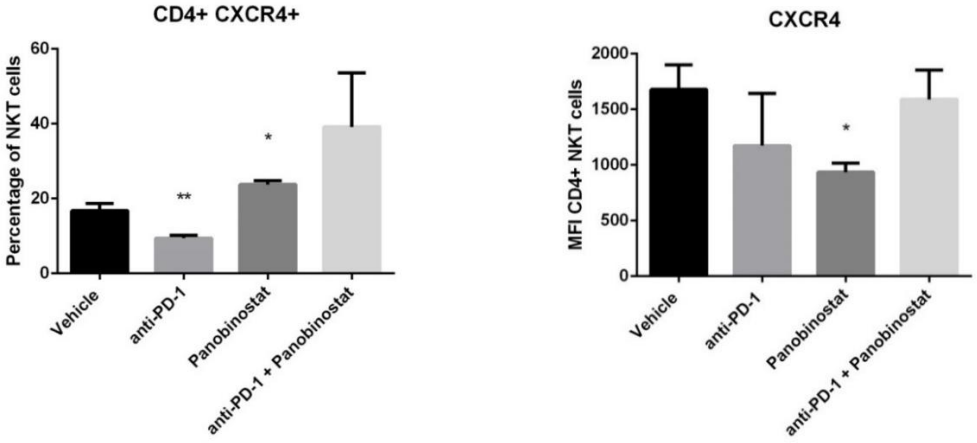

Fig. 3.25: CD4+ NKT cells in the spleen of mice treated with panobinostat monotherapy, anti-PD-1 monotherapy, combinatorial treatment and vehicle control. a) Significant decrease in the number of CD4+ CXCR4+ NKT cells the anti-PD-1 mAb treated mice ($p<0.01$) while an increase in the number of CD4+ CXCR4+ NKT cells in the panobinostat only group ($p<0.05$) was observed. **b)** Significant decrease ($p<0.05$) in the number of CXCR4 receptors expressed on CD4+ NKT cells in the panobinostat only group. Flow cytometry analysis were performed at the disease end stage. 3 mice per group each were analyzed. Statistics: one-way ANOVA.

Interestingly, the group with the longest survival, using the panobinostat monotherapy, showed an increase of CD4+ CXCR4+ NKT cells in the spleen (**fig. 3.25 a**), accompanied by a downregulation of the CXCR4 receptor (**fig. 3.25 b**) that regulates bone marrow and trafficking to the inflamed tissue. As we see in the analysis four weeks post tumor cell injection, the ratio of CD4-/CD4+ NKT cells increases during tumor progression (**fig. 3.14**). Furthermore, the number of PD-L1+ CD4+ NKT cells is increased when mice are treated with panobinostat, which could also be a marker for a more activated immune microenvironment (**fig. 3.24**).

a) b)

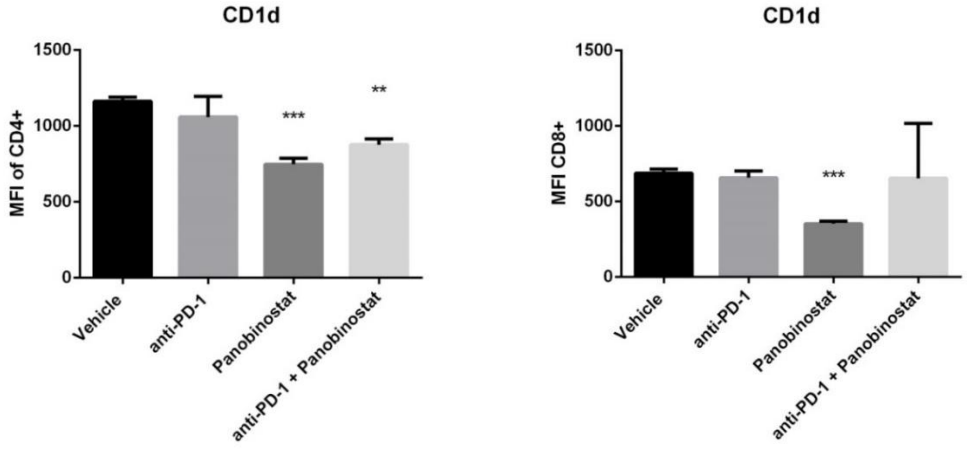

Fig. 3.26: CD1d expression on CD4+ and CD8+ T cells in the spleen of mice treated with panobinostat monotherapy is downregulated. a) A significant decrease of the number of CD1d receptor expression on CD4+ T cells was found in the panobinostat treated ($p<0.001$) and in the double treated group ($p<0.01$) compared to anti-PD-1 monotherapy and vehicle control. **b)** The number of CD1d receptor expression on CD8+ T cells was significant decreased in the panobinostat treated group ($p<0.01$) compared to anti-PD-1 monotherapy, double treated and the vehicle control. Flow cytometry analysis was performed at the disease end stage. 3 mice per group each were used. Statistics: one-way ANOVA.

a) b)

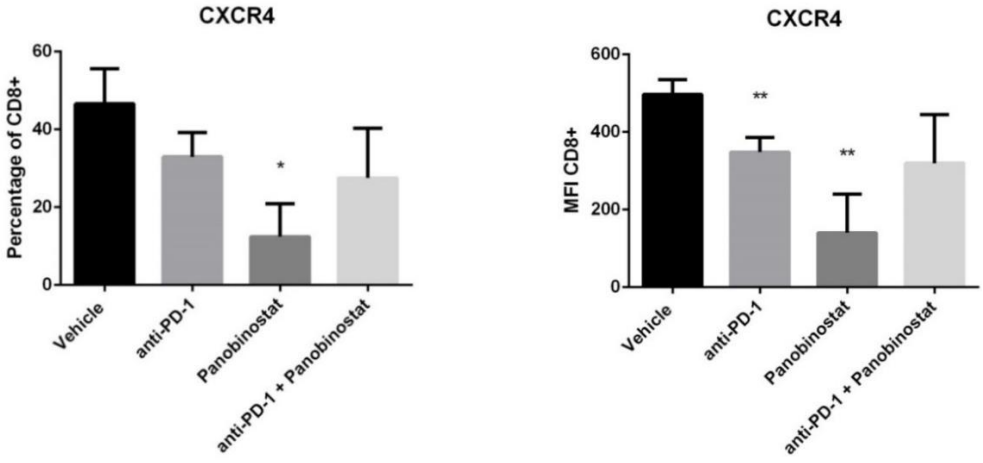

Fig. 3.27: CXCR4 expression on CD8+ T cells in the spleen of mice treated with panobinostat monotherapy is decreased. a) A significant decrease in the number of CD8+ CXCR4+ T cells in the panobinostat treated group ($p<0.05$) was found compared to the anti-PD-1, double treated and vehicle control group. **b)** The number of CXCR4 receptor expression on CD8+ T cells was significant decreased in the panobinostat ($p<0.01$) and in the anti-PD-1 treated group ($p<0.01$) compared to the double treated and the vehicle control group. Flow cytometry analysis were performed at the disease end stage. 3 mice per group each were analyzed. Statistics: one-way ANOVA.

In summary, these results indicate that panobinostat influences the immune regulated anti-myeloma properties of PD-L1 and CXCR4 receptor expression and hence the survival of myeloma bearing mice. It is known that both, panobinostat and anti-PD-1 blockade, can induce PD-L1 expression leading to diminished anti-tumor functions. Here, we show that PD-L1 is especially increased on CD4+ NKT cells in the spleen of mice treated with the combination of pan-HDAC inhibitor and ICI (**fig. 3.24**). As panobinostat is known as CXCR4 antagonist, it is remarkable that especially the CD8+ T cells show a strong reduction in their CXCR4 expression which might regulate their effector functions (**fig. 3.27**). CXCR4 enables trafficking not only to the bone marrow, but also to metastasis and inflamed tissues where the CXCR4/CXCL12 signaling axis mediates tumor invasiveness, vascularization and angiogenesis. The reduced CXCR4 expression on the CD8+ T cells might prevent their trafficking to the tumor and hence diminishing their anti-myeloma properties.

3.3.2 PD-L1 genetic knockout of 5T33 cells facilitates prolonged survival of myeloma challenged mice

Despite the strong tumor immune editing function of the PD-1/PD-L1 signaling pathway, either anti-PD-1 nor anti-PD-L1 mAbs could decelerate the tumor growth of 5T33 challenged mice. Moreover, the blockade of PD-1 diminishes the anti-myeloma efficiency of panobinostat (**fig. 3.23**). We questioned whether a genetic KO of PD-L1 of the 5T33 myeloma cells might enable a profound delay in tumor establishment through the precise and complete abrogation of the immunosuppressive PD-L1 function. Pointing towards this direction, an additional tumor cell intrinsic survival mechanism has been reported as function for PD-L1, which might enhance the anti-tumor efficiency of a genetic PD-L1 KO (Azuma et al., 2008). **Fig. 3.28** shows the significant survival prolongation of KaLwRij mice injected with 5T33 PD-L1 KO cells compared to mice challenged with 5T33 Mock transfected cells.

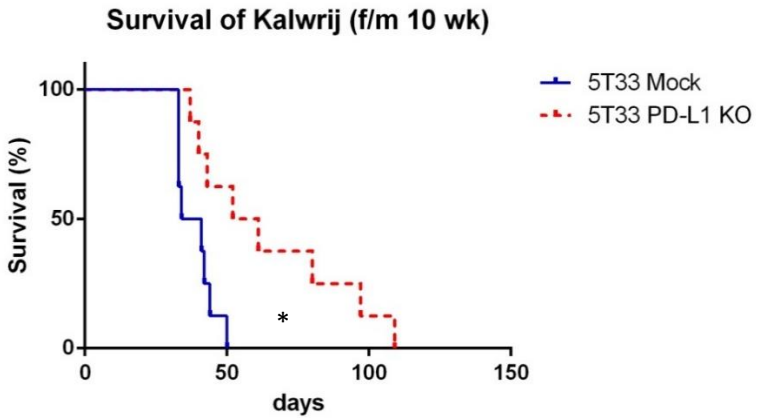

Fig. 3.28: The genetic KO of PD-L1 leads to a significant survival benefit. Survival of KaLwRij mice injected with 5T33 PD-L1 KO cells was significant increased ($p<0.01$) compared to mice inoculated with 5T33 Mock transfected cells. 8 mice per group each were used. Statistics: Log rank test (Mantel-Cox analysis): $p<0.01$.

To further investigate the molecular mechanism of the anti-tumor properties of the genetic PD-L1 KO, we analyzed immune regulatory receptors of the CD4+, CD8+ T cells and NKT cells in BM, spleen and, if available, from the tumor, at the end stage of myeloma disease. The immunoregulatory receptors PD-1, PD-L1, TIM-3, TIGIT, CD40L, CD1d and CXCR4 were measured using flow cytometry. First, we stained for co-inhibitory and immunoregulatory receptors to further analyze the T cells and their expression of PD-1, PD-L1, CXCR4, CD1d as well as the activation marker CD40L (**fig. 3.29 – 3.35**).

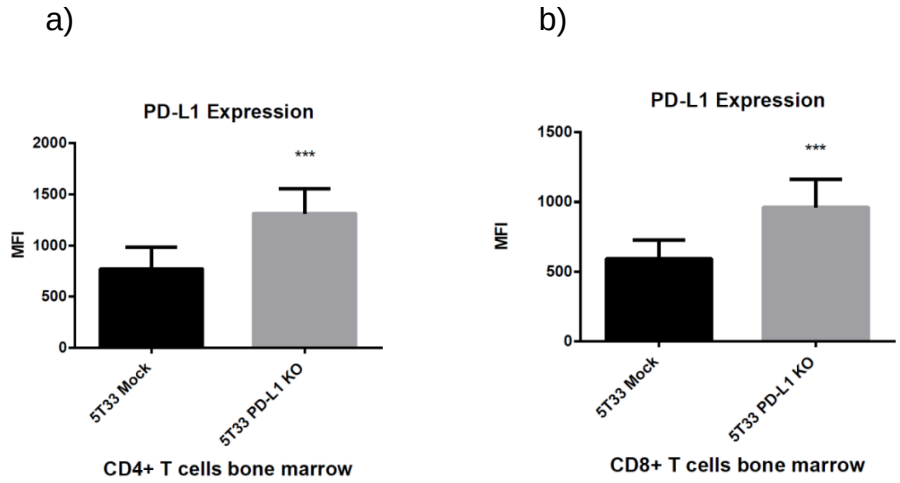

Fig. 3.29: PD-L1 expression of CD4+ and CD8+ T cells is increased in the bone marrow of mice challenged with 5T33 PD-L1 KO cells compared to the control. Analysis of CD4+ and 8+ T cells in the BM of mice inoculated with either 5T33 PD-L1 KO or 5T33 Mock transfected

cells. **a)** Significant increase ($p<0.001$) in the PD-L1 expression of CD4+ T cells and **b)** significant increase ($p<0.001$) of CD8+ T cells in 5T33 PD-L1 KO injected mice compared to the Mock control group. Flow cytometry analysis were performed at the disease end stage, (n=8). Statistics: unpaired t-test.

It is well studied that activated T cells upregulate the co-inhibitory receptors, such as PD-1 and TIGIT, following the engagement with co-stimulatory receptors to terminate the appropriate immune response. Here, we observe that especially PD-L1 is highly expressed on both, CD4+ and CD8+ T cells in the BM of the 5T33 PD-L1 KO inoculated mice (**fig. 3.29**). Additionally, the PD-1 expression is increased on CD4+ and CD8+ T cells in the BM of mice challenged with 5T33 PD-L1 KO cells compared to the Mock transfected tumor cells (**fig. 3.30**). This leads to the assumption that the PD-L1 checkpoint blockade of the myeloma cells leads to an increased activation state of the tumor-immune-microenvironment which is essential for the inhibition of the disease progression.

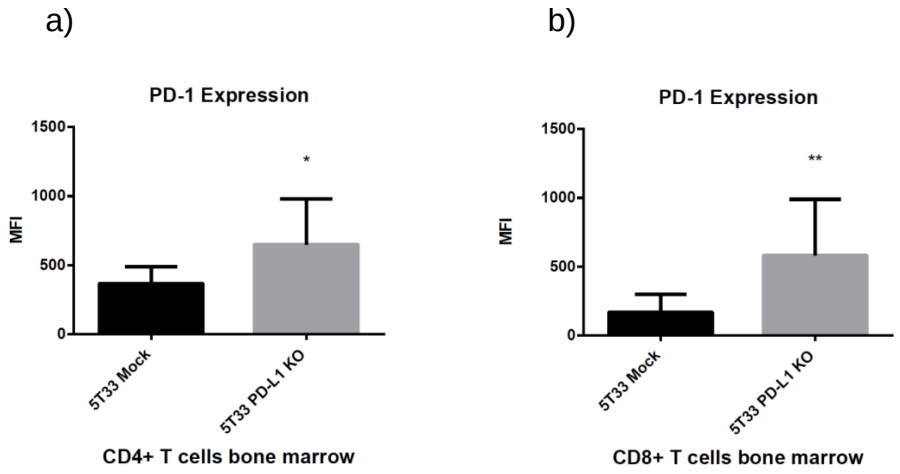

Fig. 3.30: PD-1 expression of CD4+ and CD8+ T cells is increased in the bone marrow of mice challenged with 5T33 PD-L1 KO cells compared to the control. Analysis of CD4+ and 8+ T cells in the BM of mice inoculated with either 5T33 PD-L1 KO or 5T33 Mock transfected cells. **a)** Significant increase ($p<0.05$) in the PD-1 expression of CD4+ T cells and **b)** significant increase ($p<0.01$) of CD8+ T cells in 5T33 PD-L1 KO injected mice compared to the Mock control group. Flow cytometry analysis were performed at the disease end stage, (n=8). Statistics: unpaired t-test.

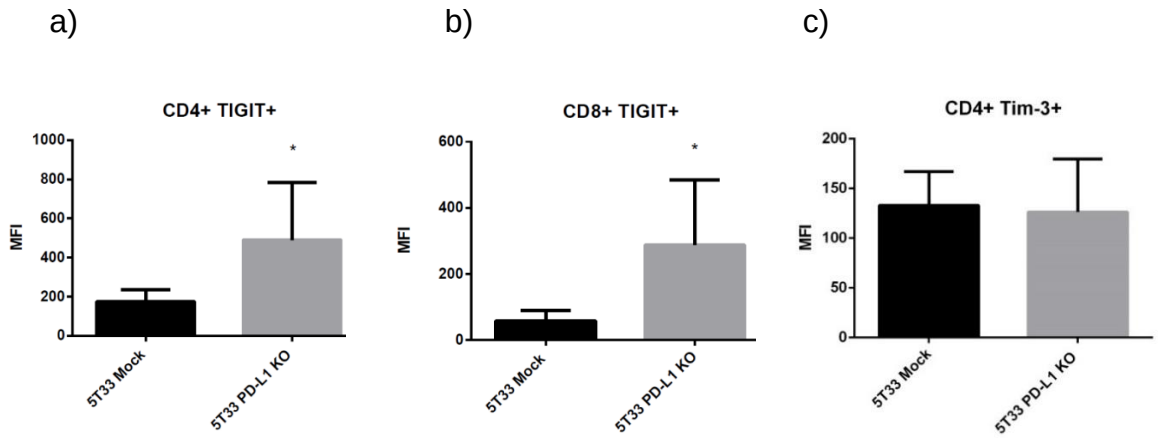

Fig. 3.31: TIGIT expression of CD4+ and CD8+ T cells is increased in the spleen of mice challenged with 5T33 PD-L1 KO cells compared to the control. Analysis of CD4+ and CD8+ T cells in the spleen of mice inoculated with either 5T33 PD-L1 KO or 5T33 Mock transfected cells. **a)** Significant increase ($p<0.05$) in the TIGIT expression of CD4+ T cells and **b)** significant increase ($p<0.05$) of CD8+ T cells in 5T33 PD-L1 KO injected mice compared to the Mock control group. **c)** No change in Tim-3 expression of CD4+ T cells in the spleen of KaLwRij mice injected with 5T33 Mock vs PD-L1 KO cells. Flow cytometry analysis was performed at the disease end stage, (n=6). Statistics: unpaired t-test.

While the co-inhibitory receptor TIGIT is significantly increased on CD4+ and CD8+ T cells in the spleen of mice challenged with the PD-L1 KO myeloma cells (**fig. 3.31**), we observed that the T cells of both groups show the same expression level of the exhaustion marker Tim-3, which is mainly upregulated in chronic activated immune cells (Han et al., 2013).

To identify if the PD-L1 expression on the tumor cells directly modulates the CXCR4 expression on the immune cells and therefore their trafficking and effector functions at the tumor sides, we further analyzed the CXCR4 expression on CD4+ and CD8+ T cells. As shown in **fig. 3.32**, both CD4+ and CD8+ T cells in the spleen of mice challenged with the 5T33 PD-L1 KO tumor cells show reduced level of CXCR4 expression at the disease end stage.

a) b)

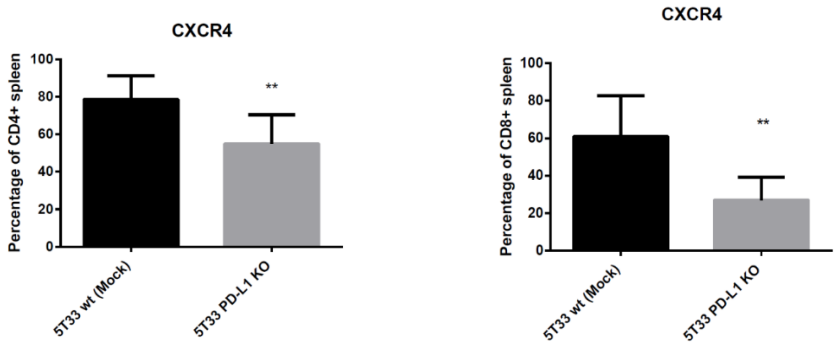

Fig. 3.32: The number of CXCR4+ CD4+ and CD8+ T cells is decreased in the spleen of mice challenged with 5T33 PD-L1 KO cells compared to the control. Analysis of CD4+ and 8+ T cells in the spleen of mice inoculated with either 5T33 PD-L1 KO or 5T33 Mock transfected cells. **a)** Significant reduction ($p<0.01$) of the number of CD4+ T cells and **b)** significant reduction ($p<0.01$) of CD8+ T cells expressing CXCR4 in 5T33 PD-L1 KO injected mice compared to the Mock control group. Flow cytometry analysis was performed at the disease end stage, (n=7). Statistics: unpaired t-test.

Since it is important that myeloma cells expressing PD-L1 have impact on the regulation of the immune microenvironment, we further analyzed the CD1d expression of the T cells in the spleen and BM, which might give insights into the complex communication interplay with the NKT cells.

a) b)

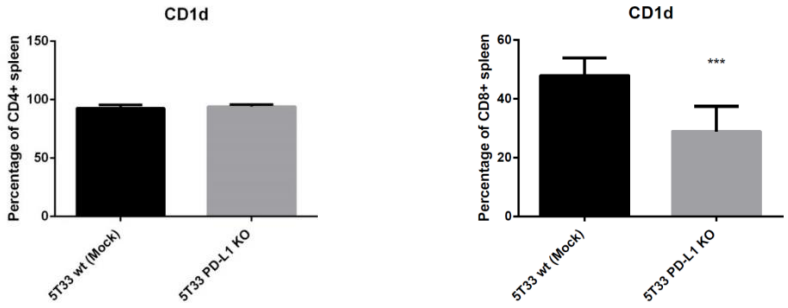

Fig. 3.33: The number of CD1d+ CD8+ T cells is decreased in the spleen of mice challenged with 5T33 PD-L1 KO cells compared to the control. Analysis of CD4+ and CD8+ T cells in the spleen of mice inoculated with either 5T33 PD-L1 KO or 5T33 Mock transfected cells. **a)** No difference in the number of CD1d+ CD4+ T cells in the spleen. **b)** Significant reduction ($p<0.001$) in the number CD1d expressing CD8+ T cells in the spleen of 5T33 PD-L1 KO injected mice compared to the Mock control group. Flow cytometry analysis was performed at the disease end stage, (n=7). Statistics: unpaired t-test.

As seen in **fig. 3.33**, the number of CD8+ T cells expressing CD1d is significantly reduced in the spleen of mice challenged with 5T33 PD-L1 KO cells compared to the mice challenged with the 5T33 Mock cells. We could not identify a modulation of the number of NKT cells. The reduction of CD1d on CD8+ T cells might point to the importance of their effector functions and NKT cell - tumor cell communication via PD-L1 during the shift into a pro-tumorigenic microenvironment.

Besides HLA like molecules and co-inhibitory receptors, also co-stimulatory receptors such as CD40L modulate the immune homeostasis (Zhang and Vignali, 2016). Here, we show that the number of CD40L+ CD4+ T cells in the spleen of mice injected with PD-L1 KO myeloma cells are significant increased compared to the CD4+ T cells in the group with 5T33 Mock tumor burden (**fig. 3.34**).

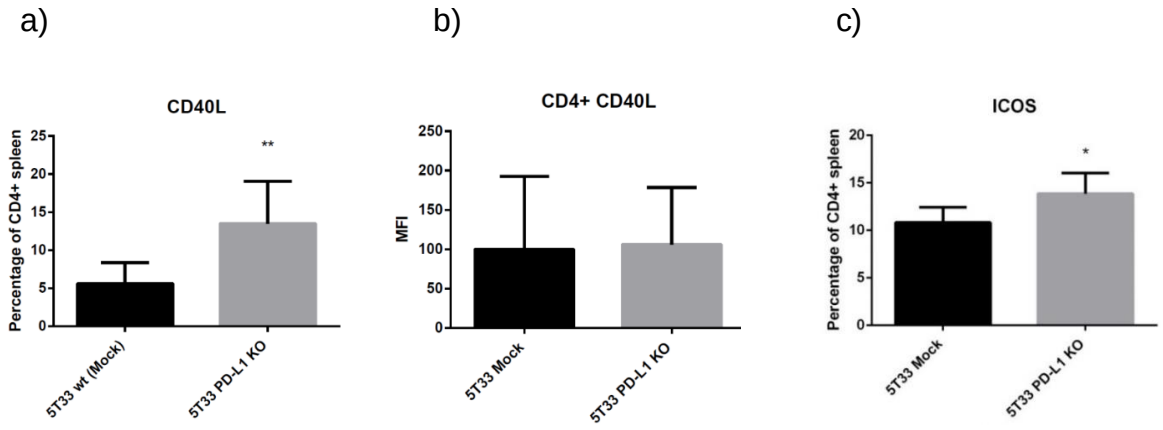

Fig. 3.34: The number of CD40L+ CD4+ T cells is increased in the spleen of mice challenged with 5T33 PD-L1 KO cells compared to the control. Analysis of CD4+ T cells in the spleen of mice inoculated with either 5T33 PD-L1 KO or 5T33 Mock transfected cells. **a)** Significant increase ($p<0.01$) in the number of CD40L expressing CD4+ T cells in the spleen compared to the Mock control group. **b)** No change in the MFI of CD40L on CD4+ T cells in the spleen of mice challenged either with 5T33 Mock or PD-L1 KO cells. **c)** ICOS expression is significant increased in CD4+ T cells in the spleen of KaLwRij mice challenged with 5T33 PD-L1 KO cells ($p<0.05$). Flow cytometry analysis was performed at the disease end stage, (n=6). Statistics: unpaired t-test.

3.3.3 No difference of MM disease progression in NOD.*scid*.*Il2Rγc*^{null} mice using 5T33 Mock vs PD-L1 KO cells

The unexpected result of the CD8+ T cell depletion in the survival of myeloma challenged mice lead us to the question whether the KO of PD-L1 on the tumor cells enables a survival benefit because of the modulated tumor- immune- cell interactions. Hence, we used NOD.*scid*.*Il2Rγc*^{null} (NSG) mice with severe immunodeficiency to ask if the survival prolongation is a result of the immunological response to PD-L1 and that otherwise both tumor cell lines enable the same tumor burden *in vivo*. In **fig. 3.35** we show that immunodeficient NSG mice experience the same tumor burden post 5T33 Mock and PD-L1 KO challenge. This suggests that the previously observed survival prolongation is dependent on the PD-L1 tumor interaction with immune cells. Moreover, as expected, immunodeficient NSG mice show an overall shorter survival than KaLwRij mice challenged with 5T33 cells.

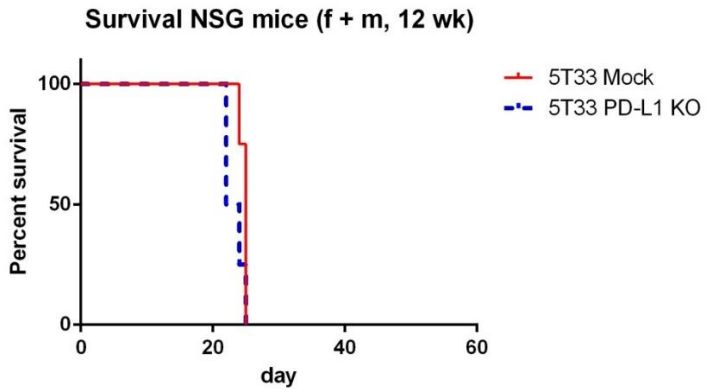

Fig. 3.35: Survival of NOD.*scid*.*Il2Rγc*^{null} (NSG) mice inoculated with 5T33 Mock vs 5T33 PD-L1 KO cells. There is no difference in the life span of NSG mice challenged either with 5T33 Mock or PD-L1 KO cells. Tumor burden in immunodeficient mice leads to shorter survival compared to KaLwRij mice, (n=4). Statistics: Log rank test (Mantel-Cox analysis): ns.

4. Discussion

CPI has shown dramatic improvements in overall survival in many malignant diseases including melanoma and Hodgkin lymphoma (Hodi et al., 2010) (Ansell et al., 2015). However, in MM the outcomes were disappointing resulting in an early termination of clinical trials (Mateos et al., 2019). Despite the continuing recent advances in therapy the disease remains incurable and especially the development of therapy resistances means that additional treatment modalities are urgently needed.

Here, we aimed to investigate the efficiency and immunological mechanisms of CPI with focus on PD-1/PD-L1 blockade using KaLwRij mice that develop MM upon injection of 5T33 murine myeloma cells. For this purpose, BM, lymph nodes and spleen cells were analyzed by flow cytometry for the appearance and phenotype of different immune subpopulations.

Immune checkpoint blockade using mAbs targeting PD-1 in MM treatment were either not effective or lead to severe adverse effects including CRS and sudden unexpected deaths (Mateos et al., 2019) (Tremblay-Lemay et al., 2018). The exact molecular mechanisms leading to the serious adverse events and diminishment of established MM therapy are still in early investigations and not well understood. In this thesis we aimed to identify immunoregulatory changes in the TME during MM disease progression and immune checkpoint blockade.

Treatment of mice with mAbs blocking PD-1 or CD47 alone or in combination had no effect on tumor growth and survival (**fig. 3.22**). Comprehensive immunophenotyping of T cell and NK cell subpopulations revealed no differences between early disease stage MM bearing mice and healthy control groups. Surprisingly, the combined application of the anti-PD-1 blocking antibody with the pan-HDAC inhibitor panobinostat reduced the anti-myeloma effect of the compound and resulted in decreased survival (**fig. 3.23**).

Panobinostat is approved for the treatment of RRMM patients and beside its direct effect on myeloma cells, it increases the immunogenicity of malignant cells by improving the presentation of tumor antigens and modulates the immunological composition of the TME (Bailey et al., 2015) (Sun et al., 2019). By analyzing the phenotype of immune cells

in the different populations, we found an increase in the CXCR4 expressing CD4+ NKT cells in the panobinostat treated animal group (**fig. 3.25**). Additionally, the CD8+ T cells expressing CD1d and CXCR4 decreased compared to the other groups in the spleen (**fig. 3.26** and **3.27**). CD1d is a MHC like receptor for glycolipids activating NKT cells, whereas CXCR4 is a BM homing receptor and linked to metastasis and tumor aggressiveness (Venkataswamy and Porcelli, 2010) (Furusato et al., 2010).

It has already been demonstrated that pan-HDAC inhibitors can induce antigen presentation not only via MHC but also via CD1d and therefore restore the NKT cell mediated anti-tumor response (Tiper and Webb, 2016). In this context, Tiper, Webb and coworkers postulated that mantle cell lymphoma cells use epigenetic mechanisms to diminish antigen presentation via CD1d. The epigenetic modulating enzyme HDAC2 can directly bind and repress the CD1d gene. By the treatment with pan-HDAC inhibitor, they showed that CD1d was upregulated and both MHC class II and CD1d mediated antigen presentation was increased *in vitro*. NKT cells have a profound impact on anti-tumor immunity. The cancer escape mechanisms by diminishing CD1d mediated antigen presentation leads to new suggestions of combining HDAC inhibitors with NKT cell-based immunotherapy (Tiper and Webb, 2016). Here, we show that this assumption might apply for multiple myeloma, as panobinostat modulates the CD1d expression both *in vitro* and *in vivo.* Therefore, the compound strongly regulates the glycolipide dependent immunogenicity and hence aberrant NKT cell activation and trafficking. As demonstrated in our *in vitro* data (**fig. 3.12**) panobinostat induces CD1d expression. In contrast, CD4+ and CD8+ T cells in the spleen of mice treated with panobinostat showed a decreased CD1d expression at the disease end stage (**fig. 3.26**), while CD4+ CXCR4+ NKT cells increase as potential marker for prolonged survival (**fig. 3.25**).

We show that CD4+ CXCR4+ NKT cells in the spleen of mice treated with anti-PD-1 mAbs are significantly reduced compared to untreated control and panobinostat or double treated animal groups (**fig. 3.25**). As CXCR4 is sufficient for the trafficking of NKT cells, this influence might shift the focus on the tumor responsive NKT cells and a potential negative interaction of ICI (Beider et al., 2003). In the panobinostat treated group, the amount of CD4+ NKT cells expressing CXCR4 was increased in contrast to the CPI treated group. However, the cell surface receptor expression level of CXCR4

was significantly downregulated on these cells. Recently it was found that panobinostat serves, beside its gene activation and various immunoregulatory functions, also as selective CXCR4 antagonist (Mandawat et al., 2010). Therefore, the efficiency of panobinostat´s direct non-epigenetic modulations might also result from the modulation of NKT cell trafficking to the inflamed tissue. Additionally, panobinostat has direct cell killing properties such as cell cycle arrest and apoptosis induction, and hence might increase the antigen release from dying tumor cells which further primes and attracts effector cells, including NKT cells (Klein et al., 2013) (Mandawat et al., 2010). As most of the tumor cells grow within the BM, NKT cells expressing CXCR4 might be especially sensitive to this mechanism of panobinostat. But why is the blockade of PD-1 abrogating the assumed CD4+ CXCR4+ NKT cell modulating anti-tumor effect?

This hypothesis needs further investigations, which include analysis if NKT cells in the BM characterized by CD4 and CXCR4 expression might obtain different tumor responses during disease progression. CD4+ NKT cells might be associated with anti-tumor capabilities (**fig**. **3.14, fig. 3.17** and **fig. 3.20**), while strong CXCR4 expression might be associated with tumor progression (**fig. 3.18 - 3.21, fig. 3.27**). As CXCR4 antagonist, panobinostat can prevent the homing to the BM by both, high expressing MM and NKT cells. On the other hand, panobinostat can induce CD1d on MM cells, but as shown in our *in vitro* analysis, in dependence of the PD-L1 gene (**fig. 3.11** and **3.12**). By applying anti-PD-1 mAbs, the PD-L1 signaling is attenuated, CD1d on MM cells less expressed and hence NKT cells are less attracted to the tumor site. This shield blocking NKT cells from the tumor could be enabled by both mechanisms: less CD1d on MM cells is expressed because of the PD-1 blockade and second, because of panobinostat´s antagonistic function to CXCR4, which prevents the effector cells to migrate to the BM where most of the MM cells are growing. In case of the panobinostat monotherapy, CD1d expression of MM cells might be enhanced and is sufficient to prime and attract NKT cells. This anti-myeloma effect might be abrogated in case of simultaneously blocking the PD-1/PD-L1 signaling during HDACi therapy.

CXCR4/CXCL12 signaling regulates the dissemination of cancer cells due to the degradation of the ECM by MMPs, which allows mobilized cancer cells not only to establish in other BM niches, but also through invading the blood stream to reach distant

organs. This advanced and aggressive disease stage with tumor cell manifestations outside the BM leads to the EMD (Ullah, 2019). Typically, MM cells disseminate to secondary lymphoid organs like lymph nodes and the liver. Approximately 8% of patients develop EMD during disease progression which is strongly associated with very poor prognosis (Rajkumar, 2016).

Following drug treatment, stem-like cancer cells can survive in a low proliferative stage of dormancy. Here, the bone marrow microenvironment serves an ideal surviving niche, as the stromal cells secrete survival factors and the hypoxic microenvironment promotes aggressive tumor cell capabilities. Activation of osteoclasts can lead to reactivation and proliferation of these dormant cancer cells (Lawson et al., 2015a). Upon the outgrowth of the remaining tumor cells, this dormancy stage of minimal residual disease (MRD) is characterized by high CXCR4 expression leading to the refractory and relapsed MM phenotype. This phenotype is associated with multidrug resistance and still making the disease incurable (Paiva et al., 2016).

As only those cancer clones will survive, which are best adapted to the tissue in which they grow and bear a phenotype which allows them to hide from the immune attack. These clones display a decreased immunogenicity that allows them to hide from the immune system. They can survive in a stage of dormancy and that underlines why the equilibrium stage is also referred to tumor cell dormancy (Sistigu et al., 2020).

Regarding MM, this is the early disease stage of MGUS and SMM, where the tumor is beginning to be clinically measurable but still asymptomatic. Similarly, after successful therapy induction in patients with active MM, the disease stage is described as MRD which refers to dormancy (Almog, 2010) (Balayan and Guddati, 2022). While osteoclast activation allows MM cells to regrow, a low blood supply can cause tumor cell dormancy (Lawson et al., 2015). Due to the activation of tumor associated macrophages (TAMs) and endothelial cells, which both secrete VEGF, the angiogenic switch is induced. In turn, so called neovascularization leads to increased supply with nutrients and oxygen enabling the fast proliferation of the malignant clone (Y. Lin et al., 2019). However, referring to the MM BM niche, the most important reason for dormancy and disease progression might be the shifting tumor immune microenvironment (Nakamura et al., 2020).

In our 4 wk analysis we observed MM disease progression accompanied by BM NKT cell infiltration (**fig 3.14 and 3.16**). These NKT cells have a strong PD-L1 and CXCR4 expression compared to healthy control and analysis three wk post tumor cell injection. Moreover, their PD-L1 CXCR4 expression pattern was phenotypically less divers as this NKT cell subpopulation and showed a more homogenous PD-L1 CXCR4 expression pattern as the healthy control (**fig 3.18**). By analyzing MM bearing mice in disease end stage, we found that the BM infiltration of NKT cells were only found in mice developing signs of leg paraplegia (**fig. 3.15**). In contrast, those mice developing a solid tumor, did not show an increased NKT cell number in the BM, but instead in the tumor tissue. Interestingly, the disease end stage was characterized by stronger PD-L1+ CXCR4+ NKT cell infiltration in the BM compared to the analysis five weeks post tumor cell inoculation corresponding to an advanced disease stage (**fig 3.19**). The significant increase of CXCR4 expression in the BM of NKT cells four wk post tumor cell injection was only observed for the CD4- NKT cell subpopulation (**fig 3.17**).

As BMSC secrete pro-survival and anti-apoptotic factors due to the adherence of MM cells via CXCR4, they promote a protective niche for tumor growth. Upon adherence, CXCL12 up-regulates its own expression and this in turn enhances IL-6 expression, which is known to stimulate osteoclastogenesis. Therefore, osteoclastogenesis is activated by high CXCR4 expression of the tumor cells (Aggarwal et al., 2006) (Tang et al., 2008). Our results lead to the hypothesis that also CXCR4 expression on CD4- NKT cells is associated with enhanced osteolytic bone lesions.

In summary, we found a gradually increase of CXCR4+ NKT cells in the BM corresponding to MM disease progression. Interestingly, we measured a shift in the CD4+/CD4- NKT cell ratio during disease progression, whereby the CXCR4+ CD4- NKT cells seem to be associated with advanced tumor growth, while the increase of CXCR4+ CD4+ NKT are associated with prolonged survival as observed in in the panobinostat treated group.

In the complex cross talk of immune cell and tumor cell interactions, NKT cells modulate the development of both the adaptive and innate immune cells, to become either immunosuppressive or to increase the effector functions to keep the tissue homeostasis balanced (Terabe and Berzofsky, 2008). The pattern of their immunomodulatory cytokine

cocktail including Th1 cytokines such as IFNγ and TNF-α or typical Th2 cytokines such as IL-4 and IL-10 are dependent on the type of activation of the NKT cells (Liao et al., 2013). Following the activation of their semi-invariant T cell receptor, NKT cells are modulated by the binding of either co-stimulatory receptors, including but not limited to CD40L, or by co-inhibitory receptors such as PD-1 (Shissler et al., 2017). Recently it was found that by analyzing the gene expression profile database of gastric cancer and gastric precancerous lesions there is a significant decrease of NKT cell infiltration which negatively correlates with CXCR4 expression during tumorigenesis. The authors postulate that NKT cells and CXCR4 expression might serve as biomarkers to monitor gastric cancer progression (Jiang et al., 2020) (Jiang et al., 2021). Furthermore, CXCR4 is associated with cancer growth, invasion and metastasis and clinical studies are ongoing that evaluate the effect of CXCR4 antagonists preventing cancer dissemination (Cortés et al., 2018). Recently a new immunotherapeutic approach in a Sarcoma model showed promising results with the combination of activated and expanded NK cells with anti-CXCR4 antibodies to prevent tumor implantation as well as metastasis outgrowth (Vela et al., 2019). It was further demonstrated that the genetic KO of CXCR4 in myeloid cells reactivated the anti-tumor activity on NK cells depending on the Fas/FasL signaling pathway in a melanoma mouse model (J. Yang et al., 2018). As NKT cells have both properties of T and NK cells, it might therefore be possible that CXCR4, in dependence of NKT cells, enables a pro-tumorigenic microenvironment especially in the bone marrow where the myeloma cells evoke.

TGF-β is known to promote tumor progression e.g. by stimulating metastasis, chemoresistance and is associated with osteolytic bone disease in MM. Myeloma cells secrete TGF-β and hence promote a pro-tumorigenic and immunosuppressive bone marrow niche (Lu et al., 2016) (Urashima et al., 1996). It is known that pan-HDAC inhibitors can attenuate the TGF-β induced tumor promoting processes relating to EMT (Sakamoto et al., 2016) (Wawruszak et al., 2019), we show that panobinostat inhibits the secretion of TGF-β from 5T33 myeloma cells *in vitro* **(fig. 3.13b)**. The downregulation of TGF-β by panobinostat might therefore partly restore the effector function of NKT cells by promoting a Th1 friendly microenvironment.

Interestingly, TGF-β is further known to induce EMT and tumor aggressiveness via Zeb1 induction (Joseph et al., 2014). As shown in **fig. 3.13a** panobinostat reduces the expression of the transcription factor Zeb1 in 5T33 cells assuming a myeloma promoting signaling pathway which can be attenuated by the pan-HDAC inhibitor.

Zeb1 can serve as link between the PD-1/PD-L1 signaling and the induction of metastasis and drug resistance. The tumor suppressor miRNA200c and the proto-oncogene Zeb1 negatively regulate each other. The miRNA200c inhibits Zeb1 and the PD-L1 induction, whereas Zeb1 induces PD-L1, angiogenesis via VEGF and PDGF as well as induces CXCR4 transcription. Interestingly, Zeb1 is also induced by HIF-1α, which has a critical pro-tumorigenic role in the hypoxic bone marrow niche of the myeloma cells (L. Chen et al., 2014) (Beji et al., 2017) (J. Zhu et al., 2018). Moreover, Zeb1 is also known to induce CD47 making it a promising target in tumor immunotherapy (Y. Guo et al., 2021).

As Zeb1 is known to induce PD-L1 and CXCR4 directly (Y. Guo et al., 2021) (Beji et al., 2017), this might be another hind that the function of CXCR4 is critical to the MM tumor progression. Up-stream of the signaling pathway of CXCR4, which might include Zeb1, there might be a critical switcher for the tumor manifestation and aggressiveness regulating various pro-tumorigenic processes.

CXCR4 is linked to tumor aggressiveness and we see in **chapter 3.2.3** how CXCR4 expression increases particularly on NKT cells in the bone marrow during tumor progression and extramedullary disease manifestation in correspondence to PD-L1 expression.

Interestingly, our *in vivo* analysis show, that treating 5T33 wt injected mice with either panobinostat, anti-PD-1 or the combination of both, those mice receiving only panobinostat had the most beneficial effect on the survival. As we will discuss later, the analysis of CD4+, CD8+ T and NKT cells in spleen, BM and, if available, tumor of different co-stimulatory, co-inhibitory as well as metastasis and NKT cell regulating receptors showed, that CXCR4 is significant decreased on CD8+ T cells in the spleen in mice treated with panobinostat compared to PD-1 treated, double treated or the untreated group (**fig. 3.27**).

As we see in **chapter 3.3.2**, mice challenged with 5T33 PD-L1 KO cells demonstrate a significant longer survival than mice challenged with 5T33 Mock cells (see **fig. 3.28**). The hypothesis that a downregulation of CXCR4 on CD8+ T cells in the spleen of myeloma bearing mice is a possible marker of longer survival might be underpinned by the finding, that one particular difference of the immune cells analyzed in the disease end stage of 5T33 Mock and PD-L1 KO injected mice, was that, again, the CD8+ T cells in the spleen of 5T33 PD-L1 KO challenged mice had a significant reduced expression of CXCR4 compared to the T cells in the Mock transfected mice (**fig. 3.32**). **Figure 3.32** demonstrates that both CD4+ and CD8+ T cells in the spleen of mice challenged with the 5T33 PD-L1 KO tumor cells show a strong reduction of their CXCR4 expression at the disease end stage. Hence, the genetic KO of PD-L1 on myeloma cells leads to a decrease of T cells expressing CXCR4, which is strongly associated with tumor progression and aggressiveness (Chang et al., 2017). Interestingly, it was recently shown that CXCR4 overexpression on B cells is associated with a highly aggressive form of chronic lymphatic leukemia (Lewis et al., 2021).

These results demonstrate the interplay of the PD-1/PD-L1 and CXCR4/CXCL12 signaling axis on the myeloma and T cells in the tumor immune microenvironment. As both, PD-L1 and CXCR4 are potential targets in anti-tumor therapy, these insights into the pathomechanism of multiple myeloma might be used for the development of new therapeutic approaches (Jelinek et al., 2017) (Liang et al., 2004). Especially the tumor protecting bone marrow niche, which is a critical component of the process of cancer immunoediting and tumor outgrowth, is influenced by the interaction of PD-1/PD-L1 and CXCR4/CXCL12 signaling.

Appropriately, we showed for the first time that CD1d expression was approximately 50% reduced in 5T33 PD-L1 KO cells compared to the Mock transfected 5T33 cells (**fig. 3.11**). The dependency of CD1d expression on the PD-L1 gene further links NKT cell mediated anti-tumor response to the immune escape mechanism as well as to CPI therapy. Even though it is quite unexpected that CD1d is associated with tumor progression as it supports immunogenicity by activating NKT cells. Nonetheless, CD4+ and CD8+ T cells in the spleen of mice treated with panobinostat, which had the longest

tumor free survival, also showed a decreased CD1d expression at the disease end stage (**fig. 3.26**).

It would be interesting to analyze the expression pattern of the myeloma cells in upcoming *in vivo* studies at different disease time points and treatment modalities. Here, a further analysis that combines the application of panobinostat and anti-CD1d depleting mAbs in KaLwRij mice injected with either 5T33 Mock or PD-L1 KO cells would give more insights into the mechanism. Such a study might help to investigate the dependency of MM tumor growth from NKT cells in relationship to PD-1/PD-L1 signaling and the extended MM specific mechanism of the pan-HDAC inhibitor.

Besides the highly immunoregulatory properties of PD-L1 due to its engagement with PD-1, it has recently been discovered that also its intracellular signaling is crucial for tumor survival and progression. Various studies have demonstrated that PD-L1 serves as “protective shield” for cancer cells faced with a cytotoxic drug. This cell intrinsic survival function of PD-L1 is independent of its receptor binding activities and points to the reason why it is commonly upregulated on many tumor entities (Chen et al., 2008). As mentioned before, interferons are important cytotoxic cytokines that are secreted by effector cells to kill their targets (Bhat et al., 2017). PD-L1 is known to be upregulated upon IFNγ stimulation and as in many regulatory circuits, PD-L1 intracellular signaling then interferes with the IFN pathway leading to apoptosis (Lee et al., 2006). Remarkably, while the engagement of PD-L1 with its receptor PD-1 leads to the downregulation of activation, inhibition of proliferation and finally apoptosis in the PD-1 expressing cell, the PD-L1 expressing cell protects itself from precisely those events when faced with cytotoxic stress (Yadollahi et al., 2021). As activation of PD-1 leads to the interference and attenuation of the proliferation and survival pathway Ras/AKT/mTor in effector immune cells, PD-L1 expressing tumor cells activate specifically this pathway (Clark et al., 2016). In this context it has been found that in IFN resistant tumors, PD-L1 is crucial for their survival. Instead of activating caspases, which leads to the activation of the apoptosis pathway, cellular stress, e.g. though the application of cytotoxic drugs, induces pro-survival pathways and DNA damage response in the tumor cell if PD-L1 is upregulated in the tumor cell (Cheon et al., 2021). This protective mechanism clearly shows that PD-L1 upregulation on tumor cells not only dampens the immune attack, but

also protects the tumor cell itself from apoptosis induction by activating survival pathways and underlines the importance of PD-L1 in drug resistance. It has to be mentioned that PD-L1 is not directly increasing the proliferation (also see **fig. 3.7**), instead it promotes survival after cytotoxic stress (Gato-Cañas et al., 2017) (Escors et al., 2018).

It might be interesting to repeat the panobinostat and anti-PD-1 therapeutic approach with the inoculation of 5T33 Mock vs PD-L1 KO myeloma cells which we discuss later with **fig. 3.28**.

It is well studied that a prerequisite for sufficient immunotherapy is a high immunogenicity, or, in other words, a hot tumor (Bonaventura et al., 2019). By combining CPI with different drug classes of ICD inducer, the immunogenicity and therefore the infiltration of effector immune cells into the TME is enabling an effective anti-tumor response (J. Zhou et al., 2019). CPI in myeloma therapy remains challenging. Even if four RRMM patients are a very limited sample size and with already having diagnosed with EMD demonstrating a poor prognosis, a clinical study using PD-L1 CPI combined with radiotherapy has shown only modest effect as treatment modality (Kazandjian et al., 2021). As shown in solid tumors, RRMM patients who are considered for pan-HDAC inhibitor therapy might benefit from additional NKT cell-based immunotherapy as well as drugs that induce ICD such as daratumumab or radiotherapy (P. M. Yang et al., 2012).

To establish an efficient CPI therapy in MM, we can learn from mechanisms of actions in cancer types where PD-1 blockade has shown dramatic success, such as melanoma (Pico de Coaña et al., 2020). In melanoma it was shown that PD-1 blockade can lead to the phenomenon of epitope spreading. Epitope spreading is comparable to the abscopal effect, where an ICD inducer unleashes various new antigens that prime not only immune cells at the primary tumor site, but also in the periphery to enable a systemic anti-tumor immune response. The main difference of epitope spreading is that especially self-antigens are the new targets for the effector cells (Lo et al., 2021). As tumors include self-antigens, this phenomenon, which occurred post PD-1 blockade in melanoma, highly enhances the anti-tumor responses. Especially subdominant CD8+ T cell clones, which can escape the tumor-immune editing mechanism and target self-

antigens, can thus grow out and attack the cancer cells (Memarnejadian et al., 2017). As MM has a lower immunogenicity, it might be promising to combine this effect with an ICD inducer such as radiotherapy or daratumumab. Additionally, vaccination strategies might also benefit from this approach. It has been shown to be more effective using a mixture of target antigens compared to a single one, e.g. by using DCs fused with myeloma cells (Rosenblatt et al., 2011). A main difference in the process of epitope spreading in MM compared to melanoma could be that also self-antigens from general healthy B and plasma immune cells can evoke as new immunologic target. As self-antigens might increase the effective anti-tumor response, they might also increase the risk for immune related adverse events (irAE) including autoimmune and rheumatic AE. Interestingly, it has been shown that especially the application of nivolumab and pembrolizumab, in contrast to ipilimumab, lead to deeper response rates and longer PFS in melanoma patients compared to other tumor entities and that the response rates correlated with the occurrence of autoimmune related AEs (Z. Khan et al., 2019) (Verspohl et al., 2021). While ipilimumab inhibits CTLA-4 in early T cell activation and priming steps mainly in secondary lymphoid organs, nivolumab and pembrolizumab inhibit PD-1 in later T cell activation phases, which can lead to reactivation of effector T cells in the TME (June et al., 2017). Hence, PD-1 blockade might unleash a broader range of tumor associated epitopes and self-reacting subdominant T cell clones that at the same time where they target the tumor, also attack the host tissue due to epitope spreading.

While ICI can not reduce MM progression in clinical practice (Zanwar et al., 2020), moreover, PD-1 blockade diminished the anti-myeloma effect of the HDAC inhibition in our *in vivo* model. Therefore, we questioned if the complete abrogation of PD-L1 specific on the MM tumor cells could have a better effect on the survival. PD-L1 is known to be overexpressed in plasma cells of many MM patients (Liu et al., 2007) and similarly the 5T33 MM cells have a strong surface expression of PD-L1. To analyze the role of PD-L1 expression on myeloma cells we generated a PD-L1 KO of the 5T33 cell line using the CRISPR/Cas9 technology (**fig. 3.4**). We found no differences in the expression of surface molecules such as co-stimulatory, MHC or adhesion molecules, proliferation (**fig. 3.7**) and migration (**fig. 3.8**) of the genetically engineered cells in comparison to the Mock control *in vitro*.

Interestingly, mice inoculated with the 5T33 PD-L1 KO cells showed a significant longer survival compared with the 5T33 Mock injected mice (**fig. 3.28**). This indicates that blocking of the PD-L1 molecule on myeloma cells plays an important role in the pathogenesis of MM and its direct blocking on malignant cells rather than in the TME might have an impact on the clinical efficiency.

By analyzing the immune cell subpopulations of both groups, we found a higher immune cell activation state in the tumor microenvironment of the PD-L1 KO myeloma cell injected mice. Especially the CD4+ T cells in BM and spleen show an increased PD-L1, PD-1 and TIGIT expression, whereas Tim-3, which severs more as an exhaustion marker, has the same expression in both groups (**fig. 3.29 – 3.31**).

Additionally, co-stimulatory receptors such as CD40L modulate the immune homeostasis. Plasma cells mature in the germinal center of the lymph nodes in strong intercommunication with T follicular helper (T_{fH}) cells where first oncogenic events can occur (Ise et al., 2018) (Silva & Gatenby, 2011). This maturing process is basically positively regulated by CD40/CD40L and negatively regulated by PD-1/PD-L1 interaction (Mintz and Cyster, 2020). When B cells mature into plasma cells, the CD40/CD40L signaling transduction increases the ability of antigen presentation and therefore has an impact on the immunogenicity of the myeloma cells (Elgueta et al., 2009). Furthermore, the CD40/CD40L signaling is essentially for dendritic cells during T cell priming and antigen presentation. By forming the immunological synapsis following the first antigen contact, DCs increase their CD40L expression which stimulates the activation of CD4+ and CD8+ T cells (Vonderheide, 2018). Furthermore, agonistic anti-CD40 antibodies can induce ADCC in CD40 expressing MM cells, upregulate FasL on the tumor cells and thereby increase apoptosis and inhibit proliferation (van de Donk et al., 2012). Interestingly, it was recently shown that CD40L stimulation of T cells in the combination with anti-PD-L1 blockade can rescue the exhaustion of CTLs during chronic infection (Bhadra et al., 2011). As seen in **fig. 3.34** we show that the co-stimulatory receptors CD40L and ICOS are increased on CD4+ T cells in the tumor microenvironment of the PD-L1 KO myeloma injected group. As the increased survival is associated with a higher activation state of the immune cells in the tumor microenvironment, we assume that PD-L1 on the tumor cells is essential for the tumor progression and that the inhibition of the

receptor on the myeloma cells rather than the checkpoints in the tumor microenvironment might be efficient in future therapy approaches.

When analyzing the spleen of the Mock vs PD-L1 KO myeloma inoculated mice, we found the same downregulation of CXCR4 and CD1d on CD8+ T cells in the PD-L1 KO myeloma group (**fig. 3.32** and **3.33**) as observed in the panobinostat treated group with extended survival (**fig. 3.26** and **3.27**). Nonetheless, we could not observe any difference in mice challenged with either 5T33 Mock or PD-L1 KO cells regarding the number of NKT cells in the BM or spleen at the disease end stage. Even though there are contrary data available, it might be in line with the literature, that it is more the function of the NKT cells rather than their number that makes the difference (Dhodapkar et al., 2003).

To investigate the mechanism of the survival prolongation in the PD-L1 KO group, we used anti-CD8 mAbs to deplete the appropriate T cells following myeloma challenge. Surprisingly, the CD8+ T cell depletion did not show a negative effect on the survival rates of both groups.

To proof that the survival prolongation of the 5T33 PD-L1 KO inoculated mice is a result of the immunological response to PD-L1, we used immunodeficient NOD.*scid*.*Il2Rγc*^{null} (NSG) mice and verified that the tumor cell lines enable the same tumor burden *in vivo*. We show that NSG mice experience the same tumor burden post 5T33 Mock and PD-L1 KO challenge (**fig. 3.35**), assuming that the previous observed survival prolongation is exclusively dependent on the PD-L1 tumor- immune cell interaction. As expected, NSG mice show an overall shorter survival than KaLwRij mice challenged with 5T33 cells demonstrating the importance of the immune system in the fight against cancer.

Conclusion

In conclusion, this thesis shows for the first time that the genetic knock out of PD-L1 on the 5T33 cells enables a significant increase of the survival of myeloma bearing mice. These results demonstrate that the specific inhibition of the immune checkpoint on the tumor cell, rather than in the tumor microenvironment using ICI, displays a new rationale for therapeutic developments in the clinic. Furthermore, NKT cells increase during MM disease progression and shift their phenotype in the KaLwRij MM model, proposing these immune cells as potential biomarker for MM disease progression and manifestation.

5. Abstract

Check point inhibition (CPI) has shown dramatic improvements in overall survival in many malignant diseases. However, in multiple myeloma (MM) the results were disappointing resulting in an early termination of clinical trials. Despite the advantages in therapy the disease remains incurable.

Aim of this work was to seek the possibilities of CPI in MM and evaluate its efficiency and immunological mechanisms with focus on PD-1/PD-L1 blockade.

To do so, we used KaLwRij mice that develop MM upon injection of 5T33 myeloma cells. Bone marrow (BM), lymph nodes and spleen cells were analyzed by flow cytometry for the phenotype of immune cells. Mice were either treated with combined CPI or with mAbs blocking PD-1 and panobinostat to evaluate a potential synergistic therapeutic effect.

Our results showed that comprehensive immunophenotyping including the analysis of T and NK cell subpopulations revealed no differences between early disease stage MM bearing mice and healthy control groups. Treatment of mice by blocking the PD-1/PD-L1 signaling pathway alone had no effect on tumor growth and survival.

As it was demonstrated that HDAC inhibitors beside their direct effect on malignant cells may increase the immunogenicity of malignant cells by improving the presentation of tumor antigens and modulate the immunological composition of the tumor microenvironment (TME), we questioned whether a synergistic therapeutic effect might be achieved by combining HDAC inhibition with CPI.

We found that the pan-HDAC inhibitor panobinostat that is approved for the treatment of myeloma patients inhibited the development of myeloma in treated mice. Surprisingly, the combined application of the anti-PD-1 blocking antibody with panobinostat reduced the anti-myeloma effect of the compound and resulted in decreased survival.

By analyzing the phenotype of immune cells in the different populations, we found in the panobinostat treated animal group an increase in the CXCR4 expressing CD4+ NKT cells. Additionally, the CD8+ T cells expressing CD1d and CXCR4 decreased compared

to the other groups in the spleen. CD1d is a MHC like receptor for glycolipids activating NKT cells, whereas CXCR4 is a BM homing receptor and linked to metastasis and tumor aggressiveness.

The second aim of this work was to investigate the MM pathomechanism in relation to the PD-1/PD-L1 singnaling pathway and to identify potentially new drug targets for MM therapy.

Again, we used KaLwRij mice that develop MM upon injection of 5T33 myeloma cells and analyzed primary and secondary lymphoid organs by flow cytometry for the phenotype of immune cells. Different disease stages of mice were evaluated.

We found a gradually increase of CXCR4+ NKT cells in the BM corresponding to MM disease progression. Interestingly, we observed a shift in the CD4+/CD4- NKT cell ratio during disease progression, whereby the CXCR4+ CD4- NKT cells seem to be associated with advanced tumor growth, while the increase of CXCR4+ CD4+ NKT are associated with prolonged survival as observed in in the panobinostat treated group.

To analyze the role of PD-L1 expression on myeloma cells in tumor progression and in the cross talk with the immune microenvironment, we generated a PD-L1 KO of the 5T33 cell line using the CRISPR/Cas9 technology.

We found no differences in the expression of surface molecules such as MHC class I and II, co-stimulatory or adhesion molecules, proliferation and migration of the genetically engineered cells in comparison to the mock control. Interestingly, mice inoculated with the 5T33 PD-L1 KO cells showed a significant longer survival compared with the 5T33 mock injected, indicating that blocking of the PD-L1 molecule on myeloma cells plays an important role in the pathogenesis of MM and its direct blocking on malignant cells rather than in the TME might have an impact on the clinical efficiency.

When analyzing the spleen of the mock vs PD-L1 KO myeloma inoculated mice, we found the same downregulation of CXCR4 and CD1d on CD8+ T cells in the PD-L1 KO myeloma group as observed in the panobinostat treated group with extended survival.

Furthermore, we used NOD.*scid*.*Il2Rγc*^{null} (NSG) mice with severe immunodeficiency to proof that the survival prolongation is a result of the immunological response to PD-L1 and that otherwise both tumor cell lines enable the same tumor burden *in vivo*.

We show that immunodeficient NSG mice experience the same tumor burden post 5T33 mock and PD-L1 KO challenge, assuming that the previous observed survival prolongation is dependent on the PD-L1 tumor- immune cell interaction and that the myeloma tumor cells are not otherwise effected in their tumor cell properties. Moreover, as expected, NSG mice show an overall shorter survival than KaLwRij mice challenged with 5T33 cells.

6. List of Figures

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8. Acknowledgements

Sometimes, you are lucky, and work is more than just a job. It is your goal, your vision, your fascination and your guidance – for me, it is the passion for science.

To make this possible, I would like to especially thank the wonderful people I met in my life who have encouraged and supported me during my scientific career.

Thank you so much, Ruth Muschel. You are such a great role model for me. I will never forget how overwhelmed I was when I first came to your lab. I have learned so many things in this exceptional time – scientifically and personally. It was really an extraordinary experience, and I will never forget that you gave me this opportunity. I would further like to thank you for the extensive proofreading of this work. I wish you all the very best from my heart.

Thank you so much, Peter Brossart. Of course, I would first like to thank you for allowing me to conduct our research in your lab. You have an impressive overview of what really matters, and you have always helped and supported me in working on the scientific topics and all the questions that came up. However, I would like to say a special thank you for the freedom you have given me and the trust you have placed in me. Despite all the hard work and long hours that research brings with it, I can honestly say that it was such an exciting and fascinating time and so much fun! Thank you 😊

Such a good time can only be created with the right people around you, day by day. That is why a special thanks goes to all my colleagues: Sascha Kampmann, Sebastian Oberbeck, Solveig Daecke, Corinna Bons and Sonny Leopold. Best science and the funniest lunch times of my life!

Thank you, Heike Lerbs, for all the work you do. The clinic wouldn´t work this way without you. Thank you, Sebastian Schlaweck and Stefan Juranek, for the very good scientific advices and supervision. Thank you, Savita Bisht-Feldmann, for the deep discussions we had that helped a lot! I would also like to thank Stefanie Held for the impact she had during this time. And thank you, Annkristin Heine and Crystel Flores, for the meaningful collaboration and support.

Some people are role models even if they don´t know it. They are so strong and brave and show you the route to follow, sometimes even in an abstract way targeting every domain in life. They give you support, joy and confidence, just because of who they are. They shape you, and they can heal you. And that will not pass with this work. I am humbled to have met you.

Last but not least, an essential thank you goes to my entire family who supported me during this intense time and during my whole life. Thank you, Michi Heider, my parents Rita and Jürgen, my stepmother Fina, my brother Robert and the best grandparents on earth, Marta and Werner. And thanks to all my friends who helped me with great discussions and activities at that time.

“If we knew what it was we were doing, it would not be called research, would it?”

Albert Einstein (1879-1955)