DISSERTATION

Augmenting DC function for rational immunotherapies in multiple myeloma
Verstärkung der DC-Funktion für rationale Immuntherapien beim Multiplen Myelom

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ADC	Antibody-drug Conjugate
AE	Adverse Events
APC	Antigen Presenting Cell
BCMA	B cell Maturation Antigen
CAR-T	Chimeric Antigen Receptor T
CCL	Chemokine Ligand
CCR7	CC-type chemokine receptor 7
cDC	Conventional DC
CFSE	Carboxyfluorescein Diacetatesuccinimidyl Ester
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Antigen 4
DAMP	Damage-associated Molecular Pattern
DC	Dendritic Cell
DMFI	Delta of Median Fluorescence Intensity
FDA	Food and Drug Administration
FLT3L	FMS-like Tyrosine Kinase 3 Ligand
FSC	Forward Scatter
Gal-3	Galectin-3
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HLA	Human Leukocyte Antigen
HSC	Hematopoietic Stem Cell
ICD	Immunogenic Cell Death
ICI	Immune Checkpoint Inhibitor
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
IMiD	Immunomodulatory Drug
KRD	Carfilzomib/ Lenalidomide/ Dexamethasone
LAG-3	Lymphocyte Activation Gene 3
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MGUS	Monoclonal Gammopathy of Undetermined Significance
MHC	Major Histocompatibility Complex
MLR	Mixed Lymphocyte Reaction
MM	Multiple Myeloma
moDC	Monocyte-derived DC
OR	Objective Response
ORR	Overall Response Rate
OS	Overall Survival
PBMC	Peripheral Blood Mononuclear Cell
pDC	Plasmacytoid DC
PD-L1	Programmed Death Ligand 1
RFS	Recurrence-free Survival
R-ISS	Revised International Staging System
RR	Relapsed or Refractory
pre-DC	DC Precursors
SD	Stable Disease
SSC	Side Scatter
TAA	Tumor-associated Antigen
TCR	T cell Receptor

Th	T Helper
TIM-3	T cell Immunoglobulin and Mucin Domain 3
TNF	Tumor Necrosis Factor
Treg	Regulatory T cells
TSA	Tumor-specific Antigen
UPP	Ubiquitin-Proteasome Pathway

Abstract

Background: Multiple myeloma (MM) is a hematological malignancy characterized by the infiltration of the bone marrow by malignant plasma B cells. Despite the approval of novel agents, MM remains an incurable disease and side effects of treatment seriously impair quality of life. Novel therapeutic approaches are needed comprising long-lasting response and low toxicities. Dendritic cells (DC)-based cancer vaccines are a promising immunotherapeutic strategy able to elicit long-lasting anti-cancer immunity and are well tolerable. DC are the most potent antigen-presenting cells (APC) that coordinate innate and adaptive immune responses and hence play a central role in immunotherapeutic approaches. However, cancer vaccines alone have failed to achieve expectations with respect to clinical outcomes. Bortezomib and carfilzomib are proteasome inhibitors that are approved as powerful anti-tumor agents in MM treatment. Recently, they were further shown to be immunogenic cell death (ICD) inducing drugs capable of inducing a type of immunostimulatory apoptosis. However, studies demonstrate that bortezomib significantly impairs DC phenotype and functions. Carfilzomib, a second-generation proteasome inhibitor with less off-target effects, could be a promising combinatory agent. The efficacy of the vaccine may further be augmented by immune checkpoint inhibition (ICI). Our goal is to investigate the effect of carfilzomib on DC combined with ICI to provide more evidence for the exploration of a combinatory approach in MM treatment.

Methods: Effects of carfilzomib and bortezomib on human monocyte-derived DC (moDC) differentiation, phenotype including immune checkpoints, and function were comparatively analyzed by FACS, ELISA, and MLR in vitro. Additionally, the quantity of peripheral blood DC in newly diagnosed carfilzomib-treated MM patients was measured and data was compared to healthy donors.

Results: Carfilzomib had only minor effects on DC differentiation, phenotype, and function. The expression of immune checkpoint programmed death ligands 1 (PD-L1) was significantly upregulated under carfilzomib exposure. Carfilzomib-exposure led to reduced T cell proliferation in MLR which could not only be leveled out, but enhanced by the addition of an anti-PD-1 antibody. Analyses of patient samples showed reduced numbers of peripheral blood and bone marrow DC at diagnosis compared to healthy donors. However, numbers recovered under treatment with carfilzomib-containing regimens.

Conclusions: Carfilzomib represents an appropriate standard of care drug for combinatorial approaches with a DC-based cancer vaccine. The efficacy of the vaccine can possibly be augmented by the combination with approved ICI. Such combinatory approaches have the potential to induce long-term immune responses and disease control without increasing toxicity.

Zusammenfassung

Hintergrund: Das Multiple Myelom (MM) ist eine hämatologische Erkrankung, die durch die Infiltration des Knochenmarks durch maligne Plasma-B-Zellen gekennzeichnet ist. Trotz der Verfügbarkeit neuer Wirkstoffe ist weiterhin keine Heilung möglich. Die Lebensqualität der Patienten wird durch Nebenwirkungen deutlich beeinträchtigt und die Entwicklung von Resistenzen stellte eine weitere Herausforderung dar. Es besteht also ein großer Bedarf an innovativen Therapieansätzen. Auf dendritischen Zellen (DC) basierende Tumorvakzinen sind ein derartiger immuntherapeutischer Ansatz. Sie sind in der Lage, langanhaltende spezifische Immunantworten gegen Tumore zu induzieren und zeichnen sich durch gute Verträglichkeit aus. DC sind die wirksamsten Antigen-präsentierenden Zellen (APC) und spielen bei diesen Ansätzen eine zentrale Rolle. Mit Tumorvakzinen allein konnte bislang keine überzeugende klinische Wirksamkeit erreicht werden.

Unser Ziel liegt in der Entwicklung einer Tumorvakzine, die in Kombination mit etablierten Therapien verabreicht wird. Eine mögliche Substanzgruppe sind Proteasominhibitoren (PI), die standardmäßig zur Therapie des MM eingesetzt werden. Kürzlich wurde nachgewiesen, dass PI in der Lage sind einen immunogenen Zelltod (ICD) auszulösen, eine Form des Zelltods, der zur Aktivierung des Immunsystems führt. Derartige Substanzen stellen daher optimale Kombinationspartner für immuntherapeutische Strategien dar. Auf der anderen Seite bergen sie aber das Risiko, über Off-Target Effekte Zellen des Immunsystems zu hemmen. Studien belegen beispielsweise, dass der PI Bortezomib den Phänotyp und die Funktionen der DC erheblich beeinträchtigt. Um zu evaluieren, ob sich der Zweitgenerations-PI Carfilzomib als möglicher Kombinationspartner eignet, wurde in dieser Arbeit dessen Einfluss auf humane DC

analysiert. Ferner wurde analysiert, ob sich die durch DC induzierte Immunantwort durch die Hinzunahme sogenannter Checkpointinhibitoren steigern lässt.

Methoden: Der Einfluss von Carfilzomib und Bortezomib auf die Differenzierung, den Phänotyp einschließlich der Expression von Checkpointmolekülen, und die Funktion von humanen aus Monozyten-abgeleiteten DC (moDC) wurde in vitro mittels FACS, ELISA und MLR vergleichend analysiert. Zusätzlich wurde die Quantität der DC im peripheren Blut bei neu diagnostizierten sowie mit Carfilzomib behandelten MM-Patienten gemessen und mit gesunden Spendern verglichen.

Ergebnisse: Carfilzomib hatte im Gegensatz zu Bortezomib nur geringe Auswirkungen auf die Differenzierung, den Phänotyp und die Funktion humaner moDC. Die Expression des Checkpointmoleküls Programmed-Death-Liganden 1 (PD-L1) war aber unter Carfilzomib-Exposition signifikant hochreguliert. Eine Carfilzomib-Behandlung der DC führte zu einer verringerten T-Zell-Proliferation im MLR, die jedoch durch Zugabe von Anti-PD-1-Antikörpern nicht nur wiederhergestellt, sondern sogar gesteigert werden konnte. Bei neu diagnostizierten MM Patienten zeigte sich eine im Vergleich zu gesunden Spendern reduzierte Anzahl peripherer Blut- und Knochenmarks-DC. Unter der Behandlung mit Carfilzomib-haltigen Therapieregimen kam es aber zur Normalisierung der Frequenzen.

Schlussfolgerungen: Carfilzomib stellt ein geeignetes Standardmedikament für kombinatorische Ansätze mit einem DC-basierten Krebsimpfstoff dar. Die Wirksamkeit kann möglicherweise durch den Einsatz zugelassener ICI erhöht werden.

1 Introduction

1.1 Multiple myeloma

1.1.1 Epidemiology and diagnosis

MM, the second most common hematological malignancy, accounts for around 1.6 % of all cancers worldwide and about 13 % of hematological neoplasms, and the incidence has been steadily increasing in recent years [1]. The median age at diagnosis is around 65 years and slightly more common in males than females [2].

MM is a malignant monoclonal plasma cell disorder, which originates from a post-germinal lymphoid B cell lineage and develops after lineage differentiation in the bone marrow [3]. It is characterized by the overproduction of abnormal monoclonal antibodies of an immunoglobulin (Ig) subclass or immunoglobulin-free light chains. These proteins are termed M-proteins. Accumulation of the M-proteins and the infiltration of myeloma cells in the bone marrow lead to a series of symptoms including anemia, bone lesions, pain, infections, weight loss, renal impairment, and fatigue [4]. MM usually develops from an asymptomatic premalignant stage called monoclonal gammopathy of undetermined significance (MGUS), which is defined as plasma cell disorder with elevated immunoglobulin but without organ damage [5]. Epidemiological studies demonstrate that 3.2 % of elderly individuals (>50 y) are affected by MGUS while most MM patients have MGUS precancerous stage. The risk of transformation to MM or other hematological malignancies is estimated to be 1 % per year [2, 5].

As the symptoms of MM are usually non-specific, they may be easily overlooked. Therefore, in addition to medical history and physical examination, special laboratory tests including analysis of monoclonal Immunoglobulins or light chains, determination of bone marrow infiltration by plasma cells, cytogenetic analyses, and imaging for bone lesions are required for diagnosis of MM.

Cytogenetic abnormalities are found in over 90% of MM patients when analyzed by the FISH method. Chromosome alterations associated with a high risk of unfavorable prognosis include the presence of translocations t (4;14) and t (14;16); del (17p); and amplifications of 1q21 [4, 6].

Table 1 summarizes established diagnostic criteria for the diagnosis of suspected MM [6, 7].

Table 1: Diagnosis of MM

Monoclonal bone marrow myeloma cells ≥10% or biopsy-proven bony or extramedullary plasmacytoma with any MM-defining events

• Presence of related organ or tissue impairment
  • Anemia – hemoglobin< 100 g/l or > 20 g/l below normal
  • Hypercalcemia – serum calcium > 2.75 mmol/l
  • Renal insufficiency – creatinine clearance < 40 ml/min or serum creatinine > 177mmil/l
  • Bone lesions – one or more osteolytic lesions on skeletal radiography, CT, or PET-CT
• Presence of a biomarker associated with near inevitable progression to end-organ damage
  • ≥ 60 % monoclonal myeloma cells infiltrated in the bone marrow
  • Involved/ uninvolved free light chain ratio of 100 or more (the involved free light chain level must be ≥ 100 mg/l)
  • MRI with more than one focal lesion

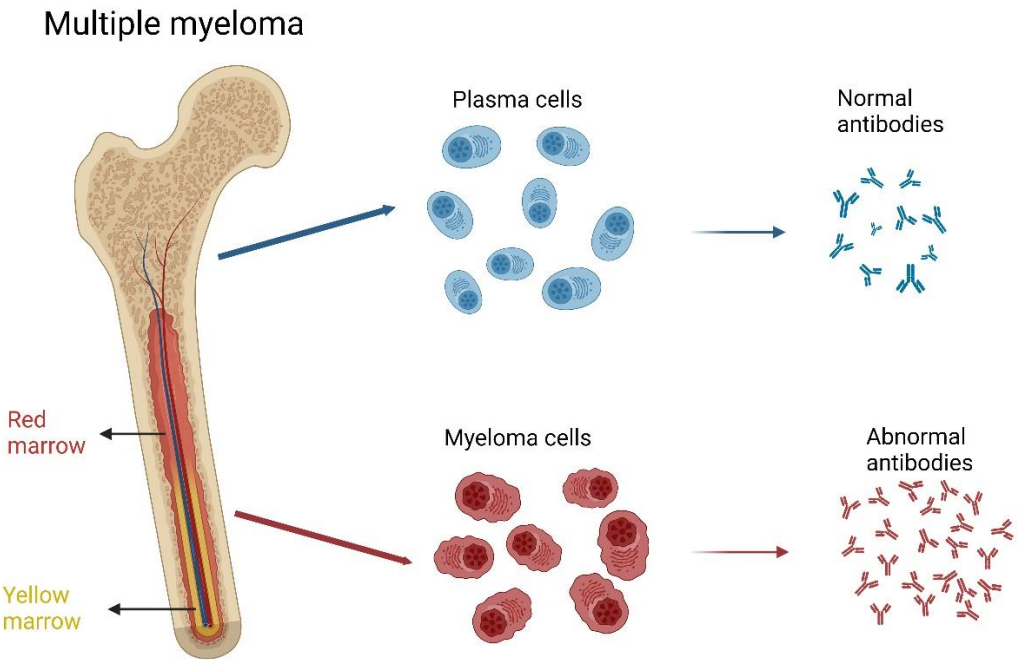


Figure 1: Multiple myeloma: The bone marrow is primarily categorized into red bone marrow, which is the main site for hematopoiesis, and yellow bone marrow, which transforms into fat tissue in adulthood. In patients with MM, abnormal myeloma cells replace normal plasma cells and hamper hematopoiesis in general. Myeloma cells produce abnormal inoperable monoclonal antibodies of the immunoglobulin subclasses IgG, IgA, and IgM and κ- and λ free light chains, respectively. Created in BioRender.com.

1.1.2 Treatment and prognosis

The treatment of MM typically consists of 3 parts, including induction, consolidation, and maintenance therapy. Induction therapy usually consists of a triple or quadruple combination of different drugs with the goal of rapidly reducing tumor burden. Consolidation therapy aims at further eliminating remaining malignant cells while maintenance therapy is used to help maintain and deepen remission and prevent disease recurrence. There are a variety of therapeutic agents approved for inducing and maintaining remission of MM. Traditional chemotherapy, radiotherapy, and dexamethasone remain important therapeutic approaches. New therapies, including proteasome inhibitors, immunomodulatory drugs (IMiD), and monoclonal antibodies, have shown promising clinical efficacy and have been included in the combination regimens. For transplant-eligible patients, especially patients who are young and physically strong, high-dose chemotherapy (melphalan) followed by autologous hematopoietic stem cell transplantation, as well as the induction and consolidation of daratumumab/ bortezomib/ thalidomide/ dexamethasone, has led to long-term survival [8]. For transplant-ineligible patients, less-intensive regimes are applied, for instance, the following combinations: daratumumab/ lenalidomide/ dexamethasone and bortezomib/ lenalidomide/ dexamethasone [8]. Approval of B cell maturation antigen (BCMA)-targeted chimeric antigen receptor (CAR-T) cell therapy and bispecific antibodies such as BCMA-CD3-directed bispecific antibody have improved therapeutic options considerably in relapsed or refractory MM [9].

Despite significant advancements in the treatment of multiple myeloma, leading to improved outcomes and quality of life, relapse occurs especially in high-risk patients and long-lasting remissions are rare with 5-year survival rates of less than 60 % [10].

Table 2 summarizes the revised international staging system (R-ISS), which serves as a risk stratification tool.

Table 2: Risk stratification

Stage	R-ISS	Prognosis [11]
I	· Serum β2-microglobulin levels <3.5 mg/l and serum albumin levels ≥3.5 g/dl · standard-risk cytogenetics · normal lactate dehydrogenase (LDH) levels.	· Estimated OS and PFS at five years were 82 and 55 %, respectively. Median OS was not reached. Median PFS was 66 months (n=871).

II	·	not stage I or stage III	·	Estimated OS and PFS at five years were 62 and 36 %, respectively. Median OS and PFS were 83 and 42 months (n=1894).
III	·	Serum β2-microglobulin ≥5.5 mg/l · either higher than normal LDH levels or high-risk cytogenetics.	·	Estimated OS and PFS at five years were 40 and 24 %, respectively. Median OS and PFS were 43 and 29 months (n=295).

High-risk cytogenetics detected using FISH include the presence of at least one of the following: del (17p), t (4;14), and t (14;16).

Novel agents are now being explored including antibody-drug conjugates (ADC), immune checkpoint inhibitions (ICI), and cancer vaccines. Challenges such as treatment-related adverse events (AE) and drug resistance emphasize future developments need to focus on higher efficacy, lower toxicity, and less burdensome treatment regimens.

1.2 Proteasome inhibitors

1.2.1 The ubiquitin-proteasome pathway (UPP)

The UPP, discovered in the 1980s, is a major pathway of intracellular protein degradation by which misfolded and damaged proteins are removed to maintain homeostasis [12]. The UPP represents an extensive and complex intracellular protein degradation pathway regulating more than 80 % of proteins in not only normal cells but also tumor cells. A variety of cell biological processes and physiological functions are regulated via UPP including cell survival, cell-cycle progression, apoptosis, DNA repair, signal transduction, protein quality control, and antigen presentation [13]. The proteasome plays a central role in this pathway and maintains homeostasis in every eukaryotic cell.

The human 26S proteasome is formed by 20S functional core particle catalytic sites and 19S regulatory subunits. Structurally, the 20S proteasome is a cylinder molecule made up of four highly homologous stacked rings that surround a central catalytic compartment with a proteolytic active site. Each of the rings contains seven distinct but related subunits α and β. Two identical outer α-rings encircle a small opening which only allows polypeptide substrates to pass and two identical inner β-rings consisting of multiple proteolytic sites that induce protein degradation [13, 14]. Each of these two β rings comprises three

proteolytic sites that present different substrate preferences, including post-glutamyl peptide hydrolyzing or caspase-like (β1), trypsin-like (β2), and chymotrypsin-like (β5) [12]. Ubiquitination is an important post-translational modification, which includes the covalent binding of ubiquitin to the target protein, which is thereby marked for degradation by the proteasome. The synergistic effect of ubiquitination of a series of enzymes and proteolytic hydrolysis of proteasome complexes is collectively referred to as the ubiquitin-proteasome system [14]. Some tumors especially MM cells largely depend on proper proteasome function. Myeloma cells produce proteins that promote both cell survival and proliferation and inhibit mechanisms of cell death. This notion set the stage for preclinical testing of proteasome inhibitors as a means to shift this fine equilibrium toward cell death [12].

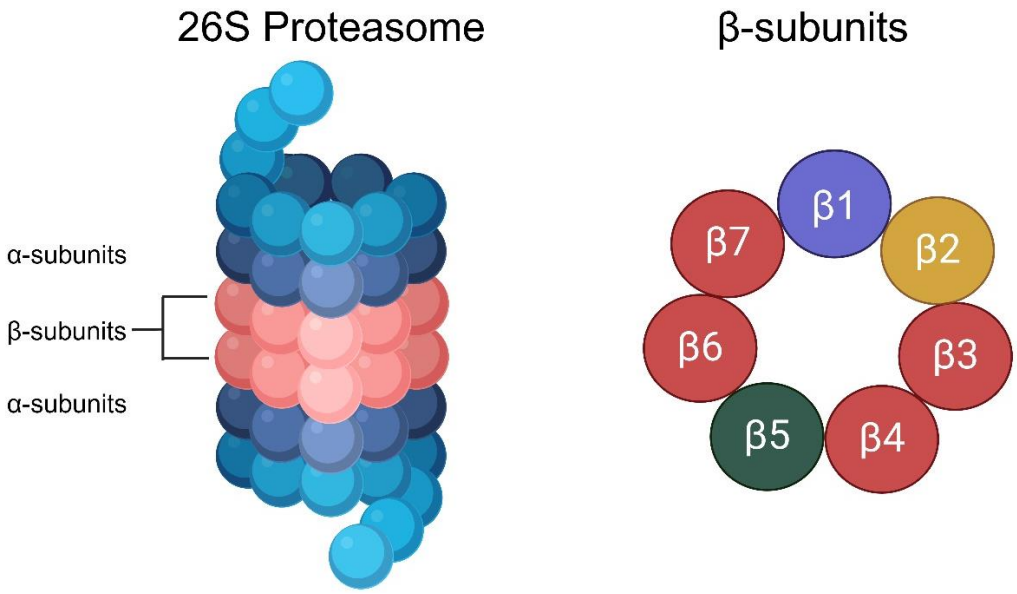


Figure 2: Structure of the 26S proteasome and its β catalytic subunits. The 26S proteasome is formed by a 20S functional proteolytic core region containing four ring-shaped subunits (arranged as α-β-β-α) and a 19S regulatory particle. Each β ring comprises three proteolytic sites that present different substrate preferences, including post-glutamyl peptide hydrolyzing or caspase-like (β1), trypsin-like (β2) and chymotrypsin-like (β5). Created in BioRender.com.

1.2.2 Proteasome inhibition as a treatment strategy

It is known that defects within the UPP are associated with cancers, especially in myeloma cells with high abnormal antibody production [12]. Inhibition of proteasome function in MM cells results in the accumulation of misfolded Ig within the endoplasmic reticulum.

Such stress of the endoplasmic reticulum hyperactivates unfolded protein response pathways and inhibits nuclear factor-κB activity leading to cell cycle arrest and induction of specific cell apoptosis [15].

Bortezomib, the first-class proteasome inhibitor approved in 2003 by the Food and Drug Administration (FDA) for the treatment of refractory MM and in 2008 for first-line treatment of MM, remarkably improved the overall response rate (ORR) and overall survival of MM patients [14]. Bortezomib is a slowly reversible inhibitor that binds to the catalytic site of the 26S proteasome and displays a potent inhibitory effect on β5 and, to a lesser extent, β2 and β1 activities [13]. Bortezomib is commonly used as a first-line treatment combined with other anti-tumor agents including lenalidomide and dexamethasone [12]. Although bortezomib has achieved powerful clinical efficacy, it still has several limitations, for example, peripheral neuropathy, the most common side effect. Around 80 % of patients treated with bortezomib will develop any grade of neuropathy, a dose-limiting factor. This problem has motivated the development of next-generation proteasome inhibitors with more favorable safety profiles and fewer side effects.

In 2012, carfilzomib, a second-in-class proteasome inhibitor, received fast-track US FDA approval, based on its safety and clinical efficacy in relapsed and refractory MM patients. In contrast to the competitive inhibitor bortezomib, carfilzomib selectively and irreversibly binds to the proteasome catalytic subunit (β5) with epoxyketone as its pharmacophore. This selective mechanism profoundly improves the safety of carfilzomib. In addition, the irreversibility of the interaction of carfilzomib with the proteasome allows it to achieve long-lasting inhibition efficacy, even in cases of bortezomib resistance [14]. Due to the tremendous improvements brought by proteasome inhibitors, carfilzomib or bortezomib are recommended as the first-line treatment in the NCCN guideline [16].

Furthermore, carfilzomib is recognized as an emerging immunogenic cell death (ICD) inducing drug. ICD represents a form of regulated cell death that is capable of eliciting an adaptive immune response in an immunocompetent environment [17]. Legrand et al. proposed a definition of ICD based on the interaction between dying and immune cells, characterizing it as a successful dialog between a dying cell and an appropriately responsive immune system [18]. Certain chemotherapeutic drugs, oncolytic viruses, physicochemical therapies, photodynamic therapy, and radiotherapy are known ICD inducers. They are able to induce the release of tumor-associated or -specific antigens (TAA) and damage-associated molecular patterns (DAMP) while killing tumor cells. DC contribute to antigen recognition and presentation, thereby helping the host to develop adaptive immunity

against tumor cells [19]. Furthermore, ICD inducers were shown to selectively deplete immunosuppressive cell populations, such as myeloid-derived suppressor cells and regulatory T cells (Treg) [20]. These mechanisms play a crucial role in antitumor immunity and hence ICD inducers represent optimal partners for combinatorial approaches in cancer immunotherapy in general, e.g. they are able to improve the efficacy of ICI [20, 21]. Recently, endoplasmic reticulum stress was identified as a central factor associated with the occurrence of ICD, which also represents the primary anti-myeloma mechanism of carfilzomib [22]. Therefore, carfilzomib could be expected to function as an in-situ tumor vaccine and endogenous immune adjuvant [23]. Although proteasome inhibitors made a breakthrough in the therapy of MM, the disease remains incurable, and side effects of long-term drug treatment seriously affect patients' quality of life. Novel approaches being explored focus on exploiting the patients´ immune system.

1.3 The immune system

The immune system consists of a wide variety of cells and proteins that aim to protect human beings from pathogenic infection and cancer. The immune system is divided into two general types: the innate immune system and the adaptive immune system [24].

1.3.1 The innate immunity

The innate system represents the first line of defense against an invading pathogen and is not dependent on prior antigen sensitization, hence it is present at birth. It includes the following types of defensive barriers: physical barriers (e.g. skin and mucous membranes), chemical barriers (e.g. temperature, low pH), phagocytic barriers (phagocytosis of pathogens by phagocytes e.g. neutrophils, macrophages, natural killer cells, mast cells, basophils, dendritic cells, eosinophils) and the inflammatory barriers (e.g. serum proteins) [24, 25].

A central characteristic of the innate response is the aggregation of neutrophils to the infection site to eliminate pathogens. From the start of the pathogen invasion or tissue injury, a great number of cytokines are released by activated macrophages nearby. Two of them, granulocyte and granulocyte-macrophage colony-stimulating factors (G- and GM-CSF) stimulate the proliferation and maturation of myeloid precursors in the bone marrow, release millions of cells into the peripheral blood and cause typical neutrophil

leukocytosis [25]. The killing of the pathogen occurs by two mechanisms named the oxygen-dependent response or respiratory burst in neutrophils. The oxygen-dependent response includes the production of hydrogen peroxide, hydroxyl radicals, and singlet oxygen by NADPH oxidase while the oxygen-independent response is conveyed by the highly toxic cationic proteins and enzymes in the granules of neutrophils [25].
Moreover, macrophages secret pro-inflammatory cytokines like tumor necrosis factor (TNF)-α and Interleukin (IL)-6 which can accumulate C-reactive protein resulting in the activation of the lytic complement cascade [26].
Traditionally, innate immune recognition and response are characterized as fast, unspecific, non-adaptive, and memory-free [27]. The innate immune system is able to activate the adaptive immune response.

1.3.2 The adaptive immunity

The adaptive immunity, also known as acquired immunity is characterized by targeted effector responses driven by antigen-specific T cells and B cells. Unlike innate immunity, the components of the adaptive immune system display high levels of specificity, long-term immune memory, and efficient secondary immune responses [24].
B cells can, on the one hand, act as antigen-presenting cells (APC), which present specific antigens to T cells [28]. After activation by a particular antigen, B cells transform into plasma cells that produce and secret antibodies against the respective antigen. The central function of antibodies is to neutralize toxins, prevent pathogens from adhering to mucous membranes, activate complement, and label infected cells as targets for innate immune cell-mediated killing [28].
T cells play a key role in the cell-mediated specific immune system. T cells derive from progenitors in the bone marrow and further develop while migrating to the thymus gland. In the thymus, they express their T cell receptor (TCR), a specific antigen-recognition molecule on the cell surface that can recognize restrictive antigens presented by major histocompatibility complex (MHC) molecules termed human leukocyte antigens (HLA) in humans [24]. While passing the thymus, T cells undergo a selection process to avoid recognition of self-antigens presented on MHC. CD3 is a pan T cell identification marker, while CD4 and CD8 are surface markers expressed on different subpopulations. CD4+ T cells respond to pro-inflammatory signals and differentiate into effector cells, including the T helper (Th) cell lineage (Th1, Th2, and Th17 subsets) and thymic-derived Foxp3+ Treg. Their TCR are restricted to MHC class II [29]. The function of Th cells, as their name “helper”

reveals, is to help CD8+T cell and B cell respond to bacteria, fungal infections, and tumors. CD8+ T cells mainly act as specific target killing cells. When CD8+ T cells are activated and differentiate into CTL, they express surface molecules, including ligands for death receptors to induce apoptosis in target cells, and also secret large amounts of cytolytic molecules and inflammatory cytokines including protease granzyme B, perforin, interferon (IFN)-γ and TNF-α, which act directly to kill infected or tumorigenic cells [28, 30]. CTL are restricted to antigen presentation via MHC I.

1.3.3 DC - professional APC

DC were identified for the first time in 1973 by Dr. Ralph Steinman, who solved the question of how innate immunity connected to adaptive immunity, which had puzzled immunologists for decades. Dr. Steinman was awarded the Nobel Prize for Medicine in 2011 for this ground-breaking finding [31].

Figure 3 illustrates the DC life cycle. DC derive from hematopoietic stem cells (HSC) in the bone marrow. With the support of FMS-like tyrosine kinase 3 ligand (FLT3L), which serves as the growth factor regulating survival, proliferation, and differentiation of the DC lineage, HSC ultimately differentiate into DC precursors (pre-DC) [32]. When pre-DC egress from the bone marrow to the peripheral tissue, they are called immature DC.

Once DC reach peripheral tissues, they behave as sentinels, the main actors of the innate immunity recognizing antigens derived from pathogens or tumor cells [33]. When antigens, for instance, pathogen-associated molecular patterns, DAMP, and TAA, or TSA, are sensed, DC can engulf extracellular antigens via endocytosis and process them, which induces DC maturation [32].

During maturation, DC lose their ability to sense their surroundings for pathogens and their endocytic capacity. Engulfed antigens are processed, and mature DC are then specialized in the production and secretion of proinflammatory cytokines, such as tumor necrosis factor-α, transforming growth factor-β, MCP-1/CCL2, MIP-1α/CCL3, IL-6, IL-12 and IL-15 [32]. Figure 4 illustrates the mechanisms of antigen processing and cross-presentation.

In addition, mature DC upregulate the expression of various surface molecules, such as MHC class I and class II, costimulatory molecules (CD80, CD86, CD40), and the CC-type chemokine receptor 7 (CCR7) [34]. The increased expression of CCR7 allows their migration towards secondary lymphoid organs towards high expression of chemokine ligand (CCL)19 or CCL21. Once arrive in the secondary lymphoid organs, DC present antigens

to CD4+ Th cells and CD8+ effector T cells to induce specific adaptive immune responses [33].

Mature DC excel at antigen presentation, especially processing exogenous antigens into peptides and presenting them via MHC class I molecules to CD8+ T cells with the support of costimulatory molecules. This process is known as cross-presentation, which is essential for initiating CD8+ T cell responses against tumors and intracellular pathogens [32]. Moreover, DC present extracellular antigens on MHC class II molecules to prime CD4+ T cells. Therefore, DC are regarded as the most potent APC that link innate and adaptive immunity (Figure 5) [34].

Several DC subsets have been identified to date according to their tissue distribution, ontogeny, phenotypical features, and functions [35]. DC are generally divided into CD11c+ conventional DC (cDC), also known as classical DC, which are subdivided into cDC1 (CD141+) and cDC2 (CD1c+) and the subtype of plasmacytoid DC (pDC) [36]. In anti-tumor immunity, cDC1 efficiently process and present intracellular antigens and initiate immune responses by cross-presenting TAA or TSA to CD8+ T cells via MHC class I signaling [37]. cDC2 mainly focus on presenting MHC class II-associated antigens to CD4+ Th cells, promoting Th1, Th2, and Th17 activation and polarization [37]. pDC (CD123+ and CD303+) are specialized in IFN production and induce anti-viral and anti-tumor immunity [38]. Monocyte-derived DC (moDC) represent a specific subset of DC that differentiate from monocytes in response to inflammatory cytokines and chemokines and accumulate at inflammatory sites like the tumor microenvironment [39]. In addition, moDC can also be generated in vitro in sufficient numbers hence serving as an important cell type for DC in vitro studies.

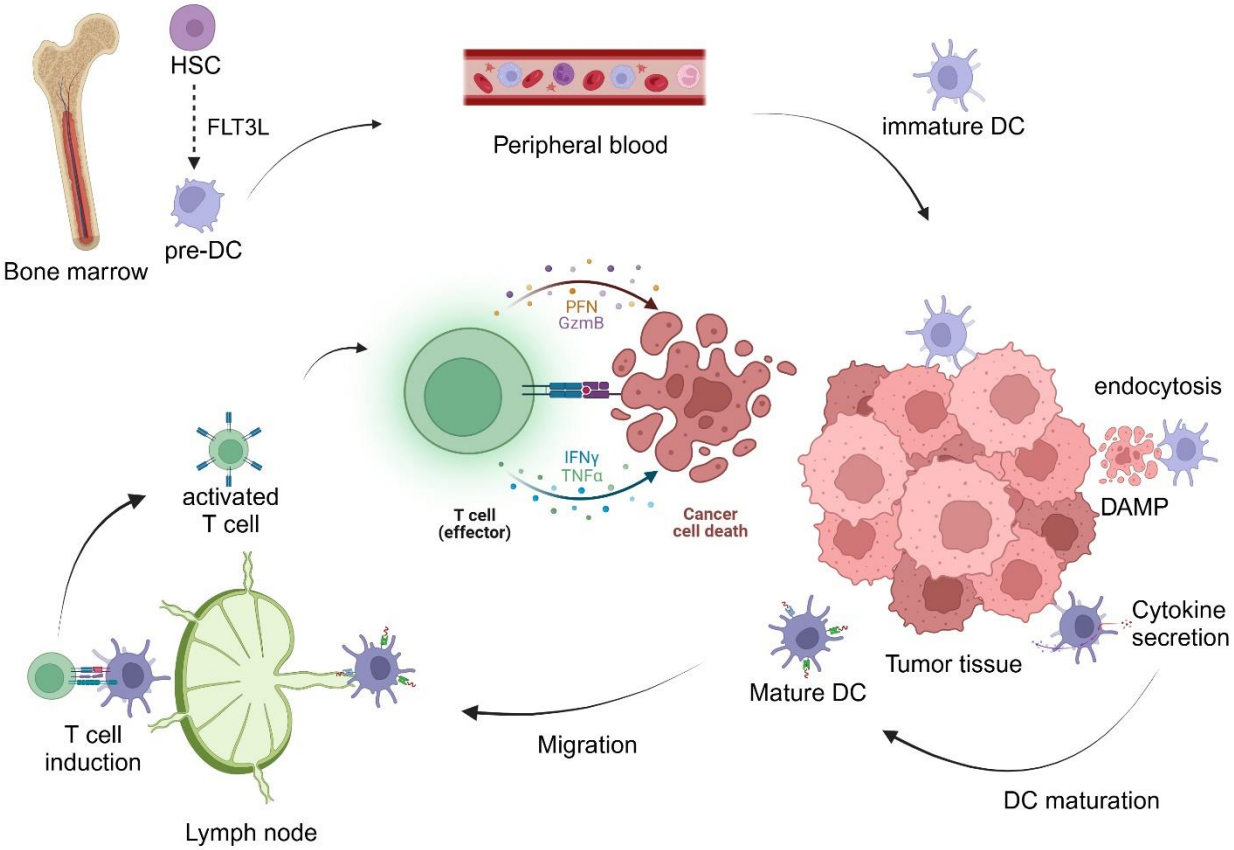


Figure 3: DC life cycle and function. DC derive from HSC and differentiate into pre-DC under the control of FLT3L in the bone marrow. When pre-DC enter the peripheral blood and colonize peripheral tissues, they are then called immature DC, which excel at antigen recognition, endocytosis, and processing. Having been activated by antigens like DAMP, DC migrate to secondary lymphoid organs while processing the antigens. The enhanced expression of the molecule CCR7 guides migration. During this process, immature DC mature, with profoundly enhanced capacities concerning antigen presentation, co-stimulation, T cell induction, and cytokine secretion. In the draining lymph node, mature DC present antigens via MHC class I and class II to CD8+ and CD4+T cells, respectively with the support of co-stimulatory molecules and induce specific adaptive immune response. Activated CD8+ effector T cells, also called CTL then migrate towards pathological tissue to eliminate tumor cells. Adapted from Moussion, et al., 2024 [32], created in BioRender.com.

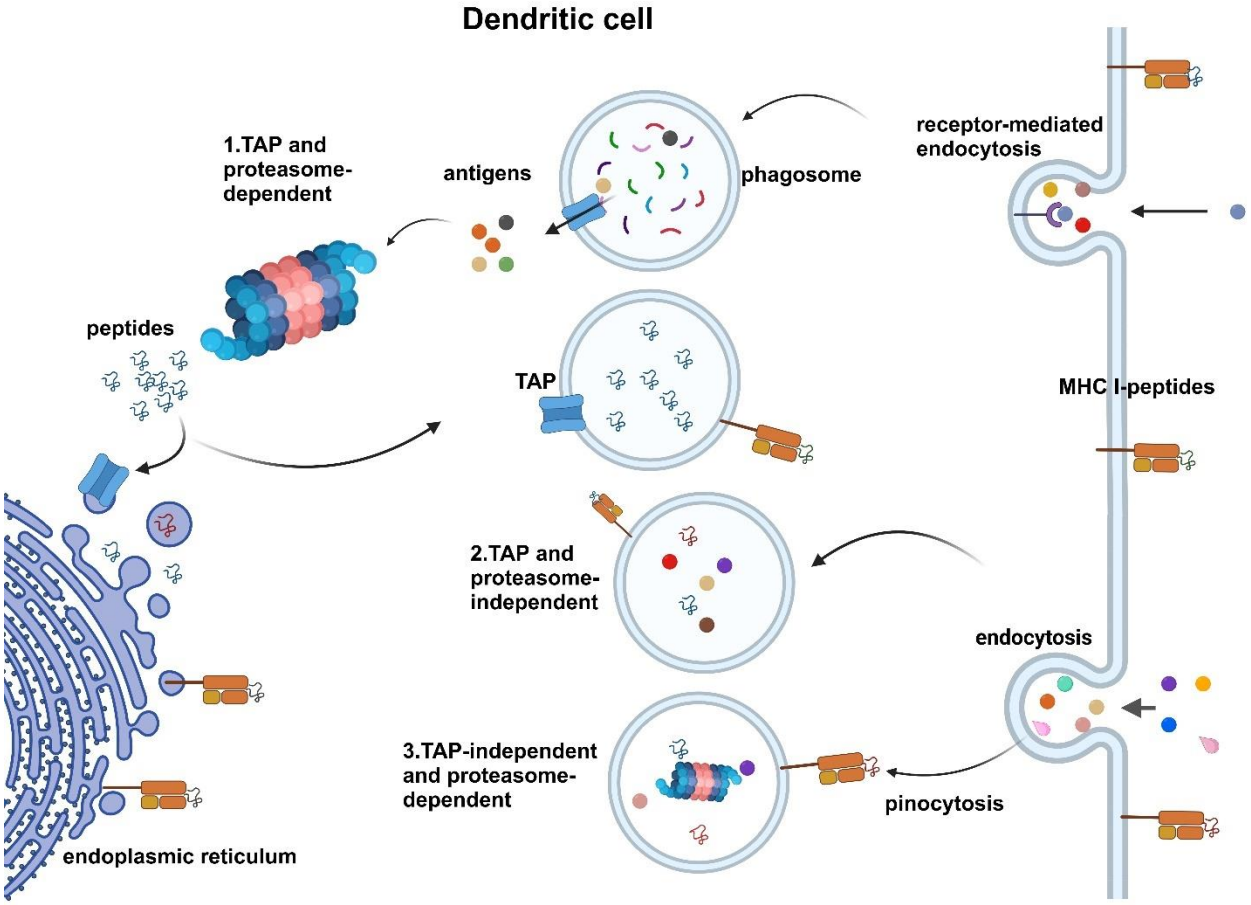


Figure 4: Three pathways of antigen procession and cross-presentation in DC. 1. Transporter associated with antigen processing (TAP) and proteasome-dependent pathway: the exogenous antigens uptaken via receptor-associated endocytosis are released into the cytoplasm. They pass the proteasome for the procession to peptides, which are then transported to the endoplasmic reticulum or phagosomes by TAP for their loading to MHC I. The peptide-MHC complex is then transported to the cell surface via the golgi apparatus. 2. The TAP and proteasome-independent pathway involves the cleavage of exogenous antigens within endosomes by cathepsin S, and binding of antigenic peptides with MHC I molecules. 3. TAP-independent and proteasome-dependent pathways: exogenous antigens are internalized via endocytosis and subsequently degraded by proteasome subunits into peptides, which are then loaded onto MHC I molecules within phagosomes. Adapted from Zhang et al., 2023 [40], created in BioRender.com.

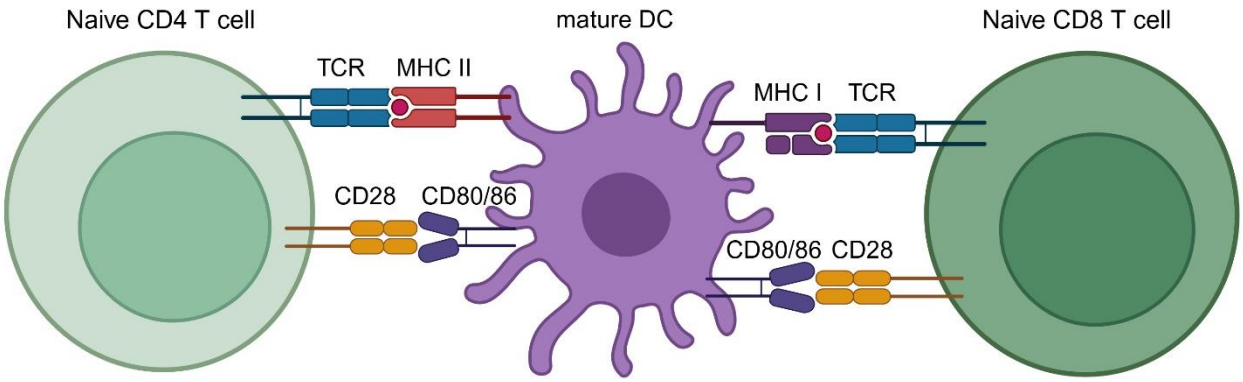


Figure 5: Mature DC in antigen presentation. DC maturation is characterized by the upregulation of costimulatory molecules (CD80 and CD86), and MHC class I and II molecules, essential for induction of the adaptive immune response. When DC migrate to the draining lymph node, processed antigens are either presented on MHC class I molecules to $CD8^+$ T cells or presented on MHC class II molecules to the $CD4^+$ T cells with the help of costimulatory molecules making stable and long-term connections with T-cells. Through specific TCR, T cells recognize antigens presented on MHC molecules on DC. Adapted from Jhunjhunwala et al., 2021 [41], created in BioRender.com.

1.4 Immunotherapy

1.4.1 Systemic approaches to immunotherapy

In recent decades, immunotherapy has revolutionized cancer treatment. The key point of this method is using the patient's immune system to eliminate tumors and prevent recurrence. Most immunotherapeutic methods are characterized by lower side effects compared to classical chemotherapy. Nowadays, multiple immunotherapies are being investigated in many cancers, some of which have been FDA-approved for first-line treatment [42]. Cancer immunotherapy comprises various approaches, amongst them (DC-based) cancer vaccines, adoptive T cell transfer including CAR-T cell therapy, monoclonal antibodies (mAb), ICI, and immunomodulators including cytokines [43].

1.4.2 DC-based cancer vaccination

The ability of DC to connect innate with adaptive immunity and especially their potent antigen-presenting capacity is exploited for cancer immunotherapy, especially in cancer vaccines. In recent years, therapeutic DC-based cancer vaccination has made considerable progress and bears the huge potential to further improve the clinical efficacy of cancer immunotherapy with rare dose-limiting toxicity.

With DC vaccination, modified TAA or TSA or mature DC loaded with tumor antigens ex vivo are injected into cancer patients to induce tumor-specific T cell response that aims to recognize and eliminate cancer cells, as well as induce immunological memory to achieve a durable immune response [44].

So far, various methods of generating DC have been used in the studies, including the generation of autologous DC developed from CD14+ monocytes or CD34+ progenitor cells, in vivo expansion of circulating blood DC, and most recently, isolation and enrichment of naturally circulating blood DC subsets [45].

Classically, moDC have been used in a majority of preclinical and clinical DC vaccination studies. Phase III clinical trials using moDC-based vaccination are being conducted in uveal melanoma (NCT01983748), castration-resistant prostate cancer (NCT02111577), and metastatic colorectal cancer (NCT02503150) [46], and promising results of a large multicenter trial (NCT00045968) where an autologous tumor lysate-loaded moDC vaccine (DCVax-L) was added to the treatment of both newly diagnosed and recurrent glioblastoma demonstrate a potential increase in OS and low side effects [47]. Despite the significant progress being made, sipuleucel-T remains the only approved moDC-based vaccination. The vaccine was designed to treat asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer. Only about 10 % of patients benefit from this vaccination [34, 48]. Researchers found that moDC in sipuleucel-T vaccination have decreased migratory capacities towards the site of T cell interaction by exhaustion of the cells [44]. Furthermore, selecting the most appropriate tumor-specific immunogenic antigens according to gene mutation needs more study for the development of DC vaccination [49]. The greatest challenge that cancer vaccine monotherapy faces is the severe immunosuppression within the tumor microenvironment [34]. Tumor cells elaborate cytokines like IL-10 and TGF-β, recruit immunosuppressive cells like myeloid-derived suppressive cells, impair DC and T cell function, and therefore limit the clinical efficacy of DC-based vaccination [49]. Meanwhile, it was shown that blood DC bear superior antigen-presentation capabilities compared to moDC, e.g. due to the more abundant MHC expression [46].

Driven by the development of efficient isolation and enrichment techniques, vaccination using circulating blood DC has recently become feasible [44]. A phase I/II clinical trial reported the safety and feasibility of patient blood CD1c+ cDC2 loaded ex vivo with TAA in metastatic melanoma, which demonstrated induction of vaccine-specific multifunctional CD8+ T-cell-mediated immune response and improved PFS [50]. A phase III clinical trial NCT02993315 performed in stage IIIB and IIIC melanoma patients using a combination

of both pDC and cDC is ongoing, 2 years of recurrence-free survival (RFS) rate, OS, and tumor-specific T cell response will be reported.

Several DC vaccination strategies with potential clinical efficacy have been clinically tested in MM. In a phase I/II clinical trial in relapsed or refractory MM patients, vaccination consisted of DC loaded with MM cell lysate, and stable disease (SD) was observed in 66.7 % of patients, with 77.8 % showing measurable immunological response [51]. However, Röllig et al. reported on the immune effects induced by a DC-based vaccine in MM patients without previous systemic treatment. Although specific cytokine secretion by CD8+ T cells was reported, CD4+ Th cell responses were not sustained. Therefore, long-term immune response and survival could not be achieved [52].

DC deficiencies in cell number and T cell stimulatory capacity were shown to play a key role in MM pathophysiology, contributing to the subsequent failure of certain immunotherapeutic approaches in MM [53]. Increasing attention is being paid to overcoming T cell exhaustion via blocking immune checkpoint pathways to improve anti-tumor immunity.

1.4.3 Immune checkpoint inhibitors

Innate and adaptive immunity synergy to recognize and eliminate genetically mutated or malignant T cells to maintain health. Under most circumstances, they are highly efficient in eradicating mutated or cancer cells at their early stages [54]. In most cases, immune checkpoints are crucial for maintaining self-tolerance and modulating the duration and intensity of immune responses of effectors in various tissues to minimize tissue damage [55]. However, tumor cells develop multiple mechanisms to evade immune surveillance, which shuts down immune functions and allows the disease to progress. Immune checkpoints are well-known mechanisms cancer cells take advantage of to disguise themselves as a normal part of the human body [54].

Within the tumor microenvironment, CTL undergo transformation, leading to decreased cytokine secretion and killing capacity and sustained expression of inhibitory receptors, a process termed T cell exhaustion [56]. These inhibitory surface molecules expressed on T cells include PD-1, cytotoxic T lymphocyte antigen 4 (CTLA-4), lymphocyte activation gene 3 (LAG-3), and T cell immunoglobulin and mucin domain 3 (TIM-3), which are defined as immune checkpoints [57]. When the checkpoint receptors on the surface of tumor cells or DC bind with their cognate ligands (PD ligand 1/2 [PD-L1, PD-L2]) for PD-1, B7-1 (CD80), and B7-2 (CD86) for CTLA-4, galectin-3 (Gal-3) for LAG-3, galectin-9 for TIM-3, this interaction leads to T cell deactivation [58].

The introduction of ICI, which block the binding of immune checkpoints to their ligands, has led to profoundly improved survival over conventional treatments for various cancers with fewer side effects. Therefore, ICI emerge as promising agents that control anti-tumor immune responses. Currently, various ICIs have been approved for clinical use, such as PD-1 antibodies (nivolumab and pembrolizumab), PD-L1 antibodies (durvalumab and atezolizumab), CTLA-4 antibody (ipilimumab) and the LAG-3 antibody relatlimab. Prolonged PFS and OS are shown in melanoma, advanced non-small-cell lung cancer, advanced renal cancer carcinoma, and relapsed or refractory Hodgkin's lymphoma with tolerable immune-related AE [57].

Despite compelling preclinical evidence suggesting that immune checkpoint inhibitors could be effective treatments in MM, no objective responses (OR) were observed in a phase I trial using pembrolizumab for relapsed or refractory MM, with the best response being SD [59]. In another phase I clinical trial analyzing the efficacy of nivolumab in hematologic malignancies for relapsed or refractory MM ($n = 27$), the response with nivolumab monotherapy remained only SD (63 %), with full recovery noted in only one patient from bone lesion after radiation therapy (4 %) [60]. These studies demonstrate the limited efficacy of ICI monotherapy and suggest that these inhibitors may require combinatory approaches with other agents to achieve convincing clinical responses in MM.

1.5 Combinatory approach in MM - project objective

Current treatments for MM include proteasome inhibitors, immunomodulatory drugs, specific antibodies, and immunotherapies [61]. Despite the approval of innovative immunotherapeutic approaches, including BCMA CAR-T therapy and bispecific antibodies, a significant percentage of MM patients will be under continuous lifelong treatment and suffer from toxicity or experience repeated relapse. The combinatory use of drugs targeting multiple myeloma and the immune system represents an emerging therapeutic approach and yields improved efficacy and synergistically enhanced control of MM [59].

As mentioned before, carfilzomib represents an ICD inducer and therefore could be a potential drug to trigger immunity for combinatory approaches. However, due to its irreversible binding to the proteasome, it could blunt the immune response by off-target effects on immune cells including DC, as previously demonstrated for bortezomib [62]. Therefore, the first objective of this project was to compare the immunomodulatory effects of clinical concentrations of carfilzomib with bortezomib on moDC differentiation, phenotype,

expression of checkpoint molecule, and function. A further goal was to analyze the effect of the addition of the anti-PD-1 antibody nivolumab on T cell proliferation induced by moDC. Thereafter, moving on to translational studies the question was whether it is possible to generate sufficient numbers of moDC with normal phenotype from peripheral blood mononuclear cells (PBMC) of MM patients under carfilzomib treatment. Additionally, possible effects of carfilzomib treatment on the frequency of circulating blood DC subsets were investigated. The general objective was to generate data allowing the future development of a combinatorial strategy using a DC-based cancer vaccine, carfilzomib, and ICI to define a novel approach with high clinical efficacy, long-term response, and limited toxicities in MM patients.

2 Materials and methods

2.1 Materials

2.1.1 Medium and reagents

Product	Manufacturer	Cat. #
Gibco RPMI 1640 cell culture medium	Thermo Fisher Scientific, Waltham, MA, USA	11875093
X-VIVO 20 cell culture medium	Lonza, Basel, Switzerland	04-448Q
Fetal bovine serum (FBS)	Thermo Fisher Scientific, Waltham, MA, USA	A5256701
Antibiotics: penicillin/streptomycin	Thermo Fisher Scientific, Waltham, MA, USA	1140122
GM-CSF	ImmunoTools, Friesoythe, Germany	11343128
IL-4	PeproTech, Cranbury, NJ, USA	200-04
IL-10	R&D Systems, Wiesbaden, Germany	217-IL-010
Lipopolysaccharide (LPS)	Merck, Darmstadt, Germany	L2654-1MG
CCL19/MIP-3β	R&D Systems, Wiesbaden, Germany	361-MI-025/CF
Carfilzomib	SelleckChem, Munich, Germany	S2853
Bortezomib	SelleckChem, Munich, Germany	S1013
DMSO	Sigma-Aldrich, Deisenhofen, Germany	D8418-100ML
CFSE	BD Biociences, San Jose, CA, USA	565082
Nivolumab (anti-PD-1)-1mg	MedChemExpress	HY-P9903A
Human IgG4 kappa, Isotype Control-1mg	MedChemExpress	HY-P99003
Ficoll paque plus	Thermo Fisher Scientific, Waltham, MA, USA	GE17-1440-03
DPBS (1X)	Thermo Fisher Scientific, Waltham, MA, USA	14190144
Staurosporine	R&D Systems, Wiesbaden, Germany	1285/100U
CD3/CD28 Dynabeads	Thermo Fisher Scientific, Waltham, MA, USA	11161D

FITC-dextran	Sigma-Aldrich, Deisenhofen, Germany	60842-46-8
Trypsin	Thermo Fisher Scientific, Waltham, MA, USA	15090046
ACK lysing buffer	Thermo Fisher Scientific, Waltham, MA, USA	A1049201

2.1.2 Antibodies for flow cytometry

Product	Manufacturer	Cat. #
FITC Mouse Anti-Human CD1a	BD Biociences, San Jose, USA	555806
FITC Mouse Anti-Human CD80	eBioscience, San Diego, USA	11-0809-42
FITC Mouse Anti-Human TIM-3	BioLegend, San Diego, USA	345022
FITC Mouse Anti-Human CD16	BioLegend, San Diego, USA	360715v
FITC Mouse Anti-Human CD11c	BioLegend, San Diego, USA	337214
FITC Mouse Anti-Human CD123	BioLegend, San Diego, USA	306014
FITC Mouse IgG1	BD Biociences, San Jose, USA	555748
PE Mouse Anti-Human CD83	eBioscience, San Diego, USA	12-0839-42
PE Mouse Anti-Human CCR7	R&D Systems, Minneapolis, MN, USA	FAB197P-100
PE Mouse Anti-Human GPNMB	eBioscience, San Diego, USA	12-9838-42
PE Mouse Anti-Human Gal-3	BD Biociences, San Jose, USA	565676
PE Mouse Anti-Human CD141	BioLegend, San Diego, USA	344104
PE Mouse Anti-Human HLA-DR	BioLegend, San Diego, USA	307606
PE Mouse IgG2a	R&D Systems, Minneapolis, MN, USA	IC003P
PE Mouse IgG1	BD Biociences, San Jose, USA	551436
PerCP-cy5.5 Mouse Anti-Human CD14	BD Biociences, San Jose, USA	562692
PerCP-cy5.5 Mouse Anti-Human CD209	BD Biociences, San Jose, USA	562692
PerCP-cy5.5 Mouse Anti-Human PD-L2	BD Biociences, San Jose, USA	564256

PerCP-cy5.5 Mouse Anti-Human CD3	BioLegend, San Diego, USA	300430
PerCP-cy5.5 Mouse Anti-Human CD19	BioLegend, San Diego, USA	302230
PerCP-Cy5.5 Mouse IgG1	BD Biociences, San Jose, USA	552834
PerCP-cy5.5 Mouse IgG2b	BD Biociences, San Jose, USA	558304
APC Mouse Anti-Human CD86	BD Biociences, San Jose, USA	555660
APC Mouse Anti-Human PD-L1	BD Biociences, San Jose, USA	563741
APC Mouse Anti-Human HLA-DR	eBioscience, San Diego, USA	17-9956-42
APC Mouse Anti-Human HLA-ABC	BD Biociences, San Jose, USA	555555
APC Mouse Anti-Human CD303	BioLegend, San Diego, USA	354206
APC Mouse Anti-Human CD1c	BioLegend, San Diego, USA	331524
APC Mouse IgG1	BD Biociences, San Jose, USA	550854
FcR Blocking Reagent	Miltenyibiotec, Bergisch Gladbach, Germany	422302
FITC Annexin-V	BD Biociences, San Jose, USA	556420
PI Staining Buffer	BD Biociences, San Jose, USA	550825

2.1.3 Commercial kits

Product	Manufacturer	Cat. #
Human MIP-1α/CCL3 ELISA kit	R&D Systems, Wiesbaden, Germany	DMA00
Human RANTES/CCL5 ELISA kit	R&D Systems, Wiesbaden, Germany	DRN00B
Human MCP-1/CCL2 ELISA kit	R&D Systems, Wiesbaden, Germany	DCP00
Human IL-6 ELISA Set	BD Biociences, San Jose, USA	555220
Human IL-12 ELISA Set	BD Biociences, San Jose, USA	559258

2.1.4 Consumables

Product	Manufacturer
Tissue culture flask 25 cm^2	Thermo Fisher Scientific, Waltham, MA, USA
Conical centrifuge tubes, 15/ 50 ml	Corning Falcon, Corning, NY, USA
Microcentrifuge tubes, 1.5 ml	Sigma-Aldrich, Deisenhofen, Germany
Pasteur pipettes, sterile	Roth, Karlsruhe, Germany
Falcon tissue culture plate, 96 well, round bottom	Corning Falcon, Corning, NY, USA
Falcon tissue culture plate, 24 well, flat bottom	Corning Falcon, Corning, NY, USA
Falcon cell culture inserts, 6.4 mm, 8.0 µm pores	Corning Falcon, Corning, NY, USA
SurPhob SafeSeal LOW BINDING filtered tips sterile 10 µl, 100 µl, 1000 µl	Biozym Scientific, Hessisch Olendorf, Germany
5/10/25-ml serological pipettes	Sarstedt, Nümbrecht, Germany

2.1.5 Devices

Product	Manufacturer
Centrifuge 5810R	Eppendorf, Hamburg, Germany
Pipettes 10 µl, 100 µl, 1000 µl, Handystep repetitive pipette	Eppendorf, Hamburg, Germany
Pipetboy	INTEGRA Biosciences GmbH, Biebertal, Germany
Flow cytometer: CytoFLEX LX	Beckman, Indiana, USA
Optical microscope: Axiovert 25 CFL	Carl Zeiss AG, Oberkochen, Germany

2.2 Methods

2.1.1 Generation of moDC

Fresh buffy coats or peripheral blood from anonymous healthy donors obtained from the blood bank in Charité – Universitätsmedizin Berlin or MM patients under carfilzomib/ lenalidomide/ dexamethasone (KRD) treatment after signing informed consents was used for the generation of moDC. The experimental analyses were approved by the local ethics committees (Charité: EA4/237/21 and University of Tuebingen: 344/2008BO2). 35 ml of PBS-diluted buffy coat (ratio 1:4) or PBS-diluted peripheral blood (ratio 1:2) was slowly layered over 15 ml Ficoll-Paque solution in 50 ml Falcon tubes. After 30 minutes of density gradient centrifugation at 1460 rpm (without brake and acceleration), the PBMC layer was slowly transferred to a 50 ml tube using squeeze Pasteur pipettes. To remove any remnants of Ficoll, cells were washed once by adding PBS up to 50 ml and centrifugated at 1500 rpm for 8 minutes. To remove most of the platelets that have the potential to activate moDC, PBMC were washed twice with 40 ml PBS and centrifugated at 200 g for 10 minutes. Cells were then counted and resuspended in serum-free X-VIVO 20 cell culture medium at 0.6-1*10^7/ml. Thereafter, 5 ml of the cell suspension was seeded into T25 cell culture flasks and allowed to adhere under 37 °C and 5 % CO_2. After 90-120 minutes of incubation, the non-adherent cells were rinsed off twice with warm (37°C) PBS. The adhered monocytes were incubated in RPMI 1640 medium (with 10 % FBS and 1 % penicillin/streptomycin) for further 6 days. GM-CSF (100 ng/ml) and IL-4 (20 ng/ml) were added to the culture every second day from day 0 to obtain immature moDC. LPS (100 ng/ml) was added to moDC 16 hours before harvest to induce maturation. On day 7, suspended and semi-suspended moDC were separated from the medium by centrifugation. Cell culture supernatant was stored at -80 °C for further cytokine secretion analysis. The remaining adherent immature and mature moDC were harvested by thoroughly rinsing twice with 5 ml cold (4 °C) PBS. Harvested moDC were immediately processed for further experiments.

Bortezomib and carfilzomib were dissolved in DMSO. Bortezomib (1 ng/ml) was added to the moDC culture 24 hours before harvest, as previously published [62]. Carfilzomib (100 nM) was added on day 6 and cells were exposed for 1 hour. Carfilzomib was added to the cells by replenishing the fresh cell culture medium with GM-CSF and IL-4. In brief, cells were centrifuged to separate the previous cell culture medium. The medium was

preserved and added to the cells after carfilzomib incubation. Cells were resuspended in the medium with carfilzomib for 1 hour at 37 °C and 5 % CO_2. Thereafter, the medium with carfilzomib was separated from the cells via centrifugation, and the adhering cells were washed twice with 5 ml room temperature fresh RPMI 1640 cell culture medium to remove carfilzomib. The cells were immediately resuspended into the preserved medium. The carfilzomib concentration and duration of exposure were defined according to previous publications and our cell apoptosis experiment on immature and mature moDC and the IC50 concentration for multiple myeloma cell lines, as well as the pharmacokinetics of carfilzomib [63, 64]. DMSO serving as a negative vehicle control and IL-10 (10 ng/ml) for positive control were added to the culture every other day from day 0, respectively.

2.2.2 Flow cytometry (FACS)

The expression of cell surface markers was investigated via FACS which allows determination of the intensity of the antibody-labeled fluorescent signals. Harvested moDC were washed once with cold PBS to remove most of the culture medium. $5*10^5$ cells were transferred into each FACS tube and resuspended in 50 µl FACS binding buffer with 0.5 µl Fc blocking agent to avoid unspecific binding by the Fc segment of the antibody. 0.5 µl FACS antibodies as well as their corresponding mouse IgG isotype controls were added to each tube (table 3). After 30 minutes of incubation in the dark and on ice, moDC were washed once with 1 ml PBS to remove unbound antibodies. MoDC were analyzed on a FACSCalibur cytometer. The delta of median fluorescence intensity (DMFI) between isotype control and a specific molecule served as a stand-in value for the expression of the analyzed surface molecules. To enhance comparability across experiments, normalized DMFI was calculated by dividing the DMFI of the treated cells by that of the DMSO control cells.

Table 3: FACS antibodies panel design

	FITC	PE	PerCP-Cy5.5	APC
Isotype 1, 2, 4	FITC-IgG1	PE-IgG1	PC5.5-IgG2b	APC-IgG1
Sample 1	CD1a	CD83	CD14	CD86
Sample 2	CD80	GPNMB	CD209	CD274 (PD-L1)
Isotype 3	FITC-IgG1	PE-IgG2a	PC5.5-IgG1	APC-IgG2b
Sample 3	TIM-3	CCR7	CD273 (PD-L2)	HLA-DR
Sample 4		Gal-3		HLA-A, B, C

2.2.3 Migration assay

After harvest, pretreated mature moDC were resuspended in serum-free RPMI medium at a concentration of $2*10^5$/ml. Each well of a 24-well plate was filled with 1 ml serum-free RPMI 1640 medium enriched with CCL19 (100ng/ml). Then, the 8 µm micro-pore cell culture inserts were placed into each well and the inserts were filled with 1 ml of the prepared cell suspension. Cells were allowed to migrate for 16 hours under 37 °C and 5 % CO_2. Each condition was performed in triplicates. Thereafter, the inserts were removed and discarded. The remaining culture medium and cells within the well were transferred to FACS tubes. Semi-adherent moDC were rinsed off with 1 ml cold (4 °C) PBS. The cells were centrifuged for 5 minutes at 400g and resuspended in 50 µl PBS. The number of migrated cells was measured during 1 minute by FACS in a previously established moDC gate.

2.2.4 Endocytosis assay

$1*10^5$ pretreated immature moDC and respective controls were incubated with 5 µl FITC-dextran for one hour at 37 °C, 5 % CO_2. Then, cells were washed twice using PBS and transferred into FACS tubes. FITC-dextran uptake was analyzed by FACS.

2.2.5 Analysis of cytokine levels

The analysis of moDC-secreted cytokine levels was performed using sandwich- (enzyme-linked immunosorbent assay) ELISA. – the level of color produced by the binding of the tested molecule to the antibody reflects the quantification of the molecule. Cell culture supernatants from pretreated mature moDC medium were collected on d7 at harvest and stored at -80 °C for later analysis. Cytokine levels of CCL-2, CCL-3, CCL-5, IL-6, and IL-12 of several samples were analyzed simultaneously using ELISA kits according to the manufacturer's instructions. In brief, after thawing the samples, 200 µl of standard, control, or sample were added into pre-coated wells and incubated at room temperature for 2 hours. After incubation, wells were washed three times, and 200 µl of detection solution was added to the wells followed by another 1 hour of incubation. After washing three times, 200 µl of the substrate solution was added and the plate was incubated for 20 minutes in the dark at room temperature. After incubation, 50 µl of stop solution was added to each well. The optical density was measured by a microplate reader at a 450 nm wavelength immediately.

2.2.6 Mixed lymphocyte reaction (MLR)

Allogeneic human T cells from peripheral blood of healthy donors were isolated via density gradient centrifugation as mentioned above. Cells were cultured in RPMI 1640 medium with 10 % heat-inactivated FBS and 1 % penicillin/streptomycin and activated by adding CD3/CD28 dynabeads for 24 hours. After activation, cells were transferred into a 15 ml conical centrifuge tube and centrifuged at 1600 rpm for 5 minutes. 1 ml RPMI 1640 medium (with 10 % FBS and 1 % penicillin/streptomycin) was added to the cell pellet and cells were pipetted up and down 10 times to be dissociated from dynabeads. Cells were transferred to a 1.5 ml microcentrifuge tube and the tube was placed in the magnetic rack for 1 minute. Then, the supernatant with cells was transferred to a T25 cell culture flask. Added RPMI 1640 cell culture medium(with 10 % FBS and 1 % penicillin/streptomycin) up to 5 ml. After 3 days of culture, preactivated T cells were washed once with PBS to remove any residual serum proteins. Thereafter, cells were resuspended in PBS at a concentration of 10^7 cells/ml. Carboxyfluorescein diacetatesuccinimidyl ester (CFSE) was diluted with PBS to achieve a 2.5 µM CFSE working solution. 0.5 ml of the cell suspension was mixed with 0.5 ml of the CFSE stain solution and incubated in a 37°C water bath for 10 minutes in the dark. After incubation, cells were washed twice with 10ml PBS (supplemented with 10% FBS) and resuspended in RPMI 1640 cell culture medium (with 10 % FBS and 1 % penicillin/streptomycin) at a concentration of 2×10^6 cells/ml. PD-1 inhibitor nivolumab (10 µg/ml) or human IgG4 isotype control, respectively was added to the stained T cells to block their PD-1 receptor. Harvested mature moDC were inactivated by 30Gy irradiation (duration 10-15 minutes) and resuspended in fresh RPMI 1640 cell culture medium with 10 % FBS and 1 % penicillin/streptomycin (2×10^5 cells/ml). 100 µl of the moDC suspension was co-cultured with 100 µl of the T cell suspension (ratio 1: 10) in a 96-well round bottom plate. Each condition was tested in triplicates. After 5 days of incubation in the dark under 37 °C and 5 % CO_2, responder T cell proliferation was assessed by flow cytometer.

2.2.7 Patient and healthy donor samples

Human peripheral blood and bone marrow were obtained from MM patients treated at Charité Universitaetsmedizin Berlin (Campus Benjamin Franklin, Department of Hematology, Oncology and Cancer Immunology) between September 2022 and December 2023. Newly diagnosed MM patients, patients undergoing treatment with a carfilzomib-

containing regimen, and patients in remission were included. Approval for experimental analysis of the patient samples was provided by the Charite ethics committee (EA4/237/21) in accordance with the latest Helsinki declaration. Informed consent was obtained from all participants.

In newly diagnosed MM patients, the following laboratory tests and imaging examinations were performed in the clinical routine before initiation of any anti-myeloma treatment: serum monoclonal protein, free light chains, calcium, creatinine, and lactate dehydrogenase (LDH) levels; type of M-protein; percentage of clonal bone marrow plasma cells; scan for osteolytic bone lesions; chromosome analysis; hemoglobin and white blood cell counts. Patients were staged according to R-ISS.

For analyses in carfilzomib-treated patients, peripheral blood samples were obtained from patients in first-line treatment after at least 4 cycles of the carfilzomib-containing regimen.

All samples from healthy donors used in this study were collected at Charité Universitaetsmedizin Berlin.

2.2.8 Quantification of blood DC subtypes

The enumeration of different subtypes of circulating blood DC and bone marrow DC was performed by analyzing specific cell surface markers by FACS. 10 ml ACK lysing buffer was added to 3 ml of the fresh blood and bone marrow samples (room temperature) to lyse red blood cells. After 5 minutes, cells were washed once using 35 ml of PBS. Another 1 ml ACK lysing buffer was added to the cell pellet and incubated for 5 minutes. Cells were washed again with 40 ml of PBS. White blood cells were then resuspended in FACS staining buffer with FcR Blocking Reagent at a ratio of 1:100 and transferred into 3 FACS tubes. Thereafter, cells were stained with the antibodies and analyzed by FACS (table 4). CD138 antibody was added for the staining of plasma and myeloma cells in bone marrow analysis. Data analysis was performed using FlowJo software.

Table 4: FACS antibodies panels for DC subtypes

DC subtype	FACS antibodies
cDC1	CD45, HLA-DR, CD16, CD14, CD19, CD3, CD141
cDC2	CD45, HLA-DR, CD14, CD19, CD3, CD11c, CD1c

pDC	CD45, HLA-DR, CD14, CD19, CD3, CD123, CD303

2.2.9 Statistical analysis

Experimental data was presented as mean ± SD. Outliers were identified via ROUT method in GraphPad Prism and excluded from the final statistical analysis. The relative differences among 3 or more groups within one variable factor were analyzed by one-way ANOVA comparing treatment conditions to the DMSO negative control. Mann-Whitney U test was used for non-parametric values within 2 groups. SPSS software program was used for clinical data analysis. For analysis of the relation between MM parameters and total DC in PBMC, continuous variables were compared using Spearman test. Binary variables were compared by Mann-Whitney U test, while multiple categorical variables were compared using one-way ANOVA. P-values <0.05 were considered statistically significant.

3 Results

3.1 Influence of carfilzomib on moDC phenotype and checkpoint molecules

3.1.1 Carfilzomib does not affect moDC phenotype

Carfilzomib, the second-generation proteasome inhibitor, does not selectively target MM cells and hence could possibly affect differentiation, phenotype, and function of moDC. MoDC were exposed to 100 nM carfilzomib or 1 ng/ml bortezomib on day 6, respectively. moDC were harvested on d7 and possible effects on moDC phenotype were assessed by FACS.

Only minimal alterations were observed in immature and mature moDC morphology including cell size and granularity under carfilzomib and bortezomib treatment compared to the DMSO negative control. IL-10 served as a positive control since it blunted moDC differentiation [65]. IL-10 exposure led to significantly smaller cell size and fewer intercellular granules as determined by FACS (figure 6A).

A

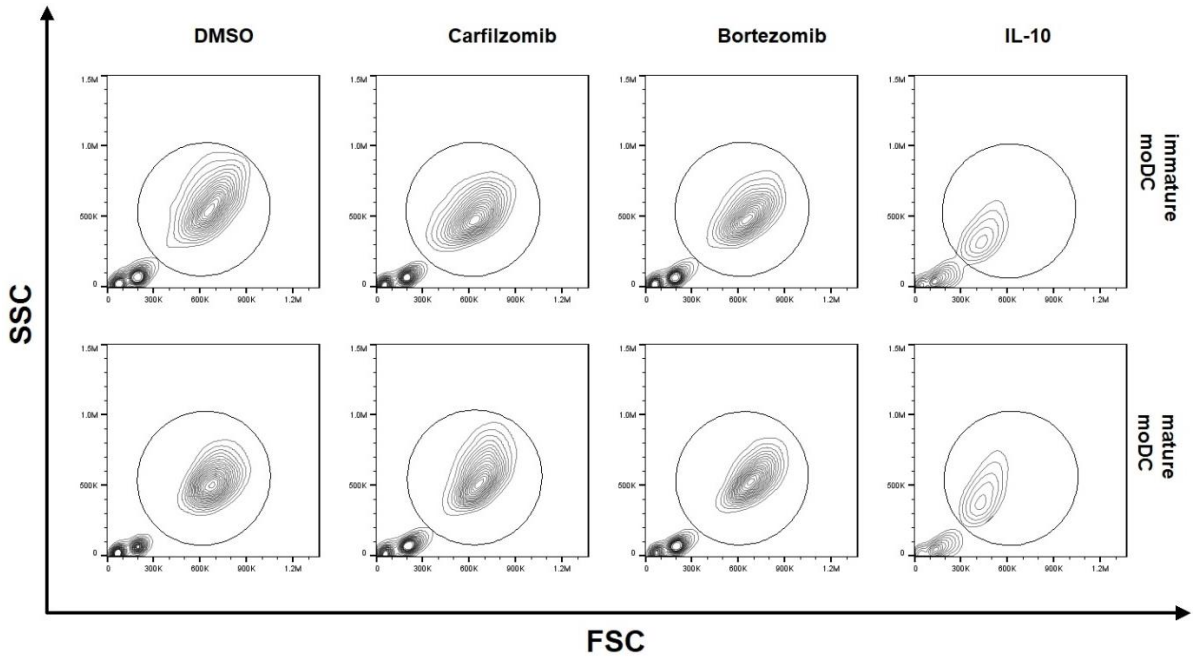


Figure 6A: Effects of carfilzomib and bortezomib on moDC morphology. Size is determined by forward scatter (FSC) on the X axis with the size of depicted cells increasing to the right. The side scatter (SSC) on the Y axis represents the granularity of cells. Cells depicted in the upper part of the graph harbor more intracellular granules. One representative of at least seven replicable experiments is depicted.

Moreover, the expression of moDC surface markers was analyzed by FACS to investigate the effect of carfilzomib and bortezomib. Figures 6B and D display exemplary FACS data of immature and mature DC phenotypes, while figures 6C and E show the statistical analyses of these surface molecules (n=7-15).

CD14 is identified as a human monocyte differentiation antigen [66]. In successful differentiation of monocytes to moDC CD14 expression diminishes. No significant effect on CD14 expression was observed after exposure to the two proteasome inhibitors and the negative control, respectively. However, CD14 remained highly expressed in cells under IL-10 treatment indicating the impairment of moDC differentiation by this cytokine.

After differentiation, CD1a, a classical moDC marker, is highly expressed on moDC. CD1a is a surface protein that plays a crucial role in the presentation of self-derived or pathogen-derived lipids to T cells. A specific subtype of DC with high expression of CD1a is known to induce substantial production of IL-12, a key factor for T cell stimulation [67, 68]. Carfilzomib treatment had no statistically significant effect on CD1a expression in immature and mature moDC. Bortezomib exposure led to significantly downregulated CD1a expression. This was observed to a greater extent under IL-10 treatment.

CD209, also called DC-sign, belongs to the C-type lectin superfamily and is predominantly expressed in dendritic cells. The main function of CD209 is the recognition of mannose-based microbial DAMP and the promotion of T cell presentation [69, 70]. Neither carfilzomib nor bortezomib had a statistically significant effect on CD209 expression.

CCR7 is a high-affinity receptor of CCL21 and CCL19, which promotes migration to the lymph node [71]. After maturation, the expression of CCR7 on DC is significantly increased. In immature moDC. Here, a statistically significant increase in its expression following carfilzomib treatment was observed, while in mature moDC, bortezomib hampered CCR7 expression.

CD83 is a surface molecule that is highly and stably expressed on completely matured DC. It plays a central role in the regulation of T and B cell maturation and mediates adaptive immune responses [72]. A significant increase of CD83 expression in immature moDC was observed under carfilzomib treatment. In line with Nencioni et al., exposure to bortezomib led to reduced expression in moDC stimulated with LPS [62].

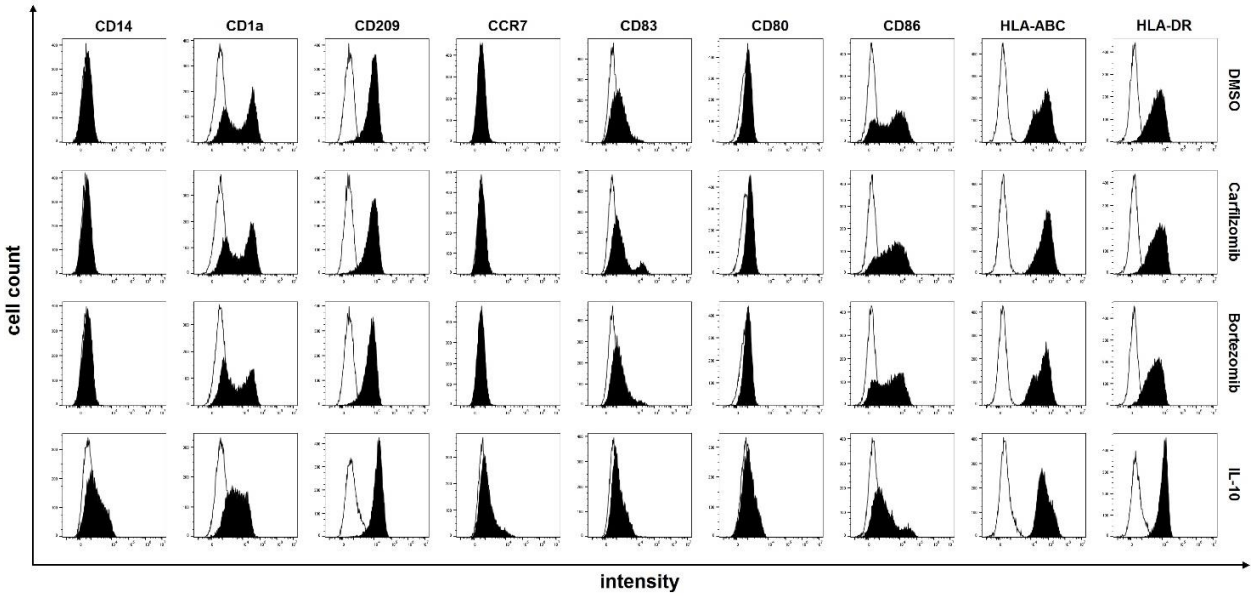
CD80 and CD86 are co-stimulatory molecules expressed on mature DC. They are ligands of CTLA-4 that mainly regulate T cell immune response. In this study, bortezomib treatment significantly blunted the expression of CD80 and CD86 in mature moDC. Only a

minor reduction of CD80 expression was observed in mature but a statistically significant increase was observed in immature moDC exposed to carfilzomib.

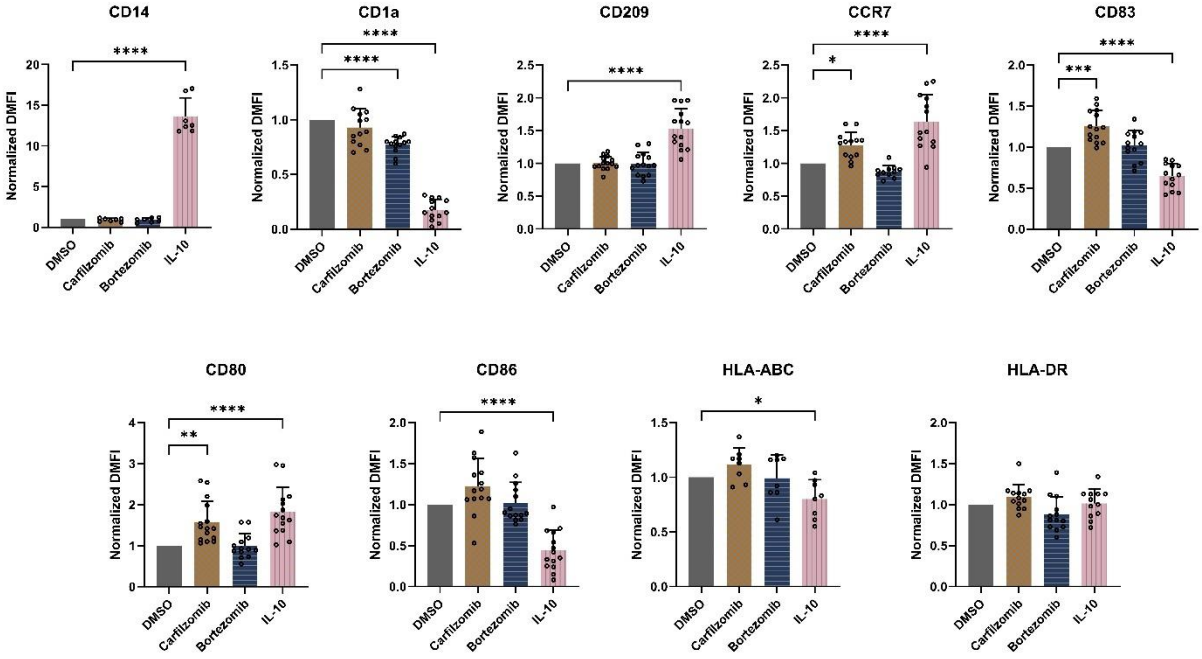
We also analyzed the effect of carfilzomib and bortezomib on two antigen-presenting molecule classes HLA-ABC (MHC class I) and HLA-DR (MHC class II). Results demonstrated no significant effect on HLA-ABC in moDC treated with carfilzomib and bortezomib, however concerning HLA-DR, bortezomib exposure led to a significant decrease in moDC after LPS stimulation.

In conclusion, bortezomib significantly impaired the expression of moDC surface molecules, while carfilzomib had only minimal influence on moDC phenotype.

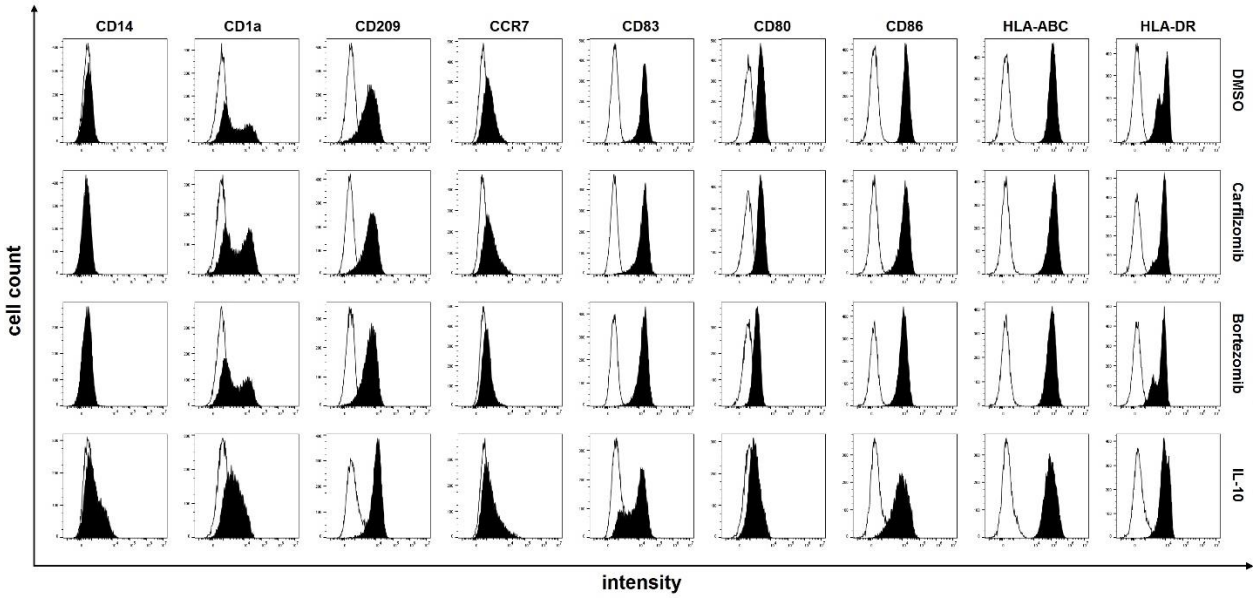
B Exemplary data of immature moDC phenotype



C Statistical analysis of immature moDC phenotype



D Exemplary data of mature moDC phenotype



E Statistical analysis of mature moDC phenotype

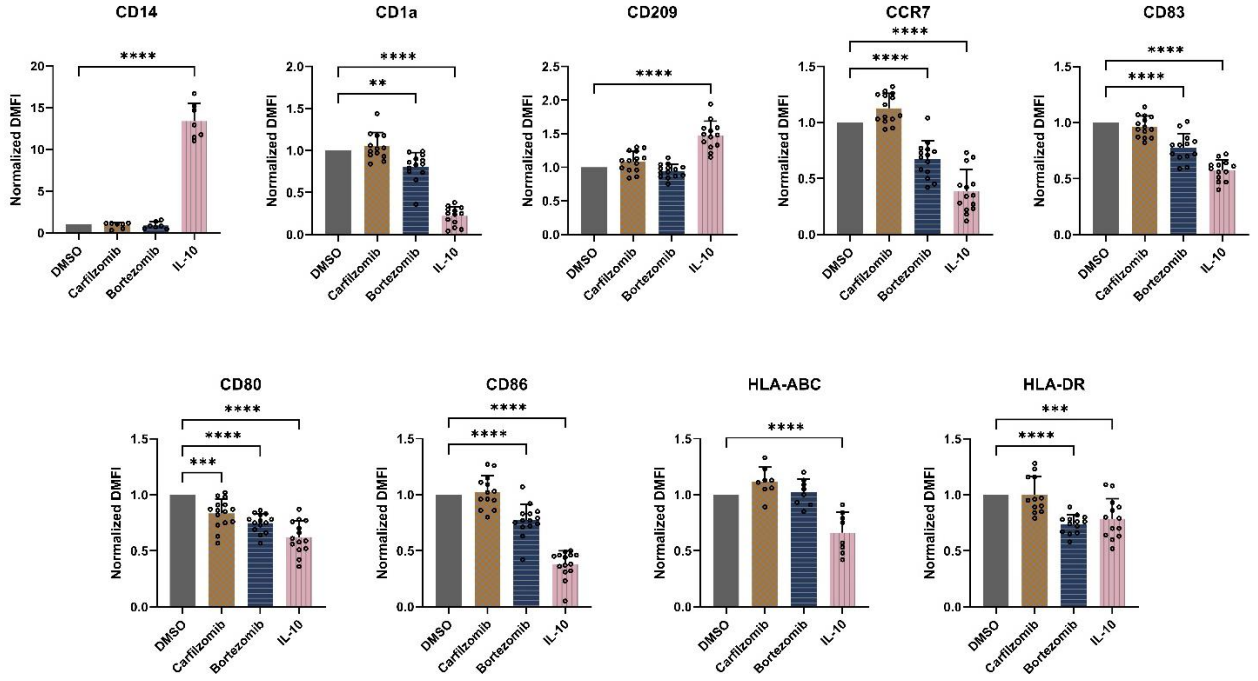


Figure 6B-E: Effect of carfilzomib and bortezomib on moDC phenotype. MoDC surface markers characterizing moDC phenotype were measured after carfilzomib and bortezomib treatment by FACS. Data is shown as normalized DMFI. DMSO served as a negative, IL-10 as a positive control. Exemplary data B (immature moDC) and D (mature moDC) are presented as FACS histograms. C (immature moDC) and E (immature moDC) display the statistical analysis of the surface markers. N= 7 - 15 (One-way ANOVA, Mean ± SD, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

3.1.2 Carfilzomib influences checkpoint molecule expression in moDC

The discovery of immune checkpoint molecules such as PD-1/ PD-L1 and the implication of monoclonal antibodies blocking these molecules represents a remarkable breakthrough in cancer treatment [73]. Checkpoint molecules are also expressed by DC and are used to regulate the immune response [74]. As mentioned above now new generations of DC-based cancer vaccines are being tested in clinical trials. The goal of these approaches is to overcome DC dysfunction in the TME by adding ICI to the strategy and thereby enhancing the efficacy of the vaccine [75]. However, in MM the use of ICI has so far failed to achieve convincing clinical response [76]. To shed more light on the possible reasons for this failure T cell the expression of several immune checkpoint molecules on the surface of moDC exposed to the carfilzomib and bortezomib was investigated. One hypothesis is that the DC capacity to induce T cells can be augmented by ICI.

PD-L1 and PD-L2 are the ligands of PD-1. The binding of PD-1 with its ligands, PD-L1 and PD-L2, delivers inhibitory signals that regulate the balance between T cell activation, tolerance, and immunopathology [77]. Notably, we observed a statistically significant upregulation of PD-L1 expression under carfilzomib and bortezomib treatment in moDC with and without LPS stimulation. After moDC maturation, the expression of PD-L1 was significantly increased. For PD-L2 expression, carfilzomib exposure demonstrated a slight but statistical increase in immature moDC (figure 7).

TIM-3 is a newly defined immune checkpoint molecule as a negative regulator of Th cells expressed on DC [78, 79]. It could impair DNA recognition and endocytosis function of cDC1 via inhibiting the cGAS-STING pathway as well[80]. The expression of TIM-3 was significantly lower in immature moDC exposed to carfilzomib. Moreover, the expression of TIM-3 was significantly decreased after DC maturation.

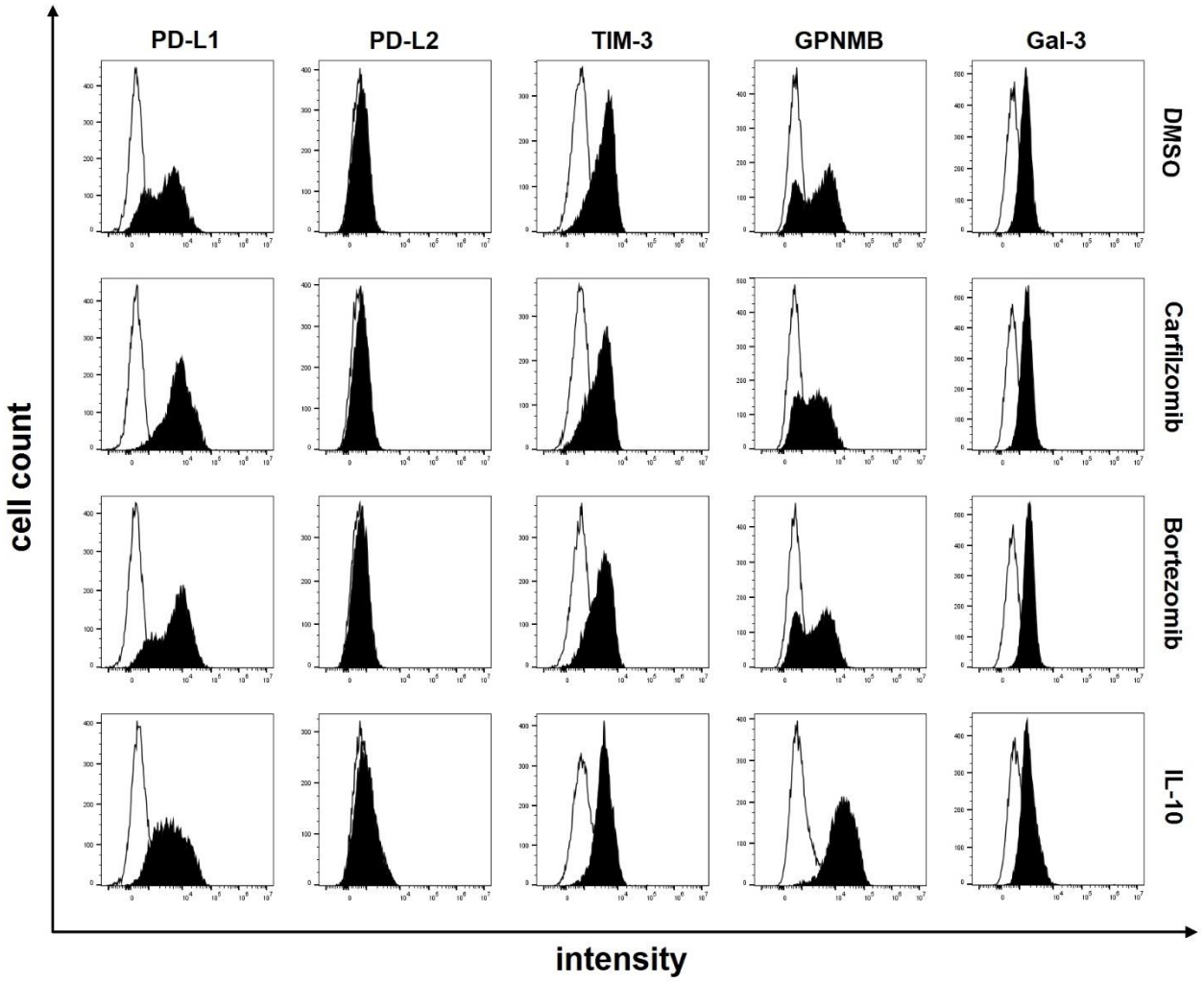
GPNMB is an immune checkpoint molecule lately demonstrated to be expressed on DC [81]. Our group has previously shown when tyrosine kinase inhibitors (imatinib and Nilotinib) and IL-10 profoundly increased the expression of GPNMB, the T cell stimulation capacity of DC was significantly blunted T cell in MLR assay, which could be restored by adding anti-GPNMB antibody [82]. Here, exposure to proteasome inhibitors carfilzomib and bortezomib did not significantly alter GPNMB expression on moDC. During maturation, the expression of GPMNB was downregulated.

Gal-3 is the ligand of LAG-3, which impairs the activation of CTL by distancing the TCR from CD8 leading to immunosuppression. Moreover, when incubated with CD4 T cells,

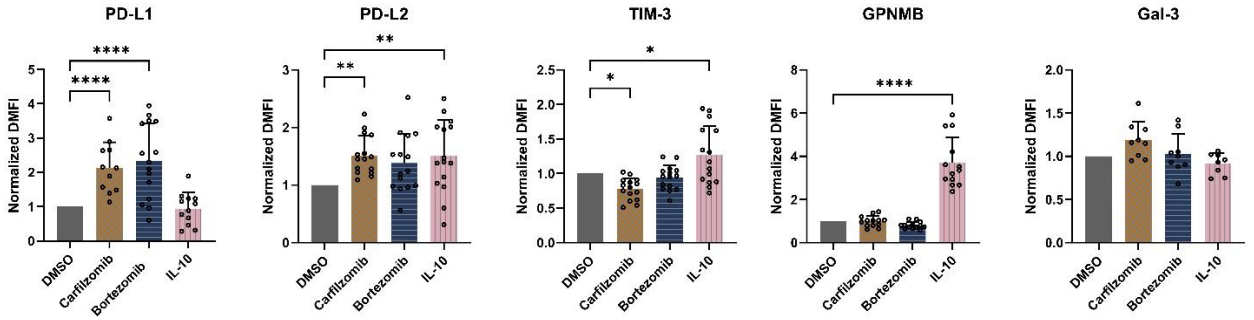
DC with downregulated Gal-3 could induce more IFN-γ and less IL-10 production [83]. In 2022, the US FDA approved anti-LAG-3 (relatimab) combined with anti-PD-1 (nivolumab) for the treatment of unresectable or metastatic melanoma [84]. No significant effect on Gal-3 expression was observed by carfilzomib and bortezomib exposure.

In conclusion, both carfilzomib and bortezomib treatment elicited an augmented expression of PD-L1 expression, whereas TIM-3 was decreased by carfilzomib exposure in immature moDC (figure 7A-E).

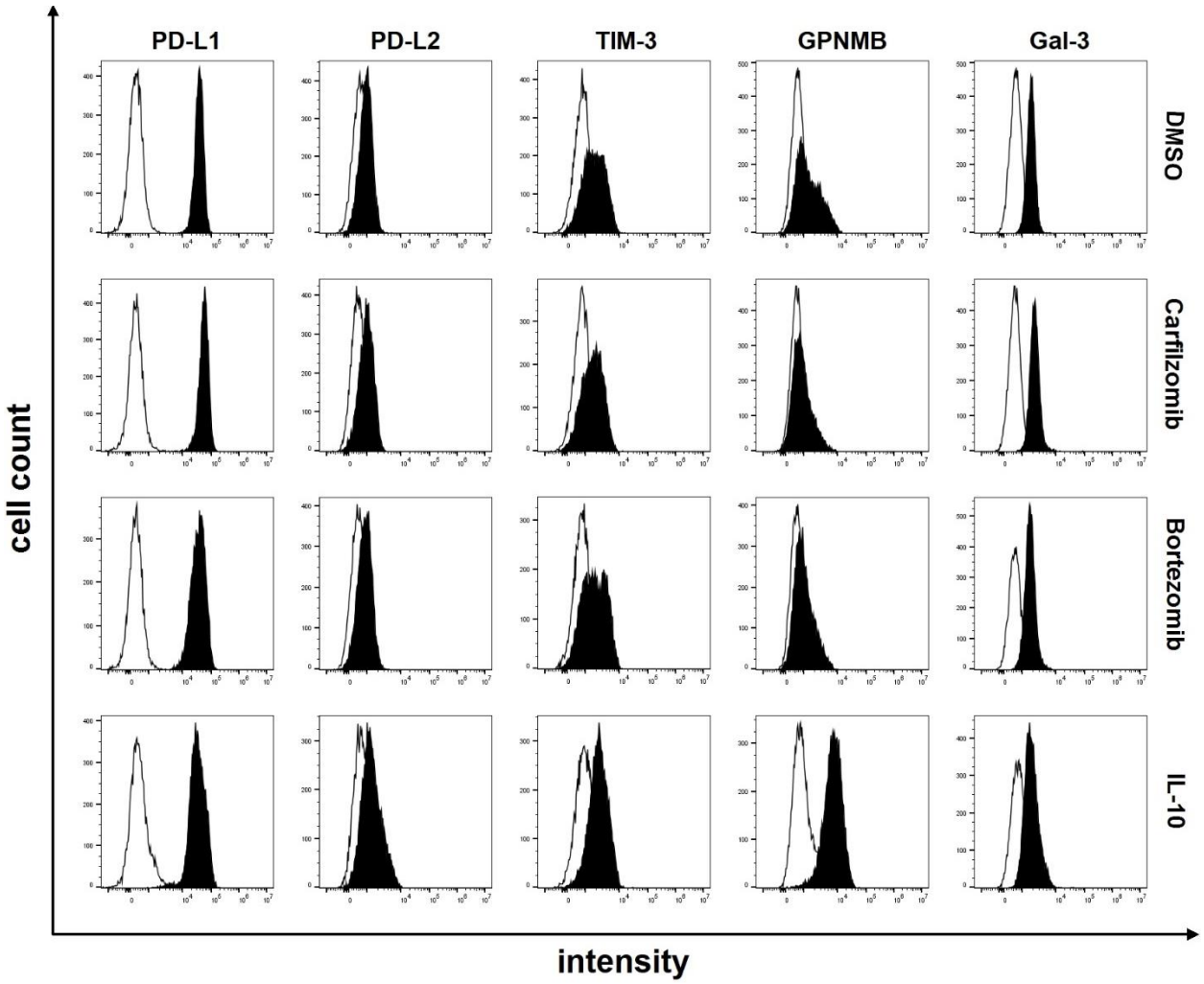
A Exemplary data of checkpoint molecule expression in immature moDC



B Statistical analysis checkpoint molecule expression in immature moDC



C Exemplary data of checkpoint molecule expression in mature moDC



D Statistical analysis of checkpoint molecule expression in mature moDC

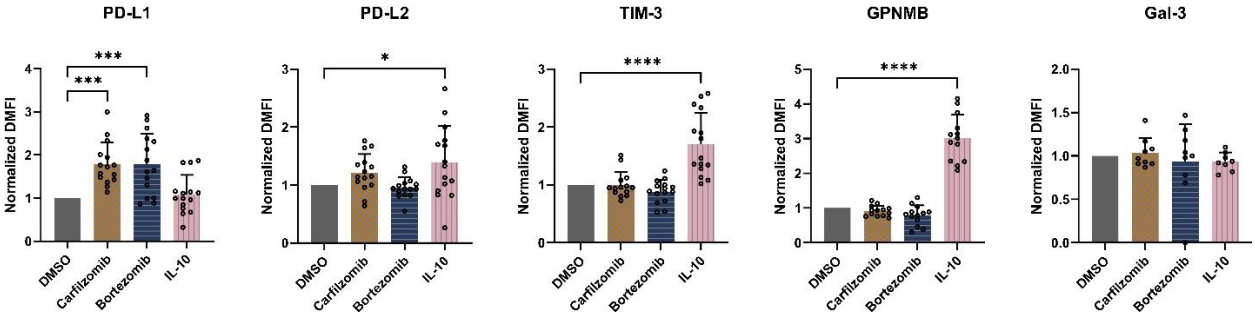


Figure 7A-D: Effect of carfilzomib and bortezomib on moDC immune checkpoint molecules. Expression of the indicated checkpoint molecules was measured by FACS after exposure to carfilzomib and bortezomib. Results are shown as normalized DMFI which was calculated via dividing by DMSO. Exemplary data (A immature moDC and C mature moDC) and statistical analysis (B immature moDC and D mature moDC) are shown. (One-way ANOVA, Mean ± SD, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001) n=9-15.

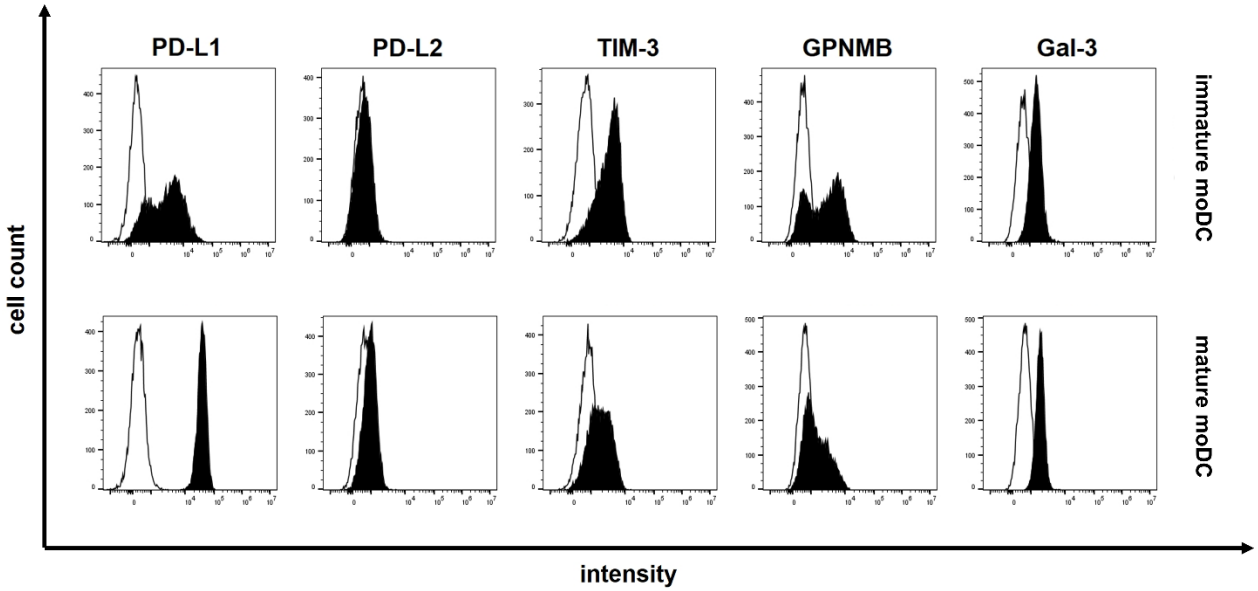


Figure 7E: Comparison of checkpoint molecules in immature and mature moDC in DMSO negative control. After maturation, a tendency towards increased level of PD-L1 expression was observed, while a decreased tendency in the expression of TIM-3 and GPNMB. Regarding PD-L2 and Gal-3, no significant difference was detected.

3.2 MoDC function

3.2.1 Carfilzomib does not affect moDC endocytosis

Immature moDC uptake pathogens or antigens via endocytosis, which is a key function of the initiation of DC-mediated antigen presentation and adaptive immunity. During the maturation process, DC lose their ability of endocytosis. Hence, the endocytic capacity was analyzed through FITC-labeled dextran internalization assay in immature moDC. No statistically significant difference was observed in endocytosis in carfilzomib-treated immature moDC compared to the DMSO negative control and bortezomib, respectively (figure 8).

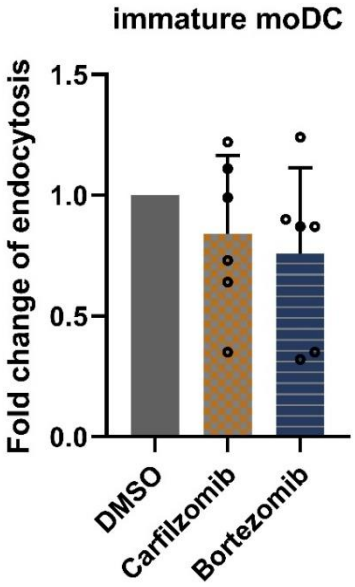


Figure 8: Effect of carfilzomib and bortezomib on endocytosis in immature moDC. Analysis of endocytic capacity using an FITC dextran assay. MFI levels of FITC were measured by flow cytometry. Data was compared to normalized DMSO. Statistical analysis of six experiments is shown and compared to DMSO control (One-way ANOVA) (experimentsperformed by Marius Dahlfrancis).

3.2.2 Carfilzomib does not blunt migration of moDC

After antigen uptake, DC upregulate the expression of CCR7 and gain the ability to migrate to the second lymphoid organs in response to CCL19/MIP-3β. The migratory capacity was assessed in transwell assays using mature moDC. Carfilzomib treatment had no statistically significant effect on migration. However, in line with the previous publication, bortezomib significantly impaired this function [62] (figure 9).

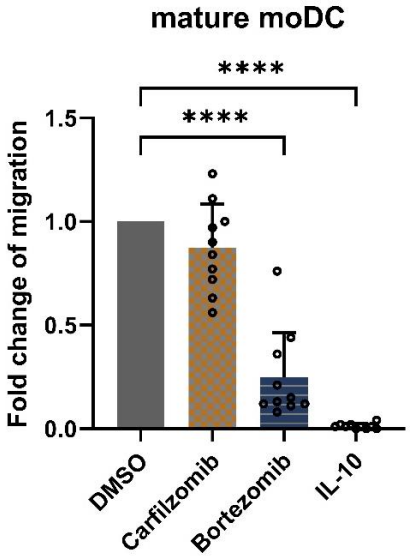


Figure 9: Effect of carfilzomib and bortezomib on the migration of mature moDC. Evaluation of migratory capacity towards CCL19 was performed by transwell assays and cell counts of migrated cells were measured by FACS. Data are shown as fold change compared to normalized DMSO. Pooled data of ten independent experiments are shown (One-way ANOVA, ****P<0.0001) (2 of 8 experiments performed by Marius Dahlfrancis).

3.2.3 Carfilzomib does not impair cytokine secretion of DC

During their maturation process, DC obtain a key function to regulate the immune response, which is the secretion of inflammatory cytokines. Levels of MCP-1/CCL2, MIP-1α/CCL3, Rantes/CCL5, IL-6, and IL-12 in the supernatants of cell cultures of mature moDC were analyzed by ELISA. Results demonstrated a significant decrease of MIP-

1α/CCL3 level in bortezomib-treated moDC, while carfilzomib showed no statistically significant influence compared to the DMSO vehicle control. A trend toward reduced levels of IL-12 by exposure to carfilzomib and bortezomib was observed, respectively (figure 10).

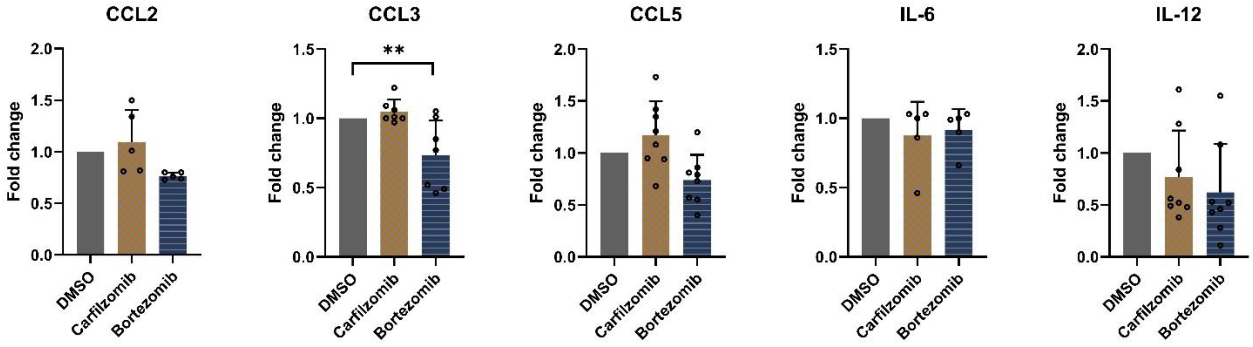


Figure 10: Effect of carfilzomib and bortezomib on cytokine secreting capacity of mature moDC. Levels of CCL2, -3, -5, and IL-6 and- 12 released by moDC were assessed using ELISA. Data was compared to normalized DMSO. Statistical analysis of 5 to 8 replicable experiments is displayed (One-way ANOVA, **P<0.01) (experiments performed by Marius Dahlfrancis).

3.2.4 Carfilzomib-induced impairment of moDC allostimulatory capacity is restored by nivolumab

Their ability to induce T cell responses is the most relevant function of DC also with regard to their use in cancer vaccines. To investigate the influence of carfilzomib exposure on the priming of allogeneic T cell responses, MLR assays were performed. For this carfilzomib pretreated or non-treated (DMSO control) mature moDC were co-cultured with CFSE-stained pre-activated allogeneic T cells. Carfilzomib hampered moDC allostimulatory capacity, which was statistically significant. Consistent with previous findings by Nencioni et al. this was also true for bortezomib-exposed moDC (figure 11).

In the next step, with the aim to confirm the hypothesis that augmentation of DC T cell stimulatory capacity in this setting might be possible by the use of ICI, the anti-PD-1 antibody nivolumab was added to the MLR assay. The addition of nivolumab not only abrogated the impairment induced by carfilzomib but led to a statistically significant increase in T cell proliferation compared to the DMSO negative control ($p<0.01$, figure 11A).

Moreover, T cell proliferation could also be enhanced in untreated moDC (DMSO control) by the addition of nivolumab to the coculture. Interestingly, the extent of augmentation of T cell proliferation was even higher in carfilzomib pretreated moDC under the influence of the ICI nivolumab compared to the combination of DMSO-treated moDC and nivolumab and this was statistically significant ($p < 0.5$, figure 11A, B, n=8).

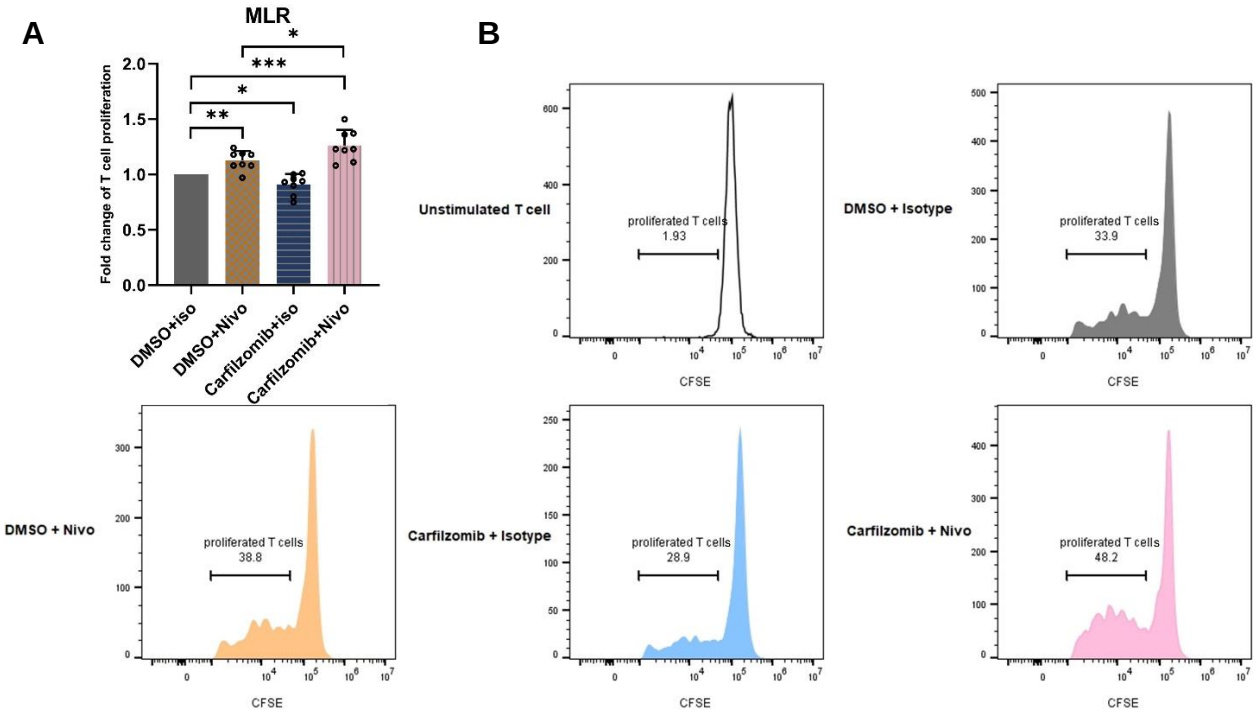
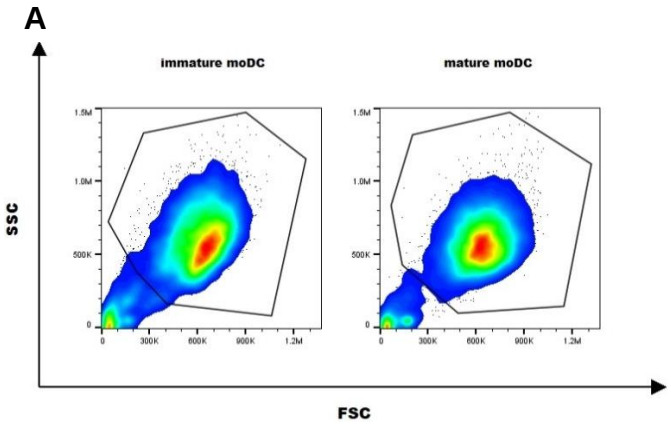


Figure 11: Effect of carfilzomib and anti-PD-1 antibody (nivolumab) on the allostimulatory capacity of mature moDC. MoDC pretreated with carfilzomib or DMSO control were co-cultured with allogenic T cells which were treated in the presence of nivolumab or human IgG4 serving as isotype control. Statistical analysis of eight independent experiments (A) and exemplary data (B) are shown. (Mann-Whitney U test, Mean ± SD, *P<0.05, **P<0.01).

3.3 Patient results

3.3.1 Generation of moDC from carfilzomib-treated MM patients is feasible

A prerequisite for the development of a DC-based vaccine is the generation of sufficient amounts of autologous DC. Traditionally monocyte-derived DC are used in cancer vaccines. As a translational step, both immature and mature moDC were generated using peripheral blood monocytes (from 70 ml peripheral blood)of 3 MM patients under first-line treatment with the carfilzomib-containing regimen KRD. During cell culture, no extra carfilzomib was added to the medium. DAPI staining has no significant effect on moDC viability (data not shown). Compared to healthy donors, no significant decrease in moDC cell count was observed in carfilzomib-treated patients (data not shown). No statistically significant differences in cell size and intracellular granularity were shown by FSC/SSC gating (Figure 12A) and the expression of 9 phenotype markers and 5 checkpoint molecules was observed compared to healthy donors especially no significant upregulation of CD80 and CCR7 in immature moDC. However, in PD-L1 expression, no difference was observed in immature and mature moDC, which is different from the in vitro data (figure 12B).



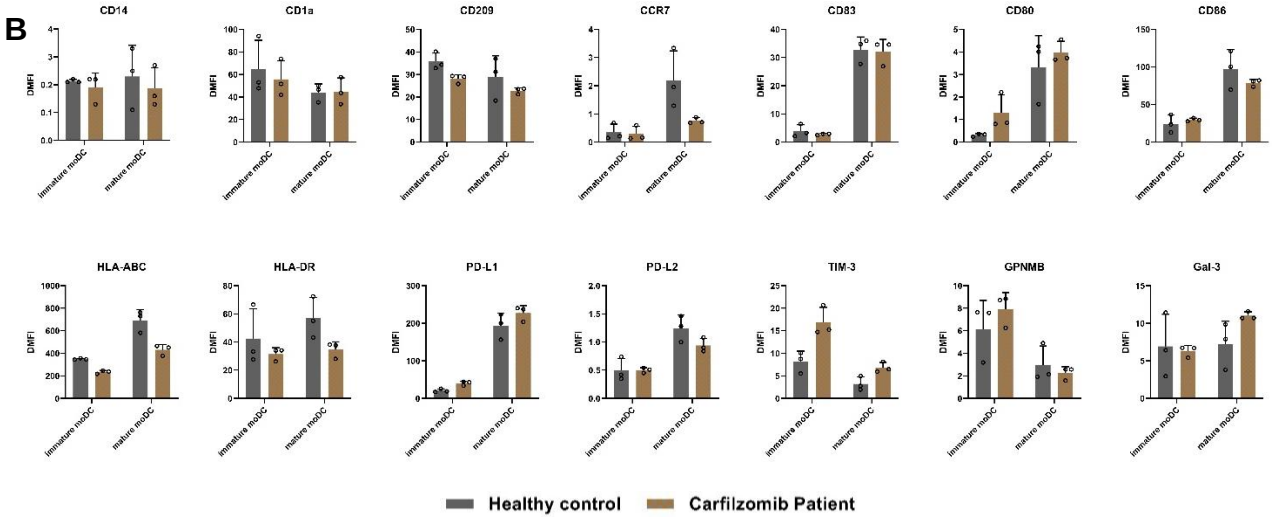


Figure 12: MoDC phenotype in carfilzomib-treated MM patients. MoDC were generated from patients' peripheral blood using GM-CSF and IL-4. LPS was used to induce maturation (mature DC). (A) Cell size and intracellular granularity were assessed by FACS. Exemplarily, one representative sample is depicted. (B) Expression of the indicated cell surface markers characterizing moDC phenotype and checkpoint molecules was investigated by FACS. Levels of expression are shown as DMFI. Statistical analysis is shown in comparison to healthy donors (N = 3, Mann–Whitney U test, no statistical significance).

3.3.2 Blood DC numbers are influenced by the disease itself, but not by carfilzomib containing regimen

In the next translational step, frequencies of blood DC subsets were analyzed in 44 MM patients and compared to 22 healthy controls. Among the analyzed MM patients, 15 were newly diagnosed, 11 undergoing carfilzomib-containing regimens, and 18 patients were in remission.

Blood routine tests were performed by the laboratory department of Charité – Universitätsmedizin Berlin. Cell populations were identified by the expression of the specific cell surface markers. cDC1 were defined as CD45+/CD3-/CD19-/CD14-/CD16- with a specifically high expression of CD141. cDC2 were defined as CD45+/CD3-/CD19-/CD14-/HLA-DR+ with specific expression of CD11c+ and CD1c+. pDC were characterized based on their high CD123 and CD303 expression with CD45+/CD3-/CD19-/CD14-/HLA-DR+ (figure 13A). The total blood DC number was calculated as the sum of these subsets.

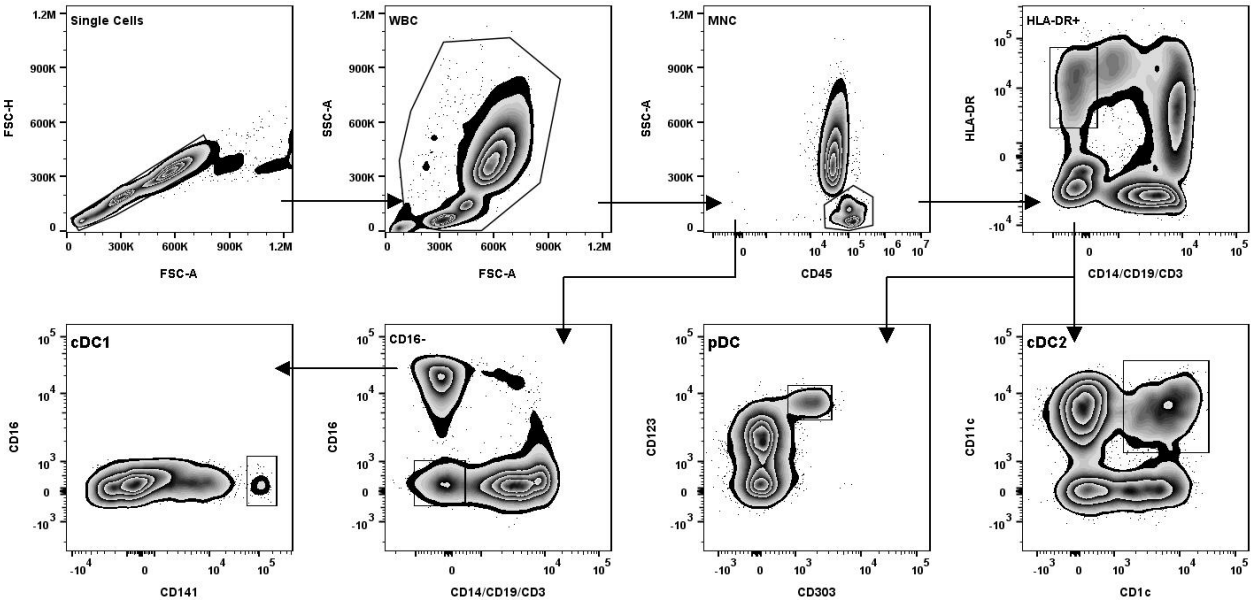


Figure 13A: FACS Gating strategy of blood DC subpopulations. White blood cells (WBC) were gated after doublet and debris exclusion. Mononuclear cells (MNC) were gated as the cell population located in the lower part of the graph harbor less intracellular granules and CD45 high expression. Thereafter, lymphocytes (CD3+ and CD19+ cells) and monocytes (CD14+ cells) were excluded. DC subsets were identified based on the expression of specific surface markers as follows: CD16-, CD141+ (cDC1); CD11c+, CD1c+ (cDC2); CD123+, CD303+ (pDC).

In newly diagnosed MM patients, a significant decrease in the percentage of PBMC as well as the absolute numbers of total DC and their subsets including cDC2 and pDC compared to healthy controls was observed (table 5, figure 13B). For cDC1, the absolute cell counts were significantly reduced. Although no statistical significance, for cDC1, a trend for a reduced percentage of PBMC in newly diagnosed MM was observed. Under carfilzomib treatment, percentages of total DC and their subsets cDC1 and cDC2 recovered without significant difference in comparison to healthy controls, however, the absolute numbers remained significantly diminished (with the exception of cDC1 which showed a trend to reduced absolute numbers). The percentages as well as absolute numbers of pDC remained statistically lower under carfilzomib treatment compared to healthy donors. In remission patients, the frequencies of pDC and absolute counts of cDC2 and pDC remained diminished resulting also in slightly but significantly decreased numbers of total blood DC (table 5, figure 13B).

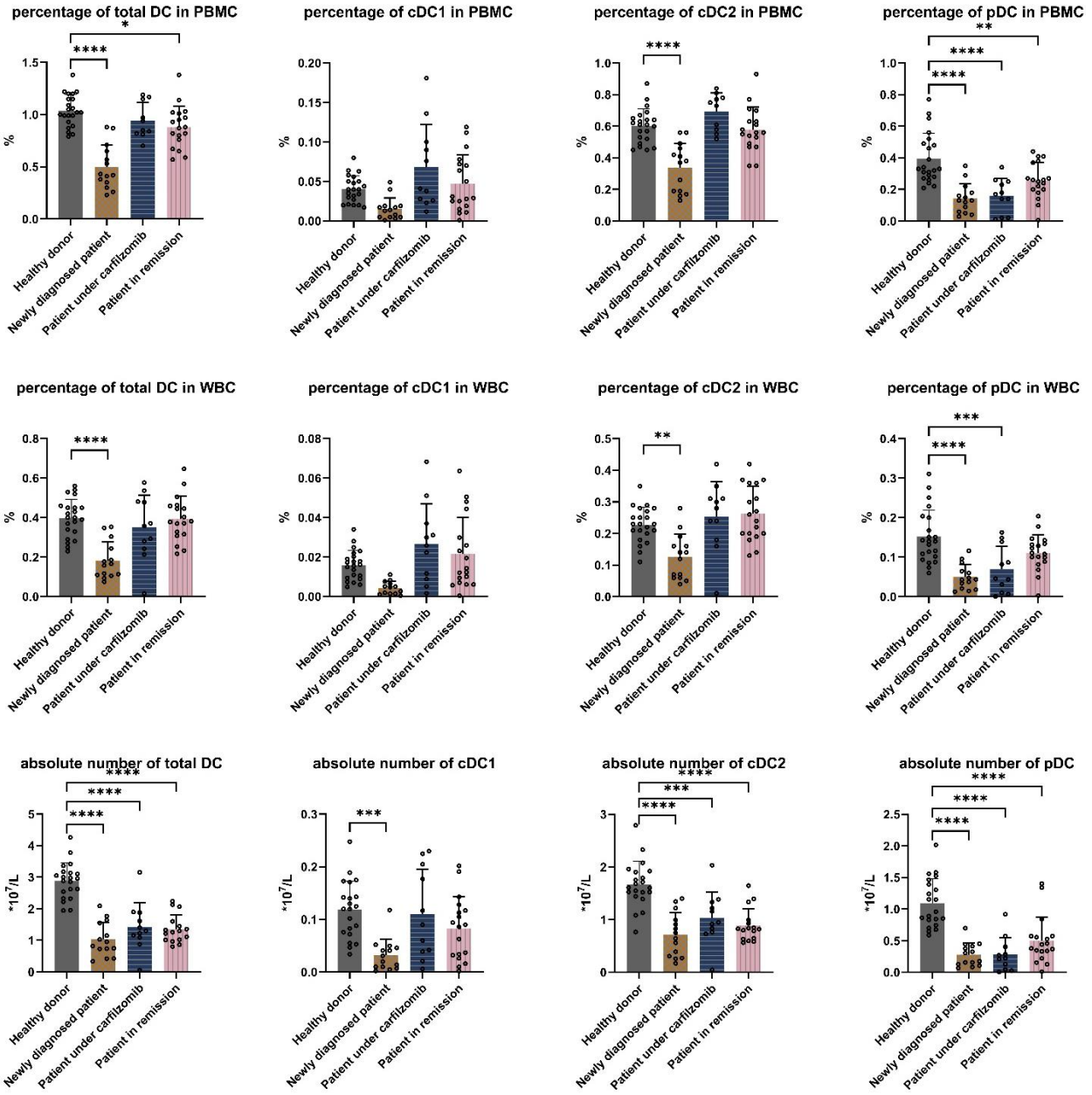


Figure 13B. Enumeration of blood DC subpopulations in healthy donors and MM patients. Comparison of cell number of blood DC subsets between healthy controls, newly diagnosed MM patients, carfilzomib-treated patients, and remission patients. Mean percentages and absolute cell numbers are shown in table 5. (One-way ANOVA, Mean ± SD, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001)

To unravel possible factors contributing to changes in blood DC frequencies data were correlated to the clinical characteristics of newly diagnosed MM patients. Results demonstrated that β2-microglobulin (p=0.021, correlation coefficient r=-0.587) and the percent-

age of plasma cells bone marrow infiltration (p=0.016, r=-0.610) were negatively correlated to the total blood DC frequency in PBMC. Patients´ clinical characteristics are listed in table 6.

Bone marrow serves as the tumor microenvironment in MM. Myeloma cells are able to produce cytokines like IL-6 and TNF-α forming an immune suppressive tumor microenvironment and we found a negative correlation between blood DC counts and plasma cells bone marrow infiltration [85]. Thereafter, we investigated bone marrow DC frequency through exception CD138+ myeloma cells by FACS gating. The gating strategy was consistent with that applied for blood DC analysis. Cell frequency of bone marrow DC subgroups in 6 newly diagnosed MM patients was compared to historical data of healthy controls (derived from previous publications). DC frequencies in bone marrow were determined by excluding CD138+ myeloma cells from the total population of CD45+ bone marrow mononuclear cells. Data is shown as median with range (table 7). All bone marrow DC subpopulations in newly diagnosed MM patients showed a significant decrease (cDC1 in MM BM 0.030% (0.016-0.040%) vs. 0.081% in healthy BM, cDC2 in MM BM 0.43% (0.26-0.77%) vs. 0.60% in healthy BM [86], pDC in MM BM 0.11% (0.06-0.37) vs. 4.35% in healthy BM [87].

Table 5: Relative and absolute blood DC numbers

	in PBMC (%)		**in WBC (%)**		**absolute number (*10^7/L)**	
	Mean(range)	**P-value**	**Mean(range)**	**P-value**	**Mean(range)**	**P-value**
Total DC						
Healthy donors	1.04 (0.79-1.38)		0.40 (0.23-0.56)		2.88 (1.96-4.27)	
Newly diagnosed patients	0.51 (0.23-0.88)	<0.0001	0.19 (0.07-0.35)	0.0001	0.98 (0.34-2.09)	<0.0001
Patients under carfilzomib	0.92 (0.11-1.19)	0.5483	0.40 (0.21-0.58)	0.9995	1.54 (0.88-3.15)	<0.0001
Remission patients	0.77 (0.57-1.38)	0.0010	0.32 (0.22-0.65)	0.0684	1.18 (0.80-4.46)	<0.0001
cDC1						
Healthy donors	0.041 (0.017-0.080)		0.016 (0.005-0.034)		0.122 (0.047-0.248)	

Newly diagnosed patients	0.019 (0.001-0.040)	0.3013	0.007 (0.000-0.011)	0.2332	0.037 (0.002-0.118)	0.0054
Patients under car-filzomib	0.064 (0.023-0.181)	0.2976	0.026 (0.009-0.068)	0.2474	0.119 (0.021-0.230)	0.9996
Remission patients	0.045 (0.001-0.119)	0.9717	0.018 (0.000-0.063)	0.9759	0.071 (0.010-0.202)	0.0313
cDC2						
Healthy donors	0.60 (0.45-0.73)		0.23 (0.11-0.35)		1.746 (0.765-2.790)	
Newly diagnosed patients	0.31(0.16-0.56)	<0.0001	0.12 (0.04-0.28)	0.0023	0.608 (0.258-1.401)	<0.0001
Patients under car-filzomib	0.68 (0.55-0.84)	0.5264	0.31 (0.16-0.42)	0.0589	1.130 (0.764-2.033)	0.0076
Remission patients	0.53 (0.35-0.93)	0.3045	0.22 (0.13-0.42)	0.9664	0.800 (0.564-2.972)	<0.0001
pDC						
Healthy donors	0.41 (0.21-0.77)		0.16 (0.06-0.31)		1.139 (0.588-2.015)	
Newly diagnosed patients	0.18 (0.03-0.35)	0.0009	0.06 (0.01-0.11)	0.0010	0.333 (0.068-0.698)	<0.0001
Patients under car-filzomib	0.14 (0.02-0.34)	0.0001	0.07 (0.01-0.11)	0.0017	0.292 (0.026-0.918)	<0.0001
Remission patients	0.20 (0.01-0.44)	<0.0001	0.08 (0.00-0.20)	0.0003	0.363 (0.011-1.403)	<0.0001

P-value for one-way ANOVA.

Table 6: MM patient characteristics and correlation with DC numbers

Total number, N		15	P-value
Male		13	
Female		2	
Age, years median, range		65 (42-87)	0.071[a]
Monoclonal-Ig (Immunoglobulin), n	IgG	6	
	IgM	0	
	IgA	3	
	Light chain only	6	
Light chain, n	Kappa	6	
	Lambda	4	
	none	5	
Serum M-Ig median, range (g/L)		17.43 (0-70.00)	0.262[a]
β2-microglobulin (mg/L) median, range		3.1 (1.8-41.6)	0.021[a] r=-0.587
Myeloma cell bone marrow infiltration (%) median, range		50 (4-90)	0.016[a] r=-0.610
Calcium (mmol/L) median, range		2.30 (2.17-2.67)	0.514[a]
Creatinine (mg/dL) median, range		0.87 (0.52-6.68)	0.090[a]
LDH (U/L) median, range		241 (103-495)	0.825[a]
Hemoglobin (g/dL) median, range		10.2 (7.6-14.2)	0.408[a]
Osteolytic bone lesion, n	None	5	0.839[b]
	One or more	10	
Chromosome analysis, n	Standard risk	8	0.875[b]
	High risk	7	
R-ISS classification, n	1	3	0.565[c]
	2	8	
	3	4	

[a]P-value for Spearman test. [b]P-value for Mann-Whitney U test. [c]P-value for one-way ANOVA.

Table 7: Bone marrow DC frequency in newly diagnosed MM patients

	cDC1	cDC2	pDC
Median (range) %	0.030 (0.016-0.040)	0.43 (0.26-0.71)	0.11 (0.06-0.37)

4 Discussion

Despite more and more therapeutic regimens being available, multiple myeloma remains an incurable disease. The immune response is known to be impaired in MM patients. Therefore, overcoming the exhaustion of immune effector cells is essential for promising treatment options in the future [88].

4.1 Short summary of results

The proteasome inhibitor carfilzomib, in contrast to bortezomib, has negligible impact on moDC differentiation, phenotype, and function. The expression of the checkpoint molecule PD-L1 is upregulated in moDC under carfilzomib exposure and allostimulatory capacity is impaired, as shown in MLR. This effect can not only be restored by the addition of nivolumab, checkpoint blockade can even significantly enhance the T cell induction capacity of moDC. Blood DC frequencies are reduced in newly diagnosed MM patients, presumably due to the disease itself and not attributed to carfilzomib, as numbers recover under carfilzomib treatment. It is feasible to generate moDC in sufficient numbers and with typical phenotype in vitro using peripheral blood monocytes of MM patients under carfilzomib treatment, a prerequisite for the development of a moDC-based cancer vaccine.

4.2 Discussion of results

Carfilzomib, a next-generation PI, is approved for first-line treatment of MM and has shown higher efficacy and fewer side effects compared to bortezomib [14]. This study shows that carfilzomib does not hamper morphology, differentiation, phenotype, and functions including migration, endocytosis, and cytokine secretion of immature or mature moDC. In line with previous data, bortezomib had significant effects on moDC phenotype and function: It impaired moDC activation and viability, blunted the expression of maturation markers, caused a decline in migration towards CCL19/MIP-3β, led to lower immunostimulatory capacity and less secretion of IL-12 and TNF [62]. One explanation of why carfilzomib has less effect on moDC than bortezomib could be the higher selectivity of binding to the proteasomal catalytic subunit (β5) of carfilzomib [14]. In addition, the irreversible binding of carfilzomib demonstrated growing evidence for clinical benefit in MM treatment without increasing side effects [14, 89]. Moreover, carfilzomib exposure caused a

statistically significant increase of CCR7 in immature moDC. However, the increase is probably not relevant with respect to the development of DC-based cancer vaccines as the expression of CCR7 is generally low in immature DC compared to mature DC, and an increase will moreover presumably have no negative effect concerning the upregulation of CCR7 is related to the enhanced migration capacity. In carfilzomib-treated patients, no significant change was observed. One explanation is that dexamethasone which is included in the carfilzomib-containing regimen reversed the increase [90].

Comparison of moDC phenotype of healthy controls and carfilzomib-treated MM patients revealed no statistically significant difference in moDC cell viability and phenotype, which was consistent with the previous publication by Shinde et al [91]. However, this group also described compromised moDC cell yields, migration, and cytokine secretion capabilities in MM patients undergoing transplantation. In my results, no moDC number decrease in carfilzomib-treated MM patients was observed. One possible explanation is that, in Shinde's study, the peripheral blood of MM samples was gained from the leftover of transplantation tubing. Patients before transplantation have undergone intense chemotherapy which is harmful to immune cells, especially DC [92, 93].

Natural blood DC show an outstanding capability in antigen presentation. More and more clinical trials on DC vaccines focus on peripheral blood DC than traditional moDC [34, 94]. However, it has been reported that in the tumor microenvironment, several tumor- or stromal-derived cytokines, such as IL-10 and vascular endothelial growth factor could reduce the numbers of infiltrating and circulating DC and inhibit DC function, resulting in immunosuppression and immune escape [37]. Moreover, isolation of autologous DC from peripheral blood in sufficient numbers and loading the specific antigen in vitro could technically improve immune response [75]. Therefore, it seems to be necessary to investigate the blood DC number to determine the best time point for using cancer vaccines to achieve a powerful and long-term anti-tumor immune response. Concerning blood DC counts of healthy donors, data gained here are consistent with van Leeuwen-Kerkhoff's study [86]. Blood DC numbers were reduced in newly diagnosed MM patients, especially those of the cDC2 subtype, which plays a central role in immune responses against cancer. By correlation exploration, I found that myeloma cell infiltration in bone marrow significantly decreased cDC2 number in blood and bone marrow. For pDC number, in line with a previous publication, a significant decrease was observed in newly diagnosed MM patients [87]. In non-

small cell lung cancer, a decrease in pDC was also reported and the pDC numbers negatively correlated with the cell frequency of HLA-DR (low) myeloid-derived suppressive cells [95]. After more than 4 cycles of carfilzomib anti-myeloma treatment, the DC frequency was restored, however, the absolute number remained lower than healthy due to the leukopenia. Here, in patients with complete remission, I still observe fewer DC numbers than in healthy controls. One explanation could be that the hematopoietic capacity of the bone marrow is destroyed by multiline therapies. Despite the elimination of immunosuppressive factors, DC fails to reach the normal number. Based on our observations, carfilzomib has no adverse effect on blood DC numbers. MM patients are routinely treated with a triple or quadruple combination including immunomodulatory drugs (IMiD) like KRD in our studies. Immunotherapy is emerging as a promising treatment for a variety of cancers, and researchers have focused on investigating new tools to trigger efficient immune responses. Tumors with elevated mutational loads, such as melanoma and lung cancer, and those with a high frequency of mutational lesions, tend to respond better to ICI treatment [54]. However, ICI monotherapy failed to achieve convincing clinical efficacy in MM, although PD-L1 is highly expressed in myeloma cells [96]. Liu et al. demonstrated that in the tumor microenvironment, bone marrow mesenchymal stem cells can exhaust CD8 + T cells in MM, even though under the treatment of ICI [59]. Therefore, inhibiting the binding of PD-1 and PD-L1 in the bone marrow microenvironment in combination with other therapeutic regimens provides new therapeutic concepts for MM and has achieved numerous results in recent years. Studies such as NCT03707808 and NCT02993315 have shown that combinatorial treatment strategy using DC-based vaccine and other treatments such as chemotherapy, radiation therapy, or ICI can achieve extended PFS and even OS benefits in various cancers [44, 97]. DC-based cancer vaccines in combination with standard-of-care treatment could overcome tumor-induced immunosuppression without significantly increasing toxicity [44, 98]. ICI used in combination with DC-based vaccines was shown to enhance the efficacy of the vaccine in a multiple myeloma mouse model. Chu et al. in a preclinical study showed that a combination of DC vaccination + pomalidomide with dexamethasone + PD-L1 inhibitor achieved initial success in anti-myeloma treatment [99]. Rosenblatt et al. blocked PD-1 signaling using the CT-011 in an MM mouse model, showing enhanced T cell responses following DC vaccination [100]. Other researchers also conducted clinical trials focusing on the combination of DC vaccines with a companion drug and ICI to reach a high anti-tumor effect [34, 43]. For example, in the phase I trial

NCT03707808, a cancer vaccine containing autologous CD1c+ myeloid DC was combined with ipilimumab and avelumab with systemic nivolumab for the treatment of advanced solid tumors, which demonstrated a safe antitumor response even in patients who have progressed on ICI [101].

We analyzed the immune checkpoint expression on moDC and conducted MLR assays to evaluate the contribution of immune checkpoints on moDC function while pretreated with proteasome inhibitors. Our expectation was that the allostimulatory function of DC would be augmented when combined with carfilzomib and ICI.

TIM-3 was down-regulated by carfilzomib in immature moDC, while had no functional impairment on moDC, since the high level of TIM-3 expression on DC could suppress extracellular DNA uptake [80].

PD-L1 and PD-L2 are the most well-known immune checkpoints related to T cell deactivation and exhaustion when binding with PD-1 expressed on T cells [102]. Accumulating studies proved that PD-L1/2 can inhibit anti-tumor immune response by exhausting specific CD4+ and CD8+ T cell functions [103, 104]. Specifically, highly expressed PD-L1 and PD-L2 on tumor cells and APC infiltrated in the tumor microenvironment, lead to immune evasion and tumor growth [105]. PD-L1 is reported to be highly expressed in myeloma cells, rather than in normal plasma cells [106]. Inhibition of the PD-1 signaling pathway using anti-PD-1 or PD-L1 antibodies represents a potential anti-myeloma therapeutic strategy, in spite of little clinical evidence. Therefore, the PD-1 pathway needs more exploration. In this study, carfilzomib exposure resulted in a significant increase in the expression of PD-L1. We also observed an immune suppressive effect on moDC T cell stimulatory capacity. In previous studies, Nencioni et al. observed bortezomib treatment significantly blunted the immunostimulatory capacity of DC while the expression of PD-L1 was significantly upregulated by bortezomib in our study [62]. This could indicate that the higher PD-L1 expression on DC, the lower T cell stimulation of DC. However, rare publications demonstrated the mechanism of this phenomenon. We further used PD-1 antibody - nivolumab to block the binding of PD-1 with PD-L1/ L2. The T cell induction capacity of moDC could be restored and even significantly enhanced by blocking with PD-1 antibody nivolumab in vitro. In the DMSO control group, we could also observe an enhancement of allostimulatory capacity by adding nivolumab. We also observed the higher PD-L1 expression on moDC, the stronger T cell induction of moDC. Therefore, results could probably demon-

strate the level of T-cell activation is related to the expression of PD-L1 with PD-1 inhibition. In the future, we could extensively analyze checkpoint expression at primary diagnosis, under treatment, and compare to healthy donors and the effects of ICI addition in MLR in these conditions. This phenomenon also be found in our other study, which demonstrated that GPNMB served as a novel immune checkpoint molecule [82]. Thus, our results indicate that combinatory approaches of carfilzomib with anti-PD-1 immunotherapy may serve to augmentation DC function in vitro. With these findings, we hypothesize carfilzomib plus PD-1 antibody could represent a promising combinatory approach with DC vaccine in MM treatment.

In our translational step, we generated moDC from KRD-treated MM patients. In comparison with our in vitro data, no significant difference in surface marker and checkpoint molecules was observed, especially the increase in PD-L1 expression. Costa et al. demonstrated that lenalidomide and dexamethasone significantly influence moDC phenotype and function. Although rare data related to immune checkpoint molecules, we hypothesize that in clinical treatments, other medications may influence DC.

Newly diagnosed MM patients were reported that suffered from an impaired immune response including blunted blood DC antigen presentation capacity by Ratta et al. and Brimnes et al. [107, 108]. There are several advantages to the use of moDC as DC-based cancer vaccination. Firstly, they are generated in vitro and hence they are not influenced by other drugs the patient is exposed to. Then, moDC are derived from blood monocytes. Sufficient numbers can be generated which allows repeated vaccinations. And they can be optimized in vitro. We hypothesized that ex-vivo generated moDC with PD-1 antibody augmentation could overcome this issue.

4.3 Strengths and weaknesses of the study

In this project, we conducted a high sample size in vitro experiment, making results much more reliable. Regarding the translational steps, we performed analysis on patients from different time points (newly diagnosed, under carfilzomib treatment, and in remission). We also explored the correlation between clinical characteristics and the blood DC subset. Further, we analyzed the DC number in TME (bone marrow) of MM patients.
We acknowledge the following limitation in this study. One limitation is that in the investigation of the effects of MM disease and carfilzomib on blood DC, we did not analyze the checkpoint expression and the function of blood DC and bone marrow DC subsets. For

bone marrow DC analysis, we only compared our patient data with historical controls. Moreover, the sample size of generation of moDC from carfilzomib-treated MM patients was low. We didn’t analyze moDC function especially allostimulatory capacity of these moDC. Therefore, this study needs further exploration and verification.

4.4 Implications for clinical practice and future research

In future studies, we could further perform animal experiments and if possible clinical trials to gain more data for our designed combinatory approach.

5 Conclusions

In conclusion, these results expand the knowledge to allow the development of a DC-based cancer vaccine and demonstrate that carfilzomib is a suitable drug for a combinatory approach. The addition of an ICI could represent a potent approach capable of increasing clinical efficacy, long-term immunity, and tolerable toxicity in MM treatment.

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Eidesstattliche Versicherung

Ich, Luojia Wang, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: Augmenting DC function for rational immunotherapies in multiple myeloma (Verstärkung der DC-Funktion für rationale Immuntherapien beim Multiplen Myelom) selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich
gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.

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Datum Unterschrift

Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

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Marius performed	Endocytosis assays (figure 8), ELISAs (figure 10), and 8 of 10 transwell experiments (migration, figure 9)
Luojia performed	FACS analysis of phenotype and checkpoint molecule expression (figure 6A-E and 7A-E), MLR (figure 11), 2 of 10 transwell experiments (migration, figure 9), patient moDC analysis, blood and bone marrow DC counts, and patient clinical data analysis