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Modulated function of dendritic cells in multiple myeloma patients

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List of abbreviations

APC	antigen-presenting cell
BM	bone marrow
BM-MNC	bone marrow mononuclear cell
BMEF	bone marrow extracellular fluid
CCR	C-C chemokine receptor
CD	cluster of differentiation
cDC	conventional/classical dendritic cell
cDC1	conventional/classical dendritic cell type 1
cDC2	conventional/classical dendritic cell type 2
CDP	common dendritic cell progenitor
CTLA	cytotoxic T-lymphocyte-associated protein
DAPI	4′,6-diamidino-2-phenylindole
DC	dendritic cell
del	deletion
Dexa	dexamethasone
ELISA	enzyme-linked immunosorbent assay
f	female
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FLC	free light chains
FLT3L	FMS-like tyrosine kinase 3 ligand
FMO	fluorescence minus one-control
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMDP	granulocyte-monocyte-dendritic-cell progenitor
HD	healthy donor
HLA-DR	human leukocyte antigen - DR isotype
HSC	hematopoietic stem cell
iFISH	interphase fluorescence in situ hybridization
IFN	interferon
IgA	immunoglobulin A
IgG	immunoglobulin G

IL	interleukin
IMWG	International Myeloma Working Group
infDC	inflammatory dendritic cell
ISS	international staging system
IU	international unit
KLRG	killer cell lectin-like receptor, subfamily G
LDH	lactate dehydrogenase
m	male
M-CSF	macrophage colony-stimulating factor
MACS	magnetic activated cell sorting
MDP	monocyte-dendritic-cell progenitor
MGUS	monoclonal gammopathy of undetermined significance
MHC	major histocompatibility complex
MM	multiple myeloma
mo-DC	monocyte derived dendritic cell
MS-5	murine stromal cell line 5
n. g.	not given
NOD	nucleotide-binding oligomerization domain
PB	peripheral blood
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PD	programmed cell death protein
PD-L	programmed death-ligand
pDC	plasmacytoid dendritic cell
PGE2	prostaglandin E2
PMT	photomultiplier tube
pre-cDC	conventional/classical dendritic cell precursor
R-ISS	revised international staging system
RT	room temperature
SCF	stem cell factor
SD	standard deviation
SEM	standard error of the mean

SMM	smoldering myeloma
t	translocation
T_{CM}	central memory T cell
T_{EM}	effector memory T cell
T_{EMRA}	terminally differentiated effector memory T cell re-expressing CD45RA
TGF	transforming growth factor
TME	tumor microenvironment
T_N	naïve T cell
VC	bortezomib, cyclophosphamide
VCD	bortezomib, cyclophosphamide, dexamethasone
VD	bortezomib, dexamethasone
VEGF	vascular endothelial growth factor
VRD	bortezomib, lenalidomide, dexamethasone
WBC	white blood cell

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1 Introduction

1.1 Multiple myeloma

1.1.1 Epidemiology, etiology and pathophysiology

Multiple myeloma (MM) is a heterogeneous tumor disease of malignant, differentiated plasma cells. It is defined through mostly multiple plasma cell clones in the bone marrow (BM), while also solitary clones (= plasmacytoma) and diffuse infiltration of the BM by malignant plasma cells occur.

In Germany, approximately 3,900 men and 3,000 women are diagnosed per year. Median age of diagnosis is at 72 years for men and at 74 years for women (Robert Koch-Institut und Gesellschaft der epidemiologischen Krebsregister in Deutschland e.V. 2019).

The etiology is mainly unknown, apart from exposure to ionizing radiation and chemicals, chronic infection and adiposis are discussed as possible risk factors (Landgren et al. 2006, DGHO Deutsche Gesellschaft für Hämatologie und Medizinische Onkologie e.V. 2018).

Pre-stages of MM are monoclonal gammopathy of undetermined significance (MGUS) and the smoldering myeloma (SMM). Genetic aberrations play a crucial role in development, therapy and prognosis (Kortum et al. 2016). Furthermore, interactions with the immune system as well as the microenvironment of the BM influence tumor evasion, progression and extension, mediated through both cytokines and cellular interactions (Kawano et al. 2017).

1.1.2 Symptoms, clinical features, diagnosis and classification

Patients with MM often present various non-specific symptoms, among these are bone pain, weight loss, fatigue and susceptibility to infection (DGHO Deutsche Gesellschaft für Hämatologie und Medizinische Onkologie e.V. 2018). In many cases, patients are symptom-free at time of diagnosis (Friese et al. 2009). Common clinical features are summed up as the CRAB-features: increased blood-calcium-levels, renal failure, anemia and bone lesions.

Diagnosis is made according to the guidelines of the International Myeloma Working Group (IMWG) (Rajkumar et al. 2014), to avoid unnecessary treatment of premalignant MGUS and SMM on the one hand but to prevent end organ damage and to improve outcome by early diagnosis of MM and high-risk pre-stages (defined as > 80 % risk of progression to MM within two years) on the other hand. Beside CRAB-criteria, histological findings and levels of free-light-chains (FLC) in serum as well as imaging are considered (Rajkumar et al. 2014). Classification of the disease is important for prognosis and choice of therapy. Therefore, patients are grouped by the revised international staging system (R-ISS, see Table 1), (Palumbo et al. 2015).

Table 1 – R-ISS classification

R-ISS Stage	Criteria
I	• serum β_2-microglobulin < 3.5 mg/l and • serum albumin ≥ 3.5 g/dl and • standard-risk chromosomal abnormalities by iFISH and • normal LDH
II	not R-ISS I or III
III	• serum β_2-microglobulin ≥ 5.5 g/dl and • either high-risk chromosomal abnormalities by iFISH or • high LDH

Table according to Palumbo et al. (2015)
R-ISS = revised international staging system, iFISH = interphase fluorescence in situ hybridization, LDH = lactate dehydrogenase

Chromosomal abnormalities are classified as high- or standard-risk abnormalities as shown in Table 2. Sonneveld et al. (2016) give an overview of important genetic abnormalities in MM also making other abnormalities a subject of discussion.

Table 2 – Chromosomal abnormalities

high-risk	standard-risk
• del(17p) • t(4;14) • t(14;16)	• all others
Table according to DGHO Deutsche Gesellschaft für Hämatologie und Medizinische Onkologie e.V. (2018) del = deletion, t = translocation	

1.1.3 Established therapies

Decision for treatment should be made according to the DGHO-guidelines (DGHO Deutsche Gesellschaft für Hämatologie und Medizinische Onkologie e.V. 2018), based on the diagnosis criteria of the IMWG (see above and Rajkumar et al. (2014)). DGHO-guidelines give detailed information for treatment: For patients suitable for autologous stem-cell transplantation, high-dose therapy followed by autologous stem-cell transplantation is treatment of choice as first-line therapy. In addition, and for patients not suitable for stem-cell transplantation (mainly determined by their condition to stand adverse side effects of high-dose induction), different drugs are used. Among these are proteasome inhibitors (e. g. bortezomib), immunomodulatory drugs (e. g. thalidomide), alkylating agents (e. g. melphalan, cyclophosphamide), monoclonal antibodies (e. g. daratumumab) and others (e. g. dexamethasone) (Kumar et al. 2017, DGHO Deutsche Gesellschaft für Hämatologie und Medizinische Onkologie e.V. 2018). Patients should be included to studies whenever possible (DGHO Deutsche Gesellschaft für Hämatologie und Medizinische Onkologie e.V. 2018). Even though, with a relative five-year survival rate of 49 % and a relative ten-year survival rate of slightly above 30 % (Robert Koch-Institut und Gesellschaft der epidemiologischen Krebsregister in Deutschland e.V. 2019), prognosis is rather poor with the given therapy options.

1.2 Dendritic cells

Dendritic cells (DCs) are key players in the immune system. As they are capable of taking up antigens, detect danger patterns and prime T cells through antigen presentation, their role in connecting the innate and adaptive immune system is of great importance. Different DC-subsets with particular functions have been discovered over the last decades: classical or conventional DCs (cDCs) which can be further divided into cDCs type 1 (cDC1s) and cDCs type 2 (cDC2s) excel in antigen-uptake and -presentation while plasmacytoid DCs (pDCs) are effective producers of type I interferons (Collin and Bigley 2018).

1.2.1 Differentiation of dendritic cells

DCs in the steady state derive from the hematopoietic stem cell (HSC). In addition, distinct monocyte-derived DCs (mo-DCs) or inflammatory DCs (infDCs), most likely related to monocytes, occur in the context of inflammation (Collin and Bigley 2018). The overlap between monocyte-derived DCs and HSC-derived DCs is still not fully resolved.

While over a long time, differentiation models with strict hierarchies of progenitors gradually losing their differentiation potential have been discussed (Guilliams et al. 2014), nowadays models state the early predetermination of progenitors to their specific pathway of differentiation (Paul et al. 2015, Notta et al. 2016, Collin and Bigley 2018).

Differentiation of DCs (Figure 1) takes primarily place in the BM, yet later precursors of cDCs are also found in the peripheral blood (PB), where final developmental steps may take place (Breton et al. 2015b, See et al. 2017). Descending from the $CD34^+$ hematopoietic stem cell (HSC) via granulocyte-monocyte-DC progenitors (GMDP) and monocyte-DC progenitors (MDP), common DC progenitors (CDPs) contain precursors determined to both cDCs and pDCs (Lee et al. 2015). While cDC precursors (pre-cDCs) and – later on – different pre-cDC1s and pre-cDC2s as precursors of cDCs can be identified, pDC differentiation does not share this pathway of pre-cDCs (Breton et al. 2015b, Breton et al. 2016, See et al. 2017). It is also presumed that pre-cDCs are *“functional in their own right”* (See et al. 2017), with features as T-cell stimulation

and cytokine secretion, stressing their potential relevance for both physiological and pathological processes.

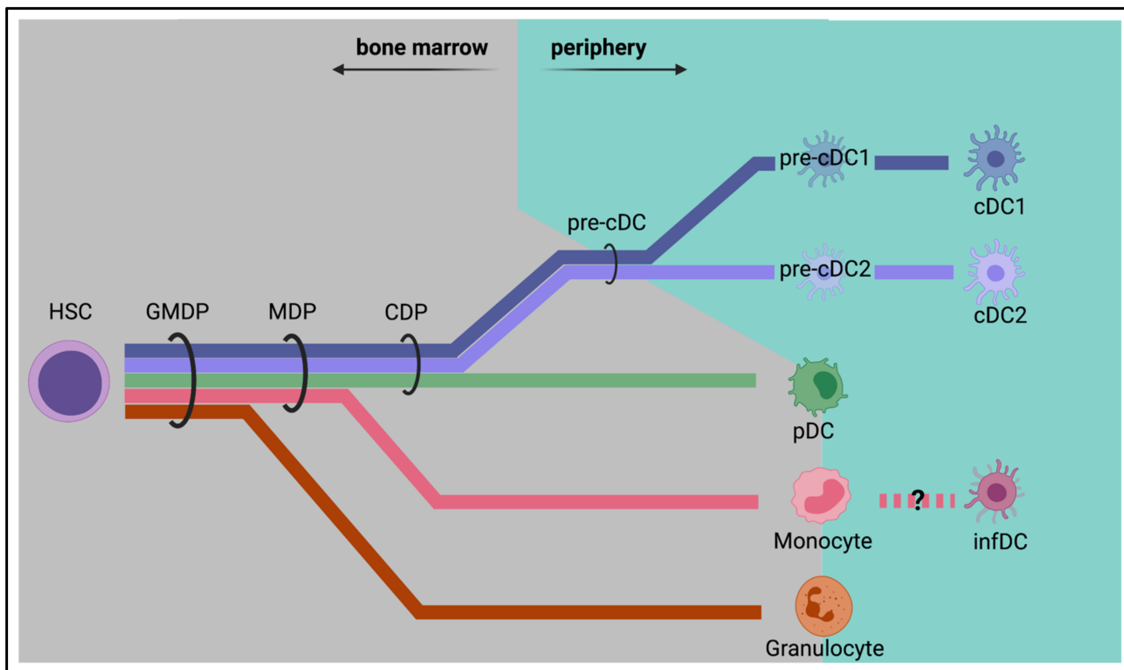


Figure 1 – Differentiation of dendritic cells

Schematic view of the differentiation of DC-subsets. HSC = hematopoietic stem cell, GMDP = granulocyte-monocyte-dendritic cell progenitor, MDP = monocyte-dendritic cell progenitor, CDP = common dendritic cell progenitor, pre-cDC = conventional/classical dendritic cell precursor, cDC1 = classical/conventional dendritic cell type 1, cDC2 = classical/conventional dendritic cell type 2, pDC = plasmacytoid dendritic cell, infDC = inflammatory dendritic cell. Figure according to Breton et al. (2015a), Puhr et al. (2015), Notta et al. (2016), Collin and Bigley (2018). Figure created with Biorender.com.

1.2.2 Function of dendritic cells

As in their differentiation, DC-subsets also differ in their phenotype and function. cDCs are essential for priming T cells. In steady state, immature cDCs perform high antigen-uptake and processing. As they sense molecular danger signals through Toll-like-receptors, nucleotide-binding oligomerization domain-(NOD-)-like-receptors and others, they mature and thereby alter their phenotype by upregulation of major histocompability complex (MHC-) molecules class -I and -II, various co-stimulatory molecules (e. g. CD40, CD80, CD86) and secretion of interleukin-(IL-)12. Also, they acquire chemokine receptors (e. g. C-C chemokine receptor (CCR)7) for homing to secondary lymphoid organs, all in all leading to

suitable capabilities of antigen presentation and T-cell activation (Collin and Bigley 2018). While cDC2s excel in presentation of antigens via MHC-II, priming $CD4^+$ T-helper cells and eliciting immune responses against extracellular pathogens, cDC1s are able to cross-present exogenous antigens via MHC-I for the activation of $CD8^+$ cytotoxic T cells and immune responses against intracellular pathogens and tumor disease (Dudziak et al. 2007, Haniffa et al. 2012, Merad et al. 2013). cDC1s are phenotypically characterized by the expression of CD141, while cDC2s express CD1c and CD11c (Collin and Bigley 2018). pDCs differ from cDCs in function and phenotype: in reaction to danger signals, pDCs are capable to secret type I interferons and other cytokines, especially in context of viral infections (Swiecki and Colonna 2015). Their phenotype is characterized by the expression of CD123, CD303 and CD304 (Collin and Bigley 2018). infDCs – mainly present in inflammatory context – share functional aspects with cDCs but seem to be more relevant at the inflamed tissue itself than at the secondary lymphoid organs. Their phenotype is characterized through expression of CD1c and CD14, pointing out their close relation to monocytes (Collin and Bigley 2018).

1.2.3 Dendritic cells in multiple myeloma

In general, DCs are important players in anti-tumoral immune responses. Nevertheless, their capability to induce those responses is dependent on their functional state and phenotype, which is – among others – regulated through cells and factors of the tumor microenvironment (TME) (Dhodapkar et al. 2008b). Through this crosstalk, DCs can become strong inducers of immune responses but also suppressors of those, contributing to insufficient immune control (Fricke and Gabrilovich 2006).

In MM, the role of DCs seems to be connected to the fate of disease progression even more closely: DCs and their precursors are assumed to play a crucial role in the pathogenesis and progress of MM (Tucci et al. 2011b). DCs in general are reduced in the PB of MM-patients (Ratta et al. 2002, Do et al. 2004, Martin-Ayuso et al. 2008) and their phenotype and cytokine secretion seem to be altered. While some authors describe a shift to an impaired phenotype with reduced expression

of MHC and co-stimulatory molecules and impaired cytokine secretion (Brown et al. 2001, Ratta et al. 2002, Brimnes et al. 2006, Tucci et al. 2011b), others on the contrary describe a phenotype with higher expression of co-stimulatory molecules (Martin-Ayuso et al. 2008, Leone et al. 2015). Reduced DC-frequencies in patients are reported to correlate with progression of MM (Pasiarski et al. 2013).

In the BM-TME of MM-patients, DCs are involved in the complex crosstalk between tumor cells and immune cells. Thereby they promote tumor growth on the one hand (Kukreja et al. 2006, Chauhan et al. 2009, Tucci et al. 2011a), on the other hand they are suspected to modulate immune responses and thereby support immune evasion of MM (Banerjee et al. 2006, Dhodapkar et al. 2008a, Chauhan et al. 2009). Especially the expression of programmed death-ligand 1 (PD-L1) is known to play an important role in suppressing T-cell responses by checkpoint-blockade and thereby leading to immune evasion of tumors (Curiel et al. 2003). DCs appear to interact with malignant MM-cells via the CD28-CD80/86-axis (Nair et al. 2011, Nair et al. 2012). As their activation of T-cell responses depends on their expression of surface molecules and their cytokine secretion, changes in DC phenotype show important consequences (Veglia and Gabrilovich 2017).

Interleukin-(IL-)6 is suspected to be a central component of these dysregulations in the TME. IL-6 is known as an important growth factor for plasma cells, thereby supporting tumor growth directly (Klein et al. 1995). Furthermore, IL-6 influences the differentiation process of $CD34^{+}$-progenitors to DCs: differentiation of DCs is reduced, while differentiation to a monocyte-like phenotype with higher phagocytotic function but a lack of antigen presentation was found (Menetrier-Caux et al. 1998, Ratta et al. 2002). Additionally, IL-6 was reported to influence the maturation of DCs, leading to an impaired phenotype (Hayashi et al. 2003, Park et al. 2004).

Further on, DCs of MM-patients showed transformation to osteoclasts *in vitro* (Tucci et al. 2011a), suggesting a direct contribution to osteolytic bone disease in MM-patients.

However, the detailed situation in the BM-TME and the transferability especially of the results gained *in vitro* remain unclear. There is still the need to identify major players and their particular pathways and networks of cellular communication. It becomes apparent, that DCs are widely involved in the TME of MM and that they are thereby substantial for a better understanding of MM, being fundamental for improved therapeutic control.

1.3 T cells

T cells are essential effector cells of the immune system. They can be further divided into $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells.

1.3.1 Function and interaction with dendritic cells

T cells depend on antigen presentation for their activation, clonal expansion and proper function. DCs are the most important antigen-presenting cells (APCs). Via MHC-molecules class-I and -II, they present antigen-fragments to T-cell receptors on T cells. Proper activation, precise modulation and restriction of T cells is mediated through co-stimulatory molecules (e. g. CD40, CD80, CD86) and co-inhibitory molecules (e. g. PD-L1) interacting with corresponding receptors on T cells (e. g. CD28, CD40L, PD-1) (Chen and Flies 2013). Thereby, T-cell responses are regulated to achieve strong anti-infectious and anti-tumorous reactions but sustain self-tolerance and prevent exuberant responses (Chen and Flies 2013).

T cells can be characterized by various surface molecules to determine their differentiation state and functionality (Larbi and Fulop 2014): mature, naïve T cells (T_N) express CCR7 and CD45RA, CD27 and CD28. They recirculate through secondary lymphoid tissues in search of their specific antigen (Gattinoni et al. 2011). Through presentation of their specific antigen and costimulatory signals by APCs, T cells get activated which leads to clonal expansion and differentiation to T effector and T memory cells (Kumar et al. 2018).

T effector cells are short-dated cells serving acute responses to eliminate infected or tumorous cells by cytotoxicity and the release of cytokines (Murphy and Reiner 2002, Trambas and Griffiths 2003). In addition, there are different T-cell memory subsets persisting for longer time spans (Golubovskaya and Wu

2016). These T memory cells serve specific functions of immunological memory. Central memory T cells (T_{CM}) are defined by expression of CD45RO, CCR7, CD27 and CD28; they are capable of migration and homing to secondary lymphoid tissues by their CCR7-expression. T_{CM} cells show extensive proliferative capacity, while their direct effector function may be less distinct (Sallusto et al. 1999, Mueller et al. 2013). They are equipped by CD27 and CD28 seeking for further co-stimulation (Mahnke et al. 2013). Effector memory T cells (T_{EM}) express CD45RO, CD27 and CD28, but not CCR7. They seem to be strong effectors in inflamed tissues (Sallusto et al. 1999) as they are capable to produce effector cytokines (e. g. interferon-(IFN-)γ) (Mueller et al. 2013). Terminally differentiated effector memory T cells re-expressing CD45RA (T_{EMRA}) are – besides their CD45RA-re-expression – characterized by the expression of markers which predict cellular senescence and low immunologic functionality as CD57 and killer cell lectin-like receptor, subfamily G-1 (KLRG-1) (Zhang and Bevan 2011, Larbi and Fulop 2014).

There are strong indications that the different functional statuses may follow one after the other from T_N via T_{CM} and T_{EM} to T_{EMRA} (Mahnke et al. 2013, Durek et al. 2016, Abdelsamed et al. 2017, Moskowitz et al. 2017).

1.3.2 T cells in multiple myeloma

T cells, especially $CD8^+$ T cells, are major players in anti-tumoral immune responses. But their proper functioning is dependent on antigen presentation and additional signals as the supply of co-stimulatory/inhibitory molecules and cytokines (Schietinger and Greenberg 2014). In tumor disease, T cells often fail to eliminate tumor cells. There are different underlying mechanisms which favor this failure (Crespo et al. 2013).

T-cell senescence is an alteration of T cells mainly through (physiological) aging processes (Effros 1998), but also in context of tumor disease (Crespo et al. 2013) and leads to T-cell dysfunction (Akbar and Henson 2011). Senescent T cells have lost their expression of CD28, which is an important counterpart to co-stimulatory molecules on APCs, but express markers associated with cellular aging like CD57 and inhibitory receptors like KLRG-1 (Crespo et al. 2013).

T-cell anergy is an inhibition of T-cell function induced by presentation of inhibitory molecules and a lack of co-stimulatory molecules by APCs. It leads to hyporesponsive T cells and may serve immunosuppressive aims, e. g. self-tolerance (Schwartz 2003). Anergic T cells express surface molecules of inhibitory axes as programmed cell death protein-1 (PD-1) and cytotoxic T-lymphocyte-associated protein (CTLA-)4 (Crespo et al. 2013). By interaction with the counterparts of these molecules (PD-L1, CD80, CD86) presented on APCs, their functional and proliferative capacity is restricted (Chen and Flies 2013).

T-cell exhaustion is caused by continuous activation and exposure to antigens as in chronic inflammation or tumor disease leading to impaired T-cell function (Wherry 2011). Exhausted T cells express CD27 and CD28 but also inhibitory receptors (PD-1, KLRG-1) (Crespo et al. 2013).

In MM, different types of T-cell dysfunction seem to be relevant, yet previous results are not completely consistent. While on the one hand, senescence is suspected to be highly relevant (Suen et al. 2016), on the other hand also exhaustion of T cells is reported additionally (Zelle-Rieser et al. 2016). Further on, the mechanisms inducing T-cell dysfunction remain unclear. While crosstalk between T cells and MM cells induces dysfunction (Kawano et al. 2017), a contribution of DCs is also most likely (Turtle et al. 2004, Chauhan et al. 2009). Different T-cell dysfunctions are accessible to therapeutic approaches (as immune checkpoint blockade, autologous adoptive T-cell transfer and others), but accurate characterization is necessary for effective therapy.

1.4 Aim of this thesis

Multiple myeloma (MM) is a tumor disease of malignant plasma cells in the bone marrow (BM). Dendritic cells (DCs) are key players in anti-tumoral immune responses as they are capable to present tumor-antigens to T cells which eventually kill tumor cells. MM patients show reduced DC numbers in the peripheral blood (PB) and an impaired immune response. As DCs originate from hematopoietic stem cells (HSCs) in the BM and MM-tumor cells reside in the BM as well, an interdependency of DCs, their progenitors and MM-cells in the MM-tumor microenvironment (TME) – leading to DC dysfunction on the one hand and tumor progression on the other hand – is most likely.

The hypothesis of this thesis was that possible interactions of DC progenitors and MM-cells in the BM lead to reduced DC numbers as well as to DC dysfunction. To address this, the numbers and functional phenotype of the distinct DC-subpopulations (conventional/classic dendritic cells type 1 (cDC1s), conventional/classic dendritic cells type 2 (cDC2s), plasmacytoid dendritic cells (pDCs), inflammatory dendritic cells (infDCs)) in the PB of MM-patients were analyzed in comparison to healthy donors (HDs). Moreover, $CD4^+$ and $CD8^+$ T-cell subsets were also analyzed by multicolor flow cytometry with specific focus on markers predicting T-cell anergy, exhaustion and senescence.

Fundamental changes in DC numbers and limitations of DC function might be due to impaired DC differentiation from progenitors in the TME of MM-patients. Thus, another question of this thesis was if soluble factors present in the bone marrow extracellular fluid (BMEF) and PB-serum of MM-patients, in particular interleukin-(IL-)6, affect the differentiation of $CD34^+$-progenitors into DCs. Therefore, a 7-day culture-assay was used to analyze the differentiation of sorted BM-$CD34^+$-progenitors from HDs to DCs in the presence of BMEF and PB-serum from MM-patients as well as HDs.

In total, this thesis aimed to give closer insights to immunological dysfunction of both DCs and T cells in MM and thereby help to identify possible targets for therapeutic interventions.

2 Material and methods

2.1 Patient cohort and healthy donors, processing of samples

Within this present thesis, blood and BM samples of both patients suffering from MM and voluntary, healthy controls were studied. Patients enclosed were consent giving high-risk patients at initial diagnosis of MM at R-ISS stage II or III. Unless otherwise indicated (see Table 4 at page 14), no treatment of the MM disease was performed prior to sample collection.

Voluntary blood donors (DRK Blutspendedienst Baden-Württemberg – Hessen gGmbH) and patients of the cardiological day-care hospital (Klinik für Innere Medizin III für Kardiologie und Angiologie des Universitätsklinikums Tübingen) served as healthy controls for analysis of PB. Exclusion criteria for HDs were infectious, autoimmune and tumor disease. Patients of the BG Unfallklinik Tübingen, Klinik für Unfall- und Wiederherstellungschirurgie, Abteilung für Endoprothetik, undergoing endoprosthetic joint replacement served as healthy controls for BM samples under the given exclusion criteria.

Ethics were approved by the ethics commission of the medical faculty Tübingen (549/2016BO2). All patients and donors gave informed consent according to the declaration of Helsinki.

There was a total of 39 healthy donors (each providing PB or BM) serving as controls (see Table 3). 22 of them were female, while 17 were male. The mean age was 57 years with a standard deviation (SD) of 17 years. A total of 37 MM-patients (each providing PB and/or BM) was included in the investigation (see Table 4). 22 of them had already received therapy, when samples were taken; samples of 15 patients were taken before therapy was started. 22 patients were female, 15 were male. The mean age of MM-patients was 58 years with a SD of 9 years.

Table 3 – Healthy donors

#	sex	age		#	sex	age
1	f	48		21	f	76
2	f	45		22	m	57
3	f	43		23	f	67
4	f	50		24	f	28
5	f	77		25	f	32
6	m	65		26	m	23
7	f	74		27	m	33
8	f	57		28	m	59
9	m	63		29	m	61
10	m	37		30	f	19
11	f	57		31	f	51
12	m	56		32	f	69
13	f	55		33	m	72
14	m	36		34	f	73
15	f	65		35	m	80
16	f	78		36	f	56
17	m	76		37	m	67
18	m	79		38	m	62
19	m	70		39	f	79
20	f	47				

f = female; m = male

Table 4 – MM-patients

#	sex	age	previous treatment	treatment specifi-cation	M Protein heavy chain	M Protein light chain	high risk criteria	ISS-stage
1	m	42	yes	VCD	Light Chain Myeloma	Kappa	del(17p)	ISS2/3
2	m	56	no		IgA	Kappa	del(17p), t(4;14)	ISS2
3	f	45	no		Light Chain Myeloma	Lambda	del(17p)	ISS2
4	f	54	no		IgA	Kappa	del(17p)	ISS3
5	m	69	yes	VD	IgA	Lambda	> 3 copies +1q21	ISS3
6	f	48	no		IgA	Lambda	> 3 copies +1q21	ISS2
7	m	50	yes	VCD	IgA	Kappa	t(4;14), > 3 copies +1q21	ISS2
8	f	67	no		Light Chain Myeloma	Kappa	del(17p), > 3 copies +1q21	ISS2
9	m	55	yes	VCD	IgA	Kappa	> 3 copies +1q21	ISS2
10	f	60	yes	VCD	IgG	Lambda	t(4;14)	ISS2
11	f	46	yes	VCD	IgA	Kappa	del (17p)	ISS2
12	m	50	no		IgG	Kappa	del (17p), > 3 copies +1q21	ISS1
13	m	45	no		IgA	Kappa	t(4,14)	ISS2
14	f	59	yes	VCD	IgG	Kappa	t(4;14), > 3 copies +1q21	ISS3
15	f	64	no		IgG	Lambda	t(4;16), > 3 copies +1q21	ISS3
16	f	82	yes	Dexa	IgG	Kappa	del (17p)	ISS3
17	f	64	yes	VD	IgG	Lambda	t(4,14)	ISS2
18	f	67	no		Light Chain Myeloma	Kappa	del(17p), > 3 copies +1q21	ISS3
19	f	67	yes	VD	IgA	Kappa	del(17p)	ISS3
20	m	56	no		IgA	Lambda	t(4;14), > 3 copies +1q21	ISS3

#	sex	age	previous treatment	treatment specifi-cation	M Protein heavy chain	M Protein light chain	high risk criteria	ISS-stage
21	f	48	yes	VCD	IgA	Kappa	del(17p), t(4;14)	ISS2
22	f	57	yes	VCD	IgG	Kappa	t(4,14)	ISS3
23	m	60	yes	VCD	Light Chain Myeloma	Lambda	> 3copies +1q21	ISS3
24	f	67	yes	VCD	IgG	Kappa	> 3copies +1q21	ISS3
25	f	55	yes	VD	IgG	Lambda	del(17p), t(4;14)	ISS2
26	m	61	yes	VRD	IgG	Kappa	del(17p)	ISS2
27	f	66	yes	VRD	IgG	Kappa	t(4;14)	ISS2
28	m	58	yes	Dexa	IgA	Kappa	del(17p)	ISS3
29	m	47	yes	n. g.	Light Chain Myeloma	Kappa	del(17p)	ISS3
30	f	60	no		IgA	Lambda	t(4;14)	ISS3
31	m	62	no		IgG	Kappa	del(17p), t(4;14)	ISS2
32	m	55	yes	VCD	IgA	Lambda	t(4;14)	ISS3
33	m	72	no		IgG	Kappa	del(17p)	ISS3
34	f	47	yes	n. g.	IgG	Kappa	t(4;14)	ISS2/3
35	f	49	no		IgG	Lambda	t(4;14)	ISS2
36	f	66	yes	Dexa	IgA	Kappa	t(4;16)	ISS2/3
37	f	55	no		Light Chain Myeloma	Kappa	t(4;14)	ISS2

f = female; m = male; Dexa = dexamethasone; VCD = bortezomib, cyclophosphamide, dexamethasone; VD = bortezomib, dexamethasone; VC = bortezomib, cyclophosphamide; VRD = bortezomib, lenalidomide, dexamethasone; IgA = immunoglobulin A; IgG = immunoglobulin G; del = deletion; t = translocation; ISS = international staging system; n. g. = not given

Before processing, all BM and whole blood samples gained in Tübingen were stored overnight at room temperature (RT) protected from light to equalize conditions for samples gained within a multicenter study, being transported overnight. For blood serum samples, this condition could not be met as samples of healthy controls were processed the same day.

2.1.1 *Peripheral blood*

PB of both patients and healthy controls was collected in EDTA-tubes and serum-tubes without separating gel (both Sarstedt, Nümbrecht, Germany or Greiner Bio-One, Kremsmünster, Austria). Blood cell counts of EDTA-whole blood samples were determined using a Sysmex cell counter (KX-21N, Sysmex, Kobe, Japan). Automated quantification of different cell fractions (lymphocytes, monocytes, granulocytes) was used. When the instrument could not determine those different fractions, the involved samples were excluded from further analysis depending on those quantifications.

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-whole-blood using density gradient centrifugation: the blood was diluted 1:2 using Dulbecco's phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, USA), then a maximum of 20 ml blood-PBS-mixture was stacked on 15 ml of Biocoll cell-separation solution (Merck Millipore, Billerica, USA). Centrifugation was performed at 450xg, 20 min, 21 °C. The interphase was taken and washed twice with PBS.

Blood serum was collected using the above-named serum-tubes. After blood drawing, tubes were stored preferably vertically during coagulation. After centrifugation (3,500xg, 10 min, RT), serum was transferred to cryotubes (Greiner Bio-One, Kremsmünster, Austria) and stored at -80 °C until further usage.

2.1.2 *Bone marrow*

Preparation of BM differed between patients and HDs due to different collection of samples: while BM of MM-patients was collected through puncture of the iliac crest and aspiration, BM of HDs was gained as residual material out of the opened bone shaft during joint replacement operations (mainly hip-total-endoprosthesis).

The BM of HDs taken during operation was anticoagulated using 5,000 IU of Natrium-Heparin (Ratiopharm, Ulm, Germany). For the purification of bone marrow mononuclear cells (BM-MNCs), samples were diluted 1:2.5 with Dulbecco's PBS (Life Technologies, Carlsbad, USA). Afterward, the suspension was mixed thoroughly and strained using a 100 µm cell strainer (Greiner Bio-One,

Kremsmünster, Austria). Next, a density gradient centrifugation was performed, stacking a maximum of 20 ml bone marrow-PBS-mixture on 15 ml of Biocoll cell-separation solution (Merck Millipore, Billerica, USA). Centrifugation was performed at 850xg, 30 min, 21 °C. The interphase containing the BM-MNCs was taken and washed twice with PBS. BMEF was taken from the upper phase after the removal of the fat layer. The BMEF was centrifuged twice at 2,000xg, 10 min, RT to remove potential cell contamination before freezing at -80 °C.

BM of MM-patients was aspirated in 20 ml-syringes (B. Braun, Melsungen, Germany). After 1:2.5 dilution with Dulbecco's PBS (Life Technologies, Carlsbad, USA), density gradient centrifugation, isolation of cells and BMEF and further handling was performed as described above for BM of HDs.

2.1.3 Cryopreservation of PBMCs and BM-MNCs

For cryopreservation, PBMCs and BM-MNCs were resuspended in ice-cold freezing-medium (RPMI-1640 (Sigma-Aldrich, St. Louis, USA), 10 % DMSO (Honeywell, Morristown, USA), 20 % fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, USA)) with a concentration from $0.5x10^7$/ml to $5x10^7$/ml and frozen (for 24 h at -80 °C, using a Mr. Frosty™ freezing container (Thermo Scientific, Waltham, USA), afterwards at -150 °C) until further usage.

Thawing of samples was performed at room temperature, until the core of the sample was only slightly frozen. Through slow pipetting, the sample was transferred into 10 ml of 4 °C thawing medium (RPMI-40 (Sigma-Aldrich, St. Louis, USA), 10 % FCS (Sigma-Aldrich, St. Louis, USA)). After centrifugation (480xg, 5 min, RT), cells were resuspended in 5 ml of thawing medium containing 200 KU DNaseI (Merck-Millipore, Billerica, USA). After incubation for 20 minutes at 4 °C protected from light, cells were strained (100 µm cell strainer (Greiner Bio-One, Kremsmünster, Austria)), counted, and washed with RPMI-40-medium. Next, cells were resuspended in needed concentration and correct buffer for following experiments (either FACS-buffer (Dulbecco's PBS w/o Mg^{2+}, Ca^{2+} (Life Technologies, Carlsbad, USA) + 1 % FCS (Sigma-Aldrich, St. Louis, USA) + 0.09 % NaN_3 (Sigma-Aldrich, St. Louis, USA) + 2 mM EDTA (Merck-Millipore, Billerica, USA)) for staining and flow cytometry or MACS-buffer (Dulbecco's PBS

w/o Mg^{2+}, Ca^{2+} (Life Technologies, Carlsbad, USA) + 2 mM EDTA (Merck-Millipore, Billerica, USA) + 0.05 % BSA (Biomol, Hamburg, Germany)) for MACS-cell-separation.

2.2 DC-differentiation-assay using CD34^{+}-progenitors

A differentiation assay for DCs published by Breton et al. (2015a) was used and adapted to the needs of the issue of this thesis.

2.2.1 Purification of CD34^{+}-progenitors

CD34^{+}-progenitors were purified from BM-MNCs. Collection, freezing and thawing of samples was performed as described above (sections 2.1.2 and 2.1.3). For the first step of purification a column-based cell separation was performed, using a CD34^{+}-UltraPure-Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Therefore, the thawed cells were resuspended in MACS-buffer (see above), incubated with Fc-block and CD34-UltraBeads for 20 minutes at 4 °C, protected from light. The cell suspension was transferred onto a prepared, once washed (MACS-buffer), LS-MACS-column (Miltenyi Biotec, Bergisch Gladbach, Germany), placed in the magnetic field of a MACS-separator (Miltenyi Biotec, Bergisch Gladbach, Germany). After three times washing with MACS-buffer, the column was moved out of the magnetic field. By rapidly flushing, the CD34^{+}-fraction of cells was collected. For further purification, cell-sorting was performed. Therefore, cells were incubated with human IgG (100 µg in 1 ml of FACS-buffer; Sigma-Aldrich, St. Louis, USA) for 20 minutes at RT protected from light to prevent unspecific antibody-binding. Afterwards, cells were stained according to Table 5 for 20 minutes at 4 °C protected from light. 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, USA) was applied after washing once more. Cells were sorted at an Aria IIIu cell-sorter (Becton Dickinson Biosciences, Franklin Lakes, USA). CD34^{+}-cells were collected in MS-5-Medium (Alpha-Medium (Merck-Millipore, Billerica, USA), 10 % FCS (Sigma-Aldrich, St. Louis, USA), 100 U/ml Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, USA), 2 mM L-Glutamine (Thermo Fisher Scientific, Waltham, USA), 2 mM Natrium-Pyruvate (Sigma-

Aldrich, St. Louis, USA). A flow cytometric reanalysis of a proportion of the collected cells was performed to prove proper purification.

Table 5 – Staining CD34$^+$ purification

Antibody			
Fluorochrome	**Antigen**	**Clone**	**Manufacturer**
DAPI	live/dead		Sigma-Aldrich
BV510	CD3	OKT3	Biolegend
	CD19	HIB19	Biolegend
	CD20	2H7	Biolegend
	CD56	HCD56	Biolegend
APC	CD34	581	BD Biosciences

2.2.2 Cultivation of CD34$^+$-progenitors

Cultivation of CD34$^+$-cells was performed using murine stromal cells (murine stromal cell line 5-(MS-5-)cells) as feeder cells. Those MS-5-cells were cultivated in MS-5-medium (see above) in 750 cm³ cell-culture-flasks (Greiner Bio-One, Kremsmünster, Austria). Cells were splitted at approximately 80 to 90 % confluence every three to five days. For detaching of the adherent cells, Trypsin-EDTA (Biozym, Hessisch Oldendorf, Germany) was used. Prior to seeding, MS-5-cells were treated with 10 µg/ml Mitomycin C (Sigma-Aldrich, St. Louis, USA), in the above-mentioned medium for three hours at 37 °C to prevent a proliferation of those cells in further culture. Subsequently, the cells were detached, transferred to a 15 ml falcon, washed and resuspended at a concentration of $2.5x10^5$/ml. 100 µl of cell suspension were seeded into each well of a tissue-culture treated 96-well-flat-bottom-plate (Corning, Corning, USA). After cultivation overnight, seeding of CD34$^+$-cells was performed the next day: FACS purified cells were adjusted at a concentration of $1x10^5$/ml in MS-5-medium, containing additional cytokines:

- granulocyte-macrophage colony-stimulating factor (GM-CSF) (Biolegend, San Diego, USA): 50 ng/ml medium
- stem cell factor (SCF) (Biolegend, San Diego, USA): 50 ng/ml medium

- FMS-like tyrosine kinase 3 ligand (FLT3L) (Biolegend, San Diego, USA): 250 ng/ml medium.

Furthermore, 50 µl either blood serum or BMEF of either patients or healthy controls were added to each well. For control wells 50 µl additional medium without cytokines were added. BMEF and blood serum were thawed at RT. The cultivation was conducted for a period of seven days at 37 °C, 5 % CO_2.

2.2.3 Harvesting, staining of the cells and quantification of cell populations by flow cytometry

At day 7 cells were harvested by pipetting and transferred into a 96-well-U-bottom-plate (Greiner Bio-One, Kremsmünster, Austria). After centrifugation and discarding of the supernatant, fluorescent antibodies were added according to Table 6. Cells were incubated for 20 minutes at 4 °C protected from light, washed once with FACS-buffer and transferred to mini-FACS-Tubes (Greiner Bio-One, Kremsmünster, Austria). Measurement was performed at a LSRFortessa flow cytometer (Becton Dickinson Biosciences, Franklin Lakes, USA). The used panel allows to distinguish between $CD45^+$ lymphocytes and $CD45^-$ MS-5-cells. Other lymphoid cell subsets than DCs can be excluded using the markers CD3, CD20, CD56, CD66b and CD14. The three different steady state DC-populations can be identified:

- cDC1s ($CD45^+live/dead^-CD66b^-CD3^-CD20^-CD56^-HLA\text{-}DR^+CD141^+$),
- cDC2s ($CD45^+live/dead^-CD66b^-CD3^-CD20^-CD56^-HLA\text{-}DR^+CD1c^+$) and
- pDCs ($CD45^+live/dead^-CD66b^-CD3^-CD20^-CD56^-HLA\text{-}DR^+CD1c^-CD14^-CD123^+CD303^+$).

Table 6 – Staining culture output

Antibody			
Fluorochrome	**Antigen**	**Clone**	**Manufacturer**
PacificBlue	CD20	2H7	Biolegend
	CD56	MEM-188	Biolegend
Zombie-Aqua	live/dead		Biolegend
BV605	CD3	OKT3	Biolegend
BV650	HLA-DR	L243	Biolegend
BV786	CD14	M5E2	Biolegend
FITC	CD66b	G10F5	Biolegend
PerCP-Cy5.5	CD303	201A	Biolegend
PE	CD123	6H6	Biolegend
PE-Cy7	CD1c	L161	Biolegend
APC	CD141	AD5-14H12	Miltenyi Biotec
APC-Cy7	CD45	HI30	Biolegend

2.3 Characterization of cells and cell populations in peripheral blood by flow cytometry

2.3.1 General procedure

2.3.1.1 Flow cytometer and performance tracking

Blood cells and blood cell populations of both MM-patients and healthy controls were analyzed by flow cytometry. All measurements were performed on a LSRFortessa flow cytometer (Becton Dickinson Biosciences, Franklin Lakes, USA) equipped with four lasers (violet 402 nm, blue 488 nm, yellow-green 561 nm, red 640 nm). For precise differentiation between positive signals and spillover, fluorescence minus one-controls (FMOs) were used, when useful.

For detection of altering flow cytometer performance during the measurement time span of several weeks, both CS&T beads (Becton Dickinson Biosciences, Franklin Lakes, USA) and 8-peak-rainbow-calibration-beads (Becton Dickinson Biosciences, Franklin Lakes, USA) were used daily before measurements. CS&T beads were used for manufacturer-provided, automatic performance adaption.

The 8-peak-beads were used according to the EuroFlow Consortium SOP ((2018), for further information see also Kalina et al. (2012)) for validating steady performance. A maximum deviation of ±15 % was accepted as “steady performance”. If larger deviations occurred, cleaning of the flow cytometer was repeated. If deviations persisted, photomultiplier tube (PMT) voltages were adapted.

2.3.1.2 Blocking and staining of cells

Thawing of frozen PBMCs was performed as described in section 2.1.3. If available, $3x10^6$ cells (for analysis of T cells) and up to $6x10^6$ cells (for analysis of DCs) were set in each well of a 96-well-round-bottom-plate (Greiner Bio-One, Kremsmünster, Austria). If only lower counts of cells were available, at least $2x10^6$ cells were used per well. After centrifugation, cells were incubated with 200 µl of human IgG-solution (100 µg/ml, Sigma-Aldrich, St. Louis, USA) for 20 minutes at RT to prevent unspecific binding. Subsequently, antibody staining was performed as specified in Table 7 and Table 8. Buffer used for the following steps of washing and incubating was FACS-buffer (see above), containing 50 KU DNaseI (Merck-Millipore, Billerica, USA). Staining of CD197 (= CCR7) was carried out for 30 minutes at 37 °C, 5 % CO_2 in the incubator. After washing, the remaining markers were stained for 20 minutes at 4 °C protected from light. Cells were washed twice, resuspended in 150 µl FACS-buffer (without DNaseI) and transferred to a FACS-Tube (Corning, Corning, USA) for measurement.

2.3.2 Panel for staining of DC subpopulations in peripheral blood

To examine myeloid cell subsets and functional status (particularly DCs), a 14-color panel was used (see Table 7). Through use of the different fluorescent antibodies, firstly different DC subpopulations can be identified:

- infDCs: lineage$^-$live/dead$^-$CD14$^+$CD16$^-$HLA-DR$^+$CD1c$^+$
- cDC1s: lineage$^-$live/dead$^-$CD14$^-$CD16$^-$HLA-DR$^+$CD1c$^-$CD141$^+$
- cDC2s: lineage$^-$live/dead$^-$CD14$^-$CD16$^-$HLA-DR$^+$CD1c$^+$CD11c$^+$
- pDCs: lineage$^-$live/dead$^-$CD14$^-$CD16$^-$HLA-DR$^+$CD1c$^-$CD303$^+$.

In addition, monocyte populations can be examined:

- classical monocytes: lineage$^-$live/dead$^-$CD14$^+$CD1c$^-$
- intermediate monocytes: lineage$^-$live/dead$^-$CD14$^+$CD16$^+$
- non-classical monocytes: lineage$^-$live/dead$^-$CD16$^+$.

Beyond that, the panel allows studying markers that predict functionality and state of maturation of DCs: the surface markers HLA-DR, CD40, CD80, CD86 and CD274 (= PD-L1) are of great interest as they are essential for DC-T-cell crosstalk. CD197 (= CCR7), indicating the ability to migrate to lymphoid tissues, provides information concerning the activation status of DCs.

Table 7 – Staining myeloid cells

Antibody			
Fluorochrome	**Antigen**	**Clone**	**Manufacturer**
BV421	CD80	2D10	Biolegend
BV510	CD3	OKT3	Biolegend
	CD19	HIB19	Biolegend
	CD20	2H7	Biolegend
	CD56	HCD56	Biolegend
Zombie Aqua	live/dead		Biolegend
BV605	CD86	IT2.2	Biolegend
BV650	HLA-DR	L243	Biolegend
BV786	CD197 (CCR7)	3D12	BD Biosciences
FITC	CD14	M5E2	Biolegend
PerCP-Cy5.5	CD303	201A	Biolegend
PE	CD274 (PD-L1)	29E.2.A3	Biolegend
PE-Dazzle	CD1c	L161	Biolegend
PE-Cy7	CD11c	Bu15	Biolegend
APC	CD141	AD5-14H12	Miltenyi Biotec
AlexaFluor 700	CD16	3G8	Biolegend
APC-Cy7	CD40	5C3	Biolegend

2.3.3 Panel for staining of T-cell subpopulations in peripheral blood

Analysis of T-cell subpopulations was performed using a 13-color panel (see Table 8). It allows the identification of different functional cell subsets according to Larbi and Fulop (2014), both for $CD4^+$ and $CD8^+$ T cells:

- naïve T cells (T_N): $CD197^+CD45RA^+CD27^+CD28^+CD45RO^-CD57^-$ $KLRG1^-$
- central memory T cells (T_{CM}): $CD197^+CD45RA^-CD27^+CD28^+CD45RO^{+-}$ $CD57^-KLRG1^-$
- effector memory T cells (T_{EM}): $CD197^-CD45RA^-CD27^+CD28^+CD45RO^{+-}$ $CD57^-$, for $CD4^+$ T_{EM} $KLRG1^-$ and for $CD8^+$ T_{EM} $KLRG1^{\pm}$
- terminally differentiated effector memory T cells re-expressing CD45RA (T_{EMRA}): $CD197^-CD45RA^+CD27^-CD28^-CD45RO^-CD57^+KLRG1^+$

Analysis of the of CD279 (= PD-1) also allows to investigate T-cell dysfunction more precisely.

Table 8 – Staining T cells

Antibody			
Fluorochrome	**Antigen**	**Clone**	**Manufacturer**
BV421	KLRG-1	2F1/KLRG1	Biolegend
BV510	CD11b	ICRF44	Biolegend
	CD14	M5E2	Biolegend
	CD15	W6D3	Biolegend
	CD19	HIB19	Biolegend
	CD56	HCD56	Biolegend
ZombieAqua	live/dead		Biolegend
BV711	CD279 PD-1	EH12.1	BD Biosciences
BV786	CD197 CCR7	3D12	BD Biosciences
BB515	CD27	M-T271	BD Biosciences
PerCP-Cy5.5	CD3	UCHT1	Biolegend
PE	CD45RO	UCHL11	Biolegend
PE-Dazzle	CD57	HNK-1	Biolegend
PE-Cy5	CD28	CD28.2	BD Biosciences
APC	CD8	SK1	Biolegend
AlexaFluor 700	CD4	SK3	Biolegend
APC-H7	CD45RA	HI100	BD Biosciences

2.4 Interleukin-6-ELISA

For the quantification of IL-6 in BMEF and blood serum, an enzyme-linked immunosorbent assay (ELISA Ready-SET-Go! kit; Thermo Fisher Scientific, Waltham, USA) was used according to the manufacturer's instructions. The day before the experiment, 100 µl of capture-antibody-solution were applied to each well of a 96-well-ELISA-plate (Thermo Fisher Scientific, Waltham, USA) and incubated at 4 °C overnight protected from light. After washing five times with washing buffer (1x PBS w/o Ca^{2+} (Merck Millipore, Billerica, USA) with 0.05 % Tween20 (Serva, Heidelberg, Germany)), 100 µl/well of assay diluent were applied and incubated for 60 minutes. Again, five washing steps were performed. Next, 100 µl of standard-solution or sample were added. Samples were diluted

using assay diluent, if necessary. After incubation for two hours, seven washing steps were performed. Following, 100 µl/well of detection-antibody-solution were applied, incubated for 1 hour and again washed five times. After adding each 100 µl of avidin-HRP-antibody-solution, 30 minutes incubation and washing five times, each 100 µl of substrate were added. After 15 minutes of incubation protected from light, the reaction was stopped using 50 µl 2 M H_2SO_4 per well. The plate was read out using a Tecan Magellan Sunrise ELISA-reader (Tecan, Männedorf, Schweiz).

2.5 Laboratory devices

- Sterile bench: Herasafe KS12 (Thermo Scientific, Waltham, USA)
- Centrifuge: Heraeus Multifuge 3 S-R (Thermo Scientific, Waltham, USA)

2.6 Analysis of flow cytometry data

2.6.1 Preparation of data for analysis

Data of peripheral blood samples was expurgated by the FlowAI-algorithm, (FlowJo-plugin, version 1.8, algorithm developed by Monaco et al. (2016)) to eliminate statistical anomalies due to measurement conditions. The following parameters were used:

- Anomalies to exclude: signal acquisition, dynamic range
- Dynamic range check side = both
- Second fraction FR = 0.1
- Alpha FR = 0.01
- Maximum changepoints = 3
- Changepoint penalty = 200

Cleaning was performed under involvement of all fluorochrome-channels and the time-channel.

2.6.2 Manual analysis

Manual analysis of flow-cytometry data was performed using FlowJo, version 10.6.1 (Becton Dickinson Biosciences, Franklin Lakes, USA). Cell populations were identified through bivariant gating. Median fluorescent intensities were determined to analyze expression of surface markers.

2.6.3 Automated analysis of T cells

To face pitfalls and limits of manual gating e. g. bias through subjective expectations, automated analysis was performed. Both challenges of and approaches to analysis of multiparameter flow cytometry data have been discussed by inter alia Saeys et al. (2016), Mair et al. (2016) and Mair (2019). In this present thesis T cells were analyzed using the CITRUS algorithm (Bruggner et al. 2014) on the cloud-based platform cytobank.org (Beckman Coulter, Brea, USA). Therefore, files were prepared by using FlowJo and the FlowAI plug-in. After exclusion of anomalies, gating on $CD3^+$ T cells was performed. These populations of interest were exported from FlowJo containing the compensations. Files were uploaded to the cytobank-platform where first $CD4^+$ and $CD8^+$ T cells were gated and then the automated algorithm was applied separately on $CD4^+$ and $CD8^+$ T cells with the following settings:

- Compensation: File-internal
- Minimum cluster size: 3.5 %
- Cross validation folds: 5
- False discovery rate: 1
- Association model: pamr
- Clustering characterization: abundances
- Event sampling method: equal
- Normalize scales: false

2.7 Statistical analysis

The Mann-Whitney rank sum-test was performed to test for significance for unpaired samples. Differences were marked as ns for $p \geq 0.05$, * for $p<0.05$, ** for $p<0.01$, *** for $p<0.005$ and **** for $p<0.001$.

3 Results

3.1 Similar numbers of white blood cells and lymphocytes in blood of MM-patients and healthy donors

To assess the fundamental differences in the immune status of MM-patients compared to HDs, first white blood cell (WBC) and lymphocyte counts per ml whole blood were compared between the patient and donor cohorts (Figure 2). Therefore, whole blood samples of both groups were analyzed using a Sysmex cell counter. There were no significant differences in numbers of WBCs per ml whole blood between patients and HDs with only a very slight trend to reduced counts in patients. In HDs, there was an average of $7 \pm 2.84 \times 10^6$ WBCs/ml while in MM-patients an average of $6.5 \pm 2.60 \times 10^6$ WBCs/ml was found. Furthermore, also for the lymphocyte fraction, no difference was found. Both HDs and MM-patients showed similar numbers of 1.5×10^6 lymphocytes/ml whole blood (SD was $\pm 0.55 \times 10^6$ lymphocytes/ml for HDs and $\pm 0.77 \times 10^6$ lymphocytes/ml for MM-patients). Thus, both WBC counts, and lymphocyte counts were roughly on the same level in MM-patients and HDs.

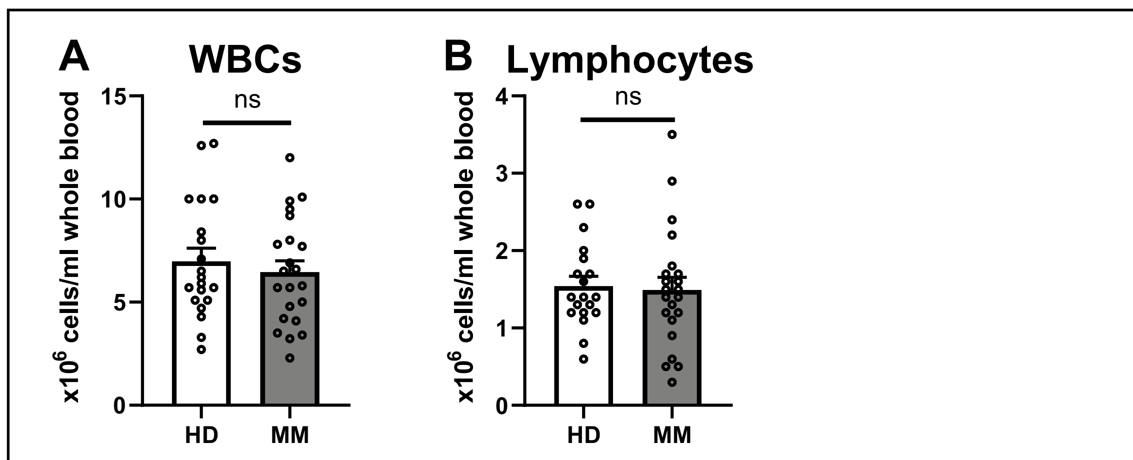


Figure 2 – White blood cell and lymphocyte counts in whole blood of multiple myeloma-patients and healthy donors were similar

Whole blood was drawn from both multiple myeloma-(MM-)patients and healthy donors (HDs). Cell counts of 20 HDs and 22 MM-patients were analyzed using a Sysmex cell counter. **A** White blood cell (WBC) counts per ml whole blood **B** Lymphocyte counts per ml whole blood. Bar diagrams display mean ±SEM with each dot representing one HD or MM-patient, respectively. Mann-Whitney rank sum-test was performed to test for statistical significance, ns = $p \geq 0.05$.

3.2 Myeloid cell populations in the peripheral blood

3.2.1 All dendritic cell subsets were reduced in PB of MM-patients

To investigate myeloid cell populations, cryopreserved PBMCs purified from whole blood of HDs and MM-patients were thawed and analyzed by 14-color flow cytometry. Gating was performed according to Figure 3A with infDCs: lineage$^-$live/dead$^-$CD14$^+$CD16$^-$HLA-DR$^+$CD1c$^+$, cDC1s: lineage$^-$live/dead$^-$CD14$^-$CD16$^-$HLA-DR$^+$CD1c$^-$CD141$^+$, cDC2s: lineage$^-$live/dead$^-$CD14$^-$CD16$^-$HLA-DR$^+$CD1c$^+$CD11c$^+$ and pDCs: lineage$^-$live/dead$^-$CD14$^-$CD16$^-$HLA-DR$^+$CD1c$^-$CD303$^+$. Cell counts per ml whole blood of the different DC-subsets (Figure 3B) were calculated using the frequencies of the DC-subsets of all lymphocytes determined by gating of flow cytometry data (gating not shown) and the lymphocyte counts of the Sysmex measurements (see Figure 2).

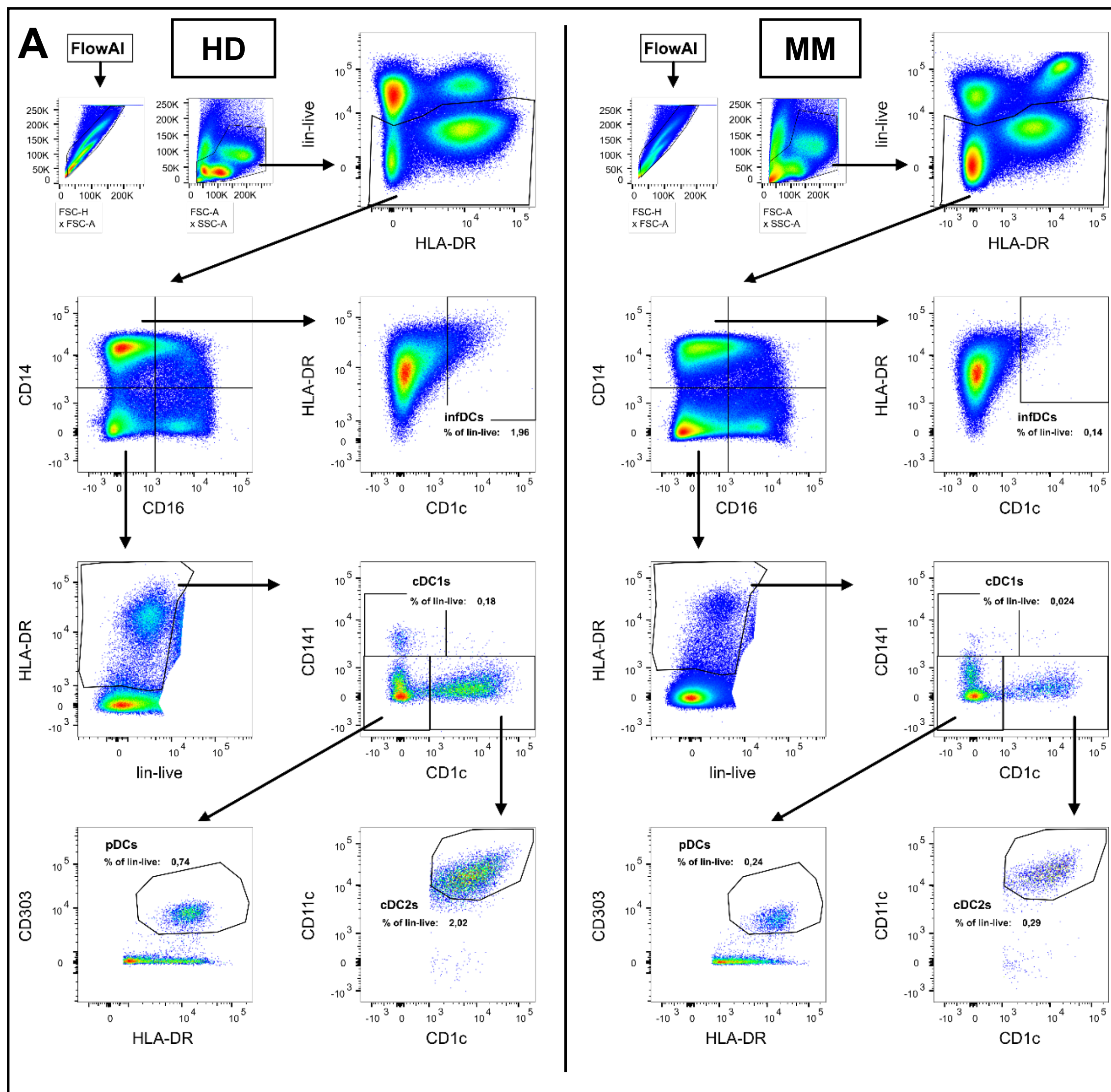
The count per ml whole blood of infDCs showed a trend to reduced values in MM-patients while the other dendritic cell subsets (i. e. cDC1s, cDC2s and pDCs) were significantly reduced compared to HDs. This was strongest for pDCs with a decrease by factor 3.6, followed by cDC2s (factor 3.3) and cDC1s (factor 2.6).

The frequencies of the different DC-subsets and monocyte subsets of lineage$^-$live cells were analyzed (Figure 3C and D). All DC-subsets of MM-patients were reduced in their frequency of lineage$^-$live cells compared to HDs (Figure 3C). This decrease was strongest in cDC1s, where cells in MM-patients were reduced by factor 6.3. For cDC2s and pDCs the effects were lower with a factor of 4.3 and 3.1, respectively. For infDCs the effect was weakest with a factor of 1.5.

Monocyte subsets were gated as classical monocytes: lineage$^-$live/dead$^-$CD14$^+$CD16$^-$CD1c$^-$, intermediate monocytes: lineage$^-$live/dead$^-$CD14$^+$CD16$^+$ and non-classical monocytes: lineage$^-$live/dead$^-$CD14$^-$CD16$^+$ (Figure 3A). For the different monocyte subsets, no general decrease in their frequency of lineage$^-$live cells was found (Figure 3D). While classical CD14$^+$ monocytes were indeed significantly reduced in their frequency of lineage$^-$live cells by factor 1.4 in MM-patients, intermediate CD14$^+$CD16$^+$ and non-classical CD16$^+$ monocytes showed no different frequencies compared to those of HDs.

In summary, looking at frequencies of lineage$^-$live cells, MM-patients showed a significant decrease of all DC-subsets and classical monocytes while intermediate and non-classical monocytes were unaltered compared to HDs. Thus, MM-patients had substantially lower numbers of DC-subsets in the PB.

Find Figure 3A on this page and Figure 3B, C, D and legend on page 31.



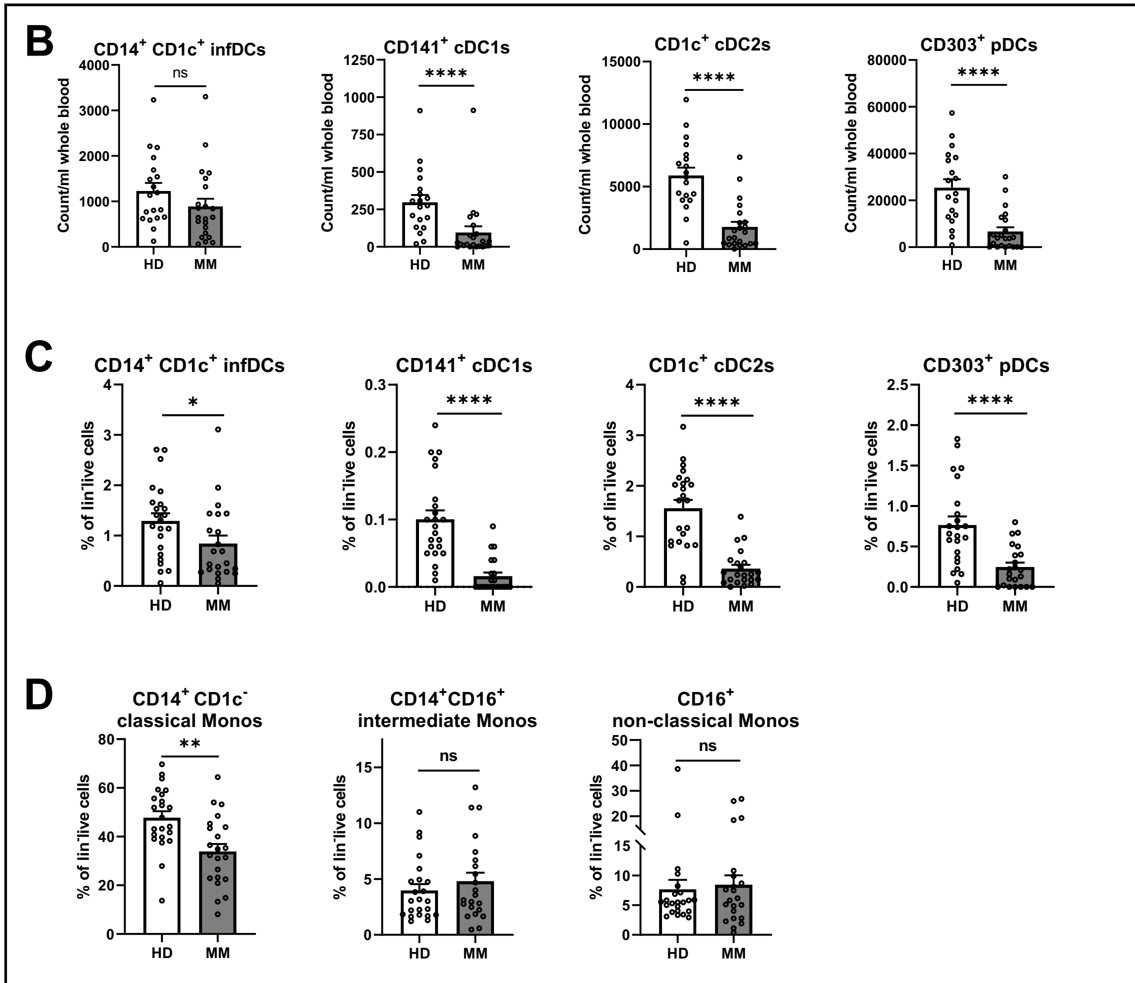


Figure 3 – Decreased numbers of all dendritic cell-subsets in multiple myeloma-patients

Frozen peripheral blood mononuclear cells (PBMCs) were thawed and stained for flow cytometry. **A** Comparative gating of cells to determine fractions of myeloid cell subsets of one of 23 representative healthy donors (HDs) (left) and one of 22 multiple myeloma-(MM-)patients (right). Dendritic cell-(DC-)subsets were identified as inflammatory DCs (infDCs): lineage(CD3, CD19, CD20, CD56)$^-$live/dead$^-$CD14$^+$CD16$^-$CD1c$^+$, conventional DCs type 1 (cDC1s): lineage(CD3, CD19, CD20, CD56)$^-$live/dead$^-$CD14$^-$CD16$^-$HLA-DR$^+$CD141$^+$CD1c$^-$, conventional DCs type 2 (cDC2s): lineage(CD3, CD19, CD20, CD56)$^-$ live/dead$^-$CD14$^-$CD16$^-$HLA-DR$^+$CD141CD1c$^+$CD11c$^+$ and plasmacytoid DCs (pDCs): lineage(CD3, CD19, CD20, CD56)$^-$ live/dead$^-$CD14$^-$CD16$^-$HLA-DR$^+$CD141$^-$CD1c$^-$CD303$^+$). Monocyte subsets were identified as classical monocytes: lineage(CD3, CD19, CD20, CD56)$^-$live/dead$^-$CD14$^+$CD16$^-$CD1c$^-$, intermediate monocytes: lineage(CD3, CD19, CD20, CD56)$^-$live/dead$^-$CD14$^+$CD16$^+$, and non-classical monocytes: lineage(CD3, CD19, CD20, CD56)$^-$live/dead$^-$CD14$^-$CD16$^+$. **B** Total numbers of DC-subsets per ml whole blood. **C** Fraction of DCs of lineage(CD3, CD19, CD20, CD56)$^-$ live/dead$^-$ cells. **D** Fraction of monocytes of lineage(CD3, CD19, CD20, CD56)$^-$live/dead$^-$ cells. Bar diagrams display mean ±SEM with each dot representing one healthy donor or ►

MM-patient, respectively. Mann-Whitney rank sum-test was performed to test for statistical significance, ns = $p \geq 0.05$, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$, **** = $p<0.001$.

3.2.2 Phenotype of DCs was altered in MM-patients

Expression of different surface molecules plays a crucial role in DC function, especially of those which are involved in T-cell activation. Therefore, the expression of different surface molecules (CD40, CD80, CD86, HLA-DR, CCR7 and PD-L1) on DC-subsets of HDs and MM-patients was analyzed by flow cytometry (Figure 4).

All DC-subsets of MM-patients showed an increase in the percentage of CD40 high-expressing cells compared to HDs. Greatest differences were found in infDCs, followed by pDCs and cDC2s. For cDC1s there was no significant increase, yet a sharp trend.

CD80 expression revealed no significant differences. Very slight trends to increased median expression in MM-patients were visible for infDCs and cDC2s while for the other subsets, expression was basically on the same level.

Likewise, there were no significant differences in CD86 expression with merely slight trends to increased expression on all subsets in MM-patients compared to HDs.

Looking at HLA-DR, there were tendencies to decreased median expression in MM-patients for all subsets compared to HDs. This trend was strongest in cDC1s.

The proportion of CCR7 high-expressing infDCs and pDCs was slightly – yet not significantly – increased in MM-patients compared to HDs.

The proportion of PD-L1 positive cells was increased in MM-patients compared to HD. These results were significant for infDCs with over a third of increase compared to HDs and as well for cDC1s and cDC2s with each almost a twofold. In pDCs this effect was much weaker and not significant.

In conclusion, the phenotype of DC-subsets was altered in MM-patients compared to HDs, displaying increased frequencies of $CD40^{+}$ and $PD\text{-}L1^{+}$ cells and a trend to lower expression of HLA-DR in MM-patients.

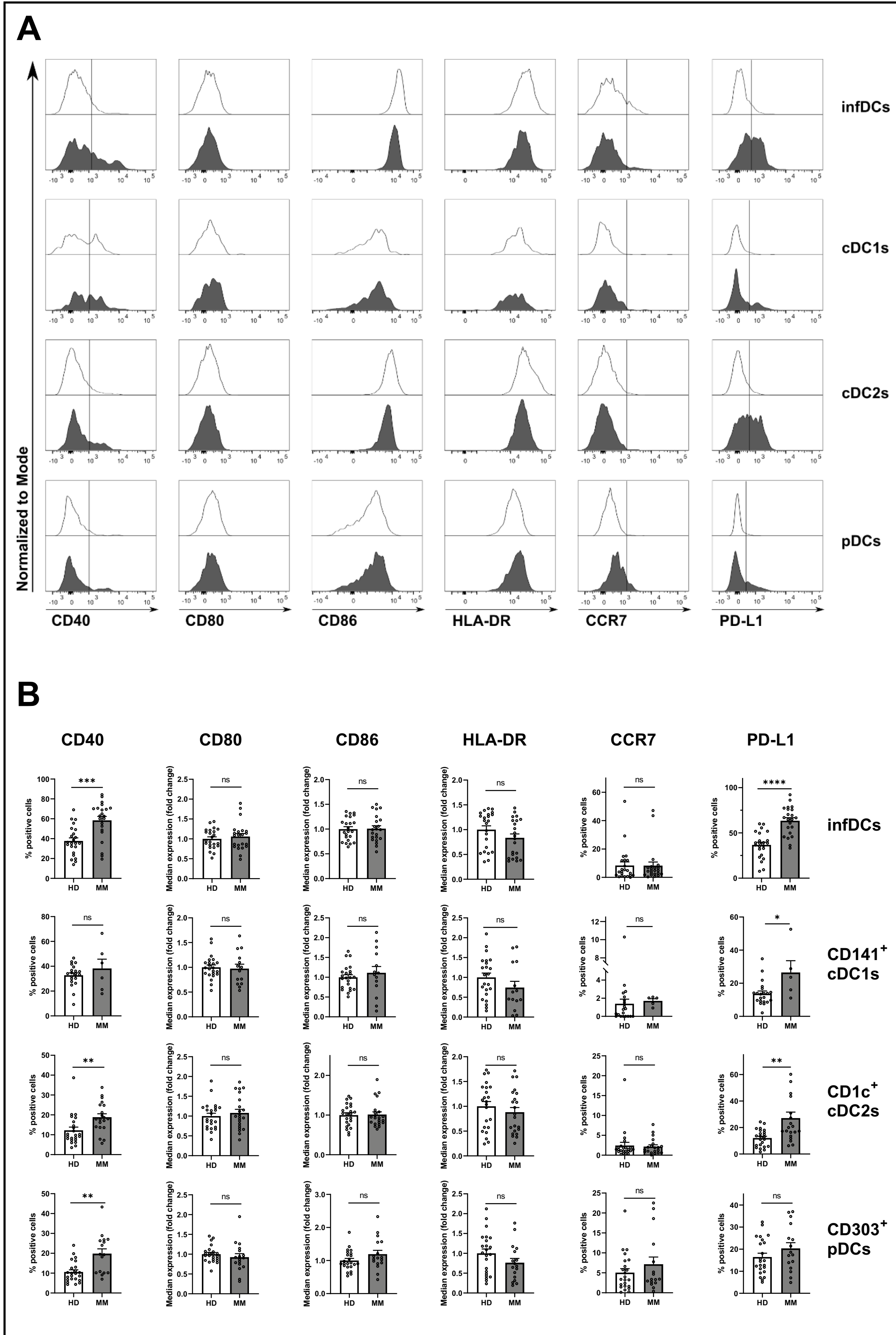

A
infDCs
cDC1s
cDC2s
pDCs
Normalized to Mode
CD40
CD80
CD86
HLA-DR
CCR7
PD-L1
B
CD40
CD80
CD86
HLA-DR
CCR7
PD-L1
infDCs
CD141+ cDC1s
CD1c+ cDC2s
CD303+ pDCs
% positive cells
Median expression (fold change)
HD
MM
ns
*
**
***
****

Figure 4 – Dendritic cell phenotype was altered in multiple myeloma-patients

Peripheral blood mononuclear cells (PBMCs) were thawed and stained for flow cytometry. Expressions of surface markers (CD80, CD86, human leukocyte antigen - DR isotype (HLA-DR)) were detected as medians of measured fluorescence. Fractions of positive cells (CD40, C-C chemokine receptor 7 (CCR7), programmed death-ligand 1 (PD-L1)) were determined through gating using FMO-controls. Cells were gated as shown in Figure 3A. **A** The histograms show expression of different surface molecules (columns) for the dendritic cell-(DC-)subsets (rows) of one exemplary healthy donor (HD) (upper histograms, black/white) and one multiple myeloma-(MM-)patient, respectively (lower histograms in each graph, grey) **B** The bar charts show mean fold change of median marker expression ±SEM or fraction of marker positive DC-subsets in MM-patients (n=22) compared to HDs (n=23). Each data point represents at least ten measured events of one individual for median marker expressions and at least 50 measured events for fractions of positive cells. Lower subset counts were excluded from statistical data analysis. Mann-Whitney rank sum-test was performed to assess for statistical significance with ns = $p \geq 0.05$, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$, **** = $p<0.001$. infDC = inflammatory dendritic cell; cDC1 = conventional/classical dendritic cell type 1, cDC2 = conventional/classical dendritic cell type 2; pDC = plasmacytoid dendritic cell.

3.3 T cells in the peripheral blood

T cells responses are induced through DCs while T cells themselves are major players in anti-tumoral immune responses. Therefore, T cells were analyzed by flow cytometry as restrictions in DC number and function might lead to impaired function of T cells and thereby contribute to immune dysfunction in MM-patients.

3.3.1 MM-patients showed a reduced frequency of T cells in peripheral blood

To analyze numbers and fractions of T cells, PBMCs isolated from patients' and HDs' whole blood were thawed and stained for flow cytometry. Gating was performed as shown in Figure 5A. Lymphocyte counts per ml blood were determined using a Sysmex cell counter (Figure 2). T-cell counts per ml blood were calculated based on gating of flow cytometry data and those lymphocyte counts.

T cells tended to reduced numbers per ml blood in MM-patients, this trend was stronger in $CD4^+$ than in $CD8^+$ T cells (Figure 5B). Analyzing the frequencies of T cells of $lineage^-$live cells, a significant decrease of $CD3^+$ T cells appeared in MM-patients compared to HDs (Figure 5C). When analyzing $CD4^+$ and $CD8^+$ T cells separately, $CD4^+$ T cells also showed a significant decrease of frequency in MM-patients compared to HDs, while for $CD8^+$ T cells there was merely a trend to decreased frequencies.

To address T-cell function in detail, different T-cell subsets were analyzed (Figure 5D). During an immune response, T cells undergo different functional states from naïve, circulating T_N cells via T_{CM} and T_{EM} cells to terminally differentiated effector memory T cells, displaying features of exhaustion (T_{EMRA}). Analysis of these different T-cell subsets showed no significant differences between MM-patients and HDs for T_N and T_{CM} cells. $CD8^+$ T_{EM} cells were significantly decreased in MM-patients, while for the $CD4^+$ subset a congruent trend could be described. T_{EMRA} showed strong trends to increased frequencies in MM-patients for both $CD4^+$ and $CD8^+$ subsets compared to HDs.

While T cells were generally reduced in their frequencies, there was a trend to a different distribution of the T_{EM} and the T_{EMRA} subsets in MM-patients compared to HDs.

Find Figure 5A on page 38 and Figure 5B, C, D and legend on page 39.

A

FlowAI

CD4+ T cells

CD8+ T cells

Median : PD-1: 3420

Central Memory

KLRG-1 negative

Median : PD-1: 3597

Naive

KLRG-1 negative

Median : PD-1: 5359

Terminally Differentiated Effector Memory Reexpressing CD45RA

KLRG-1 positive

Median : PD-1: 2697

Effector Memory

KLRG-1 negative

Median : PD-1: 4965

Central Memory

KLRG-1 negative

Median : PD-1: 3518

Naive

KLRG-1 negative

Median : PD-1: 5018

Terminally Differentiated Effector Memory Reexpressing CD45RA

KLRG-1 positive

Median : PD-1: 4263

Effector Memory

KLRG-1 indifferent

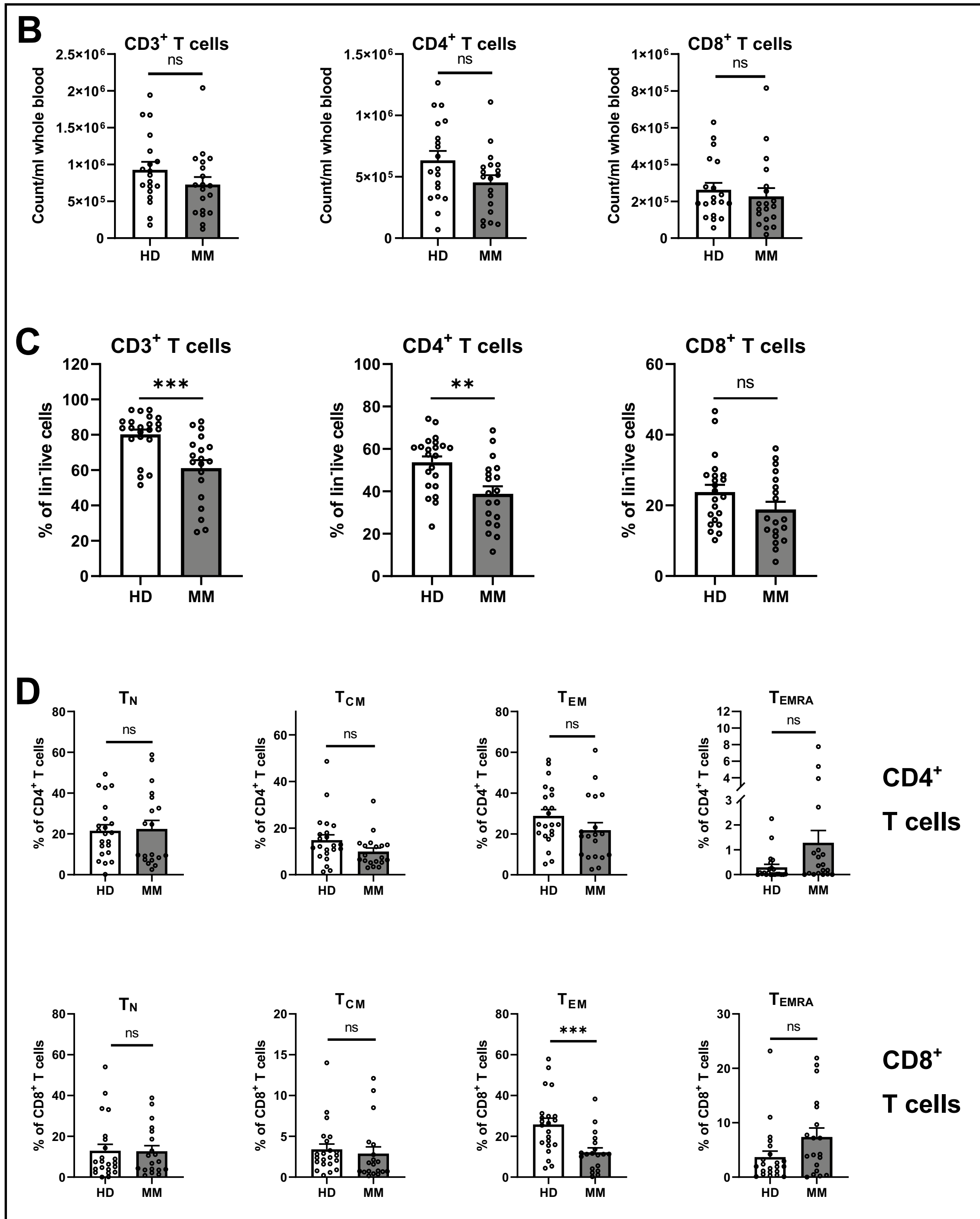


Figure 5 – Multiple myeloma-patients showed reduced fractions of T cells in peripheral blood

Cryopreserved peripheral blood mononuclear cell (PBMCs) of 22 healthy donors (HDs) and 19 multiple myeloma-(MM-)patients were stained for flow cytometry. **A** Gating of cells to determine cell counts and fractions of T-cell subsets. **B** Cell counts of $CD3^+$ T cells, $CD3^+CD4^+$ T helper and $CD3^+CD8^+$ cytotoxic T cells per ml blood. **C** Fraction of $CD3^+$ T cells, $CD3^+CD4^+$ T helper and $CD3^+CD8^+$ cytotoxic T cells of lineage(CD11b, CD14, CD15, CD19, CD56)$^-$live/dead$^-$ cells. ►

D Fractions of T-cell subsets (naïve T cells (T_N), central memory T cells (T_{CM}), effector memory T cells (T_{EM}), terminally differentiated effector memory T cells re-expressing CD45RA (T_{EMRA})) of each $CD3^+CD4^+$ (upper row) and $CD3^+CD8^+$ (lower row) T cells. Bar diagrams display mean ±SEM, each dot representing one individual. Mann-Whitney rank sum-test was performed to test for statistical significance, ns = p≥0.05, * = p<0.05, ** = p<0,01, *** = p<0.005.

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3.3.2 *Similar PD-1 expression in MM-patients and HDs*

To address phenotypic characteristics of T cells in MM-patients that might show influence of impaired DC function on T cells and as well predict T-cell dysfunction, the expression of PD-1 (= CD279) as part of the PD-1-PD-L1-immunomodulatory axis was analyzed.

Comparing the median expression of PD-1 on the different T-cell subsets, significant differences between HDs and MM-patients could neither be found for $CD4^+$ nor for $CD8^+$ subsets (Figure 6). Merely for T_{EMRA} subsets, there was a slight trend to lower expression of PD-1 in MM-patients with a mean fold change of 0.79±0.30 for $CD4^+$ T cells and 0.84±0.31 for $CD8^+$ T cells in comparison to HDs.

Generally, PD-1 expression on T cells was comparable in MM-patients and HDs.

Find Figure 6A on this page and Figure 6B and legend on page 43.

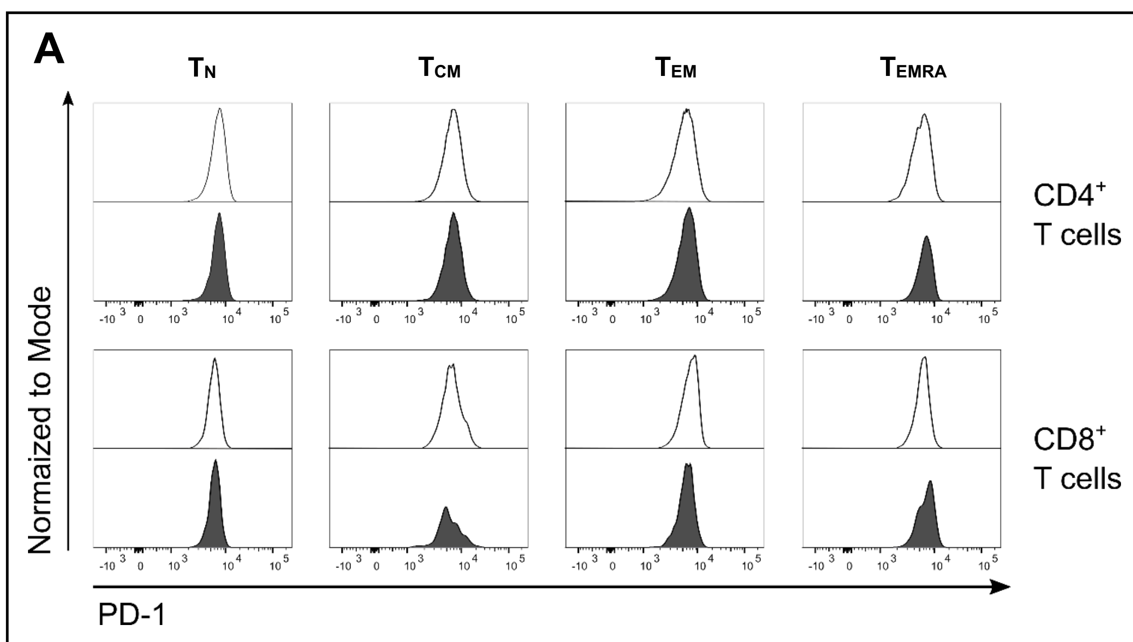


Figure 6 – Similar PD-1 expression on T-cell subsets in multiple myeloma-patients and healthy donors

Peripheral blood mononuclear cell (PBMCs) from healthy donors (HDs) and multiple myeloma-(MM-)patients were analyzed for T-cell subsets by flow cytometry. Programmed cell death protein-1 (PD-1) expression was detected as median of measured fluorescence. T-cell subsets were gated as shown in Figure 5A. **A** The histograms show PD-1 expression of $CD4^+$ (upper row) and $CD8^+$ subsets (lower row) of one representative MM-patient (lower histograms in each graph, grey) and one representative HD (upper histograms, black/white) **B** The bar charts show mean fold change ±SEM of PD-1 expression in MM-patients (n=19) compared to HDs (n=22). Each dot represents at least ten measured events of one individuum. Lower subset counts were excluded from statistical data analysis. Mann-Whitney rank sum-test was performed to test for statistical significance with ns = $p \geq 0.05$. T_N = naïve T cells; T_{CM} = central memory T cells; T_{EM} = effector memory T cells; T_{EMRA} = terminally differentiated effector memory T cells re-expressing CD45RA.

3.3.3 *Unsupervised gating by CITRUS-algorithm revealed no explicit differences in CD4$^+$ T cells and supported key findings of manual gating for CD8$^+$ T cells*

To analyze flow cytometry data of T-cell subsets in the peripheral blood, a second approach was applied. The CITRUS algorithm was used to cluster cells in an automated, unsupervised manner. This proceeding is helpful to overcome bias through subjective expectations of the investigator as well as to detect unexpected results which might not be obvious in manual gating strategies, e. g. because of the restricted possibilities to display the high dimensionality of data. CD4$^+$ and CD8$^+$ T cells were analyzed separately in this approach. Data of both CD4$^+$ and CD8$^+$ T cells is presented in two figures each. The first figure displays the clustering made by the algorithm. Here, pre-gated CD4$^+$ or CD8$^+$ cells were grouped together according to their marker expression. Each upstream cluster contains all downstream clusters (which means, that in the central cluster, all events are present). By providing the algorithm the information which cells originate from HDs and which from MM-patients, predictive differences in the dataset are determined by the algorithm. All clusters for which differences between the two groups were found, are displayed in red in Figure 7A and Figure 8A. Connected clusters with differences are grouped by a blue background area. In the second part of the figure (Figure 7B and Figure 8B), relative expressions of different markers are shown based on the same clustering hierarchy. By combining information of both figures, cluster which are predictive for either HDs or MM-patients can be identified and further characterized by their marker expression.

For CD4$^+$ T cells, predictive differences were found at seven areas of the CITRUS map: three single clusters (94456 at top-center, 94439 on the left-hand side and 94498 at the center) and four groups of clusters (top-left, top-right, bottom-left and bottom-right). Cluster 94498 is located at an early level of the hierarchical tree and both the top-right cluster-group and cluster 94456 are therefore dependent on this cluster.

The abundance of cluster 94498 was increased in MM-patients compared to HDs. For the cluster 94456 and the top-right cluster-group, abundance was increased

in HDs compared to MM-patients. The clusters showed higher expression levels of CD45RA, CCR7, CD27, KLRG-1 and CD57 but lower expression of CD28 and PD-1 on $CD4^+$ T cells. The top-right cluster group also showed higher expression levels of CD45RO and CD27 when compared to cluster 94456.

The bottom-right cluster group was also more abundant in HDs. In general, it showed high expressions of CD45RO, CCR7, CD27, CD28 and KLRG-1. Expression of PD-1 ranged from medium to high levels.

The bottom-left cluster group showed higher abundance in MM-patients. Its phenotype was characterized by high expression of CD45RA, CCR7, CD27, CD57 and medium to high expression of PD-1. CD28 and KLRG-1 were expressed on lower levels.

Cluster 94439 was more abundant in HDs. Its expression profile was comparable to the bottom-left cluster group with higher expression of CD45RO and CD28.

The top-left group was more abundant in HDs. Expression levels differed between the clusters of this cluster group. There were both high expressions of CD45RA and CD45RO for some clusters, CCR7 and KLRG-1 were expressed high for all of the clusters while PD-1 and CD57 were expressed low. For both CD27 and CD28 expression covered wide ranges.

In conclusion, there were no strong differences in the phenotypes of clusters associated to HDs or MM-patients for $CD4^+$ T cells. Nevertheless, in the bottom-left cluster group some clusters presented a PD-1^+, $CD57^+$, $CD28^-$ phenotype, which might match the tendencies of higher T_{EMRA} frequencies in MM-patients in manual analysis. Corresponding clusters to this were also found for the $CD8^+$ T cells (see following section).

Find Figure 7A on this page and Figure 7B and legend on page 47.

A

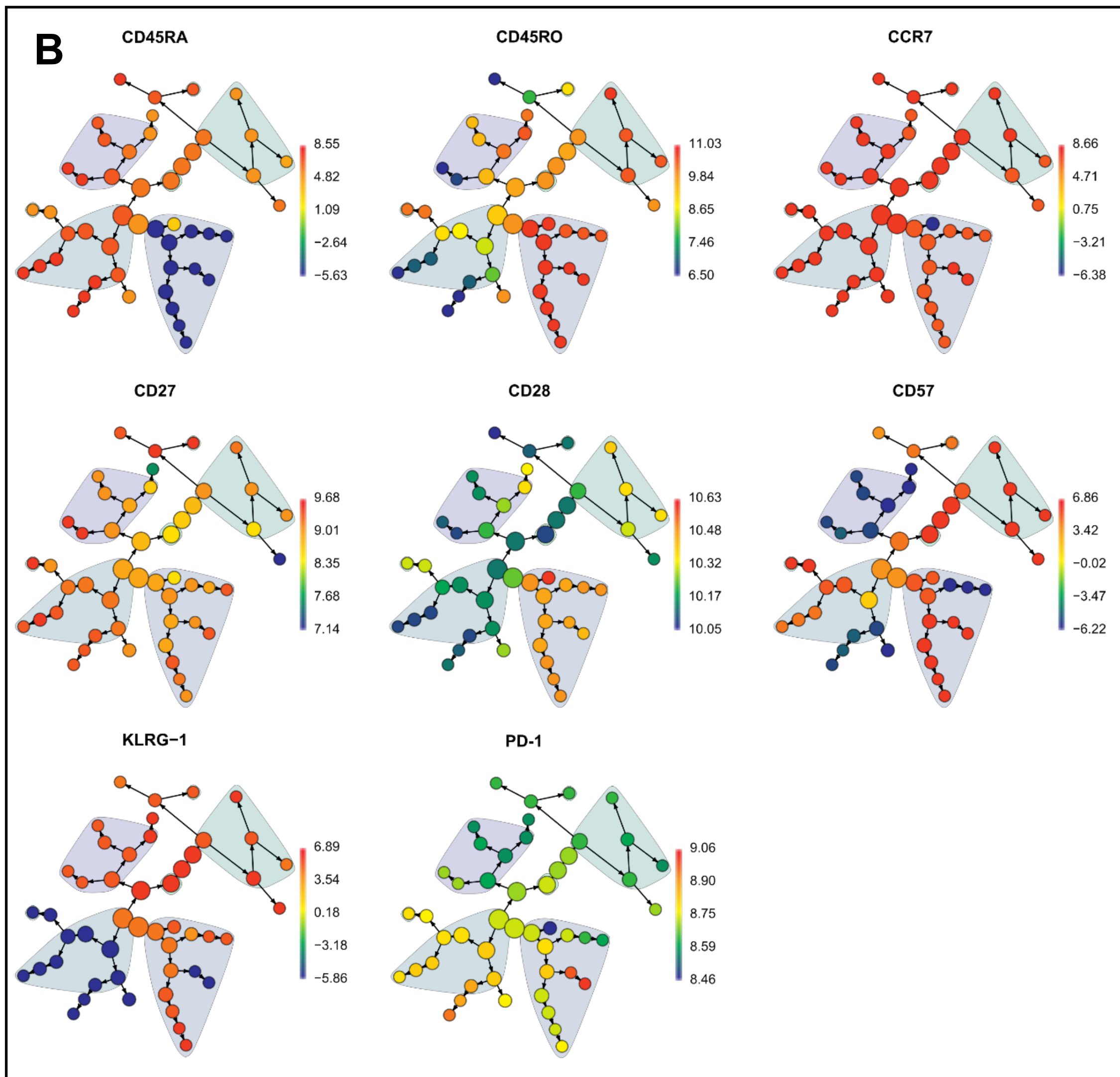


Figure 7 – CITRUS analysis of CD4⁺ T cells

Compensated flow cytometry data of 22 healthy donors (HDs) and 19 multiple myeloma-(MM-)-patients, pre-gated on CD4⁺ T cells, analyzed using the CITRUS algorithm run on the cytobank.org platform. **A** CITRUS map, clustering based on expression of CD45RA, CD45RO, CCR7, CD27, CD28, CD57, killer cell lectin-like receptor, subfamily G-1 (KLRG-1) and programmed cell death protein-1 (PD-1). Clusters showing predictive differences between HDs and MM-patients (pamr-association model) are colored red. **B** Median expression of markers within the different clusters of the CITRUS map.

For CD8^{+} T cells, the CITRUS map revealed predictive differences at five areas of the map. These are cluster 7952 (at the top), the grouped clusters 7993 and 7991 (at the center), the grouped clusters 7990 and 7989 (right below the previous mentioned), the bottom-right group (clusters 7977, 7944 and 7918) and the bottom-left group (clusters 7956, 7953 and 7947).

Cluster 7952 was more abundant in HDs. Its expression was characterized by medium to high levels of CD45RA, CD45RO and CCR7, CD27, CD28 and KLRG-1 while PD-1 and CD57 were expressed on lower levels.

The cluster group 7993/7991 also was more abundant in HDs. Its expression profile showed high expressions of CD45RO (esp. for cluster 7991), CCR7, CD28 and KLRG-1. CD45RA was expressed on a medium level in cluster 7993 while expression was lower for cluster 7991. CD27 was expressed on a medium level. CD57 and PD-1 were both expressed on medium levels, both showing a higher expression in cluster 7991.

The abundance of cluster group 7990/7989 was higher in MM-patients. Expression was high for CD45RA, CCR7 and KLRG-1. CD45RO, CD28, and CD57 were expressed on medium levels, whereas CD27 and PD-1 were expressed on lower levels.

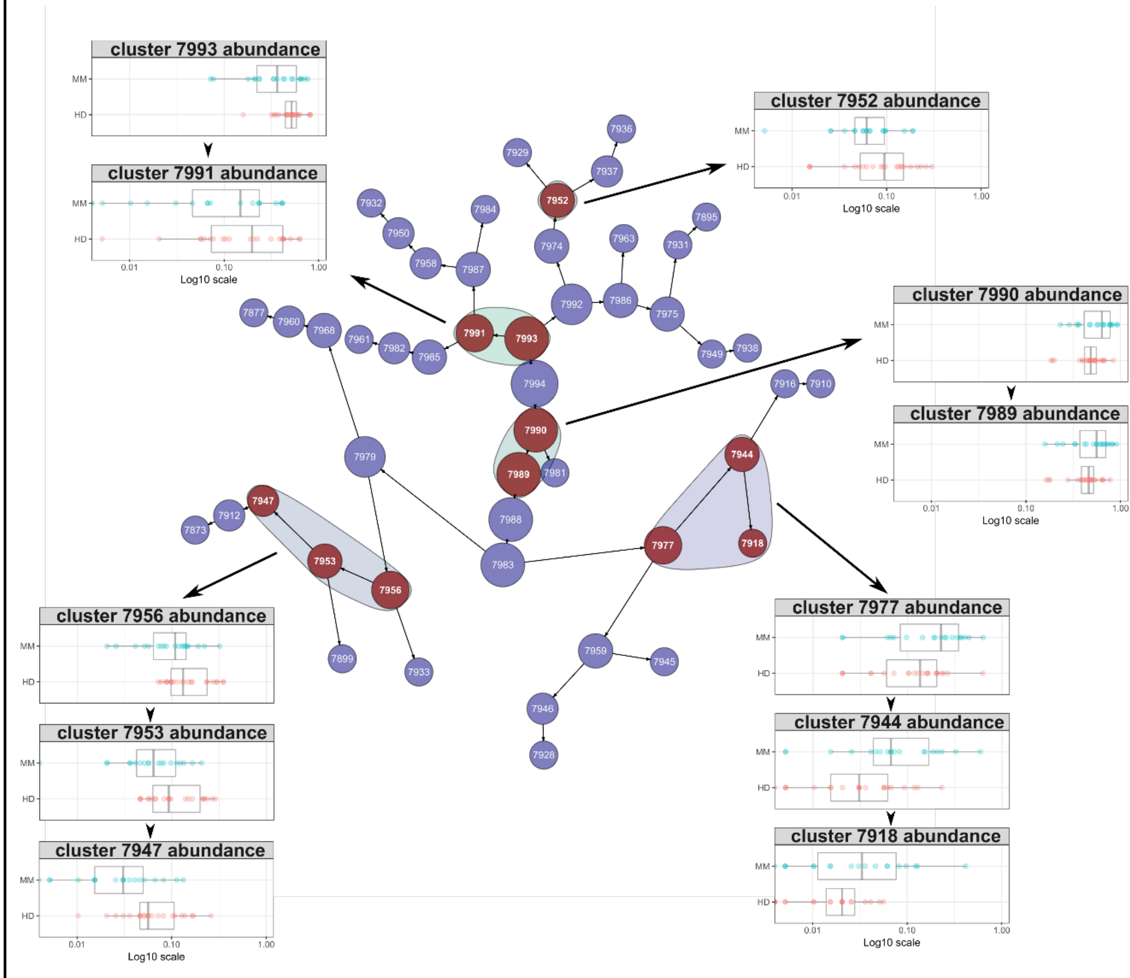
The bottom-right group showed higher abundance in MM-patients. The phenotype was characterized by a high expression of CD45RA, CCR7, CD57 and KLRG-1. CD45RO and PD-1 were expressed on medium to high levels. CD27 and CD28 were expressed lower.

The bottom-left group was more abundant in HDs. Expression of CD45RA and CD45RO, CCR7, CD27, CD28 and KLRG-1 were high. CD57 was expressed on a medium level, PD-1 was expressed low.

Summing up the CITRUS results for the CD8^{+} T cells, differences between HDs and MM-patients in the bottom-right group and the bottom-left groups were most prominent. Differences for the abundance of cells were rather strong for those cluster groups. The bottom-right group was represented stronger in MM-patients. The phenotype indicates that these T cells might be less effective players in

immune responses: CD27 and CD28 were expressed low, PD-1 was expressed on a medium level that was not found very much in the map. Expression of CD57 and KLRG-1 was among the strongest for all clusters of the map. Compared to that, the bottom-left cluster (more abundant in HDs) represented a more favorable phenotype for immune responses of T cells. CD27 and CD28 were expressed much stronger, PD-1 was expressed on lower levels. Also, CD57 was expressed on lower - yet high - levels. These results correspond to the findings of manual gating, where $CD8^+$ T_{EM} cells were reduced in MM-patients and $CD8^+$ T_{EMRA} cells showed trends to increased frequencies.

Find Figure 8A on page 50 and Figure 8B and legend on page 51.


A
cluster 7993 abundance
cluster 7991 abundance
cluster 7952 abundance
cluster 7990 abundance
cluster 7989 abundance
cluster 7956 abundance
cluster 7953 abundance
cluster 7947 abundance
cluster 7977 abundance
cluster 7944 abundance
cluster 7918 abundance
MM
HD
Log10 scale

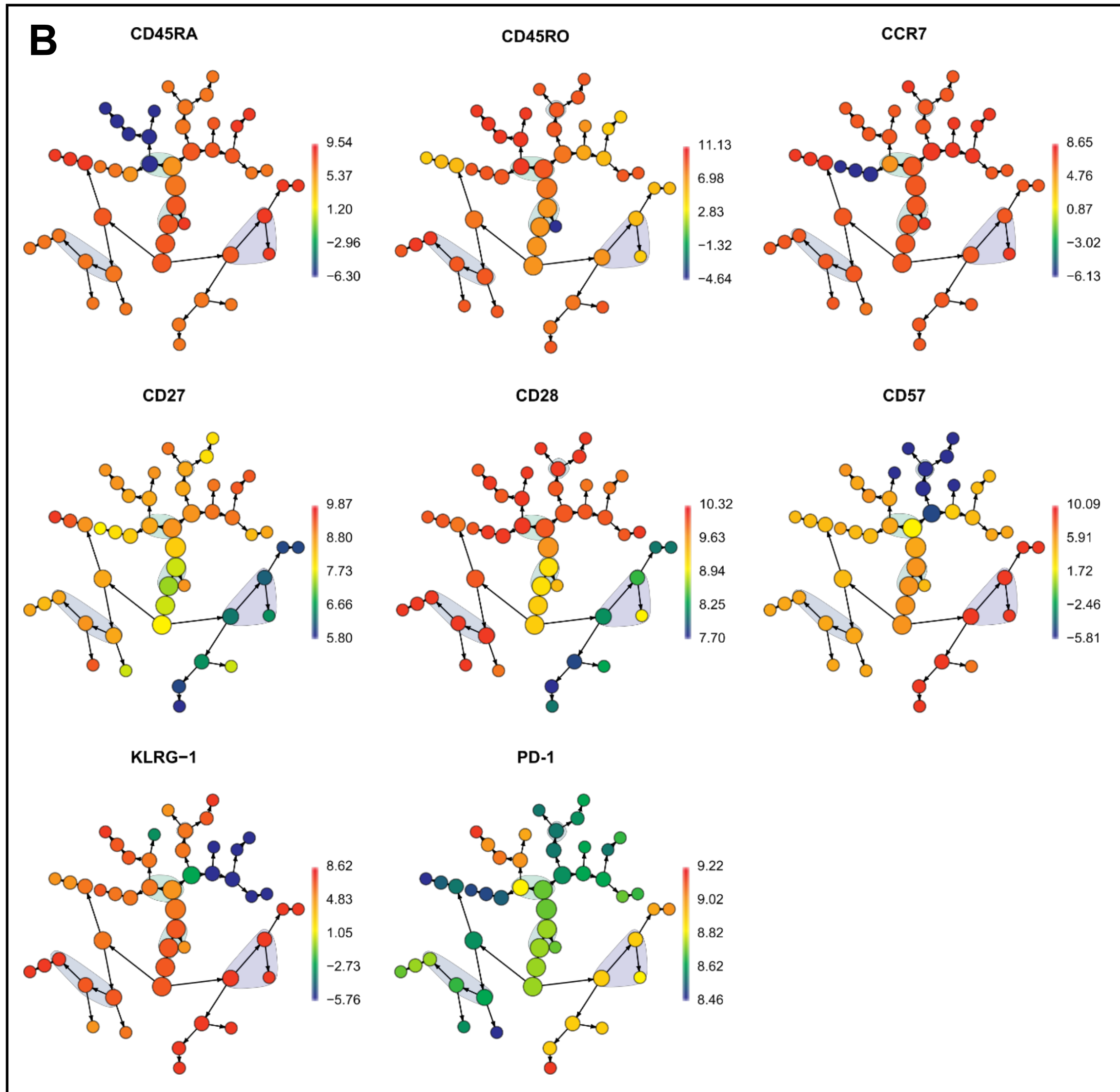


Figure 8 – CITRUS analysis of CD8$^+$ T cells

Compensated flow cytometry data of 22 healthy donors (HDs) and 19 multiple myeloma-(MM-)-patients, pre-gated on CD8$^+$ T cells, analyzed using the CITRUS algorithm run on the cytobank.org platform. **A** CITRUS map, clustering based on expression of CD45RA, CD45RO, CCR7, CD27, CD28, CD57, killer cell lectin-like receptor, subfamily G-1 (KLRG-1) and programmed cell death protein-1 (PD-1). Clusters showing predictive differences between HDs and MM-patients (pamr-association model) are colored red. **B** Median expression of markers within the different clusters of the CITRUS map.

3.4 MM-Bone marrow extracellular fluid influenced the differentiation of CD34^{+}-progenitors to DCs with higher numbers of cDCs and phenotypical changes

3.4.1 Cultivation of CD34^{+}-progenitors in the presence of MM-BMEF led to higher numbers of cDCs compared to HD-BMEF

To analyze factors which possibly influence the differentiation from CD34^{+}-progenitors to DCs, progenitor cells of HDs were exposed to BMEF of either HDs or MM-patients in a 7-day-culture-assay. After purification by FACS, CD34^{+}-progenitors were cultured in the presence of murine MS-5-cells, GM-CSF, SCF and FLT3L. BMEF containing soluble factors present in the BM of HDs or MM-patients was added to the culture system. At day 7, flow cytometry was used to determine DC subpopulations in the culture output. Gating was performed according to Figure 9A, cell numbers were determined by flow cytometry.

Numbers of CD45^{+} cells differentiated from CD34^{+}-progenitors were higher in the presence of BMEF compared to cultivation without BMEF (dotted line). When exposed to BMEF of HDs their number was 2-fold higher compared to BMEF of MM-patients (Figure 9B).

Next, numbers and frequencies of the DC-subsets defined as

- cDC1s: CD45^{+}live/dead^{-}CD66b^{-}CD3^{-}CD20^{-}CD56^{-}HLA-DR^{+}CD141^{+},
- cDC2s: CD45^{+}live/dead^{-}CD66b^{-}CD3^{-}CD20^{-}CD56^{-}HLA-DR^{+}CD141^{-}CD1c^{+} and
- pDCs: CD45^{+}live/dead^{-}CD66b^{-}CD3^{-}CD20^{-}CD56^{-}HLA-DR^{+}CD141^{-}CD14^{-}CD1c^{-}CD123^{+}CD303^{+}

were analyzed (Figure 9C).

cDC1-numbers and frequencies were significantly increased with exposure to MM-BMEF compared to HD-BMEF, being on a medium level when cultured without BMEF (dotted line).

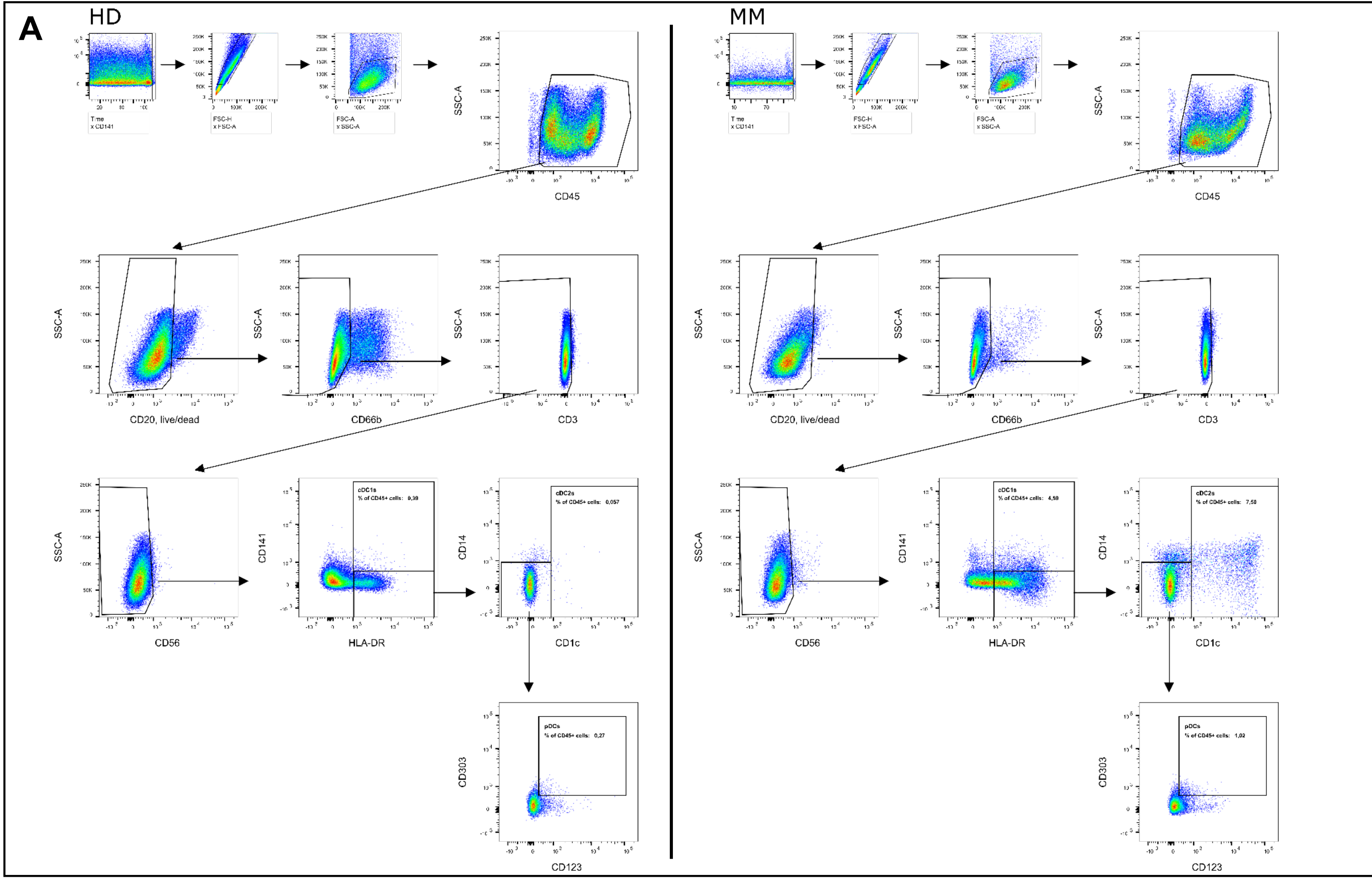
For the cDC2-subset, numbers and frequencies were lower under exposure to both HD- and MM-BMEF than without BMEF, but both numbers and frequencies

of the cDC2-subset were significantly higher when cultured in the presence of MM-BMEF compared to HD-BMEF.

For the pDC-subset, numbers were slightly higher after exposure to HD-BMEF compared to MM-BMEF, while without exposure to BMEF, numbers were in between. Yet, no similar difference between MM-BMEF and HD-BMEF was found for the pDC-frequencies of $CD45^+$ cells, as numbers of those $CD45^+$ cells were also higher under this condition. Frequencies of pDCs were lower under exposure to both MM- and HD-BMEF compared to no BMEF.

While no significant differences were found for pDCs, both cDC-subsets showed significantly higher numbers and frequencies cultured in the presence of MM-patients' BMEF compared to HD's BMEF.

Find Figure 9A on page 54 and Figure 9B, C and legend on page 55.


A
HD
MM
Time x CD141
FSC-H x FSC-A
FSC-A x SSC-A
SSC-A
CD45
CD20, live/dead
CD66b
CD3
CD56
CD141
HLA-DR
CD14
CD1c
CD303
CD123
cDC1s % of CD45+ cells: 0,39
cDC2s % of CD45+ cells: 0,057
pDCs % of CD45+ cells: 0,27
cDC1s % of CD45+ cells: 4,59
cDC2s % of CD45+ cells: 7,59
pDCs % of CD45+ cells: 1,02

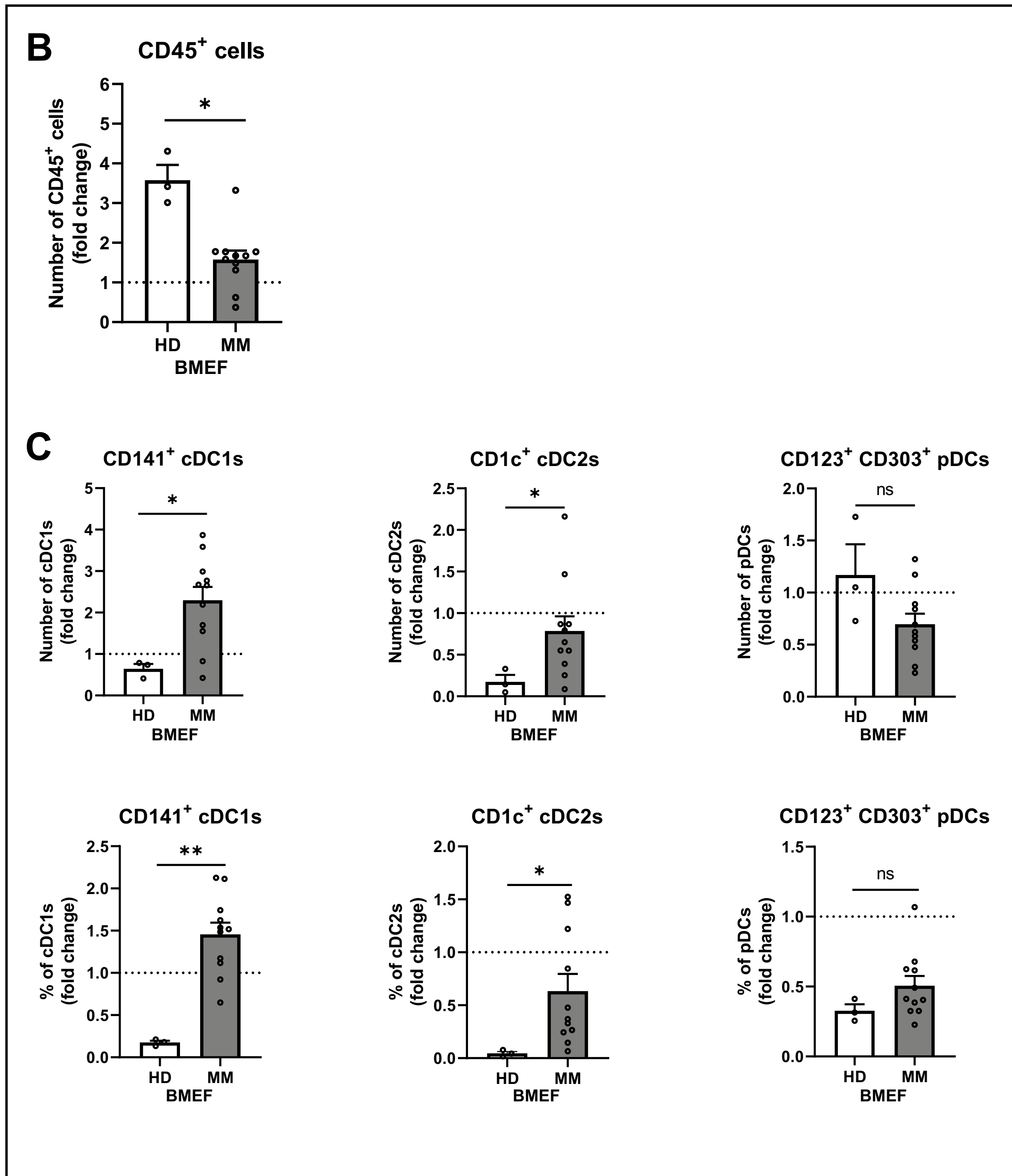


Figure 9 – cDCs were increased in number and frequency when cultured in the presence of bone marrow extracellular fluid from multiple myeloma-patients

CD34⁺-progenitors from healthy donors (HDs) purified by magnetic activated cell sorting (MACS) and fluorescence activated cell sorting (FACS) were cultured for seven days in the presence of bone marrow extracellular fluid (BMEF) of HDs (n=3) or multiple myeloma-(MM-)patients (n=11). **A** Gating of CD45⁺ cells and dendritic cell-(DC-)subsets. **B** Count of CD45⁺ cells after seven days of culture as fold change of control containing no BMEF (dotted line). **C** Count (upper diagrams) and proportion of CD45⁺ cells (lower diagrams) of the three DC-subsets as fold change of control containing no BMEF (dotted line). Each dot represents triplicates of one individual. ►

Mann-Whitney rank sum-test was used to test for statistical significance with ns = $p \geq 0.05$, * = $p<0.05$ and ** = $p<0.01$. cDC1 = conventional/classical dendritic cell type 1, cDC2 = conventional/classical dendritic cell type 2; pDC = plasmacytoid dendritic cell.

3.4.2 Cultivation of CD34⁺-progenitors in the presence of MM-BMEF led to an altered phenotype of DCs compared to HD-BMEF

To examine the possible influence of soluble components on the phenotype of DCs differentiated from $CD34^+$-progenitors in culture, the expressions of HLA-DR (Figure 10A) and each subset-defining marker (Figure 10B) were analyzed for cDC1s, cDC2s and pDCs separately.

All subsets showed lower expression of HLA-DR cultured in the presence of BMEF compared to absence of BMEF (dotted line). Furthermore, expression of HLA-DR was significantly higher cultured in the presence of MM-BMEF compared to HD-BMEF. This was strongest in cDC2s showing a 4-fold, followed by cDC1s (2-fold) and pDCs (1.4-fold).

cDC1s had similar expressions of the subset-defining marker CD141 cultured in absence of BMEF as well as in the presence of HD- and MM-BMEF.

The subset-defining marker CD1c was expressed significantly higher by a 7-fold on cDC2s after culture in the presence of MM-BMEF compared to HD-BMEF, yet both lower than cultured in absence of BMEF (dotted line).

For the pDC-subset, expression of CD303 was lower when cultured in the presence of BMEF compared to no BMEF, further on there was a slight, but significantly lower expression of CD303 when cultured in the presence of MM-BMEF compared to HD-BMEF. Levels of CD123-expression were on similar levels in absence of BMEF and the presence of both MM- and HD-BMEF.

HLA-DR-expression was significantly higher for all three subsets after culture in the presence of MM-BMEF compared to HD-BMEF. Also, the CD1c-expression was higher for the cDC2-subset after presence of MM-BMEF and CD303-expression was slightly decreased for the pDC-subsets, each time compared to HD-BMEF.

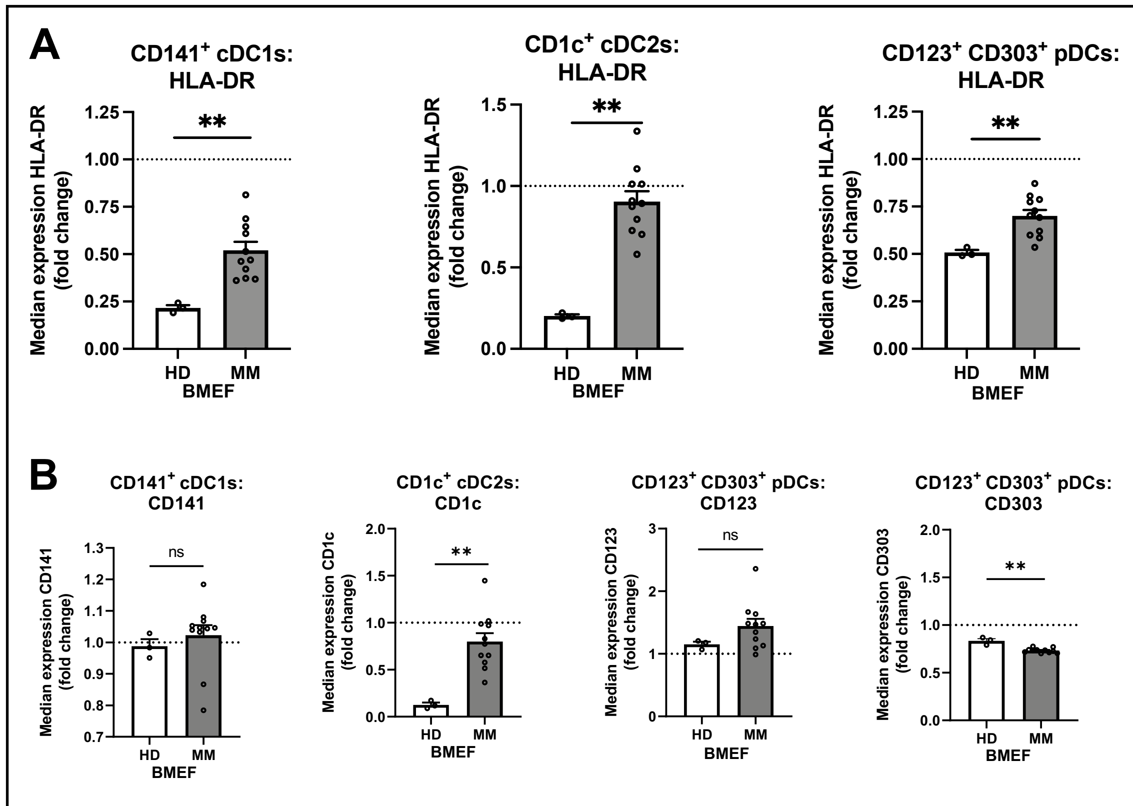


Figure 10 – Alterations in phenotype of cDC2s and pDCs after cultivation in the presence of bone marrow extracellular fluid from multiple myeloma-patients compared to bone marrow extracellular fluid from healthy donors

CD34$^+$-progenitors from healthy donors (HDs) purified by magnetic activated cell sorting (MACS) and fluorescence activated cell sorting (FACS) were cultured for seven days in the presence of bone marrow extracellular fluid (BMEF) of HDs (n=3) or MM-patients (n=11). **A** Median expression of human leukocyte antigen - DR isotype (HLA-DR) on the different dendritic cell-(DC-)subsets as fold change of control (CD34$^+$ cells of HDs) containing no BMEF (dotted line). **B** Median expression of subset-defining markers for the different DC-subsets as fold change of control containing no BMEF (dotted line). Each dot represents triplicates of one individual. Mann-Whitney rank sum-test was used to test for statistical significance with ns = $p \geq 0.05$, * = $p<0.05$ and ** = $p<0.01$. cDC1 = conventional/classical dendritic cell type 1, cDC2 = conventional/classical dendritic cell type 2; pDC = plasmacytoid dendritic cell.

3.4.3 *Interleukin-6 was strongly increased in BMEF of HDs*

To analyze differences in the BMEF between MM-patients and HDs an IL-6-ELISA was performed. The ELISA revealed significant differences in IL-6 levels in the BMEF of HDs and MM-patients (Figure 11). For HDs, the mean of five donors equals 7,531±2,020 pg/ml undiluted BMEF, for six samples of MM-patients a mean of 1.986±1.755 pg/ml was measured.

Values of IL-6-levels in HD-BMEF were significantly increased by 3,800-fold in comparison to MM-BMEF.

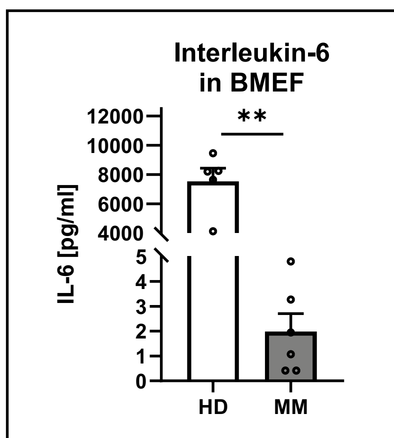


Figure 11 – Interleukin-6-levels were increased in bone marrow extracellular fluid of healthy donors
Bone marrow extracellular fluids (BMEFs) of 1:2.5 phosphate-buffered saline-(PBS-)diluted bone marrow samples were thawed; samples of healthy donors (HDs) were further diluted 1:20 and 1:40 with assay diluent. Interleukin-6-(IL-6-)ELISA was performed as stated in section 2.4. Bar charts displays mean ±SEM, each dot representing technical quadruplicates for HDs and technical duplicates for multiple myeloma-(MM-)patients of each one of five individuals for HDs and one of six individuals for MM-patients. Mann-Whitney rank sum-test was performed to test for statistical significance, * = $p<0.05$, ** = $p<0.01$.

3.5 MM-PB-serum impaired the differentiation of CD34$^+$-progenitors to DCs

3.5.1 Cultivation of CD34$^+$-progenitors in the presence of MM-PB-serum led to reduced numbers of cDC1s and pDCs compared to HD-PB-serum

To further analyze the influence of soluble factors on the differentiation of CD34$^+$-progenitors to DCs, progenitors were cultured in an equivalent 7-day-differentiation assay under exposure to PB-serum of either HDs or MM-patients. After purification, CD34$^+$-progenitors from HDs were cultured in the presence of murine MS-5-cells, GM-CSF, SCF and FLT3L. PB-serum of either MM-patients or HDs was added. Gating of cells, harvested at day 7, was performed as shown in Figure 9A (page 54).

The number of CD45$^+$ cells was significantly lower by nearly 2-fold after culture in the presence of MM-serum, compared to HD-serum. In comparison to absence of serum (dotted line), numbers were higher in the presence of HD-serum while lower in the presence of MM-serum (Figure 12A).

When analyzing numbers of the different DC-subsets separately (Figure 12B), two differences occurred. Numbers of cDC1s were significantly lower by nearly factor three after being cultured with MM-serum, compared to both HD-serum and absence of serum. Likewise, numbers of pDCs were lowered more than 2-fold when exposed to MM-serum, compared to HD-serum. In comparison to absence of serum, pDC numbers were 2-fold higher being cultured in the presence of HD-serum and slightly lower in the presence of MM-serum. There were similar trends in the proportion of those subsets of all CD45$^+$ cells, showing no significance: While frequencies of cDC1s were lower after culture in the presence of both HD- and MM-serum, compared to absence of serum, in pDCs the frequencies were higher cultured in the presence of HD- and MM-serum, compared to absence of serum.

For the cDC2-subset there was a trend contrary to the one described above showing no significance; both number and frequency were increased after being

cultured in the presence of MM-serum compared to HD-serum, yet both number and frequency lower than in absence of serum.

While after cultivation in the presence of MM-serum numbers of cDC1s and pDCs were reduced significantly in comparison to HD-serum, cDC2s showed an opposite trend.

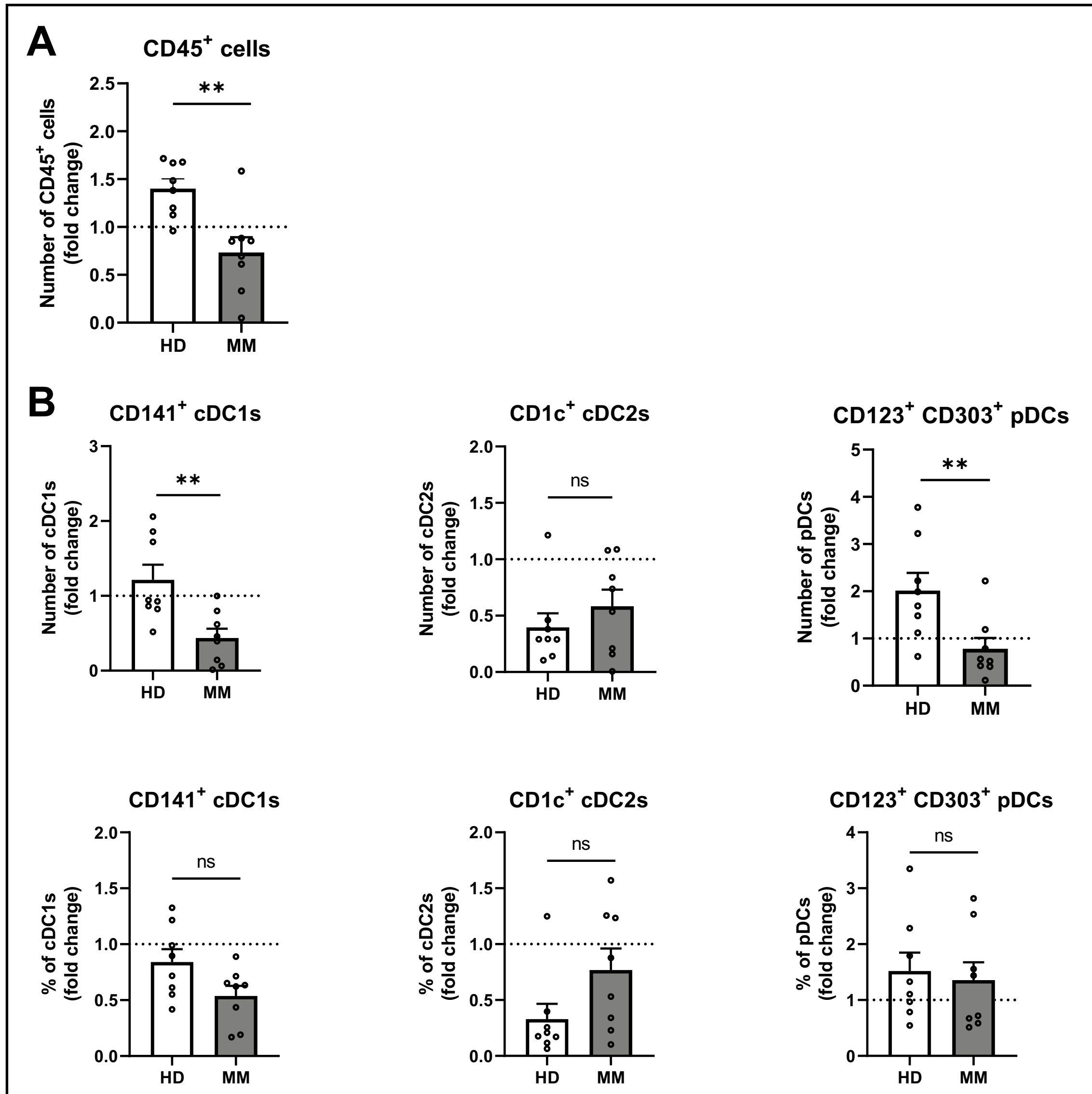


Figure 12 – Less cDC1s and pDCs after culture in the presence of peripheral blood-serum from multiple myeloma-patients compared to peripheral blood-serum from healthy donors

CD34$^+$-progenitors from healthy donors (HDs) purified by magnetic activated cell sorting (MACS) and fluorescence activated cell sorting (FACS) were cultured for seven days in the presence of peripheral blood-(PB-)serum of HDs (n=8) or multiple myeloma-(MM-)patients (n=8). **A** Count of CD45$^+$ cells after seven days of culture as fold change of control containing no PB-serum (dotted line). **B** Count (upper diagrams) and proportion of CD45$^+$ cells (lower diagrams) of the three dendritic cell-(DC-)subsets as fold change of control containing no PB-serum (dotted line). Each dot represents triplicates of one individual. Mann-Whitney rank sum-test was used to test for statistical significance with ns = $p \geq 0.05$, * = $p<0.05$ and ** = $p<0.01$. cDC1 = conventional/classical dendritic cell type 1, cDC2 = conventional/classical dendritic cell type 2; pDC = plasmacytoid dendritic cell.

3.5.2 Cultivation of $CD34^+$-progenitors in the presence of MM-PB-serum led to lower expression of CD141 in cDC1s compared to HD-PB-serum

Further on, the phenotype of the DC-subsets was analyzed to uncover possible influences of PB-serum of MM-patients, compared to HDs.

There were no significant differences in HLA-DR-expression between culture in the presence of PB-serum from HDs and MM-patients, yet expression was lower in both conditions than after culture without PB-serum (dotted line), (Figure 13A).

For the cDC1-subset, the expression of the subset-defining marker CD141 was approximately 0.75-fold lower being cultured with MM-serum, compared to HD-serum. Expression after culture in the presence of MM-serum was similar to no PB-serum (Figure 13B).

For the other subset-defining markers (CD1c for cDC2s, CD123 and CD303 for pDCs), there were no significant differences between culture in the presence of HD-serum and MM-serum: here, all marker-expressions were on comparable levels. Expression of CD1c on cDC2s and expression of CD303 on pDCs were lower when cultured with PB-serum, compared to culture without PB-serum, for expression of CD123 this was inverse (Figure 13B).

After differentiation under exposure to MM-serum, cDC1s showed reduced expression of CD141 compared to HD-serum, while for HLA-DR and the other subset-defining markers (CD1c, CD123, CD303) no differences in expression were found in comparison of culture with HD- and MM-PB-serum.

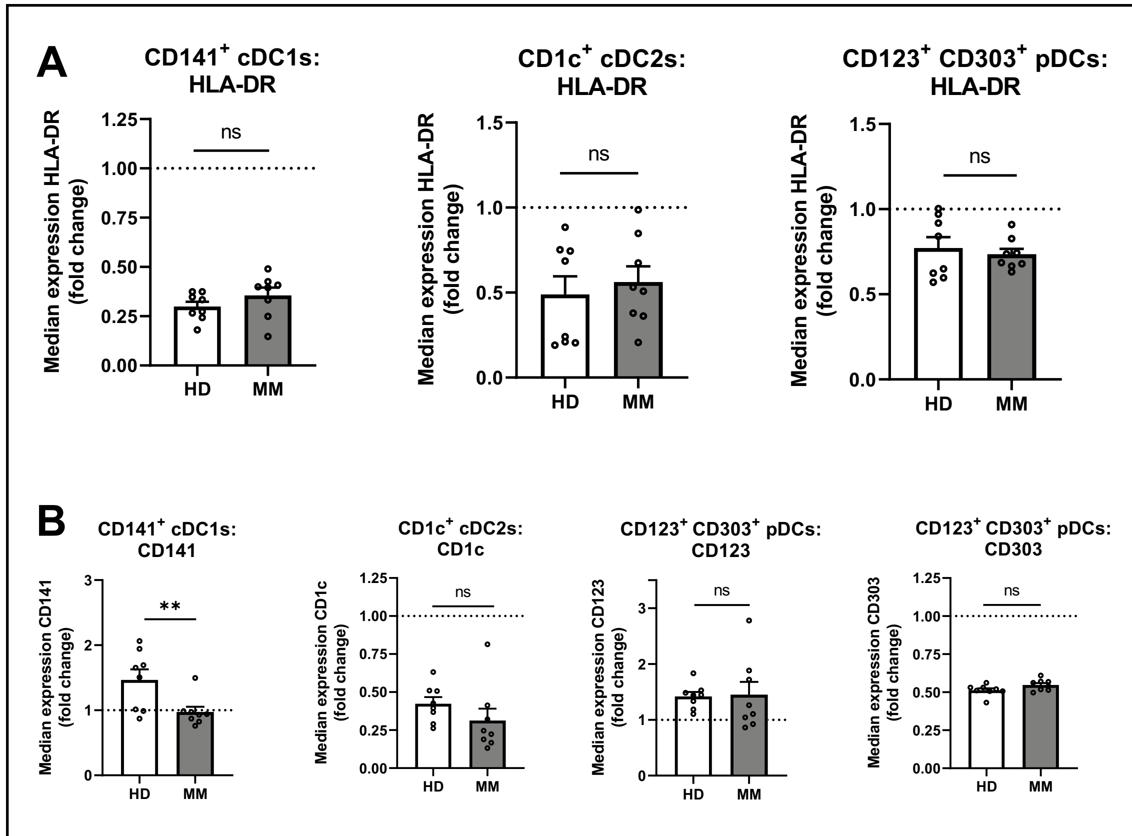


Figure 13 – Expression of CD141 was decreased on cDC1s being cultured in the presence of peripheral blood-serum from multiple myeloma-patients, compared to serum from healthy donors

CD34⁺-progenitors from healthy donors (HDs) purified by magnetic activated cell sorting (MACS) and fluorescence activated cell sorting (FACS) were cultured for seven days in the presence of peripheral blood-(PB-)serum of HDs (n=8) or multiple myeloma-(MM-)patients (n=8). **A** Median expression of human leukocyte antigen - DR isotype (HLA-DR) on the different dendritic cell-(DC-)subsets as fold change of control containing no PB-serum (dotted line). **B** Median expression of subset-defining markers for the different DC-subsets as fold change of control containing no PB-serum (dotted line). Each dot represents triplicates of one individual. Mann-Whitney rank sum-test was used to test for statistical significance with ns = $p \geq 0.05$, * = $p<0.05$ and ** = $p<0.01$. cDC1 = conventional/ classical dendritic cell type 1, cDC2 = conventional/classical dendritic cell type 2; pDC = plasmacytoid dendritic cell.

3.5.3 Similar IL-6 levels in blood serum of HDs and MM-patients

To investigate differences in blood serum of MM-patients and HDs, an IL-6-ELISA was performed (Figure 14). No significant different results were observed with healthy controls showing a mean of 6.78±3.07 pg/ml IL-6 compared to a mean of 5.02±4.39 pg/ml in MM-patients.

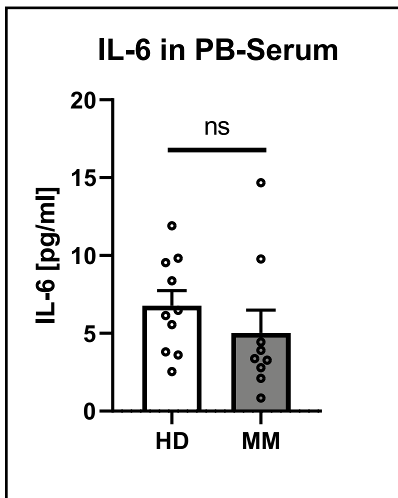


Figure 14 – Slight trends to increased interleukin-6-values in peripheral blood of healthy donors compared to multiple myeloma-patients

Peripheral blood-(PB-)serum of multiple myeloma-(MM-)patients and healthy donors (HDs) was gained through blood drawing. Interleukin-6-(IL-6-)ELISA was performed as stated in section 2.4. Bar charts display mean ±SEM, each datapoint representing technical triplicates for HDs and MM-patients. Biological n=10 for HDs and n=9 for MM-patients. Mann-Whitney rank sum-test was performed to test for statistical significance, ns = $p \geq 0.05$.

4 Discussion

Within this present thesis, different aspects of DC differentiation and function in patients suffering from MM have been explored. In this context, DCs and T cells from PB have been analyzed, as well as *in vitro* differentiation of $CD34^+$-progenitors to DCs. Previous work has revealed several alterations in MM-patients yet being not in full agreement and with questions remaining unanswered.

The comparison of the analyzed WBC- and lymphocyte counts between MM-patients and HDs revealed no significant differences. Mean values of both HDs and MM-patients were located within the physiological range of about 4,000/µl to 10,000/µl. Even though leukopenia as a manifestation of hematopoietic insufficiency appears in MM-patients principally (Christen et al. 2017, DGHO Deutsche Gesellschaft für Hämatologie und Medizinische Onkologie e.V. 2018), the MM-patients examined in this thesis were not affected severely. Further on, the comparable lymphocyte counts indicate that there is no general quantitative impairment in the proliferation of lymphocytes.

4.1 MM-patients have lower DC numbers with an immunosuppressive phenotype

Hitherto, a general reduction of DCs in the PB of MM-patients has been described by several groups (Ratta et al. 2002, Do et al. 2004, Martin-Ayuso et al. 2008). Yet, different DC-subsets in MM-patients have not been analyzed in detail. Within this thesis, separate analysis of DC-subsets in the PB of MM-patients was performed.

cDC1s, cDC2s and pDCs were reduced highly significant in MM-patients in both their count per ml whole blood and in their proportion of all $lineage^-$live cells. infDCs were only slightly reduced. Furthermore, $CD14^+$ classical monocytes showed reduced frequencies of $lineage^-$live cells in MM-patients. In contrast, numbers of $CD14^+CD16^+$ intermediate and $CD16^+$ non-classical monocytes were comparable in MM-patients and HDs. These results indicate that the impairment in DC abundance affects the DC-subsets cDC1s, cDC2s and pDCs equally. This is consistent with previously reported results of pDCs and cDCs showing similar

impairments (Leone et al. 2015). For those cell subsets a (partly) shared differentiation pathway is known (Lee et al. 2015, See et al. 2017).

For infDCs, results go along with alterations of $CD14^+$ classical monocytes, which points out their closer relationship. These results also suggest that infDCs could possibly descent at least in parts from $CD14^+$ classical monocytes at sites of inflammation (Guilliams and van de Laar 2015).

For a better understanding of the relations between monocytes, infDCs, DC-subsets and probably even osteoclasts, a detailed characterization of the cellular components in the TME of the BM of MM-patients could deliver valuable insights. The performed analysis of DCs from PB gives information about a general DC-impairment, though.

Different alterations of the DC phenotype in MM-patients have been described by different authors. It remains unclear if MHC-II and co-stimulatory molecules are expressed on higher (Martin-Ayuso et al. 2008), comparable (Brown et al. 2001) or lower (Ratta et al. 2002, Brimnes et al. 2006, Tucci et al. 2011b) levels.

Within this thesis, expressions of CD40, CD80, CD86, HLA-DR, CCR7 and PD-L1 were analyzed. The expressions of CD80, CD86 and the proportion of CCR7 positive cells were almost on the same level for all DC-subsets in MM-patients and HDs. For CD80 and CD86 this is consistent with the work of Brown et al. (2001) which showed similar results and further on a reduced upregulation of CD80 and CD86 in DCs from MM-patients after stimulation. In contrast Martin-Ayuso et al. (2008) showed higher expression of CD86. Further, Brimnes et al. (2006) showed higher expression of CD86 and lower expression of CCR7 while Ratta et al. (2002) and Tucci et al. (2011b) reported lower expression of CD80 in MM-patients. As the work of Brown et al. (2001) suggested the relevance of stimulation to reveal possible impairments, these inconsistent results might be due to varying conditions in patients and/or experimental setup. The results in this thesis regarding CD80, CD86 and CCR7 indicate a comparable maturation of DCs from MM-patients and HDs.

In further studies the issue of stimulation needs to be addressed in detail for a better understanding of possible DC dysfunction only apparent in a DC-stimulatory context.

Next, the expression of CD40 was significantly increased in infDCs, cDC2s and pDCs from MM-patients, while cDC1s showed a similar trend. CD40 is part of the CD40-CD40L-axis, which shows high importance for maturation of DCs to proper functioning APCs (van Kooten and Banchereau 2000). For other tumor entities, it has been shown that this CD40-CD40L-axis is crucial for activation of both DCs and T cells (Hillebrand et al. 2019). Marigo et al. (2016) highlighted the additional relevance of the CD40-CD40L-axis for successful T-cell therapies in tumor disease in an EG7 lymphoma mouse model. The higher expression of CD40 on DCs in PB of MM-patients could indicate a disorder in this axis leading to upregulation by a negative feedback-loop. Brown et al. (2001) showed that by stimulation of CD40, DCs from MM-patients did not upregulate CD80 and CD86 sufficiently, compared to HDs. These results support the considerations of an impairment in the CD40-CD40L-axis. Thus, this disorder indicates an alteration in maturation of DCs in MM-patients, compared to HDs.

The expression of HLA-DR was slightly - yet not significantly - lower on all DC-subsets of MM-patients than on those of HDs. This slight trend corresponds with previous results (Ratta et al. 2002, Brimnes et al. 2006), indicating an impaired antigen presentation through DCs in MM. In contrast, Martin-Ayuso et al. (2008) showed higher expression of HLA-DR, considerations concerning this inconsistency are mentioned above.

Analysis of PD-L1 expression revealed significantly higher proportions of PD-L1 positive cells within the infDC-, cDC1- and cDC2-subset. For the pDC-subset a similar trend was observed. Interestingly, the infDC-subset is affected by this alteration in the same way as the cDC-subsets are. Therefore, it is reasonable to suggest that this alteration of increased proportion of PD-L1-positive cells – as well as the increased in CD40 expression – might not depend on the specific differentiation of those subsets as infDCs do not share the pathway of the other subsets. Ray et al. (2015) and Sponaas et al. (2015) reported increased

proportions of PD-L1 on DCs in the BM of MM-patients but Sponaas et al. (2015) reported increased proportions in the PB only for few patients, thus, the results described here are contrary. Sponaas et al. (2015) already highlighted the inter-individual differences of PD-L1-expression in their patient cohort; those differences might also explain the contrary results. Eventually, patients' proportions of PD-L1-high-expressing cells vary depending on their state of disease or other factors. The immune modulatory PD-1-PD-L1-axis plays a crucial role in the regulation of immune responses and is closely linked to immune dysfunction in tumors (Keir et al. 2008). Also, this axis is a promising target for therapies (immune checkpoint blockade) in different tumor diseases (Sunshine and Taube 2015). Nevertheless, blockade of PD-1 through the checkpoint inhibitor nivolumab did not lead to satisfying results in MM-patients yet (Suen et al. 2015, Lesokhin et al. 2016). As T cells are as well important for the success of immune checkpoint blockade, also analysis of T cells might contribute to a better understanding of this therapeutic failure (see below).

To conclude, all DC-subsets are reduced in PB of MM-patients, compared to HDs with a more severe decrease in cDCs and pDCs than in infDCs. The phenotype of DCs is altered in an immunosuppressive way in MM-patients. These alterations might contribute to both impaired anti-MM immune responses and a general immunodeficiency in MM-patients. A closer look on the abundance and phenotype of DCs in the TME of the BM might be a promising approach for further investigations.

4.2 T cells from MM-patients show a shift towards a more senescent phenotype

Besides DCs, also T cells seem to play an important role in tumor evasion of MM (Joshua et al. 2016), while general DC dysfunction might lead to general T-cell dysfunction beyond the borders of anti-tumoral responses.

First, abundance of T cells in PB was analyzed. While the counts per ml whole blood of $CD3^+$ T cells and both $CD4^+$ T helper and $CD8^+$ cytotoxic T cells showed only slight trends to reduced values in MM-patients, the percentages of $CD3^+$

T cells and the $CD4^+$ T helper cell subsets of all $lineage^-$live cells were significantly lower in MM-patients, compared to HDs. For the $CD8^+$ T-cell subset there was a similar trend of reduced percentage. These results could be an indication for impaired activation and clonal expansion of T cells.

Next, T cells were analyzed with a focus on their functional state in T-cell memory. While there were no differences between MM-patients and HDs for T_N and T_{CM} cells, analysis of the T_{EM} and T_{EMRA} subsets revealed noteworthy trends. $CD4^+$ T_{EM} cells showed a trend to reduced abundance in MM-patients, for $CD8^+$ T_{EM} cells, this reduction was significant. In contrast, T_{EMRA} cells showed trends to increased abundance in MM-patients for both the $CD4^+$ and $CD8^+$ subset. These observations of a shift towards the more senescent T_{EMRA} subset go along with previous findings: Zelle-Rieser et al. (2016) showed significantly reduced proportions of $CD8^+$ T_{EM} cells in the PB from MM-patients as well as increased expression of the senescence marker CD57 in $CD8^+$ T cells in MM-patients' BM. Suen et al. (2016) identified T-cell senescence as a major impairment of T cells in MM showing higher expression of CD57 and KLRG-1 as well as a lack of CD27 and CD28 in MM-patients' clonal T cells. While senescence appears physiologically in the context of cellular aging (Larbi and Fulop 2014) it is also most likely that T-cell senescence occurs in the context of tumors and may be induced by tumor cells (Montes et al. 2008, Crespo et al. 2013). Senescent T cells are impaired in their function (Appay et al. 2000, Crespo et al. 2013). This might lead to tumor immune evasion and also general immunodeficiency.

As the PD-1-PD-L1-axis is a central element to modulate T cell driven immune responses through DCs (Freeman et al. 2000, Curiel et al. 2003), PD-1 expression was analyzed on T cells. PD-1 expression was comparable on all T-cell subsets in the PB from MM-patients and HDs. This is, on the one hand, contrary to previous reported results, as Zelle-Rieser et al. (2016) showed a significantly increased PD-1 expression on both $CD4^+$ and $CD8^+$ T cells from both PB and BM of MM-patients. On the other hand, the results shown in this thesis are consistent with other results and concepts. Suen et al. (2016) reported that PD-1- (as well as CTLA-4-) expression on T cells of MM-patients is comparable to HDs. In general, the blockade of the PD-1-PD-L1-axis turned out to be an

effective therapy in different tumor diseases (reviewed in Sunshine and Taube (2015)). However, in MM PD-1-PD-L1-blockade monotherapy showed to be a less promising approach (Armand 2015, Suen et al. 2015) while combined treatment options might indeed lead to new therapeutic options (Jelinek et al. 2018). One reason for the failure of monotherapeutic approaches might be the type of T-cell impairment. While in T-cell exhaustion PD-1 expression is increased and immune checkpoint blockade is effective (Crespo et al. 2013), targeting T-cell senescence with no increase in PD-1 expression is not expected to be successful (Suen et al. 2016). Therefore, the results of a more senescent T-cell phenotype without any alteration in PD-1 expression go mainly along with other authors' results and assumptions.

With an increasing number of parameters analyzed by methods as flow cytometry, reasonable strategies for evaluation of these growing datasets need to be established. At some point, the risks of manual analysis – e. g. overlooking of connections and patterns through information overload – gain relevance. In addition, by using new concepts of data analysis and visualization, known disadvantages like expectation bias can be met (Mair et al. 2016, Saeys et al. 2016, Montante and Brinkman 2019). By the sensible use of automated tools and artificial intelligence, new opportunities to detect correlations in big datasets and interpret those in a meaningful way are accessible. A requirement for the use of automated tools is the steady quality of the acquired data. Within this thesis it was attempted to match those requirements of data quality (amongst others by using 8-peak-calibration beads) and introduce automated data analysis (by applying the CITRUS algorithm on the T-cell dataset). These aims could partly be met. While ensuring steady instrument performance and data quality through calibration worked out well, automated data analysis brought several challenges. Especially the continuous expression of markers like CD45RO and CD45RA led to insufficient separation of cell clusters by different algorithms. This goes along with the reported experiences of other authors as Conrad et al. (2019). Further on, the interpretation of the created visualizations and linkage to current models was a central issue of this approach. Finally, the application of the CITRUS algorithm provided a largely satisfying output. While detected differences

between HDs and MM-patients were rather limited in the analysis of CD4$^+$ T cells, the detected differences in the analysis of CD8$^+$ T cells went along well with the results in manual gating and current knowledge about T cells in tumor disease and MM: the automated approach revealed indications for the above discussed senescence of CD8$^+$ T cells.

In total, analysis of T cells in the blood of MM-patients revealed impairments mainly in form of T-cell senescence. This impairment can be crucial for both anti-tumoral and anti-infectious immune responses in MM-patients. For an advanced understanding of interactions between T cells and DCs in MM – probably leading to this impairment – studying DCs and T cells in the BM-TME in MM-patients will be necessary. In addition, functional assays concerning both proliferative capacity of T cells given their more senescent phenotype as well as the capacity of DCs to stimulate T-cell expansion might deliver useful insights.

4.3 Differentiation of dendritic cells

4.3.1 Influence of BMEF

Cultivation of CD34$^+$-progenitors in the presence of BMEF revealed several differences between BMEF of HDs and MM-patients. While CD45$^+$ cell counts were higher when cultured in the presence of HD-BMEF, cDCs were significantly reduced, compared to MM-patients' BMEF.

The IL-6-ELISA though showed a massive increase of IL-6-levels in the BMEF of HD compared to MM-patients. This sows serious doubts, if the used BMEF was comparable material and therefore suitable to detect MM specific differences. As IL-6-levels are also increased through trauma and surgery (Jawa et al. 2011) and in comparison to MM-BMEF, lower levels of IL-6 would have been expected in HD-BMEF (Kyrstsonis et al. 1996, Gado et al. 2000), it is likely that the observed levels in "healthy" donors do not reflect the physiological situation of "healthy" BMEF. As BMEF of MM-patients was gained through puncture of the iliac crest (a less invasive procedure with less intensive tissue trauma), and BMEF of HDs was gained through open joint surgery (a more invasive procedure with substantial tissue trauma), the measured differences probably reflect differences of those procedures.

For further investigations, a more comparable procedure to gain biological samples is needed, begging the question, if even different iliac crest punctures performed by different personal on different patients are comparable in their level of tissue damage and therefore will lead to comparable samples.

Under those given limitations, the results partly go along with previous publications: cultured in the presence of the HD-BMEF with high IL-6-levels, the numbers of cDC1s were significantly lower than cultured in the presence of MM-BMEF influence and slightly lower than without added BMEF. For cDC2s, numbers were lowest under the influence of HD-BMEF but both under influence of HD- and MM-BMEF lower than without BMEF. Given these varying results for cDC1s and cDC2s, it is very likely that other factors than IL-6 contribute to the impairments in differentiation.

Nevertheless, IL-6 might play a partial role in the impaired differentiation in the presence of HD-BMEF. This influence of IL-6 was also described by Menetrier-Caux et al. (1998) and Ratta et al. (2002) who showed, that IL-6 restricts the differentiation of $CD34^+$-progenitors to DCs but favors the differentiation towards monocytic cells. Within this thesis, pDCs-numbers, however, were not lowered, which indicates that mainly cDC-progenitors might be affected by a potential impairment.

The phenotype of the DC-subsets was also changed. When cultured in the presence of HD- and MM-BMEF, expression of HLA-DR was lower, compared to no BMEF. Moreover, the expression was significantly lower under the influence of HD-BMEF, compared to MM-BMEF. This decreased expression of HLA-DR in context with the high IL-6-levels in HD-BMEF goes along with the work of Ratta et al. (2002), showing that presence of IL-6 during DC differentiation leads to lower expression of HLA-DR. This suggests that IL-6 affects DC-phenotype in a negative way. Moreover, cDC2s showed lower expression of their subset-defining marker CD1c when exposed to HD-BMEF compared to both MM-BMEF and no exposure to BMEF during the differentiation process. Possible explanations for this phenomenon of different susceptibility in terms of numbers and phenotype could be that (quantitative) proliferation and (qualitative)

differentiation of phenotype are affected by IL-6 through different mechanisms and that therefore progenitors of different subsets are vulnerable in different degrees. Furthermore, other cytokines may be important for those effects as well.

To sum up, the influences of higher IL-6-levels in HD-BMEF go along with impairments of the DC-subsets in different manifestations. These IL-6-related influences also match with previous work of other groups. Yet it remains unclear, why subsets are affected in different intensity and manner. Nevertheless, it is most likely that the used HD-BMEF does not indeed reflect the situation within an intact BM microenvironment.

4.3.2 Influence of PB-serum

As BMEF turned out to be not fully reliable, especially in representing the conditions in healthy BMEF, PB-serum was used to further inspect the influence of soluble factors on differentiation of $CD34^+$-progenitors to DCs.

After cultivation of $CD34^+$-progenitors from HDs in the presence of PB-serum from MM-patients, $CD45^+$ cell counts where significantly lower compared to cultivation in the presence of HDs' PB-serum. Further on, counts of cDC1s and pDCs were significantly lower when cultivated with MM-patients' PB-serum, while cDC2s showed no significant difference, compared to HDs' PB-serum. The proportion of those DC-subsets of all $CD45^+$ revealed no significant differences but trends corresponding with the counts.

The phenotype of DCs showed a lower expression of CD141 on cDC1s after cultivation with PB-serum of MM-patients. The levels of the other subset-defining markers (CD1c, CD123, CD303) and HLA-DR were on comparable levels after cultivation with HDs' and MM-patients' PB-serum. These results support the hypothesis that DC differentiation is impaired by soluble components present in the PB of MM-patients.

As IL-6-levels where comparable in HDs' and MM-patients' PB-serum, it is very likely that – besides the reported influence of IL-6 (Ratta et al. 2002) – other components are responsible for impairments of DC differentiation as well. These components seem to have greater quantitative than qualitative influence, as

alterations in DC phenotype were less striking. Menetrier-Caux et al. (1998) identified macrophage colony-stimulating factor (M-CSF) to be responsible for misguided DC differentiation towards monocytic cells. Further on IL-10, vascular endothelial growth factor (VEGF), transforming growth factor-(TGF-)β and prostaglandin E2 (PGE2) are discussed as possible suppressors of DC differentiation and function (Zou 2005, Motta and Rumjanek 2016). On the other hand, the lack of DC favoring components could also contribute to impairments in differentiation and phenotype. As the used culture system partly provides necessary factors as GM-CSF, this kind of interference might by hard to detect with the used method. Nevertheless, IL-4 and -12 as well as IFN-γ are known to favor DC differentiation and function (Zou 2005, Motta and Rumjanek 2016).

In conclusion, PB-serum of MM-patients showed negative influence on DC differentiation, even though IL-6-levels were comparable to that of HDs. This suggests that also other soluble factors are involved in DC-impairment. Further work is necessary to identify the pathways regulating the TME in the BM of MM-patients and possibly affecting DC differentiation. Besides examination of soluble components and their effects, taking a closer look on direct cell-cell-interactions might also lead to a better understanding of the pathological condition in the MM-TME; e. g. co-cultures of MM-cells and $CD34^+$-progenitors might be a promising approach.

4.4 Conclusion

The role of DCs in MM is of great interest, yet important questions remain unanswered. At least two major issues highlight the interest in DCs in MM-patients:

First, DCs seem to play an important role in the TME. Through interaction with other cells like MM tumor cells and T cells they probably support tumor immune invasion, favor disease progression and potentially contribute to osteolysis (Kukreja et al. 2006, Tucci et al. 2011a, Leone et al. 2015, Kawano et al. 2017).

Secondly, impairment of DCs beyond the boundaries of tumors can have serious consequences for immune responses in general (Banchereau and Steinman 1998).

The results of this thesis show that DCs and T cells in the PB of MM-patients were altered in both their abundance and their phenotype. The abundance of all DC-subsets was significantly lower in MM-patients. Further on, the phenotype of the DC-subsets was shifted towards a more immunosuppressive phenotype. These results indicate, that DCs are most likely impaired in their function as APCs. Further studies need to address the functional consequences of this impairment in detail.

In addition, *in vitro* differentiation of $CD34^{+}$-progenitors to DCs was impaired when PB-serum of MM-patients was added, suggesting the influence of soluble factors on this differentiation. As IL-6-levels were not elevated in MM-patients' PB-serum, further analysis of PB-serum and also BMEF might identify other components responsible for this impairment.

Also, T cells in the PB of MM-patients were reduced in their abundance; their phenotype was shifted to a more senescent phenotype. The changes in T-cell phenotype are consistent with tumor-related alterations (Crespo et al. 2013, Schietinger and Greenberg 2014), yet the appearance in the PB could also indicate a close relation to the DC-impairments. Again, the analysis of the functional interplay between DCs and T cells is needed to clarify these open questions.

Immunodeficiency is a common feature in MM-patients leading to infections with a substantial contribution to morbidity and mortality (Augustson et al. 2005, Pratt et al. 2007, Blimark et al. 2015). Besides iatrogenic immunodeficiency through therapy, especially in the context of autologous stem cell transplantation (Blimark et al. 2015), other mechanisms are subject of discussion. Yet, it is unclear which mechanisms contribute to this immunodeficiency to which extent. While hypogammaglobinemia is a common feature of MM and suspected to be responsible for increased susceptibility to infections (Joshua et al. 2016), also impairments of DC and T-cell function are potential contributors. Even though the immunodeficiency in MM-patients seems not to be mainly characterized by typical disease associated with T-cell impairment (Joshua et al. 2016), higher incidences

of viral infections as herpes zoster and other viral infections are reported (Blimark et al. 2015, Joshua et al. 2016) pointing out a potential T-cell deficiency.

Even though the present thesis does not provide proof for a general functional impairment of immune responses in MM-patients caused by DC- and/or T-cell dysfunction, the described results in the PB of MM-patients indicate that far-reaching alterations in DCs and T cells are not limited to the TME. To further address this issue, comparative analysis of immune cells present in different tissues and the TME of MM and functional examination could deliver substantial information. Also, the correlation of patients' conditions with their immunoprofile might clarify the role of different alterations in MM-disease and their contribution to immunodeficiency.

5 Summary

Multiple myeloma (MM) is a tumor disease of malignant plasma cells in the bone marrow (BM). Among others, major clinical features are osteolysis and related elevation of calcium levels in the peripheral blood (PB). Further on, immunodeficiency contributes to morbidity and mortality of patients. Dendritic cells (DCs) are key players of the immune system as they are able to effectively initiate T-cell responses through presentation of antigens and co-stimulatory molecules. DCs are grouped into different subsets with distinct phenotype and function as conventional DCs (cDC1s, cDC2s), plasmacytoid DCs (pDCs) and inflammatory DCs (infDCs). In MM, DCs appear to be tightly connected to MM pathogenesis. By interactions with other cells of the tumor microenvironment (TME), they are assumed to contribute to disease progression and tumor immune evasion. Further, they are suspected of being linked to osteoclastogenesis and immunodeficiency. This thesis aimed to investigate DC-subsets in PB of MM-patients, characterize T-cell dysfunction in detail and examine the possible influence of soluble factors present in MM-patients on DC differentiation. By using multi-color flow cytometry, DCs and T cells in the PB of MM-patients were analyzed in regard of abundance and phenotype. Furthermore, the *in vitro* differentiation of $CD34^+$-progenitors to DCs was studied. Therefore, $CD34^+$-progenitors were purified by fluorescent activated cell sorting (FACS) and cultured in a 7-day-culture-system in the presence of soluble components present in the PB of MM-patients. All DC-subsets were reduced in PB of MM-patients compared to healthy donors (HDs). Moreover, their phenotype was altered to increased expression of CD40 and programmed death-ligand (PD-L)1, suggesting immunosuppressive functional alterations. T cells were also reduced in the PB of MM-patients compared to HDs, with a slightly more senescent phenotype, also suggesting impaired immune function. The differentiation of $CD34^+$-progenitors of HDs to DCs was partly impaired by PB-serum of MM-patients. As interleukin-(IL-)6-levels were similar in the PB-serum of HDs and MM-patients, it is likely that other factors than IL-6 are responsible for this impairment. All in all, DC differentiation, abundance and phenotype are impaired in MM-patients with possible effects on tumor immune evasion and general immunodeficiency.

6 Deutsche Zusammenfassung

Das Multiple Myelom (MM) ist eine Tumorerkrankung maligner Plasmazellen im Knochenmark. Wichtige klinische Symptome sind - neben weiteren - Osteolysen und damit verbunden erhöhte Calcium-Spiegel im Blut. Weiterhin trägt eine Immunschwäche zur Morbidität und Mortalität der Patient:innen bei. Dendritische Zellen (DCs) sind wichtige Akteure des Immunsystems, da sie in der Lage sind, durch Präsentation von Antigenen und co-stimulatorischen Molekülen effektiv T-Zell-Antworten auszulösen. DCs werden in verschiedene Subtypen mit verschiedenen Funktionen eingeteilt: konventionelle DCs (cDC1s, cDC2s), plasmazytoide DCs (pDCs) und inflammatorische DCs (infDCs). DCs sind vermutlich eng mit der Pathogenese des MM verbunden. Es wird angenommen, dass DCs durch Interaktion mit anderen Zellen der Tumorumgebung zum Fortschreiten der Erkrankung und zur Immunevasion des Tumors beitragen. Weiterhin wird vermutet, dass DCs mit Osteoklastogenese und Immundefizienz in MM-Patient:innen verbunden sind. Ziel der vorliegenden Arbeit war die Untersuchung der verschiedenen DC-Subtypen im peripheren Blut von MM-Patienten und die genaue Charakterisierung von T-Zell-Dysfunktion; weiterhin wurde der mögliche Einfluss löslicher Faktoren, die in MM-Patienten vorkommen, auf die Differenzierung von DCs untersucht. Die Häufigkeit und der Phänotyp von DCs und T-Zellen im peripheren Blut von MM-Patient:innen wurden durchflusszytometrisch analysiert. Weiterhin wurde die *In-vitro*-Differenzierung von $CD34^+$-Vorläufern zu DCs untersucht. Dazu wurden $CD34^+$-Vorläufer mittels fluorescent activated cell sorting (FACS) aufgereinigt und in einer 7-tägigen Kultur unter dem Einfluss von löslichen Komponenten, die im Blut von MM-Patient:innen vorkommen, kultiviert. Im Blut von MM-Patient:innen war die Anzahl aller DC-Subtypen gegenüber dem Blut gesunder Spender:innen verringert, wobei sie vermehrt CD40 und programmed death-ligand (PD-L)1 exprimierten, was auf immunsuppressive funktionelle Veränderungen der DCs hindeutet. Ebenso war die Anzahl von T-Zellen im PB von MM-Patient:innen gegenüber gesunden Proband:innen vermindert. Sie zeigten Hinweise auf eine leichte Veränderung zu einem seneszenten Phänotyp, was ebenfalls auf eine gestörte Immunfunktion hindeutet. Die Differenzierung von

$CD34^+$-Vorläuferzellen gesunder Spender:innen zu DCs wurde teilweise durch PB-Serum von MM-Patient:innen beeinträchtigt. Da die Interleukin-(IL-)6-Spiegel im PB von MM-Patient:innen und gesunden Proband:innen auf vergleichbarem Niveau waren, ist anzunehmen, dass andere Faktoren als IL-6 für diese Einschränkung verantwortlich sind. Insgesamt zeigten sich Einschränkungen von DC-Differenzierung, -Häufigkeit und -Phänotyp in MM-Patient:innen, was möglicherweise Einfluss auf die Tumor-Immunevasion und generelle Immundefizienz haben könnte.

7 Literature

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8 Erklärung zum Eigenanteil

Die Arbeit wurde in der Medizinischen Universitätsklinik und Poliklinik Tübingen, Abteilung Innere Medizin II für Hämatologie, Onkologie, klinische Immunologie und Rheumatologie, unter Betreuung von Frau Professorin Dr. rer. nat. Stella Autenrieth durchgeführt.

Die Konzeption der Studie erfolgte durch Frau Prof. Dr. rer. nat. Stella Autenrieth.

Die Versuche wurden nach Einarbeitung durch Labormitglieder (Frau Dr. rer. nat. Jennifer Richardson, Frau Manina Günter, Frau Marion Strauss, Frau Carolin Langnau, Frau Simone Pöschel, Frau Sabrina Grimm) von mir eigenständig durchgeführt. Die Patientenaufklärung und Probenentnahme erfolgte durch verschiedene Ärzte des Universitätsklinikums Tübingen, der BG Unfallklinik Tübingen, des DRK Blutspendedienstes Baden-Württemberg - Hessen und anderer Kliniken. Das Cell-Sorting (FACS) im Rahmen der Differenzierungsassays wurde durch die FACS-Core-Facility (Frau Dr. rer. nat. Kristin Bieber, Frau Simone Pöschel, Frau Sabrina Grimm) der Abteilung Innere Medizin II des Universitätsklinikums Tübingen durchgeführt.

Die statistische Auswertung erfolgte nach Anleitung durch Frau Prof. Dr. rer. nat. Stella Autenrieth eigenständig durch mich.

Ich versichere, das Manuskript selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

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