Charles University

Faculty of Science

Study program: Molecular and Cell Biology, Genetics and Virology

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Pathophysiological development and differentiation of cells during hematopoiesis

Patofyziologický vývoj a diferenciace buněk v krvetvorbě

Doctoral thesis

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Prague, 2019

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Podpis

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List of abbreviations

5-AC	5-azacytidine
AIMT	antigen-inexperienced memory T-cells
AML	acute myeloid leukemia
AP-1	activator protein 1
APC	antigen presenting cells
ARE	antioxidant response element
BM-MNC	BM mononuclear cells
BrdU	bromodeoxyuridine
CCR7	C-C Motif Chemokine Receptor 7
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate succinimidyl ester
CML	chronic myeloid leukemia
CRBN	cereblon
CRL4	E3 Ubiquitin Ligase Cullin 4A
CSNK1A1	casein kinase 1A1
CTA	cancer testis antigen
CXCR2	C-X-C Motif Chemokine Receptor 2
DAMP	danger associated molecular patterns
DC	dendritic cell
DIAPH1	Diaphanous (mDia)-related formin mDia1
DN	double negative
DP	double positive
FoxP3	Forkhead Box P3
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HO-1	Heme Oxygenase 1
HSC	hematopoietic stem cell
IFNγ	interferon γ
IL	interleukin
IM	innate memory
iNKT	invariant natural killer T-cells
IP-10/CXCL10	Interferon γ inducible protein 10/C-X-C Motif Chemokine Ligand 10

IPSS-R	Revised International Prognostic Scoring System
Keap1	Kelch Like ECH Associated Protein 1
Ki-67	antigen Ki-67
KIRs	Killer-cell immunoglobulin-like receptor
Lck	LCK Proto-Oncogene, Src Family Tyrosine Kinase
LIM	lymphopenia induced memory
MCP-1/CCL2	Monocyte Chemoattractant Protein-1/ C-C Motif Chemokine Ligand 2
MDS	myelodysplastic syndrome
MDSC	myeloid derived suppressor cells
MHC	major histocompatibility complex
miRNA	microRNA
MSC	mesenchymal stem cell
mTEC	medullary thymic epithelial cells
NFAT2	nuclear factor activated T-cells 2
NHEJ	non-homologous end joining
NK	natural killer
NKG2A	Killer Cell Lectin Like Receptor C1
NKG2D	Killer Cell Lectin Like Receptor K1
NOS3	Nitric Oxide Synthase 3
NQO1	NAD(P)H dehydrogenase (quinone 1)
Nrf2	Nuclear Factor, Erythroid 2 Like 2
OS	overall survival
OVA	ovalbumin
PI3K	phosphoinositide-3 kinase
PMA	phorbol 12-myristate 13-acetate
RAG	recombination activating gene
RNF41	Ring Finger Protein 41
S100A8 // S100A9	S100 calcium-binding protein A8 // A9
SP	single positive
TCR	T-cell receptor
TGFβ	transforming growth factor β
Th	T helper
Th17	T helper 17
TNFα	tumor necrosis factor α

Treg	T regulatory
VDJ	variable-diversity-joining
VM	virtual memory
WT1	Wilms' tumor 1 antigen

1 Acknowledgements

Firstly, I would like to thank my supervisor Dr. Ondřej Štěpánek for guidance and valuable advice. I would also like to thank my consultant Dr. Zdeněk Hodný as well as Dr. Anna Jonášová for giving me the opportunity to work on an interesting and challenging project.

Moreover, I am thankful to all the collaborators that contributed to the publications that are part of my dissertation thesis and for the continuing support of the members of the Facility of Light Microscopy and Flow Cytometry at the Institute of the Molecular Genetics at the Czech Academy of Sciences, especially to Zdeněk Cimburek for sorting my cells and giving me useful advice regarding my project.

Last but not least, I am grateful to the human volunteers and patients at the Hematology department of the General University Hospital for their generous donation of blood and bone marrow for my research and to the mice who were sacrificed for the sake of science.

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3 Abstract

In recent years, a great effort has been deployed towards a better understanding of the molecular changes in cells and in the bone marrow (BM) environment that contribute to the development and progression of myelodysplastic syndrome (MDS) to acute myeloid leukemia (AML). Among others, the aberrant hematopoietic stem cells in MDS often display increase in DNA double strand breaks, genomic instability with common loss or rearrangement of chromosomes and an ineffective response to DNA damage, a phenomenon that has been linked to the onset of cellular senescence. Additionally, the BM microenvironment can become more pro-inflammatory.

In our effort to better understand the contribution of the BM microenvironment on MDS progression, we analyzed the expression profiles of cytokines in the BM microenvironment in all stages of MDS/AML and found several proinflammatory cytokines that increase with disease progression. Also, by repeated sampling of patients over the course of 5-azacytidine therapy, we were able to assess the changes in the proinflammatory cytokine milieu with the progression of the disease.

Additionally, we aimed to identify the candidate markers for the improvement of MDS prognosis. We focused on naturally occurring germline polymorphism of NAD(P)H dehydrogenase (quinone 1) gene (NQO1*2) that diminishes the ability to reduce oxidative stress. We uncovered that patients with NQO1*2 genotype progress faster and have a shorter overall expression, especially pronounced in low-risk MDS with normal karyotype. Moreover, the NQO1*2 genotype was associated with higher sensitivity to increased ferritin levels. We suggest that NQO1*2 polymorphism can be used as a prognostic marker.

Further, in a subset of low-risk MDS patients treated with lenalidomide, we found that the combination of lenalidomide and prednisone and/or erythropoietin had a positive impact on relapsed and refractory patients with del(5q) MDS.

Next, we focused on the characterization of virtual memory (VM) $CD8^+$ T-cells. The diminishment of this population was recently identified in chronic myeloid leukemia. Thus it is possible that VM T-cells could play a role in MDS pathology as well. We discovered that VM T-cell development is determined by their T-cell receptor self-reactivity. Moreover, by analyzing the expression profiles of antigen-experienced memory T-cells and VM T-cells, we uncovered that VM T-cells acquire a partial memory program. However, this does not make them more potent in inducing autoimmunity when compared to naïve cells with the same T-cell

receptor. Thus, we postulate that VM T-cell formation from highly self-reactive T-cell receptor clones could function as a novel self-tolerance mechanism.

Finally, we compared findings of the different subsets of antigen-inexperienced memory T-cells and concluded that VM T-cells and lymphopenia-induced memory T-cells likely represent one subset of antigen-inexperienced memory T-cells. We have proposed to call this subset "homeostatic memory T-cells".

4 Abstract (Czech version)

V posledních letech byla vynaložena velká snaha o lepší pochopení molekulárních změn v buňkách a v prostředí kostní dřeně, které přispívají k rozvoji a progresi myelodysplastického syndromu (MDS) do akutní myeloidní leukémie (AML). Aberantní hematopoetické kmenové buňky v MDS často vykazují nárůst dvojitých zlomů DNA, genomovou nestabilitu s běžnou ztrátou nebo přestavbou chromozomů a neúčinnou odpověď na poškození DNA. Navíc může být mikroprostředí kostní dřeně více prozánětlivé.

Pokusili jsme se lépe porozumět příspěvku mikroprostředí kostní dřeně při progresi MDS. Analyzovali jsme expresní profily cytokinů v mikroprostředí kostní dřeně u všech stadií MDS/AML a našli jsme několik prozánětlivých cytokinů, jejichž hladiny se zvyšují s progresí onemocnění. Opakovaně jsme odebírali vzorky kostní dřeně od MDS pacientů v průběhu léčby 5-azacytidinem a podařilo se nám stanovit změny v cytokinovém prostředí v průběhu progrese onemocnění.

Dále jsme se zaměřili na identifikaci kandidátních markerů pro zlepšení prognózy MDS. Zaměřili jsme se na přirozeně se vyskytující polymorfismus genu NAD (P) H dehydrogenázy (chinonu 1) (NQO1*2), který snižuje schopnost redukovat oxidační stres. Zjistili jsme, že pacienti s genotypem NQO1*2 se zhoršují rychleji a mají kratší celkovou dobu přežití, což bylo zvláště patrné u MDS pacientů s nízkým rizikem progrese do AML a normálním karyotypem. Navíc byl genotyp NQO1*2 spojený s vyšší citlivostí na zvýšené hladiny feritinu. Navrhujeme tedy použití polymorfismu v NQO1*2 jako prognostického markeru.

Navíc jsme zjistili u skupiny MDS pacientů léčených lenalidomidem, že kombinace lenalidomidu a prednisonu a/nebo erytropoetinu má pozitivní dopad na relapsované a refrakterní pacienty s MDS del(5q).

Dále jsme se zaměřili na charakterizaci virtuálních paměťových (VM) $CD8^+$ T-buněk. Snížení této populace bylo nedávno zjištěno u chronické myeloidní leukémie. Je tedy možné, že VM T-buňky mohou hrát také roli v patologii MDS. Zjistili jsme, že vývoj VM T-buněk je determinován autoreaktivitou jejich T-buněčného receptoru. Porovnáním expresních profilů pravých paměťových T-buněk a VM T-buněk jsme zjistili, že VM T-buňky získávají částečný paměťový program. To však neznamená, že jsou účinnější při indukci autoimunity v porovnání s naivními buňkami se stejným T-buněčným receptorem. Předpokládáme tedy, že tvorba VM T-buněk z vysoce autoreaktivních T-buněk by mohla fungovat jako nový mechanismus autotolerance.

Nakonec jsme porovnali poznatky o různých podskupinách paměťových T-buněk, které vznikly bez předchozího kontaktu s antigenem a dospěli jsme k závěru, že VM T-buňky a paměťové T-buňky vzniklé díky lymfopenii pravděpodobně představují jednu podskupinu antigen-nezkušených paměťových T-buněk. Navrhujeme pro ně souhrnný název "homeostatické paměťové T-buňky".

5 Preface

During my graduate studies at the PhD program in Molecular and Cellular Biology, Genetics and Virology at the Faculty of Sciences, Charles University, I have worked in two research laboratories at the Institute of Molecular Genetics of the Czech Academy of Sciences.

I started my PhD studies in the Laboratory of Genome Integrity, where I studied the effects of 5-azacytidine on the progression of myelodysplastic syndromes. This project was in collaboration with 1st Department of Medicine - Department of Hematology, First Faculty of Medicine, Charles University in Prague and General University Hospital. My project focused on the role of bone marrow proinflammatory cytokines on the progression of the disease and the impact of the genetic polymorphism of the NAD(P)H dehydrogenase (quinone 1) on the survival of patients with myelodysplastic syndrome.

However, since the project had reached its completion and had no further continuation, I had decided to improve my understanding of immunology and to transfer during my 2nd year of PhD studies to the Laboratory of Adaptive Immunity, where I focused on studying antigen-inexperienced $CD8^+$ T-cells with the memory phenotype. My primary interest was to characterize the impact of T-cell receptor self-reactivity on development of antigen-inexperienced $CD8^+$ T-cells with the memory phenotype and their role in the induction of the autoimmunity. The last project focuses on the comparative analysis of several subsets of antigen-inexperienced memory $CD8^+$ T-cell and the role of hygienic status, age and genetic background on their development.

My dissertation thesis includes the following chapters: introduction summarizing current knowledge about the topic, aims of my research projects, brief description of the most important methods and discussion of the particular projects and resulting publications, conclusions, and reprints of manuscripts that were published.

6 Introduction

Hematopoiesis is a hierarchical process that begins in the bone marrow (BM) when hematopoietic stem cells (HSC) enter the cell cycle and give rise to the lineage precursors that further differentiate into more specific cell types of myeloid, erythroid or lymphoid lineage. In normal hematopoiesis, this process is tightly regulated to replenish the blood cells within the organism while sustaining a sufficiently large HSC pool for proper blood production.

Although the majority of blood cells absolve their entire differentiation process in the BM tissue, for T-cell precursors it is necessary to leave BM and travel to the thymus, where they can fully mature. It is within the confinement of the thymus, where thymocytes go through the process of recombining the genes encoding for T-cell receptor chains. T-cell receptor recombination is accompanied by a selection process ensuring that only cells with relatively low but sufficient self-reactivity are released into the periphery.

6.1 Role of mature T-cells in hematopoiesis

The mature T-cells populate different niches within the whole body including the BM. However, the abundance of cell types in the lymphoid organs and in the BM differ significantly. It has been noted that there is a substantial population of T-cells within the BM and that the BM T-cell compartment has a higher proportion of cells with memory phenotype than in peripheral blood or lymph nodes. However, there is some controversy regarding the recirculation and homeostatic proliferation of these cells. So far, the main approaches deployed in assessing the proliferation status of these cells were *in vivo* labelling with bromodeoxyuridine (BrdU) [1, 2], adoptive transfer of carboxyfluorescein diacetate succinimidyl ester (CFSE) labelled cells [3], staining with proliferation marker Ki-67 [4, 5] and *in vivo* stable radioisotope labelling [6].

Because of the known side-effects of long-term BrdU labelling [7] and the need to label cells with CFSE *ex vivo*, the studies performed using the latter methodologies give more precise results without perturbing the BM niche. Studies using Ki-67 staining concluded that more than 90% of murine memory $CD8^+$ T-cells are in the G_0 phase of the cell cycle, in accordance with the analysis of human BM T-cells. Moreover, the majority of T-cells in BM are of the memory phenotype and lack the ability to leave the BM tissue due to the expression of CD69 [8].

Recently, *in vivo* murine studies of normal hematopoiesis have shown that the presence of $CD8^+$ T-cells with the memory phenotype enhances the proliferation and self-renewal of the healthy HSC. Also, the mice lacking $CD8^+$ T-cells with the memory phenotype have

significantly fewer HSC, a situation that can be remedied by the adoptive transfer of CD8^{+} memory T-cells [9]. Moreover, the dynamic alterations in the T-cell compartment have been shown to contribute to the pathological manifestations of myeloid leukemias.

6.2 Functional integrity of HSC

During aging, the functional integrity of HSC can be influenced by acquiring somatic mutations that could give them a selective advantage in entering the cell cycle and can result in the pathophysiological differentiation and development of hematologic malignancies.

Additionally, HSC cell fate is regulated in response to chronic inflammatory stimuli. The long-term inflammation in the BM has been shown to skew the differentiation of HSC, to impair the fitness of normal HSC and to contribute to the development of diseased states, such as in the myelodysplastic syndromes (MDS) or in acute myeloid leukemia (AML). Inflammation is one of the most adverse co-founding in MDS diagnosis that is found in 10% patients [10-12]. The impact of the chronic inflammation is demonstrated in the increase of the incidence and earlier onset of the MDS [13, 14].

Both MDS and AML are characterized by aberrant expansion of immature hematopoietic precursors called blasts, defined by expression of CD34 molecule. The MDS is a very heterogeneous HSC disease with various disease manifestations that is classified into several subtypes [15] and stratified with regard to the risk of progression into AML. In low-risk MDS, the expansion of blasts is localized to the BM and the process of maturation is inefficient due to increased apoptosis of blasts. This leads to insufficient peripheral blood cell counts, i.e. cytopenia (anemia, thrombocytopenia, neutropenia). In high-risk MDS, the aberrant HSC becomes resistant to the induction of apoptosis and increasingly proliferate. Ultimately, in the final stage of the disease (AML), the blasts are released to the bloodstream, which is correlated with the short overall survival (OS) of the affected individuals.

6.3 The immune system dysregulation in MDS

Interestingly, 28% of MDS cases present autoimmune manifestations [16] and the history of autoimmune diseases increases the risk of developing MDS or AML [13, 14].

Multiple immune cell subsets have been recognized to contribute to the development and manifestations of the MDS. The proinflammatory microenvironment contributes to the expansion of the aberrant MDS blasts [17], causes abnormalities in natural killer (NK) cells

[18] and T-regulatory lymphocytes (Treg) and supports the development of specialized myeloid derived suppressor cells (MDSC) [19, 20].

6.3.1 Myeloid-derived suppressor cells (MDSC)

MDSCs are a group of immature myeloid cells that develop over the course of major inflammatory processes and infections in order to alter the response of the innate immune system to stress. MDSCs have the ability to suppress T-lymphocyte function. Interestingly, MDSCs have been found in increased numbers in MDS and it has been suggested that these cells can play a role in the development [21] and progression [22] of MDS, where the increase in MDSC correlates with the expansion of Tregs and the progression of MDS [22].

The $Gr1^{+}$ $CD11b^{+}$ myeloid precursors give rise to the MDSCs via reprogramming induced by S100 calcium-binding protein A9 (S100A9) [23]. The S100A8/S100A9 heterodimer (calprotectin) belongs to a group of danger associated molecular patterns (DAMP) that are abundantly expressed in the myeloid cells. The formation and excretion of calprotectin are increased during the inflammation, where it stimulates the leukocyte recruitment to the site of inflammation [24, 25]. The importance of the calprotectin in the development of MDS has been shown using S100A9 transgenic mice overexpressing the S100A9 protein in the cells of HSC origin. These S100A9 transgenic mice accumulate MDSCs in BM and gradually develop progressive multilineage cytopenias and dysplasia resembling the phenotype of MDS [21].

6.3.2 Natural killer cells

Recently, the role of NK cells in the pathology of MDS has been thoroughly examined. Several reports have noted a reduction in the absolute counts of peripheral NK cells among MDS patients compared to age-matched healthy controls [18, 26, 27], as well as their lower cytotoxicity [28] that was especially pronounced in high-risk MDS. Moreover, the decreased cytotoxicity of NK cells has been confirmed in the BM [29].

The loss of cytotoxicity was mainly due to the reduced expression of activating natural cytotoxicity receptors NKp30 and NKG2D (Killer Cell Lectin Like Receptor K1) [28], possibly due to the chronic exposure to their ligands on leukemic blasts [30]. This hypothesis was corroborated by the inability of NK cells to directly kill autologous MDS blasts [29]. The worsening of NK cell function might be correlated with the progression of the disease, as the 20-50% of circulating NK cells have been shown to share karyotype abnormalities with the MDS blasts [27].

6.3.3 T-cells

Several subsets of T-cells have been shown to play a role in the development and progression of MDS. Among them are $CD8^+$ T-cells and several subsets of $CD4^+$ T-cells, such as regulatory T cells (Tregs) and T helper 17 (Th17) T-cells.

Tregs ($CD4^+$ $CD25^+$ $FoxP3^+$) dampen adaptive immune response by suppressing autoreactive T-cells (FoxP3, Forkhead Box P3). Consequently, the contraction of Treg compartment is associated with autoimmunity [31], while the expansion of Tregs is intrinsic in the loss of immunosurveillance and promotion of tumorigenesis [32]. Both of these manifestations are present in MDS. In low-risk MDS low numbers of Tregs have been reported, whereas in high-risk MDS and AML are Tregs expanded [33, 34].

Interestingly, the quantitative imbalance of Tregs in low-risk MDS correlates with the increased proportion of proinflammatory Th17 T-cells and higher levels of apoptosis in the BM [35]. This imbalance between Tregs and Th17 numbers has been observed in many diseases of the BM with inflammation as one of the symptoms [36]. However, the percentage of the Th17 cells later decreases to normal levels as the disease progresses [35].

A portion of low-risk MDS patients presents a skewed ratio of $CD4^+$ and $CD8^+$ T-lymphocytes in the BM [33], and in some cases also in the peripheral blood [26, 37], which has been ascribed to the increased proliferation of $CD8^+$ T-cells [37, 38]. A recent study using peripheral blood from a younger cohort of MDS patients has shown that it is the depletion of $CD4^+$ T-cells rather than the enlargement of $CD8^+$ compartment that contributes to this abnormality [39]. This finding however still awaits its confirmation in the BM niche as well as the inclusion of more aged patients. The analysis of T-cell repertoire in MDS patients has indicated the oligoclonal character of T-cells across all subtypes and stages of MDS [40-43]. In aplastic anemia, a BM failure disorder that can progress into MDS, the enlarged oligoclonally expanded $CD8^+$ T-cell compartment consists of cells with the effector phenotype [44]. Similar findings were observed in childhood refractory cytopenia [45].

MDS patients with abnormal proliferation of the $CD8^+$ T-cells in BM can benefit from using the immunosuppressant therapy by cyclosporine A or anti-thymocyte globulin that can for some time subdue the progression of the disease [37, 38].

Additionally, several *in vitro* studies have demonstrated the role of $CD8^+$ T-cells in the suppression of malignant hematopoietic precursors [46]. Indeed, in MDS patients with trisomy 8, the $CD8^+$ T-cells have been shown to suppress the aberrant HSC clones via antigen-specific response to Wilms' tumor 1 antigen (WT1), since WT1 is located on chromosome 8 and is

overexpressed in cells with trisomy 8 [47]. Recently, $CD4^+$ T-cells from AML have been shown to suppress the proliferation of the mesenchymal stem cells (MSC) *in vitro* via secreting miRNA 10a [48].

Moreover, a subset of T-cells in some cases shares chromosomal abnormalities [49] or somatic mutations [50] with the malignant clone. It is however unclear, whether the T-lymphocytes with the suppressive activity on HSC or MSC also share the chromosomal abnormalities with the aberrant HSC clones.

6.4 Alterations of the BM niche

Changing spectrum of the cell types within the BM niche can have an impact on the cytokines secreted into the BM microenvironment and thus can influence the development and progression of the disease.

6.4.1 Stromal cells

MSC form an important part of the BM niche and an increasing number of studies shows that they might contribute to the development of MDS.

MSC derived from *in vitro* cultures originating in BM aspirated from MDS patients (MDS-MSC) are characterized by slower proliferation and induce the program of senescence earlier than MSC derived from healthy subjects [51-54]. Recently, the group of Blau *et al*. analyzed the chromosomal aberrations in MSC and found out that the MDS-MSC bear identical chromosomal aberrations as the aberrant MDS clones. Additionally, they uncovered that the same patient can have a set of distinctly different chromosomal aberrations in HSC and in MDS-MSC [55].

Moreover, isolated MSC from newly diagnosed MDS patients were able to support the *in vitro* growth and differentiation of HSC into osteoblasts, adipocytes, and chondrocytes, but their ability to react to proinflammatory cytokines has been modified. The *in vitro* grown unstimulated MDS-MSC were producing twice as much IL-1β (interleukin 1β) as healthy MSC and after addition of proinflammatory cytokines IL-1β and TNFα (tumor necrosis factor α), the MDS-MSC increased the production of GM-CSF (granulocyte-macrophage colony-stimulating factor) three times more than the healthy MSC [56].

One of the reasons that the MDS-MSC behave differently than the healthy MSC could be the reduction in their ability to express Dicer1. Dicer1 is endoribonuclease that cleaves RNA and catalyzes the production of short inhibiting miRNA (microRNA). The consequence of the

deletion of Dicer1 is the loss of miRNAs that exert various regulatory functions in the cell. The experimental loss of miRNAs induced by the inhibition of Dicer1 in MSC leads to the induction of senescence [57].

The modified MSC with lower expression of Dicer1 as well as MDS-MSC are producing lower levels of G-CSF (granulocyte colony-stimulating factor) and much higher levels of IL-6 and TGFβ (transforming growth factor β) when compared to healthy MSC. These changes in the expression of the cytokines contribute to the reduced ability of MDS-MSC to support the growth of HSC in *in vitro* co-culture [57]. Further, the deletion of Dicer1 in mouse osteoprogenitors induces secondary MDS-like neoplasia [58].

6.4.2 The changes in the cytokine profiles

In the last 20 years, there have been a number of studies focusing on changes in the expression and secretion profiles of the cells in BM niche, either by studying the changes in cytokine profiles in peripheral blood plasma [59-61] or in the plasma of the BM [62, 63].

The most prominent members among those aberrantly expressed cytokines are TNFα, IFNγ (interferon γ) TGFβ, IL-4, IL-6 and IL-10. From this list, IL-6 and IL-1 belong to a group of proinflammatory cytokines that are involved in acute inflammatory response, whereas IL-4, IL-10 and TGFβ are cytokines with anti-inflammatory properties.

IFNγ plays an important role during the regulation of innate immune response and it impacts the regulation of adaptive immunity as well. IFNγ is produced mainly by $CD4^+$ Th1 lymphocytes and by NK cells and its elevated expression is characteristic for autoimmune diseases. Furthermore, the increased levels of IFNγ are often identified in peripheral blood and BM plasma of low-risk MDS patients [64]. One of the possible reasons for the increased levels of aberrantly expressed cytokines is the influence of polymorphisms in their receptors and promoters of the genes coding for cytokines, such as IL-6, TGFβ and TNFα [65, 66].

6.5 The choice of therapy in MDS

The best supportive care for MDS patients consists of transfusions of platelets and/or erythrocytes and prophylactic treatment with antibiotics. Another line of treatment is based on the score of their Revised International Prognostic Scoring System (IPSS-R) and the patient's co-morbidities and may involve HSC transplantation, the use of immunosuppressant, erythropoietin, or treatment with drugs such as lenalidomide, 5-azacytidine (5-AC) or decitabine/5-aza-2′-deoxycytidine [67, 68].

Lenalidomide is primarily used for the treatment of multiple myeloma, chronic lymphocytic leukemia and in MDS patients with del(5q). Lenalidomide binds to a cereblon (CRBN) protein that works as an adaptor protein for E3 ubiquitin ligase Cullin 4A (CRL4) and modifies its substrate specificity, thus modulating the proteasomal degradation of its target proteins. CRBN retargeting induces the degradation of transcription factors Ikaros and Aiolos in T-cells, leading to enhanced secretion of IL-2 and augmentation of immune function.

Moreover, CRBN retargeting specifically repairs the formation of immunological synapse in defective T-cells in chronic lymphocytic leukemia and lymphoma via restoring the normal Rho GTPase activation [69]. Interestingly, one of the haploinsufficient genes in del(5q) MDS is *DIAPH1* (Diaphanous (mDia)-related formin mDia1), a Rho GTPase effector protein. Murine knockout of mDia1 results in the age-dependent myelodysplastic phenotypes [70] that can be rescued by treatment with lenalidomide [71].

Both 5-AC and decitabine are derivatives of cytidine that have the ability to incorporate into DNA in place of cytosine. The alteration of the chemical structure of 5-AC and decitabine compared to cytidine then modifies the CpG site structure so that the DNA methyltransferases are able to recognize them, but they are entrapped on the DNA during the process of the methylation and subsequently degraded, leading to the inhibition of DNA methylation [72-74]. The idea for using the hypomethylating agents was brought about in response to the discovery that the malignant tumor cells change the methylation profile of the cell via the hypermethylation of the tumor suppressor genes and/or miRNAs [75, 76]. In Europe, the use of 5-AC has been approved for the treatment of high-risk MDS with blasts within a range of 10 – 30% and has been the first therapy that significantly prolongs the OS of high-risk MDS patients [77]. Both 5-AC and decitabine are inherently unstable compounds that are degraded via cytidine deaminase within a couple of hours after the injection. Besides, only about 10-20% of 5-AC can be incorporated into DNA via the conversion by ribonucleotide reductase while the remaining 80-90% is incorporated into RNA [74]. Additionally, the 5-AC incorporated into RNA results in the reduction of the RNA stability. One of the proteins specifically affected by this incorporation is the subunit 2 of ribonucleotide reductase [78]. The resulting reduction of the deoxyribonucleotide pools affects the rate of DNA replication and repair. Thus, 5-AC and decitabine have mostly non-overlapping effects on the gene expression profiles [79].

6.6 Impact of MDS therapy on the immune cells

Numerous studies have shown that the therapy with lenalidomide, 5-AC and decitabine has an impact on the immune cells. However, *in vivo* effects in patients often differ from the effects of these drugs on healthy *in vitro* stimulated cells or *in vitro* studies on the cell lines.

5-AC treatment increases the expression of cancer testis antigens (CTAs) on aberrant CD34$^+$ myeloid blasts. Aberrant CTA expression is a known marker for cancer recognition by the immune system [80], which correlates with the increase of the proportion of CD8$^+$ T-cells able to recognize CTAs when compared to the state prior to starting of the treatment. Further, the longitudinal analysis of high-risk MDS and AML patients has not found any differences in the CD4$^+$, CD8$^+$, Tregs or MDSC cell counts in the peripheral blood over the course of therapy with 5-AC, when compared to the numbers before the treatment [81]. These outcomes are in striking difference to the results obtained by *in vitro* stimulation of peripheral blood cells from healthy donors that yielded the inhibition of the CD8$^+$ T-cell growth, reduction in their ability to kill the leukemic cell line *in vitro*, reduction of CD4$^+$ Th1-cell numbers and induction of the suppressive capacity of Tregs [82].

Similarly, *ex vivo* analysis of NK cells from the peripheral blood of MDS patients treated with 5-AC has not generated any functional impairment of NK cells [81]. Further analysis elucidated that the reduction in the NK cell cytotoxicity is concentration-dependent and apparent only when using the doses that are much higher than the approved clinical regimen dosage [81-83]. Additionally, several *in vitro* studies suggested that the lenalidomide exposure induces activation of phosphoinositide-3 kinase (PI3K), AP-1 (activator protein 1) and nuclear translocation of transcription factor NFAT2 (nuclear factor activated T-cells 2) resulting in the augmentation of IL-2 secretion by T-cells and phenotypic changes in NK cells, increasing their proliferation and cytotoxicity potential [84, 85]. However, the longitudinal analysis of NK cells in the blood of multiple myeloma patients has not shown any effect of the lenalidomide on the NK cell function [86]. Nevertheless, *in vivo* studies using murine models of lymphoma show that lenalidomide treatment results in the decreased numbers of MDSC and Tregs [87].

6.7 T-cell development

Cancer is one of the many perturbations of the organism that affect the immune system homeostasis. For better understanding of the role of T-cells in the adaptive immunity and surveillance of HSC neoplasias, it is necessary to discuss the process of their development. Firstly, HSC give rise to the lymphoid progenitors that migrate from the BM to the thymus,

where they undergo a series of developmental changes characterized by acquiring the T-cell specific surface markers. Upon arrival to the thymus, T-cell precursors pass through the cortex to the medulla of the thymus, where they are being exposed to the different microenvironments that help to guide their maturation.

6.7.1 T-cell development in the thymus

The T-cell precursors inside the thymus are called thymocytes. Shortly upon the arrival to the thymus, thymocytes have not yet started to express the lineage defining co-receptors CD4 or CD8, and are thus called double negative (DN) cells. The DN developmental stage can be further subdivided by their expression of the α-chain of IL-2 receptor CD25 and by the glycoprotein CD44.

Firstly, DN thymocytes start expressing CD44, followed by the co-expression of CD44 and CD25 at DN2 stage, while the third stage of the DN is defined as CD25+ CD44-. DN3 thymocytes initiate the process of recombination of their T-cell receptor (TCR) that will define their specificity to recognize foreign antigens [88]. TCR is a membrane protein comprised of two subunits, typically α and β that are covalently linked via a disulfide bond. The well-defined sequential order of VDJ recombination begins with the rearrangement of the β-chain of TCR in DN3 thymocytes, specifically by the joining of D (diversity) and J (joining) segments, followed by the V-DJ segment rearrangement (V, variable).

The VDJ rearrangement is initiated by the recombination activating genes RAG1 and RAG2 that cooperatively bind the recombination signal sequences surrounding the segments to-be-rearranged. The RAG proteins then initiate DNA double strand breaks at the recognition sequences between the segments of VDJ genes [89]. The rearranged segment ends are then processed and rejoined through the non-homologous end joining (NHEJ) DNA repair pathway [90]. The lack of precision of NHEJ can result in the addition or deletion of several nucleotides which subsequently shifts the open reading frame, and the rearranged TCRβ is not able to yield in-frame protein product [91].

In the event of the first attempt to rearrange TCRβ chain being unsuccessful, the rearrangement is initiated at the second segment. The DN thymocytes that fail to rearrange TCRβ chain for the second time are eliminated by the apoptosis [92].

After the successful rearrangement, TCRβ chain is expressed and transported onto the cell membrane, where it pairs with the surrogate chain pre-Tα and forms a pre-TCR complex with the newly expressed CD3 molecules. The signaling through pre-TCR triggers maturation of

DN thymocyte that inhibits any further rearrangement of TCRβ chain, a process called allelic exclusion [93]. Thymocytes then initiate the expression of both CD4 and CD8 co-receptor molecules and are called double positive (DP) thymocytes [94].

Another defining characteristic of DP cells is the loss of CD25 expression and re-expression of the RAG genes that are required for the TCRα chain rearrangement. Interestingly, TCRα chain rearrangement can occur simultaneously at both chromosomes. It is however very unlikely that both TCRα chains will rearrange productively. Additionally, two resulting TCRα chains will have different functional receptor specificities and binding affinities [95].

6.7.2 Positive and negative selection

After successful TCRα rearrangement, DP thymocytes migrate to the cortico-medullary junction in the thymus, where they go through the process of positive and negative selection. The positive selection is mediated via the cortical epithelial cells that express Class I or Class II of major histocompatibility complex (MHC) proteins on their surface. Additionally, the MHC proteins are embedded with the spectrum of self-peptides. In order to survive and to proceed through the maturation, the DP thymocytes have to be able to bind to these self-MHC proteins with a sufficient affinity to receive the survival signal. The ability of DP thymocytes to bind to the Class I MHC will result in the maturation to $CD8^+$ T-cell, while the positive selection on Class II MHC will produce a $CD4^+$ T-cell. The remaining functionally incompetent DP thymocytes that are unable to receive the survival signal will be eliminated via a process called "death by neglect" [96].

Next, the positively selected surviving cells will migrate further towards the cortico-medullary junction and undergo the process of negative selection with the purpose of eliminating the highly self-reactive cells. In this new microenvironment, the self-antigens are presented on a different subset of antigen presenting cells (APC) called medullary thymic epithelial cells (mTEC) that have the ability to express a vast range of tissue specific antigens due to the activation of autoimmune regulator gene [97]. Additionally, the tissue specific antigens are transferred to the nearby thymic dendritic cells (DC) that are present in higher numbers [98]. The strong-enough reaction of DP thymocytes with the mTEC will culminate in the induction of apoptosis. This process should theoretically result in the elimination of all highly auto-reactive T-cells and is backed-up by the mechanism of production of Tregs from the highly self-reactive T-cells [99].

At the late stage of the positive selection, DP thymocytes terminate the expression of CD8 co-receptor molecule and give rise to $CD4^{int}$ $CD8^{-}$ thymocytes [100, 101]. The subsequent upregulation of the CD4 co-receptor on thymocytes that were previously able to recognize Class II MHC molecules will result in the continuation of TCR signaling. These cells will mature into the single positive CD4-expressing thymocytes (SP4) and later into $CD4^{+}$ T-cells. The $CD4^{int}$ $CD8^{-}$ thymocytes with the ability to recognize Class I MHC molecules will have disrupted TCR signaling and will have to undergo co-receptor expression switch, where the $CD4^{int}$ $CD8^{-}$ thymocytes downregulate CD4 and upregulate CD8 expression. These single positive CD8 thymocytes will ultimately give rise to $CD8^{+}$ T-cells upon their egress onto periphery.

6.8 T-cell memory

After the successful T-cell maturation process in the thymus, newly formed T-cell is released to the periphery as a naïve antigen inexperienced T-cell. On the periphery, a majority of mature αβ T-cells recirculate between blood and the lymph nodes where they sample the antigens of various origins embedded on the MHC molecules of the APC. When the organism encounters a pathogen infection, the exogenous antigens are processed by the DC with the ability to present these pathogen-derived antigens to T-cells. Mature DC then enter the draining lymph node and present the processed exogenous antigens to T-cells [102].

Over the course of a given infection, only a few T-cell clones have the ability to recognize particular antigen derived from the pathogen via their TCRs. This recognition bias is caused by the enormous variability of the TCR repertoire. The responsive T-cell is activated via the binding of the cognate antigen presented on the MHC to the TCR. The resulting TCR signaling pathway induces a rapid proliferation and differentiation into short-lived effector T-cells and memory T-cells. The function of these T-cells with a memory phenotype is to facilitate long-term protection against the possible reinfection with the same pathogen.

The populations of different subtypes of memory T-cells can be distinguished based on the differential expression of the specific surface markers. Two of the most important murine surface markers are the adhesive glycoprotein CD44 that is found on the antigen-experienced T-cells and the L-selectin (CD62L) that is lost upon the T-cell activation and allows for the discrimination between the short-lived effectors and long-lived memory T-cells [103, 104].

6.8.1 Murine antigen-inexperienced memory T-cells (AIMT)

It has been noted previously that some of the $CD8^+$ T-cells that have never encountered their cognate antigen can exhibit an apparent memory phenotype – and are thus antigen-inexperienced memory T-cells (AIMT) [105]. Three subtypes of the AIMT cells with a different mechanism of generation have been identified in murine studies: (i) the innate memory (IM) cells that originate in the thymus [107], (ii) the memory-phenotype cells generated on the periphery due to experimental lymphopenia-induced proliferation (LIM) [108, 109], and (iii) the virtual memory (VM) cells that are converted to the memory phenotype on the periphery [110]. The AIMT cells have been found in young adult unchallenged murine strains, where they form 10-20% of all $CD8^+$ T-cells.

Up to now, the only well-established marker for differentiating between the murine antigen inexperienced and 'true' antigen-experienced central memory $CD8^+$ T-cells is the reduced expression of the α4-integrin chain (CD49d) in the AIMT cells, which is common for all three subtypes of these cells [111, 112]. Additionally, it has been described in unchallenged murine C57Bl/6J strain that the percentage of AIMT cells increases during aging [113, 114].

Two key cytokines, IL-4 and IL-15, were reported to play a pivotal role in the conversion of naive T-cells into the AIMTs. However, until recently, it was unclear which one of these cytokines was the main player during the conversion. The question was recently elucidated by the group of Tripathi *et al*., who demonstrated that the cytokine dependency of the AIMTs in mice is strain specific. In the Balb/c mouse strain, the AIMT cells are largely dependent on the presence of IL-4 supplied by a subset of invariant NK T-cells (iNKT) in the thymus [115] – leading to the development of thymic-derived IM cells. Thus, the IL-15-deficiency has only partial impact on the AIM T-cell compartment in the Balb/c strain. Meanwhile, the C57Bl/6J strain has a very limited number of iNKT cells in the thymus and their AIMT cells are formed on the periphery due to the effects of IL-15 [116-118].

Unfortunately, the functional properties of VM T-cells have not been clearly defined yet. Recently, a seminal work by Lee *et al*. suggested the enhanced protective role of VM T-cells compared to naïve T-cells during infection with *Listeria monocytogenes* [119]. On the other hand, far less known is about the possible role of IM cells [120]. Besides the cytokine requirements and the site of origin, the differences between VM and IM T-cells are largely unknown.

6.8.2 Human antigen-inexperienced memory T-cells

Recently, it has been described that upon adoptive transfer into lymphopenic humanized mouse strains, the naïve $CD8^+$ T-cells from humans are able to expand and acquire the memory phenotype [121], suggesting that the AIMT cells can develop in humans as well.

In line of this evidence, a population of a human $CD8^+$ T-cells with some similar characteristics to the murine VM $CD8^+$ T-cells has been described in human cord blood as well as in adult human blood [113, 122]. The authors have named this population innate-like $CD8^+$ T-cells [122] or NK-like $CD8^+$ T-cells [123], since this population expresses characteristic NK-receptors NKG2A (Killer Cell Lectin Like Receptor C1) and/or killer-cell immunoglobulin-like receptors (KIR). Furthermore, NK-like $CD8^+$ T-cells express high levels of transcription factors Eomes and T-bet that are linked to the $CD8^+$ T-cell differentiation, as well as the IL-2 receptor subunit β (CD122). Moreover, NK-like $CD8^+$ T-cells were shown to be able to rapidly produce high levels of IFNγ after the stimulation with either IL-12/IL-18 or PMA/ionomycin (PMA, phorbol 12-myristate 13-acetate) [113, 122]. Based on these features, White *et al.* proposed that these cells might be the human equivalent to the murine VM T-cells [113]. Similarly to the murine VM T-cells, the putative human VM T-cells accumulate during aging [113, 124] and it has been suggested that VM T-cells acquire the markers of the senescence with age as they lose their proliferative capacity in response to the TCR signals [125]. However, unlike the murine VM T-cells, these putative human VM T-cells express low levels of CD5. More importantly, these cells are negative for the CD27 and CCR7 (C-C Motif Chemokine Receptor 7) markers and positive for the CD57 and CD45RA surface markers [113, 122], suggesting that these cells are terminally differentiated cells rather than the central memory-phenotype T-cells [126, 127]. It is however possible that discrepancies in the expression of these particular surface markers can be caused by the inter-species differences.

6.8.3 Antigen-inexperienced memory T-cells in leukemia

Moreover, one of the recently published studies on putative human VM T-cells focuses on the possible defect in the VM T-cell compartment during chronic myeloid leukemia (CML), a myeloproliferative neoplasm affecting HSC.

In this study, the patients that have reached complete remission after the treatment with tyrosine kinase inhibitor imatinib, were able to restore the numbers of VM T-cells in the peripheral blood to the normal levels [128]. Thus VM T-cells could conceivably contribute to the development and progression of MDS.

7 Aims of the study

Firstly, the MDS project was focused on better understanding of the progression of MDS, specifically how treatment with the hypomethylating drug 5-AC affects the BM microenvironment. Therefore, it was necessary to collect a vast number of BM biopsies from MDS patients over the course of the disease progression and to analyze the BM plasma from the patients treated with 5-AC before and during therapy. In parallel, we have tested the effect of the 5-AC treatment *in vitro* using multiple cell lines of different origin.

Among others, the occurrence of DNA damage, senescence and the changed cytokine profile in the BM microenvironment of MDS patients have been linked to the onset and progression of the disease. Currently, the first-choice therapy for the treatment of high-risk MDS is 5-AC – a hypomethylating agent with an impact on the epigenetic regulation. Therefore, we have investigated the effects of 5-AC on other changes, such as the induction of cell senescence, the changes of cytokine levels and the immunomodulation effect.

Second, we investigated the impact of the common single nucleotide polymorphism C609T of the NAD(P)H dehydrogenase (quinone 1) (NQO1) on incidence and survival of MDS patients that have reduced levels of the functional NQO1 protein due to this $NQO1^{C609T}$ (NQO1*2) polymorphism. The reduction of NQO1 protein levels affects their ability to detoxify benzene compounds and to react to oxidative stress. This analysis was done by genotyping the archived BM aspirate smears or peripheral blood samples from MDS patients at the Hematology department of the General University Hospital in Prague and by analyzing how the NQO1*2 polymorphism affects the parameters of the progression of the disease.

Additionally, we inspected the possible augmentation of lenalidomide therapy in relapsed and refractory patients with del(5q) MDS by addition of erythropoietin and anti-inflammatory glucocorticoid prednisone.

The third project was focused on deciphering of the driving forces beyond the cell fate choice in AIMT $CD8^+$ T-cells. The cell fate choices are driven by the extracellular stimuli such as cytokines as well as by the specificity of a TCR expressed in a particular T-cell clone. We have hypothesized that VM T-cells might originate from a relatively highly self-reactive $CD8^+$ T-cells clones. The aim of this project was to uncover the origin of VM T-cells and to characterize the function and differentiation program triggered in this T-cell subset. Moreover, in order to better understand the AIMT cell compartment, we have reviewed the current literature on the topic and summarized the published works into a review article.

7.1 Specific aims

1. Characterization of the effect of 5-AC therapy on the expression profile of cytokines in the BM microenvironment, the answer to DNA damage in the BM of MDS patients by using the *in vitro* models.
2. Utilization of the candidate markers for the improvement of diagnosis and prognosis of MDS patients.
3. Analysis of the gene expression of naive, VM and antigen-experienced T-cells using deep mRNA sequencing to clarify whether VM T-cells use a unique differentiation program.
4. Phenotypic and functional characterization of the monoclonal T-cell populations expressing TCRs cloned from the naive or VM T-cell subpopulations.
5. Comparison of the TCR repertoires expressed by naive and VM T-cell subpopulations by sequencing of the TCR-encoding genes to clarify whether TCR specificity drives the differentiation of T-cells into VM cells.

8 Materials and methods

The experiments performed for the research described in my dissertation thesis span a wide range of methodologies. For the majority of the experiments, either human or murine cells were used.

For *in vitro* experiments primary BM-MNC and human cell lines were used. For this purpose, we have obtained fresh BM samples aspirated from the posterior iliac crest of healthy volunteers. The BM plasma was separated by centrifugation and the cytokine milieu was analyzed to establish the baseline levels of proinflammatory cytokines. Subsequently, erythrocytes were lysed and the BM-MNC were cultivated *in vitro* and treated with 5-AC. Additionally, a selection of human cancer cell lines or immortalized normal cells was utilized for the experiments studying the effects of 5-AC administration *in vitro*.

In vivo experiments were performed using various transgenic mouse models, mainly derived from C57Bl/6 strain. One of those models was C57BL/6-Tg(Ins2-OVA)59Wehi/WehiJ mouse (commonly referred to as RIP.OVA^{hi}, here as RIP.OVA) [129]. This mouse strain carries a transgene comprising of the full-length chicken ovalbumin cDNA under the control of rat insulin promoter, resulting in the soluble form of ovalbumin (OVA) that is highly expressed in pancreatic β-cells. We used this model for studying the autoimmune potential of OVA-specific VM T-cells. Firstly, we adoptively transferred sorted OVA-specific CD8^{+} T-cells into RIP.OVA mice by intravenous injection into the tail vein. This was followed by intravenous injection of either *Listeria monocytogenes* expressing OVA epitope or DC loaded with OVA peptide (SIINFEKL). We utilized a selection of OVA peptide derivatives with variable strength of binding to OVA-specific TCR. In case of sufficient strength of binding of OVA epitope/peptide to OVA-specific TCR, the stimulated adoptively transferred CD8^{+} T-cells expanded and induced apoptosis in OVA-expressing pancreatic β-cells, subsequently leading to the development of experimentally induced autoimmune diabetes due to the lack of insulin. We monitored the development of diabetes by measuring blood glucose levels and the presence of glucose in urine.

The experimental data were collected via a wide range of individual protocols. The five key approaches for the data collection included: (1) multicolor flow cytometry and fluorescence-activated cell sorting, (2) a multiplex bead-based assay for evaluation of cytokines, (3) immunofluorescence and immunoblotting, (4) quantitative real time-PCR and (5) RNA sequencing.

The additional methodologies are further described in detail in their respective publications.

9 Results and discussion

In recent years, a great effort has been deployed towards better understanding the molecular changes in the cells and in the environment that contribute to the development and progression of the MDS. In order to gain a better comprehension of the contribution of BM microenvironment on the progression of MDS, we investigated the changes in proinflammatory cytokine milieu in the MDS BM plasma. We then concentrated our efforts toward finding possible prognostic markers for disease progression by analyzing the impact of NQO1*2 polymorphism on the development and progression of MDS. Additionally, we investigated whether the augmentation of lenalidomide treatment by addition of erythropoietin or prednisone can increase the response rate in refractory and relapsed del(5q) MDS patients.

Second arm of my research focused on the characterization of VM CD8$^+$ T-cells. The depletion of this population was recently identified in the CML. Thus VM T-cells could as well contribute to the MDS pathology. By analyzing this cell population in several mouse models, we were able to identify the developmental cues for the formation of VM T-cells, analyze their expression profiles and characterize this population with regards to self-tolerance. Finally, we compared multiple aspects of the biology between different subsets of AIMT cells and concluded that VM T-cells and LIM T-cells likely represent a single subset of AIMT cells. Further details are discussed below.

9.1 Changes of the BM cytokine milieu in MDS

To provide the insights into alterations of the proinflammatory cytokines during MDS progression, we measured the proinflammatory cytokine changes in MDS patients from the entire spectrum of the IPSS-R score (low-risk, high-risk and AML). We were able to find several elevated proinflammatory cytokines in the BM of MDS patients. Specifically, we found elevated levels of IL-8, IP-10/CXCL10 (Interferon γ inducible protein 10/C-X-C Motif Chemokine Ligand 10), MCP-1/CCL2 (Monocyte Chemoattractant Protein-1/ C-C Motif Chemokine Ligand 2) and IL-27 and reduction in the levels of IL-12p70 in the BM of MDS patients compared to healthy donors (**Publication #1**).

The elevated levels of IL-8 were described in multiple works studying the inflammatory diseases and cancers. Under normal conditions, IL-8 promotes trafficking of neutrophils to the site of injury and stimulates the formation of neutrophil extracellular traps. In pathogenesis, tumor-derived IL-8 promotes an influx of MDSCs into tumor microenvironment, leading effectively to diminishing of the anti-tumor immune responses [130]. Recently, CD34$^+$ blasts

were identified as the main source of IL-8 in AML. Subsequently, the inhibition of IL-8 receptor CXCR2 (C-X-C Motif Chemokine Receptor 2) leads to the alleviation of the disease via inhibiting the proliferation of aberrant HSC cells [131], making IL-8 axis inhibition an ideal candidate for therapeutic intervention in MDS. Additionally, IL-8, IP-10 and MCP-1 contribute to the senescence-associated secretome [132, 133].

IL-12 is produced primarily by the functional APCs after activation via Toll-like receptor signaling and is needed for the maturation of DCs. Healthy MSC have the ability to suppress the proliferation of nearby T-cells via suppressing the maturation of DCs. *In vitro* co-culture of MDS-MSC with mature monocyte-derived DCs yielded lower secretion of IL-12 than in healthy MSC, suggestive of the compromised functions of DCs [134]. Measured decrease in the production of IL-12 in all MDS groups in our cohort thus indicates an impaired capacity to induce effective adaptive immune responses in MDS patients.

Another member of the IL-12 family, IL-27, is produced by APCs and is involved in the immune homeostasis via inhibition of Th17 differentiation [135]. Additionally, IL-27 modulates Treg responses by inhibiting the production of IL-2 [136, 137]. Both of these cell types play a major role in the progression of MDS [36].

In the effort to better understand the effects of 5-AC on the BM, we assessed the proinflammatory cytokine secretion profiles of the several cell lines and the healthy BM mononuclear cells (BM-MNC) from fresh BM aspirates after the treatment with 5-AC *in vitro*. Indeed, we showed that 5-AC induces expression of proinflammatory cytokines both in several cancer cell lines as well as in immortalized MSC and in BM-MNCs. The levels of IL-8, IL-27 and MCP-1 were further elevated in BM plasma of high-risk MDS patients during 5-AC therapy, with moderately higher levels of IL-8 and IL-27 in the patients not responding to the therapy.

Additionally, the inflammatory co-morbidities such as diabetes mellitus or rheumatoid arthritis contributed to the elevated levels of IL-8 and MCP-1 in low-risk MDS patients.

Notably, the levels of cytokines measured from the peripheral blood plasma at the time of BM aspiration did not strictly correlate with the cytokine levels in the BM plasma, indicating that the estimation of cytokines from the PB plasma could be insufficient to obtain an accurate projection of the cytokine milieu in the BM niche.

Overall, all forms of MDS feature a deregulated proinflammatory cytokine landscape in the BM and such alterations are further augmented by the 5-AC therapy of high-risk MDS patients.

9.2 Utilization of candidate markers for the improvement of diagnosis and prognosis of MDS patients.

Although MDS is a serious illness known for several decades, the molecular pathogenesis and the question why the disease evolves to AML remain unclear. Genetic, epigenetic, and immunopathological factors most likely contribute to the pathogenesis of MDS [138-140].

About 50% of MDS patients have cytogenetic abnormalities, mostly unbalanced chromosomal losses and gains with profound impact on the expected OS of these patients. Recently, a lot of effort has been directed towards the identification of somatic mutations in stem cells and other progenitors that could influence the initiation and progression of MDS [141, 142]. Somatic mutations occur in about 80% of MDS patients [140, 143]. The presence of somatic mutations can lead to the dysplastic hematopoiesis caused by the apparent growth advantages of the mutation-ridden aberrant HSC clones. These somatic mutations could be a consequence of the BM exposure to the toxic chemicals, as in the case of secondary MDS.

Inborn changes that lead to the subsequent MDS evolution were so far only described in rare inherited BM failure syndromes such as Fanconi anemia, Diamond-Blackfan anemia, congenital dyskeratosis, and Shwachman-Diamond syndrome [144].

In our effort to find a prognostic marker for the development and progression of MDS, we decided to focus on the NQO1, an enzyme involved in detoxification of quinones, reduction of oxidative stress, and stabilization of p53 (**Publication #2**).

Under normal conditions NQO1 protein is not detected in human BM cell aspirates or in the purified CD34^{+} blast cells, however upon benzene exposure, both of these cell types are inducible for the NQO1 activity [145]. The *NQO1* gene contains the Nrf2-binding site in its promoter region and its expression is regulated by the Keap1/Nrf2/ARE pathway that is essential for the adaptation to oxidative stress (Keap1, Kelch Like ECH Associated Protein 1; Nrf2, Nuclear Factor, Erythroid 2 Like 2; ARE, antioxidant response element). The NQO1 protein is predominantly present in the BM stroma, endothelial cells and in BM adipocytes [146] and plays a role in endothelial adhesion of hematopoietic cells [147].

The naturally occurring germline polymorphism of NQO1^{C609T} (NQO1*2) results in loss of the NQO1 activity and rapid degradation of the NQO1*2 protein due to the formation of an unstable tertiary structure [148]. The individuals homozygous for this polymorphism have no NQO1 activity, while the heterozygotes have low to intermediate activity that can be insufficient during high genotoxic stress, e.g. anthracycline chemotherapy and exposure to toxic radicals caused by iron overload [149]. Recently, several studies indicated that

individuals with NQO1 deficiency may be at increased risk for the development of MDS and leukemia [150-152].

In our study, we focused on the possible influence of NQO1 enzyme activity on the course of the disease itself, the impact of NQO1*2 polymorphism on the severity of the disease, and the overall survival. Considering the clinical heterogeneity of the MDS, we have analyzed the correlation of NQO1*2 polymorphism with the various IPSS-R score groups and known prognostic factors.

Firstly, we have found a higher presence of NQO1*2 among MDS/AML patients in comparison to a large population cohort of previously published healthy controls. Therefore, in agreement with previous studies our data indicate that there is a predisposition to the MDS development among NQO1*2 bearers.

Moreover, our results revealed the higher presence of NQO1*2 in the MDS subgroups with higher BM blasts percentage and with karyotype abnormalities. However, we did not find any correlation with the specific cytogenetic aberrations, as was described by Zachaki *et al.* [153], probably due to the relatively smaller sample size of patients with cytogenetic aberrations.

Further, we examined the impact of NQO1*2 polymorphism on the OS in MDS patients with normal karyotype and found a striking difference in the OS among low-risk patients. In our cohort, the patients with fully functional NQO1 survived from the date of diagnosis on average nearly twice as long as the patients with NQO1*2. Moreover, the NQO1*2 polymorphism impacted the OS among 5-AC-treated MDS patients. The patients with NQO1*2 progressed faster than NQO1 and thus were eligible for the 5-AC treatment sooner after the diagnosis than NQO1.

Recently, Rassool *et al*. showed in the murine model that an increase in oxidative stress plays a role in the development and progression of myeloid leukemia [154]. One of the sources of the oxidative stress in MDS is iron overload induced by regular erythrocyte transfusions that are affecting over 60% of MDS patients and shortening the expected OS [155].

Superabundant intracellular ferric ions give rise to the production of highly reactive hydroxyl radicals that can induce the oxidative damage of lipids, proteins, and DNA and can trigger cell death [156]. Production of toxic radicals and the development of oxidative stress can inflict the damage of hematopoietic progenitors, genetic instability, and worsen the BM microenvironment, resulting in the emergence of novel mutations and genesis of additional clonal aberrations [157].

NQO1 is one of the enzymes needed for the regulation of oxidative stress caused by the accumulation of hydroxyl radicals during the iron overload. The presence of several

polymorphisms in the genes coding for the enzymes in the antioxidant defense system (Nrf2, NQO1, NOS3, and HO-1) concurrent with the iron overload can have impact on the etiology of breast cancer [158]. Since the expression of Nrf2 and NQO1 can be upregulated by the dietary iron overload [159], we wanted to ascertain whether the decreased NQO1*2 cytoprotective function can play a role in the progression of MDS in patients burdened with increased ferritin levels and iron overload. Indeed, we have found significantly shortened OS among patients affected by both the presence of NQO1*2 polymorphism and increased serum ferritin levels when compared to other patient subgroups.

In conclusion, the presence of NQO1*2 alleles is associated with a higher probability of MDS development, faster disease progression, sensitivity to blood transfusion-provoked iron overload, and shorter expected OS indicating that the knowledge about NQO1 polymorphism among MDS patients at the time of the diagnosis, as well as the early administration of iron-chelating agents to patients affected both by the dysfunctional NQO1 allele and iron overload, might be beneficial for the control of disease progression.

Another approach on how to improve the response rate in low-risk MDS with del(5q) chromosomal aberration focused on the combination of lenalidomide therapy with erythropoietin and/or glucocorticoid prednisone (**Publication #3**). The use of lenalidomide is approved for low-risk MDS patients with del(5q) chromosomal aberrations. This group of MDS patients is very specific because of their long OS, progressive macrocytic anemia with increased platelet count and a high rate of transfusion dependency.

By binding to CRBN, lenalidomide modifies the substrate specificity of E3 ubiquitin ligase CRL4 and influences the proteasomal degradation of its target proteins. One of these proteins is casein kinase 1A1 (CSNK1A1) that is haploinsufficient in del(5q) cells. The relative lack of CSNK1A1 results in the increased levels of β-catenin that support the proliferation of aberrant HSC with del(5q) [160]. However, when the levels of CSNK1A1 drop beyond a certain level, this induces the p53-dependent apoptosis. The treatment with lenalidomide thus selectively induces apoptosis in the del(5q) HSCs [161].

An additional effect of lenalidomide is the stimulation of erythropoiesis, leading to the common loss of transfusion dependency [162]. Despite its efficacy and the initial response in 60-70% of the patients, approximately 30% of the initially responding patients eventually relapse [163]. Recently, the group of Basiorka *et al.* explained the possible mechanism for the restorative ability of lenalidomide on erythropoiesis. Lenalidomide treatment of normal BM-MNC *in vitro* increased the erythropoietin sensitivity by inhibiting the E3 ubiquitin ligase RNF41 that regulates the erythropoietin receptor turnover (RNF41, Ring Finger Protein 41) [164].

Simultaneously, Toma *et al*. successfully utilized the combination of erythropoietin and lenalidomide in transfusion-dependent refractory low-risk MDS without del(5q) [165]. Moreover, *in vitro* study by Narla *et al.* demonstrated that the treatment with lenalidomide with corticosteroid drug dexamethasone has an additive effect on the expansion of erythroid progenitors [166].

Consequently, we postulated that the combination of corticosteroid prednisone, erythropoietin and lenalidomide should lead to the reduction of transfusion dependency in refractory MDS and in relapsed patients with del(5q) MDS. The success of this clinical management was confirmed in our study cohort. Five out of seven relapsed del(5q) MDS patients treated with lenalidomide in our cohort achieved transfusion independence in response to the addition of either erythropoietin alone or in combination with prednisone. As per non del(5q) MDS group, only one out of ten treated MDS patients achieved transfusion independence after combination therapy with lenalidomide and erythropoietin.

Hence, the treatment with lenalidomide represents an effective solution for del(5q) MDS group and the combination with prednisone and erythropoietin may be beneficial for initial non-responders or patients after therapy failure.

9.3 The role of homeostatic TCR signals in the formation of VM CD8$^+$ T-cells

As discussed previously, T-cells represent a hematopoietic lineage that is involved in most adaptive immune responses and play a role in hematopoiesis. T-cells make multiple cell-fate decisions during their maturation and life cycle. The cell-fate choices are driven by the extracellular stimuli (e.g. cytokines) as well as by the specificity of a TCR expressed in individual T-cell clones.

In this project (**Publication #4**), we focused on the effect of homeostatic TCR signals on the development of VM T-cells. Although constituting 10-20% of all peripheral CD8$^+$ T-cells in mice, the origin, biological roles, and relationship of VM T-cells to naïve and foreign antigen-experienced memory T cells are incompletely understood.

By analyzing the gene expression profiles of naive, VM and antigen-experienced T-cells via deep mRNA sequencing, we were able to uncover that VM T-cells represent a distinct T-cell population. Further assessment of the previously established signature genes for memory program and for naïve program uncovered that VM T-cells represent an intermediate stage between naïve and memory T-cells.

Next, we intended to better understand the forces driving differentiation to the VM T-cell program. We tested a hypothesis that the level of self-reactivity of a given T-cell has an impact on the formation of VM T-cell. For this, we benefited from the previously published observations that the coupling frequency between CD8 co-receptor and Lck kinase (LCK Proto-Oncogene, Src Family Tyrosine Kinase) is a limiting factor for TCR signaling in thymocytes [167, 168].

We used a transgenic mouse model expressing a CD8.4 chimeric co-receptor, consisting of the extracellular portion of CD8 fused to an intracellular part of CD4 [168]. This fusion leads to a supraphysiological coupling of the CD8 co-receptor to Lck, a kinase that initiates a TCR signal transduction. The resulting CD8.4 T-cells have enhanced TCR signaling to self-antigens [169], which allowed us to address the role of homeostatic TCR signaling in VM T-cell formation.

We found that strong homeostatic TCR signaling introduced by a higher CD8-Lck coupling in CD8.4 T-cells leads to a higher frequency of differentiation into VM T-cells in a polyclonal repertoire. Furthermore, by introducing the CD8.4 transgene into mice with monoclonal T-cell populations, we ascertained that the high level of self-reactivity predetermines the VM differentiation program in $CD8^+$ T-cells.

Next, we compared the TCR repertoires expressed by naive and VM T-cell subpopulations by sequencing the TCR-encoding genes to elucidate whether the TCR-specificity drives the differentiation of T-cells into VM developmental program. Indeed, we found that the naïve and VM T-cells use distinct TCR repertoires, which we directly confirmed by generating retrogenic monoclonal T-cell populations expressing TCRs cloned from the naive or VM T-cell subpopulations [170] that recapitulated the same cell fate choice.

Until recently, it was generally acknowledged that the antigen-experienced memory T-cells surpass the naïve T-cells and induce a much faster and stronger response to cognate antigen stimulation [171-173]. Tearing down the scientific dogma, it was shown that the response of naïve T-cells to antigen stimulation can be under certain conditions stronger than the response of memory T-cells [174-176]. In line of this evidence, the comparison between VM T-cells and naïve T-cells showed that VM T-cells better respond to inflammatory cytokines IL-12 and IL-18 [110], more rapidly generate short-lived effectors [177], and better protect against *Listeria monocytogenes* infection [177, 178] than naïve T-cells. However, our results clearly showed that the VM T-cells were less efficient than naïve T-cells with the same TCR specificity in inducing the experimental autoimmune diabetes. A partial explanation of this phenomenon lies in the lower upregulation of CD49d and CD25 in VM T-cells than in naïve T-cells upon the activation with sub-optimal antigen. Our findings thus demonstrate that the induction of VM

developmental program in the highly self-reactive T-cells can serve as a compensatory mechanism in the protection against autoimmunity.
Subsequently, we summarized the published findings about different subtypes of AIMT cells, such as cytokine requirements for their formation, the impact of TCR specificity on the differentiation process, gene expression signature markers and strength of the immune response. Based on these parameters, we concluded that VM T-cells and LIM T-cells likely represent a single subset of AIMT cells. We have proposed to call this subset “homeostatic memory T-cells” (**Publication #5**).

10 Conclusion

In my dissertation thesis, I discussed five projects dealing with the pathophysiological development and differentiation of cells during hematopoiesis. Three projects were focused on the pathophysiological changes of the hematopoiesis during the development and progression of MDS.

In these projects, we showed that the proinflammatory cytokine milieu in the BM changes during the progression of the disease. We found an increase in IL-8, IP-10, MCP-1 and IL-27 and decrease in IL-12 levels compared to healthy controls. Repeated sampling of high-risk MDS patients showed an increase in IL-8, IL-27 and MCP-1 over the course of 5-AC therapy. Moreover, we identified NQO1*2 as a novel marker for the prediction of the pace of MDS progression and expected OS. Third, we confirmed that the addition of erythropoietin and/or prednisone improves the response rate of relapsed and del(5q) MDS patients treated with lenalidomide.

Second arm of my research focused on the characterization of VM $CD8^+$ T-cells. We established the VM T-cell development as a novel cell-fate decision checkpoint, determined by their TCR self-reactivity. Next, we described the molecular mechanisms driving the formation of VM T-cells and discovered that although VM T-cells develop from the highly self-reactive cells and acquire a partial memory program, they are less efficient in inducing the experimental autoimmune diabetes than naïve T-cells.

Finally, we compared multiple aspects of the biology between different subsets of AIMT cells and concluded that the VM T-cells and LIM T-cells likely represent a single subset of AIMT cells. We have proposed to call this subset “homeostatic memory T-cells”.

10.1 Summary of major findings

1. The proinflammatory cytokine milieu in the BM changes during the progression of the MDS. The patients undergoing the 5-AC therapy experienced an increase in the levels of proinflammatory cytokines IL-8, IL-27 and MCP-1 over the course of therapy.
2. NQO1*2 is a novel candidate marker for the prediction of the pace of MDS progression.
3. Addition of erythropoietin and prednisone improves the response rate of relapsed and del(5q) MDS patients treated with lenalidomide.
4. Naïve, antigen-experienced and VM T-cells represent distinct cell populations with unique expression profiles. The expression profile of VM T-cells corresponds to the intermediate stage between naïve and antigen-experienced memory T-cells.
5. VM T-cell development is a novel cell-fate decision checkpoint, determined by their TCR self-reactivity. TCR specificity drives differentiation of T-cells into VM cells.
6. VM T-cells acquire partial memory program, but they are less efficient in inducing the experimental autoimmune diabetes than naïve T-cells.

11 Publications

The fulltexts of the publications included in this thesis can be found in the last section of the thesis.

11.1 List of the publications included in the thesis

#1 Dynamic alterations of bone marrow cytokine landscape of myelodysplastic syndromes patients treated with 5-azacytidine. **Moudra A**, Hubackova S, Machalova V, Vancurova M, Bartek J, Reinis M, Hodny Z, Jonasova A. Oncoimmunology. 2016 May 13;5(10):e1183860. PMID: 27853634

#2 NQO1*2 polymorphism predicts overall survival in MDS patients. **Moudra A**, Minarik L, Vancurova M, Bartek J, Hodny Z, Jonasova A. British Journal of Haematology. 2019 Jan;184(2):305-308. PMID: 29363755

#3 Lenalidomide treatment in lower risk myelodysplastic syndromes-The experience of a Czech hematology center. (Positive effect of erythropoietin ± prednisone addition to lenalidomide in refractory or relapsed patients). Jonasova A, Neuwirtova R, Polackova H, Siskova M, Stopka T, Cmunt E, Belickova M, **Moudra A**, Minarik L, Fuchs O, Michalova K, Zemanova Z. Leukemia Research. 2018 Jun;69:12-17. PMID: 29614393

#4 Strong homeostatic TCR signals induce formation of self-tolerant virtual memory CD8 T cells. Drobek A*, **Moudra A***, Mueller D, Huranova M, Horkova V, Pribikova M, Ivanek R, Oberle S, Zehn D, McCoy KD, Draber P, Stepanek O. EMBO Journal. 2018 Jul 13;37(14). PMID: 29752423 *** Equal contribution**

#5 Opinion: Virtual memory CD8 T cells and lymphopenia-induced memory CD8 T cells represent a single subset: Homeostatic memory T cells. Pribikova M, **Moudra A**, Stepanek O. Immunology Letters. 2018; 203:57-61. PMID: 30243945

11.2 Contribution

Ad #1 **Dynamic alterations of bone marrow cytokine landscape of myelodysplastic syndromes patients treated with 5-azacytidine.**

I performed all experiments, sample collection and analysis of the data (Figure 1-4) and wrote the manuscript under the supervision of Dr. Zdeněk Hodný, Dr. Anna Jonášová and Dr. Jiří Bartek.

Ad #2 **NQO1*2 polymorphism predicts overall survival in MDS patients.**

I performed the genotyping, sample collection, created the database of the patient data, designed the study and performed the statistical analysis (Figure 1-2) as well as wrote the manuscript under the supervision of Dr. Zdeněk Hodný, Dr. Anna Jonášová and Dr. Jiří Bartek.

Ad #3 **Lenalidomide treatment in lower risk myelodysplastic syndromes-The experience of a Czech hematology center. (Positive effect of erythropoietin ± prednisone addition to lenalidomide in refractory or relapsed patients).**

I contributed to the project by performing the statistical analysis of the patient data (Figure 1) and wrote the manuscript under the supervision of Dr. Anna Jonášová.

Ad #4 **Strong homeostatic TCR signals induce formation of self-tolerant virtual memory CD8 T cells.**

I performed the research, data collection (Figure 3 - 6), and data analysis of the experiments and wrote the manuscript under the supervision of Dr. Ondřej Štěpánek.

Ad #5 **Opinion: Virtual memory CD8 T cells and lymphopenia-induced memory CD8 T cells represent a single subset: Homeostatic memory T cells.**

I contributed to the review by writing parts of the manuscript and the reviewing the source data for the publication under the supervision of Dr. Ondřej Štěpánek.

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