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Activated lymphocytes and monocytes in myelodysplastic syndromes

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LIST OF ABBREVIATIONS

AML	acute myeloid leukemia
ANC	absolute neutrophil count
ATG	antithymocyte globulin
BCR/ABL	breakpoint-cluster region/Abelson leukemia
bFGF	basic fibroblast growth factor
BFU-E	burst-forming unit of erythrocytes
BM	bone marrow
CD	cluster of differentiation
CD40L	CD40 ligand
CDR	complementarity determining region
CFC	colony forming cell
CFU-GM	granulocyte-monocyte colony forming unit
CRP	C-reactive protein
CsA	cyclosporine A
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
FAB	French-American-British (classification)
FACS	fluorescent activated cell sorting
FasL	Fas-ligand
FCS	fetal calf serum
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
FSC	forward scatter
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GPI	glycosylphosphatidylinositol
Hb	hemoglobin
HPC	hematopoietic progenitor cell
HUMARA	Human androgen receptor
IFN-γ	interferon-gamma
IL	interleukin
IMiDs	immune-modulatory drugs
Int-1/2	intermediate risk 1/2
IPSS	International Prognostic Scoring System
mAb	monoclonal antibody
LPS	lipopolysaccharide
MACS	magnetic activated cell sorting
MDS	myelodysplastic syndromes
MNC	mononuclear cells
MoDC	monocyte derived dendritic cell
MPD	myeloproliferative disorders
NK	natural killer

PB	peripheral blood
PBS	phosphate-buffered saline
PE	phycoerythrin
PerCP	peridinin-chlorophyl protein
PIG-A	phosphatidyl inositol glycan-class A
PNH	paroxysmal nocturnal hemoglobinuria
RA	refractory anemia
RAEB	refractory anemia with excess blasts
RAEB-t	refractory anemia with excess blasts in transformation
RARS	refractory anemia with ringed sideroblasts
RCMD	refractory cytopenia with multilineage dysplasia
RCMD-RS	refractory cytopenia with multilineage dysplasia and ringed sideroblasts
RIC	reduced-intensity conditioning
SAA	severe aplastic anemia
SCF	stem cell factor
SSC	side scatter
TCR	T cell receptor
TGF-β	transforming growth factor-beta
T-LGL	T-cell large granular lymphocyte
TNF-α	tumor necrosis factor-alpha
TRAIL	TNF-related apoptosis inducing ligand
Treg	regulatory T cell
VEGF	vascular endothelial growth factor
XCI	X-chromosome inactivation
WHO	World’s Health Organization
WT-1	Wilms’ tumor antigen-1

Chapter 1

General Introduction

The myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal hematological diseases. Cytopenia is the presenting symptom in most patients diagnosed with MDS and is the consequence of impaired production of mature blood cells in the bone marrow (BM). The variable degree of BM failure and the increased tendency of patients to develop acute leukemia account for the wide clinical heterogeneity of the disease. Acquired defects in the hematopoietic progenitor cells play a central role in the development of MDS. This is supported by the observation that the mean age of patients at diagnosis is 70 years. The annual incidence of MDS ranges from 2 – 12 cases/100.000 per year, but increases to 50 cases per 100.000 among persons aged 70 or older (Aul et al, 2001). Some environmental risk factors have been identified, including occupational exposure to benzene. Also medical therapies (such as alkylating agents, topoisomerase II inhibitors and ionizing radiation) are related with the development of MDS (Steensma and Bennett 2006).

There is currently no standard treatment for MDS. The only putative curative treatment is allogeneic stem cell transplantation, but only a minority of patients is eligible for this treatment. Although promising recent results with immunomodulatory drugs (List et al, 2006, Raza et al, 2008) and methyltransferase-inhibitors (Silverman et al, 2006, Wijermans et al, 2005), the majority of patients will only receive supportive treatment with regular transfusions, antibiotics and hematopoietic growth factors.

In recent years it has become clear that also immune mechanisms contribute to the clinical shaping of the disease. Furthermore, it has become clear that immunosuppressive treatment modalities can alleviate cytopenias in selected patients. The further clarification of these immune processes was the main object of this doctoral project. Before getting into this subject, an introduction into the physiopathology and the different MDS classification systems is in order.

1.1. Myelodysplastic syndromes: Classification and Prognosis

The heterogeneity in the clinical evolution and morphological findings in MDS is reflected in the existence of different classifications and prognostic scores. In 1982, the French-American-British (FAB) cooperative group proposed guidelines for the diagnosis and classification of MDS (Bennett et al, 1982). This FAB-classification was primarily based on morphological findings. Five clinical subgroups were proposed, based on the finding of dysplasia in the myeloid lineage, the percentage of blood and marrow blasts, the presence of ringed sideroblasts and the finding of Auer rods (see table I.1). In general, RA and RARS are considered as “low-risk” disease, whereas RAEB and RAEB-t are associated with a poor prognosis and have a higher risk to progress towards leukemia.

In 2001, the FAB guidelines were revised and updated in the World Health Organization (WHO) Classification of MDS (Vardiman et al, 2002). This classification was not only based on morphology, but also incorporated prognostic factors like degree of dysplasia and cytogenetics to define MDS entities (see table I.2). The WHO classification has lead to the introduction of subgroups like refractory cytopenia with multilineage dysplasia (RCMD) with or without ringed sideroblasts and the 5q- syndrome. It also reclassified RAEB-t in the AML-group and in addition classified chronic myelomonocytic leukemia (CMML) as an “overlap” syndrome between MDS and myeloproliferative disorders (MPD).

Table I.1: *French-American-British (FAB) Classification*

Disease	PB findings	BM findings
RA	Cytopenia of 1 lineage <1% blasts	Normo- or hypercellular with dysplasia <5% blasts
RARS	Cytopenia of 1 lineage <1% blasts	Normo- or hypercellular with dysplasia <5% blasts Ringed sideroblasts >15% of nucleated cells
RAEB	Cytopenia of 2 or more lineages <5% blasts	Dysplasia involving all 3 lineages 5-20% blasts
RAEB-t	Cytopenia of 2 or more lineages >5% blasts or Auer rods	Dysplasia involving all 3 lineages 21-30% blasts or presence of Auer rods in blasts
CMML	Monocytosis (> 1000/µl) <5% blasts	<20% BM blasts

Table I.1: FAB-classification (Bennett *et al*, 1982)
RA: refractory anemia, RARS: refractory anemia with ringed sideroblasts; RAEB: refractory anemia with excess blasts; RAEB-t: RAEB in transformation; CMML: chronic myelomonocytic leukemia

Table I.2: *World Health's Organization Classification*

Disease	PB findings	BM findings
RA	Anemia no or rare blasts < 10^9/L monocytes	Erythroid dysplasia only <10% granulocytes or MGK dysplastic <5% blasts <15% ringed sideroblasts
RARS	Anemia No blasts	Erythroid dysplasia only <10% granulocytes or MGK dysplastic >15% ringed sideroblasts <5% blasts
RCMD	Cytopenia 2 or 3 lineages No or rare blasts No Auer rods < 10^9/L monocytes	Dysplasia in >10% of cells in 2 or more myeloid cell lines <5% blasts No Auer rods <15% ringed sideroblasts
RCMD-RS	Cytopenia 2 or 3 lineages No or rare blasts No Auer rods < 10^9/L monocytes	Dysplasia in >10% of cells in 2 or more myeloid cell lines <5% blasts No Auer rods >15% ringed sideroblasts
RAEB-1	Cytopenias <5% blasts No Auer rods < 10^9/L monocytes	Unilineage or multilineage dysplasia 5-9% blasts No Auer rods
RAEB-2	Cytopenias 5-19% blasts Auer rods +/- < 10^9/L monocytes	Unilineage or multilineage dysplasia 10-19% blasts Auer rods +/-
MDS-U	Cytopenias No or rare blasts No Auer rods	Unilineage granulocytic or MGK dysplasia <5% blasts No Auer rods
5q- syndrome	Anemia <5% blasts Platelets normal or increased	Normal to increased MGK with hypolobulated nuclei <5% blasts No Auer rods Isolated del (5q)

Table I.2: WHO classification (Vardiman *et al*, 2002)
RA: refractory anemia; RARS: refractory anemia with ringed sideroblasts; RAEB: refractory anemia with excess blasts; RAEB-t: refractory anemia with excess blasts in transformation; CMML: chronic myelomonocytic leukemia; RCMD: refractory cytopenia with multilineage dysplasia; MDS-u: MDS unclassified; MGK: megakaryocyte

Cytopenia is the presenting symptom in most patients and the majority of patients will succumb to complications of cytopenia rather than to acute leukemia. The significance of cytopenia is recognized in the International Prognostic Scoring System (IPSS) (Greenberg et al, 1997). The IPSS categorizes patients into 4 prognostic subgroups based on the percentage of BM blasts, the presence and the type of cytogenetic abnormalities and the degree of cytopenia (see table I.3). Besides these factors, several other disease-related characteristics have been shown to have an impact on clinical outcome. These include morphological characteristics (e.g. atypical localization of immature progenitors, "ALIP" (Tricot et al, 1984, Verburgh et al, 2003)), lactate dehydrogenase (Germing et al, 2005), transfusion dependency (Malcovati et al, 2005), angiogenic markers (Lundberg et al, 2006), immunophenotype (Stetler-Stevenson et al, 2001), *in vitro* clonogenic myeloid assays (Aul et

al, 1992) and telomere dynamics (Ohyashiki et al, 1994). Therefore, new prognostic models have been suggested (Bernasconi et al, 2007, Germing et al, 2005, Malcovati et al, 2007, Malcovati et al, 2005). Prognostic classification scores are important to optimize treatment strategies. Patients with low risk e.g. have a median survival reaching to 10 years and are therefore probably not eligible for prompt aggressive treatment strategies.

Table I.3: *International Prognostic Scoring System*

	Survival and AML evolution score value				
Prognostic variable	**0**	**0.5**	**1.0**	**1.5**	**2.0**
Marrow blasts (%)	<5	5-10	-	11-20	21-30
Karyotype[a]	Good	Intermediate	Poor		
Cytopenias[b]	0/1	2/3			

Table I.3: International Prognostic Scoring System (Greenberg *et al*, 1997)
[a]Good: normal, -Y, del(5q) only, del(20q) only; Poor: complex (≥ 3 abnormalities) or chromosome 7 anomalies; Intermediate: +8, single miscellaneous abnormality or double abnormalities
[b]Absolute neutrophil count < 1500/mm³; platelet count <100.000/mm³; hemoglobin <10g/dl

Risk group	Combined score	Median survival
Low	0	5.7 y
Intermediate-1	0.5-1.0	3.5 y
Intermediate-2	1.5-2.0	1.2 y
High	≥ 2.5	0.4 y

1.2. Pathogenesis of BM failure

The pathogenesis of marrow failure in MDS is complex, but the mystery is progressively unraveled. Although patients present with PB cytopenias, the BM cellularity is usually increased. This apparent paradox has been interpreted as a sign of intra-medullary destruction of a substantial portion of developing hematopoietic cells by apoptosis or programmed cell death (Yoshida 1993). Excessive apoptosis in MDS marrow cells has been ascribed to abnormalities of growth and differentiation, intrinsic to the myelodysplastic clonal population. But cell death is also triggered by the BM microenvironment, either through a relative deficiency of growth factors, through an overproduction of inhibitory cytokines, or through immune-mediated mechanisms (see figure I.1).

Figure I.1: *Pathogenesis of MDS-related marrow failure*

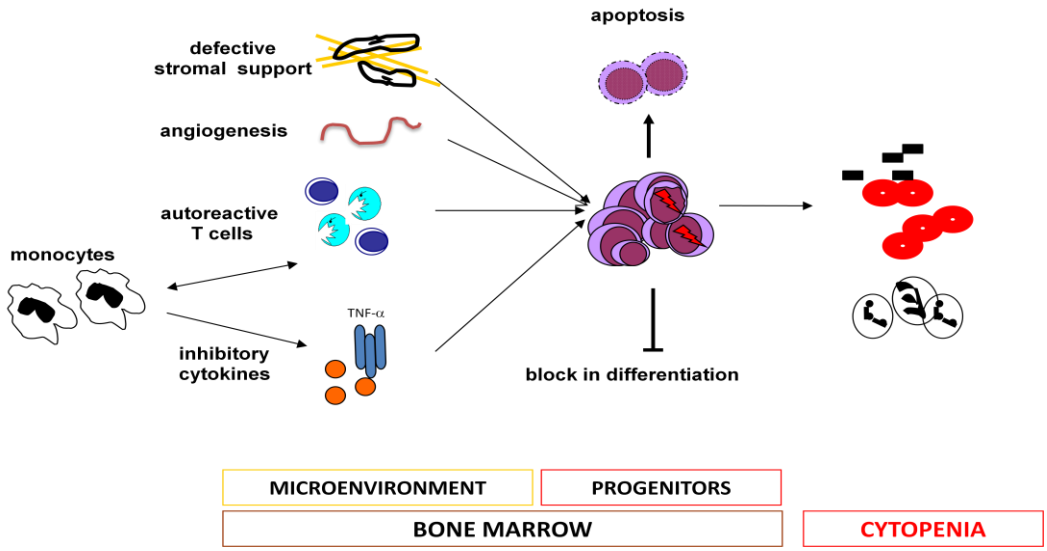


Figure I.1: Pathogenesis of MDS-related marrow failure
PB cytopenias result from intrinsic abnormalities within the hematopoietic progenitor pool leading to impaired differentiation and increased apoptosis. In addition, several factors within the BM microenvironment influence this process. These factors include a defective stromal supportive layer with lower production of growth-promoting cytokines, increased angiogenesis, the presence of growth-inhibiting cytokines and auto-reactive T cells. The influence of monocytes and the interplay between monocytes and T cells in the BM microenvironment were the subject of this study.

1.2.1. Excessive apoptosis of progenitor cells

Excessive apoptosis occurs in all FAB subtypes and affects erythroid, myeloid and megakaryocytic lineages (Lepelley et al, 1996, Raza et al, 1995). Apoptosis can be triggered by signals from outside the cell through the death receptor pathway, as well as through the intracellular mitochondrial pathway with subsequent caspase activation.

Supporting the involvement of the **death receptor pathway**, hematopoietic progenitor cells from MDS have been shown to over-express death receptors including CD95 (Fas) and TRAIL (TNF-related apoptosis-inducing ligand), as well as their ligands (Bouscary et al, 1997, Gersuk et al, 1998, Reza et al, 1999, Ribeiro et al, 2004, Zang et al, 2001). In normal circumstances, primitive HP cells express only low amounts of Fas. However different cytokines (including TNF-α and IFN-γ) have been shown to up-regulate Fas expression on $CD34^+$ cells (Dai et al, 1998, Maciejewski et al, 1995) and these cytokines are found to be up-regulated in MDS patients. In addition, MNC from MDS patients undergo increased apoptosis compared to normal HPC after treatment with anti-CD95-activating antibody or TRAIL activating antibody (Schmidt et al, 2001, Tehranchi et al, 2003). Cross-linking of Fas by its natural ligand results in the sequential triggering of caspases, which eventually leads to apoptotic cell death (De Maria et al, 1999, Krammer et al, 1994, Tanaka et al, 1996). The source of the stimuli that increase death-receptor signaling in MDS cells is not entirely clear. Macrophages (Allampallam et al, 2002), other stromal elements (Tauro et al, 2001, Tauro et al, 2002) or cytotoxic T cells (Kook et al, 2001) all might produce excess death-receptor stimuli such as TNF-α, which lead to increased apoptosis. Alternatively, the increased levels of TNF-α and FasL on the surface of hematopoietic progenitor cells from patients might induce autocrine stimulation of the death-receptor pathway.

There is also scientific evidence that implicates the **mitochondrial pathway** in modulating the increased levels of apoptosis (Tehranchi et al, 2003). However, there is uncertainty whether this activation is a primary event or an amplification of signals emanating from the death receptor pathway. The anemia seen in RARS can be interpreted as being induced by apoptosis driven by mitochondria. The increased iron deposits in perinuclear mitochondria leads to the generation of reactive oxygen species that disrupt the mitochondria (Tehranchi et al, 2005b).

Most reports argue in favor of the highest apoptotic rates in RA, RARS and RAEB with a progressive decline in apoptosis when the disease acquires a more leukemic phenotype (Bouscary et al, 1997, Parker and Mufti 1998). A shift in the ratio of pro-apoptotic (Bax, Bad) vs. anti-apoptotic (Bcl2, Bcl-X) proteins during disease evolution supports this observation (Parker et al, 1998).

Nevertheless, some questions remain regarding apoptosis. There are conflicting results in literature, largely due to different techniques used to determine apoptosis and whether the results were determined on BM biopsies or on aspirates. Reports on increased apoptosis after culture could also represent artifacts caused by the culture conditions. And although most studies report statistically different increments in the amount of apoptotic cells, the absolute increase in the number of apoptotic cells can be limited (Albitar et al, 2002, Claessens et al, 2005, Shimazaki et al, 2000). For example, the biological relevance of a 3-5% increase in the absolute percentage of apoptotic cells can be questioned (Corey et al, 2007).

1.2.2. Genomic abnormalities of progenitor cells

MDS is characterized by clonal hematopoiesis. Whereas in the normal situation mature cells arise from different BM hematopoietic stem cells, “MDS” clones dominate the normal stem cells that can still be found in the BM (Delforge et al, 1995). Intrinsic abnormalities within the progenitor cells promote aberrant proliferation and differentiation of MDS progenitor cells. Numerous genetic and epigenetic changes in key genes involved in proliferation and differentiation have been identified, including key transcription factors (Harada et al, 2004, Nakao et al, 2004), signal transduction pathways (Lyons et al, 1988), DNA repair enzymes (Lepelley et al, 1994) and cell-cycle regulators (Quesnel and Fenaux 1999). Average telomere length has been shown to be reduced in MDS progenitors and short telomere length coincides with complex cytogenetic abnormalities (Boultwood et al, 1997, Ohyashiki et al, 1994). None of these gene defects have however been identified as solely responsible for the MDS phenotype.

Cytogenetic abnormalities in hematopoietic cells are seen in 60% or more of patients at some point in their illness. The detection of these cytogenetic changes is important not only for diagnostic purposes, but also because they relate to prognosis (Haase et al, 2007). The IPSS acknowledges this importance and categorizes karyotypes with good, intermediate and poor prognosis (see table I.3).

These chromosomal abnormalities are not random and have also helped to further understand the pathogenesis of the disease. For example, the typically deleted regions of chromosome 5 contain genes for hematopoietic growth factors and their receptors (Pellagatti et al, 2007). Clonal cells lacking chromosome 7 appear to have a proliferation advantage due to over-expression of the short isoform of the granulocyte colony-stimulating factor (G-CSF) receptor (Sloand et al, 2006).

1.2.3. BM microenvironment

In addition to intrinsic defects in the progenitor cell pool leading to impaired production of mature blood cells, there is increasing evidence that influences from the hematopoietic microenvironment can aggravate this cytopenia in MDS. The role of immune cells in the physiopathology of MDS is discussed separately in the next paragraph.

Several groups showed that **stromal cells** derived from MDS marrow are impaired in their ability to support hematopoietic progenitor cells (Aizawa et al, 1999, Flores-Figueroa et al, 2002). These MDS-derived stromal cells were found to induce apoptosis of normal $CD34^+$ cells, while long-term marrow cultures of MDS patients are characterized by decreased or absent stromal growth. An altered production of hematopoietic growth factors together with an imbalanced responsiveness to these factors contribute to the marrow failure.
Another biological mechanism that can induce apoptosis is a lack of appropriate adhesive interactions between cells and their stroma. It has been shown that MDS progenitor cells down-regulate expression of α4β1 and α5β1 resulting in decreased adhesion to fibronectin and in increased apoptosis (Delforge et al, 2005).

Also contributing to the inhospitable marrow environment are the increased levels of inhibitory and inflammatory **cytokines**. IL-1β, IL-6, IL-8, SCF, GM-CSF, EPO and TGF-β have been measured in serum or marrow of patients with various, even conflicting results (Bowen et al, 1993, Fontenay-Roupie et al, 1999, Maurer et al, 1993, Verhoef et al, 1992). Several independent groups have reported elevated levels of TNF-α mRNA and protein in BM correlating with raised rates of apoptosis (Gersuk et al, 1998, Kitagawa et al, 1997, Maciejewski et al, 1995). In accordance with these findings, suppression of TNF-α, either by pentoxifylline or anti-TNF-α antibody resulted in a reduction of apoptosis, enhanced *in vitro* clonal culture and a favorable clinical response in some patients (Gersuk et al, 1998, Raza et al, 1996).

There is also evidence that **angiogenesis** contributes to MDS pathogenesis. Angiogenesis is the formation of new blood vessels from an existing vasculature (Cines et al, 1998). Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are the strongest inducers of angiogenesis (Aguayo et al, 2003, Albitar 2001). Besides promoting the growth of endothelial cells, most of the angiogenic factors also promote the growth of hematopoietic cells. Most of these factors (including VEGF, bFGF) have been reported to be increased in the sera of patients with MDS and AML (Kitagawa et al, 1997, Salven et al, 1999). Autocrine production of VEGF enhances leukemia colony formation and "ALIP" in CMML and other MDS subtypes (Bellamy et al, 2001). There is conflicting evidence regarding angiogenesis in MDS. Some studies have proposed that BM micro-vessel density increases with MDS progression (Moehler et al, 2003, Wimazal et al, 2006) whereas others report increased vascularity in early stages (Campioni et al, 2004, Lundberg et al, 2006). Increased angiogenesis has been associated with poor survival (Albitar 2001, Ribatti et al, 2002). Over-expression of angiogenic factors in leukemia is believed to serve two purposes: firstly, they promote growth and proliferation of the hematopoietic cells through direct effects, and secondly, they provide neo-vascularisation which then in turn helps the growth and proliferation of the hematopoietic cells. But, as most angiogenic factors are produced in response factors to inflammation, it is possible that neovascularization in AML and MDS is simply an inflammatory response rather than an active and necessary process for the neoplasia.

1.2.4. Multistep pathogenesis

No single gene defect appears to be solely responsible for MDS and chromosomal abnormalities can be observed at the time of diagnosis and at disease progression. This has lead to the proposal of a multistep model of MDS disease pathogenesis. (1) A first event of targeted injury within the hematopoietic stem cell leads to clonal proliferation. (2) These damaged progenitor cells then evoke an immunological response compromising progenitor cell growth and differentiation on one hand and lead to an overproduction of pro-apoptotic cytokines on the other hand. (3) Accelerated proliferation and apoptosis may give rise to a reduction of telomere length. Telomere shortening is connected with genomic instability, which then results in cytogenetic evolution and probably represents an important step in development of pathological clone and disease progression. (4) Finally, the transformation to advanced MDS and AML is linked to inactivation of the tumor suppressor genes that promotes an abnormal proliferation of immature precursors belonging to the pathological clone (Rosenfeld and List 2000).

1.2.5. Immune mechanisms

Immune cells such as lymphocytes and monocytes constitute an important part of the hematopoietic microenvironment. There is increasing evidence that these cells are not just innocent bystanders, but that they can contribute to the clinical shaping of the disease. The involvement of immune mechanisms in the pathogenesis of MDS is supported by different observations.

1.2.5.1. Association with autoimmune diseases

About 10% of MDS patients have overt manifestations of an autoimmune disorder in the course of their disease (Billstrom et al, 1995, Castro et al, 1991, Enright et al, 1995, Hamblin 1996). Asymptomatic immunological abnormalities have been reported too (Mufti and Galton 1986). In a large Japanese single institution collection of 153 patients, 63% had some abnormal immunological tests: hyper-gammaglobulinemia was the most common, followed by hypo-gammaglobulinemia, the presence of antinuclear antibodies, rheumatoid factor, anti-DNA antibodies and a positive Coombs' test (Okamoto et al, 1997). Presentation of autoimmune symptoms was previously considered as a bad prognostic factor (Billstrom et al, 1995, Enright et al, 1995, Hamidou et al, 2001, Okamoto et al, 1997). But it was shown that patients with autoimmune manifestations had no worse survival than patients without these autoimmune disorders, if one normalizes for IPSS score (Giannouli et al, 2004).

1.2.5.2. *In vitro* suppression of progenitor cells

Smith et al were the first to demonstrate that autologous PB lymphocytes from MDS patients could suppress BM CFU-GMs *in vitro* (Smith and Smith 1991). This was later substantiated by Molldrem and co-workers in patients undergoing treatment with ATG (Molldrem et al, 1998). They showed that removal of $CD3^+$ (and more specifically $CD8^+$) cells from BMMNC resulted in increased numbers of CFU-GM grown *in vitro* in 3 patients responding to treatment with ATG. In addition, this *in vitro* inhibitory effect of T cells disappeared after successful treatment with ATG. The observation that MHC class I blocking antibodies reversed the effect, suggests that the $CD8^+$ inhibitory effect is MHC class I dependent. Later, it was substantiated by Kochenderfer that treatment with ATG reduces or completely eliminates the inhibitory effect of T cells (Kochenderfer et al, 2002).
Baumann has demonstrated that lymphocyte-depleted ($CD2^+$, $CD5^+$, $CD7^+$) MNC from patients with RA and RARS showed a significantly increased generation of GM-CFC in long

term cultures compared to non-depleted MNC, an effect not seen in MNC from normal controls (Baumann et al, 2002). In addition, in that particular study it was shown that these CFC were non-clonally derived, indicative of a selective suppression of clonal cells.

1.2.5.3. Clonal TCR repertoire

Clonal T cells can be identified through analysis of the complementarity determining region (CDR)-3 of the T cell receptor (TCR), which binds to antigenic peptides presented by HLA molecules. Gene arrangement of different V, D, J and C regions leads to the assembly of receptors of various length. During the course of an antigen-driven immune response, clonal expansion of antigen-specific T cells leads to increased representation of specific Vβ families (McHeyzer-Williams et al, 1999). Such (oligo)clonal T cell expansions have been demonstrated by several independent groups to be present in about half of the patients with MDS (Epling-Burnette et al, 2007b, Epperson et al, 2001, Melenhorst et al, 2002, Sloand et al, 2005, Wlodarski et al, 2006). In the hypothesized immune pathogenesis of MDS, $CD8^+$ T cells of a specific Vβ subfamily might expand in response to a neo-antigen, to a quantitatively up-regulated antigen, or to an aberrantly expressed normal protein (Young and Maciejewski 1997). In other myeloid malignancies, $CD8^+$ T cell responses against a number of antigens presented by myeloid cells have been discovered, including proteinase 3 (Molldrem 2002, Sloand et al, 2005) and WT-1 (Gao et al, 2000) or neoantigens created by chromosomal translocations such as breakpoint-cluster region/Abelson leukemia (BCR-ABL) (Bocchia 2002). It has been shown that MDS patients over-express WT-1 and proteinase 3 and can have circulating proteinase 3 and WT-1 specific T cells (Rosenfeld et al, 2003).

There is an association between MDS and T-cell large granular lymphocyte (LGL) leukemia (Sauntharajah et al, 2001). T-LGL leukemia is a clonal disorder of $CD8^+$ T cells associated with BM failure. T-LGL disease is characterized by a circulating T cell repertoire dominated by a single $CD8^+$ T cell clone representing a mixed population of effector-memory and end-effector T cells. As such, this could be interpreted as an exaggerated T cell response in these patients.

1.2.5.4. Relation with severe aplastic anemia, paroxysmal nocturnal hemoglobinuria

In *severe aplastic anemia* (SAA) an immune-mediated process leads to progressive BM destruction (Young and Maciejewski 1997). There is considerable overlap between MDS and SAA, and it is often a challenge to differentiate between these two entities. Furthermore, the immune-mediated process in SAA can itself drive clonal change and development of MDS by selective pressure. This is substantiated by the acquisition of patients with SAA of trisomy 8 positive progenitors (Maciejewski et al, 2002a).
Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal hematopoietic stem cell disorder caused by an acquired gene mutation in the phosphatidylinositol glycan-class A (PIG-A) gene. This leads to a decrease or absence of glycosylphosphatidylinositol (GPI)-anchored proteins CD55 and CD59 on neutrophils and red blood cells. This in turn leads to the classical clinical triad of intravascular hemolysis, thrombosis and BM failure. PNH abnormalities can be found in up to 25% of patients with MDS and the PNH abnormality has been shown to be a favorable factor for response to ATG (Dunn et al, 1999). Current understanding of the PNH defect is that PIG-A gene mutations are present in normal marrow. PIG-A mutations result in a failure of the PNH cell to anchor critical cell surface molecules. In a healthy marrow environment these minor clones have no growth advantage. As PNH cells do not express co-stimulatory molecules, in conditions of an immune-mediated process they overcome cytolytic attack by T cells for which co-stimulatory molecules are needed. In these conditions, PIG-A mutations provide a selective growth advantage. This hypothesis is substantiated by recent data demonstrating that GPI-anchored protein (AP) positive $CD34^+$ cells are more likely to express apoptotic markers than GPI-anchor-deficient (Ismail et al, 2003). The fact that GPI-AP-deficient cells showed decreased growth in culture, proves that they have no intrinsic growth advantage, but that the microenvironment produces the right conditions for these cells to become the dominant clones.

Clonal expansions of PIG-A mutant stem cells are believed to be a marker of immune selection pressure leading to related syndromes (MDS/SAA/PNH) (Young 1992, Young and Maciejewski 2000). Young and co-workers therefore propose that SAA and MDS include a subset of individuals with a common immune-mediated etiology for the marrow failure. In this model SAA and MDS resemble at presentation because of the very low marrow cellularity. The underlying MDS is only revealed after hematological recovery, when dysplastic features and cytogenetic abnormalities become evident.

1.2.5.5. Response to anti-thymocyte globulin and cyclosporine A

Several trials have shown that about a third of patients with MDS respond to treatment with anti-thymocyte globulin (ATG) and/or cyclosporine A (CsA) (Barrett et al, 2006, Biesma et al, 1997, Broliden et al, 2006, Jonasova et al, 1998, Killick et al, 2003, Lim et al, 2007, Molldrem et al, 1997, Tichelli et al, 1988). Although the destruction of suppressive T cells is an attractive model to understand the working mechanism of these drugs, it is not fully understood. In favor of this model, Molldrem has shown that the tendency of lymphocytes to suppress myelopoiesis *in vitro* disappears after successful treatment with ATG (Molldrem et al, 1998). In addition, treatment with ATG can also lead to the disappearance of clonal T cell expansions as was shown by Kochenderfer (Kochenderfer et al, 2002). Apart from its effect on T cells, ATG also has non-T cell mediated effects (Killick et al, 2000b). In long term culture conditions ATG favored the persistence of normal $CD34^+$ cells from MDS marrow, which was attributed to a direct negative effect on MDS progenitors. The nature of this protective mechanism was not identified but it is possible that ATG reduced death from apoptosis by blocking the Fas receptor on progenitor cells, preventing T cell engagement via Fas-ligand (Killick et al, 2000a).
One peculiar but consistent finding is the fact that improvement in BM function does not relate to elimination of the cytogenetically abnormal clone (Molldrem et al, 1997, Sloand et al, 2005). For example, it was shown that immunosuppressive therapy even leads to increased numbers of trisomy 8 cells in the marrow (Sloand et al, 2005). This suggests that a suspected specific T cell response (directed to trisomy 8 cells) leads to global marrow failure by attacking normal residual progenitor cells. A bystander phenomenon has been suggested, and suppression can be the result of the release of cytokines by activated T cells (Maciejewski et al, 1995) or by cross-recognition of targets through molecular mimicry or epitope spreading (Eisen 2001).

Although published studies on the effectiveness of treatment with immunosuppressives comprehend relatively small numbers of patients, investigators have sought to determine factors predictive for response. Age below 60y (Sauntharajah et al, 2002) and low platelet counts were reported to be independent factors for response (Broliden et al, 2006, Molldrem et al, 2002). Other factors found to be strongly predictive for response to ATG include: the presence of a PNH clone (Dunn et al, 1999), HLA DR15 phenotype (Sauntharajah et al, 2002), low marrow cellularity and low IPSS-score (Lim et al, 2007). Based on these observations, a scoring system combining age, HLA-DR type, interval to transfusion was developed defining the probability of response (Sauntharajah et al, 2003).

1.2.5.6. Cytogenetic abnormality trisomy 8

The clearest insight in the immune-mediated pathogenesis in MDS has been established in trisomy 8 MDS and much is due to the work from Sloand and co-workers. All of the above mentioned evidence in favor of immune mechanisms has also been shown in this type of MDS. Firstly, it was shown that this subgroup of patients responded extremely well to immunosuppressive therapy (Sloand et al, 2002). In addition, Sloand has shown that all patients with trisomy 8 have $CD8^+$ T cell-expansions of one or more TCR Vβ subfamilies (Sloand et al, 2005). She was able to demonstrate that these expanded T cell populations are solely capable of inhibiting trisomy 8 cell growth in short-term hematopoietic culture. But although trisomy 8 cells show signs of apoptosis (represented by expression of cyclin D1, c-myc, Fas-receptor, Annexin V) they are actually relatively resistant to apoptosis, and this is related to upregulation of survivin. Finally, some cases with SAA will acquire clones with trisomy 8 in the course of the disease, suggesting that immune mechanisms can drive acquisition of trisomy 8-positive clones (Maciejewski et al, 2002a).

1.2.5.7. Regulatory T cells

Regulatory T cells (Tregs) are implicated in the suppression of immune responses and the maintenance of tolerance. The naturally occurring Tregs are characterized by expression of CD4, CD25 (the IL-2 receptor), GITR (glucocorticoid-induced tumor necrosis factor receptor family-related gene), cytotoxic T lymphocyte antigen-4 (CTLA-4) and the transcription factor forkhead box P3 (FOXP3). This transcription factor is a regulator for cytokine production and is necessary for cell-cell contact-dependent inhibition of effector T cell activation by Tregs (Gallimore and Godkin 2008).

Tregs play an important role in the immune surveillance of malignancies. Kordasti and co-workers have recently demonstrated that the number $CD4^+$ Tregs tends to be lower in low-risk MDS whereas it is increased in high-risk MDS (Kordasti et al, 2007). The authors speculate that expansion of Tregs in high-risk MDS could facilitate disease progression via inhibition of an effective immune response. The low numbers in low-risk MDS on the other hand might be associated with the emergence of auto-reactive T cell clones. In most patients with SAA Tregs are also decreased (Solomou et al, 2007).

1.3. Treatment of MDS

There is currently no standard therapy for MDS. A wide arsenal of therapeutic modalities exists ranging from watchful-waiting to allogeneic stem cell transplantation. As suggested by the term “syndromes”, there is substantial clinical and biological heterogeneity among patients. Furthermore, the disease is not static and can change over time within the same patient. Therefore a risk-adapted treatment strategy is warranted for patients with MDS. To date the IPSS is commonly used for predicting survival and risk for leukemic progression. If predicted survival is short, the risk of significant morbidity and mortality of an aggressive therapy may outweigh the negative outcome of a less aggressive approach. In lower-risk patients the therapeutic goal is to achieve higher blood counts and to reduce symptoms caused by the cytopenias. These “missionary” approaches attempt to improve growth and differentiation of hematopoietic cells and include hematopoietic growth-factors, immunomodulatory and immunosuppressive drugs. Three drugs are currently approved by the US Food and Drug Administration for MDS (5-azacytidine, decitabine and lenalidomide) and have altered management of MDS dramatically.
Younger patients with high risk MDS can be candidates for a more aggressive treatment (sometimes referred to as the “crusader” approach) including cytotoxic drugs or transplantation. The only known putatively curative therapy for MDS is allogeneic stem cell transplantation. But, even when using less-toxic non-myeloablative conditioning therapy, only a minority of patients will be eligible for this treatment

1.3.1. Hematopoietic growth factors

One of the properties attributed to growth factors is their ability to promote cell survival through the suppression of apoptosis (Williams et al, 1990). EPO with or w/o G-CSF improves anemia in almost half of the patients with early-stage MDS (Casadevall et al, 2004, Hellstrom-Lindberg et al, 2003, Park et al, 2008) and is a frontline treatment of early MDS. In part, these growth factors might improve pancytopenia by inhibiting spontaneous apoptosis in the MDS clone (Fontenay-Roupie et al, 1999, Hellstrom-Lindberg et al, 1997). G-CSF was shown to inhibit CD95-induced apoptosis, spontaneous release of cytochrome c and activation of caspase-9 in erythroid progenitor cells from patients with MDS (Tehranchi et al, 2003, Tehranchi et al, 2005a).

A scoring system has been constructed to predict the likelihood of response to growth factors. This model incorporates previous transfusion requirements and serum EPO levels to predict the chance of response to such therapy. Individuals most likely to respond to EPO have low transfusion requirements (< 2 U packed red cells per month) and low serum EPO levels (< 500 mU/mL) (Hellstrom-Lindberg et al, 2003).

1.3.2. Immunomodulatory drugs

Among the growth inhibitory cytokines, TNF-α has been reported to play a prominent role in MDS-related BM failure. Since *in vitro* blockade had shown an increased colony formation (Gersuk et al, 1998), inhibition of TNF-α has become one of the targets to treat MDS-related cytopenias. These anti-TNF agents include immunomodulatory drugs (IMiDs), infliximab, pentoxifylline and amifostine. The working mechanism of thalidomide and its derivatives, the IMiDs, will also be further highlighted in Chapter 7. But with the exception of lenalidomide for patients with the 5q- syndrome and non-5q- patients (List et al, 2006, List et al, 2005, Raza et al, 2008), response rates of these drugs have been rather disappointing (Deeg et al, 2002, Maciejewski et al, 2002b, Raza et al, 2004b, Rosenfeld and Bedell 2002, Stasi and Amadori 2002), either to limited *in vivo* activity or due to increased toxicity. The combination of etanercept with ATG might increase the response rate (Deeg et al, 2004).

Lenalidomide, a derivative of thalidomide has shown greater activity. In an initial study, List reported a 56% response rate in 43 patients with symptomatic anemia (List et al, 2006, List et al, 2005, Raza et al, 2008). This effect was more pronounced in patients with a deletion 5q, where an 83% response rate was observed. This high response rate was recently confirmed in 148 patients with del(5)(q31), 67% of which achieving transfusion-independence (List et al, 2006). A phase II trial in patients without a deletion 5q showed a more moderate activity in transfusion-dependent patients (Raza et al, 2008).
The apparent specific activity in patients with del(5)(q31) has recently been further clarified (Pellagatti et al, 2007). Lenalidomide has been shown to specifically inhibit the *in vitro* growth of erythroblasts containing del(5)(q31) and this coincided with significant up-regulation of several genes involved in erythropoiesis. These genes include SPARC, a gene that is of specific interest since it is located at the commonly deleted region in 5q- syndrome.

1.3.3.Epigenetic therapy

The expression of tumor-suppressor genes might be pathologically reduced by epigenetic silencing mechanisms (Yoo and Jones 2006). Of these, DNA methylation and histone deacetylation contribute to MDS progression and disease resistance (Kantarjian et al, 2006a, Kantarjian et al, 2007b, Silverman et al, 2002). Two demethylating agents have shown anti-MDS activity and have been approved by the FDA: 5-azacytidine and 5-aza-2'-deoxycytidine. In randomized studies of these agents versus best supportive care, the complete response rates were 7-10% with an overall response rate of 30-50% (Silverman et al, 2002, Wijermans et al, 2000). Responses were associated with an improved quality of life and transfusion independence. Valproic acid, a histone deacetylase inhibitor, has shown activity in low-risk MDS with improvement in cytopenias in about 40% (Kuendgen et al, 2006).

1.3.4. Intensive chemotherapy

AML-type induction regimens can be used in high risk MDS patients, but complete remissions are mainly obtained in young patients with favorable cytogenetics (Kantarjian et al, 2006b). Even so, median remission duration is usually short due to a high incidence of early relapses (Boogaerts and Demuynck 1996). Therefore, autologous stem cell transplantation after remission induction or consolidation chemotherapy has been explored with autologous BM cells (De Witte et al, 1997). Since sufficient numbers of $CD34^+$ progenitors can be mobilized in PB after intensive chemotherapy, also mobilized PB stem cells (Delforge et al, 1995, Demuynck et al, 1996) have been used.

1.3.5. Allogeneic stem cell transplantation

Allogeneic transplantation is the only curative treatment for MDS, but is generally reserved for younger patients with higher risk MDS. There is currently increasing experience with reduced-intensity conditioning (RIC) transplantation for patients with MDS. Such transplants are more easily tolerated (in the short term) and have substantial graft-versus-leukemia effect (Ho et al, 2003, Martino et al, 2006, Parker et al, 2002). Although RIC transplants have a lower transplant-related mortality, this benefit can be counterbalanced by an increased relapse rate in patients with higher IPSS scores (Martino et al, 2006). It may be that transplantation could be considered more often in some patients with low or int-1 IPSS scores.

Chapter 2

Aims of the study

As described in the previous chapter, immune mechanisms can contribute to the clinical shaping of MDS and are therefore putative targets to treat MDS-related marrow failure. But in spite of a better understanding of the role of the immune system in MDS pathogenesis, many questions remain unanswered and conflicting reports on the role of immune competent cells exist.

- Aim #1

There are conflicting results regarding the number of T, B and NK cells in patients with MDS. In addition, reports on the clonal involvement of T, B and NK cells in the disease process are highly variable. The heterogeneity of MDS might be responsible for these sometimes conflicting results and therefore large data sets are required. These data on the number and the clonal involvement of T, B and NK cells are currently lacking. The first aim of this PhD project was to provide such data in a large group of MDS patients. Using flow cytometry we quantified the number of $CD4^+$ and $CD8^+$ T cells, B cells, NK cells and monocytes in PB and BM and related these numbers to clinical and disease characteristics (Chapter 4). With FISH and HUMARA assay, we also determined the clonal involvement of these highly purified cell populations (Chapter 5).

- Aim #2

Most evidence to date points towards a pathogenic role of autologous T cells, and more specifically $CD8^+$ T cells in MDS-related marrow failure. The presence of clonal T cell populations and their suppressive role *in vitro* are the basis of this concept. In addition, these T cells have been shown to have an activated phenotype although these reports comprehend only small numbers of patients. But, some important questions remain unanswered. Firstly, it is not known if these activated T cells are restricted to a specific

clinical subgroup of patients. Furthermore, it has also not been studied if the activation phenotype of these cells relates to any *in vitro* myelosuppressive activity. We therefore determined the activation phenotype of T cells in a large group of patients and related these results to clinical and disease characteristics. We also related the activation phenotype of T cells to *in vitro* colony formation (Chapter 6).

- Aim #3

It is not known to what extent monocytes are involved in the process of MDS-related marrow failure. Monocytes have been shown to be the principal source of TNF-α, but it is currently unknown which mechanisms drive production of this cytokine. One particular interesting pathway is the CD40-CD40L pathway by which monocytes are stimulated by activated T helper cells. The role of this pathway in several auto-immune diseases has already been established. We sought evidence for the possible contribution of monocytes in the process of BM failure, and more particularly of the CD40-CD40L pathway. These results are summarized in chapter 7. We determined surface expression levels of different activation markers (including CD40) on circulatory and BM monocytes. The concomitant expression of CD40L by T helper cells was also determined and these results are summarized in Chapter 6. Clinical and disease characteristics of patients with activated monocytes were analyzed. In order to determine their capacity to produce inflammatory cytokines, monocytes from patients were submitted to different stimuli (LPS and CD40-receptor agonists). Finally, we looked at the *in vitro* effects of agonizing or antagonizing the CD40 receptor on *in vitro* colony formation.

- Aim #4

Thalidomide and its derivatives are used in the treatment of MDS patients. Inhibition of TNF-α production is one of the proposed working mechanisms, but this has never been studied *in vitro* in MDS patients. We subjected monocytes from patients and controls to different stimuli (LPS and CD40-receptor agonists) in the presence of increasing concentrations of thalidomide and analyzed its effect on the production of different cytokines (Chapter 8).

Chapter 3

Patients, materials and methods

3.1. Patient data and samples

All sampling and handling were conducted in accordance with the guidelines of the local ethical committee of the University Hospital of Leuven, which comply with the Helsinki declaration. Only samples from patients with morphologically proven MDS were used for this study. The characteristics of the patients used in the analysis are summarized in each chapter. Samples were obtained at routine PB and BM controls and after informed consent. Patients with a concomitant or recent history (within last month) of infection were excluded from this analysis. Data on cytogenetic analysis were provided by the Centre for Human Genetics of the University Hospital of Leuven and the Cytogenetic Lab of the Bordet Institute of Brussels. In addition, PB and BM samples were obtained from healthy volunteers to serve as control. The PB control group consisted of a group of young (below 50y) and of age-matched controls.

3.2. Blood counts, C-reactive protein

Hematological values were determined with a Sysmex XE-2100 automated Hematology Analyzer. Serum C-reactive protein (CRP) concentration was measured on an Hitachi Modular Analytics D automated analyzer with a particle-enhanced immunoturbidimetric method (CRPLX, Roche Diagnostics).

3.3. Flow cytometry

PB and BM samples were collected in sodium EDTA coated tubes and were processed immediately. After lysis with 10:1 NH_4Cl buffer and washing with phosphate buffered saline (PBS, Bio-Whittaker Europe, Cambrex, Belgium), cells were immunostained with the

following fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinin chlorophyll (PerCP) conjugated monoclonal antibodies (MoAbs): anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD19, anti-CD16/56, anti-CD45, anti-HLA-DR, anti-CD62L, anti-CD57, anti-CD28, anti-CD40L, anti-CD16, anti-CD40, anti-CD69, anti-CD80, anti-CD86 (all from Becton Dickinson, Erembodegem, Belgium). Appropriate isotype-matched control antibodies against irrelevant antigens (Becton Dickinson, Erembodegem, Belgium) were used as negative controls. Cells were incubated with 10 µl of the monoclonal antibody for 30 minutes at 4°C in the dark, subsequently washed once and resuspended in 400 µl CellFIX (Becton Dickinson). Samples were analyzed on a FACScan or FACSCanto Flowcytometer (Becton Dickinson). Analysis of the data was performed with CELLquest and BD FACSDiva software (Becton Dickinson). Lymphocyte subsets were identified with the following MoAb panels: B cells ($CD3^-/CD19^+$), helper T cells ($CD3^+/CD4^+$), cytotoxic T cells ($CD3^+/CD8^+$), and NK cells ($CD3^-/CD16^+56^+$). Monocytes were determined as the $CD14^+$ population with intermediate side scatter (SSC).

To determine the phenotype of lymphocytes, we gated on the $CD3^+$ population with low SSC and enumerated the percentage of cells in this population that was double positive for CD3 and HLA-DR, CD57, CD28 and CD62L (cf. chapter 5). Similarly, we gated on the population with low SSC that was CD4 and CD3 double-positive, to determine the percentage of this population that expressed CD40L. To determine the phenotype of monocytes, we gated on the $CD14^+$ population with intermediate SSC and enumerated the percentage of cells in this population that was double-positive for CD14 and CD16, CD40, CD69, CD80, and CD86 (cf. chapter 6).

3.4. Monocyte isolation and stimulation

PB mononuclear cells (PBMNCs) were isolated over a Ficoll-Hypaque (Lymphoprep, Nycomed Pharma, Oslo, Norway) step gradient and washed once in PBS and subsequently in MACS buffer. $CD14^+$ cells were isolated by MACS columns using $CD14^+$ magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Isolated $CD14^+$ cells were enumerated using trypan blue exclusion. They were subsequently plated in Iscove's modified Dulbecco's Medium (IMDM, Gibco BRL Life Technologies, Paisley, UK) supplemented with penicillin and streptomycin (Gibco BRL Life Technologies, Paisley, UK) and 15% fetal bovine serum (FBS, Stem Cell Technologies, Vancouver, BC, Canada) in wells of 24-well plates (Falcon Multiwell, Becton Dickinson) at a density of $3x10^5$ cells per mL per well. After 1 week of culture in a humidified incubator at 37°C with 5% CO_2,

the medium was replaced with fresh medium supplemented with either lipopolysaccharide (LPS, Santa Cruz Biotechnology, Germany) at 1µg/mL or a mixture of clone 64 (agonist anti-human CD40 monoclonal antibody, PanGenetics, The Netherlands) at 10µg/mL, and interferon-gamma (IFN-γ, Santa Cruz Biotechnology, Germany) at a final concentration of 1000 IU/mL. After 24h, supernatant was harvested and stored at -20°C until further analysis.

3.5. Colony forming assay

Following the enumeration of viable cells, BMMNC ($5x10^5$ for MDS patients, 2.5×10^5 for controls) were prepared in MethoCult H4434 media containing 30% FBS, 50 ng/mL stem cell factor, 10 ng/mL GM-CSF, 10 ng/mL interleukin-3, and 3 U/mL EPO (StemCell Technologies, Vancouver, BC, Canada). Following 14 days of incubation, plates were scored using an inverted microscope. Groups of 40 or more cells were scored as a colony. Granulocyte, monocyte and granulocyte-monocyte colonies were scored as CFU-GM. Erythroid colonies, either single or clustered, were each scored as a BFU-E.
To assess to effects of CD40-antagonists on *in vitro* colony formation, from each sample a tube without and a tube with ch5D12 (antagonist chimeric anti-human CD40 antibody, PanGenetics, The Netherlands) at a final concentration of 10 µg/mL was prepared and plated in duplicate wells. A similar strategy was used to assess to effects of CD40-agonists. For this purpose, we used sCD40LT (Bender Med Systems, Austria) at a final concentration of 25 pg/mL) or clone 64 (agonist anti-human CD40 monoclonal antibody, PanGenetics, The Netherlands) at a final concentration of 10 µg/mL.

3.6. Enzyme-linked immunosorbent assay

We used commercially available ELISA-kits for the detection of TNF-α (BD, Pharmingen), IL-1β (R&D Systems), IL-6 (PeproTech Ltd, UK) and IL-10 (PeproTech Ltd, UK) per manufacturer's recommendations. Nunc maxisorp plates were used and were read on an ELISA reader. The sensitivity limits of the dilutions were 1.25 pg/mL, 4 pg/mL, 16 pg/mL and 24 pg/mL, respectively.

3.7. Clonality studies: HUMARA PCR

Clonality of purified cell fractions was assessed by PCR amplification of polymorphic short tandem repeats of the human androgen receptor (HUMARA) gene. Cell fractions ($CD3^+/CD4^+$, $CD3^+/CD8^+$, $CD3^-/CD19^+$, $CD14^+$, $CD3^-/CD16^+CD56^+/CD14^-$) of 12 female patients were sorted on a FACSVantage flow cytometer supplemented with Lysis II software (Becton Dickinson) and equipped with a Cell Concentrator (Becton Dickinson). Sorted cells were processed immediately. A mean of 30.000 sorted cells was used for each assay.

DNA extraction, restriction enzyme digestion and HUMARA PCR were performed as previously described (Delforge et al, 1995). Briefly, DNA was resuspended in 30 µl of H_20. After overnight incubation at 37°C with 20 U of HpaII and 20 U of CfoI (HhaI) (MBI Fermentas, Germany) for the digestion of unmethylated (or active) alleles, residual non-digested DNA was amplified by PCR. For PCR amplification, 2.5 µl DNA template was added to 1.54 mM MgCl2, 200 µM dNTP's, 12 pM of FAM labelled primer 5'-CCG-AGG-AGC-TTT-CCA-GAA-TC-3', and primer 5'-TAC-GAT-GGG-CTT-GGG-GAG-AA-3', 2.5 U Amplitaq Gold and Gene Amp buffer (Perkin-Elmer, The Netherlands) to a final reaction volume of 25 µl. Amplification of DNA was performed for 34 cycles (30s at 95°C, 30s at 60°C, 30s at 72°C) with an initial denaturation step of 10 min at 95°C and a final elongation step of 5 min at 72°C. PCR products were mixed with formamide and GENESCAN 400 HC-Rox size standard (Perkin-Elmer), denaturated and run automatically for 30 min at 15 kV on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer) containing a Performance Optimized Polymer – 4 capillar. GeneScan software (Perkin-Elmer) was used for analysis of the peak area and height of individual alleles. A corrected allelic ratio (CrR) was calculated by dividing the ratio of the predigested sample (upper/lower allele) by the ratio of the non-predigested sample. A cell fraction was considered to be clonal if the Lyonization of one allele exceeded 75%. This corresponded to CrR values of <0.33 and >3.

3.8. Clonality studies: FISH

FISH analyses were performed on purified PB subpopulations, purified with a FACS Vantage flow cytometer (cf. 3.7.). Interphase cells of MDS patients with numerical cytogenetic abnormalities were used for this study. DNA probes were purchased from Vysis Inc. (Downers Grove, IL, USA). The commercial probes included: dual-color probe LSI ERG1/LSI D5S721:D5S23 for bands 5q31 (Spectrum orange LSI ERG1) and 5p15.2 (Spectrum green LSI D5S721: D5S23) of chromosome 5; CEP for Y III alpha satellite; dual-color probe MLL

(11q23) for MLL gene in chromosome 11; CEP 8 targeting chromosome 8 centromere; CEP X targeting chromosome X centromere; directly labeled locus-specific DNA probes targeting 13q14 (LSI 13 retinoblastoma gene) of chromosomes 13 and LSI D20S108 spectrum orange targeting 20q12 of chromosome 20. The FISH procedure was performed as described by the manufacturer's instructions.

Before hybridisation, slides were incubated in pepsin solution (0.0005% pepsin-0.01 N HCL) at 37°C for 10 min. Then, the slides were rinsed in PBS at room temperature for 5 min and denaturated with 70% deionized formamide/2 X SSC at 75°C for 3 min, then successively passed through cold ethanol (70%, 90% and 100%) for 3 min respectively. Following air-drying of the slides, the denatured probe (75°C for 5 min) was added onto the slides and then covered with a coverslip. The hybridisation reaction was carried out in a humidity chamber at 37° overnight. Post-hybridisation wash was made with 0.4 X SSC/0.3 % NP40 for 1 min at 73°C, 2 X SSC/0.1% NP40 for 1 min at room temperature. Finally, the slides were dehydrated in ethanol (70%, 90% and 100%) for 3 min respectively and were air-dried in the dark, counterstained with DAPI II, and covered with a coverslip.

Each FISH assay was performed following probes suppliers' instruction. Fluorescence signals were visualized with Leitz DMRB fluorescence microscope equipped with a cooled black and white CCD camera (Photometrics) run by Smartcapture software (Vysis, Stuttgart,Germany). A total of 200 nuclei were scored per specimen by independent observation. In all experiments, background levels were no higher than 5.5% in agreement with the specifications for the probes set.

3.9. Statistics

All statistics was performed using GraphPad Prism version 3.00 for windows (GraphPad Software, San Diego CA, USA). Mann-Whitney u-tests were used to compare means of two populations. The Wilcoxon matched pairs test was used to analyze results of cytokine production and results of the BM cultures. Spearman's tests were used to analyze covariance between two continuous variables. Kruskal-Wallis non-parametric ANOVA tests with Dunns post tests were used to compare the medians of three or more groups (chapter 4).

For this study, we determined expression of activation markers by lymphocytes and monocytes in a large patient group. In order to compare differences in prevalence of "meaningful" categorical variables (gender, treated vs. untreated, FAB, WHO, IPSS,

cytogenetic abnormalities) we needed to categorize patients and compare relative frequencies of these variables in these defined groups. Since age below 60y has been shown to predict response to immune suppressive therapy, we categorized into a group ≤ 60y and a group > 60y. Patients were also grouped as having a “high” and “low” prevalence of activated lymphocytes/monocytes. Since there are no published cut-off values, the median value of expression of a specific marker was used as cut-off. We also categorized patients using the 95^{th} percentile in the control distribution as cut-off value. Relative frequencies of categorical variables were compared using X^2-tests or Fisher exact-tests for low frequencies (below 5). Medians between two groups were compared using Mann-Whitney u-tests. P-values represent double-sided tests and values below 0.05 were considered significant.

Chapter 4

White blood cell populations in PB and BM of MDS patients

4.1. Introduction

Despite improvements in supportive care management of MDS patients, complications of cytopenia remain a major cause of morbidity and mortality of patients. As will be discussed in more detail in chapter 5, the myeloid lineage is consistently clonally involved in patients with MDS. Hence, due to impaired differentiation and increased apoptosis of MDS progenitor cells there is decreased production of mature myeloid cells. The vast majority (60-80%) of patients develops symptomatic *anemia*. *Neutropenia* occurs in about half of the patients which together with dysfunctional neutrophils leads to an increased incidence of infections (Greenberg et al, 2002). The prevalence of *thrombocytopenia* in MDS ranges from 40 to 65% and hemorrhagic death is reported in 10% of patients (Kantarjian et al, 2007a).

Notwithstanding convincing evidence on the occurrence of cytopenias in the myeloid lineages, there is limited data on the numbers of lymphocytes and NK cells. Reported data show conflicting results and firm conclusions cannot be drawn because of the limited number of patients included in these reports, and by patient selection.

4.2. Specific aims studied in this chapter

1. To enumerate PB and BM $CD4^+$ T cells, $CD8^+$ T cells, NK cells and B cells in a large group of predominantly untreated patients.

2. A/ To enumerate mature PB myeloid cells (monocytes, neutrophils, hemoglobin levels and platelet counts) in these patients.
   B/ To correlate numbers of myeloid cells with the number of lymphoid cells.

3. To compare PB values from myeloid, lymphoid and NK cell origin with relevant disease- and patient-related characteristics.

4.3. Experimental design and results

4.3.1. Experimental design

With flow cytometry the percentage of monocytes, $CD4^+$ T cells, $CD8^+$ T cells, B cells and NK cells was determined. Hemoglobin concentration, platelet count, white blood cell count and neutrophil count were analyzed with a Sysmex XE-2100 automated Hematology Analyzer. We chose to compare *absolute* counts of the different cell populations. As neutropenia is common in patients and as neutrophils make up 50-70% of mature blood cells in PB, this would automatically result in increased percentages of other populations. This is supported e.g. by the inverse correlation between ANC and the percentage of $CD4^+$ (r=-0.31, p=0.01) and $CD8^+$ (r= -0.26, p=0.002) T cells in our patient population.

4.3.2. Characteristics of patients and controls

We analyzed PB (n=101) and BM (n=30) of a total of 131 patients. Patient characteristics are summarized in table IV.1. The majority of patients was treatment-naïve. In parallel, PB (n=92) and BM (n=41) aspirates from healthy controls were analyzed following the same protocol. The PB control group consisted of 60 predominantly young subjects (mean age 26, range 19-45) and 32 age-matched controls (mean age 67, range 50-82). The BM control group consisted solely of young subjects (mean age 22, range 19-24).

4.3.3. PB results

4.3.3.1. Myeloid lineage

As expected, hemoglobin concentration (9.9 ± 0.2 vs. 13.9 ± 0.2 g/dL, p<0.0001), platelet count (211 ± 21 vs. 232 ± 10 x10^3/µL, p=0.003) and ANC (2525 ± 200 vs. 3604 ± 207/µL, p<0.0001) were significantly lower in patients compared to control subjects, irrespective of their age. Monocyte counts were comparable (see table IV.2).

4.3.3.2. Lymphoid lineage

As is summarized in table IV.2, we demonstrate that T cell counts were influenced by age. In patients, neither the total number of T cells, nor the number of $CD4^+$ and $CD8^+$ T cells were significantly different from those of age-matched controls. Notwithstanding lower B cell numbers with ageing (Lazuardi et al, 2005), B cell counts were significantly lower in MDS patients.

Table IV.1: *Clinical characteristics of patients*

	PB (n=101)	BM (n=30)
Age (years) mean (range)	70 (29-94)	71 (45-90)
Gender (male/female)	66/35	17/13
FAB		
RA	57	17
RARS	33	8
RAEB	11	5
WHO		
RA	1	
RCMD	50	15
RARS	1	
RCMD-RS	32	8
5q- syndrome	5	1
RAEB-1	7	1
RAEB-2	4	4
MDS-u	1	1
Cytogenetics		
Normal	45	11
Abnormal	42	16
Good[1]	12	5
Intermediate[1]	19	6
Poor[1]	11	5
N.A.[2]	14	3
IPSS		
Low	39	8
Int-1	39	12
Int-2	7	7
High	2	
MDS treatment		
None	70	24
rhEPO/rhG-CSF/both	7/1/1	
Iron chelation	9	
Transfusions	20	6

Table IV.1: Clinical characteristics of patients

MDS, myelodysplastic syndromes; FAB, French-American-British; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB: refractory anemia with excess blasts; WHO: World Health Organization; RCMD, refractory cytopenia with multilineage dysplasia; RCMD-RS, refractory cytopenia with multilineage dysplasia and ringed sideroblasts; MDS-u, MDS-unclassified; IPSS, International Prognostic Scoring System

[1] Good: -Y, del(5q) only, del(20q) only; Poor: complex (≥ 3 abnormalities) or chromosome 7 anomalies; Intermediate: +8, single miscellaneous abnormality or double abnormalities

[2] Not available

4.3.3.3. NK cells

NK cell numbers were also significantly lower in patients compared to young or aged controls.

Table IV.2: *Absolute counts of PB white blood cell populations*

	Mean ± SEM (/µL)	p-value MDS vs. control	p-value young vs. aged controls
Neutrophils			
MDS	2636 ± 230		
Control (age-matched)	3604 ± 207	<0.0001	
Control (young)	4392 ± 532	0.0008	0.10
Monocytes			
MDS	260 ± 39		
Control (age-matched)	229 ± 19	0.26	
Control (young)	188 ± 14	0.88	0.32
$CD3^+$ cells			
MDS	1060 ± 139		
Control (age-matched)	1038 ± 98	0.38	
Control (young)	1903 ± 153	<0.0001	<0.0001
$CD4^+$ T cells			
MDS	551 ± 38		
Control (age-matched)	680 ± 84	0.22	
Control (young)	907 ± 42	<0.0001	0.005
$CD8^+$ T cells			
MDS	334 ± 38		
Control (age-matched)	260 ± 29	0.52	
Control (young)	526 ± 74	<0.0001	0.0002
B cells			
MDS	103 ± 19		
Control (age-matched)	128 ± 12	0.0003	
Control (young)	186 ± 12	<0.0001	0.001
NK cells			
MDS	247 ± 23		
Control (age-matched)	348 ± 32	0.0008	
Control (young)	272 ± 17	0.002	0.43

Table IV.2: Absolute counts of PB white blood cell populations
Absolute numbers of different white blood cell populations in PB of 101 MDS patients, 29 age-matched controls and 31 young controls. P-values represent Mann-Whitney u-tests comparing MDS with specific donor-group (row 3) or comparing the two different donor groups (row 4).

4.3.4. Subgroup-analysis of PB results

The large group of analyzed patients enabled us to compare numbers between the following relevant clinical subgroups: FAB-subclasses, WHO-subclasses, IPSS-risk categories, recurrent cytogenetic abnormalities, treated vs. untreated patients, male vs. female gender.

4.3.4.1. Myeloid lineage

The IPSS recognizes cytopenia is a risk factor for worse prognosis, which is translated in scoring cytopenia in 2 or 3 lineages with 0.5 point (cf. chapter 1). This explains the significant lower Hb, platelet levels and ANC with increasing disease severity (table IV.3 d-g). Although there are no significant differences between patients and controls, the number of monocytes also decreases with increasing disease severity (see table IV.3 h-i). We demonstrated that RARS patients had significantly lower Hb levels than RA patients. In contrast, the platelet numbers in these patients were significantly higher. These significances were also found when comparing the WHO subgroups RCMD and RCMD-RS. In addition, we found that patients with 5q- syndrome had a significantly lower Hb-level (8.6 vs. 10.7 g/dL, $p<0.05$) but a higher platelet count (446 vs. $139x10^3/\mu L$, $p=0.005$) than patients with RCMD.

Anemia did not coincide with lower platelet numbers or low ANC. Platelet counts correlated well with ANC ($r = 0.32$, $p=0.001$). Although monocytes and neutrophils originate from the same myeloid progenitor cell (CFU-GM), we were unable to demonstrate correlation between the numbers of these 2 cell types. High variability and differences in kinetics (e.g. differences in life-span) could account for the absence of covariance.

4.3.4.2. Lymphoid lineage

$CD4^+$ and $CD8^+$ T cell numbers were generally unaltered throughout different FAB and WHO subclasses. Only patients with poor cytogenetic risk score had lower $CD4^+$ and $CD8^+$ T cell counts. As these patients predominantly had an int-2 IPSS risk score, this risk group had lower $CD8^+$ T cell counts compared to other IPSS risk categories. Although the number of B cells did not differ significantly between different FAB subgroups (Kruskal Wallis test $p=0.059$), we found that patients with RARS had significantly lower numbers than patients with RA ($p=0.02$, Mann-Whitney u-test).

Table IV.3: *Subgroup analysis of different white blood cell populations*

	Hb	Plt	ANC	Mono	$CD4^+$ T	$CD8^+$ T	B	NK
	Mean ± SEM g/dL	Mean ± SEM $x10^3/\mu L$	Mean ± SEM /µL	Mean ± SEM /µL	Mean ± SEM /µL	Mean ± SEM /µL	Mean ± SEM /µL	Mean ± SEM /µL
FAB								
RA	10.5 ± 0.3	170 ± 20	2454 ± 230	257 ± 34	557 ± 50	347 ± 64	109 ± 17	241 ± 26
RARS	***9.0 ± 0.3***[a]	***314 ± 51***[c]	3110 ± 416	185 ± 29	569 ± 67	330 ± 53	54 ± 8	266 ± 45
RAEB	9.8 ± 0.5	111 ± 36	***1283 ± 245***[e]	130 ± 82	474 ± 116	282 ± 55	78 ± 38	227 ± 86
IPSS								
Low	10.3 ± 0.3	***252 ± 26***[d]	***3095 ± 355***[f]	***334 ± 46***[h]	539 ± 52	329 ± 38	89 ± 17	278 ± 44
Int-1	9.6 ± 0.4	223 ± 48	2313 ± 324	171 ± 31	526 ± 63	296 ± 36	94 ± 17	266 ± 37
Int-2	9.6 ± 0.7	89 ± 23	1177 ± 264	70 ± 14	400 ± 139	***159 ± 27***[k]	53 ± 27	177 ± 43
Gender								
Male	9.9 ± 0.2	202 ± 29	2347 ± 186	277 ± 31	528 ± 46	356 ± 54	84 ± 12	256 ± 27
Female	10.1 ± 0.3	224 ± 29	2850 ± 440	222 ± 36	602 ± 63	310 ± 38	93 ± 20	244 ± 44
Karyotype								
Normal[1]	9.9 ± 0.3	198 ± 20	2520 ± 254	420 ± 102	567 ± 49	365 ± 38	88 ± 15	308 ± 39
Good[1]	9.6 ± 0.6	261 ± 73	2150 ± 374	328 ± 80	557 ± 149	214 ± 40	109 ± 32	184 ± 41
Intermediate[1]	9.7 ± 0.5	350 ± 87	3121 ± 643	257 ± 158	486 ± 82	263 ± 42	87 ± 27	239 ± 51
Poor[1]	10.7 ± 0.7	117 ± 30	***1293 ± 285***[g]	***63 ± 6***[i]	***299 ± 55***[j]	217 ± 49	64 ± 25	***130 ± 27***[l]
Treatment								
None	10.6 ± 0.2	197 ± 22	2550 ± 232	222 ± 29	559 ± 43	342 ± 49	99 ± 14	237 ± 26
Transfusions	***8.0 ± 0.3***[b]	262 ± 76	2619 ± 492	171 ± 40	637 ± 113	265 ± 40	62 ± 12	226 ± 49
Other	9.4 ± 0.4	282 ± 33	2571 ± 594	294 ± 67	454 ± 99	390 ± 112	43 ± 15	344 ± 98

[1] Good: -Y, del(5q) only, del(20q) only; Poor: complex (≥ 3 abnormalities) or chromosome 7 anomalies; Intermediate: +8, single miscellaneous abnormality or double abnormalities

Medians of >3 subgroups were compared with Kruskal Wallis non-parametric ANOVA tests with Dunns post tests. Results with P-values <0.05 are marked in the table and are:

[a] **K.W. p=0.004 (RARS vs. RA (p<0.01))**
[b] **K.W. p<0.0001 (None vs. transfusions (p<0.001))**
[c] **K.W. p=0.0002 (RARS vs.RA (p<0.01); RARS vs.RAEB (p<0.001))**
[d] **K.W. p=0.002 (Low vs. int-1 (p<0.05); Low vs. int-2 (p<0.001))**
[e] **K.W. p=0.008 (RAEB vs.RA (p<0.05); RAEB vs.RARS (p=0<01))**
[f] **K.W. p=0.002 (Low vs. int-1 (p<0.05); low vs. int-2 (p<0.001))**
[g] **K.W. p=0.03 (Poor vs. normal (p<0.01))**
[h] **K.W. p=0.0007 (Low vs. int-1 (p<0.05); low vs. int-2 (p<0.01))**
[i] **K.W. p=0.002 (Poor vs. normal (p<0.01); poor vs.good (p<0.01))**
[j] **K.W. p=0.1 (Poor vs. normal (p<0.05))**
[k] **K.W. p=0.09 (Int-2 vs. low (p<0.05))**
[l] **K.W. p=0.03 (Poor vs. normal (p<0.05))**

4.3.4.3. NK cells

NK cell numbers were significantly lower in patients with poor cytogenetic risk score.

4.3.5. BM counts in MDS patients compared to healthy controls

The control group consisted exclusively of young volunteers, so any influence of age on BM counts could not be analyzed. Thirty-one BM samples from patients were compared to results of 41 controls. Patients had significantly lower marrow $CD4^+$ T cells (491 ± 82 vs. 671 ± 56 per µL, p=0.04), $CD8^+$ T cells (285 ± 48 vs. 516 ± 44 per µL, p=0.002) and B cells (133 ± 35 vs. 237 ± 16 per µL, p=0.005). Both groups had similar numbers of NK cells (260 ± 60 vs. 261 ± 17 per µL, p=0.28).

4.4. Discussion

Cytopenia is the leading cause of MDS-related morbidity and mortality and this has been appreciated by inclusion of cytopenia in the IPSS score as an unfavorable risk factor (Greenberg et al, 1997). In our series we could demonstrate that the overall numbers of neutrophils and platelets decrease with increasing IPSS score. We could also show this for the number of monocytes, even though MDS patients in general, did not have lower numbers than age-matched controls.

Patients with RARS stand out compared to other FAB-subtypes regarding their hemoglobin levels and platelet counts. A high platelet count is more frequently observed in RARS patients and a new provisional entity in the WHO classification has been described and called “refractory anemia with ringed sideroblasts associated with marked thrombocytosis (RARS-T)” (Cabello et al, 2005). Patients with this condition present with signs of essential thrombocytosis (ET), including marked increase in platelet count (platelets > 600.000/µL), hypercellular marrow and increased megakaryocytes. In addition, these patients have ringed sideroblasts which is usually associated with MDS (Cabello et al, 2005, Van Etten and Shannon 2004). Recently, numerous reports have demonstrated the presence of a point-mutation in DNA coding for the Jak homology domain 2 (JH2) pseudokinase domain of Janus kinase 2 (JAK2) in a significant proportion of patients with MPD. The *JAK2* V617F mutation is present in polycythemia vera (65-97%), essential thrombocytosis (23-57%) and chronic idiopathic myelofibrosis (34-50%). The same mutation has been found in 6 of 9 RARS-T patients (Szpurka et al, 2006). Within our patient group, 3 of the 33 RARS patients

could be assigned as RARS-T with having platelets >600.000/µL. But, even the other 30 "normal" RARS patients still had a significantly higher platelet count compared to the other MDS subgroups.

One of the aims of this study was to evaluate numbers of lymphocytes and NK cells in MDS patients. Firstly, we demonstrated in our group that age had a major influence on the number of circulatory T cells. Despite involution of the ageing thymus and a decreased T cell output, the number of peripheral T cells is considered not to change much with age (Douek et al, 1998, Hulstaert et al, 1994). Our observations do not fully support this, as we found significantly higher T cell numbers in our young control group compared to the aged controls. When it comes to patients with MDS, we did not find any numerical differences between the number of $CD4^+$ and $CD8^+$ T cells between patients and age-matched controls. However, other authors have reported reduced numbers of $CD4^+$ T cells and increased $CD8^+$ T cell counts. Differences in control groups ("adult" donors (Bynoe et al, 1983)) and patient groups (30 of 36 patients were RAEB (Colombat et al, 1988)) could account for these apparent differences. The finding of low $CD8^+$ T cells in intermediate-2 risk patients for instance is probably in line with Colombat's observations, as most of the patients in that particular population would have had a higher IPSS score (but was not yet applied at the time of this publication). The same observation was made by Amin, who demonstrated that "high" risk MDS patients (RAEB-t, CMML) had lower T cell numbers (Amin et al, 2003). This group even demonstrated that a T cell count below 800/µL was an independent risk factor for worse prognosis.
In contrast to the PB results, we found fewer $CD4^+$ and $CD8^+$ T cells in the BM of patients. But as the control group consisted solely of young individuals, the importance of this observation is not clear.

Hematopoietic stem cells in the BM give rise to early B cells through common lymphocyte precursors. These common lymphocyte precursors become Pro-B cells in the BM by successful immunoglobulin heavy-chain gene rearrangements and subsequently differentiate into Pre-B cells, which then migrate to the periphery. Humoral immunity in aged individuals is severely compromised as a result of (1) decreased production of B lymphocytes because of intrinsic and microenvironmental defects and (2) a disrupted germinal centre formation due altered T cell/B cell interactions (Lazuardi et al, 2005).
We show similar results of lower B cell counts in aged individuals in our control group (see table IV.2). Notwithstanding this, patients with MDS have even lower B cell counts. Intriguingly, RARS patients had even lower B cell numbers than RA patients.

Reduced numbers can be related to impaired production or to increased apoptosis and supportive data for both have been published. Amin reported a higher apoptotic rate (Annexin V-positive) in the $CD19^+$ population of patients (Amin et al, 2003). Sternberg demonstrated that patients have reduced numbers of B-cell progenitors and in a gene expression array, patients have been found to have marked reduced expression of B-affiliated genes (Sternberg et al, 2005). B cell function is also altered in patients. MDS B cells are impaired in stimulating autologous or allogeneic T cells to proliferate in mixed lymphocyte reactions.

NK cells are critical in host defense against malignant transformation and are potent anti-leukemic cytotoxic effectors. Their anti-leukemic potential has been demonstrated in AML patients, where NK cell activity positively correlated with relapse-free survival in patients in complete remission (Tajima et al, 1996). In normal individuals, it is regarded that the number of NK cells significantly increases with age (Aw et al, 2007) whereas NK cell function, both cytotoxicity and cytokine production, is impaired in aging individuals. In MDS, numerical and functional differences have been described. In contrast with previous reports (Fujii et al, 2003, Kiladjian et al, 2006, Ogata et al, 1994), we demonstrate a decreased NK cell number in PB of patients and this was more pronounced in patients with poor cytogenetic risk score. It is however not possible from this data to speculate if these low numbers contribute to the worse prognosis patients with poor cytogenetic risk. Surprisingly, we found that the number of NK cells decreased with increasing CRP levels, but any causal relationship remains elusive.

4.5. Summary of results

We quantified the number of monocytes, B, NK, $CD4^+$ and $CD8^+$ T cells in a large group of predominantly untreated patients with MDS. In addition, we correlated these data to clinical and disease characteristics. We conclude that in contrast with the general conception, lymphopenia is uncommon in patients with MDS. Only patients with a poor cytogenetic risk score do have significantly lower $CD4^+$ and $CD8^+$ T cell counts. In contrast with this observation, we found that B cell and NK cell numbers are lower in patients, irrespective of their disease status.

Chapter 5

Clonal involvement of white blood cell populations

5.1. Introduction

As was highlighted in chapter 1, MDS is a heterogeneous group of clonal disorders, characterized by ineffective hematopoiesis and frequent progression to acute myeloid leukemia (AML). Intrinsic abnormalities within the progenitor pool promote aberrant proliferation and differentiation of MDS progenitor cells, and can culminate in increased apoptosis (Parker and Mufti 2004). The numbers of the different populations of mature blood cells are not fully informative on the clonal nature of these cells. Impaired differentiation related to genomic alterations in the progenitor pool could lead to lower numbers of mature cells and, in addition, to the functional abnormalities that are observed.

Different methods can be used to determine clonality of a given population of cells. **Morphologic assessment** can give some basic information in acute leukemia since blast cells are morphologically different from normal cells and can be assumed to be derived from the same clone based on their similar appearance. In addition to having a similar morphology, blast cells also have a consistent expression of cell surface molecules, which can be examined by means of **flow cytometry**. Besides giving prognostic information, **cytogenetics** and also molecular analysis can reveal the clonal origin of the cell population under examination. With standard banding *karyotyping* of cultured cells, one can detect numerical and structural chromosomal aberrations. Fluorescence in situ hybridization (*FISH*) carried out on interphase cells, represents a more sensitive and more specific cytogenetic method enabling quantification of smaller populations of clonal cells carrying a specific cytogenetic abnormality. This technique does not require dividing cells and therefore does not require prior culture of the cells of interest. The drawback of FISH is that it is only informative on those chromosomal regions to which the probes hybridize. PCR is a very sensitive method to detect small genetic lesions or *point mutations* (e.g. ras mutations) and is an alternative method to detect clonal involvement of cells.

Finally, determination of the **X-chromosome inactivation** (XCI) patterns in the cells of female patients has been used as an effective tool for studying monoclonality, especially in cases where conventional clonality tests (karyotyping, FISH) are not informative. This will be discussed in more detail in the next paragraph.

5.2. Specific aim studied in this chapter

To determine clonal involvement of purified cell populations (monocytes, $CD4^+$ T cells, $CD8^+$ T cells, B cells and NK cells) from PB of patients, with HUMARA or FISH.

5.3. HUMARA-assay

XCI analysis is based on the concept that early in embryonic development one of both X-chromosomes in each female somatic cell becomes randomly inactivated (Lyon 1961). This process of XCI is thought to bring dosage compensation between normal XX females and XY males, but is also thought to compensate for additional X-chromosomes generated through meiotic and mitotic errors. The process of XCI is balanced, meaning that both X-chromosomes have equal chances to become inactivated. As the process in one cell is unrelated to the process occurring in other cells, the Lyonization ratio between the number of active paternally derived (Xp) and maternally derived (Xm) X-chromosomes follows a binomial distribution. Unequal distribution between Xm and Xp is referred to as *excessive Lyonization or skewing*. This can occur in normal situations (e.g. aging) but is usually the hallmark of a monoclonal cell population as daughter cells keep the XCI pattern of their precursor cell. Silencing of genes on the X-chromosome occurs through methylation of cytosine residues (CpG islands) in the promoter region of genes. Assays to assess skewing in the XCI pattern require (1) the presence of a polymorphism to be a able to distinguish between the 2 X-chromosomes and (2) a means to determine which X-chromosome is active. For our analysis of XCI skewing we used the HUMARA-assay.
The human androgen-receptor gene (HUMARA) maps to Xq11-q13 (Tilley et al, 1989) and contains a polymorphic CAG repeat in the coding region of the first exon. In case of heterozygosity in the number of CAG repeats between the 2 alleles, digestion with restriction enzymes leads to fragments of different length which can be amplified by PCR. Digestion with methylation-sensitive restriction enzymes, enables distinction between methylated (inactive) and unmethylated (active) alleles.

In figure V.1 the principle of the HUMARA assay is further elucidated. Panel A shows a capillary gel electrophoresis plot of PCR amplified products of undigested DNA of a given cell population (in this case, monocytes from patient #12 from table V.1). As can be appreciated, PCR amplification of the specific region of the HUMARA gene lead to 2 PCR products (214 bp and 244 bp in length). Following digestion with methylation-sensitive restriction enzymes (*HpaII* and *HhaI*), only inactive/methylated DNA remains intact. PCR amplification of this residual DNA revealed that the shorter allele was preferentially methylated/inactivated (panel B). In this patient, this is the hallmark of a clonal population. For interpretation of HUMARA results, the corrected allelic ratio (CrR) is calculated (panel C). This compensates for the preferential amplification of the shorter allele (and thus prevents over-estimation of skewing of the shorter allele). A population is considered clonal when the corrected allelic ratio (CrR) is <0.33 or >3.

Figure V.1: *HUMARA-assay*

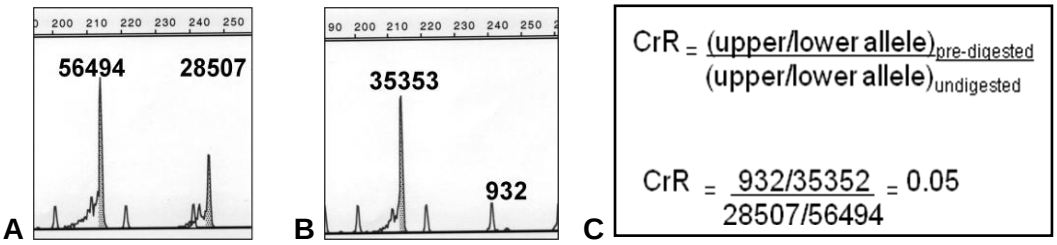


Figure V.1: HUMARA assay of purified monocytes from PB of patient #12 from table V.1.
A/ Amplification of undigested DNA reveals 2 PCR products on capillary gel electrophoresis, indicating the patient is heterozygous for the number of CAG repeats. Numbers represent the area under the curve of each peak.
B/ Following digestion of unmethylated alleles, PCR amplification reveals that 1 allele is preferentially inactivated.
C/ Calculation of corrected allelic ratio, which accounts for the preferential amplification of the shorter PCR product.

5.4. Results

5.4.1. HUMARA

The clonal involvement of different purified lymphoid populations from 12 female patients was determined with the HUMARA assay. Figure V.2 shows the plots of the capillary gel electrophoresis of the PCR products of pre-digested DNA from different PB cell populations from patient #12. As summarized in table V.1, the myeloid fraction (represented by the monocytes) was monoclonal in all cases. T cells were predominantly polyclonal in all patients, except for a 78y patient (# 10) who had skewed lionization in $CD4^+$ and $CD8^+$ T cells. In addition, one other patient had clonal $CD8^+$ T cells. In contrast, we showed clonal B and NK cells in half of the analyzed patients.

Figure V.2: *HUMARA of purified cell populations of MDS patient #12*

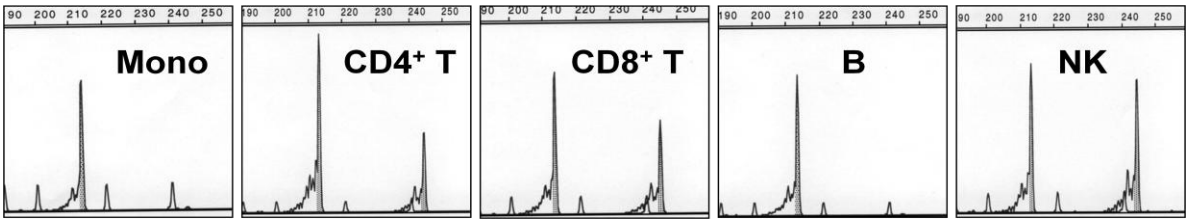


Figure V.2: HUMARA on purified PB cell populations
Plots of the different PB populations after digestion and PCR amplification, showing clonal populations of monocytes and B cells, but polyclonal populations of NK, $CD4^+$ T and $CD8^+$ T cells.

Table V.1: *Results of HUMARA on purified PB cell populations of 12 patients*

				CrR of purified populations				
Case	***Age***	***FAB***	***Cytogenetics***	***Monocytes***	***$CD4^+$T***	***$CD8^+$T***	***B***	***NK***
1	60	RARS	46,XX,del(11)(q21) [4]/46,XX [6]	27.70	0.60	0.82	0.49	0.35
2	64	RARS	46,XX [3]	129.15	0.45	0.72	4.64	90.17
3	74	RARS	46,XX,del(13)(q13q21) [3]/46,XX [9]	0.00	0.36	0.36	0.42	0.00
4	59	RA	46,XX,del(5)(q14q33) [8]/46,XX [2]	0.29	0.67	0.68	0.36	0.63
5	74	RA	46,XX,del(5)(q13q34) [7]/ 47,XX,+8 [2]/46,XX [1]	23.62	1.62	1.18	1.92	4.27
6	73	RA	46,XX [11]	0.15	0.65	1.62	0.19	0.10
7	78	RAEB	46,XX [10]	3.01	2.89	4.48	3.31	2.95
8	75	RA	46,XX [10]	14.05	1.12	1.24	2.36	45.80
9	62	RA	46,XX,del(5)(q31) [8]/46,XX [2]	0.01	2.36	2.36	0.50	1.24
10	73	RARS	46,XX,del(13)(q13q21) [2]/46,XX [8]	0.00	0.19	0.19	0.31	0.35
11	74	RAEB	46,XX [10]	93.87	0.70	0.70	21.59	9.46
12	59	RA	47,XX,+8 [7]/46,XX [3]	0.05	0.81	0.81	0.02	1.26

Table V.1: Results of HUMARA on purified PB cell populations of 12 patients.
Of 12 patients different white blood cell subpopulations were purified from peripheral blood and analyzed with HUMARA. Clonal populations are underlined in the table.

5.4.2. FISH

Cell populations from PB of patients with a known acquired cytogenetic abnormality were purified with a FACS Vantage flow cytometer. Of 12 patients, slides of sufficient quality were available for evaluation with FISH. The slides were incubated with probes specific for the detected abnormality in the original FISH analysis of the BM cells. These 12 patients were different from the patients mentioned in the HUMARA experiments. The results of the clonal involvement of these populations of nine of these patients have already been published (Ma et al, 2004). The results have been included in this manuscript together with unpublished results of 3 additional patients.

As can be appreciated in table V.2, we could detect in nearly all patients monocytes (10/11) and NK cells (11/12) derived from the clone with the cytogenetic abnormality. In contrast with the HUMARA experiments, B cells originated predominantly of cells not carrying the cytogenetic abnormality. Only in 25% of cases (3/12) we could demonstrate B cells with an abnormal FISH signal. $CD4^+$ and $CD8^+$ T cells carrying the cytogenetic abnormality were very rare, and were only found in 1 patient, be it at a low frequency.

Table V.2: *Results of FISH studies on purified PB cell populations of 12 patients*

					FISH (abnormal %)				
Case	*M/F*	*age*	*FAB*	*Cytogenetics*	*Monocyte*	*$CD4^+$T*	*$CD8^+$T*	*B*	*NK*
1	F	59	RA	46,XX,del(5)(q14q33) [3]/46,XX [2]	79	7	3.5	2	74.5
2	F	74	RARS	46,XX,del(13)(q13q21) [3]/46,XX [3]	25	2	2	1	9
3	M	84	RA	45,X,-Y [5]/46,XY [5]	2.5	3	2.5	6.5	10.5
4	M	74	RA	46,XY,del(20)(q12) [7]/46,XY [3]	NA	NA	6.5	20	97
5	F	61	RA	46,XX,del(5)(q12q34) [8]/46,XX [2]	88	1	2.5	2.5	51.5
6	M	73	RA	46,XY,del(5)(q14q34) [4]/ 46,XY,del(5)(q23q34) [3]/46,XY [3]	52	2	1.5	2.5	19
7	F	75	RARS	46,XX, del(13)(q13q21) [2]/ 46,XX [8]	7.5	3	1.6	3.3	13.3
8	M	75	RA	47,XY,+8 [9]/46,XY [1]	93.3	4.5	1.3	1	10.6
9	F	58	RA	47,XX,+8 [7]/46,XX [3]	26.5	0.5	1	2.5	5
10	M	59	RA	47,XY,+8 [6]/ 46,XX [4]	95	0.5	0	2	16.5
11	M	79	RA	46,XY,del(20)(q12) [4]/ 46,XY [8]	16	3	3.5	4	11
12	F	60	RARS	46,XX,del(11)(q21) [4]/ 46,XX [2]	52.5	2	1	7	32

Table V.2: Results of FISH on purified PB cell populations of 12 patients.
PB cell populations were purified with a FACS Vantage flow cytometer. Of 12 patients with known cytogenetic abnormalities, slides of sufficient quality were available for evaluation. The number of abnormal metaphases is given between brackets. Populations where the frequency of the cytogenetic abnormality demonstrated with FISH was higher than background, are underlined. Results of the clonal involvement of these populations of cases 1-2, 4-7 and 9-11 were previously published in Ma et al, Leukemia 2004;18:1451-6.
NA: not available

5.4.3. HUMARA and FISH on populations of same patient

Of 3 female patients with known cytogenetic abnormalities, results of both HUMARA and FISH analysis were available. These results are shown in table V.3. Since these experiments were performed on PB taken on different occasions, these results should be interpreted with caution. Monocytes were invariable of clonal origin. In spite of normal XCI patterns of B and different T cell populations in these 3 patients, we could detect cells carrying the cytogenetic abnormality within these populations, be it at low frequency. With FISH we could invariably detect the clonal cytogenetic abnormality in the NK cell population. XCI pattern in these populations were skewed in only 1 patient.

Table V.3: *HUMARA and FISH on purified populations of same patients*

			Monocyte		$CD4^+T$		$CD8^+T$		B		NK	
Nr	*FAB*	*Cytogenetics*	*FISH (%)*	*CrR*	*FISH (%)*	*CrR*	*FISH (%)*	*CrR*	*FISH (%)*	*CrR*	*FISH (%)*	*CrR*
1	RA	46,XX,del(5)(q14q33) [3]/ 46,XX [2]	79	0.29	7	0.67	3.5	0.68	2	0.36	74.5	0.63
2	RARS	46,XX,del(13)(q13q21)[3]/ 46,XX [3]	25	0.00	2	0.36	2	0.36	1	0.42	9	0.00
3	RARS	46,XX,del(11)(q21) [4]/ 46,XX [2]	52.5	27.70	2	0.60	1	0.82	7	0.49	32	0.35

Table V.3: HUMARA and FISH on purified populations of same patients
Of 3 female patients with known cytogenetic abnormalities, results of both HUMARA and FISH on the purified populations from PB were available. Clonal populations are underscored in the table. CrR (corrected allelic ratio)

5.5. Discussion

MDS is characterized by clonal hematopoiesis. Dysplastic changes and cytopenias are seen in all mature myeloid but not in lymphoid blood lineages. Together with the observation that transformation to acute lymphoblastic leukemia (ALL) is extremely rare, these findings suggest that the primary transformation event in MDS in most cases occurs at a multipotent but myeloid-restricted progenitor cell level. This notion has been supported by most clonality studies.

Indeed, the monoclonal origin of mature myelocytes, red cells and platelets has been unequivocally proven (Prchal et al, 1978, Raskind et al, 1984). The clonal nature of lymphocytes in MDS however is still a matter of debate, as controversial observations have been published. XCI studies report the existence of both polyclonal (Abrahamson et al, 1991, Anan et al, 1995, Van Kamp et al, 1992) and monoclonal (Culligan et al, 1992, Okada et al, 1999, Prchal et al, 1978, Tefferi et al, 1990, Tsukamoto et al, 1993) lymphocytes.

The method by which clonality is assessed, is a major determinant for the obtained results. Early studies using XCI methods showed none or minor clonal involvement of B lymphocytes (Culligan et al, 1992, Van Kamp et al, 1992), whereas some studies based on FISH performed on FACS sorted cell populations revealed clonal B cells in patients with 5q or 20q deletion (White et al, 1994) and in a patient with monosomy 7 (Van Lom et al, 1995). In contrast, others reported no detectable karyotypic abnormalities with FISH in lymphoid lineages, in patients with deletion of long arm of chromosome 5 (Kroef et al, 1997, Nilsson et al, 2000), monosomy 7 (Gerritsen et al, 1992) and trisomy 8 (Abruzzese et al, 1996, Anastasi et al, 1993, Fugazza et al, 1995a, Fugazza et al, 1995b, Kibbelaar et al, 1993).

There are several caveats in interpreting results from clonality assays and more specifically regarding lymphocytes. Firstly, XCI assays reveal an increasing incidence of skewed patterns in both myeloid and lymphoid cells with age in healthy females (Busque and Gilliland 1998, Busque et al, 1996, Champion et al, 1997). Secondly, the fact that mature lymphocytes are not being derived from the MDS clone does not exclude the possibility that the primary transformation event in MDS can occur at the HSC level. For the transformation event in MDS may be incompatible with further lymphoid differentiation and if so, the lymphocytes in patients with MDS may be derived from residual normal HSCs or long-lived lymphoid progenitor cells (Boultwood and Wainscoat 2001). Finally, polyclonal PB lymphocytes do not exclude the presence of a minor monoclonal lymphoid population.

A way to overcome these problems, is analyzing BM progenitor cells for clonal involvement, or performing functional assays *in vitro* or *in vivo* in order to see if these progenitor cells produce mature cells. Nilsson demonstrated that a multipotent (lympho-myeloid) HSC is likely the primary target for transformation in most MDS cases with 5q deletions (Nilsson et al, 2000). This was based on the observation that more than 95% of the cells in the $CD34^+CD38^-$ HSC compartment of all investigated patients carried the 5q deletion, as did $CD34^+CD19^+$ pro-B-cell progenitors in most patients. In addition, he demonstrated that the HSC pool in all these patients was severely compromised in its ability to reconstitute hematopoiesis in vitro and in vivo. Miura et al provided similar evidence that monosomy 7 MDS arises at the common lympho-myeloid progenitor cell level (Miura et al, 2000).

Saitoh and co-workers investigated cases with trisomy 8 and demonstrated in striking contrast to what was observed in cases with 5q deletions that whereas myeloid committed progenitor cells were invariably part of the +8 clone, there was no evidence for involvement of the phenotypically ($CD34^+Thy\text{-}1^+$) defined HSC pool (Saitoh et al, 1998). They raised the possibility that the acquisition of the +8 aberration could be a secondary or late event in MDS and this was further substantiated by Nilsson in 2002 (Nilsson et al, 2002). Notwithstanding variable frequencies of +8 cells in the $CD34^+CD38^-$ fraction (0-80%), little or no normal HSC activity in the $CD34^+CD38^-$ population was detected, suggestive that this pool is part of the MDS clone. Interestingly, in cases with combined +8 and del(5q) the primitive $CD34^+CD38^-$ fraction invariably carried the del(5q) deletion, whereas mature myeloid cells carried del(5q) with or without an extra chromosome 8.

This indicates that the acquisition of +8 is acquired at a later stage during MDS pathogenesis. This is in line with the multistep pathogenesis of MDS where cytogenetic abnormalities are thought to represent a secondary oncogenic hit in a myeloid population which is already clonally mutated (see figure V.3).

Figure V.3: *Clonal evolution in MDS*

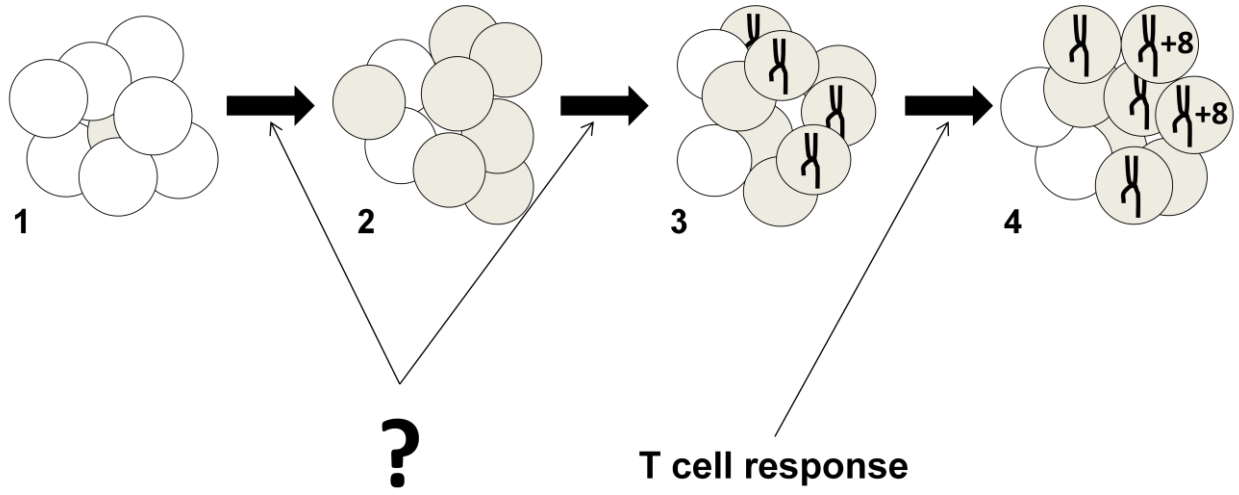


Figure V.3: Clonal evolution in MDS
(1) Represents normal hematopoiesis in the bone marrow, where normal progenitor cells (here all represented in white) randomly develop into mature blood cells. **(2)** As a result of a yet unknown transitory event, a specific progenitor cell develops growth advantage which leads to clonal expansion of this progenitor cell (represented in grey). **(3)** Within this clonally expanded pool, numeric cytogenetic abnormalities can develop, e.g. deletion of long arm of chromosome 5. **(4)** It has been shown that the acquisition of an extra chromosome 8 (represented by +8) can occur in marrows with an acquired T cell response/immune response. The acquisition of this extra chromosome 8 can also occur in progenitor cells without other detectable cytogenetic abnormalities.

FISH is a more sensitive method than XCI assays to detect monoclonality in MDS. This is supported by Van Dijck and co-workers, who demonstrated a 5q deletion in 2 RA patients without signs of clonality in XCI assay (van Dijk and de Witte 2004). Also our combined data on FISH and HUMARA on 3 patients are supportive of this. NK cells were invariably found to be of clonal origin, whereas XCI patterns revealed clonality in only 1 case. However, since FISH and HUMARA was performed on PB samples taken on different occasions, these results should be interpreted with caution.

In agreement with previously published results, T cells were found to be polyclonal in the vast majority of the patients we analyzed, irrespective of the technique we used. Only two patients were found to have skewed lyonization in the $CD4^+$ T cell and $CD8^+$ T cell population. Skewed hematopoiesis has been shown to occur spontaneously in ageing women (Busque et al, 1996, Champion et al, 1997). Since the allelic ratio was borderline in all cell fractions of these two patients, this phenomenon could have been responsible for the finding of skewed $CD4^+$ T cell and $CD8^+$ T cell populations in these patients. With FISH, we could only detect the known cytogenetic abnormality in $CD4^+$ T cells and $CD8^+$ T cell in one patient.

We demonstrate with HUMARA clonal involvement of B cells in half of our patients. In contrast, with FISH only 25% had B cells carrying the abnormal karyotypes. In the previous chapter we demonstrated that B cells are generally in lower numbers in patients. Although clonal involvement is an attractive explanation for decreased numbers of B cells, we did not see different B cell numbers between patients with clonal and polyclonal B cell populations (data not shown).

NK cells are usually found to be polyclonal in most patients (Ogata et al, 1994), but there have been reports of clonal involvement (Kiladjian et al, 2006, Ma et al, 2004, Miura et al, 2000). We found a high prevalence of clonal NK cells using the HUMARA assay (6/12) and FISH (11/12). Although the number of patients was relatively low, we did not find that clonal involvement of NK cells led to lower overall NK cell numbers.

5.6. Summary of results

With FISH and HUMARA we determined clonal involvement of purified white blood cell fractions (monocytes, B, NK, $CD4^+$ and $CD8^+$ T cells) from PB of patients with MDS. We provide further evidence that the transformation events in MDS are not restricted to the myeloid lineage. Also B cells and NK cells are shown to be of clonal origin in a substantial part of patients. Although we did not demonstrate clonal $CD4^+$ T or $CD8^+$ T cells with the HUMARA assay, this does not rule out the possibility of minor populations of clonally derived T cells.

Chapter 6

Activated lymphocytes in blood and marrow

6.1. Introduction

Increasing evidence exists that autologous T cells participate in the process of MDS-related marrow failure. This evidence comes from several observations. Firstly, autologous T cells from MDS patients have been shown to **inhibit *in vitro* myelopoiesis** (Baumann et al, 2002, Molldrem et al, 1998). In addition, it has been shown that this inhibitory T cell activity can disappear after successful treatment with ATG (Molldrem et al, 1998). T cells are the most important effector cells of the acquired immune system. Activation of T cells requires recognition of specific antigenic peptides presented on MHC molecules. Upon recognition of antigenic peptides on MHC molecules by their specific T cell receptor, naïve T cells undergo clonal expansion. This activation also induces phenotypic changes on the surface of these T cells. **Clonal expansions** of specific T cells lead to skewing in TCR spectratypes. Many authors have reported these T cell expansions to be present in MDS patients, predominantly in the $CD8^+$ T cell fraction (Epling-Burnette et al, 2007b, Epperson et al, 2001, Kook et al, 2001, Melenhorst et al, 2002, Sloand et al, 2005, Wlodarski et al, 2006). In addition, it was shown that these T cells had an **activated phenotype** ($CD57^+$, $CD28^-$) (Epling-Burnette et al, 2007b, Kook et al, 2001).

Nevertheless, these observations on T cell involvement in MDS pathogenesis required further investigation. Firstly, the published studies on activated T cells only comprehend a limited number of patients. It is currently unknown if T cell activation is restricted to a specific subgroup of patients. In addition, it is still unknown to what extent expression of these activation markers relates to their supposed myelosuppressive activity. Therefore, we have determined the activation phenotype of T cells in a large cohort of MDS patients and have tried to find evidence for a relation between the presence of activated T cells and disease- and patient-related characteristics.

Although most evidence points at involvement of the $CD8^+$ T cell fraction, there is also evidence of a T helper-1 (T_H1) response in immune-mediated marrow failure syndromes (Young and Maciejewski 1997). Cytokines such as IFN-γ, TNF-α and IL-2 are increased and are typical for T_H1 responses. CD40-CD40L interactions have also been shown to play a primordial role in such T_H1 responses. In this perspective, the expression levels of CD40L by $CD4^+$ T cells and the role of monocyte activation/inactivation via CD40-receptor interactions were also studied and respectively described in chapters 6 and 7.

6.2. Specific aims studied in this chapter

1. A/ To determine surface expression of activation markers ($HLA\text{-}DR^+$, $CD57^+$, $CD28^-$, $CD62L^-$) on PB and BM T cells of patients with MDS.
   B/ In addition, to determine the CD40L expression on the surface of $CD4^+$ T cells.

2. To correlate these findings with relevant disease- (FAB, WHO, IPSS, recurrent cytogenetic abnormalities) and patient-related characteristics.

3. To correlate these findings to *in vitro* colony forming capacity of BMMNC.

6.3. Flow cytometric analysis of surface phenotype of lymphocytes

6.3.1. Experimental design

Upon activation, T cells gain expression of HLA-DR and CD57 whereas they lose expression of CD28 and CD62L. Therefore, the fraction of T cells that are $HLA\text{-}DR^+$, $CD57^+$, $CD28^-$ and $CD62L^-$ respectively, represent the fraction of T cells that are activated. With flow cytometry we determined the percentage of T cells that expressed HLA-DR, CD57, CD28, CD62L. CD40L is transiently expressed on activated T helper cells. The percentages throughout this manuscript represent the fraction of T cells (total or T helper cells, as indicated) that express the specific marker.

6.3.2. Patient characteristics

The patient and control group were the same as the groups discussed in chapter 4. The expression of CD40L by $CD4^+$ T cells was measured in the same patient population that was used to determine the phenotype of monocytes (chapter 7).

6.3.3. Results

6.3.3.1. Lymphocyte phenotype in PB and BM of MDS patients compared to controls

Firstly, we determined if our results were influenced by age. Expression of the above-mentioned markers was not significantly different between the age-matched group and the group consisting of predominantly young subjects. We therefore used the whole control group as reference group.

MDS patients had increased numbers of HLA-DR$^+$, CD57$^+$, CD28$^-$ and CD62L$^-$ T cells compared to controls, in PB as well as in BM. In figure VI.1, representative dot plots are shown. The results are summarized in table VI.1. We demonstrated a highly significant correlation between co-expression of these markers. Although we did not determine co-expression of these markers on the same T cells, the observation is highly suggestive that these markers are over/under-expressed in the same patients.

Figure VI.1: *Expression of activation markers on T cells*

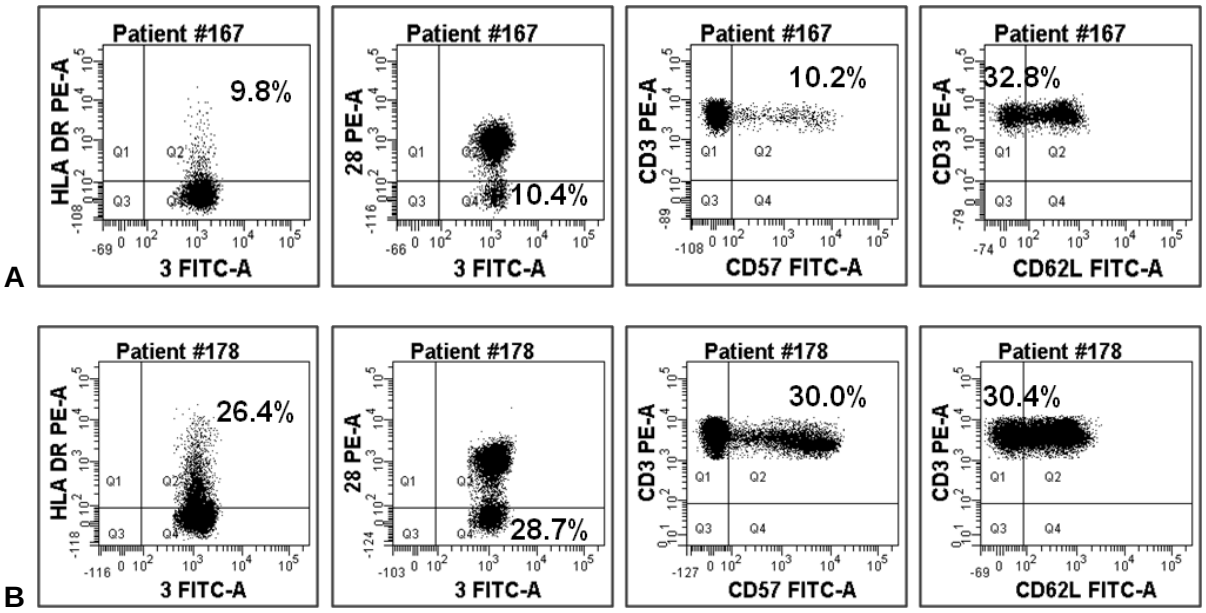


Figure VI.1: Expression of activation markers on T cells
Total PB cells were double-stained with PE- or FITC-conjugated mouse antihuman anti-CD3, anti-HLA-DR, anti-CD28, anti-CD57 and anti-CD62L mAb. Panel **(A)** represents a 55y female patient with RAEB-2 with abnormal karyotype (46, XX, 5q-, 11q-) with low expression of activation markers on circulating lymphocytes; Panel **(B)** represents a 51y male patient with RAEB-1 with complex karyotype with high expression of activation markers on circulating lymphocytes.

Figure VI.2: *Expression of CD40L by T helper cells*

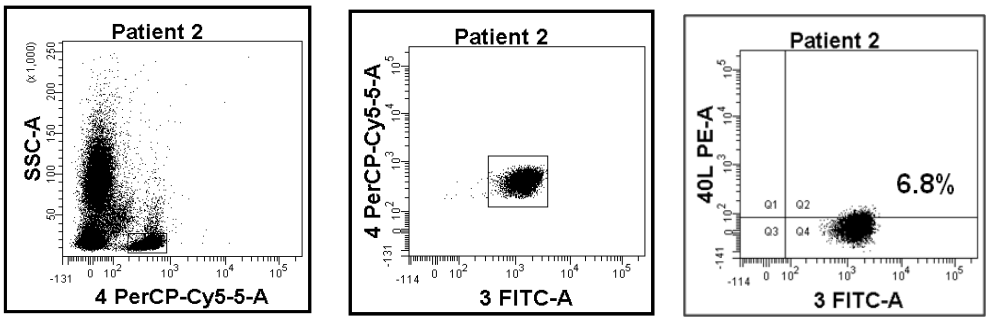


Figure VI.2: Expression of CD40L by T helper cells
Total PB cells were stained with anti-CD4-PerCP, anti-CD3-FITC and anti-CD40L-PE. Representative dot plot of determination of the fraction of T helper cells ($CD4^+/CD3^+$) expressing CD40L.

Table VI.1: *Frequency of surface expression of HLA-DR, CD57, CD28 and CD62L by T cells*

	PERIPHERAL BLOOD		BONE MARROW	
	Mean ± SEM (%)	p-value	Mean ± SEM (%)	p-value
$HLA\text{-}DR^+$				
Control	9.9 ± 0.5		12.8 ± 1.1	
MDS	18.7 ± 1.1	<0.0001	27.7 ± 2.9	<0.0001
$CD28^-$				
Control	11.0 ± 0.9		15.7 ± 1.2	
MDS	20.2 ± 1.5	<0.0001	27.7 ± 3.9	0.02
$CD57^+$				
Control	6.2 ± 0.6		9.7 ± 1.2	
MDS	18.0 ± 1.6	<0.0001	20.1 ± 3.8	0.009
$CD62L^-$				
Control	26.4 ± 1.3		28.7 ± 1.7	
MDS	43.3 ± 2.2	<0.0001	43.8 ± 5.3	0.02

Table VI.1: Frequency of surface expression of HLA-DR, CD57, CD28 and CD62L by T cells
Table summarizes results of determination of T cell phenotype by flow cytometry of peripheral blood of 101 patients and 92 controls and bone marrow of 30 patients and 42 controls. P-values represent results of Mann-Whitney u-test used to compare results of MDS patients and controls.

In addition, we demonstrated that patients have more $CD40L^+$ T helper cells in PB (4.2 % ± 0.4 vs. 2.2 % ± 0.2, $p<0.0001$) and in BM (5.0 % ± 0.8 vs. 2.6 % ± 0.5, $p<0.01$) compared to controls. CD40L expression by T helper cells coincided with expression of HLA-DR by total T cells ($r = 0.43$, $p=0.0007$).

Three patients had their PB and BM examined on the same day. These results are summarized in table VI.2. As is appreciable, expression of the markers in BM and PB are similar. Although one might argue that these BM aspirates could have been “contaminated” by PB cells, it rather suggests that T cell activation in PB is representative for the activation status of T cells in the BM.

Table VI.2: *Concomitant analysis of PB and BM samples*

Sample	FAB		HLA-DR$^+$ T cells (%)	CD28$^-$ T cells (%)	CD40L$^+$ Thelper cells (%)
MDS 1	RA	PB	24,5	26,5	8,0
		BM	21,0	21,3	16,8
MDS 2	RA	PB	8,4	6,6	3,2
		BM	17,3	5,5	3,8
MDS 3	RAEB	PB	12,0	7,8	4,3
		BM	10,2	5,7	4,6

Table VI.2: Concomitant analysis of PB and BM samples
Of 3 patients, PB and BM samples taken on the same day were available for analysis. Expression of CD57 and CD62L was not tested in these samples.

6.3.3.2. Correlation with clinical and disease characteristics

- **Laboratory values: blood counts, CRP**

We did not observe any correlation between the expression of activation markers and blood counts in any defined clinical subgroup. Nor was there a correlation between expression of the above mentioned markers and serum CRP levels. We found that the number of CD8$^+$ T cells significantly correlated with the percentage of CD28$^-$ ($r = 0.30$, $p<0.005$), CD57$^+$ ($r = 0.33$, $p<0.01$) and CD62L$^-$ ($r = 0.37$, $p<0.005$) T cells.

- **Disease characteristics: FAB, WHO, IPSS, blast count, cytogenetics, treatment**

The percentage of lymphocytes with an activated phenotype did not differ between FAB or WHO subgroups. Furthermore, IPSS risk score and BM blast count had no influence on expression of activation markers by lymphocytes, nor did supportive care or presence of abnormal cytogenetics. Surprisingly, we found that only gender had an influence on our results as males had significantly higher percentages of HLA-DR$^+$ (20.2 % ± 1.4 vs. 15.4 % ±

1.7, p=0.01) and $CD28^-$ (22.6 % ± 1.0 vs. 16.1 % ± 2.3, p=0.03) T cells. Also in the control group, males had higher percentages of $HLA\text{-}DR^+$ and $CD28^-$ T cells, but this did not reach statistical significance (data not shown).

- **Subgroup analysis**

Finally, we categorized our patient population into relevant groups in order to compare relative frequencies of categorical variables in these groups (e.g. FAB, WHO, IPSS, recurrent cytogenetic abnormalities, treatment). We compared patients younger and older than 60y, since age under 60 has been identified in multivariate analysis as a good prognostic factor to predict outcome to immunosuppressive therapy (Sauntharajah et al, 2003). Similarly, we compared patients with “high” and “low” levels of T cell activation. We therefore divided our patient population into two groups, using the median as cut-off point. In the same way we grouped our patient population using the 95^{th} percentile in the control group as cut-off point. The only consistent finding was a significantly higher number of $CD8^+$ T cells in patients with high prevalence of lymphocyte activation.

6.4. *In vitro* colony formation assays

6.4.1. Experimental design

We cultured BMMNC of patients in a standard clonogenic assay. The production of hematopoietic colonies was scored after 14 days. To evaluate the possible myelosuppressive properties of activated T lymphocytes, we correlated the numbers of colonies scored, with the percentage of activated T lymphocytes in the sample.

6.4.2. Patient characteristics

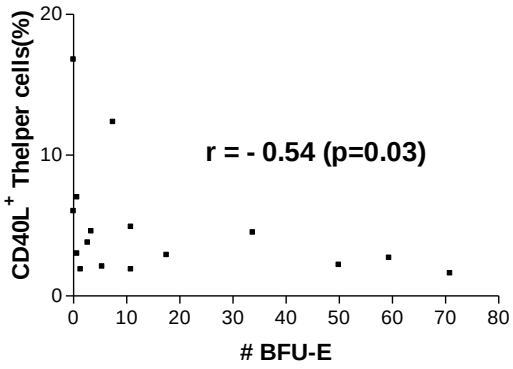
BMMNC of 18 patients were available for this analysis. Patients were categorized as RA (n=11), RARS (n=4) and RAEB (n=3) according to FAB and as RCMD (n=9), RA (n=1), 5q-syndrome (n=1), RCMD-RS (n=4), RAEB-1 (n=2) and RAEB-2 (n=1) according to WHO.

6.4.3. Results

The number of colonies formed *in vitro* was independent of the level of expression of HLA-DR, CD57, CD28 and CD62L, suggesting that the mere expression of these surface markers does not influence the clonogenic capacity of progenitor cells *in vitro*. In contrast, we found that the number of BFU-E declined with increasing numbers of $CD40L^+$ T helper cells (r = -0.54, p=0.03, see figure VI.3).

Figure VI.3: *Correlation between CD40L expression of T helper cells and BFU-E formation*

Figure VI.3: Correlation between CD40L expression by T_H cells and BFU-E formation
The number of BFU-E formed *in vitro* were correlated with the % of $CD40L^+$ expressing T helper cells. Data of 16 patients was available. The result represents a Spearman-test.



6.5. Discussion

There is evidence that an adaptive immune response develops in some patients with MDS. In a primary T cell response, a multitude of naïve T cells start proliferating in response to recognition of their antigen (expansion phase). Once the antigen has been cleared, the majority of T cells undergoes apoptosis (contraction phase), leaving behind a small population of memory cells (memory population formation). In case of a persisting antigenic stimulus, oligoclonal or even clonal populations may persist. This has been shown for other chronic inflammatory conditions (Masuko et al, 1996, Smith et al, 2000) but also for MDS, by independent authors (Epling-Burnette et al, 2007b, Epperson et al, 2001, Kook et al, 2001, Melenhorst et al, 2002, Sloand et al, 2005, Wlodarski et al, 2006) Moreover, it has been shown that the myelosuppressive properties of T cells is restricted to these specific T cell clones (Sloand et al, 2005). In addition, these T cells have been shown to have an activated phenotype. Considering the fact that TCR spectratyping is very time-consuming, we investigated if immunophenotyping of lymphocytes could serve as an alternative approach to detect patients with a presumed immune-mediated pathogenesis.

Firstly, we convincingly showed that in PB of 101 patients more T cells have an activated phenotype (HLA-DR$^+$, CD57$^+$, CD28$^-$, CD62L$^-$). The aim of this study was not to investigate kinetics of expression of these markers and therefore we did not look for co-expression of these markers on a single T cell. But, the fact that there is a highly significant correlation between expression of all these markers, is highly suggestive that these markers are over/under-expressed in the same patient.

In addition to the 101 PB samples, we have shown that in the BM also significantly more T cells have this activated phenotype. As can be observed in tables VI.1 and VI.2, the numbers of activated T cells are higher in the BM. Similar findings have been reported in patients with chronic idiopathic neutropenia (CIN), where the IFN-γ production by BM T cells was higher compared to PB T cells (Papadaki et al, 2005). Table VI.2 suggests that the values measured in PB mirror the values in BM, where they are believed to exert their function.

The finding of T cell activation is not specific for patients with MDS. Systemic T cell activation has been shown to be a common feature in other hematological and non-hematological malignancies (Van den Hove et al, 1998). Even so, several concerns about the usefulness of immunophenotyping of all T cells in MDS patients particularly, can be raised.
Firstly, one might argue that activation of T cells is just a mere result of increased prevalence of ***concomitant infection*** in this patient group. However, patients with obvious signs (or a recent history) of infection were excluded from the analysis. Moreover, overall CRP levels were low and did not correlate with the level of lymphocyte activation, making a systemic inflammatory response an unlikely explanation for our observation.
Secondly, our observation could just be a mere ***consequence of aging***. Similar concerns were raised when T cell expansions were observed in MDS patients, as one can find more T cell expansions in healthy aging persons. Melenhorst et al, however, demonstrated that patients have even more abnormally expanded TCR populations compared to age-matched controls, and that the degree of expansion was several orders greater than that seen in older normal individuals (Melenhorst et al, 2002). As for the immunophenotype of T cells, we could not observe significant differences between “young” and “old” individuals in our control group, nor could we observe a correlation between expression of these markers and age.

Another explanation is that activation of lymphocytes could be a ***result from regular blood transfusions***. The use of allogeneic blood transfusions has indeed been shown to induce immunologic effects, a phenomenon referred to as “transfusion-related immunomodulation” (TRIM) (Vamvakas and Blajchman 2007). Allogeneic mononuclear cells, soluble white blood

cell-derived mediators as well as soluble HLA molecules circulating in allogeneic plasma have been implicated in its pathogenesis. TRIM encompasses both pro-inflammatory and immunosuppressive effects. Even so, we do not believe that TRIM has influenced our results to a large extent, as the majority of patients in our analysis were not receiving any transfusion at all (81 of 101). Furthermore, we could not demonstrate more or less T cell activation in patients receiving regular blood transfusions. Similarly, others have already demonstrated that in individuals with hemoglobinopathies, regular blood transfusions did not lead to lymphocyte activation (Papadaki et al, 2005), nor to the development of TCR Vβ expansions (Melenhorst et al, 2002).

One of the main reasons to perform this study was to determine if immunophenotyping of T lymphocytes could serve as an alternative method to detect those patients with a suspected immune-mediated disease. Whereas MDS patients in general have more activated T cells, we were unable to detect a clinically relevant subgroup of patients with more/less T cell activation. Previous reports indicate that patients below 60y and patients with trisomy 8 are likely to respond to immunosuppressive therapy (cf. chapter 1). Patients under the age of 60 were not significantly different regarding T cell activation markers. In the 7 patients with trisomy 8 in our analysis, the expression levels of the above-mentioned markers was highly variable.

We also evaluated the *in vitro* properties of activated T cells on clonogenic growth of progenitor cells. We could not demonstrate a correlation between the degree of activation (i.e. expression of HLA-DR, CD28, CD57 and CD62L) and the number of colonies grown *in vitro*. Other intrinsic and extrinsic factors on progenitor growth could be a valid explanation for this absent correlation.

Finally, the question rises if immunophenotyping of T cells with these markers is of any use at all. For instance: are they a sensitive parameter to detect immune activation? There has been a report published on patients treated with an anti-TNF-α antibody. After treatment, the proportion of activated T cells (i.e. HLA-DR$^+$, CD25$^+$, CD38$^+$, CD69$^+$) decreased (Boula et al, 2006). These results thus suggest that some of these markers do mirror on-going immune activation. We also found that patients with high level of lymphocyte activation, have more CD8$^+$ T cells. This observation is in line with the current view that CD8$^+$ T cells are suppressive and have been shown to have an activated phenotype.

Maybe not all activation markers are equal, regarding the functional properties of the T cell expressing them. And the data we provide on CD40L expressed on T helper cells, is a good

example for this. CD40L (or CD154) is a 33-kDa trans-membrane glycoprotein that is transiently expressed on the surface of activated T cells, predominantly the $CD4^+$ T cells. Similarly as for the expression of the other markers, we have convincingly shown that in patients more $CD4^+$ T cells express CD40L. CD40L expression coincided with expression of HLA-DR by all T cells. In contrast with the other markers, we demonstrate that the number of BFU-E grown *in vitro* inversely correlates with the number of $CD40L^+$ T helper cells in the culture. The importance of this finding is further substantiated in the next chapter, where we will show that the receptor of CD40L is expressed to a higher extent in patients. Moreover, we will show that inhibition of this pathway can increase the production of colonies by BM cells.

6.6. Summary of results

We have determined the activation phenotype ($HLA\text{-}DR^+$, $CD57^+$, $CD28^-$, $CD62L^-$) of T cells in a large group of patients with MDS and have demonstrated that in PB and BM of these these patients more T cells have an activated phenotype than in healthy controls. We have also analysed if this T cell activation is restricted to a specific clinical subgroup. However, we were unable to demonstrate such a clinical subgroup. Nor could we demonstrate that clinical features were altered by the level of expression of these markers by T cells. In addition, we performed *in vitro* clonogenic assays to investigate a possible inhibitory potential of T cells expressing these markers on *in vitro* colony growth. But also these experiments failed to demonstrate a significant effect of the expression levels of these above-mentioned markers.

We specifically examined the expression of CD40L on $CD4^+$ T cells in patients and could demonstrated that this expression was also increased in patients. In contrast with the above-mentioned markers, we found a negative correlation between the number of $CD40L^+$ $CD4^+$ T cells and hemoglobin levels in patients. Furthermore, high expression of this marker by $CD4^+$ T helper cells coincided with lower *in vitro* production of BFU-E's.

Chapter 7

Activated monocytes in blood and marrow

7.1. Introduction

Although the role of immune-mediated processes in the pathogenesis of BM failure has been known for many years, the role of the monocytes in this process has not been extensively studied. Monocytes could play a pivotal role, as previous studies have shown that they are the principal cellular source of TNF-α production in MDS (Kitagawa et al, 1997). TNF-α has been implicated in the pathogenesis of MDS-related marrow failure because of its pro-apoptotic properties (Parker and Mufti 2004). Previous studies by Visani et al could not demonstrate that TNF-α production by MDS monocytes is different from that of normal monocytes (Visani et al, 1993).

Activation of monocytes/macrophages is a crucial event for the development of the inflammatory response *in vivo*. For monocytes are capable of synthesizing a wide array of pro-inflammatory mediators in response to not only a number of soluble factors, but also in response to direct interaction with other cells, such as activated T cells. The CD40-CD40L system appears to be important for this function (Grewal and Flavell 1998, van Kooten and Banchereau 2000) and the contribution of CD40-CD40L interactions in immune-mediated processes has been shown in several auto-immune diseases such as rheumatoid arthritis (Harigai et al, 1999, Sekine et al, 1998), inflammatory bowel disease (Carlsen et al, 2006, Kasran et al, 2005), autoimmune encephalomyelitis (t Hart et al, 2005) and thyroiditis (Carayanniotis et al, 1997). In rheumatoid arthritis patients, CD40 has been shown to be expressed by synovial macrophages (Sekine et al, 1998) whereas CD40L is expressed on the surrounding synovial fluid T cells (MacDonald et al, 1997). In addition, in these patients CD40L expression on $CD4^+$ T cells correlates with the disease activity, and blockade of this pathway causes alleviation of arthritis in mice .

In the previous chapter we have shown that CD40L expression by $CD4^+$ T cells is increased in MDS patients. In this chapter we further substantiate the role of CD40-CD40L interactions in MDS pathogenesis. We demonstrate that surface expression of the CD40 receptor is increased on PB and BM monocytes. In addition, we will discuss the effects of activation and blocking of the CD40 receptor on monocytes on cytokine production and *in vitro* colony formation.

7.2. Specific aims studied in this chapter

1. To investigate the phenotype of monocytes in PB and BM of MDS patients.

2. To study the *in vitro* effects of blocking and activating CD40 on monocytes with regard to production of cytokines and *in vitro* clonogenic growth.

3. To correlate these experimental data with relevant patient and disease characteristics.

7.3. Flow cytometric analysis of surface phenotype of monocytes

7.3.1. Experimental design

With flow cytometry we determined the expression of specific surface markers by monocytes in PB and BM. We defined monocytes as those cells with intermediate side scatter (SSC) characteristics that expressed CD14. We gated on this population to enumerate the percentage that expressed CD16, CD40 and CD69 and the co-stimulatory molecules CD80 and CD86 (see figure VII.1).

Figure VII.1: *Flow cytometric determination of phenotype of monocytes*

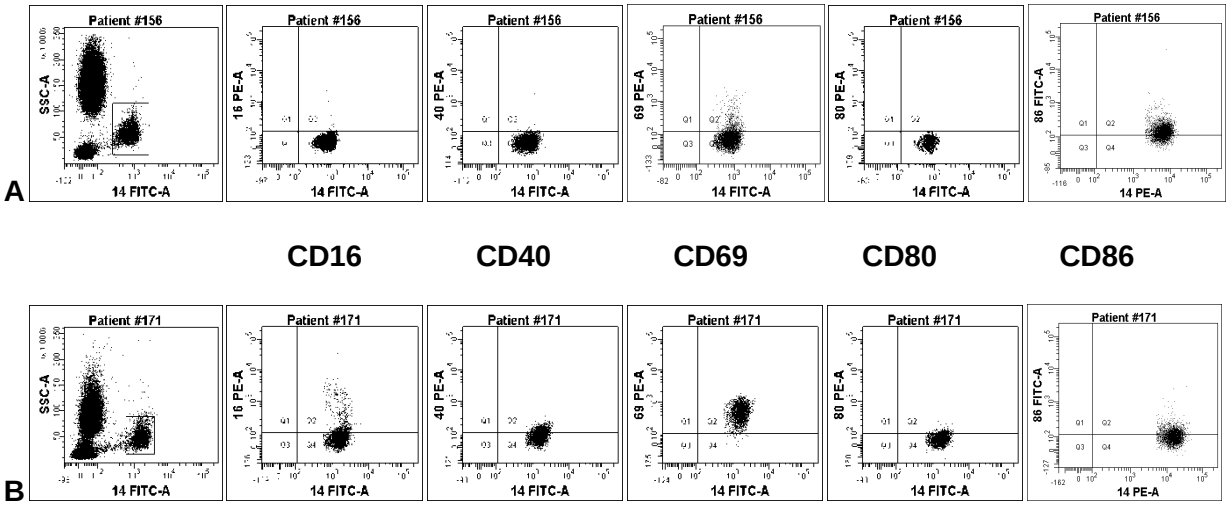


Figure VII.1: Flow cytometric determination of phenotype of monocytes
Determination of surface expression by monocytes of CD16, CD40, CD69, CD80 and CD86. **(A)** represents results of a 57y female patient with RARS/RCMD-RS with del(20)(q12) with low level of expression of activation markers (9.9 %, 2.1 %, 14.5 %, 2.4 % and 78.5 % respectively); **(B)** represents results of a 78y female patient with RA/RCMD and normal cytogenetics with high expression of activation markers (21.7 %, 24.5 %, 96.5 %, 5.1 % and 43.5 % respectively).

7.3.2. Patient characteristics

We analyzed PB samples of 61 patients and 29 age-matched controls. Also BM of 24 patients and 11 young controls was analyzed. Patient characteristics are summarized in table VII.1.

Table VII.1: *Clinical characteristics of patients*

	PB (n=61)	BM (n=24)
Age (years) mean (range)	71 (32-86)	72 (45-90)
Gender (male/female)	37/24	13/11
FAB		
RA	37	16
RARS	20	5
RAEB	4	3
WHO		
RA	1	1
RCMD	30	12
RARS	1	
RCMD-RS	19	5
5q- syndrome	3	2
RAEB-1	3	2
RAEB-2	1	1
MDS-u	3	1
Cytogenetics		
Normal	26	11
Abnormal	23	11
Good[1]	7	6
Intermediate[1]	10	1
Poor[1]	6	4
N.A.[2]	12	2
IPSS		
Low	24	9
Int-1	20	9
Int-2	4	4
High	1	
MDS treatment		
None	40	21
rhEPO/rhG-CSF/both	7/1/1	
Iron chelation	6	
Transfusions	10	3

Table VII.1: Clinical characteristics of patients
[1]IPSS defines the karyotypic abnormalities –Y, del(20q) and del(5q) as favorable; trisomy 8, single miscellaneous and double miscellaneous abnormalities as intermediate; and 3 or more abnormalities and any chromosome 7 abnormality as poor.
[2]Not available

7.3.3. Results

7.3.3.1. Phenotype of PB monocytes

As is summarized in table VII.2, we demonstrate that patients have more monocytes expressing the activation markers CD16, CD40, CD80 and CD86 in PB. Over-expression of CD16, CD40 and CD80 occurred in the same patients (correlation-coefficient $r > 0.54$, $p<0.0001$).

Table VII.2: *Phenotype of monocytes in PB and BM*

	PERIPHERAL BLOOD		BONE MARROW	
	Mean±SEM (%)	p-value	Mean±SEM (%)	p-value
CD16$^+$				
Control	9.7 ± 0.7		3.5 ± 0.6	
MDS	14.6 ± 1.1	0.01	9.1 ± 1.8	0.008
CD40$^+$				
Control	2.8 ± 0.3		8.9 ± 1.4	
MDS	10.2 ± 1.1	<0.0001	19.6 ± 2.8	0.05
CD69$^+$				
Control	67.8 ± 3.1		55.7 ± 5.9	
MDS	69.5 ± 3.1	0.3	58.4 ± 6.9	0.7
CD80$^+$				
Control	1.7 ± 0.2		1.7 ± 0.3	
MDS	3.8 ± 0.5	0.004	6.8 ± 1.6	0.0006
CD86$^+$				
Control	56.9 ± 4.3		79.9 ± 4.1	
MDS	74.1 ± 3.0	0.001	77.7 ± 5.9	0.3

Table VII.2: Phenotype of monocytes in PB and BM
Surface expression by monocytes of CD16, CD40, CD69, CD80 and CD86 was determined with FACS. PB from 61 patients and 29 age-matched controls was analyzed, as well as BM samples from 24 patients and 11 controls. P-values represent Mann-Whitney u-tests.

7.3.3.2. Phenotype of BM monocytes

Similar observations were made in BM samples of 24 patients, as is shown in table VII.2. The control group consisted solely of young individuals (n=11).

7.3.3.3. Correlation of monocyte phenotype with clinical and disease characteristics

- **Laboratory values: blood counts, CRP**

The large number of analyzed samples allowed us to corroborate clinical data to the FACS data. We first correlated hemoglobin levels, platelet, white blood cell and neutrophil counts to the percentage of monocytes expressing a specific marker. We observed a negative correlation between ANC and the percentage of circulating CD80$^+$ monocytes ($r = - 0.26$, $p<0.05$). The same observation was made for marrow samples where the percentage of CD80$^+$ monocytes inversely correlated with PB ANC at the time of BM sampling ($r = - 0.51$, $p<0.05$). There was no significant correlation between other markers and hematological parameters. Of note, in healthy donors we also found an inverse correlation between ANC and the percentage of CD80$^+$ monocytes ($r= - 0.49$, $p<0.01$). Although this type of analysis does not allow demonstration of a causal relationship, this suggests that activation of monocytes

can be related to lower ANC values, irrespective of disease status. CRP levels were generally low and independent of expression of co-stimulatory molecules.

- **Lymphocyte activation markers**

We correlated expression of monocyte activation markers with lymphocyte activation markers and could demonstrate that CD40L expression by T helper cells coincides with CD40 expression by monocytes (Spearman r = 0.32, p=0.02, see figure VII.2). We observed the same correlation with CD69 (r = 0.31, p=0.02) and CD86 (r = 0.39, p=0.004).

Figure VII.2: *Correlation between expression of CD40L by T helper cells and CD40 by monocytes*

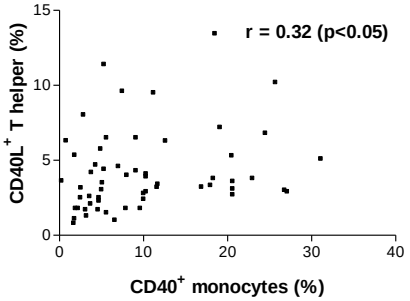


Figure VII.2: Correlation between expression of CD40L by T_H cells and CD40 by monocytes
The percentage of PB $CD40L^+$ T helper cells was correlated with the percentage of PB $CD40^+$ monocytes in the group of 61 patients. Spearman's analysis showed a significant correlation between these variables.

- **Disease characteristics: FAB, WHO, IPSS, blast count, cytogenetics, treatment**

Subsequently, we determined if expression of these markers correlated with disease status according to the FAB subtype, WHO subtype, IPSS score, cytogenetics, gender or previous treatment. We found no differences between RA, RARS and RAEB patients. Interestingly, we found that patients with the karyotypic abnormality trisomy 8 had the highest level of circulating $CD40^+$ monocytes (see figure VII.3). Patients with intermediate-1 IPSS score had significantly more $CD40^+$ monocytes than low-risk patients, but this was most likely attributable to the fact that all trisomy 8 patients had an intermediate-1 risk score. There were no differences between patients that had previously received treatment and treatment-naïve patients with regard to the expression of these markers by monocytes. We found no correlation between blast-percentage and the expression of co-stimulatory molecules. Clinical signs or a history of autoimmunity were observed in 5 patients, but did not relate to expression of activation markers by monocytes.

Figure VII.3: *Percentage of CD40$^+$ monocytes according to cytogenetic risk score*

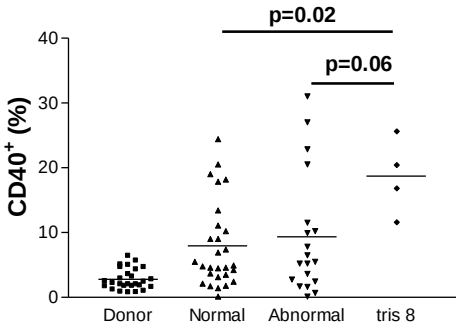


Figure VII.3: CD40$^+$ monocytes according to cytogenetic risk score
Comparison of percentages of CD40$^+$ monocytes in PB between patients with different cytogenetics. Of 49 patients cytogenetic data was available, of which 26 had a normal karyotype, 19 had a karyotypic abnormality other than trisomy 8, and 4 had trisomy 8. P-values represent Mann-Whitney u-tests.

In order to compare differences in prevalence of categorical variables (gender, treated vs. untreated, FAB, WHO, IPSS), we needed to categorize our patient group. We separated patients into two groups based on the percentage CD40 expressing monocytes, using the highest value of CD40 expressing monocytes found in the control group as the cut-off value (6.4%): one group of patients with <6.4% CD40$^+$ monocytes, and a group of patients with >6.4% CD40$^+$ monocytes. High levels of CD40$^+$ monocytes coincided with a higher IPSS score, intermediate and poor cytogenetic risk score, age below 60y and presence of trisomy 8 (see table VII.3). The latter two factors have been identified in multivariate analyses as being independent factors predictive of response to immunosuppressive therapy. Of note, patients with favorable karyotypic abnormalities (3 patients with del(5q), 2 patients with del(20q) and 2 men with –Y) had low expression of CD40 on their circulating monocytes.

Table VII.3: *Comparative analysis of laboratory findings and disease characteristics between patients with high and low levels of circulating CD40$^+$ monocytes*

	CD40 high (n=33)	CD40 low (n=28)	p-value
Continuous variables			
CD80$^+$ (% of monocytes)	5.1 ± 0.7	2.2 ± 0.3	**0.001**[a]
CD86$^+$ (% of monocytes)	77.4 ± 3.6	70.2 ± 4.9	n.s.[a]
Age (years)	68 ± 2	74 ± 2	n.s.[a]
Hemoglobin (g/dL)	10.1 ± 0.4	10.0 ± 0.4	n.s.[a]
Platelets (x10^3/µL)	185 ± 32	190 ± 34	n.s.[a]
White blood cell count (/µL)	4662 ± 357	5389 ± 631	n.s.[a]
Absolute neutrophils count (/µL)	2657 ± 327	2619 ± 333	n.s.[a]
Categorical variables			
Gender (male/female)	19/14	18/10	n.s.[b]
Age <60y	9 of 33	1 of 28	**0.01**[b]
FAB (RA/RARS/RAEB)	18/12/3	19/8/1	n.s.[b]
WHO (RA/RCMD/RCMD-RS/RARS/	0/17/11/1	1/13/8/0	
5q-/RAEB-1/RAEB-2/MDS-u)	0/2/1/1	3/1/0/2	n.s.[b]
IPSS (low/int-1/int-2/high)[1]	7/13/3/1	17/7/1/0	**0.02**[b]
Anemia (Hb<10g/dL)	19 of 33	16 of 28	n.s.[b]
Thrombocytopenia (<100x10^3/µL)	12 of 33	9 of 28	n.s.[b]
Neutropenia (<1500/µL)	8 of 33	9 of 28	n.s.[b]
Abnormal karyotype[1]	13 of 24	10 of 25	n.s.[b]
Favorable/Intermediate/Poor[2]	0/8/5	7/2/1	**0.001**[b]
Trisomy 8[1]	4 of 24	0 of 25	**0.03**[b]
MDS-treatment (yes/no)[3]	8 of 33	13 of 28	n.s.[b]
Auto-immune disease[4]	3 of 33	2 of 28	n.s.[b]

Table VII.3: Comparative analysis of laboratory findings and disease characteristics between patients with high and low levels of circulatory CD40$^+$ monocytes

Patients are grouped into a "high" and "low" group, using the highest measured value in the control group as cut-off point (6.4 %). Table summarizes results of (a) Mann-Whitney u-tests to compare means in both groups, numbers are represented as mean +/- SEM; and (b) X^2-tests and Fisher's exact tests (for frequencies <5) for categorical variables.

[1]From 49 of the 61 patients, cytogenetic data were available to determine IPSS score.

[2]IPSS defines the karyotypic abnormalities –Y, del(20q) and del(5q) as favorable; trisomy 8, single miscellaneous and double miscellaneous abnormalities as intermediate; and 3 or more abnormalities and any chromosome 7 abnormality as poor.

[3]MDS-treatment included growth-factors (4 rhEPO, 1 rhG-CSF, 1 with a combination of rhEPO and rhG-CSF), transfusions (n=9), iron chelation-therapy (n=2), or a combination (n=4).

[4]Auto-immune disease included vasculitis (n=1), rheumatoid arthritis (n=1), auto-immune hemolytic anemia (n=1), rheumatoid arthritis combined with ITP (n=1), inflammatory bowel disease (n=1).

7.4. Cytokine production of monocytes

7.4.1. Experimental design

The aim was to evaluate differences in cytokine production between monocytes from MDS patients and controls. We therefore selected $CD14^+$ cells from PB using magnetic beads (MACS). After MACS selection, purified $CD14^+$ cells were evaluated for viability and subsequently plated in 24-well plates at a density of $3x10^5$ cells per ml per well. After 7 days of culture in DMEM containing 15% FCS to induce maturation, they were subjected for 24 hours to two types of stimuli to induce cytokine production: LPS or stimulation of the CD40 receptor. For the latter, we used an agonist chimeric mouse anti-human CD40 mAb (clone 64, PanGenetics, The Netherlands) (Companjen et al, 2002, Kasran et al, 2005).
In order to maximize the response to the given stimulus, we used a high concentration of LPS (1 µg/mL). For the same reason we added IFN-γ (final concentration 1000 IU/mL) to clone 64, which is known to induce transcription of the CD40-receptor (Benveniste et al, 2004). One well only received fresh medium and served as a control to measure spontaneous cytokine production. Supernatant was harvested after 24h and kept at -20°C until analysis.

Figure VII.4: *Experimental design for assessment of cytokine production by monocytes*

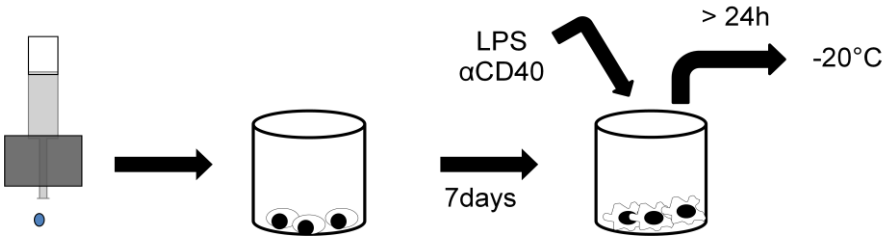


Figure VII.4: Experimental design for assessment of cytokine production by monocytes.
$CD14^+$ cells were isolated from PB using MACS. Subsequently, $3x10^5$ viable $CD14^+$ cells were subsequently plated per well of a 24 well-plate in DMEM containing 15% FCS (DMEM+) for 7 days in a humidified incubator at 37°C to induce maturation. After 7 days, the medium was replaced by fresh DMEM+ containing either LPS (1 µg/mL) or clone 64 (agonist anti-CD40 mAb, 10 µg/mL) with IFN-γ (1000 IU/mL). After 24h supernatant was collected and kept at -20°C until analysis of cytokine concentration by ELISA.

7.4.2. Patient characteristics

We isolated $CD14^+$ cells from PB of 44 patients and 44 healthy controls. Patients were diagnosed as RA (n=28), RARS (n=12), RAEB (n=4) according to FAB and as RCMD (n=23), RARS (n=1), , RCMD-RS (n=11), RAEB-1 (n=4), 5q- syndrome (n=2) and MDS-u (n=3) according to WHO.

7.4.3. Results

7.4.3.1. Purity of MACS selection of $CD14^+$ cells

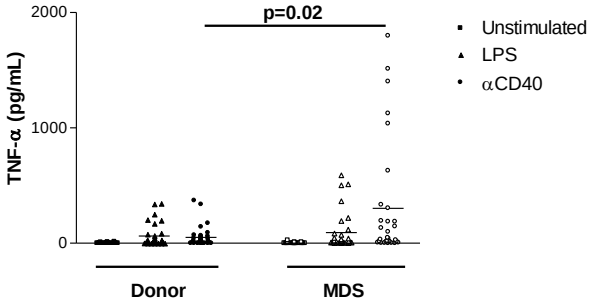
The mean purity of $CD14^+$ isolation was 97.1 % (range 91.9 – 99.7 %) for controls and 97.2 % (range 95.0 – 98.9 %) for patients. The number of dead cells was low, but higher in patients compared to the number in controls (3.0 % ± 0.5 vs. 2.1 % ± 0.4, p=0.03).

7.4.3.2. Cytokine production

- TNF-α

Spontaneous TNF-α production was low (3 ± 1 pg/mL in both groups). As is depicted in figure VII.5, we found that LPS induced similar levels of TNF-α in patients (93 ± 29 pg/mL) and controls (63 ± 17 pg/mL, p=n.s.). In contrast, clone 64 induced a significantly higher TNF-α production in patients compared to controls (302 ± 91 vs. 52 ± 15 pg/mL, p=0.02).

Figure VII.5: TNF-α production of $CD14^+$ cells
After 7 days in culture, $CD14^+$ were stimulated with LPS (1 µg/mL) or clone 64 (10 µg/mL) and IFN-γ (1000 IU/mL). After 24h supernatant was collected. Dot plots represent the individual and mean TNF-α concentrations and the p-value represents result of Mann-Whitney u-test.

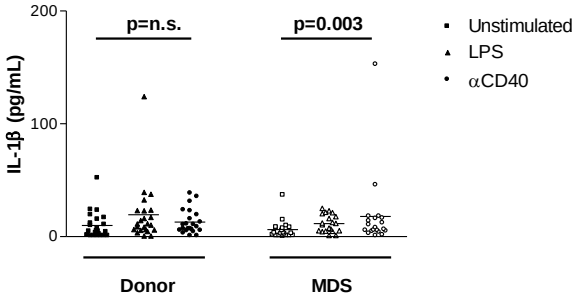


- IL-1β

Spontaneous IL-1β production was comparable (10 ± 2 pg/mL in donors, 6 ± 2 pg/mL in patients). LPS-stimulation induced increased IL-1β levels after 24h in both groups (19 ± 5 pg/mL in donors and 12 ± 2 pg/mL in patients, p=0.03 and p=0.005 respectively). IL-1β concentrations produced by donor monocytes after CD40 stimulation were not significantly

different from un-stimulated monocytes (13 ± 2 pg/mL). In contrast, the same stimulus induced significantly more IL-1β production in MDS monocytes (18 ± 7 pg/mL, p=0.003).

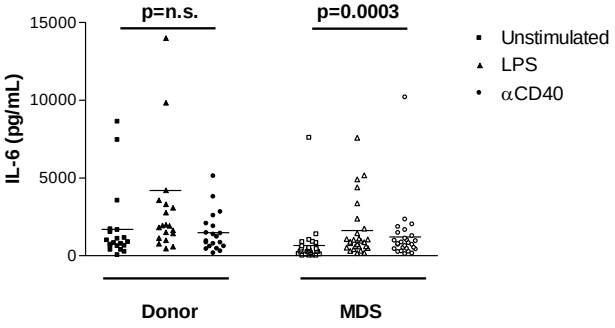
Figure VII.6: IL-1β production of CD14$^+$ cells
After 7 days in culture, CD14$^+$ were stimulated with LPS (1 µg/mL) or clone 64 (10 µg/mL) and IFN-γ (1000 IU/mL). After 24h supernatant was collected. Dot plots represent the individual and mean IL-1β concentrations and p-values represent results of Wilcoxon matched pairs tests.



- IL-6

Spontaneous IL-6 production was lower in MDS monocytes (657 ± 274 pg/mL vs. 1693 ± 514, p=0.0009). LPS induced higher IL-6 levels compared to un-stimulated monocytes, both in healthy controls (4190 ± 1370 pg/mL, p=0.009) as in patients (1618 ± 356 pg/mL, p=0.0002) and the induced concentrations were lower in patients (p=0.006). IL-6 concentrations measured after CD40 stimulation were not significantly different from base-line concentrations in controls (1486 ± 284 pg/mL). In patients however, CD40 stimulation induced increased levels of IL-6 (1207 ± 363 pg/mL, p=0.0003) compared to un-stimulated monocytes. In both patients and controls, LPS was a more powerful stimulus to induce IL-6 than CD40 stimulation (p=0.04 and p=0.007 respectively).

Figure VII.7: IL-6 production of CD14$^+$ cells
After 7 days in culture, CD14$^+$ were stimulated with LPS (1 µg/mL) or clone 64 (10 µg/mL) and IFN-γ (1000 IU/mL). After 24h supernatant was collected. Dot plots represent mean and individual IL-6 concentrations and p-values Wilcoxon matched pairs tests (un-stimulated vs. CD40 stimulation).



- IL-10

Spontaneous IL-10 production was comparable in both controls (505 ± 179 pg/mL) and patients (239 ± 76 pg/mL). IL-10 production upon stimulation with LPS was not significantly higher than spontaneous production, neither in controls (452 ± 99 pg/mL) nor in patients (371 ± 102 pg/mL). Stimulation with IFN-γ and clone 64 induced significantly higher IL-10

concentrations in controls (956 ± 250 pg/mL, p=0.005) as in patients (1646 ± 393 pg/mL, p<0.0001). In patients, the latter stimulus induced significantly higher IL-10 production than LPS (p=0.0002).

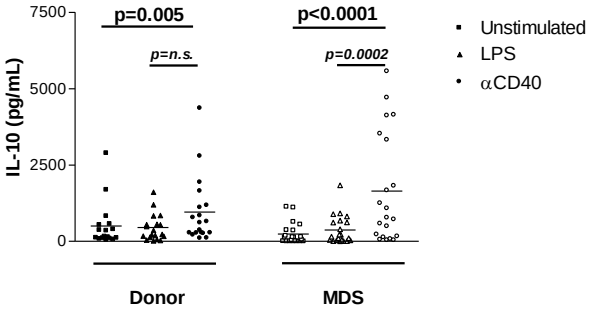


Figure VII.8: IL-10 production of CD14$^+$ cells After 7 days in culture, CD14$^+$ were stimulated with LPS (1 µg/mL) or clone 64 (10 µg/mL) and IFN-γ (1000 IU/mL). After 24h supernatant was collected. Dot plots represent mean and individual IL-10 concentrations and p-values represent results of Wilcoxon matched pairs tests.

7.4.3.3. Correlation with disease characteristics

We found no differences between different FAB-subtypes regarding the production of the above-mentioned cytokines, nor between IPSS-subgroups or males vs. females.
The amounts of TNF-α, IL-1β and IL-6 produced after LPS stimulation highly correlated (data not shown). We could not demonstrate a correlation between monocyte phenotype and subsequent in vitro production of TNF-α, IL-1β, IL-6 or IL-10.

7.5. *In vitro* colony forming assays

7.5.1. Experimental design

Different groups have evaluated the suppressive activity of autologous lymphocytes on MDS progenitor growth *in vitro*. In these experiments, colony-forming assays in the presence/absence of autologous lymphocytes were initiated. Lymphocyte depletion from whole BMMNC was achieved by means of immune-absorption (Molldrem et al, 1998) or with magnetic beads (Baumann et al, 2002). We believed that using similar strategies for monocytes was cumbersome. First of all, monocyte-depletion itself increases the relative number of progenitor cells in the BMMNC and therefore monocyte-depletion automatically results in increased numbers of CFC's. In addition, running over column with immuno-magnetic beads could lead to activation of monocytes and subsequent production of growth-inhibitory cytokines. Therefore comparing monocyte-depleted cultures to a condition where a fixed number of purified CD14$^+$ were added back, would favor the condition without monocytes in terms of colony production.

Instead, we used two other strategies to evaluate the effects of monocytes *in vitro*. As we demonstrated over-expression of the CD40-receptor on monocytes and its ligand on T helper cells, and that activation of this receptor led to increased TNF-α production, we hypothesized that interference with CD40 signaling could affect hematopoietic progenitor growth *in vitro*. We therefore used an antagonist anti-CD40 mAb (ch5D12, PanGenetics, The Netherlands) to inhibit CD40 signaling between monocytes and T helper cells and tested this in the presence/absence of monocytes. In addition, we correlated the expression of monocyte activation markers with the number of CFC's formed *in vitro*.

7.5.2. Patient characteristics

We plated BMMNCs of 26 patients in the presence or absence of ch5D12. Patients were diagnosed as RA (n=17), RARS (n=4) and RAEB (n=5) according to FAB, and as RCMD (n=14), 5q- (n=2), MDS-u (n=1), RCMD-RS (n=4), RAEB-1 (n=2) and RAEB-2 (n=3) according to WHO.

7.5.3. Results

7.5.3.1. Antagonizing CD40-CD40L interaction *in vitro*

Significantly more CFU-GM and BFU-E grew in the presence of ch5D12 compared with cultures without ch5D12 (see figure VII.9/A). In order to prove that this effect was monocyte-dependent, we also added ch5D12 to cultures of BMMNC depleted of $CD14^+$ cells with MACS columns. Colony formation of CD14-depleted BMMNC was not influenced by the presence of ch5D12, whereas ch5D12 increased colony production in the same samples in presence of all BMMNC (see figure VII.9/B). We did not observe this effect of ch5D12 on CFU-GM and BFU-E colony number in BMMNC cultures of healthy controls (n=9) (see figure VII.9/C).

Figure VII.9: *Results of BMMNC cultures*

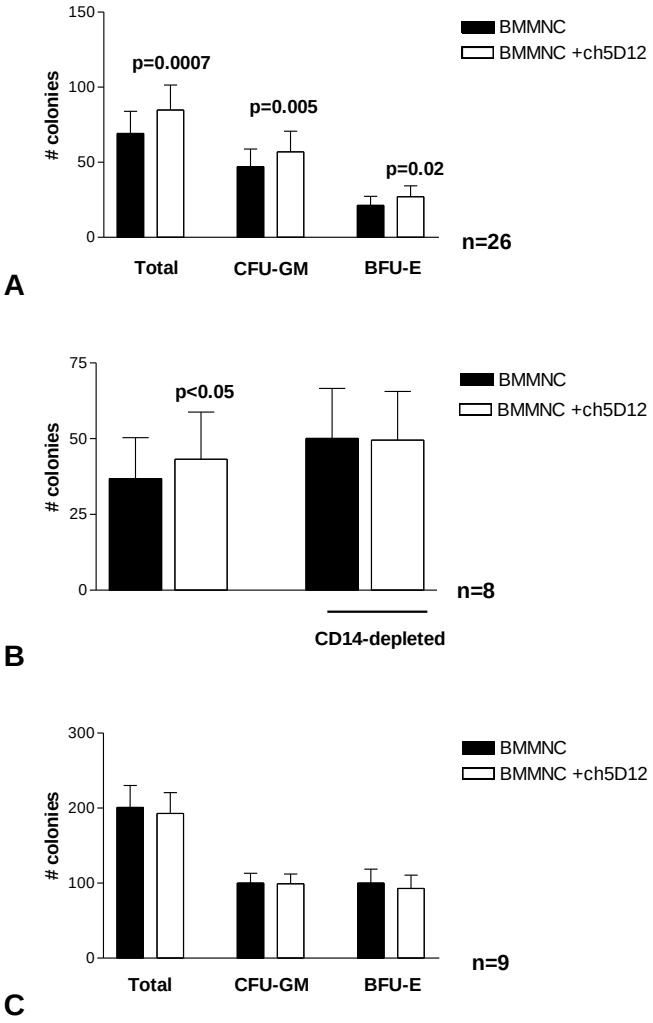


Figure VII.9: Results of BMMNC cultures. BMMNCs were grown in methylcellulose supplemented with growth factors in standard conditions or in the presence of ch5D12 (antagonist chimeric anti-CD40 mAb). After 14 days, CFU-GM and BFU-E were scored. Results are shown as number of colonies per $250x10^3$ plated BMMNCs.
(A) BMMNCs of 26 patients were cultured in M4434 in presence/absence of ch5D12. The presence of the ch5D12 significantly increased colony formation. P-values represent results of Wilcoxon matched pairs tests.

(B) Of 10 patients BMMNCs and BMMNCs depleted of $CD14^+$ cells were cultured in presence/absence of ch5D12. The significant increase in total number of colonies was not observed in CD14-depleted conditions.

(C) BMMNCs of 9 healthy controls were cultured in presence/absence of ch5D12. No significant changes in colony formation were observed.

7.5.3.2. Stimulating CD40 *in vitro*

In order to evaluate the influence of additional CD40 stimulation on *in vitro* clonogenic growth, BMMNC were cultured in presence of 2 different agonists. In an initial series of experiments, BMMNC of 11 patients were cultured in the presence of 25 µg/mL sCD40LT. This concentration was found to induce similar TNF-α concentrations (at 24h) as clone 64 (data not shown). In presence of sCD40LT, we observed a decrease in the number of colonies produced (111 ± 38 vs. 116 ± 43) but this was not significant. Subsequently, we initiated cultures in the presence of clone 64. The scarce data on 2 patients showed a decrease in the numbers of CFU's produced *in vitro* (11 → 7 and 105 → 94, respectively). Additional data on 5

patients that were not diagnosed with MDS, showed also a decrease in the number of colonies produced (79 ± 4 vs. 82 ± 7), but also this difference was not found to be significant.

7.5.3.3. Whole BMMNC

To further substantiate that CD40-CD40L interactions affect hematopoietic progenitor growth, we examined whether a correlation exists between the number of colonies produced and the number of activated monocytes within the BMMNC. We found a tendency for lower numbers of CFU-GM with increasing percentage of $CD40^+$ monocytes ($r = - 0.42$, $p=0.07$). In chapter 6, we already described a similar inverse relation between CD40L expression by T helper cells and the number of BFU-E produced *in vitro*.

7.6. Discussion

As we discussed in the previous chapter, in some patients with MDS cytotoxic T cells are capable of suppressing endogenous myelopoiesis. However, it has been shown most convincingly in trisomy 8 patients that clonal trisomy 8 cells are quite resistant to T cell mediated killing. Even worse, the established adaptive immune response can cause global marrow failure in these patients by suppressing residual normal hematopoietic progenitor cells. Several explanations have been suggested for this “innocent bystander phenomenon”: release of cytokines by activated T cells (Maciejewski et al, 1995), cross-recognition of targets through molecular mimicry and epitope spreading (Eisen 2001). Alternatively, monocytes could be responsible for this effect.
As discussed in chapter 1, monocytes have previously been shown to be the main source of TNF-α and this cytokine can inhibit hemopoiesis (Gersuk et al, 1998). As a result, anti-TNF treatment is pioneered in the clinical setting but with only limited success. However, to date it is not clear which mechanism drives this TNF-α production by monocytes. One possible explanation is interaction of monocytes with T helper cells. In addition, this interaction could serve as the link between T cell activation and inhibition of normal hematopoiesis. With our work we have provided evidence that CD40-CD40L interactions play an important role in this process.

The role of CD40-CD40L interactions between these cells has been shown in several immune-mediated diseases. The CD40 receptor is a 45-50 kDa protein and is a member of the tumor necrosis factor receptor (TNFR) superfamily. Interaction of CD40 with its ligand (CD40L) has

been shown to play a crucial role in cellular and humoral immune response. CD40 is expressed on numerous cell types: B cells, monocyte/macrophages, endothelial cells, dendritic cells, fibroblasts, vascular smooth muscle cells and neurons. CD40 signalling in monocytes results in upregulation of co-stimulatory molecules and in the production of inflammatory cytokines such as IL-1α/β, IL-6, IL-8, IL-10, IL-12 and TNF-α.

In the previous chapter we have shown that CD40L is expressed to a higher extent by T helper cells in MDS. In this chapter, we provide further evidence for the involvement of CD40-CD40L interactions. Firstly, we have shown that MDS monocytes express higher levels of CD40 receptor than age-matched controls. We have shown this in PB as well as in BM samples. In addition, we have shown that the presence of activated monocytes in PB coincides with activated monocytes in BM. We also have data to support that over-expression of CD40 is repeatable and stable. This over-expression of CD40 coincides with global over-expression of other activation markers (CD16, CD69) and the co-stimulatory molecule CD80. Importantly, over-expression of CD40L by $CD4^+$ T cells coincides with over-expression of CD40 by monocytes. This observation suggests that activation of both cell populations occurs in the same patients.

Additional *in vitro* data also support the importance of CD40-CD40L interactions in MDS. We showed that MDS monocytes produce significantly more TNF-α in response to CD40 receptor stimulation than control monocytes. Previous attempts by others could not demonstrate an increased intrinsic production of TNF-α by monocytes in response to LPS-stimulation (Visani et al, 1993). Also in our experiments, MDS monocytes behaved similarly to LPS as control monocytes regarding TNF-α production. It is, however, very unlikely that LPS-signaling itself relates to the *in vivo* setting. In addition to TNF-α production, we also demonstrated other functional differences of MDS monocytes. The production of IL-1β, IL-6 and IL-10 upon CD40 stimulation was found to be different from what control monocytes produce in response to the same stimulus.

We investigated if the surface expression of specific molecules by monocytes related to subsequent *in vitro* cytokine production. More specifically, we wondered if monocytes that express high levels of CD40, subsequently produce more TNF-α in response to stimulation of the CD40 receptor. However, we were unable to show a relation, and we think there can be several explanations for this. It was previously shown that freshly isolated monocytes need priming in order to respond to CD40 stimulation, even when they already express CD40 at the surface (Burger et al, 2004). We therefore cultured the purified monocytes for 7 days to induce maturation. This maturation will undoubtedly have resulted in up-regulation of expression of

CD40, as was shown by others (Burger et al, 2004). However, we did not consistently measure CD40 expression on these cultured monocytes with flow cytometry. As cultured monocytes are highly auto-fluorescent and in addition need rigorous manipulation and treatment with trypsin in order to dehiscent them from the wells, which can interfere with flow cytometry results. On the other hand, monocytes from healthy controls will also have up-regulated expression of CD40. But, even in the presence of the same maximized stimulus, control monocytes still produce lower amounts of TNF-α.

This observation suggests that different down-stream pathways are activated in MDS monocytes in addition to the different expression levels of CD40. It has been shown that CD40 ligation leads to activation of MAPK pathway, and eventually to NF-κB activation (Rothe et al, 1995, van Kooten and Banchereau 2000). Increased NF-kB has been described in MDS mononuclear cells (Braun et al, 2006, Sanz et al, 2002). To what extent NF-κB activation is related to CD40 expression, remains however to be explored.

The strongest evidence for the involvement of monocytes in the process of BM failure comes from the observation that blocking the CD40 receptor leads to increased colony formation. One might argue that B cells also express CD40, but we have shown that this effect is monocyte-dependent by depleting monocytes from the cultures. Other findings further substantiate the involvement of CD40-CD40L interactions on myelopoiesis. These findings include: the inverse correlations between the numbers of colonies formed *in vitro* and (1) the percentage of $CD40L^+$ T helper cells and (2) the percentage of $CD40^+$ monocytes.

We also evaluated the effects of stimulation of the CD40 receptor on myelopoiesis. We started these experiments with commercially available sCD40LT. We found that the concentration of TNF-α acquired with 25 μg/mL of added sCD40LT were similar to that seen with clone 64 (data not shown). Analysis of experiments of BMMNC's from 11 patients cultured with sCD40LT, showed a decrease in the number of colonies formed, although this decrease was not significant. Additional experiments with clone 64 also showed a decrease in the number of colonies, although only two patients' samples were available for the analysis. This absence of a more profound inhibitory effect on myelopoiesis of CD40 stimulation compared to the effects seen with ch5D12, can look contradictory at first sight. We believe that a possible explanation is the half-life of the molecule that is used to stimulate/block the CD40 receptor. As it takes 14 days for a standard colony assay, the molecule should be stable during most of this period and, in addition, must be able to continuously activate target cells. Stimulation of CD40 receptor, warrants trimerization of the receptor, which is achieved by surface bound CD40L (as on T helper cells) or with sCD40LT. As sCD40LT is unstable, surface bound CD40L or

CD40 agonist antibodies are preferred for *in vitro* experiments (Gurunathan et al, 1998). As clone 64 has an IgG1 backbone, its half-life is affected by internalization by FcRγ1 receptors. This hypothesis is substantiated by other reports. The effect of TNF-α on hematopoiesis has also been shown reversely, i.e. by the use of TNF-blocking antibodies (Gersuk et al, 1998).

In contrast to our findings on monocytes in PB and BM, it was previously shown by our group that *ex vivo* generated mature monocyte-derived dendritic cells (*m* MoDC) from MDS patients had reduced expression of co-stimulatory molecules, including CD40 (Ma et al, 2007). In addition to the altered phenotype, *m* MoDC were shown to produce more TNF-α, IL-10 and IL-12p70 than cultured *m* MoDC from controls. This observation of reduced expression of CD40 on MoDC but increased expression on monocytes are not mutually exclusive. We believe the increased expression on monocytes mirrors on-going immune activation. It remains to be explored if the differences observed in cultured *m* MoDC relate to an intrinsic maturation defect, or that previous activation of these monocytes leads to altered *in vitro* maturation to MoDC.

7.7. Summary of results

We investigated the contribution of monocytes in MDS-related marrow failure and more specifically, the role of CD40-CD40L interactions with T helper cells in this process. We demonstrate that monocytes in MDS patients have an increased expression of CD40 and other co-stimulatory molecules. High expression-levels of CD40 coincided with increased expression of CD40L by $CD4^+$ T cells. The percentage of circulating $CD40^+$ monocytes was higher in patients aged under 60y and patients with trisomy 8, which both have been shown to be independent factors predicting response to immunosuppressive treatment. We have also demonstrated that stimulation of the CD40 receptor on cultured MDS $CD14^+$ cells leads to a significantly higher TNF-α production. Finally, we have shown that *in vitro* culture of BMMNC's in presence of a blocking CD40 antibody resulted in a significant increase in the number of produced hematopoietic colonies.
These results suggest that the level of CD40 expression on circulatory monocytes could be a marker to identify those patients with a suspected immune-mediated disease. Furthermore, blocking CD40-CD40L interactions could be a new means to treat MDS-related cytopenias.

Chapter 8

In vitro effects of thalidomide on cytokine production of monocytes

8.1. Introduction

Thalidomide, a derivative of glutamic acid, was developed in the 1950s as a sedative and an anti-emetic drug. As such, it was used against morning-sickness due to pregnancy. It was withdrawn from widespread use in the 1960s because of its teratogenicity (Tough et al, 1961) affecting about 10,000 infants worldwide. After being banned for more than two decades, *in vitro* studies demonstrating that thalidomide inhibits TNF-α production (Sampaio et al, 1991) have led to its use in clinical conditions thought to be mediated by increased production of pro-inflammatory cytokines. These diseases include refractory cutaneous lupus (Holm et al, 1993), chronic graft versus host disease (Vogelsang et al, 1992), rheumatoid arthritis (Gutierrez-Rodriguez et al, 1989) and Behçet's syndrome (Hamuryudan et al, 1998).

Thalidomide is a potent immunomodulating agent with a broad spectrum of immunologic effects. *In vitro* studies have shown that thalidomide inhibits TNF-α production by LPS-stimulated macrophages and this inhibitory action is attributed to enhanced degradation of TNF-α mRNA (Moreira et al, 1993). Besides inhibiting TNF-α production, thalidomide has also been shown to inhibit IL-6 and IL-12 production (Moller et al, 1997, Rowland et al, 1998). It also influences the function of T cells by increasing IL-2 mediated T cell proliferation and IFN-γ production and co-stimulation of preferentially the $CD8^+$ subset (Haslett et al, 1998). Furthermore, thalidomide inhibits neutrophil chemotaxis, modifies surface expression of adhesion molecules such as integrin receptors on leukocytes, and can modulate dendritic cell function (Geitz et al, 1996, Mohty et al, 2002).

Besides its activity in a variety of immune-mediated conditions, thalidomide has been shown to have therapeutic effects in several malignancies. The anticancer effect of thalidomide has been ascribed to immunomodulating effects as well as to anti-angiogenic effects and direct cytotoxic properties.

Thalidomide has been used in MDS patients as single-agent therapy (Musto 2004, Raza et al, 2001, Strupp et al, 2002) and in combination with other agents such as EPO (Steurer et al, 2003), chemotherapy (Cortes et al, 2003), arsenic trioxide (Raza et al, 2004a) (Candoni et al, 2005) and etanercept (Candoni et al, 2005). Responses are seen in 20-35% of patients and include predominantly erythroid responses. The patient tolerance however is low, especially due to side effects like fatigue and poly-neuropathy. This translates in high dropout rates observed in clinical studies.

We have previously demonstrated that monocytes from MDS patients have a different biological behavior with regard to their cytokine production. Currently, *in vitro* data on the effect of thalidomide on cytokine production in MDS are lacking. We therefore subjected purified monocytes to LPS or a CD40-agonist without or with increasing doses of thalidomide and measured its effect on the levels of TNF-α, IL-1β, IL-6 and IL-10.

8.2. Results

8.2.1. Patients and controls

A median of 4.2 x 10^6 (range 1.2 – 30.0) $CD14^+$ cells was recovered from 23 patients. As described in section 7.3, $CD14^+$ cells were plated at a density of 3x10^5 in 1 mL per well in a 24-well plate. After 7 days of incubation in a humidified incubator at 37°C to induce maturation, the medium was replaced with medium containing either LPS or clone 64 with INF-γ, as described. Immediately thereafter, thalidomide was added at concentrations of 5, 10 and 25 µg/mL. Thalidomide was dissolved in DMSO (stock concentration 5 µg/µL). After 24h, supernatant was collected and kept at -20°C until analysis.
Depending on the number of recovered $CD14^+$ cells, $CD14^+$ cells were divided over 3 to 9 wells. The more $CD14^+$ cells were available, the higher the concentrations of thalidomide that we were able to test *in vitro*. This resulted in lower numbers of samples that could be incubated with 25 µg/mL thalidomide. The figures below show the number of samples. P-values represent paired t-tests compared to the condition without thalidomide.

8.2.2. Stimulation with LPS

8.2.2.1. Healthy controls

TNF-α production was lower with every concentration of thalidomide used. For the highest concentration of thalidomide (25µg/mL) this was not significant, presumably because of the low number of samples available for analysis. Concentrations of IL-1β, IL-6 or IL-10 were not significantly different with or without thalidomide, and are therefore not shown.

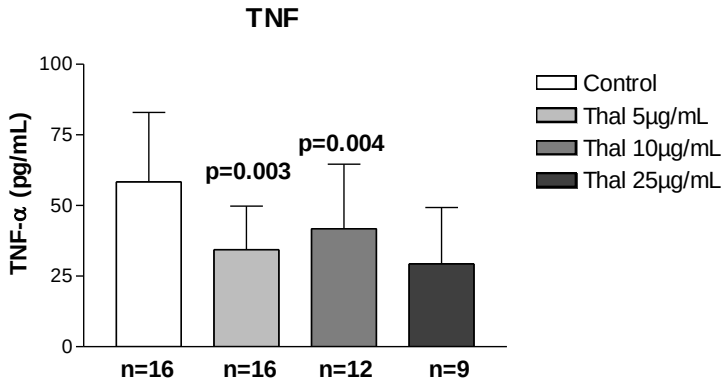


Figure VIII.1: Inhibition of TNF-α by thalidomide of LPS-stimulated control monocytes.
$CD14^+$ cells of 16 different control subjects, were subjected to 1 µg/mL LPS in presence of thalidomide in increasing concentrations. P-values represent 2-sided paired t-tests.

8.2.2.2. MDS

TNF-α production was significantly lower with every concentration of thalidomide used, as depicted in figure VIII.2. We could not demonstrate that higher concentrations of thalidomide could further down-regulate TNF-α production. IL-1β and IL-6 concentrations induced by LPS-stimulation were not blocked by thalidomide. On the contrary, with increasing concentrations of thalidomide, concentrations of IL-6 and especially IL-1β were higher than in the absence of thalidomide, unlike in donors. IL-10 concentrations were not affected by the presence of thalidomide (results not shown).

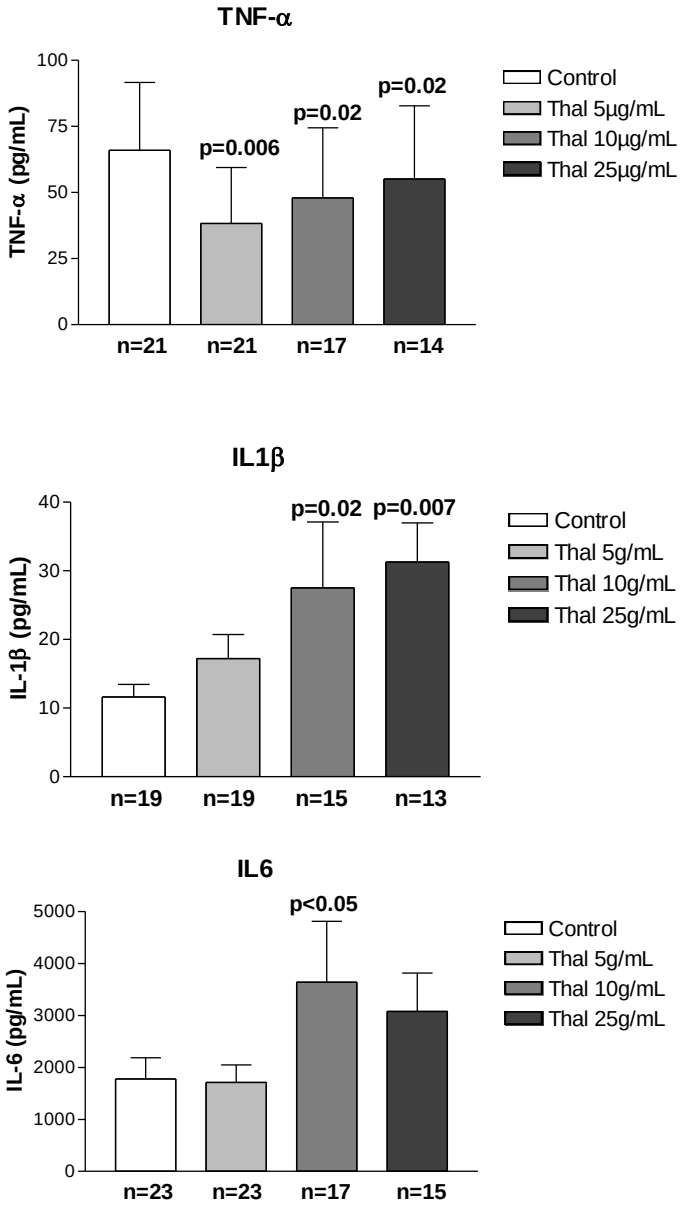


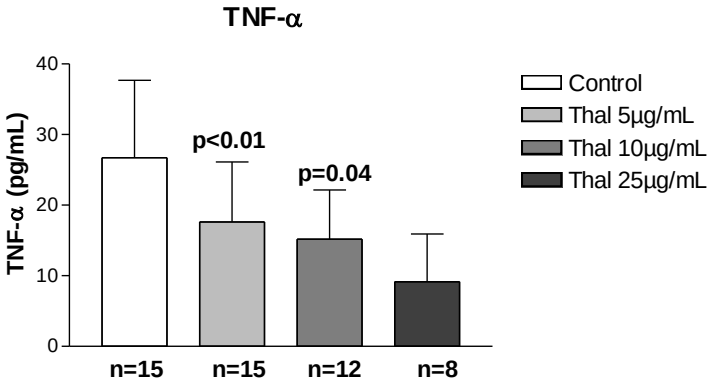
Figure VIII.2: Effects of thalidomide on LPS-stimulated MDS monocytes. $CD14^+$ cells of 23 different MDS patients were subjected to 1 µg/mL LPS in presence of thalidomide in increasing concentrations. TNF-α, IL-1β an IL-6 concentrations were determined with ELISA. P-values represent 2-sided paired t-tests.

8.2.3. Stimulation with Clone 64

8.2.3.1. Healthy controls

TNF-α concentrations were significantly down-regulated in a dose-dependent way by the presence of thalidomide, as shown in figure VIII.3. IL-1β and IL-6 production were significantly higher in the presence of thalidomide (data not shown). IL-10 concentration was not altered in presence of thalidomide.

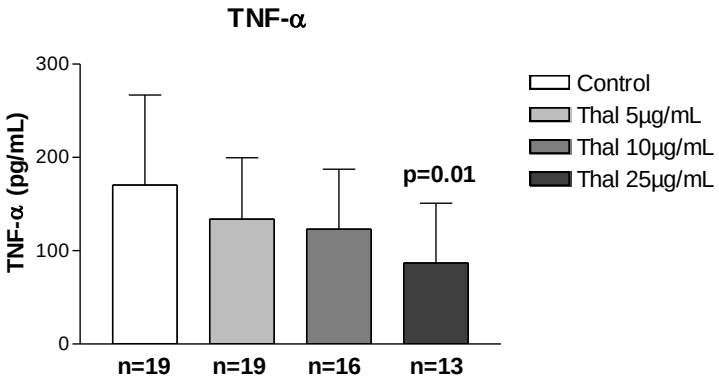
Figure VIII.3: Effects of thalidomide on CD40-stimulated control monocytes on TNF-α production. $CD14^+$ cells of 15 different control subjects were subjected to clone 64 (10 µg/mL) and IFN-γ (1000 IU/mL) in presence of thalidomide in increasing concentrations. TNF-α concentrations were determined with ELISA. P-values represent 2-sided paired t-tests.



8.2.3.2. MDS patients

TNF-α production was only significantly lower in the presence of 25 µg/mL thalidomide. IL-1β, IL-6 and IL-10 concentrations were not significantly different in the presence of thalidomide.

Figure VIII.4: Effects of thalidomide on CD40-stimulated MDS monocytes on TNF-α production. $CD14^+$ cells of 19 different MDS patients were subjected to clone 64 (10 µg/mL) and IFN-γ (1000 IU/mL) in presence of thalidomide in increasing concentrations. TNF-α concentrations were determined with ELISA. P-values represent 2-sided paired t-tests.



8.3. Discussion

TNF-α has been shown to inhibit hematopoiesis and has been implicated in the pathogenesis of MDS-related BM failure. In order to diminish the inhibitory effects of TNF-α, several therapeutic approaches have been used in patients. Non-specific TNF-α inhibitors like pentoxyfillin or more specific molecules like TNF-receptor-fusion protein (Deeg et al, 2002) have been used, but with overall modest clinical results (cfr. Chapter 1). In selected patients with MDS the use of thalidomide has resulted in clinically significant hematological responses. Thalidomide and other IMiDs have a wide mode of action, including inhibition of

TNF-α production by monocytes. Whether this mechanism is clinically important in MDS patients is not known.

We confirm that thalidomide inhibits TNF-α production of LPS-stimulated monocytes in control subjects and in patients with MDS. However, we were unable to demonstrate a concentration dependent decrease in TNF-α, but this is in line with other authors (Mohty et al, 2002). LPS is the standard stimulus to test the potency of molecules like IMiDs in their ability to modulate cytokine production by monocytes. However, it is questionable if this pathway is representative for the *in vivo* situation.

In the previous chapter we provided evidence that CD40-CD40L interactions between monocytes and T helper cells are important in the pathogenesis of MDS-related BM failure. As such, we demonstrated that CD40 stimulation of MDS monocytes led to significantly higher TNF-α levels. The inhibitory action of thalidomide on TNF-α production has been ascribed to enhance degradation of TNF-α-mRNA (Moreira et al, 1993) meaning that it is not specifically targeting LPS downstream signaling pathways. Activation of monocytes through CD40 receptor leads to activation of MAPK pathways and eventually leads to NF-kB activation (Rothe et al, 1995, van Kooten and Banchereau 2000).

Consequently, we have analyzed the activity of thalidomide on CD40-induced TNF-α production and up to this date, we have been the first to report this. We could indeed demonstrate that thalidomide is also capable of inhibiting TNF-α production induced by CD40 stimulation of monocytes from healthy controls. In patients however, significantly higher doses of thalidomide are needed to achieve a similar effect. The exact *in vivo* concentrations of thalidomide in marrow of patients has not been established, partly due to a high variability in the absolute bioavailability of thalidomide from one patient to another (Peuckmann et al, 2000). Nevertheless, this observation is in line with the observation that relatively high doses are needed to achieve responses in MDS. These doses are however hard to reach due to dose-limiting toxicity, especially neurologic side effects.
We believe that together with our result that CD40-CD40L interactions are involved in the pathogenesis of MDS-related marrow failure, this observation of the weaker *in vitro* potency of thalidomide in MDS is important.

We do realize that several concerns can be raised when interpreting results from *in vitro* experiments. A first concern is the possibility that thalidomide has additional effects on MDS monocytes than on monocytes from healthy controls. More precisely, thalidomide could induce apoptosis in MDS monocytes and hence influence the results. Recently, Gockel et al

reported that thalidomide indeed induces apoptosis in monocytes, via mitochondrial cytochrome c release followed by caspase-activation (Gockel et al, 2004). They could demonstrate this with thalidomide concentrations of 10-50 µg/mL, but this effect was only seen after 48h of incubation with the drug. This raises the possibility that an altered susceptibility to apoptosis by thalidomide could be responsible for the lower TNF-α concentrations measured in presence of thalidomide. However, in our experiments the supernatant was already harvested after 24h, thus making this hypothesis less likely. The fact that monocytes from MDS and normal controls were not significantly different in conditions of LPS-stimulation also argues against the fact that increased/decreased in apoptosis influenced our results.

In vitro observations cannot always be translated into the *in vivo* situation. Notwithstanding numerous reports on the inhibitory effect of IMiDs on TNF-α production *in vitro*, treatment with thalidomide does not always result in a measurable decrease in serum TNF-α levels in patients (Moutsopoulos et al, 2006), whereas others even report an increase in TNF-α (Jacobson et al, 1997). These inconsistent results have also been reported on the levels of several angiogenic factors, such as VEGF (Musto 2004).

Since previous reports suggested that age influences functionality of monocytes, we included age-matched controls in our experiments (Plackett et al, 2004, Plowden et al, 2004). But we could not demonstrate differences between young and aged controls regarding TNF-α production. Moreover, as the majority of the control subjects was of older age, this phenomenon is probably not responsible for the differences we observed.

In addition to the production of TNF-α by monocytes, we also evaluated the effect of thalidomide on IL-1β, IL-6 and IL-10 production. We demonstrate a gradual increase in the production of IL-1β with increasing thalidomide concentrations in both donors and patients, either stimulated with LPS or with clone 64. Previous reports did not see an increase in the IL-1β production in patients treated with thalidomide, but similar dose-dependent changes have been reported for IL-12p70 production by dendritic cells (Mohty et al, 2002). The production of IL-6 and IL-10 was not affected by the presence of thalidomide, which is in line with previous reports (Bauditz et al, 2002).

Of course, inhibition of TNF-α production is just one mechanism by which IMiDs can exert their function. And this might not be the most important effect responsible for the responses seen in MDS patients. Thalidomide has been shown to have direct effects on T cells. It stimulates cytotoxic T cell proliferation and induces secretion of IFN-γ and IL-2 in these cells.

In healthy volunteers, thalidomide significantly decreased the circulating $CD4^+$ to $CD8^+$ T cell ratio (Gad et al, 1985). However, in MDS patients treated with thalidomide, no significant change in this $CD4^+$ to $CD8^+$ ratio was observed (Strupp et al, 2005).

In addition to changes in $CD4^+$ to $CD8^+$ T cell ratio, also changes in T_H1 to T_H2 profile have been observed. In human PB mononuclear cell cultures thalidomide induced T_H2 cytokine production while concomitantly inhibiting T_H1 cytokine production (McHugh et al, 1995). As we have demonstrated that activated T_H1 cells can contribute to BM failure in MDS, this effect might also be important in patients with hematological responses with thalidomide. Thalidomide also mediates anti-tumor effects by modulating NK cell number and function (Davies et al, 2001), possibly via IL-12p70 (Lauwerys et al, 2000) in patients with multiple myeloma. If this occurs in MDS patients treated with thalidomide remains to be explored.

8.4. Summary of results

Thalidomide and other immunomodulatory drugs are used in patients with MDS. Inhibition of TNF-α is one of the proposed working mechanisms. We evaluated the effect of thalidomide on *in vitro* cytokine production by monocytes from MDS patients stimulated with either LPS or CD40 agonists. We demonstrate that thalidomide inhibits LPS-induced TNF-α production in monocytes from both patients and controls. Thalidomide also inhibits TNF-α production of control monocytes stimulated with CD40 agonists. In contrast, we demonstrate that monocytes derived from MDS patients are relatively resistant to thalidomide. Only in presence of a high concentration (25 µg/mL) thalidomide we could observe a significant inhibition of TNF-α production induced by CD40 stimulation.

Chapter 9

General discussion

MDS comprises a heterogeneous group of clonal hematopoietic disorders which are characterized by peripheral cytopenias and an increased tendency to evolve towards acute myeloid leukemia. Acquired intrinsic abnormalities in the hematopoietic progenitors are believed to play a central role in the pathogenesis of the disease, but factors from within the BM microenvironment have been shown to influence the course of the disease. Since there is no standard treatment for patients with MDS, a better understanding of these factors that play a role in the pathogenesis of MDS-related BM failure, could lead to new therapeutic modalities. As for the current therapeutic strategies, it is also largely unknown which patients will respond to these treatments. In spite of the existence of algorithms that predict favorable responses to e.g. growth-factors (Hellstrom-Lindberg et al, 2003) and immunosuppressive drugs (Sauntharajah et al, 2003), the use of these therapeutic modalities frequently relies on the experience of the clinician, the access to these treatments and patients' tolerability, and not exclusively on scientific evidence.

It has been shown that immune mechanisms can play a determinant role in the development of BM failure. This evidence comes from: (1) the observation that autologous T cells can inhibit *in vitro* progenitor growth (Baumann et al, 2002, Molldrem et al, 1998), (2) the presence of clonal T cell expansions in selected patients (Epling-Burnette et al, 2007b, Epperson et al, 2001, Melenhorst et al, 2002, Sloand et al, 2005, Wlodarski et al, 2006), (3) the close relation of MDS with other BM failure syndromes in which immune mechanisms have been shown to play a crucial role (such as SAA, PNH) (Maciejewski et al, 2002a, Young and Maciejewski 2000), (4) the response of about a third of patients to immunosuppressive therapies (Barrett et al, 2006, Biesma et al, 1997, Broliden et al, 2006, Jonasova et al, 1998, Killick et al, 2003, Lim et al, 2007, Molldrem et al, 1997, Tichelli et al, 1988). The object of this project was to further clarify the role of the immune compartment in MDS.

More or less immune cells in MDS?

Cytopenia is the leading cause of MDS-related morbidity and mortality and this has been appreciated by inclusion of cytopenia in the IPSS score as an unfavorable risk factor (Greenberg et al, 1997). Upon enumerating the number of circulatory mature blood cells in MDS patients, age-related effects should be taken into consideration, as apparent differences observed in patients could just well be a mere result of their relatively high age. We could indeed demonstrate these differences between young and age-matched controls. The IPSS only considers cytopenia in **myeloid** lineages (i.e. red blood cells, platelets, neutrophils). In our group of 101 patients, we could demonstrate that the overall number of neutrophils and platelets decreased with increasing IPSS score. We could also show this for the number of monocytes, even though MDS patients in general, did not have lower numbers than age-matched controls.

"**Lymphopenia**" is not recognized by the IPSS as a prognostic risk factor and hence it is not generally considered in the clinical setting. However, several groups have shown that lymphocytes are generally decreased in MDS patients (Bynoe et al, 1983, Colombat et al, 1988). In addition, a T cell count below 800/µL has been shown to be an independent risk factor for worse prognosis (Amin et al, 2003). In our study, we show that a low T cell number is rather uncommon in patients with MDS. Only higher risk patients show a decline in the number of $CD4^+$ and $CD8^+$ T cells (RAEB, IPSS int-2). Our findings are probably in line with previous reports, since these reports included predominantly higher risk patients (30 RAEB patients, (Colombat et al, 1988)). Differences in the control group could have accounted for these findings too, since we found that the number of T cells is age-dependent, at least in our control groups.

The number of B cells in PB depends on continuous production of pro-B cells by lymphocyte precursors in the BM, and in addition, requires successful immunoglobulin gene rearrangements. In aged individuals, humoral immunity is compromised as a result of decreased B cell production and altered T cell/B cell interactions (Lazuardi et al, 2005). We found such an age-related decline in the number of B cells in our control groups. Notwithstanding this, MDS patients were found to have even lower B cell numbers. Reduced numbers can be related to impaired production or to increased apoptosis and supportive data for both have been published. Amin reported a higher apoptotic rate (Annexin V-positive) in the $CD19^+$ population of patients (Amin et al, 2003). Sternberg demonstrated that patients have reduced numbers of B cell progenitors and in a gene expression array, patients have been found to have marked reduced expression of B-affiliated genes (Sternberg et al, 2005).

NK cells are critical in host defense against malignant transformation and are potent antileukemic cytotoxic effectors. NK cells are components of the innate immune system and hence do not require prior immunization. Their anti-leukemic potential has been demonstrated in AML patients, where NK cell activity positively correlated with relapse-free survival in patients in complete remission (Tajima et al, 1996). In normal individuals, it is regarded that the number of NK cells significantly increases with age whereas NK cell function, both cytotoxicity and cytokine production, is impaired in ageing individuals (Aw et al, 2007). In MDS, numerical and functional differences have been described (Epling-Burnette et al, 2007a, Fujii et al, 2003, Kiladjian et al, 2006, Ogata et al, 1994). In contrast with previous reports, we demonstrate a decreased NK cell number in PB of patients and this was more pronounced in patients with poor cytogenetic risk score. It is however not possible from our data to speculate if these low numbers contribute to the worse prognosis patients with poor cytogenetic risk. Surprisingly, we found that the number of NK cells decreased with increasing CRP levels, but any causal relationship remains elusive.

Are immune cells of clonal origin in MDS?

MDS is characterized by clonal hematopoiesis. Most clonality studies in MDS suggest that in the majority of patients, the primary clonal transformation event occurs at a multipotent but myeloid-restricted progenitor cell level. We evaluated clonal involvement of purified PB populations with FISH (for patients with known cytogenetic abnormalities) and HUMARA (for female patients that where shown to be heterozygous for the number of CAG repeats in the first exon of their HUMARA-alleles). In our series, we could demonstrate clonal involvement of monocytes in the vast majority of patients we analyzed. This is in line with current conceptions on the pathogenesis of MDS, and is supported by most reports in the literature showing clonal involvement of myeloid cells (Prchal et al, 1978, Raskind et al, 1984).

The clonal nature of lymphocytes in MDS however is still a matter of debate as controversial observations have been published. XCI studies report the existence of both polyclonal (Abrahamson et al, 1991, Anan et al, 1995, Van Kamp et al, 1992) and monoclonal (Culligan et al, 1992, Okada et al, 1999, Prchal et al, 1978, Tefferi et al, 1990, Tsukamoto et al, 1993) lymphocytes.

In this study, **T cells** were found to be polyclonal in the vast majority of the patients we analyzed. Only 2 patients were found to have skewed lyonization in the $CD4^+$ T cell and $CD8^+$ T cell population. But since the allelic ratio was borderline in all cell fractions of these 2 patients, this observation suggests constitutional or acquired skewing. With FISH, we could only detect the known cytogenetic abnormality in $CD4^+$ and $CD8^+$ T cells in one patient respectively. With HUMARA, we demonstrate clonal involvement of **B cells** in half of our patients. In contrast, with FISH only 25% had B cells with the known abnormal karyotypes. We found a high prevalence of clonal **NK cells** using the HUMARA assay (6/12) and FISH (11/12). NK cells have been reported to be polyclonal in most patients (Ogata et al, 1994), but there have been other reports of clonal involvement (Kiladjian et al, 2006, Ma et al, 2004, Miura et al, 2000).

There are several reasons why clonality studies on lymphocyte populations should be interpreted with care, and the following arguments can be responsible for the conflicting results found in literature. Firstly, the method by which clonality is assessed is a major determinant for the obtained results. As we have elucidated in chapter 4, FISH is a more sensitive technique to detect (known) clonal abnormalities in a given cell population. A skewed pattern of XCI warrants a minimum of 75% of cells carrying the same XCI profile, whereas with FISH one can detect abnormal cells if these cells exceed 5% of the normal cells. Van Dijk and co-workers already have provided evidence for this hypothesis, as they could demonstrate a 5q deletion in 2 patients with FISH, without evidence of signs of clonality in XCI assays (van Dijk and de Witte 2004).
We also have provided additional evidence for this, be it in only 3 patients. From these patients, we had XCI and FISH data on purified PB populations. With FISH we could invariably detect NK cells derived from the abnormal clone, whereas HUMARA only showed a skewed XCI pattern in one patient. Since these populations were purified from PB samples taken on 2 occasions, we cannot compare the results of FISH and HUMARA directly. Moreover, the results of the NK cells of patient 2 tend to show the opposite: whereas only 9% of the cells had del(13)(q13q21), the corrected ratio showed that nearly all cells had the same XCI pattern. We think that this finding rather suggest that the acquisition of the deletion of the long arm of chromosome 13 is a late event in the clonal evolution of the disease in this patient. Similar evidence for a step-wise acquisition of additional chromosomal aberrations has been provided by Nilsson and co-workers who showed that the acquisition of +8 was a secondary event in MDS pathogenesis (Nilsson et al, 2002).

A second reason why XCI assays should be interpreted with caution, is that in healthy females there is an increasing incidence of clonal patterns in both myeloid and lymphoid cells with age (Busque and Gilliland 1998, Champion et al, 1997), a phenomenon called acquired skewing. Thirdly, the fact that mature lymphocytes are not being derived from the MDS clone does not exclude the possibility that the primary transformation event in MDS can occur at the lymphoid-myeloid HSC level, for the transformation event in MDS may be incompatible with further lymphoid differentiation. Finally, polyclonal PB lymphocytes do not exclude the presence of a minor monoclonal lymphoid population as the sensitivity of non of the current detection techniques is 100%.

Notwithstanding that FISH tends to be a more sensitive technique to detect clonal abnormalities, not all patients show evidence of clonal involvement of lymphocytes. B cells have been shown to be involved in patients with 5q or 20q deletion (White et al, 1994) and monosomy 7 (Van Lom et al, 1995), whereas in others with 5q deletions (Kroef et al, 1997, Nilsson et al, 2000), monosomy 7 (Gerritsen et al, 1992) they were not.

A way to overcome these problems, is analyzing BM progenitor cells for clonal involvement, or performing functional assays in vitro or in vivo in order to see if these progenitor cells produce mature cells. In this way Nilsson and Miura provided evidence that early lymphoid progenitor cells were part of the malignant clone in del(5q) (Nilsson et al, 2000) and monosomy 7 patients (Miura et al, 2000), respectively.

Relationship between clonal involvement and PB counts?

As was described in chapter 1, MDS is characterized by PB cytopenias and increased marrow cellularity. Increased intra-medullary apoptosis has been held responsible for this apparent paradox (Yoshida 1993). Intuitively, one would reason that cells belonging to the abnormal clone are intrinsically impaired to differentiate into mature cells and preferentially become apoptotic. Consequently, clonal involvement automatically leads to peripheral cytopenias. Vice versa, cytopenias in a certain lineage would imply clonal involvement of this cell type. However, our results do not totally support this. For instance, although monocytes are invariably clonally derived, the number of PB monocytes in patients is not different from age controls. We also did not find that numbers of NK or B cells were lower in patients with clonal involvement of these populations.

Given the limited number of patients studied, definite conclusions are difficult to draw. It is possible that by analyzing more samples, one might be able to find an inverse correlation between the number FISH-positive cells and the number of that population in PB. Also differences in kinetics can be responsible for the lack of covariance. Lymphocytes are long-lived and therefore a decreased production is not easily detected. The fact that the pathogenesis of MDS is multifactorial is probably the best explanation for this absent relationship, as many other factors have been shown to influence the production of mature cells.

Activated T cells in patients with MDS

It was previously shown that the immune system in patients with MDS demonstrate signs of an adaptive immune response. A chronic antigenic stimulus can lead to the development of (oligo-)clonal T cell populations. Independent investigators (Epling-Burnette et al, 2007b, Epperson et al, 2001, Kook et al, 2001, Melenhorst et al, 2002, Sloand et al, 2005, Wlodarski et al, 2006) have reported minor T cell clones to be present in MDS patients. Moreover, in patients with trisomy 8, it was shown that the myelosuppressive properties of T cells is restricted to these specific T cell clones (Sloand et al, 2005). In addition, these T cells have been shown to have an activated phenotype (Epling-Burnette et al, 2007b). Considering the fact that TCR spectratyping is very time-consuming and not widely available, we investigated if immunophenotyping of lymphocytes could serve as an alternative approach to detect patients with a presumed immune-mediated pathogenesis.

Firstly, we convincingly show that more T cells have an activated phenotype (HLA-DR$^+$, CD57$^+$, CD28$^-$, CD62L$^-$) in PB of 101 patients. We have also provided evidence that T cells in the BM have this activated phenotype, and that their expression levels mirror those of PB T cells. It was previously shown that these markers are possible good surrogates to track on-going immune activation. Boula and co-workers showed that after treatment with an anti-TNF-α antibody, the proportion of activated T cells (i.e. HLA-DR$^+$, CD25$^+$, CD38$^+$, CD69$^+$) decreased (Boula et al, 2006).

Despite this observation, one could speculate about the antigenic stimulus and the relationship between activated T cells and MDS pathogenesis. Firstly, one might argue that activation of T cells is just a mere result of increased prevalence of ***concomitant infections*** in this patient group. However, patients with obvious signs (or a recent history) of infection were excluded from the analysis. Moreover, CRP levels were low and did not correlate with

the level of lymphocyte activation, making a systemic inflammatory response an unlikely cause for our observation.
Secondly, our observation could just be a mere ***consequence of aging***. Similar concerns were raised when T cell expansions were observed in MDS patients, as one can find more T cell expansions in healthy ageing persons. However, Melenhorst et al demonstrated that patients have even more abnormally expanded TCR populations compared to age-matched controls, and that the degree of expansion was several orders greater than that seen in older normal individuals (Melenhorst et al, 2002). As for the immunophenotype of T cells, we could not observe significant differences between young and elderly individuals in our control group, nor could we observe a correlation between expression of these markers and age.
Activation of lymphocytes could also be a ***result from regular blood transfusions***, as it is known that allogeneic blood transfusions can induce immunologic effects in the recipient (Vamvakas and Blajchman 2007). However, the majority of the patients in this analysis did not receive any blood transfusions. And others have already demonstrated that in individuals with hemoglobinopathies, regular blood transfusions did not lead to lymphocyte activation (Papadaki et al, 2005), nor to the development of TCR Vβ expansions (Melenhorst et al, 2002).
When designing this study, one of our main objectives was to evaluate the usefulness of T cell immunophenotyping as a possible alternative for the detection of those patients with a suspected immune-mediated disease. Unfortunately, with the current design we were unable to detect a clinically relevant subgroup of patients with more/less T cell activation. Also in the 7 patients with trisomy 8 in our analysis, the expression levels of the above-mentioned markers was highly variable. Since highly sensitive molecular tests have given evidence that most (if not all) patients with an acquired trisomy 8 clone have an acquired immune response, our results suggest that our flow-cytometric protocol is possibly not sensitive enough to discriminate a subgroup of MDS patients with immune-mediated disease.

We also evaluated the in vitro properties of activated T cells on clonogenic growth of progenitor cells. We could not demonstrate a correlation between the degree of activation (i.e. expression of HLA-DR, CD28, CD57 and CD62L) and the number of colonies grown *in vitro*. The high variability in the number of colonies grown, which is a consequence of the heterogeneity of MDS, could be a valid explanation for this absent correlation.

Not all lymphocyte activation markers are equal.

In the previous paragraph, we discussed our results of T cells in PB and BM with regard to their expression levels of the activation markers HLA-DR, CD57, CD28, CD62L. Maybe not all activation markers are equal, regarding the functional properties of T cells expressing these antigens. The current conception is that the inhibitory effect of autologous T cells in MDS resides in the $CD8^+$ pool. The finding that patients with high expression levels of the above-mentioned markers have significantly higher numbers of $CD8^+$ T cells, is an indirect indication of an active $CD8^+$ T cell process. However, since we did not examine differential expression of these markers on $CD4^+$ and $CD8^+$ T cells respectively, this remains speculative. Most other reports in literature describe expression levels of some of these markers on $CD3^+$ cells (Boula et al, 2006) or on $CD8^+$ T cells (Baumann et al, 2002, Melenhorst et al, 2002). In patients with chronic idiopathic neutropenia, it has been shown that both $CD4^+$ and $CD8^+$ T cells have an increased expression level of activation markers (Papadaki et al, 2005). Interestingly, in this paper expression of activation markers was not restricted to expanded T cell clones.

Our results provide evidence that $CD4^+$ T cells also can be activated in MDS patients. We have shown that CD40L expression on $CD4^+$ T cells is markedly up-regulated in patients. This high percentage of circulating CD40L-expressing T helper cells is intriguing. It indicates that CD40L expression is either increased or stabilized in MDS. The increased CD40L expression may be involved through antigen presenting cells in the induction of the suppressive CD8 clonotype, however this warrants further investigation. In contrast to the other markers, we found that expression of CD40L by T helper cells was clinically relevant. The number of BFU-E formed *in vitro* was inversely related to the expression level of CD40L of T cells in culture.

The observation that also $CD4^+$ T cells can contribute to BM failure is not contradictory with previous observations. Firstly, TCR Vβ expansions can be found in $CD4^+$ as well as in $CD8^+$ T cell populations (Melenhorst et al, 2002) indicative of $CD4^+$ T cell responses in these patients. Secondly, these observations are not challenging previous *in vitro* experiments showing that only autologous $CD3^+$ and $CD8^+$ T cells are myelosuppressive (Baumann et al, 2002, Molldrem et al, 1998). We did not show that $CD4^+$ T cells are inhibitory as such, but have provided evidence that they work in concert with monocytes through CD40-CD40L interactions. The presence of monocytes is thus required to see an inhibitory effect of $CD4^+$ T cells *in vitro*.

Activated monocytes in patients with MDS

In some patients with MDS cytotoxic T cells are capable of suppressing endogenous myelopoiesis. T cell mediated killing requires antigen-recognition by specific T cell receptors and therefore implies specificity in suppressing the targeted cells. Notwithstanding this, some patients develop global marrow failure as a result of this acquired T cell response. Several explanations have been suggested for this “innocent bystander phenomenon”: release of cytokines by activated T cells (Maciejewski et al, 1995) or cross-recognition of targets through molecular mimicry and epitope spreading (Eisen 2001). Alternatively, other cells besides T cells are involved in the process. With our work, we have shown that monocytes are partners in the process of immune-mediated marrow failure.

Monocytes have previously been shown to be the main source of TNF-α, a myelosuppressive cytokine (Gersuk et al, 1998). Up till now the major driving force for the increase in TNF-α production remained elusive. We have shown that CD40-CD40L interactions between monocytes and T_H1 cells are a possible driving force, as in other immune-mediated diseases. The CD40 receptor is a 45-50 kDa protein and is a member of the tumor necrosis factor receptor (TNFR) superfamily. Interaction of CD40 with its ligand (CD40L) has been shown to play a crucial role in cellular and humoral immune responses. CD40 is expressed on numerous cell types: B cells, monocyte/macrophages, endothelial cells, dendritic cells, fibroblasts, vascular smooth muscle cells and neurons. CD40 signalling in monocytes results in up-regulation of co-stimulatory molecules and in the production of pro-inflammatory cytokines such as IL-1α/β, IL-6, IL-8, IL-12 and TNF-α.

We have shown that monocytes express higher levels of the CD40 receptor in MDS patients than in age-matched controls. We have shown this in PB as well as in BM samples. The presence of activated monocytes in PB mirrors the presence of activated monocytes in BM. This **over-expression of CD40** coincides with global over-expression of other activation markers (CD16, CD69) and the co-stimulatory molecule CD80 on monocytes. Importantly, over-expression of CD40L by $CD4^+$ T cells also correlates with over-expression of CD40 by monocytes. This observation suggests that activation of both cell populations occurs in the same patients.

Additional *in vitro* data also support the importance of CD40-CD40L interactions in MDS. We showed that MDS monocytes produce significantly **more TNF-α in response to CD40 receptor stimulation** than control monocytes. We are the first to demonstrate increased production of TNF-α by MDS monocytes. Previous attempts by others could not demonstrate

an increased intrinsic production of TNF-α by monocytes in response to LPS-stimulation (Visani et al, 1993). In our experiments, MDS monocytes behave similarly to LPS as control monocytes regarding TNF-α production. It is however very unlikely that LPS-mediated signaling itself relates to the *in vivo* setting. In addition to TNF-α production we also demonstrated other functional differences of MDS monocytes. The production of IL-1β, IL-6 and IL-10 upon CD40 stimulation is different from what control monocytes produce in response to the same stimulus.

The strongest evidence for the involvement of monocytes in the process of BM failure comes from the observation that **blocking the CD40 receptor leads to increased colony formation**, and this effect is monocyte-dependent. Other findings substantiating the involvement of CD40-CD40L interactions on myelopoiesis include: the inverse correlations between the numbers of colonies formed *in vitro* and (1) the percentage of $CD40L^+$ T helper cells and (2) the percentage of $CD40^+$ monocytes.

Which factors mirror an on-going immune attack in the bone marrow?

One of the objectives of this study was to evaluate the potential use of immunophenotyping of T cells or monocytes, as a marker for MDS patients with a suspected immune-mediated disease. Most current biological markers that are assumed as such, are those markers that are associated with a hematological response after immunosuppressive treatment. In an analysis of the patients treated at the NIH, HLA-DR15 haplotype, patient's age and interval between first transfusion and immunosuppressive treatment, were shown to be independent factors associated with a good response to ATG +/- CsA (Sauntharajah et al, 2003). Also hypocellularity of the BM has been allocated a good predictive marker for patients to respond to immunosuppression (Lim et al, 2007). Amongst these factors, hypocellularity is probably the most subjective parameter.

We have demonstrated that the presence of activated T cells ($HLA\text{-}DR^+$, $CD57^+$, $CD28^-$, $CD62L^-$) is probably not restricted to patients with an on-going immune attack in the BM. These T cells were also not found to be restricted to a specific subgroup of patients, nor to cytopenias or need for treatment, and did not relate to in vitro myelosuppressive characteristics of these cells. More importantly, in patients with trisomy 8, which are assumed to have immune-mediated disease, the expression levels of these markers was highly variable. Of course this does not fully exclude any role of these activated T cells in patients

with immune-mediated MDS. For instance, it might be that T cells were not activated at the time of sampling. Prospective studies in patients undergoing immunosuppressive treatment are warranted to make a definite judgment on this matter.

In contrast to the activation phenotype of lymphocytes, we found that the surface phenotype of monocytes was more instructive. We were able to detect patients with suspected immune-mediated disease, solely on the basis of their surface CD40 expression level by circulating monocytes. All trisomy 8 patients and all but 1 patient aged under 60y had high surface expression of CD40. The finding of low levels of CD40 expression in patients with del(20q) is also very informative. Epling-Burnette and co-workers have recently reported that clonal T cell populations were not detected in patients with del(20q) (Epling-Burnette et al, 2007b). In addition, as surface CD40 expression did not covariate with CRP levels in serum, the finding of activated monocytes was not a mere result of a systemic inflammatory response. Since monocytes are not believed to re-circulate in PB, activation of monocytes must therefore have happened inside the BM. Lymphocytes have the property to travel from tissues to blood and back. The finding of activated lymphocytes is therefore less likely to be specific for an intra-medullary process.

Which signals trigger CD40 expression by monocytes in patients with MDS remains to be explored. IFN-γ is a well-known activator of macrophages. In one study, Buhtoiarov and co-workers showed that in the absence of INF-γ, a stimulatory anti-CD40 mAb lost its ability to fully activate macrophages suggesting the primordial role of IFN-γ (Buhtoiarov et al, 2005). Alternatively, IFN-γ induced by anti-CD40 mAb may collaborate with anti-CD40 mAb or with other cytokines or downstream molecules induced by CD40 ligation, which alone are ineffective. It has been shown that CD40 and IFN-γ could be synergistic in activation of tumoricidal macrophages (Imaizumi et al, 1999): the CD40L expressed on activated $CD4^+$ T cell interacts with the CD40 molecule expressed on the membrane of the APC, resulting in the production of cytokines and expression of co-stimulatory molecules CD80 and CD86, culminating in activation of $CD8^+$ effector T cells. Activation of tumor-specific immune responses resulting in reduction of tumor growth can be achieved either by using CD40L (Gurunathan et al, 1998, Nakajima et al, 1998) or agonistic anti-CD40 mAb (Diehl et al, 1999, French et al, 1999, van Mierlo et al, 2002).

Finally, it is still hypothetic if the abnormal high surface expression of CD40 is a result of clonal involvement, and thus an abnormal phenotype resulting from the disease. From our study, we cannot formally contradict this as we did not perform clonality studies and immunophenotyping of monocytes on the same sample. The appearance of the FACS

dotplot does not suggest a relationship between clonal involvement and phenotype of monocytes. If CD40 expression was higher within an abnormal clone, one would expect clear separation of 2 different populations on a CD40/CD14 FACS-dotplot, which was never observed by us. Even so, the importance of this matter has recently been illustrated by Itawa and co-workers who demonstrated that the percentage of abnormal cells influenced specific results. The authors evaluated the *in vitro* production of matrix-metalloproteinase 9 (MMP-9) by purified $CD14^+$ cells and they found that this was highly variable in MDS patients. They convincingly showed that this variance was attributable to the percentage of abnormal (here FISH-positive cells for a known clonal marker) cells within the $CD14^+$ population: abnormal (MDS) cells were deficient in their ability to produce MMP-9 (Iwata et al, 2007).

To immunosuppress or not to immunosuppress?

MDS is a complex disease and its pathogenesis is full of paradoxes. The peripheral cytopenias that coincide with a hypercellular bone marrow is the best-known paradox. An increased rate of intra-medullary apoptosis is responsible for this observation. With our work and with recent published papers, another big issue has emerged: is it favorable or unfavorable for MDS patients to have an activated immune system?
The theme throughout this work is that immune-mediated mechanisms can contribute to BM-failure observed in patients. Most evidence points towards a determinant role of T cells in this process. Not only $CD8^+$ T cells can directly inhibit hematopoiesis, but we have shown that also $CD4^+$ T cells can act through CD40-CD40L interactions with monocytes in inhibiting myelopoiesis. On the other hand, a low T cell number (Amin et al, 2003) and especially low NK cell numbers have been associated with poor prognosis (Epling-Burnette et al, 2007a, Kiladjian et al, 2006). In our study, we also found that NK cell numbers were reduced in higher-risk patients, but a causal relationship between them, is only speculative.

As the golden rule in medicine is still “primum non nocere”, treating cytopenic patients with immunosuppressive drugs remains cumbersome. Not only the increased risk of opportunistic infections, but the possible loss of immune surveillance on leukemic transformation are of great concerns. Recently the concept of regulatory T cells has gained much interest. A recent published study reported low numbers of Tregs in lower risk MDS with an increase in numbers in high risk patients. The authors speculated that in these patients Tregs would inhibit anti-leukemic immune control and could therefore be implicated in disease progression. This would have consequences for the future treatment of patients with ATG.

Recently Feng and co-workers have shown that treatment with rabbit-ATG (currently the most frequently used in the clinical setting) increased the number of Tregs (Feng et al, 2008). This would suggest that treatment with ATG could lead to disease progression rather than alleviating cytopenia. However, the latter has not un-equivocally been shown in patients treated with ATG and/or CsA.

What is the best immunosuppressive strategy?

To date, most data exist on the use of ATG and/or CsA to achieve this. The wide range of effects of ATG have recently been reviewed by Mohty (Mohty 2007). ATG is a polyvalent IgG serum of animals immunized with thymocytes or (activated) lymphocyte cell lines. It establishes T cell depletion largely through complement-dependent lysis (in peripheral blood) and through apoptosis and subsequent phagocytosis in peripheral lymphoid tissues. It also induces apoptosis in B cell lineages and interferes with the functional properties of dendritic cells. Besides this, it also induces regulatory T (Feng et al, 2008) and natural killer T cells. Interestingly, most of these effects are apparent desirable effects in treating immune-mediated BM-failure in MDS patients. Also CD40 is targeted by ATG, but it remains unknown to what extent this effect on monocytes contributes to the treatment successes witnessed in patients.

In this study, we used the molecule ch5D12 which is an antagonist chimeric anti-human CD40 antibody with a IgG_4 backbone. Its therapeutic value is to be expected by tapering the immune response and hence limiting collateral damage on normal progenitors in order to improve blood counts. Monoclonal antibodies against CD40 are currently under investigation for use in various hematological disease, such as multiple myeloma (Tai et al, 2005, Tai et al, 2004) and non-Hodgkin's lymphoma (Fanale and Younes 2007). The mode of action of these antibodies relies on antibody-dependent cell-mediated cytotoxicity (Hayashi et al, 2003) and by a direct apoptotic effect on malignant cells (Tai et al, 2004). In other malignancies, activation of monocytes with agonist anti-CD40 Mab has been shown to inhibit tumor growth (Lum et al, 2006). For this reason, inhibition rather than stimulation of the CD40-CD40L pathways seems therapeutically relevant in MDS. Since clinical development of all other antagonist anti-human CD40L antibodies have been terminated due to thrombotic events (related to the IgG_1-backbone of these molecules), the antagonist anti-CD40 ch5D12 described here is the only current option to target inhibition of CD40-CD40L in human pathologies including MDS.

A final word on thalidomide

Thalidomide is a potent immunomodulating agent with a broad spectrum of immunologic effects, and is used with modest efficacy in the treatment of MDS. *In vitro* studies have shown that thalidomide inhibits TNF-α production by LPS-stimulated macrophages and this inhibitory action is attributed to enhanced degradation of TNF-α mRNA (Moreira et al, 1993). Since we demonstrated that in MDS signalling via the CD40-receptor is a more important trigger for TNF-α production by monocytes, we compared the efficacy of thalidomide in presence of LPS vs. a CD40 agonist. We could demonstrate that thalidomide was also capable of inhibiting TNF-α production induced by CD40 stimulation of monocytes from healthy controls. However, in patients, significantly higher doses of thalidomide were needed to achieve a similar effect. Although the exact *in vivo* concentrations of thalidomide in marrow of patients has not been established, this finding is in line with the observation that relatively high doses are needed to achieve responses in MDS. However these doses are hard to achieve in patients, mainly due to dose-limiting neurologic toxicity. Our observation might also be responsible for the conflicting *in vivo* measurements of TNF-α in patients treated with thalidomide that show decreased (Moutsopoulos et al, 2006) as well as increased levels (Jacobson et al, 1997).

With the emergence of new thalidomide-analogues such as lenalidomide that appear to be more powerful with regards to the number of responses, and which appear to cause fewer side effects (List et al, 2006, List et al, 2005, Raza et al, 2008) it is an open question if thalidomide will still be used in the treatment of MDS. It remains however to be explored if these new analogues will perform better than thalidomide in terms of blocking TNF-α production of CD40-stimulated MDS monocytes.

Conclusions

In this work we have further unraveled the pathogenesis of MDS-related marrow failure. In addition to the myelosuppressive properties of $CD8^+$ T cells, we have shown that also $CD4^+$ T cells can play a role in the inhibition of hematopoiesis, albeit indirectly through interaction with monocytes. It was also largely unknown which mechanisms activated monocytes to produce growth-inhibitory cytokines such as TNF-α. We have shown that CD40-CD40L interactions with $CD4^+$ T cells are likely to be responsible for this. Even more, our *in vitro* data support that inhibition of this particular pathway can improve colony growth. Furthermore, we have provided evidence that those patients with high surface expression of CD40 by

circulating monocytes, are those patients with a suspected immune-mediated disease. Thalidomide is used in patients with MDS and has a wide variety of effects. Inhibition of (increased) TNF-α production is believed to be an important mechanism in patients with MDS. However, we have shown in monocytes from patients that, in contrast to controls samples, very high concentrations of the drug are needed to observe *in vitro* inhibition of TNF-α production after stimulation of the CD40-receptor.

Chapter 10

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Summary

The myelodysplastic syndromes (MDS) constitute a heterogeneous group of clonal hematopoietic disorders which are characterized by peripheral cytopenias and an increased tendency to evolve towards acute myeloid leukemia. It has been shown that immune mechanisms can play a determinant role in the development of bone marrow (BM) failure and in the clinical shaping of the disease. The object of this project was to further clarify the role of lymphocytes and monocytes in the pathogenesis of marrow failure in MDS.

The first aim of this project was to quantify the number of mature immune cells in a large group of patients with MDS, since at the initiation of this project the literature data was still rather limited and showed conflicting results. We have therefore quantified the number of $CD4^+$ T cells, $CD8^+$ T cells, B cells and NK cells in peripheral blood (PB) of a large group of predominantly untreated patients with MDS (n=101) and compared these results with PB numbers in healthy age-matched controls. In our patient group, the number of $CD4^+$ and $CD8^+$ T cells was not different compared to controls. In contrast, we found that the number of B cells and NK cells were significantly lower in patients with MDS.

The second aim of our project was to determine clonal involvement of these immune cells in MDS. MDS is characterized by clonal hematopoiesis and current consensus is that, in a large majority of patients with MDS, the primary mutational event occurs in a myeloid-restricted progenitor cell. We analyzed purified cell fractions from MDS patients with the HUMARA assay (n=12) and with FISH (n=12). Monocytes were of clonal origin in the vast majority of patients, whereas $CD4^+$ and $CD8^+$ T cells were found to be of polyclonal origin. In our series we demonstrate clonal involvement of NK cells and B cells in about half of the patients. These results challenge the concept that MDS is restricted to myeloid lineages. From our limited data set we were unable to show that clonal involvement of a cell type resulted in overall lower cell counts of that cell type.

It was previously shown that clonal T cell expansions can be detected in selected patients with MDS. These T cells have been shown to suppress progenitor growth *in vitro* and these cells have an activated phenotype. The presence of these clonal T cell expansions could be indicative for those patients responding well to immunosuppressive therapies. Since T cell

receptor spectratyping is time-consuming, we investigated if immunophenotyping with flow cytometry could serve as an alternative method to identify this patient subgroup. We have therefore determined with flow cytometry the activation phenotype of T cells in a large group of patients (n=131), and correlated these results with clinical and disease characteristics. We confirmed that MDS patients have more activated T cells (i.e. HLA-DR^{+}, CD28^{-}, CD57^{+}, CD62L^{-}) in PB and BM. However using these activation parameters, we were unable to detect a relevant clinical subgroup with more T cell activation. Moreover, even in patients with the cytogenetic abnormality trisomy 8, which is believed to be related to immune-mediated marrow failure, an over-expression of these activation markers was not observed. The expression of these above-mentioned markers by T cells did also not relate to a suppressive activity on *in vitro* clonogenic growth of progenitor cells. Therefore, we believe that immunophenotyping with these above-mentioned markers is probably not a good surrogate marker to detect patients with immune-mediated disease.

Some patients with MDS have evidence of an established adaptive immune response causing global marrow failure. To date it is uncertain which factors are responsible for the suppression of residual normal hematopoietic progenitor cells. One possible explanation is that activated T cells work in concert with monocytes and with our work we have indeed provided evidence that CD40-CD40L interactions play an important role in this process. Firstly, we have shown with flow cytometry that monocytes in PB and BM of patients have a higher surface expression of CD40. Expression of the natural ligand of this co-stimulatory molecule (CD40L) on T helper cells was also increased in patients with MDS. Moreover, expression of both receptor and ligand was up-regulated in the same patients. Evidence for a role of this pathway was further substantiated by two other *in vitro* observations. Firstly, stimulation of purified monocytes with CD40-agonists lead to significantly higher production of tumor necrosis factor-alpha (TNF-α), a cytokine known to be myelosuppressive and important in MDS pathogenesis. Secondly, *in vitro* culture in the presence of an antagonistic anti-CD40 antibody significantly increased the clonogenicity of committed hematopoietic progenitor cells. This effect disappeared in the absence of monocytes, suggesting the pivotal role of monocytes in *in vitro* suppression of myelopoiesis.
The clinical relevance of our findings is further substantiated by the observation that high surface expression levels of CD40 by monocytes, could identify a subgroup with a high probability of responding to immunosuppressive therapy (i.e. age below 60y, trisomy 8). Moreover, as our *in vitro* culture data suggests, counteracting CD40-CD40L interactions *in vivo* could even be a new approach to target MDS-related cytopenia.

Thalidomide and its derivatives are used in the treatment of MDS. One of the proposed working mechanisms of these molecules is inhibition of TNF-α production by monocytes. Since we have shown that stimulation via the CD40 receptor plays a key role in the production of TNF-α in MDS, we compared the efficacy of thalidomide in inhibiting cytokine production in MDS monocytes compared to monocytes from healthy volunteers. We demonstrated that only in presence of high concentrations of the drug, TNF-α production of CD40-stimulated monocytes from MDS patients was significantly blocked, unlike in healthy controls. This observation can provide an explanation for the relatively high dosages that are needed to achieve a clinical response.

Samenvatting

De myelodysplastische syndromen (MDS) vormen een heterogene groep van klonale hematologische aandoeningen die gekenmerkt worden door perifere cytopenieën en een verhoogde intrinsieke kans om te evolueren naar acute myeloïde leukemie. Verworven afwijkingen in het genetisch materiaal van primitieve hematopoïetische stamcellen liggen aan de basis van MDS, maar de laatste jaren is duidelijk geworden dat ook immunologische processen een belangrijke rol spelen in het ontstaan van MDS-gerelateerd beenmergfalen. Het verder ontrafelen van de rol van lymfocyten en monocyten in beenmergfalen bij MDS maakte het voorwerp uit van dit doctoraatsonderzoek.

De meeste patiënten met MDS hebben cytopenieën en dit vormt de belangrijkste bron van morbiditeit en mortaliteit. Voornamelijk het aantal circulerende myeloïde bloedelementen (rode bloedcellen, neutrofielen, bloedplaatjes) is gedaald bij MDS. Er is minder eensgezindheid over het aantal lymfocyten en natural killer (NK) cellen bij patiënten met MDS. De gepubliceerde resultaten zijn immers niet éénduidend, grotendeels te wijten aan de relatief kleine patiëntenreeksen. Allereerst hebben we deze immuuncellen ($CD4^+$ en $CD8^+$ T cellen, B cellen en NK celllen) gekwantificeerd in het bloed van een grote groep (n=101) onbehandelde patiënten en de resultaten vergeleken met gezonde vrijwilligers van dezelfde leeftijd. We toonden aan dat patiënten vergelijkbare aantallen $CD4^+$ en $CD8^+$ T cellen hebben; de aantallen NK cellen en B cellen daarentegen waren significant lager in patiënten.

MDS wordt gekenmerkt door klonale hematopoiese en de huidige evidentie wijst aan dat de primaire maligne transformatie bij de meeste patiënten plaatsgrijpt in een primitieve maar myeloïde stamcel. Een tweede doelstelling van dit onderzoek was nagaan of bij patiënten met MDS de circulerende immuuncellen ook deel uitmaakten van de maligne kloon. Hiervoor onderzochten we gezuiverde celfracties uit het bloed van patiënten met de *HUMARA*-techniek (n=12) en fluorescente in situ hybridisatie (FISH, n=12). Monocyten die van myeloïde oorsprong zijn, waren zoals verwacht nagenoeg steeds van klonale origine. $CD4^+$ en $CD8^+$ T cellen waren daarentegen van polyklonale oorsprong. Opmerkelijk was de bevinding van klonale NK en B cellen in ongeveer de helft van de patiënten die we onderzochtten. Onze resultaten ondersteunen daarmee vroegere rapporten over klonale

afwijkingen in B cellen en NK cellen, en dagen het concept dat MDS louter de myeloïde stamcellen treft uit. Uit onze resultaten konden we echter niet besluiten dat wanneer een celpopulatie deel uitmaakte van de MDS-kloon, dit automatisch resulteerde in lagere bloedwaarden.

Er zijn aanwijzingen dat immuunprocessen mee verantwoordelijk zijn voor het beenmergfalen bij specifieke patiënten met MDS. Illustratief hiervoor is het feit dat immuunsuppressieve therapie bij een derde van patiënten een significante verbetering van de bloedwaarden teweegbrengt. Bij bepaalde patiënten met MDS kan men zgn. klonale T cell expansies detecteren, waarvan men heeft kunnen aantonen dat ze de groei van progenitorcellen afremmen *in vitro*. Bovendien brengen deze T cellen bepaalde activatie-merkers tot expressie op hun celoppervlak. Mogelijkerwijs zijn het deze patiënten, waarbij men deze groepjes T cellen kan detecteren, juist deze mensen die beantwoorden aan immuunsuppressieve therapie. Gezien de bepaling van het T cel receptor-repertoire zeer arbeidsintensief is, onderzochten we of er een groep patiënten kon gedefinieerd worden op basis van de expressie van activatiemerkers op circulerende T cellen. Daartoe bepaalden we het fenotype van T cellen in een grote groep patiënten met MDS (n=131) met flowcytometrie en correleerden onze bevindingen met klinische variabelen. MDS patiënten hebben significant meer geactiveerde T cellen (d.w.z. HLA-DR^{+}, CD28^{-}, CD57^{+}, CD62L^{-}) in vergelijking met gezonde leeftijdsgenoten, maar desondanks konden we geen subgroep onderkennen bij wie dit meer uitgesproken was. Bovendien vonden we geen verband tussen expressie van deze merkers door T cellen, en een groei-inhiberende activiteit van deze cellen *in vitro*.

Het is vooralsnog niet geweten hoe geactiveerde T cellen bij patiënten uiteindelijk leiden tot globaal beenmergfalen. We onderzochten de mogelijkheid dat monocyten betrokken zijn in dit proces. We hebben verschillende aanwijzingen dit monocyten, meer bepaald via CD40-CD40L-interacties met T cellen, de groei van progenitorcellen bij MDS kunnen onderdrukken. Van monocyten werd vermoed de belangrijkste bron van tumor necrosis factor-alpha (TNF-α) te zijn, waarvan geweten is dat het de groei van progenitorcellen onderdrukt en in patiënten met MDS ook meer geproduceerd wordt. We toonden aan dat MDS monocyten meer TNF-α produceren t.g.v. stimulatie van de CD40 receptor. Bovendien toonden we aan dat deze receptor meer tot expressie wordt gebracht door monocyten van MDS patiënten. Ook het ligand van deze receptor (CD40L), wordt significant meer geëxprimeerd door CD4^{+} T cellen in (dezelfde) patiënten. Meer nog, inhibitie van deze CD40-CD40L interactie *in vitro* leidde tot een significant hogere productie van hematopoietische kolonies. Het klinisch belang van onze bevinding wordt gestaafd door de vaststelling dat patiënten waarvan de circulerende

monocyten de hoogste CD40-expressie hebben, juist deze patiënten zijn die geacht worden het best te reageren op immuunsuppressieve therapie (nl. jonger dan 60j, cytogenetische afwijking trisomie 8). Anderzijds lijken onze bevindingen te suggereren dat inhibitie van CD40-CD40L-interacties tussen monocyten en T cellen een nieuwe therapeutische piste zou kunnen zijn om patiënten met beenmergfalen t.g.v. MDS te behandelen.

Tenslotte evalueerden de werkzaamheid van thalidomide op geïsoleerde monocyten van MDS patiënten. Thalidomide leidt immers bij een beperkte groep patiënten tot een verbetering van de anemie, en dit wordt o.a. toegeschreven aan het feit dat thalidomide de TNF-α productie door monocyten inhibeert. In het licht van onze bevinding dat activatie via de CD40-receptor een belangrijke rol speelt *in vivo*, stimuleerden we gezuiverde monocyten met CD40-agonisten in toenemende concentraties van thalidomide. Waar de TNF-α productie van gezonde monocyten in elke concentratie van thalidomide significant werd geïnhibeerd, waren zeer hoge concentraties van dit product nodig om de productie door MDS monocyten te onderdrukken. Deze bevinding kan een verklaring bieden waarom patiënten met MDS relatief hoge dosissen thalidomide nodig hebben voor een respons, die t.g.v. de nevenwerking van dit product vaak niet te bereiken zijn.

Professional career Stef MEERS

- Graduated Summa cum lauda in Medicine June 2001
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Laboratory Experience

- Human genetics (Prof. JJ Cassiman - 8/1996)
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