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Influence of the JAK2-V617F mutation on integrin-mediated adhesion to VCAM1 in murine and human cell lines in the context of classical Philadelphia-negative myeloproliferative neoplasms

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Abstract

JAK2-V617F is an activating point mutation of the intracellular tyrosine kinase JAK2 and the most prevalent mutation in patients suffering from Philadelphia-negative myeloproliferative neoplasm (MPN). Besides debilitating constitutional symptoms, myelofibrosis, and leukemic transformation, the MPN phenotype comprises extramedullary hematopoiesis and an increased risk for arterial and venous thrombosis. JAK2 has recently been shown to mediate chemokine-induced activation of integrin adhesion molecules thereby enabling increased cell adhesion and tissue-specific homing of leukocytes. By using an overexpression model of JAK2-V617F in murine BaF3 cells and JAK2-V617F-positive human HEL cells treated with JAK2-specific kinase inhibitors or shRNAs, we herein demonstrate that aberrant JAK2-V617F signaling is associated with increased integrin-mediated cell adhesion to Vascular Cell Adhesion Molecule 1 (VCAM1). In BaF3 cells, an increase in integrin affinity to VCAM1, but not regulation of β_1-integrin surface expression, seems to contribute to the mechanism underlying the adhesion phenotype. We argue that the observed enhancement of integrin-mediated cell adhesion in JAK2-V617F-positive cells might contribute to the clinical phenomena of thrombosis and extramedullary hematopoiesis occurring in MPN patients and, hence, deserve to be studied in more detail.

Key Words

Myeloproliferative neoplasm, JAK2-V617F, β_1-integrin, cell adhesion, VCAM1, thrombosis, extramedullary hematopoiesis

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Supplements

Abbreviations

The list below includes all abbreviations used in this dissertation. Abbreviated protein names which are commonly used instead of the respective full names (e.g. AKT) are not listed, unless they are of particular importance for this study (e.g. JAK). The proteins not listed include AKT, ASXL, CRKL, DNMT, ERK, FGR, GAPDH, HCK, IDH, IZH, MAPK, MIP, MLL-AF9, NOTCH, PI3K, PLC, PLD, RAP, RHO, SF3B1, SRC, SRSF2, STAT, TET, TGF, TYK, U2AF1, and VAV.

Protein names are generally spelled with all capital letters to avoid confusion (e.g. an antibody that binds murine and human isoforms of a particular protein or the name of a study that includes experiments with human and mouse material). If the name refers to the isoform of a particular species only, it is indicated in the text.

– R	– receptor
AML	Acute myeloid leukemia
APC	Allophycocyanin
APO-E	Apolipoprotein E
ASCT	Allogeneic stem cell transplantation
BCR-ABL	Breakpoint cluster region – Ableson murine viral oncogene homolog
BSA	Bovine serum albumin
CALR	Calreticulin
CD	Cluster of differentiation
CHIP	Clonal hematopoiesis of indeterminate potential
CXCL	CXC-motif chemokine ligand
CXCR	CXC-motif chemokine receptor
CML	Chronic myeloid leukemia
DFG	Deutsche Forschungsgemeinschaft (German Research Foundation)
(c)DNA	(complementary) Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DSMZ	Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures
DVT	Deep vein thrombosis
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EPO	Erythropoietin
ET	Essential thrombocythemia
FBS	Fetal bovine serum
FTS-A	Farnesylthiosalicylic acid-amide
G-CSF	Granulocyte – Colony stimulating factor
GPCR	G-protein-coupled receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
HSPC/HSC	Hematopoietic stem and progenitor cell / Hematopoietic stem cell
HU	Hydroxyurea
ICAM	Intercellular adhesion molecule

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
JAK	Janus kinase
LDL	Low-density lipoprotein
LFA	Lymphcyte function-associated antigen
LOH	Loss of heterozygosity
LT-HSC	Long term (repopulating) HSC
MAC	Macrophage antigen
MAdCAM	Mucosal vascular addressin cell adhesion molecule
MDS	Myelodysplastic syndrome
METS	Metabolic Equivalent of Task
MF	Myelofibrosis
MPL	Myeloproliferative leukemia virus (TPO-R)
MPN	Myeloproliferative neoplasms
MPP	Multipotent progenitors
MSCV	Murine stem cell virus
mTOR	Mammalian target of rapamycin
NET	Neutrophil extracellular trap
NFE	Nuclear factor erythroid
NK	Natural killer
P –	Phospho –
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PMF	Primary myelofibrosis
PSGL	P-selectin glycoprotein ligand
PV	Polycythemia
RCT	Randomized controlled trial
RNA	Ribonucleic acid
RNAi	RNA interference
SDS−PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
shRNA	small hairpin RNA
TBST	Tris-buffered saline/Tween 20
TNF	Tumor necrosis factor
TPO	Thrombopoietin
Tris	Tris(hydroxymethyl)aminomethane
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen
WHO	World health organization

c_i	Molar concentration
ϱ_i	Mass concentration
φ_i	Volume fraction

1. Introduction

This dissertation addresses the influence of JAK2-V617F – a constitutively active mutant of the intracellular tyrosine kinase JAK2 – on integrin-mediated leukocyte adhesion. JAK2-V617F is the most prevalent mutation of the hematopoietic system among patients with classical Philadelphia-negative myeloproliferative neoplasms (MPNs) and drives the disease in humans and mice. A JAK inhibitor is used to treat MPN patients. Integrins are transmembrane receptor proteins that mediate cell-cell and cell-matrix adhesion and play a key role in the immune system, particularly in leukocyte adhesion and extravasation.

We begin this introduction by providing a contemporary overview on MPNs as clinical entities and on the underlying pathophysiology. We then present our hypothesis that JAK2-V617F contributes to thrombosis in MPN patients by increasing integrin-mediated adhesion of leukocytes. We critically discuss this idea in the context of the existing evidence on the pathophysiology of thrombosis in MPNs. The chapter ends with the aims of this study.

1.1. MPNs and the underlying pathophysiology

1.1.1. MPN patients suffer constitutional symptoms, thrombosis, myelofibrosis, and secondary leukemia

The myeloproliferative neoplasms are clonal disorders of the hematopoietic system that are hallmarked by an excessive production of mature blood cells. Alongside Philadelphia chromosome-positive chronic myeloid leukemia (CML), Philadelphia-negative polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) constitute the main, i.e. the most frequent, diseases of this group. The latter three are often referred to as a distinct group of *classical MPNs* because they share several clinical and genetic characteristics (Rumi & Cazzola, 2017; Vainchenker & Kralovics, 2017). In this dissertation, the term *MPN* refers to those three entities only.

MPNs are rare cancers with an estimated incidence rate of 0.1 to 1 per 100,000 per year for myelofibrosis (MF)[1], 0.4 to 2.8 per 100,000 per year for PV, and 0.38 to 1.7 per 100,000 per year for ET in Europe (Moulard et al., 2014). The vast majority of cases occur in the age group of 65 years and older (Visser et al., 2012).

The clinical course of MPNs is characterized by a chronic phase of steady myeloproliferation and increased peripheral blood counts, and an advanced phase of progressive

[1] Includes all cases of myelofibrosis, irrespective of whether it was acquired primarily or secondarily to ET or PV.

myelofibrosis with concomitant cytopenias and risk for leukemic transformation. PV, ET, and PMF differ most importantly with respect to lineage-specific myeloproliferation and to the onset of fibrosis. The chronic phase of PV and ET is hallmarked by trilineage hyperplasia with leading erythrocytosis and isolated thrombocytosis, respectively. PMF can initially mimic ET by showing isolated thrombocytosis without major bone marrow fibrosis (Prefibrotic PMF). In sharp contrast to ET, PMF patients thereafter rapidly develop marked myelofibrosis with progressive anemia, splenomegaly through extramedullary hematopoiesis, and myelodysplasia (Overt PMF). Occasionally, entities blend into each other, e.g. ET patients can develop erythrocytosis, so-called masked PV, or cannot clearly be distinguished from each other, as in some cases of ET and prefibrotic PMF.

PV and ET patients typically remain in chronic phase for years during which they are strongly affected by a plethora of constitutional symptoms and vascular events. Symptoms include rather unspecific phenomena, fatigue in particular, but also bone pain, night sweats, headaches, depression and loss of sexual desire or function, as well as symptoms typically found in MPNs, like abdominal discomfort due to splenomegaly, or pruritus in PV. Although mostly unspecific, the symptoms have been shown to clearly have a higher prevalence in MPN patients and result in reduced overall quality of live compared to population controls (Anderson et al., 2015; Harrison et al., 2017). Underscoring the debilitating impact of those symptoms, MPN patients reported a leisure time activity corresponding to a mean energy consumption of 25.1 metabolic equivalents (METS), compared to 45.8 METS in the control group, being similar to scores from patients suffering Parkinson's disease (28.3 METS) (Mesa et al., 2007).

Vascular events comprise thrombosis of arteries, i.e. stroke and myocardial infarction, and of veins, including atypical forms like abdominal vein thrombosis[2], and have a complex and yet unresolved underlying pathophysiology. Less frequently, bleeding occurs in MPNs with risk factors being aspirin treatment, previous hemorrhage and diagnosis of PMF (Finazzi et al., 2012). Vascular events contribute significantly to symptom burden and, especially in young patients, to early death (Harrison et al., 2017; Hultcrantz et al., 2015).

Advanced phase MPN refers to severe complications that arise after years in chronic phase and comprise progressive myelofibrosis (Post-ET MF, Post-PV MF) and development of acute leukemia (Blast phase MPN). Since PMF has no or only a short chronic phase before the onset of fibrosis, it is understood as advanced phase MPN from the beginning. Alongside myelofibrosis, advanced phase MPN, including PMF, is characterized by an exacerbation of constitutional

[2] In a recent meta-analysis, about 41% of patients with liver vein thrombosis (Budd Chiari syndrome) and about 32% of patients with portal vein thrombosis suffered MPN at the same time (Smalberg et al., 2012).

symptoms, most importantly fatigue and splenomegaly due to extramedullary hematopoiesis, with signs of systemic inflammation. Blast phase MPN is usually refractory to standard therapy and, hence, has poor prognosis.

In all MPN patients, relative survival compared to population controls is diminished, being lower in PMF compared to PV, and in PV compared to ET (Hultcrantz et al., 2012; Maynadié et al., 2013; Price, Davis, Karve, Pohl, & Walgren, 2014).[3] The surplus mortality is attributable primarily to secondary development of acute leukemia and myelofibrosis. Bacterial infections and, in young patients, cardiovascular events contribute to a lesser extent (Hultcrantz et al., 2015). One recent large multicenter study estimated the median survival at 19.8 years in ET, 13.5 years in PV, and 5.9 years in PMF patients (Tefferi et al., 2014). The estimates pre-eminently reflected different incidences of leukemic transformation, namely 14.2% in PMF, compared to 6.8% and 3.8% in PV and ET, respectively.

The diagnosis of MPNs is based on peripheral blood counts, bone marrow histology, and the presence of recurrent mutations that drive clonal hematopoiesis according to the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia (Arber et al., 2016).

An overview on the clinical hallmarks of MPNs is given in *Table 1*.

[3] Although the population-based studies mentioned above consistently showed an impaired relative survival of ET patients, the influence of ET on long-term survival is currently under debate. First, some clinical studies found no difference in survival of ET patients compared to the general population (Cervantes, Passamonti, & Barosi, 2008). Second, recent revisions of the WHO diagnostic criteria for ET and the concomitant introduction of the new subentity "prefibrotic /early stage primary myelofibrosis" led to a reclassification of some high-risk ET patients into the PMF group. As a consequence, survival of the new "pure" ET population is speculated to resemble age- and sex-matched controls (Barbui, Thiele, et al., 2011; Hultcrantz et al., 2015).

Table 1. Clinical hallmarks of MPNs. Median survival is an estimate from (Tefferi et al., 2014). Therapeutic aims follow the suggestions of (Barbui, Barosi, et al., 2011). *MK-poiesis:* Megakaryopoiesis.

	PV	ET	Prefibrotic PMF	Overt PMF
Peripheral blood	Erythrocytes ↑↑ Platelets ↑ / - Granulocytes ↑ / -	Erythrocytes - Platelets ↑↑ Granulocytes -	Erythrocytes - / ↓ Platelets ↑↑ Granulocytes - / ↑	Erythrocytes - / ↓ Platelets ↑ / ↓ Granulocytes - / ↑↑ (potentially blasts)
Bone marrow	Trilineage growth	MK-poiesis ↑↑ Very rarely minor reticulin fibrosis	MK-poiesis ↑↑ with atypia Granulopoiesis ↑ Erythropoiesis - / ↓ Reticulin fibrosis ≤ grade 1	MK-poiesis ↑↑ with atypia Reticulin /collagen fibrosis grades 2-3
Clonal marker	JAK2-V617F or JAK2 exon 12	JAK2, CALR, or MPL	JAK2, CALR, or MPL	
Median survival	13.5 years	19.8 years	5.9 years	
Therapeutic aims	Reduce - symptom burden - vascular events	Reduce - symptom burden - vascular events	Reduce symptom burden Slow down progression / Prolong life	

1.1.2. The only approved targeted therapy for MPNs is ruxolitinib, an inhibitor of JAK1/2

The distinct clinical courses of MPN entities demand risk-adapted therapeutic strategies. In ET and PV, given the long median survival, the foremost therapeutic objective is the prevention of thromboembolic events. Furthermore, it is desirable to minimize the risk of leukemic transformation and secondary myelofibrosis. In PMF, the primary goal of therapy is prolongation of life, or, if unachievable, symptom-oriented palliation[4]. In all MPN patients, alleviation of symptom burden as a distinct therapeutic aim is becoming increasingly recognized (Scherber, Geyer, & Mesa, 2014).

Antithrombotic therapy involves low-dose aspirin and hematocrit control through venesection in PV, and low-dose aspirin alone in ET, combined with conventional cardio-vascular lifestyle interventions. In addition, high-risk patients (prior thrombosis, age over 60 years, or progressive myeloproliferation) are treated with cytoreductive agents like hydroxyurea (HU), interferon α (IFNα), or, in ET, with the platelet reducing drug anagrelide. Randomized controlled trials (RCTs) proofed the efficacy of hematocrit control, aspirin, as well as venesection and HU in PV. It merits attention that HU is usually not used in young patients due to its putative leukemogenic nature. Beyond these measures, antithrombotic therapy relies on assumptions rather

[4] Therapeutic aims follow the suggestions of the European Leukemia Net (Barbui, Barosi, et al., 2011).

than on solid data. For example, the usefulness of aspirin in ET has been inferred from successful trials in PV, but a recent retrospective observation raised concerns about an increased incidence of hemorrhage in a large subgroup of patients (Alvarez-Larrán et al., 2016). An effect of IFNα on vascular events has not been shown, but was deduced from its potential to reduce mutant clone size. However, a RCT is currently under way. Two large studies concerning the use of anagrelide (Birgegard et al., 2018; Harrison et al., 2005) in ET proved its efficacy in reducing venous thrombosis compared to HU, but also showed an association with an increased risk for arterial thrombosis, bleeding, and secondary myelofibrosis. Taken together, antithrombotic therapy in ET and PV currently remains unsatisfactory due to a limited armamentarium of unspecific agents and to incomplete assessment in high-quality studies[5].

In MF, cure can only be achieved by allogeneic stem cell transplantation (ASCT), which brings with it a considerable therapy-related mortality and is therefore unsuitable for the majority of patients (Barbui, Barosi, et al., 2011). Hence, management of PMF and advanced phase MPN in general is mostly restricted to the control of peripheral blood counts, splenomegaly, and constitutional symptoms. HU is the first-line drug for reducing excessive blood counts. The effect on symptom burden and progressive splenomegaly, however, is minimal, and not distinct from placebo (Mesa et al., 2014). Cytoreductive agents are also ineffective in preventing leukemic and myelofibrotic transformation. PV patients under HU treatment continued to progress to acute leukemia/myelodysplasia (cumulative incidence 6.6%, 16.5%, and 24% at 10, 15, and 20 years) and myelofibrosis (cumulative incidence 15%, 24%, and 32% at 10, 15, and 20 years) (Kiladjian, Chevret, Dosquet, Chomienne, & Rain, 2011). The insufficiency of MPN therapy is further emphasized by an internet-based survey of a large group of MPN patients that showed a majority, although being on therapy, was still significantly suffering from fatigue, and other disease-related symptoms (Mesa et al., 2007).

The elucidation of the genetic background of MPNs that started with the description of the mutant kinase JAK2-V617F in the majority of MPN patients in 2005 promised to be the beginning of a new era in the treatment of MPNs where kinase inhibitors specifically disrupt the aberrant signaling pathways underlying the disease. Despite significant progress in the understanding of MPN pathophysiology, ever since only one drug, the combined JAK1/2 inhibitor ruxolitinib, has been approved for MPN therapy. Most importantly, ruxolitinib prolonged overall survival, reduced spleen volume and symptom burden in intermediate-2 and high-risk MF patients (Verstovsek et al., 2017). However, inhibition of JAK2-V617F, the mutant kinase driving the disease, by ruxolitinib did only modestly decrease the clone size in MF and PV patients (Vannucchi

[5] For a detailed discussion of antithrombotic therapy in MPN, please refer to the reviews of (Barbui, Finazzi, & Falanga, 2013) and (Vannucchi & Harrison, 2017).

et al., 2017; Verstovsek et al., 2012), which is in marked contrast to Philadelphia-positive MPN (CML) where inhibition of the mutant BCR-ABL kinase diminishes clonal burden by several orders of magnitude (Baccarani & Soverini, 2014). In PV, ruxolitinib was more effective in controlling blood counts, spleen size, and symptoms as second line therapy after HU failure compared to standard therapy (Vannucchi et al., 2015). Interestingly, in this trial, ruxolitinib treatment was associated with a reduction in thrombotic events. However, since this had not been a preplanned endpoint, it has to be cautiously judged.

In summary, ruxolitinib is a valuable new instrument in the treatment of MF and PV and the first successful form of targeted therapy in MPNs. It has limited effect on clone size and is further restricted by adverse effects, including hematotoxicity and increased risk for infections. Nevertheless, it provides a solid partner for combination therapies with emerging agents. Currently, the efficacy of other JAK inhibitors as well as inhibitors of the PI3K/AKT/mTOR pathway, histone deacetylase, and telomerase are evaluated in clinical trials[6]. Ultimately, it will be vital to further deepen our knowledge about MPN pathophysiology in order pave the way for meaningful therapeutic innovation.

1.1.3. MPNs are driven by recurrent mutations that activate hematopoietic cytokine pathways

The last fifteen years have witnessed the elucidation of a hitherto unknown complex genetic landscape underlying MPN pathophysiology. The majority of MPN patients harbor a recurrent mutation in either one of the genes JAK2, myeloproliferative leukemia virus (MPL), and calreticulin (CALR). Because these mutations are mutually exclusive, mostly restricted to MPNs, and have been shown to initiate and promote the disease in mice, they are conceptualized as MPN phenotypic driver mutations (Nangalia & Green, 2017).

The most prevalent mutation JAK2-V617F occurs in about 90 % of PV, and 50 % of ET and PMF patients, respectively (Baxter et al., 2005; Horn et al., 2006). JAK2 is an intracellular tyrosine kinase that plays a pivotal role in the signal transduction of cytokine receptors, including the key cytokines of hematopoiesis erythropoietin (EPO), thrombopoietin (TPO), and granulocyte colony-stimulating-factor (G-CSF). The JAK family of kinases comprises JAK1, JAK2, JAK3, and TYK2. JAKs are closely associated with intracellular domains of cytokine receptors, which lack intrinsic catalytic activity, and are activated by auto- and transphosphorylation upon ligation of the respective receptor (Waters & Brooks, 2015). JAK activation leads to phosphorylation of associated cytokine receptors and the subsequent recruitment and activation of signaling molecules like signal

[6] Emerging treatments in MPNs are thoroughly discussed by (Bose & Verstovsek, 2017) and (Vannucchi & Harrison, 2017).

transducers and activators of transcription (STAT). By disrupting the auto-inhibitory function of the JAK2 pseudokinase domain, the JAK2-V617F mutation renders the kinase domain constitutively active (Gnanasambandan, Magis, & Sayeski, 2010; Saharinen, Vihinen, & Silvennoinen, 2003). As a result, JAK2-V617F mutated cells show permanent activation of JAK2 downstream targets including STAT5, PI3K, and MAPK/ERK, hypersensitivity to EPO, and cytokine-independent proliferation – a phenotype that is reversible upon treatment with a small molecular inhibitor of JAK2 (Dupont et al., 2007; James et al., 2005; Kralovics et al., 2005; Levine et al., 2005). Among the few JAK2-V617F negative PV patients, the vast majority harbors a functionally similar mutation in exon 12 of the JAK2 gene (Scott et al., 2007).

Similar signaling traits, cytokine-independent growth, hypersensitivity to TPO, and constitutive activation of MPL downstream targets JAK2, STAT3, STAT5, AKT, and MAPK/ERK, are conferred by an activating point mutation in the transmembrane domain of MPL (MPL-W515L), that encodes the TPO receptor (Pikman et al., 2006). MPL-W515L and few other MPL exon 10 mutations have been found in 4 to 6% of ET and PMF, but not in PV patients (Beer et al., 2008; Pardanani et al., 2006).

In most of the remaining ET and PMF patients without mutated JAK2 or MPL different mutations have been detected in exon 9 of CALR, that all resulted in a +1-base pair frame shift and the introduction of a surplus of positively charged amino acids in the mutant C-terminus (Klampfl et al., 2013; Nangalia et al., 2013). Being a protein best known as a chaperone of the endoplasmatic reticulum, CALR had not been described in the role of an oncogene before. However, the positive charge of the mutant C-terminus enables CALR an increased physical interaction with MPL that confers cytokine-independent growth through MPL activation and downstream signaling via JAK2, STAT5, PI3K, and MAPK/ERK (Chachoua et al., 2016; Elf et al., 2016).

Taken together, mutually exclusive, recurrent mutations in JAK2, MPL, and CALR are present in the majority of MPN patients. Their effects on intracellular signaling converge in the activation of downstream targets of the key hematopoietic cytokine receptors, most importantly JAK2, STATs, AKT/PI3K, and MAPK/ERK. This provides one possible explanation for the efficacy of JAK1/2 inhibitor ruxolitinib in the treatment of JAK2-V617F-negative PMF patients.

1.1.4. Differential downstream signaling of the driver mutations affect MPN phenotype

How exactly mutant JAK2, MPL, and CALR contribute to the development of distinct MPN phenotypes is incompletely understood. It has been speculated (Nangalia & Green, 2017), that

differential activation of the signaling pathways involved could, in part, account for MPN phenotypic heterogeneity.

Although, in the case of ET, patients with mutant CALR, MPL, and JAK2 have in common the defining isolated thrombocytosis, patient groups differ with respect to the age of clinical presentation and the risk of secondary myelofibrosis, i.e. CALR mutated patients are younger at diagnosis (Rumi et al., 2014) and acquire MF more often (Al Assaf et al., 2015)[7]. Furthermore, CALR patients with more extensive C-terminal alterations, putatively capable of a more intense MPL interaction, were preferentially found in PMF (Klampfl et al., 2013; Nangalia et al., 2013) and induced a more severe phenotype with myelofibrotic progression in a murine retroviral bone marrow transplant model (Marty et al., 2016). As possible explanation, Green and Nangalia suggested that higher levels of MPL signaling could lead to a more severe phenotype with early onset and (early progression to) myelofibrosis. This is in accordance with (Villeval et al., 1997), who showed induction of MF by excessive MPL signaling in mice[8], and with (Rumi et al., 2013), who found more bone marrow reticulin in patients with homozygous MPL mutations and high allele burden, compared to MPL heterozygosity and low allele burden.

Along this line, in PV, patients with JAK2 exon 12 mutations are younger and have higher erythrocyte counts than JAK2-V617F mutated patients (Scott et al., 2007)[9]. Furthermore, homozygous knockin of JAK2-V617F led to a PV phenotype in mice, while heterozygous JAK2-V617F induced a phenotype resembling ET (Li et al., 2014)[10]. Since JAK2-K539I, an exon 12 mutant, showed stronger interaction with the EPO receptor, in contrast to JAK2-V617F, which in turn was found to complex with MPL more intensively than JAK2-K539I (Yao et al., 2017), it is plausible that the level of erythrocytosis in PV is dependent on the mutation specific intensity of EPO receptor signaling.

In conclusion, Green and Nangalia proposed a phenotypic continuum of MPNs in which varying degrees of erythrocytosis or thrombocytosis are conferred depending on the intensity by which the phenotypic driver mutations signal through MPL or the EPO receptor.

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7 No increased risk for myelofibrosis was found in the retrospective studies of (Rumi et al., 2014) and (Rotunno et al., 2014). A possible explanation is the rare occurrence of secondary MF in ET. Hence, prospective reassessment is desirable.

8 The group transplanted lethally irradiated mice with bone marrow cells that had been retrovirally transduced with TPO cDNA.

9 In a larger study of (Passamonti et al., 2011) no difference could be detected in the age of disease onset. However, even this study only included about 100 patients.

10 Similar phenotypic differences were found by (Tiedt et al., 2008) in knockin mice with different ratios of JAK2-V617F to JAK2-WT copy numbers.

1.1.5. Phenotypic driver mutations are acquired at the level of hematopoietic stem cells

The term phenotypic driver mutation implies that CALR, MPL, and JAK2 mutations alone are capable of initiating and promoting clinical MPN – and indeed, this notion is in line with observations from different mouse models (bone marrow transplantation, or transgenic) where typical mutations in either gene induced fully penetrant MPN phenotypes. (Mullally, Lane, Brumme, & Ebert, 2012) provide an in-depth review of MPN mouse models before the discovery of CALR mutations, including JAK2-V617F and mutant MPL. An overview on JAK2-V617F and CALR mouse models is given in (Vainchenker & Kralovics, 2017). It merits attention however, that hematopoiesis in these mouse models was polyclonal, i.e. these models do not reflect the acquisition of a mutation in a single hematopoietic stem cell (HSC) and the monoclonal nature of human MPNs. Nevertheless, several findings suggest that driver mutations initiate the disease from HSCs.

First, the JAK2-V617F mutation was found in immunophenotypically defined HSCs of PV patients by (Jamieson et al., 2006). Second, (Delhommeau et al., 2007) showed that a fraction of JAK2-V617F positive cells residing within the $CD34^+$ compartment of PV and PMF patients were able to differentiate to T-, and B-lymphocytes, and to NK-cells, in addition to the myeloid nature of the disease[11]. Third, LT-HSCs, but not a short-term (ST) HSC/multipotent progenitor (MPP) mixed population, with conditional heterozygous knockin of JAK2-V617F were able to initiate a MPN phenotype in a murine bone marrow transplant model (Mullally, Poveromo, et al., 2012)[12].

Nevertheless, to date only one study investigated the transplantation of single mutant HSCs using limiting dilution and single cell transplantation assays (Lundberg et al., 2014). In this study, single immunophenotypically defined LT-HSCs carrying the JAK2-V617F mutation that were able to reconstitute hematopoiesis in lethally irradiated mice induced an MPN phenotype only in a subset of recipients. Together with the discovery of JAK2-V617F to be among the most frequent mutations occurring in the context of clonal hematopoiesis of indeterminate potential (CHIP)[13] (Genovese et al., 2014; Jaiswal et al., 2014; Xie et al., 2014), these results indicate that the acquisition of JAK2-V617F, and potentially further phenotypic driver mutations, in a HSC alone might be

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11 (Chaligné et al., 2012) found MPL-W515 mutations in myeloid, NK- and B-cells, but not in T-cells in 2 of 3 PMF patients. (Nangalia et al., 2013) detected mutant CALR in immunophenotypically defined HSCs of MPN patients, and in the earliest phylogenetic node of erythroid colonies derived from ET patients.

12 That is in contrast to more potent oncogenes like MLL-AF9, that is able to transform committed progenitors to leukemic stem cells (Krivtsov et al., 2006), and in line with BCR-ABL, that is not capable of such transformation (Huntly et al., 2004).

13 That is clonal hematopoiesis in individuals without overt hematological disease, a process strongly associated with increasing age.

capable of initiating clonal hematopoiesis, but insufficient to induce clinical MPN. For a detailed discussion of this matter and of MPN stem cells please refer to (Mead & Mullally, 2017).

1.1.6. Additional cell-intrinsic and environmental factors influence MPN phenotype and severity

Having said that phenotypic driver mutations engender different MPN phenotypes from HSCs, but that, at least in the case of JAK2-V617F, acquisition of the mutation alone is probably insufficient, other factors must exist that enable promotion of systemic disease beyond clonal outgrowth and influence the development of a particular phenotype.

Several cell-intrinsic factors influence the disease-initiating cell and its progeny. First, as already discussed above, some mutations favor particular phenotypes. For example, CALR and MPL mutations are not found in PV, and CALR mutants with more extensive C-terminal alterations are associated with a more severe phenotype. It remains an unresolved question, however, why JAK2-V617F is found across all MPN entities. Comparing JAK2-V617F heterozygous erythroid colonies from ET and PV patients, (Chen et al., 2010) showed differential activation of STAT1 and interferon signaling that was essential for the differences in erythroid and megakaryocytic differentiation. The underlying cause was speculated to be additional genetic or epigenetic alterations.

Second, whether a driver mutation is homozygous or heterozygous influences the resulting phenotype. JAK2-V617F homozygosity has been shown to skew hematopoiesis towards erythroid differentiation and to induce PV in mice as compared to heterozygosity, that favored megakaryocytic differentiation and an ET phenotype (Li et al., 2014). Interestingly, JAK2-V617F copy-neutral loss of heterozygosity (LOH) by mitotic recombination occurs frequently in ET and PV patients, but only in PV the homozygous clone becomes dominant (Godfrey et al., 2012). This may reflect the need for additional genetic or epigenetic lesions, or for a different environment for the homozygous clone to acquire clonal dominance[14].

Third, indeed, several additional recurrent mutations have been reported in MPNs. The vast majority of these mutations occur in epigenetic regulator genes (TET2, DNMT3A, ASXL1, EZH2, IDH1/2), and genes of the splicing apparatus (U2AF1, SF3B1, SRSF2), and are known to occur in other myeloid malignancies as well, especially in myelodysplastic syndromes (MDS) (Nangalia et al., 2013). In their review on MPN genetics, (Vainchenker & Kralovics, 2017) proposed a genetic continuum of MPN, MPN/MDS, and MDS. According to their model, in

[14] For example, additional effects might be required to overcome the stem cell defect associated with JAK2-V617F homozygosity (Li et al., 2014).

MPNs, the acquisition of mutations of the MDS spectrum in addition to the MPN phenotypic driver mutation can initially foster clonal expansion, but ultimately result in dysplastic hematopoiesis and increased risk for acute leukemia, depending on the load and the type of MDS mutations with splicing mutations being more severe. This is in line with the observations that PMF patients bear higher mutational burden compared to ET and PV patients (Nangalia et al., 2013) and that some mutations like ASXL1, SRSF2, and EZH2 are associated with poor survival in PMF (Vannucchi et al., 2013). Mechanistically, non-driver mutations are thought to either increase the mutation rate in the cell affected, or to cooperate with phenotypic driver mutations in promoting clonal expansion and disease progression[15]. Conversely, JAK2-V617F has been shown to interfere with DNA damage-activated apoptotic pathways (Zhao et al., 2008) thereby on its own favoring the acquisition of further mutations (Plo et al., 2008).

Fourth, since lineage bias has been detected in HSCs, it is tempting to speculate as to whether the acquisition of a driver mutation in a platelet-biased HSC is more likely to result in an ET phenotype, and PV is more likely to arise from a mutant myeloid-biased HSC[16].

In addition to cell-intrinsic factors, mutant cells underlie the influence of the surrounding microenvironment. Underlining the importance of the bone marrow microenvironment for MPN development, an aberrant bone marrow niche alone – depleted of retinoic acid receptor γ (Walkley et al., 2007), or with defective NOTCH activation (Y. W. Kim et al., 2008) – was sufficient to induce the disease in mice. In the context of clinical MPN, mutant HSCs are thought to remodel the surrounding niche into a self-reinforcing neoplastic niche that suppresses normal HSCs and promotes clonal expansion of mutant HSCs, myelofibrosis, and leukemic transformation. This concept is based on comprehensive studies in Philadelphia-positive MPN (Schepers et al., 2013), as well as in JAK2-V617F-driven classical Philadelphia-negative MPN (Arranz et al., 2014; Mager et al., 2015; Triviai et al., 2014). Presumably, the niche is also interfering with the treatment of MPN patients. This was indicated by the study of (Manshouri et al., 2011) that showed *in vitro* alleviation of the anti-proliferative effect of pharmacological JAK inhibition on JAK2-V617F positive cells by cytokine secretion of cocultured bone marrow stroma cells.

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15 For example, loss of function in TET2 worsened the MPN phenotype in JAK2-V617F positive hematopoiesis in a murine bone marrow transplant model (Kameda et al., 2015). Furthermore, it compensated for the impaired HSC function of JAK2-V617F positive cells in a serial competitive bone marrow transplantation assay. Interestingly, (Ortmann et al., 2015) showed striking differences in proliferative capacities, transcriptional profiles, and phenotype development in TET2 JAK2-V617F double-mutant MPN patients depending on the order in which mutations were acquired.

16 Please refer to (Sanjuan-Pla et al., 2013) for platelet-biased, and to (Yamamoto et al., 2013) for myeloid-biased HSCs. Current concepts of HSC biology are reviewed by (Eaves, 2015).

1.1.7. Systemic inflammation contributes to MPN pathophysiology

Intriguingly, the aforementioned studies showed the critical involvement of cytokines in the interplay between mutant HSCs and bone marrow niche. This raises the question as to whether the influence of cytokines from the remodeled MPN bone marrow niche extends beyond the bone marrow and, vice-versa, whether MPNs show systemic deregulation of cytokines that affects the hematopoietic microenvironment. Indeed, several observations support the idea that systemic deregulation of cytokines, i.e. systemic inflammation, significantly contributes to promotion and progression of MPNs.

First, several serum cytokines have been shown to be elevated in MPN patients with some being associated with poor outcome, e.g. IL-8, IL-2-R, IL-12, IL-15 in PMF, or MIP-1β in PV (Pourcelot et al., 2014; Tefferi et al., 2011; Vaidya et al., 2012). These studies, as well as (Tabarroki et al., 2012), also showed distinct cytokine profiles of the different MPN phenotypes.

Second, for certain cytokines mechanistic involvement in MPN pathophysiology has been suggested, among others for IL-8 and TNFα. High levels of IL-8 have been shown to contribute to dysregulation of megakaryopoiesis. In the study of (Emadi et al., 2005), disruption of IL-8 signaling by neutralizing antibodies or RNA interference restored normal proliferation and differentiation of PMF megakaryocytes. Furthermore, being an important target gene of transcription factor nuclear factor erythroid-2 (NFE2), IL-8 is likely to contribute to its disease initiating and promoting effects (Wehrle et al., 2013). NFE2 is thought to play an important role in MPN pathogenesis because MPN patients express increased levels of NFE2 (Goerttler et al., 2005; W. Wang, Schwemmers, Hexner, & Pahl, 2010), some harbor recurrent truncating NFE2 mutations that result in enhanced function of wild-type NFE2 (Jutzi et al., 2013), and because NFE2 overexpression in transgenic mice engendered a myeloproliferative phenotype mimicking many features of clinical MPN including leukemic transformation (Kaufmann et al., 2012). TNFα was shown to be a mediator of how JAK2-V617F cells obtain clonal dominance (Fleischman et al., 2011). In this study, the JAK2-V617F mutation controlled increased TNFα production in primary MPN cells. High levels of TNFα hampered colony formation of hematopoietic progenitor cells from healthy controls, but not from JAK2-V617F-positive MPN patients. Absence of TNFα limited clonal expansion and mitigated disease phenotype in a murine JAK2-V617F-driven MPN bone marrow transplant model.

Third, several clinical phenomena of MPNs have been linked to deregulated cytokines. Several studies imply that myelofibrosis is essentially mediated by TGFβ produced by immature megakaryocytes (Chagraoui et al., 2002; Ciurea et al., 2007). Major thrombotic events in ET and PV patients were associated with the highest levels of C-reactive protein, an acute-phase reactant and established risk factor for atherosclerosis (Barbui, Carobbio, et al., 2011). Most importantly,

several studies imply that systemic release of certain cytokines contribute significantly to the debilitating constitutional symptoms of MPN patients – a concept that is discussed for many other cancers as well (Seruga, Zhang, Bernstein, & Tannock, 2008). For example, the aforementioned IL-8 was associated with the presence of constitutional symptoms in the study of (Tefferi et al., 2011) thereby further supporting the idea of an uniquely important role for IL-8 within MPNs. Furthermore, some argue (Hasselbalch, 2012) that the effective reduction of spleen size and symptom burden in MF and PV patients by ruxolitinib has at least in part to be attributed to an anti-inflammatory effect, which is well recognized for JAK inhibitors in several clinical settings (Schwartz et al., 2017). This is based on the observation that ruxolitinib treatment resulted only in a modest reduction of tumor burden but a pronounced reduction in certain cytokine levels (Verstovsek et al., 2010, 2012) that correlated with improvement in a MF-specific symptom score (Dueck et al., 2013). The impact of inflammatory processes on MPN symptom development is reviewed in more detail by (Geyer, Dueck, Scherber, & Mesa, 2015).

Fourth, in an epidemiological study from Sweden, the occurrence of MPNs was associated with prior history of autoimmune diseases (Kristinsson, Landgren, Samuelsson, Björkholm, & Goldin, 2010). This suggests that systemic inflammation cannot only be elicited by the mutant MPN clone, as seen for example in the release of TNFα by JAK2-V617F mutant cells, but that it may precede clinical MPN and contribute to its onset for example by fostering a mutant clone that on its own is not capable of obtaining clonal dominance. This concept is further supported by a study that found a similar association of chronic immune stimulation with another clonal malignancy, namely MDS / acute myeloid leukemia (AML) (Kristinsson et al., 2011).

All pathogenic factors discussed above that contribute to the development of different MPN phenotypes are summarized in *Figure 1*.

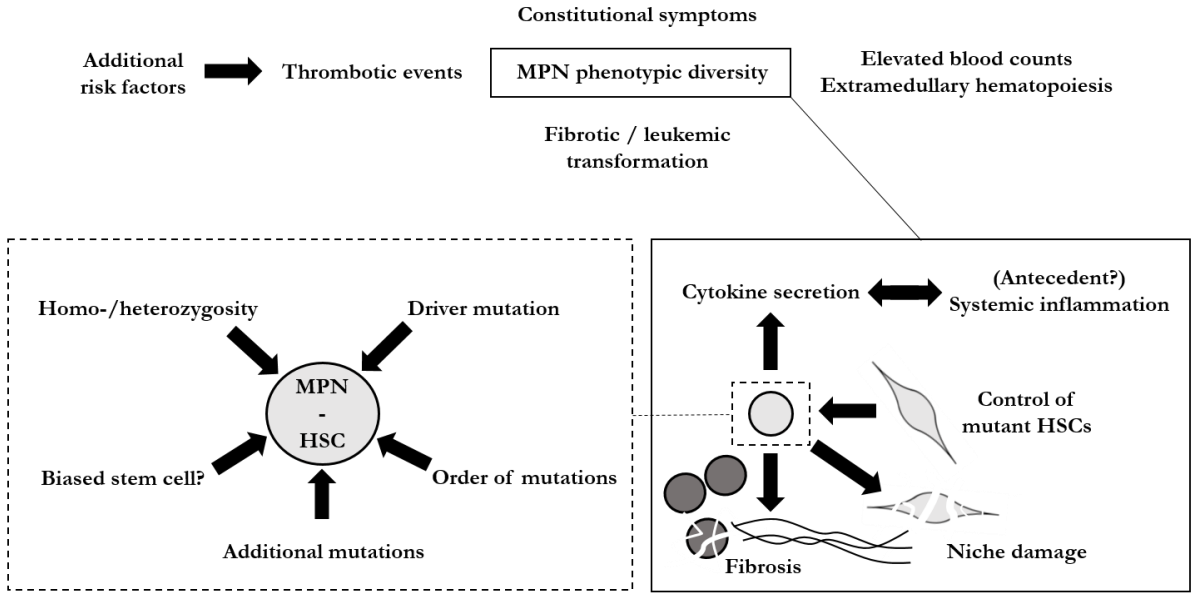

Figure 1. Pathogenesis of MPNs. This overview summarizes factors that contribute to MPN development and heterogeneity of clinical presentation on the level of the mutant HSC (MPN-HSC) driving the disease (*lower left panel*) and on tissue level of MPN bone marrow (*lower right panel*). Mutant HSCs are differentially affected by the type of MPN driver mutation (JAK2, CALR, MPL and subtype), driver mutation homo- or heterozygosity, acquisition of additional mutations, and order of acquisition. Whether the acquisition of a driver mutation in a lineage-biased HSC can skew the resulting myelopoiesis remains to be demonstrated. On tissue level, the mutant clone is evading the control of the surrounding bone marrow niche, particularly mesenchymal stem cells (spindle-shaped cells), by secreting niche-damaging cytokines (Arranz et al., 2014). Primary or secondary myelofibrosis impairs physiological hematopoiesis (dark cells). It remains to be further elucidated, how antecedent systemic inflammation (e.g. in autoimmune diseases) could promote MPN development. Myeloproliferation and systemic elevation of cytokines are reflected in a plethora of clinical consequences ranging from fatigue (constitutional symptoms), elevated or reduced blood counts (myeloproliferation or fibrosis), and splenomegaly (extramedullary hematopoiesis) to cardiovascular events. The pathogenesis of the latter is particularly poor understood. It is likely that individual risk factors like age, gender, and comorbidities impact disease presentation. Please refer to the text for a detailed description of MPN pathogenesis and respective literature references.

1.2. Hypothesis

Herein, we hypothesize that constitutive activation of JAK2 by the V617F mutation leads to activation of leukocyte integrins. We believe, that this results in increased cell adhesion and could play a role in vascular occlusion and aberrant homing of hematopoietic progenitors to the spleen and other extramedullary locations – major causes of disease-related morbidity and mortality in MPN patients.

1.2.1. Leukocytosis is associated with increased risk for vascular occlusion in MPNs

MPN patients have a high risk for thrombosis, but also for hemorrhage – a paradox that remains poorly understood. In the past, much attention has been payed to increased blood counts, the clinical hallmark of MPNs, and their relationship to vascular events, specifically based on the idea that hyperviscous blood could be prone to coagulation. Indeed, in PV, reduction of hematocrit below 45% was associated with a marked decrease in the rate of cardiovascular death and major thrombosis (Marchioli et al., 2013). Nevertheless, it remains questionable how much erythrocytosis and hyperviscosity contributed to the vascular phenotype in this study since patients with high hematocrit were less frequently treated with HU and hence also exhibited more pronounced leukocytosis (Barbui et al., 2015). Moreover, mice with EPO-induced erythrocytosis did not show defective hemostasis as compared to erythrocytosis in a JAK2-V617F-driven PV mouse model (Lamrani et al., 2014).

In contrast, high platelet counts were associated with increased bleeding, but not thrombosis, in ET patients during the prospective PT-1 trial (P. J. Campbell et al., 2012). The cohort study of (Carobbio et al., 2008) even found a lower incidence of thrombosis in ET patients with high platelet counts (>1000 x 10^9/L), compared to a group with modestly elevated platelets (650-1000 x 10^9/L). Concerning the underlying cause, different factors likely act together including an acquired von Willebrand syndrome in patients with extreme thrombocytosis (Budde et al., 1984, 1993), production of defective platelets (see below), and aspirin treatment (Finazzi et al., 2012).

Strikingly, leukocytosis during follow-up correlated with thrombosis and major hemorrhage in the PT-1 trial (P. J. Campbell et al., 2012). Accordingly, in the study of (Carobbio et al., 2008), the group with the highest risk for thrombosis comprised patients with low platelet counts, leukocytosis and JAK2-V617F positivity. In PV, leukocytosis also correlated with thrombotic risk (Barbui et al., 2015; Landolfi et al., 2007; Marchioli et al., 2013). Taken together, this data strongly implicates leukocytosis as major factor in the pathogenesis of thrombosis in MPNs.

1.2.2. The effect of JAK2-V617F on coagulation has been studied in platelets, but not in leukocytes

Considering that high allele burden of JAK2-V617F was accompanied by an increased risk for experiencing major cardiovascular events in PV (Vannucchi et al., 2007) and that JAK2 mutated ET and PV patients had comparable thrombotic risk being twice that of CALR mutated patients (Rumi et al., 2014), it is reasonable to ask as to whether JAK2-V617F in particular induces certain procoagulant features in mutant leukocytes.

In the past, however, most attention has been payed to the role of JAK2-V617F mutant platelets, yielding mixed results. Platelet reactivity to several agonists was enhanced in JAK2-V617F-positive ET patients (Arellano-Rodrigo et al., 2006) and in platelets from conditional JAK2-V617F-positive mice, that also showed decreased tail bleeding volume (Hobbs et al., 2013). In contrast, (Lamrani et al., 2014) observed prolonged tail bleeding time and instable thrombus formation *in vitro* and *in vivo* in two different JAK2-V617F knockin models concomitant with a decreased proportion of high molecular weight von Willebrand factor multimers. Interestingly, it took two to eight weeks to develop this phenotype after tamoxifen-mediated induction of JAK2-V617F suggesting that the hemostatic dysfunction might not have been a direct consequence of the mutation but rather be attributed to systemic processes in MPNs.

(Etheridge et al., 2014) showed that JAK2-V617F-positive endothelial cells and not mutant platelets were essential for severely attenuated thrombosis after injury observed in their MPN mouse model. Since JAK2-V617F has been detected in liver and spleen endothelial cells, as well as in circulating endothelial progenitor cells in MPN patients (Rosti et al., 2013; Sozer et al., 2009; Teofili et al., 2011), ongoing investigations of the role of mutant endothelial cells in aberrant hemostasis deserve further attention.

Taken together, these studies show the difficulties of consistently delineating the effects of JAK2-V617F on platelets in the context of systemic MPN. Nevertheless, given the *in vivo* analyses, they rather support the idea that mutant platelets in MPNs are primarily associated with defective hemostasis and bleeding, rather than thrombosis, which is in line with the clinical observations mentioned above[17].

1.2.3. The role of leukocytes in arterial and venous thrombosis

Beyond MPNs, the involvement of leukocytes in both, arterial and venous thrombosis, increasingly attracts scientific attention. This development is based on the clinical association of high leukocyte counts with cardiovascular events (Coller, 2005; Swystun & Liaw, 2016) and driven by an increased understanding of leukocyte subsets (Weber, Zernecke, & Libby, 2008) and the improving *in vivo* imaging techniques. Of course, thrombosis is not a single process that either occurs in an artery or a vein, but it comprises several situations of coagulation being induced by different stimuli in different environments. Arterial thrombosis is often induced by erosion or rupture of an atherosclerotic plaque in a coronary or cerebral artery, whereas venous thrombosis is commonly

[17] Nevertheless, impaired hemostasis and thrombosis might even arise from the same source. This is indicated by the mouse model of (Lamrani et al., 2014) where emboli, accelerated thrombus lysis, and instable thrombi were found at the same time.

found in association with patient immobilization and occurs in the deep veins of the lower extremities and the pelvis. Mouse models indicate that leukocytes play important roles in both scenarios.

On the one hand, it is known for some time that, in early atherosclerosis, circulating monocytes are recruited to activated endothelium of the arterial intima where they ingest lipids and become foam cells thereby forming fatty streaks, i.e. early atherosclerotic plaques (Hansson, 2005). Furthermore, it has been shown in mice that myocardial infarction is followed by an exacerbation of atherosclerosis which is mediated by recruitment of neutrophils and inflammatory monocytes to atheromata (Dutta et al., 2012). When spleens of these mice, the source of excessive monocytes in plaques, were removed, atherosclerosis did not accelerate following myocardial infarction. Similarly, leukocytosis induced by disruption of CXCR4 function in mice was associated with expansion of atherosclerotic plaques, whereas depletion of neutrophils, which were found to accumulate in expanding plaques, by specific antibodies prevented this exacerbation (Zernecke et al., 2008). Apart from promoting formation and expansion of atherosclerotic plaques in the animal models described, neutrophils have been detected in eroded or ruptured lesions of patients with acute coronary syndrome (Naruko et al., 2002). In this case, however, it remains unclear as to whether neutrophil infiltration is cause or consequence of late stage plaque progression. On the other hand, in a physiological murine model of deep vein thrombosis (DVT), monocytes and neutrophils have been shown to be recruited first to endothelium activated by restricted blood flow, to outnumber the platelets involved, and to provide the initiating stimulus for thrombus formation (von Brühl et al., 2012). Moreover, the study showed that neutrophils were indispensable for thrombus propagation since they bound factor XII and fostered its activation by neutrophil extracellular trap (NET) formation. Taken together, high-quality mouse studies showed that leukocytes are indispensable not only for initiation and propagation of DVT, but also for formation and expansion of atherosclerotic plaques.

Considering that arterial and venous thrombosis show considerable differences, it remains elusive why MPN patients are prone to both. That leukocytes are crucially involved in either process points towards a fundamental similarity that, hence, could be of particular importance in MPNs: leukocytes have to adhere to vascular endothelium. Whether leukocytes home to atherosclerotic lesions of the arterial intima or roll on and crawl along venous endothelium (von Brühl et al., 2012) they must establish physical contact to endothelial cells firm enough to withstand the shear forces of blood flow. The direct interaction of leukocytes with endothelial cells occurring in leukocyte extravasation has been intensively studied and will be approached further below. It merits attention, that leukocytes can also be recruited to activated endothelium by bridging

platelets[18], although, to our knowledge, the role of this interaction in health and disease has not been delineated.

In summary, leukocytes play a pivotal role in the development of DVT and atherosclerosis, a prerequisite for the most common form of arterial thrombosis. The cell-cell adhesion of leukocytes to vascular endothelium, or to platelets, is a mechanistic key point in both processes. If deregulated, it could thus contribute to the increased occurrence of arterial and venous thrombosis in MPN patients.

1.2.4. Integrins mediate leukocyte adhesion and are regulated by JAK2

Leukocytes exit the blood stream by a complex interaction with vascular endothelium that is well-known as the leukocyte adhesion cascade and, briefly, comprises slow rolling, adhesion strengthening, intraluminal crawling, paracellular and transcellular migration, and migration through the basement membrane (Ley, Laudanna, Cybulsky, & Nourshargh, 2007; Vestweber, 2015). For the first encounter, subsequent slow rolling, and adhesion strengthening two proteins are of paramount importance, namely selectins and integrins. When a blood-borne leukocyte flows past activated vascular endothelium, it is captured by bonds between endothelial selectins and their leukocyte ligands, pre-eminently P-selectin glycoprotein ligand-1 (PSGL1), causing it to roll over the intima[19]. During this rolling, leukocyte integrins bind their endothelial ligands. Finally resulting in leukocyte arrest, the integrin-ligand bond is then gradually strengthened by conformational changes of the integrin that increase affinity of the individual protein (activation) on the one hand, and by redistribution and clustering of integrins in the plasma membrane (valency) on the other. Integrin activation is mainly driven by chemokine-stimulated G-protein-coupled receptor (GPC-R) signaling (inside-out signaling), as well as by integrin ligand-induced conformational changes (outside-in signaling) (Alon & Feigelson, 2012).

Independently from GPC-R signaling, JAK2 has recently been described to mediate chemokine-induced integrin activation and downstream functional consequences including adhesion and homing in human T-lymphocytes (see below). Thus, the constitutively active mutant JAK2-V617F of MPN patients could foster integrin-mediated leukocyte adhesion to vascular endothelium.

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[18] This notion is based on *in vitro* studies showing the recruitment of flowing leukocytes to surface-immobilized, activated platelets (Buttrum, Hatton, & Nash, 1993), to platelets adhering to endothelial cells (Kuijper et al., 1996), or to surface-immobilized platelets induced by turbulent blood flow (Skilbeck, Walker, David, & Nash, 2004). Furthermore, it was shown that neutrophils migrating on a platelet surface under flow formed embolising aggregates (Rainger, Buckley, Simmons, & Nash, 1998).

[19] Some leukocytes also express certain selectin subtypes. This is of particular importance when lymphocytes exit through high endothelial venules of lymph nodes (Alon & Feigelson, 2012).

Integrins are transmembrane receptor proteins that most importantly mediate cell-cell and cell-matrix adhesion, but are, moreover, critically involved in signaling pathways of cell survival, proliferation, differentiation and organ development. In line with their diverse roles, integrins comprise 24 different receptors assembled as heterodimers from 18 α-subunits and 8 β-subunits. In leukocytes, β_1-integrin and β_2-integrin are predominantly expressed forming VLA4 (very late antigen 4 ($\alpha_4\beta_1$)), and LFA1, MAC1 (lymphocyte function-associated antigen 1 ($\alpha_L\beta_2$), macrophage antigen 1 ($\alpha_M\beta_2$)), respectively, and others. Most importantly, those integrins are critically involved in several steps of leukocyte extravasation including slow rolling and induction of firm arrest on the endothelium following selectin-mediated capture. During these processes, integrins pass through at least three different conformations with distinct affinities – low, intermediate, and high – for their endothelial ligands ICAM1 (ligand to LFA1) and VCAM1 (ligand to VLA4) (Alon & Feigelson, 2012; I. D. Campbell & Humphries, 2011; Vestweber, 2015).

Several studies indicate that JAK2 might be involved in integrin activation in MPN leukocytes. (Gotoh, Ritchie, Takahira, & Broxmeyer, 1997) stimulated hematopoietic cell lines with EPO or TPO and observed a rapid increase in adhesion to fibronectin, a main component of the extracellular matrix of the bone marrow. This adhesion was not associated with a change in β_1-integrin expression, but could be abrogated by antibodies targeting β_1-integrin, and was dependent on PI3K activation. This is in accordance with the study of (Arai, Nosaka, Kohsaka, Miyasaka, & Miura, 1999), that also showed β_1-integrin activation downstream of CRKL, an adaptor protein of EPO-R and IL-3 receptor (IL-3-R). Since JAK2 is the central mediator of intracellular EPO-R, MPL, and IL-3-R signaling involving PI3K activation, constitutive activation of JAK2 by the V617F mutation could possibly result in a similar adhesion phenotype dependent on β_1-integrin.

In human T-lymphocytes, JAK2, together with JAK1 and JAK3, has been established as a crucial mediator of chemokine-induced integrin activation, adhesion, and homing to secondary lymphoid organs upstream of RHO and RAP modules (Mirenda et al., 2015; Montresor et al., 2013, 2015; Pérez-Rivero et al., 2013). Besides supporting the abovementioned hypothesis, these studies also suggest that permanent activation of JAK2 in JAK2-V617F mutated cells could possibly result in aberrant homing – a phenomenon possibly underlying extramedullary hematopoiesis of MPN patients.

1.3. Aims

In this study, we investigate the influence of the JAK2-V617F mutation on integrin-mediated cell adhesion. We focus on the interaction of leukocyte integrins with the ligand VCAM1, which plays a crucial role in endothelial adhesion and homing of a broad leukocyte spectrum including

lymphocytes, macrophages, and hematopoietic progenitors (Dutta et al., 2015; Herter & Zarbock, 2013; Ramos et al., 1999). Furthermore, we assess as to whether JAK2-V617F affects VCAM1-specific affinity of integrins based on the observation that JAK2 activation induces adhesion to VCAM1 in lymphocytes by increasing integrin affinity (Montresor et al., 2013).

As *in vitro* models of JAK2-V617F-positive MPN, we use an overexpression model of murine BaF3 and 32D hematopoietic progenitor cell lines (*Chapter 3.1.*), and human erythroleukemia (HEL) cells that endogenously express homozygous JAK2-V617F (*Chapter 3.2.*). In the murine models, overexpression of wild-type JAK2 (JAK2-WT) serves as control. In HEL cells, JAK2-V617F function is disrupted using kinase inhibitors or shRNA-mediated knockdown.

To investigate integrin-mediated adhesion, we employ a static adhesion assay where cells adherent to a VCAM1-coated surface are exposed to shear stress. The affinity of leukocyte integrins is measured by flow cytometric quantification of cell-bound soluble VCAM1. The involvement of β_1-integrin is assessed in particular because it is the best described ligand of VCAM1. Its activation and expression in JAK2-V617F-positive cells is determined by flow cytometry-based assays.

This *in vitro* study shall provide first evidence on the influence of JAK2-V617F on integrin-mediated adhesion to VCAM1. To our knowledge, this relation has hitherto not been investigated. If JAK2-V617F induced integrin-mediated adhesion, this would contribute to our understanding of the potential involvement of leukocytes in MPN thrombosis and would provide a solid basis on which studies in more physiological models could be founded.

2. Materials and methods

The greater part of experiments has been performed during a twelve-month research stay in 2013/2014 and the rest during the following two years. Here, we describe the methodology underlying our experiments and enclose a list of the specific materials used at the end of each subchapter. Standard laboratory equipment and common materials used in biological research are not listed. All experiments were conducted in accordance with the provisions of the German Ordinance on Biological Substances, the German Labor Protection Act, and the German Law on Genetic Engineering.

The term *incubation (at 37 °C)* refers to storage in a conventional cell culture incubator in humid atmosphere of 100 % relative humidity supplemented with a volume fraction of 5 % CO_2 at 37 °C, unless otherwise indicated. To assess viable cell density, cells were counted using a light microscope, Trypan blue and a counting chamber.

2.1. Cell culture

2.1.1. Cell lines

All cell lines were processed under sterile conditions of a biological safety cabinet and routinely maintained in sterile cell culture flasks for suspension culture in an atmosphere of 100 % relative humidity supplemented with a volume fraction of 5 % CO_2 at 37 °C. BaF3 and 32D cells stably overexpressing EPO-R and murine JAK2-WT and JAK2-V617F, respectively, have been described previously (Schnöder et al., 2015). They were cultured in modified RPMI medium (*Table 3*) and passaged every second day. BaF3 JAK2-WT and 32D JAK2-WT cells were cultured in the presence of EPO (ϱ_{EPO} = 1 IU/ml). HEL cells (Quentmeier, MacLeod, Zaborski, & Drexler, 2006) were cultured in modified RPMI medium and passaged every third day. HEK293T cells were maintained in sterile dishes for adherent cell culture in modified DMEM medium (*Table 3*) and passaged every second day. All cell lines were kept in culture for a maximum period of 8 weeks.

For long-term storage, cells were cryopreserved in liquid nitrogen. For freezing, $5 \cdot 10^6$ cells were resuspended in 0.8 ml of modified RPMI medium supplemented with 0.1 ml FBS and 0.1 ml dimethyl sulfoxide (DMSO) and slowly cooled down to − 80 °C using an isopropanol-isolated storage box. After 24 hours, cells were transferred to a liquid nitrogen tank. Cells were thawed in a 37 °C warm water bath, washed with phosphate-buffered saline (PBS), and maintained as described above.

2.1.2. Inhibitor treatment

BaF3 cells were harvested, washed three times with PBS, counted, and seeded in serum-reduced RPMI medium (*Table 3*) at a density of $1 \cdot 10^6$ cells/ml for 4 hours. Afterwards, cells were either treated with EPO for 10 minutes (ϱ_{EPO} = 3 IU/ml), JAK inhibitor I for 30 minutes ($c_{JAK\ inhibitor\ I}$ = 2 µmol/l), or both. An equivalent volume of the solvents DMSO and ddH_2O were used as control for JAK inhibitor I and EPO treatment, respectively.

HEL cells were harvested, washed three times with PBS, and counted. They were seeded at a density of $0.5 \cdot 10^6$ cells/ml in modified RPMI medium supplemented with either DMSO, JAK inhibitor I, or NVP-BSK805, and incubated for 16 hours at 37 °C. Except in dose titration experiments, inhibitors were used at standard concentrations of 1 000 nmol/l (JAK inhibitor I) and 1 500 nmol/l (NVP-BSK805).

Table 2. Cell lines.

Cell line	Source
BaF3 EpoR JAK2-V617F BaF3 EpoR JAK2-WT 32D EpoR JAK2-V617F 32D EpoR JAK2-WT	BaF3 and 32D cells were obtained from DSMZ. Cells were stably transfected earlier by our group with pMSCV-EpoR-Neo and either pMSCV-JAK2V617F-Puro or pMSCV-JAK2WT-Puro. Vectors encoded murine JAK2 sequences.
HEL	HEL cells were obtained from the DSMZ.
HEK293T	HEK293T cells were obtained from the DSMZ.

Table 3. Solutions/Buffers for cell culture.

Solution/Buffer	Ingredients
Modified RPMI medium	- 500 ml RPMI 1640 medium - 50 ml fetal bovine serum (FBS) - 29 ml additive solution - 100 µl Plasmocin™ ($\varrho_{stock\ solution}$ = 2.5 mg/ml)
Serum-reduced RPMI medium	- 500 ml RPMI 1640 medium - 5 ml fetal bovine serum (FBS) - 100 µl Plasmocin™ ($\varrho_{stock\ solution}$ = 2.5 mg/ml)
Additive solution	- 100 ml PBS - 100 ml PBS supplemented with 35 µl 2-sulfanylethan-1-ol (2-mercaptoethanol) - 100 ml HEPES buffer ($c_{stock\ solution}$ = 1 mol/l, pH = 7.2)

Solution/Buffer	Ingredients
Additive solution (continued)	- 100 ml sodium pyruvate ($c_{\text{stock solution}}$ = 100 mmol/l) - 100 ml L-glutamine ($c_{\text{stock solution}}$ = 200 mmol/l) - 60 ml MEM Non-Essential Amino Acids Solution (100X) - 20 ml asparagine ($\varrho_{\text{stock solution}}$ = 10 mg/ml) - pH = 7.2 - Additive solution is sterile filtered
Modified DMEM medium	- 500 ml DMEM medium - 50 ml FBS - 5 ml penicilline/streptomycin (activity of penicillin = 5 000 U/ml, $\varrho_{\text{streptomycin}}$ = 5 000 µg/ml) - 5 ml L-glutamine ($c_{\text{stock solution}}$ = 200 mmol/l) - 100 µl Plasmocin™ ($\varrho_{\text{stock solution}}$ = 2.5 mg/ml) - Adjust pH to 7.4 with NaOH - 5 ml HEPES buffer ($c_{\text{stock solution}}$ = 1 mol/l, pH = 7.2) - Modified DMEM medium is sterile filtered

Table 4. Chemicals/Equipment for cell culture.

Chemical/Equipment (as mentioned in the text)	Product Name	Company
RPMI 1640 medium	(GIBCO) RPMI 1640 Medium	Thermo Fisher Scientific, Inc.
EPO	Recombinant EPO	Janssen-Cilag GmbH
DMEM medium	(GIBCO) Dulbecco's Modified Eagle Medium	Thermo Fisher Scientific, Inc.
DMSO	Dimethyl sulfoxide	Carl Roth GmbH + Co. KG
PBS	(GIBCO) Dulbecco's Phosphate-Buffered Saline	Thermo Fisher Scientific, Inc.
JAK inhibitor I	JAK Inhibitor I	Calbiochem (Merck KGaA)
NVP-BSK 805	NVP-BSK805 2HCl	Selleck Chemicals
FBS	(GIBCO) Fetal bovine serum	Thermo Fisher Scientific, Inc.
Plasmocin™	Plasmocin™ prophylactic	InvivoGen
2-sulfanylethan-1-ol	2-mercaptoethanol	Sigma-Aldrich, Inc.
HEPES	HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Carl Roth GmbH + Co. KG
Sodium pyruvate	(GIBCO) Sodium pyruvate	Thermo Fisher Scientific, Inc.
L-glutamine	(GIBCO) L-glutamine	Thermo Fisher Scientific, Inc.

Chemical/Equipment (as mentioned in the text)	Product Name	Company
(GIBCO) MEM Non-Essential Amino Acids Solution (100X)	(GIBCO) MEM Non-Essential Amino Acids Solution (100X)	Thermo Fisher Scientific, Inc.
Asparagine	Asparagine	Carl Roth GmbH + Co. KG
Penicillin/Streptomycin	Penicillin/Streptomycin (5 000 U/ml) (GIBCO)	Thermo Fisher Scientific, Inc.

2.2. Lentiviral transduction and shRNA-mediated knockdown

Knockdown experiments were performed using commercially available shRNA sequences targeting human JAK2, or a non-targeting control sequence (scrambled) (*Table 5*), expressed from a pLKO.1-puro vector (MISSION shRNA Plasmid DNA (TRC1/1.5 vectors), Sigma-Aldrich, Inc.). Plasmids were delivered by lentiviral transduction. For production of lentiviral particles, HEK293T cells were cultured in modified DMEM medium (*Table 3*) until 60-70 % confluency were reached. The transfection solution was prepared by supplementing 1.8 ml OptiMEM medium with 3 μg of the plasmid of interest, 3 μg of viral plasmids pMD2.G and psPAX2, respectively, and 27 μl FuGENE. After 15 minutes incubation at room temperature, the mixture was added dropwise to HEK293T cells covered with 4 ml of fresh modified DMEM medium. After 24 hours incubation at 37 °C, the supernatant was replaced with 9 ml fresh medium. After additional incubation for 16 hours, supernatant containing viral particles was harvested, 6 ml fresh medium was added and harvested once again after 8 hours. Cell debris were removed with a cellulose acetate syringe filter with a pore size of 0.45 μm and viral supernatant was stored at − 80°C.

HEL cells were infected in the evening (Day 0) by centrifuging $3{\cdot}10^6$ cells in 2 ml viral supernatant in a 6-well plate for suspension culture at 514 *g*, 33 °C for 90 minutes. Afterwards, the cells were incubated at 37 °C until the next morning (Day 1). Then, the viral suspension was discarded and replaced with modified RPMI medium (*Table 3*). In the morning of Day 2, selective pressure was established with puromycin at a mass concentration of 2 μg/ml. Cells were harvested for experiments on Day 2 (shRNA D), Day 3 (shRNA C), and Day 4 (shRNA A + B), respectively, due to differential onset of apoptosis (see *Results*). On the respective days, knockdown efficacy was controlled by Western blotting and viability was assessed by flow cytometry as described below. Before conducting adhesion assay, soluble ligand binding assay and integrin surface expression analysis, HEL cells were serum-starved in serum-reduced RPMI medium (*Table 3*) for 3.5 hours to minimize extrinsic effects by serum components.

Table 5. shRNA vectors. shRNAs A-D were targeted to human JAK2. They were obtained from Sigma-Aldrich, Inc. (MISSION shRNA Plasmid DNA (TRC1/1.5 vectors)).

shRNA title	Source
A	Sigma-Aldrich, Inc., TRCN0000003179
B	Sigma-Aldrich, Inc., TRCN0000196855
C	Sigma-Aldrich, Inc., TRCN0000003178
D	Sigma-Aldrich, Inc., TRCN0000003180

Table 6. Chemicals/Equipment for shRNA-mediated knockdown.

Chemical / Equipment (as mentioned in the text)	Product Name	Company
OptiMEM medium	(GIBCO) Opti-MEM™ I Reduced Serum Medium	Thermo Fisher Scientific, Inc.
pMD2.G lentiviral packaging	pMD2.G	Addgene #12259 (Trono Lab Packaging and Envelope Plasmids)
psPAX2 lentiviral packaging plasmid	psPAX2	Addgene #12260 (Trono Lab Packaging and Envelope Plasmids)
FuGENE®	FuGENE® HD Transfection Reagent	Promega GmbH
Cellulose acetate syringe filter	Syringe filters, cellulose acetate membrane, pore size = 0.45 µm	VWR (Avantor)
Puromycin	Puromycin dihydrochloride BioChemica	AppliChem GmbH

2.3. Western Blotting

After treatment, cells were washed in ice-cold PBS for three times and then resuspended in ice-cold, premixed 100 µl lysis buffer supplemented with 4 µl protease inhibitor and 1 µl sodium fluoride ($c_{\text{stock solution}}$ = 1 mol/l). Immediately, 40 µl phosphatase inhibitor were added. After an incubation period of 45 minutes on ice, the suspension was centrifuged at 10 000 *g* for 15 minutes at 4 °C. The supernatant protein lysate was either directly processed or stored at – 80 °C. Protein concentration was estimated by the Bradford-dye binding method compared to a serial dilution of bovine serum albumin (BSA). For each sample, lysate containing 30 µg total protein were denatured by adding one quarter sample volume of SDS denaturation buffer and boiling at 95 °C for 10

minutes. SDS-PAGE was performed using SDS-polyacrylamide gels with a polyacrylamide mass fraction of either 7.5 %, 10 %, or 12.5 %, depending on the target proteins. Proteins were electro-blotted together with a protein size marker on a nitrocellulose membrane by the wet transfer method. Unspecific antibody interactions were blocked by incubation with blocking buffer for 1 hour. After brief washing in TBST, membranes were incubated overnight at 4 °C with primary antibody solution supplemented with BSA at a mass fraction of 5 %. Used antibody solutions were stored at – 20 °C and reused thrice. The antibodies and dilutions used are depicted in *Table 7*. The next day, membranes were rinsed in TBST for three times 10 minutes, incubated with horseradish-peroxidase-labelled secondary antibody solution for 1 hour, followed by three times 10 minutes rinsing in TBST. Images of immune-reactive protein bands were developed using luminol-chemiluminescence. Antibodies were removed by washing the membranes in stripping buffer for 20 minutes at 50 °C and the staining process was repeated as described above. Image raw files were processed with Adobe Photoshop CS2 (Adobe Systems, Inc.) in the three steps of black/white inversion, auto-contrast adjustment, and cutting. No further editing steps were performed. All editing steps were always applied to the complete raw file. The only purpose of cutting was to enable neat arrangement of multiple blots in the figures. The only exception was the blot of shRNA C-mediated knockdown of JAK2 depicted in the lower left panel of *Figure 14. Western blots showing shRNA-mediated knockdown of JAK2 in HEL cells.* The raw file includes knockdown controls of two other experiments, since we used to run knockdown controls of different experiments together on one gel. We attached the complete figure as *Supplemental figure 1* for comparison.

Table 7: Antibodies for Western blotting.

Antibody	Reference for target phosphosite	Dilution	Company
p-JAK2 (3771)	(Feng et al., 1997; Gauzzi et al., 1996)	1 : 1 000	Cell Signaling Technology, Inc.
JAK2 (3230)		1 : 1 000	Cell Signaling Technology, Inc.
p-Plcγ1 (2821)	(Chang, Holtzman, & Chen, 2004; H. K. Kim et al., 1991)	1 : 1 000	Cell Signaling Technology, Inc.
Plcγ1 (2822)		1 : 1 000	Cell Signaling Technology, Inc.
p-Akt (9271)	(Jacinto et al., 2006)	1 : 1 000	Cell Signaling Technology, Inc.

Antibody	Reference for target phosphosite	Dilution	Company
Akt (9272)		1 : 1 000	Cell Signaling Technology, Inc.
p-p44/42 MAPK (9106)	(Roskoski, 2012)	1 : 1 000	Cell Signaling Technology, Inc.
p44/42 MAPK (9102)		1 : 1 000	Cell Signaling Technology, Inc.
GAPDH (H86504M)		1 : 5 000	Meridian Life Sciences, Inc.
p-Stat5 (05–495)	(Okutani et al., 2001)	1 : 1 000	Millipore (Merck KGaA)
Stat5 (sc-1081)		1 : 100	Santa Cruz Biotechnologies, Inc.
β-actin antibody (A5441)		1 : 10 000	Sigma-Aldrich, Inc.
Anti-Maus IgG – HRP conjugated		1 : 2 000	Santa Cruz Biotechnologies, Inc.
Anti-Rabbit IgG – HRP conjugated		1 : 2 000	Cell Signaling Technology, Inc.

Table 8: Solutions / Buffers for Western blotting.

Solution / Buffer	Ingredients
Lysis buffer	- HEPES at c_{HEPES} = 50 mmol/l - Glycerol at $\varphi_{Glycerol}$ = 10 % - NaCl at c_{NaCl} = 150 mmol/l - TritonX100 at $\varphi_{TritonX100}$ = 1 % - $MgCl_2$ at c_{MgCl2} = 1.5 mmol/l - EGTA at c_{EGTA} = 5 mmol/l - pH = 7.4 - Solved in ddH_2O
TBST (10x concentrated) *Tris-buffered saline with Tween 20*	- NaCl at c_{NaCl} = 150 mmol/l - Tris at c_{Tris} = 50 mmol/l - Tween 20 at $\varphi_{Tween\ 20}$ = 0.05 % - Solved in ddH_2O - For Western Blotting, 10x TBST was used in a 1:10-diluted aqueous solution (1x)

Solution / Buffer	Ingredients
SDS denaturation buffer (4x concentrated)	- Tris at c_{Tris} = 250 mmol/l - Glycerol at $\varphi_{Glycerol}$ = 50 % - SDS at φ_{SDS} = 10 % - 2-mercaptoethanol at $\varphi_{2\text{-mercaptoethanol}}$ = 25 % - Bromophenol blue at $\varrho_{Bromophenol\ blue}$ = 0.02 % - pH = 6.8 - Solved in ddH_2O
Blocking buffer	- 1x TBST - Milk powder at $\varrho_{Milk\ powder}$ = 5 %
Stripping buffer (1 000 ml)	- 7.57 g Tris - 20 g SDS - 7 ml 2-mercaptoethanol - ddH_2O

Table 9: Chemicals / Equipment for Western blotting.

Chemical / Equipment (as mentioned in the text)	Product Name	Company
Protease inhibitor	Protease Inhibitor Cocktail (Complete)	Roche Deutschland Holding GmbH
Phosphatase inhibitor	PhosSTOP	Roche Deutschland Holding GmbH
Reagent for Bradford assay	Bio-Rad Protein Assay Dye Reagent Concentrate®	Bio-Rad, Inc.
BSA (Bovine serum albumin)	Albumin, Fraktion V	Carl Roth GmbH + Co. KG
Milk powder	Milchpulver, Blotting grade	Carl Roth GmbH + Co. KG
Chemiluminescence reagents (luminol and hydrogen peroxide)	EMD Millipore™ Immobilon™ Western Chemiluminescent HRP Substrate (ECL)	Millipore (Merck KGaA)
Nitrocellulose membrane	Amersham Protran® 0.45 µm Nitrocellulose	GE Healthcare, General Electric Company
Chemiluminescence imager	Fusion FX and Fusion FX software	Vilber Lourmat

2.4. Static adhesion assay

One day prior to experiment, wells of a 96-well plate were coated with 100 µl Hank's Balanced Salt Solution (HBSS) containing VCAM1 or BSA, respectively, in pentaplicates per sample and stored in a frost-free refrigerator at 4 °C. For BaF3 cells, we used murine VCAM1 at a mass concentration of 3 µg/ml. For HEL cells, we used human VCAM1 at a mass concentration of 1 µg/ml. BSA, at a mass fraction of 5 %, was used as a control to detect adhesion not specific to VCAM1.

Cells were harvested, washed three times with HBSS and counted. $2 \cdot 10^6$ cells per sample were incubated with Calcein AM at a molar concentration of 2 µmol/l in 1 ml RPMI 1640 medium for 30 minutes followed by three washing steps with RPMI 1640 medium. 100 µl of RPMI 1640 medium containing 100 000 stained cells were then added to the respective wells of the coated 96-well plate. The plate was briefly centrifuged and then incubated at 37 °C for 25 minutes to allow firm adhesion of the cells. Afterwards, fluorescence intensity of Calcein AM in each well was determined using a plate fluorimeter. Supernatant cell suspension was discarded and 200 µl of HBSS were added to each well using a calibrated multichannel pipette. The multichannel pipette was used as depicted in *Figure 2* thereby ensuring that the same stress was applied to all samples of a particular replicate. After each washing step, the fluorescence intensity was reassessed. The results represent the ratio of the final fluorescence intensity after three washing steps to the signal obtained in the beginning.

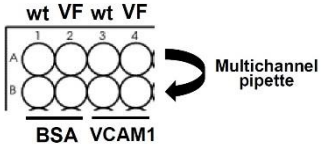

Figure 2. Pipetting regimen in the static adhesion assay. The washing steps involved the inversion of the plate on a paper towel to discard the supernatant. Then, 200 µl HBSS were added to each well row-wise using a multichannel pipette. Each row represented a complete replicate of samples. In this way it was ensured that variation of shear force during the manual pipetting process did not systematically distort the results.

Table 10: Chemicals / Equipment for static adhesion assay.

Chemical / Equipment (as mentioned in the text)	Product Name	Company
96-well plates	96-well plate, black, clear bottom, TC-treated, sterile	Corning, Inc.
Human VCAM1	Recombinant Human VCAM-1 / CD106 Fc Chimera	R&D Systems, Inc.
Murine VCAM1	Recombinant Mouse VCAM-1 / CD106 Fc Chimera	R&D Systems, Inc.

Chemical / Equipment (as mentioned in the text)	Product Name	Company
BSA (Bovine serum albumin)	Albumin, Fraktion V	Carl Roth GmbH + Co. KG
Calcein AM	Vybrant™ Cell Adhesion Assay Kit (V-13181)	Thermo Fisher Scientific, Inc.
RPMI 1640 medium	(GIBCO) RPMI 1640 Medium	Thermo Fisher Scientific, Inc.
HBSS	(GIBCO) Hank's Balanced Salt Solution without Magnesium and Calcium	Thermo Fisher Scientific, Inc.
Plate fluorimeter	Synergy™ HT	BioTek Instruments, Inc.
Multichannel pipette	Eppendorf Research® plus 8 channel pipette, 30-300 uL	Eppendorf AG

2.5. Flow cytometry

All flow cytometric assays were performed on a FACS Canto II (Becton Dickinson GmbH). Raw files were processed using the FlowJo software (Tree star, Inc.).

2.5.1. Soluble VCAM1 binding assay

Cells were harvested, washed thrice with HBSS, and counted. $1 \cdot 10^6$ cells per sample were resuspended in 100 µl RPMI 1640 medium. Each sample included a positive control with manganese chloride, which was described to induce high levels of VCAM1 ligand binding and β_1-mediated adhesion (Mould, Akiyama, & Humphries, 1995). Manganese chloride was added to the positive controls generating a molar concentration of 1 mmol/l and all samples were incubated for 15 minutes at 37 °C. Afterwards, VCAM1 was added and the incubation was continued for another 45 minutes. For BaF3 cells, we used murine VCAM1 at a mass concentration of 10 µg/ml. For HEL cells, we used human VCAM1 at a mass concentration of 5 µg/ml. Subsequently, cells were incubated on ice for 10 minutes, washed twice with ice-cold HBSS supplemented with FBS at a volume fraction of 1 % (HBSS/FBS) and were then stained with a phycoerythrin (PE)-labelled secondary antibody targeting recombinant VCAM1 for 20 minutes at 4 °C. Unbound antibody was cleared by washing three times with HBSS/FBS. PE-specific fluorescence intensity was determined by flow cytometry and the background signal generated by samples incubated with secondary antibody only was subtracted.

2.5.2. Coculture assay

32D JAK2-WT and JAK2-V617F cells were harvested, washed with HBSS and counted. $1.5 \cdot 10^6$ 32D JAK2-V617F cells in 1 ml RPMI 1640 medium were stained with CellTracker™ Blue CMAC dye at a molar concentration of 4 µmol/l at 37 °C for 30 minutes. After clearing excess dye by washing with serum-reduced RPMI medium (*Table 3*), cells were incubated in serum-reduced RPMI medium for 3.5 hours at a density of $1 \cdot 10^6$ cells/ml, either separately or in a 1:1 mixed culture. Afterwards, a soluble VCAM1 binding assay was performed as described above but with the addition of CMAC blue-specific gating.

2.5.3. Total integrin and HUTS-21 epitope surface expression analysis

Cells were harvested, washed with PBS, and counted. $1 \cdot 10^6$ cells were incubated with specific antibody or isotype control, respectively, for 90 minutes at 4 °C. Excess antibody was cleared by washing with PBS supplemented with FBS at a volume fraction of 1 % (PBS/FBS). Dye-specific fluorescence intensity was determined by flow cytometry. Values were corrected for unspecific signals of isotype controls. For HEL cells, HBSS was used instead of PBS and antibodies targeting human proteins instead of murine isoforms. The antibodies were used in 1:100-dilutions. All antibodies are depicted in *Table 9*.

2.5.4. Cell death assay

The cell death assay was used in two different variants – to assess the effects of different concentrations of JAK2 inhibitors on HEL cells and to control cell viability following JAK2 knockdown. In the former case, cells were centrifuged, resuspended in 300 µl of HBSS/FBS, and directly stained with 1 µl SYTOX™ Blue to assess the number of dead cells. No Annexin V antibody was used. Additional washing steps were not performed in order to prevent loosing cell debris resulting in an underestimation of toxicity.

In the latter case, cells were harvested after starvation, washed with HBSS, counted, and then divided in order to perform the adhesion assay and the flow cytometry-based assays in parallel with $0.5 \cdot 10^6$ cells per sample. The washing step was conducted in order to remove cell debris that could possibly feature irregular adhesion or ligand binding. In this setting, the assay included Annexin V antibody and was used to control as to whether apoptotic cells remained in the samples after washing. In this case, $0.5 \cdot 10^6$ cells were resuspended in 100 µl Annexin V binding buffer, 2.5 µl of PE-conjugated Annexin V antibody were added, and the cells were incubated for

15 minutes at room temperature, protected from light. 200 µl of Annexin V binding buffer were added. Shortly before the measurement, 1 µl SYTOX™ Blue was added.

Table 11: Chemicals / Equipment for flow cytometry.

Chemical / Equipment (as mentioned in the text)	Product Name	Company
HBSS	(GIBCO) Hank's Balanced Salt Solution without Magnesium and Calcium	Thermo Fisher Scientific, Inc.
RPMI	(GIBCO) RPMI 1640 Medium	Thermo Fisher Scientific, Inc.
Human VCAM1	Recombinant Human VCAM-1 / CD106 Fc Chimera	R&D Systems, Inc.
Murine VCAM1	Recombinant Mouse VCAM-1 / CD106 Fc Chimera	R&D Systems, Inc.
Manganese chloride	Manganese chloride	Carl Roth GmbH + Co. KG
VCAM1 secondary antibody	R-Phycoerythrin-conjugated AffiniPure F(ab')$_2$ Fragment Goat Anti-Human IgG, Fc$_\gamma$ Fragment Specific (minimal cross-reactivity to Bovine, Horse, and Mouse Serum Proteins)	Jackson ImmunoResearch Laboratories, Inc.
FBS	(GIBCO) Fetal bovine serum	Thermo Fisher Scientific, Inc.
CellTracker™ Blue CMAC	CellTracker™ Blue CMAC Dye	Thermo Fisher Scientific, Inc.
PBS	(GIBCO) Dulbecco's Phosphate-Buffered Saline	Thermo Fisher Scientific, Inc.
PE Mouse Anti-Human CD29, clone HUTS-21	PE Mouse Anti-Human CD29, clone HUTS-21, isotype IgG2a-κ, BD Pharmingen™, Material Number 556049	Becton Dickinson GmbH
HUTS-21 isotype control	PE Mouse IgG2a, κ Isotype Control, BD PharmingenTM, Material Number 558595	Becton Dickinson GmbH
Anti-murine CD29 antibody	APC anti-mouse/rat CD29 Antibody	BioLegend, Inc.
Isotype control for anti-murine CD29 antibody	APC Armenian Hamster IgG Isotype Ctrl Antibody	BioLegend, Inc.

Chemical / Equipment (as mentioned in the text)	Product Name	Company
Anti human CD29	PE anti-human CD29 Antibody	BioLegend, Inc.
Isotype control for anti-human CD29 antibody	PE Mouse IgG1, κ Isotype Ctrl Antibody	BioLegend, Inc.
Annexin V	PE Annexin V	BioLegend, Inc.
Annexin V binding buffer	Annexin V binding buffer	BioLegend, Inc.
SYTOX™ Blue	SYTOX™ Blue Dead Cell Stain	Thermo Fisher Scientific, Inc.
Flow cytometer	FACS Canto II	Becton Dickinson GmbH
FlowJo software	FlowJo, Version 8.8.6	Tree star, Inc.

2.6. Data analysis, representation, and statistical methodology

Data was analyzed with help of Microsoft Excel 2013 and GraphPad PRISM, Version 7.02. The latter was used to generate the figures depicting data. All other figures and tables, as well as this text, were generated using Microsoft PowerPoint and Word 2013.

Our use of descriptive and inferential statistics follows the suggestions of (Vaux, 2012). Briefly, this means recognizing that statistics can be helpful and necessary to represent and interpret large data sets, but also misleading if applied in settings they have not been intended for. The latter case most importantly refers to very small data sets, as often used in molecular or cell biology, which can be easily interpreted by the reader himself/herself. In this context, the use of statistics can rather distract and inappropriately skew the results in a certain direction.

Therefore, in our study,

- we always depict all data points because our sample size never exceeds five,
- we only provide a measure of central tendency where sample size exceeds three,
- and we do not use inferential statistics.

We abstain from using inferential statistics in our study, because we agree with Vaux in that biological knowledge rather has to be gained from the coherence of several different models of the issue of interest, than from chasing p-values by repeating many times few experiments of limited external validity. As a consequence, we emphasize the limitations of our results and the imperative to validate them in more physiological models, especially animal studies (see *Discussion*). The value of our study lies in providing a basic body of evidence serving to test initial hypotheses, evaluate whether they should be tested in other models at all, and plan those physiological models as thoroughly as possible since they usually involve animal life and are cost-intensive. Those very sophisticated physiological models, which are attributed high external validity, of course have to be carefully planned with regard to sample size and analyzed using inferential statistics.

3. Results

3.1. JAK2-V617F overexpression in the murine pro-B cell line BaF3

During the first part of this project, we studied an overexpression model of JAK2-V617F and JAK2-WT in BaF3 cells. BaF3, a murine pro-B cell line, is often used as a robust and easy-to-handle model of hematopoietic progenitors and has been employed to model the effects of the JAK2-V617F mutation in MPNs since its discovery (James et al., 2005). Because JAK2-V617F requires the coexpression of a type I cytokine receptor to unfold its constitutive downstream signaling (Lu et al., 2005), we used a BaF3 cell line that stably expressed EPO-R as described earlier (Schnöder et al., 2015).

3.1.1. Integrin-mediated adhesion is enhanced in JAK2-V617F mutated cells

To investigate as to whether constitutively active JAK2-V617F influences the key function of integrins, cell adhesion, in hematopoietic cells, we compared BaF3 cells stably transfected with overexpression vectors encoding JAK2-V617F and JAK2-WT, respectively, in a static adhesion assay. Permanent activation of the key JAK2 downstream effector molecules STAT5, ERK, PLCγ1, and AKT (Kota, Caceres, & Constantinescu, 2008; Schnöder et al., 2015) in JAK2-V617F cells, as indicated by activating tyrosine phosphorylation, was verified by Western blotting (*Figure 3*). For references showing the relevance of target phospho-sites, please refer to Table 7. JAK2-V617F and JAK2-WT cells both showed activation of JAK2 downstream targets upon EPO treatment, which could be abrogated by JAK inhibitor I. BaF3 JAK2-V617F cells featured JAK2 downstream target phosphorylation even in the absence of EPO stimulation.

For the static adhesion assay, cells were seeded in a 96-well plate coated with VCAM1 and incubated for 25 minutes at 37 °C to enable proper adhesion. Subsequently, shear stress was applied repeatedly by gently pipetting saline to the wells and discarding supernatant cell suspension. To assess cell density, cells were stained with a fluorescent dye and fluorescence intensity was measured before and after shear stress application. The proportion of remaining fluorescence intensity after washing to the initial value was interpreted as proportion of adherent cells to the initial cell number and is depicted in *Figure 4*. Plausibility was controlled by light microscopy.

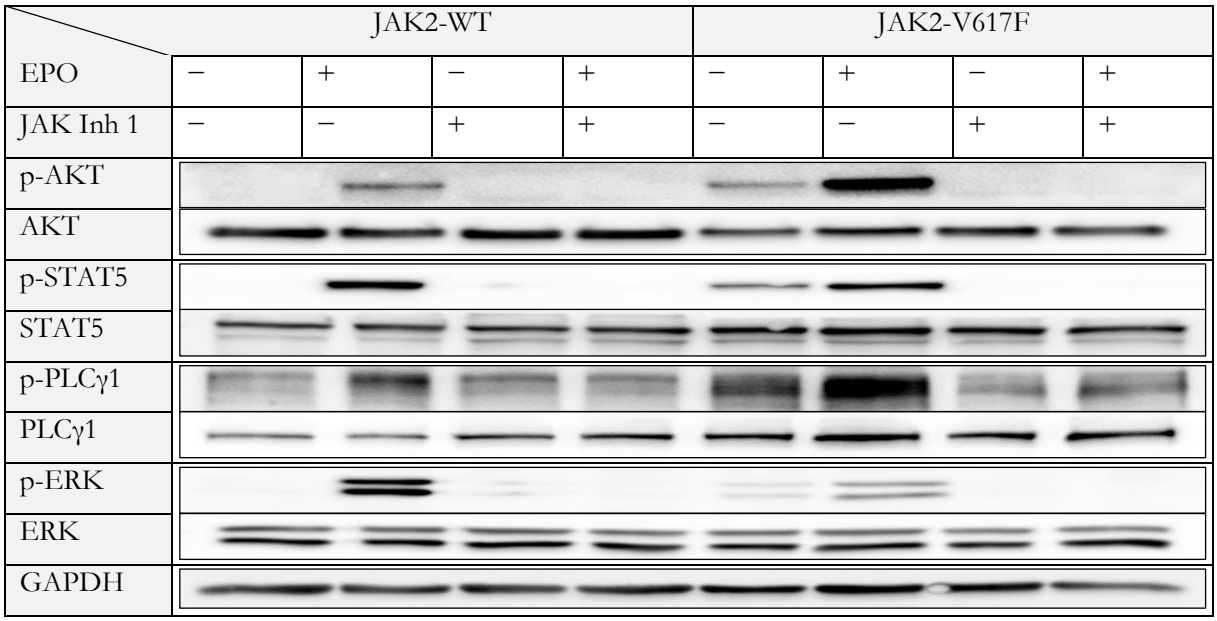

Figure 3. Activation of JAK2 signaling cascade by the JAK2-V617F mutation in BaF3 cells. BaF3 JAK2-V617F and BaF3 JAK2-WT cells were seeded in serum-reduced RPMI medium at a density of $1 \cdot 10^6$ cells/ml for 4 hours. Cells were either treated with EPO (ϱ_{EPO} = 3 IU/ml) for 10 minutes, JAK inhibitor I ($c_{JAK\ inhibitor\ I}$ = 2 µmol/l) for 30 minutes, or both. DMSO was used as control for JAK inhibitor I. Western blot analysis was performed for JAK2 downstream targets AKT, STAT5, PLCγ1, ERK1/2, and respective phosphoproteins, as well as for GAPDH as loading control. For references showing the relevance of target phospho-sites, please refer to *Table 7*.

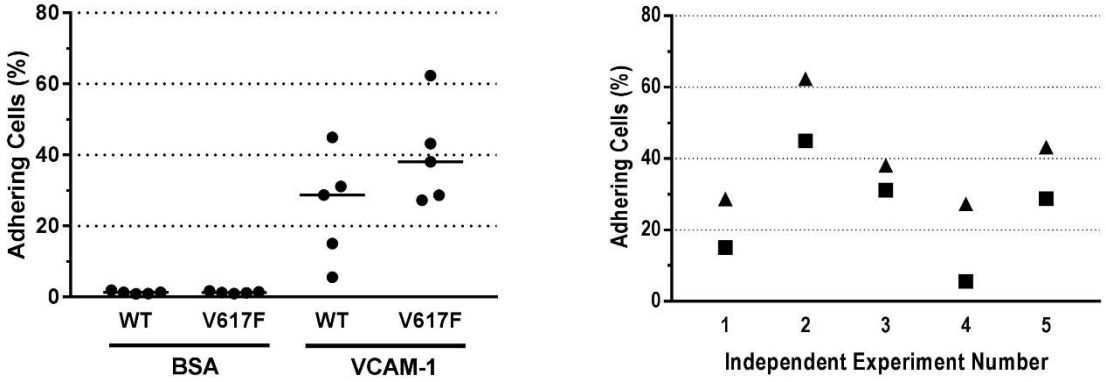

Figure 4. Static adhesion assay comparing BaF3 JAK2-V617F and JAK2-WT cells. BaF3 cells stably transfected with JAK2-V617F and JAK2-WT overexpression vectors, respectively, were stained with Calcein AM and seeded in wells of a 96-well plate in pentaplicates. The wells had been coated with VCAM1 or BSA as control. After 25 minutes of incubation, dye-specific fluorescence intensity was measured using a plate fluorimeter. Wells were then rinsed three times with HBSS and a final measurement was performed. The ratio of final to initial value was depicted as "Adhering Cells (%)". *Left panel.* Shows medians of five independent experiments. *Right panel.* Representation of adhering cells as measured in each individual experiment. BaF3 JAK2-V617F cells are depicted as ▲, JAK2-WT control cells as ■.

Initially, three independent experiments were performed. However, due to considerably high inter-experimental variation (15.1 % compared to 45.0 % adhering JAK2-WT cells in the first and second experiment, respectively (*Figure 4, right panel*), we decided to conduct two additional experiments. This did not change the variation observed. We consider the manual application of shear stress, although carried out carefully, to be the main reason. Nevertheless, we can exclude that manual pipetting distorted the relation between JAK2-V617F and JAK2-WT samples because of our pipetting regimen. It involved a calibrated multichannel pipette which allowed variation of shear stress between replicates and experiments, but not between the JAK2-V617F and JAK2-WT samples (see *Figure 2* in *Chapter 2.4. Static adhesion assay*).

Overall, the proportion of adhering cells harboring the JAK2-V617F mutation clearly exceeded the percentage of adhering JAK2-WT cells in each individual experiment. Therefore, we conclude that activation of JAK2 by the V617F mutation increased cell adhesion. To control unspecific adhesion effects, an equivalent number of wells coated with BSA instead of VCAM1 were included. Neither cell type showed significant adhesion to BSA (*Figure 4, left panel*).

3.1.2. Affinity of leukocyte integrins is increased in JAK2-V617F-positve cells

We next investigated as to whether the increase in integrin-mediated adhesion could be explained by a change in integrin affinity to VCAM1 in JAK2-V617F cells.

To determine integrin affinity, we incubated cells with a soluble recombinant VCAM1 chimera protein followed by staining with a phycoerythrin (PE)-conjugated secondary antibody which specifically binds to the human Fc_{γ} fragment of the chimera protein (soluble VCAM1 binding assay). The signal was quantified by flow cytometry. In order to eliminate unspecific signals, we included a sample stained with the secondary antibody only. The signal obtained was defined as background and subtracted from the actual values. Moreover, we used manganese chloride as a positive control, since it was described to promote high levels of VCAM1-specific ligand binding and adhesion (Mould et al., 1995). The resulting median fluorescence intensities of four independent experiments are depicted in *Figure 5*.

BaF3 JAK2-V617F cells showed increased binding of soluble VCAM1 compared to BaF3 JAK2-WT cells. As expected, incubation with manganese chloride led to elevated levels of bound soluble VCAM1 in both samples. After manganese chloride treatment, the difference in VCAM1 binding between the JAK2-WT and JAK2-V617F condition became more pronounced.

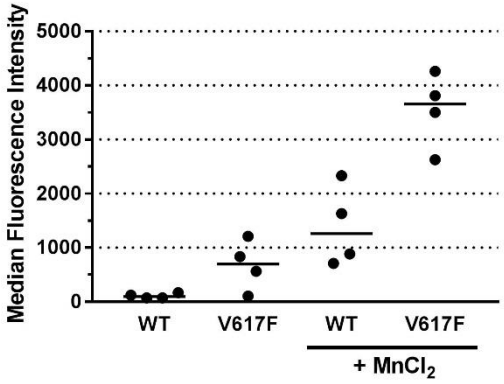

Figure 5. Soluble VCAM1 binding assay comparing BaF3 JAK2-V617F and JAK2-WT cells. For each sample, $1 \cdot 10^6$ cells were incubated in the presence or absence of manganese chloride (c_{MnCl2} = 1 mmol/l) for 15 minutes. After addition of 10 µg/ml VCAM1, incubation was extended for another 45 minutes. Subsequently, cells were incubated on ice for 10 minutes, washed twice with ice-cold HBSS/FBS and then stained with PE-conjugated secondary antibody for 20 minutes at 4 °C. Unbound antibody was cleared by washing three times with HBSS/FBS. PE-specific fluorescence intensity was determined by flow cytometry and the background signal generated by samples incubated with secondary antibody only was subtracted. Four independent experiments were performed. Bars represent medians.

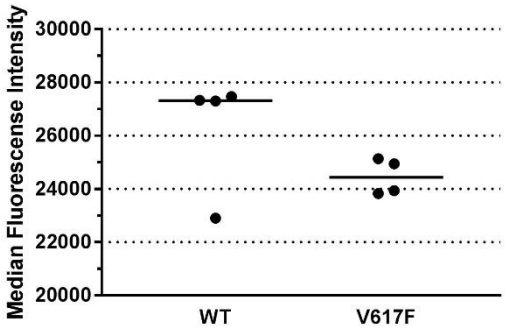

Figure 6. β_1-integrin (CD29) surface expression on BaF3 JAK2-V617F and JAK2-WT cells. For each sample, $1 \cdot 10^6$ cells were incubated with APC-conjugated anti-murine CD29 antibody for 90 minutes at 4 °C. Unbound antibody was cleared by washing with PBS/FBS. APC-specific fluorescence intensity was determined by flow cytometry and corrected for unspecific binding by subtracting the signal generated by an antibody isotype control. Four independent experiments were performed. Bars represent medians.

3.1.3. Surface expression of β_1-integrin is not increased by JAK2-V617F

Beside enhanced integrin affinity, increased adhesion and binding of soluble VCAM1 could also be caused by elevated surface expression of VCAM1 ligands on BaF3 JAK-V617F cells. In order to examine this possibility, we assessed the expression of β_1-integrin (CD29), the best described ligand of VCAM1 in leukocytes, on the surface of BaF3 JAK2-V617F and BaF3 JAK2-WT cells by flow cytometry. We used a CD29-specific, allophycocyanin (APC)-conjugated antibody and a respective isotype control. The introduction of JAK2-V617F mutation did not lead to an increase in β_1-integrin surface expression compared to JAK2-WT (*Figure 6*).

3.1.4. JAK2-V617F mutation increases integrin affinity by an intrinsic mechanism

According to our hypothesis, constitutive activation of the signaling cascade downstream of JAK2-V617F promotes integrin activation. However, because JAK2-V617F is well known for inducing secretion of several cytokines (see *Chapter 1.1.7. Systemic inflammation contributes to MPN pathophysiology.*), it is not unlikely that enhanced integrin affinity observed in BaF3 JAK2-V617F cells was rather caused by extracellular mediators than by a direct intracellular effect. In order to assess the influence of such paracrine effects in our experiments, we designed a coculture model of 32D JAK2-V617F and 32D JAK2-WT cells used it to perform a soluble VCAM1 binding assay. If the JAK2-V617F mutation caused the release of extracellular mediators affecting integrin affinity, cocultured JAK2-WT cells would be expected to show an increase in soluble VCAM1 binding. By staining 32D JAK2-V617F cells with a fluorescent dye, we were able to distinguish them from their JAK2-WT counterparts in the flow cytometry-based soluble VCAM1 binding assay. To diminish the influence of residual serum components of the modified RMPI medium, cells were seeded in serum-reduced medium for 3.5 hours. By the time these experiments were performed, our group had shifted its focus from the BaF3 model to the 32D myeloid progenitor cell model. Besides behaving similarly to BaF3 cells in terms of adhesion and integrin activation when stably transfected with a JAK2-V617F overexpression vector (Gupta et al., 2017), murine 32D cells offered the advantage of being derived from the myeloid lineage. Hence, they seemed more appropriate to study myeloproliferative neoplasms.

Cultured separately, 32D cells showed the same pattern of soluble VCAM1 binding as BaF3 cells (*Figure 7*). Namely, overexpression of JAK2-V617F was associated with a marked increase in VCAM1 binding compared to JAK2-WT cells. Values of 32D JAK2-WT cells from mixed culture with 32D JAK2-V617F did not differ from cells that had been separately cultured. Hence, JAK2-V617F signaling did not appear to result in secretion of extracellular mediators capable of inducing a change in integrin affinity in JAK2-WT cells. Interestingly, 32D JAK2-V617F cells showed an increase in integrin affinity when cultured with 32D JAK2-WT cells.

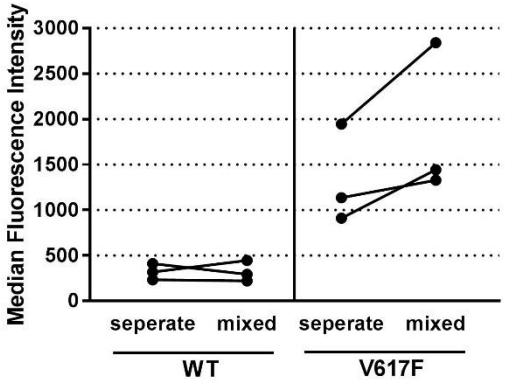

Figure 7. Soluble VCAM1 binding assay of separate and mixed cultures of 32D JAK2-WT and JAK2-V617F cells. 32D JAK2-V617F cells were stained with CellTracker™ Blue CMAC dye (c_{Dye} = 4 µmol/l) for 30 minutes at 37 °C. Thereafter, 32D JAK2-V617F and 32D JAK2-WT cells were cultured at a concentration of $1 \cdot 10^6$ cells/ml for 3.5 hours in serum-reduced medium either separately or in a 1:1 mixed culture, followed by a soluble VCAM1 binding assay which was performed as described above (*Figure 5*). Three independent experiments are shown. Lines connect values obtained in the same experiment.

3.2. Inhibition of JAK2-V617F activity in HEL cells

Although the BaF3 cell line is well-established in hematological research, it involves several limitations. Among others, these are mainly the murine origin and the lymphoid background. Therefore, we chose to complement the data derived from the BaF3 model with a series of experiments using human erythroleukemia cells (HEL). This cell line has been established from a patient with erythroleukemia, shows globin synthesis, indicating an erythroid and hence myeloid origin, and is homozygous for the JAK2-V617F mutation (Quentmeier et al., 2006).

3.2.1. Pharmacological inhibition of JAK2 kinase activity

As one pillar of our experimental design we used kinase inhibitors to abolish the effects induced by constitutive downstream signaling of JAK2-V617F in HEL cells. We selected JAK inhibitor I, an established inhibitor of JAK2 and, although less effectively, of other members of the JAK family (JAK1, JAK3, TYK2), and NVP-BSK805, a novel agent, targeting JAK2 with about 20-fold selectivity towards other JAK family kinases (Baffert et al., 2010).

We first conducted dose titration experiments to determine concentrations effectively disrupting JAK2-V617F downstream signaling. Cells were incubated with various doses of the respective agents or the solvent DMSO. Western blotting was performed for JAK2, its downstream targets STAT5 and ERK1/2, and their key phosphorylation sites.

As expected, in HEL cells, JAK2 downstream targets STAT5 and ERK1/2 were constitutively phosphorylated (*Figure 9*). Upon treatment with either inhibitor the signals declined in a dose dependent manner, NVP-BSK805 requiring a higher concentration compared to JAK inhibitor I. In contrast to its downstream targets, JAK2 itself remained unphosphorylated under control conditions and underwent progressive phosphorylation upon exposure to increasing doses of either inhibitor. This seemingly paradox behavior has been described before for JAK inhibitor I treatment of HEL cells (Haan et al., 2009). To our knowledge, the underlying mechanism remains unclear.

In addition to effectively abrogating JAK2-V617F downstream signaling, inhibitor treatment for 16 hours did not lead to a relevant increase in dead cell counts, as determined by SYTOX™ Blue staining (*Figure 8*). We therefore decided to use the most potent molar concentrations for our further experiments, 1 000 nmol/l of JAK inhibitor I and 1 500 nmol/l of NVP-BSK805.

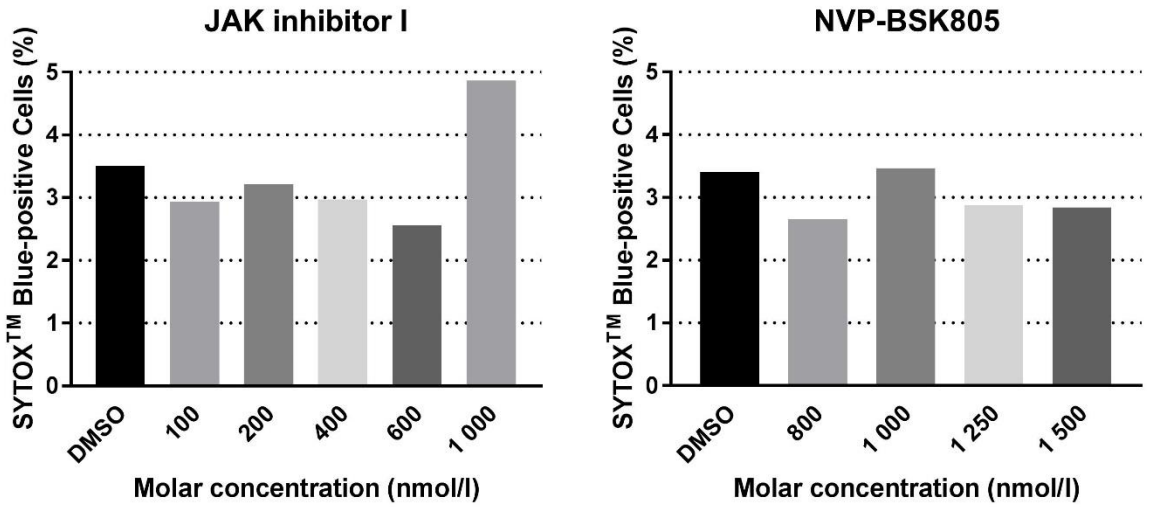

Figure 8. Cell death induced by different doses of pharmacological JAK2 inhibitors in HEL cells. HEL cells were incubated at a cell density of $0.5 \cdot 10^6$ cells/ml with JAK inhibitor I, NVP-BSK805, or DMSO in modified RPMI medium for 16 hours at indicated concentrations. Graphs show the percentage of cells that incorporated SYTOX™ Blue, a dye that only penetrates compromised membranes of dead cells. Flow cytometric assay. *Left Panel.* HEL cells treated with JAK inhibitor I. *Right panel.* HEL cells treated with NVP-BSK805.

3.2.2. Adhesion to VCAM1 is reduced in HEL cells after pharmacological inhibition of JAK2

Since HEL cells had not been used in the static adhesion assay in our group before, we performed a preliminary assay on a 96-well plate coated with various concentrations of VCAM1 in order to evaluate and optimize experimental conditions. However, the concentration of VCAM1 did not

affect the adhesion kinetics of HEL cells (*Figure 10, left panel*). Therefore, a mass concentration of 1 μg/ml VCAM1 was used in all subsequent adhesion assays using HEL cells.

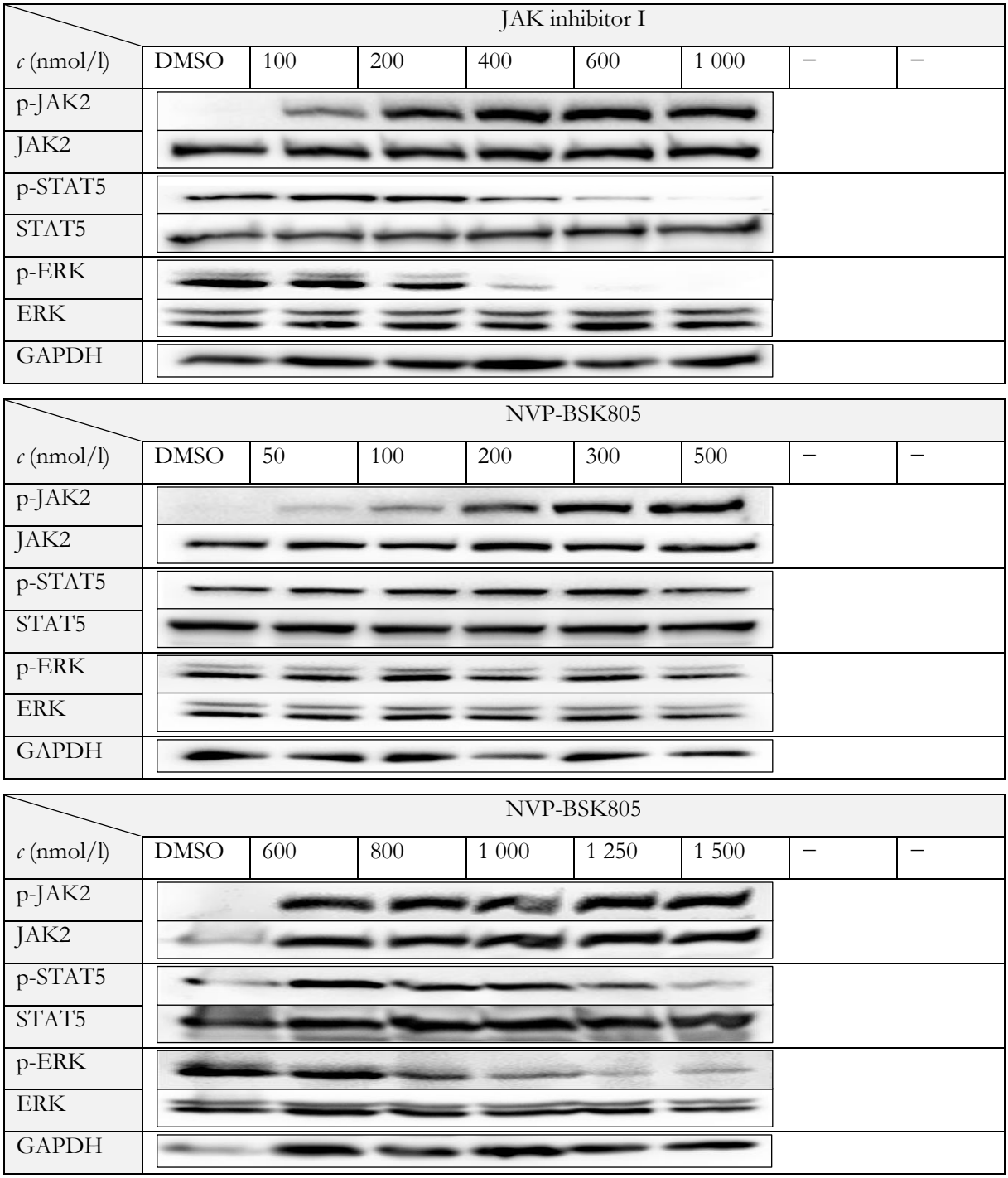

Figure 9. Disruption of JAK2 signaling cascade by different doses of pharmacological JAK2 inhibitors in HEL cells. HEL cells were incubated at a cell density of $0.5 \cdot 10^6$ cells/ml with JAK inhibitor I, NVP-BSK805, or DMSO in modified RPMI medium for 16 hours at indicated concentrations. Western blots showing phosphorylation status of JAK2, STAT5, ERK1/2, and GAPDH as loading control. *Upper panel.* Effects of JAK inhibitor I. *Middle and lower panel.* Effects of NVP-BSK805 at a wider range of concentrations.

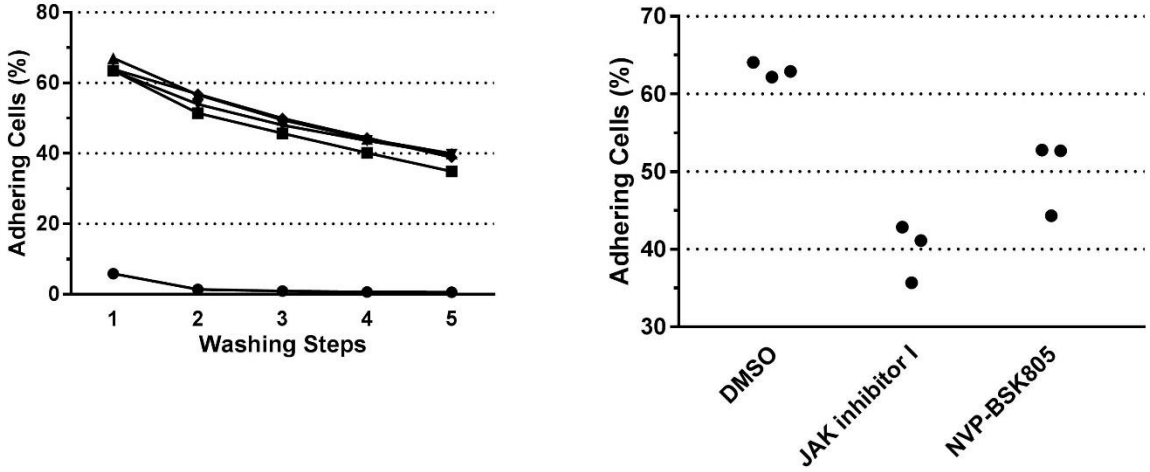

Figure 10. Static adhesion assay in HEL cells: VCAM1 dose titration (left) and pharmacological abrogation of JAK2 signaling (right). Static adhesion assay was performed as described above (*Figure 4*). *Left panel.* Plates were coated with VCAM1 at different concentrations, or BSA. One static adhesion assay performed with untreated HEL cells. The symbols represent the following mass concentration of VCAM1. ■ 1 μg/ml, ▲ 2.5 μg/ml, ▼ 5 μg/ml, ♦ 10 μg/ml, ● BSA control. *Right panel.* HEL cells were seeded at a density of $0.5 \cdot 10^6$ cells/ml in modified RPMI medium and treated with either DMSO, JAK inhibitor I ($c_{\text{JAK inhibitor I}}$ = 1 000 nmol/l), or NVP-BSK805 ($c_{\text{NVP-BSK805}}$ = 1 500 nmol/l). After 16 hours of incubation at 37 °C, static adhesion assay was conducted as described above (*Figure 4*). Three independent experiments were performed.

To investigate the influence of constitutively active JAK2-V617F signaling on the ability of HEL cells to adhere to VCAM1, we abrogated JAK2 kinase activity by exposing the cells to either JAK inhibitor I or NVP-BSK805 for 16 hours. We included DMSO treated cells as a control. Both inhibitors markedly decreased the number of cells adhering to a VCAM1 coated surface (*Figure 10, right panel*).

3.2.3. Integrin affinity to VCAM1 in HEL cells is not reduced by treatment with JAK2 inhibitors

We next investigated whether the reduced adhesion of JAK2-inhibited HEL cells was accompanied by impeded integrin affinity. In advance, we titrated the concentration of VCAM1 in the soluble ligand binding assay to exclude any experimental limitations due to insufficient saturation or other dose related effects (*Figure 11*). The amount of VCAM1 bound by untreated HEL cells was low, however, it dramatically increased upon treatment with manganese chloride at a molar concentration of 1 mmol/l. Elevation of VCAM1 concentration was associated with a steady rise in soluble VCAM1 binding. We decided to use VCAM1 at a mass concentration of 5 μg/ml in subsequent experiments because it provided sufficient amount of ligand to indicate a marked

increase in affinity, as was demonstrated by manganese chloride treatment. Furthermore, it would, rather than a mass concentration of 1 µg/ml, allow to detect a possible decline in affinity.

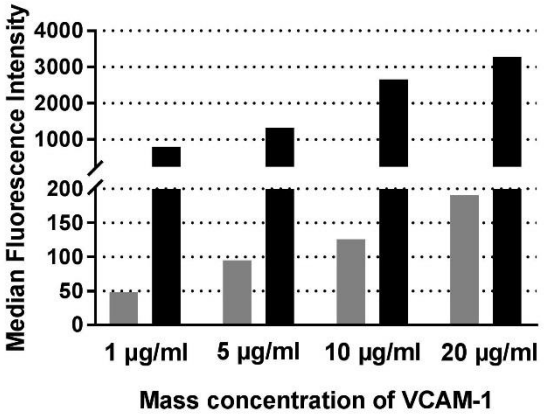

Figure 11. Optimization of VCAM1 concentration in soluble ligand binding assay with HEL cells. A single soluble ligand binding assay was performed as described above (*Figure 5*). Instead of the murine isoform, we used human VCAM1 at mass concentrations of 1, 5, 10, and 20 µg/ml. Black bars represent samples treated with manganese chloride, whereas grey bars depict untreated samples.

HEL cells were treated with JAK inhibitor I and NVP-BSK805 under the same conditions as in the static adhesion assay (*Figure 10*). However, neither inhibitor resulted in reduced VCAM1 binding (*Figure 12*). While medians showed only slight changes that could be considered experimental variation (*Figure 12, left panel*), VCAM1 binding was sparsely, but consistently higher compared to control in each individual experiment with exception of one value in the NVP-BSK805 group (*Figure 12, right panel*). Thus, we consider a small effect of JAK2 inhibition fostering integrin affinity to VCAM1 possible, at least by JAK inhibitor I.

Complementary to the soluble VCAM1 binding assay, we used an anti-HUTS-21 antibody as an additional approach to study integrin affinity to VCAM1 in JAK2 inhibited HEL cells. Anti-HUTS-21 is an antibody that was described to specifically bind β_1-integrin, the best described ligand of VCAM1, in an open, high-affinity conformation that was associated with increased cell adhesion (Luque et al., 1996). We exposed HEL cells with and without JAK2 inhibitor treatment to PE-conjugated anti-HUTS-21 antibody in a flow cytometric assay. Neither JAK inhibitor I nor NVP-BSK805 led to consistent changes in surface levels of the HUTS-21 epitope (*Figure 13, left panel*).

As a last point, total surface expression of β_1-integrins was determined by flow cytometry (*Figure 13, right panel*). In three independent experiments, expression levels were reduced in HEL cells treated with JAK2 inhibitors as compared to controls. However, interexperimental variation

was considerably high making it difficult to estimate the effect size. Values for JAK inhibitor I and NVP-BSK805 were slightly reduced in two cases and markedly diminished in the third.

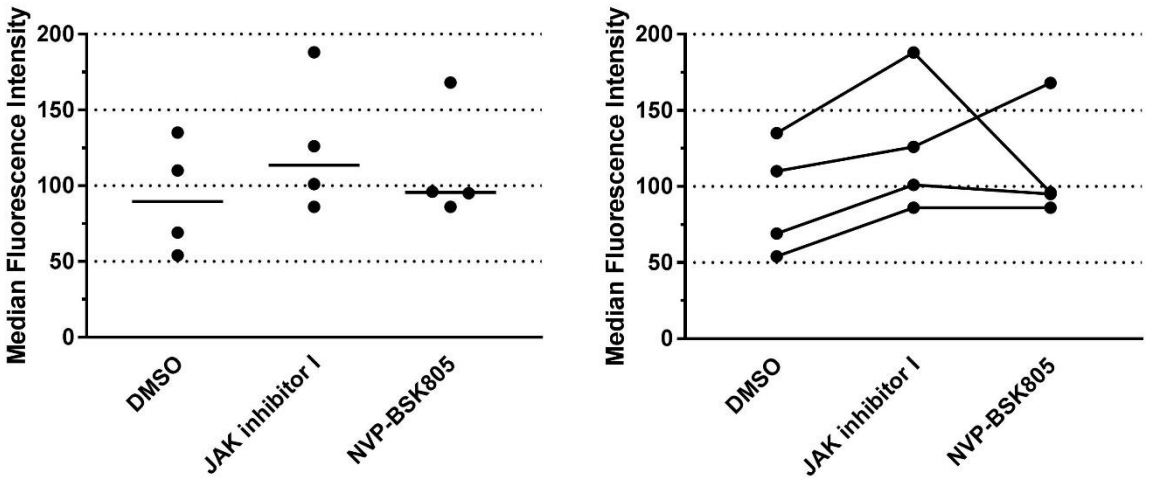

Figure 12. Soluble VCAM1 binding assay comparing HEL cells with and without pharmacological JAK2 inhibition. HEL cells were seeded at a density of $0.5 \cdot 10^6$ cells/ml in modified RPMI medium and treated with either DMSO, JAK inhibitor I ($\iota_{\text{JAK inhibitor I}}$ = 1 000 nmol/l), or NVP-BSK805 ($\iota_{\text{NVP-BSK805}}$ = 1 500 nmol/l). After 16 hours of incubation, soluble ligand binding assay was performed as described above (*Figure 5*) using human VCAM1 at a mass concentration of 5 μg/ml. Four independent experiments were performed. Results are depicted with bars representing medians (*left panel*) and with lines interconnecting values obtained in the same experiment (*right panel*).

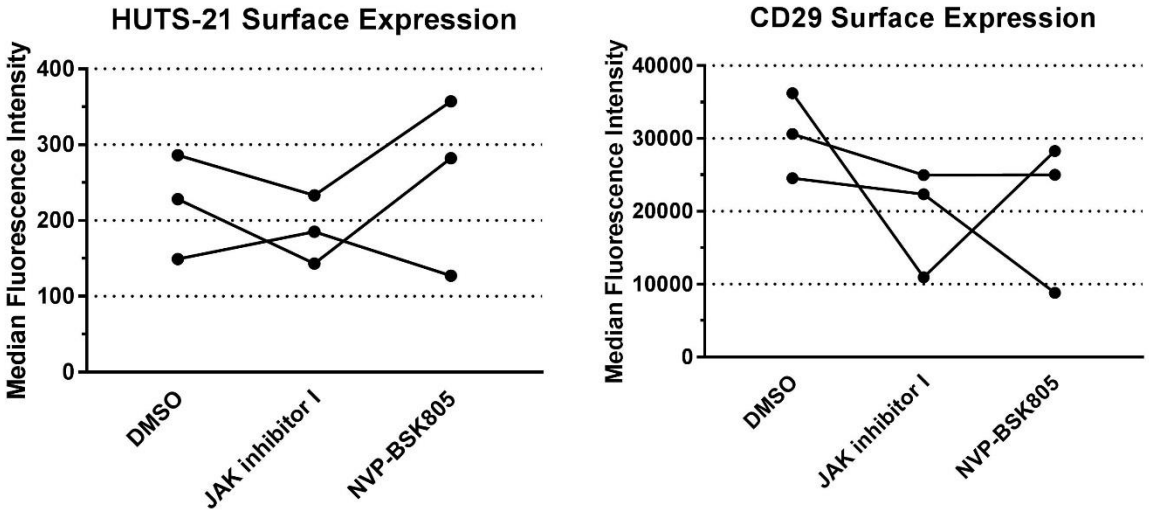

Figure 13. Flow cytometric quantification of high-affinity conformation-specific (HUTS-21) and total β_1-integrin (CD29) cell surface expression on HEL cells during pharmacological inhibition of JAK2. HEL cells were treated with JAK inhibitors for 16 hours as described above (*Figure 12*). Thereafter, $1 \cdot 10^6$ cells each were exposed to PE-conjugated anti-human HUTS-21, PE-conjugated anti-human CD29 (total) antibody, or respective isotype controls for 90 minutes at 4°C. Residual antibody was cleared by washing three times with HBSS/FBS. Dye-specific fluorescence was measured by flow cytometry. Values were corrected using isotype control signals. Three independent experiments were performed. Lines connect values of the same experiment. *Left panel.* HUTS-21 surface expression. *Right panel.* Total CD29 surface expression.

3.2.4. Genetic inhibition of JAK2 by shRNA-mediated knockdown

The second pillar of our endeavor to elucidate the relation between JAK2-V617F constitutive signaling and integrin-mediated adhesion in HEL cells was to genetically knockdown JAK2 by specific shRNA. We made use of lentiviral transduction to deliver pLKO.1-puro vectors expressing shRNA sequences targeting JAK2. After infection, HEL cells were kept under selective pressure with puromycin. While we strived for a long incubation period in order to increase selection of successfully transduced cells, the sudden onset of apoptosis after a few days proved to be an obstacle. This effect, which we attributed to the phenomenon of oncogenic addiction (Jain et al., 2002; Weinstein, 2002), was different amongst various shRNA sequences resulting in the incubation period to range from two to four days. Nevertheless, in one case even reduction to the shortest reasonable time of two days failed to prevent a fraction of cells from transitioning to apoptosis (*Figure 15*, shRNA D). The remainder of shRNA sequences did not significantly diminish cell viability (*Figure 15*). As cells were harvested on the respective days, all of them showed markedly reduced JAK2 expression (*Figure 14*). For all experiments, cells were infected with lentivirus in the afternoon of Day 0 and incubated overnight. In the morning of Day 1, virus medium was exchanged for standard cell culture medium and cells were given time to recover from transduction stress. Puromycin was added on Day 2, except in the experiment using shRNA D. Cells were harvested on Day 2 (shRNA D), Day 3 (shRNA C) and Day 4 (shRNA A + B). On the respective day, cells were seeded in medium with serum-reduced RPMI medium for 3.5 hours to minimize extrinsic effects by residual serum ingredients. Afterwards, all assays were carried out in parallel (static adhesion, soluble VCAM1 binding, integrin antibody assays).

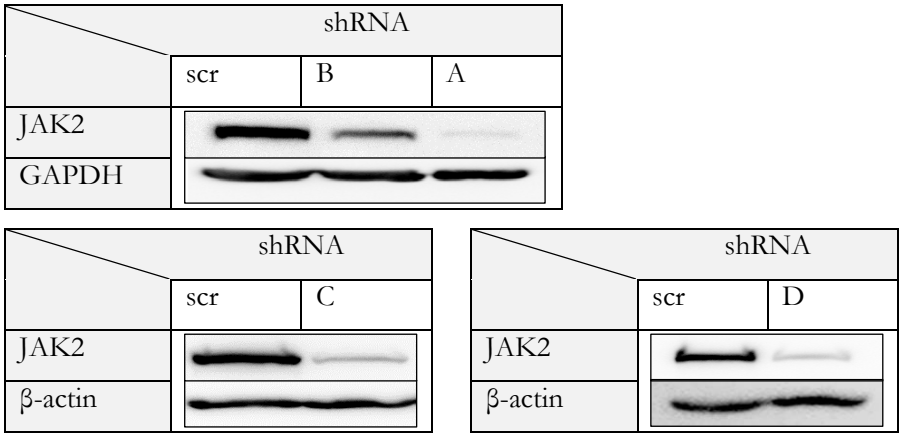

Figure 14. Western blots showing shRNA-mediated knockdown of JAK2 in HEL cells. HEL cells were transduced with lentivirus that delivered one out of four different JAK2-specific shRNA sequences (A-D), or a scrambled control. After one day of rest, cells were selected with puromycin at a mass concentration of 2 μg/ml. Cells were harvested on Day 2 (shRNA D, *lower right panel*), Day 3 (shRNA C, *lower left panel*) and Day 4 (shRNA A + B, *upper panel*). JAK2 expression was determined by Western blotting. GAPDH or β-actin served as loading control.

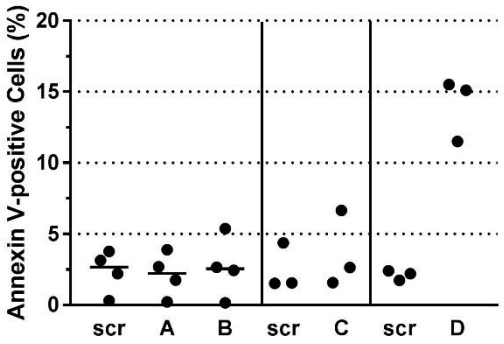

Figure 15. Apoptosis as quantified by Annexin V-positivity in HEL cells expressing JAK2 shRNA. HEL cells were prepared as described in *Figure 14* to knockdown JAK2. Thereafter, they were incubated in serum-reduced RPMI medium for 3.5 hours, and then stained with PE-conjugated Annexin V antibody and SYTOX™ Blue. Dye-specific fluorescence was determined by flow cytometry. The number of SYTOX™ Blue-positive cells was negligibly low in all samples (data not shown). In contrast to SYTOX™, which stains cells with a compromised cell membrane, i.e. dead cells, Annexin V stains phosphatidylserine that is exposed on the membranes of apoptotic cells.

3.2.5. JAK2 knockdown reduces integrin-mediated adhesion in HEL cells

HEL cells were treated with four different shRNA sequences against JAK2 and a scrambled control, respectively, as described above. Adhesion was assessed by static adhesion assay (*Figure 16*). Knockdown of JAK2 by all four shRNA sequences was associated with a reduction in adhesion compared to control, although to different extent. Concerning the marked effect of shRNA D, the associated induction of apoptosis (*Figure 15*) should be considered as possible bias.

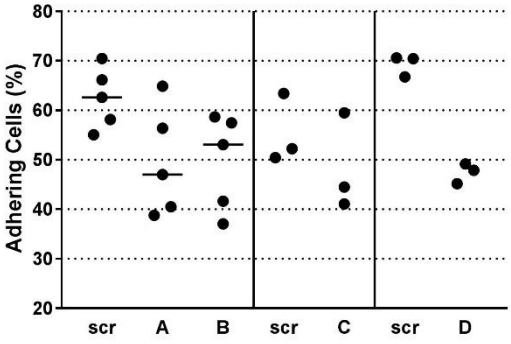

Figure 16. Static adhesion assay in HEL cells expressing various JAK2-specific shRNA species. HEL cells were prepared as described in *Figure 13* to knockdown JAK2. Thereafter, they were incubated in serum-reduced RPMI medium for 3.5 hours. Thereafter, a static adhesion assay was performed as described in *Figure 4* using human VCAM1 at a mass concentration of 1 µg/ml. Bars represent medians. Five (shRNA A + B) and three (shRNA C + D) independent experiments, respectively, were performed.

3.2.6. Different JAK2 shRNA sequences do not uniformly affect integrin affinity and expression in HEL cells

According to our hypothesis, integrin affinity to VCAM1 was expected to diminish upon JAK2 knockdown as did adhesion of HEL cells. The results of the soluble VCAM1 binding assay however were inconclusive (*Figure 17*). While results for shRNA B were potentially indicative of a reduction of integrin affinity compared to control, there seemed to be no effect of shRNA A and C. Due to restricted viability and proliferation, only a limited number of cells treated with shRNA D were available for experiments after two days. Therefore, we did not perform the soluble VCAM1 binding assay in favor of the static adhesion assay. Noticeably, the experiment with shRNA C – including the scrambled sequence – clearly yielded higher values than the other experiments. This can most likely be ascribed to the circumstance that the former was performed about half a year later and with a different batch of VCAM1 chimera protein.

We also determined surface expression of high-affinity β_1-integrin by anti-HUTS-21 antibody binding. HEL cells treated with shRNA A and B showed a trend towards reduced HUTS-21 epitope exposure which was more pronounced in samples treated with shRNA B (*Figure 18, left panel*). Samples treated with shRNA C and D did not contain enough cells to perform the anti-HUTS-21 binding assay in addition to the other assays.

For interpretation of integrin affinity and HUTS-21 exposure, total β_1-integrin surface expression had to be taken into consideration. Surface expression of β_1-integrin remained unaffected by shRNA A, was reduced by shRNA B and showed a trend towards reduction by shRNA C and D (*Figure 18, right panel*). Therefore, it is likely that the pronounced change in integrin affinity (soluble VCAM1 binding and HUTS-21 expression) induced by shRNA B was at least in part caused by a downregulation of β_1-integrin surface expression.

Taken together, the experiments did not show a consistent effect of shRNA-mediated knockdown of JAK2 on integrin affinity to VCAM1 in HEL cells. Although cells treated with shRNA A showed a modest decrease in HUTS-21 epitope exposure, without concomitant changes in total β_1-integrin surface expression, the majority of shRNA experiments together with the results obtained from pharmacological inhibition of JAK2 do not point towards a consistent interdependence between disruption of JAK2 signaling and integrin affinity in HEL cells. Nevertheless, they show that disruption of JAK2-V617F signaling impairs adhesion without decreasing integrin affinity to a similar extend. Therefore, it seems likely that a different mechanism underlies the adhesion phenotype observed in JAK2-V617F-positive HEL cells.

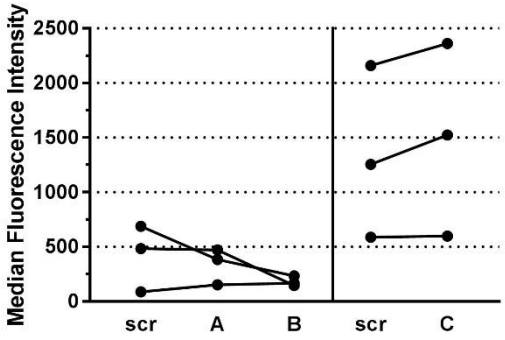

Figure 17. Soluble VCAM1 binding assay in HEL cells expressing JAK2 shRNA species. HEL cells were prepared as described in *Figure 14* to knockdown JAK2. Thereafter, they were incubated in serum-reduced RPMI medium for 3.5 hours. Soluble VCAM1 binding assay was performed as described above (*Figure 5*) using human VCAM1 at a mass concentration of 5 µg/ml. Three independent experiments were performed. Lines connect values of the same experiment.

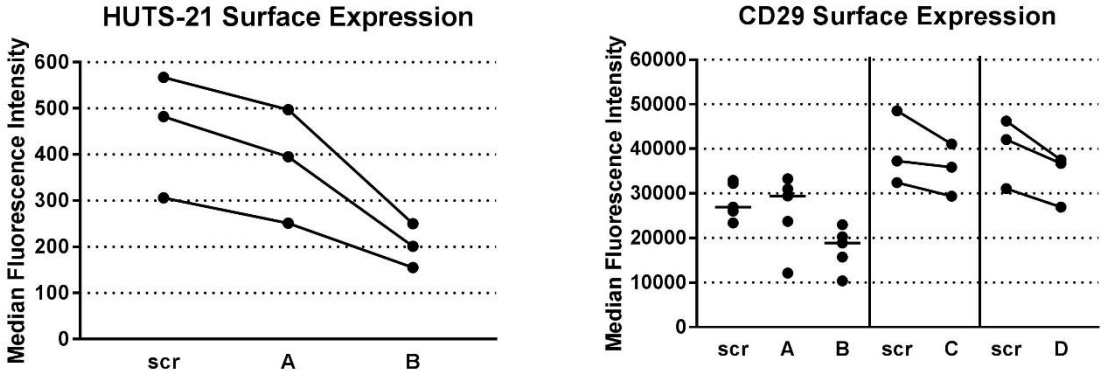

Figure 18. Flow cytometric quantification of high-affinity conformation-specific (HUTS-21) and total β_1-integrin (CD29) cell surface expression in HEL cells treated with JAK2-specific shRNA. HEL cells were prepared as described in *Figure 14* to knockdown JAK2. Thereafter, they were incubated in serum-reduced RPMI medium for 3.5 hours. Cells were stained for flow cytometric assay as mentioned in *Figure 13*. Lines connect values of the same experiment. Five (CD29 surface expression for shRNA A + B) and three independent experiments, respectively. *Left panel.* HUTS-21 surface expression. *Right panel.* Total CD29 surface expression.

4. Discussion

Here, we first discuss the results presented in this dissertation with respect to their applicability and limitations. Moreover, we involve further progress that our group has made recently. Finally, we discuss possible implications of those new findings for future research.

4.1. The present study

In this study, we report that activation of JAK2 by the V617F mutation is responsible for promoting high adhesion to VCAM1, which is mediated by integrins, in hematopoietic cell lines. This has been demonstrated in murine BaF3 cells where overexpression of JAK2-V617F resulted in increased adhesion compared to JAK2-WT, as well as in human HEL cells, where adhesion was impaired when inherent JAK2-V617F signaling was abolished by pharmacological or genetic inhibition. By demonstrating increased binding of soluble VCAM1 to JAK2-V617F-positive BaF3 cells, we furthermore provide a potential mechanism of increased integrin-mediated adhesion, namely enhancement of integrin affinity. This enhanced affinity does not seem to be induced by secretion of extracellular mediators by JAK2-V617F-positive cells since affinity to VCAM1 remained unchanged in JAK2-WT cells that were cocultured together with JAK2-V617F cells. These results suggest that aberrant JAK2-V617F signaling directly induced increased integrin affinity to VCAM1 in the mutant cells.

In the following, we discuss some aspects and limitations of our experiments that merit particular attention.

4.1.1. Increased adhesion is induced by overexpression of JAK2-V617F

All our cell lines expressed non-physiological levels of JAK2-V617F. In BaF3 and 32D cells, overexpression was conferred by a MSCV promotor and HEL cells are known to exhibit an ~10-fold genetic amplification of JAK2 (Quentmeier et al., 2006). On the one hand, it therefore remains to be demonstrated as to whether physiological levels of JAK2-V617F increase integrin-mediated adhesion at all. On the other hand, since marked phenotypic differences have been observed in MPN mouse models depending on the expression level of JAK2-V617F relative to JAK2-WT expression (Tiedt et al., 2008), it is tempting to speculate as to whether adhesion to VCAM1 changes in intensity depending on JAK2-V617F expression level. In the study of (Tiedt et al., 2008), JAK2-V617F expression below JAK2-WT level resulted in an ET phenotype and increased spleen weight, while higher expression of JAK2-V617F was associated with a PV

phenotype and an even larger spleen. Possibly, increasing level of JAK2-V617F relative to JAK2-WT could be associated with progressive integrin-mediated adhesion and thus contribute to the differences observed for example by increasing aberrant homing of hematopoietic progenitors to the spleen. This also is a relevant issue in MPN patients because they exhibit phenotypic differences depending on their JAK2-V617F mutation status being heterozygous or homozygous (see *Introduction*).

4.1.2. Integrins beyond β_1-integrin could contribute to adhesion induced by JAK2-V617F

Herein, we used VCAM1 to assess integrin-mediated adhesion. According to (Humphries, Byron, & Humphries, 2006), ligands of VCAM1 comprise integrins $\alpha_4\beta_1$, $\alpha_9\beta_1$, $\alpha_4\beta_7$, and $\alpha_D\beta_2$. Among them, $\alpha_4\beta_1$-integrin (VLA4) has been most intensively studied and is recognized as the predominant integrin mediating adhesion of mature leukocytes (Herter & Zarbock, 2013). We hence focused on $\alpha_4\beta_1$-integrin when we tried to shed first light upon the mechanisms underlying integrin-mediated adhesion to VCAM1 in JAK2-V617F-positive cells by assessing the surface expression of β_1-integrin as well as the exposure of the HUTS-21 epitope, which is specific for the activated conformation of β_1-integrin. However, we cannot exclude that other heterodimers like $\alpha_4\beta_7$-integrin and $\alpha_D\beta_2$-integrin were involved in the effect observed. To our knowledge, expression of $\alpha_4\beta_7$-integrin has been demonstrated in monocyte-like cells (Fernekorn, Butcher, Behrends, Hartz, & Kruse, 2004), in NK cells (Perez-Villar et al., 1996), and in eosinophils (Walsh, Symon, Lazarovits, & Wardlaw, 1996). Furthermore, it has been shown to play a critical role in integrin-mediated homing of HSCs to the bone marrow (Katayama, Hidalgo, Peired, & Frenette, 2004). $\alpha_D\beta_2$-integrin is expressed on most human polymorphonuclear cells and monocytes (Miyazaki et al., 2014) and is able to bind to VCAM1 (Van der Vieren et al., 1999), although its role in leukocyte-endothelial interaction remains to be further elucidated. Future studies investigating the influence of JAK2-V617F on integrins should therefore involve $\alpha_D\beta_2$-integrin and $\alpha_4\beta_7$-integrin.

4.1.3. JAK2-V617F increases integrin affinity and involves additional mechanisms of enhancing adhesion

Principally, the avidity of integrin-mediated cell adhesion depends on the affinity of individual heterodimers to their ligands (affinity) on the one hand, and on redistribution of integrins on the cell surface and clustering in the membrane areas involved in adhesion contacts (valency) on the other (Ley et al., 2007).

In our study, we report that in BaF3 cells integrin affinity to VCAM1 was increased in JAK2-V617F overexpressing cells as determined by a soluble ligand binding assay. At the same time, surface expression of β_1-integrin remained unchanged. Because β_1-integrin plays a major role in adhesion of lymphocytes (Herter & Zarbock, 2013), it is thus likely that, in lymphoid BaF3 cells, enhancement of β_1-integrin affinity is the main reason for increased adhesion induced by JAK2-V617F. This notion is further supported by studies showing that JAK2 activation following chemokine stimulation induced β_1-integrin-mediated adhesion of lymphocytes (Mirenda et al., 2015; Montresor et al., 2013, 2015). We cannot exclude, however, that enhancement of affinity or expression of $\alpha_4\beta_7$- and $\alpha_D\beta_2$-integrin, or increased valency also contributed to the adhesion phenotype observed. Interestingly, it has been shown that stimulation with particular chemokines can increase affinity of $\alpha_4\beta_7$-integrin to MAdCAM1 and suppress its affinity to VCAM1, and vice-versa, thereby providing a potential mechanism of tissue-specific homing of lymphocytes (Sun et al., 2014). Likewise, JAK2-V617F did not necessarily have to increase overall affinity of a particular integrin heterodimer, but could specifically enhance its affinity to VCAM1.

In HEL cells, impaired adhesion upon disruption of JAK2-V617F signaling was not accompanied by decreased integrin affinity as shown by soluble VCAM1 binding and HUTS-21 epitope exposure – except in the case of shRNA B, which probably was a result of decreased β_1-integrin surface expression as already discussed. Since β_1-integrin surface expression was also slightly reduced by the other shRNA sequences and the pharmacological JAK inhibitors, it probably contributed to the effect observed. Because it has not been a marked reduction, other mechanisms like decreased expression or affinity of $\alpha_4\beta_7$- and $\alpha_D\beta_2$-integrin and changes in valency could have been involved.

4.1.4. The static adhesion assay models one aspect of a complex physiological process

Finally, we put emphasis on the limitations of our static adhesion assay. In the context of MPNs we are ultimately interested in leukocyte arrest on platelets and endothelium as a prerequisite of presumable leukocyte-induced thrombus formation on the one hand and for endothelial transmigration as an essential step of aberrant homing of hematopoietic progenitors involved in extramedullary hematopoiesis on the other. In this highly complex process, integrin-mediated firm arrest following selectin-mediated capture plays a pivotal role. The static adhesion assay models this firm arrest and the resistance to shear stress. However, it does not reflect several crucial details of the physiological process.

First, capture of blood-borne leukocytes and rolling involve several different receptor ligand interactions beyond integrins that do not only form physical bonds between cells, but also

activate a complex signaling machinery upon ligation that is required for further steps of slow rolling, firm adhesion and extravasation. For example, in the case of neutrophil rolling on E-selectin, leukocyte PSGL1 and L-selectin are capable of forming catch-bonds, which are required for inducing neutrophil arrest by activating β_2-integrin (Morikis et al., 2017).

Second, during rolling, leukocytes are exposed to chemokines presented on the endothelium that activate integrins via GPC-Rs – a process that is necessary to prevent detachment under shear flow (Herter & Zarbock, 2013). Because JAK2 has been shown to contribute to CXCL12-induced VLA4 and LFA1 activation (Montresor et al., 2013, 2015), JAK2-V617F could possibly render this pathway hypersensitive for CXCL12 as it was shown for EPO-dependent proliferation (Dupont et al., 2007; Levine et al., 2005).

Third, although the static adhesion assay models the process of integrin-mediated adhesion strengthening following a weak selectin-mediated bond, the adhesion phenotype observed in this assay does not necessarily translate to shear stress resistance *in vivo*. For example, in the study of (Giagulli et al., 2006), neutrophils with knockout of SRC family kinases FGR and HCK showed no difference in static adhesion compared to control cells, but a reduction of sustained arrest to inflamed mouse muscle venules *in vivo*. From experiments that demonstrated impaired spreading upon adhesion, but unchanged integrin affinity in a soluble ligand binding assay, the authors concluded that combined FGR and HCK deficiency did not affect integrin affinity via inside-out signaling, but did hamper outside-in signaling following integrin ligation that is responsible for further strengthening and sustaining adhesion. This study exemplifies that several processes are required for sustained leukocyte-endothelium adhesion *in vivo,* of which integrin-ligand interaction modelled in the static adhesion assay is only one important step.

Taken together, the static adhesion assay models one important step involved in the complex process of leukocyte adhesion to platelets and endothelium. It is thus reasonable to assume, but not certain, that increased integrin-mediated static adhesion in JAK2-V617F-positive cells translates to adhesion *in vivo*. Therefore, it is urgently required to reassess the effect of JAK2-V617F on adhesion to VCAM1 in a more physiological model that reflects the involvement of diverse adhesion receptors, the chemokine-induced inside-out activation of integrins, and the resistance to continuous or pulsed shear flow of the blood stream.

4.2. Progress of our group

The majority of experiments presented in this dissertation were conducted during a twelve-month research stay in 2013/2014. For the submission of a doctoral thesis however, the regulations of the Medical Faculty of the Otto-von-Guericke University Magdeburg require the successful

completion of the German Licensing Examination for Physicians, which the applicant passed in November 2017. Between the completion of the experiments presented herein and the submission of the written dissertation in 2018, our group could thus make further progress in the understanding of integrin-mediated adhesion induced by JAK2-V617F. Some results have been published along with data from this dissertation (Gupta et al., 2017). Because this report contributes to an improved understanding of the issues discussed in this dissertation, we briefly summarize these published results in the following.

In accordance with the present data from the BaF3 model, our group[20] reported increased adhesion of murine 32D cells to VCAM1 in a static adhesion assay when cells were overexpressing JAK2-V617F, compared to JAK2-WT. These finding was accompanied by a marked increase in soluble VCAM1 binding, but not with a change in β_1-integrin surface expression. Moreover, the 9EG7 epitope of murine β_1-integrin, which is only exposed in the open, high-affinity conformation, was expressed at higher levels in JAK2-V617F-positive cells. Importantly, increased adhesion and increased VCAM1 binding could both be reversed by pharmacological inhibition of JAK2 kinase activity. Together, these findings underscored that overexpression of JAK2-V617F leads to increased integrin-mediated adhesion to VCAM1 *in vitro* and that enhancement of β_1-integrin affinity likely contributes to this effect.

In a second step, our group desired to find out as to whether these findings translated to JAK2-V617F-positive MPN patients. Indeed, primary granulocytes from MPN patients harboring the JAK2-V617F mutation showed enhanced adhesion to immobilized VCAM1, increased affinity to soluble VCAM1 and no change in β_1-integrin surface expression, compared to healthy donors. In line with the report of (Montresor et al., 2013), who showed the activation of RAP1 downstream of JAK2 in CXCL12-induced integrin-mediated adhesion of lymphocytes, our group found an increase in RAP1 activation in JAK2-V617F-overexpressing 32D cells. This activation was dependent on JAK2-V617F signaling since it decreased upon pharmacological inhibition of JAK2. An upregulation of RAP1 activity was also found in JAK2-V617F-positive MPN patients compared to healthy donors. Inhibition of RAP1 activity by the pharmacological inhibitor farnesylthiosalicylic acid-amide (FTS-A) or by RNAi targeting RAP1 led to reduced adhesion to VCAM1.

In summary, these findings corroborated that JAK2-V617F induces integrin-mediated adhesion to VCAM1 as assessed by a static adhesion assay. Importantly, this report provided additional insights beyond the scope of the present dissertation.

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[20] The term *our group* indicates that the work described in this chapter was performed by a group of scientists as described in the manuscript (Gupta et al., 2017), and is used in contrast to *we* in the rest of this dissertation, which indicates work performed by the author.

First, it was demonstrated that the induction of adhesion by JAK2-V617F observed in hematopoietic cell lines translates to primary granulocytes of JAK2-V617F-positive MPN patients. It remains to be investigated as to whether this finding in patients is due to a direct effect of JAK2-V617F or whether it is induced by the environment of systemic inflammation. In this context, it would be interesting to involve JAK2-V617F-negative MPN patients harboring different driver mutations.

Second, the report provided further support for the concept that JAK2-V617F induces adhesion by enhancing integrin affinity to VCAM1 by showing the induction of the 9EG7 epitope in JAK2-V617F cells and by demonstrating the parallel decline of adhesion and soluble ligand binding upon pharmacological JAK inhibition. For a more complete understanding of these processes, future studies need to assess the involvement of the other integrin ligands of VCAM1, as well as changes in integrin valency.

Third, by revealing that RAP1 is activated downstream of JAK2-V617F and mediates enhanced adhesion to VCAM1, the report shed first light on the aberrant signaling pathways involved in JAK2-V617F-dependent integrin activation. It remains to be demonstrated as to whether the rest of the signaling cascade in JAK2-V617F mutant cells also parallels the processes described downstream of wild-type JAK2 in lymphocytes, including activation of RHO-A, PLD1, and VAV1 (Montresor et al., 2013). Importantly, the incipient elucidation of the signaling pathway disclosed a first target for potential therapeutic intervention. It is thus crucial to delineate the role, if any, of JAK2-V617F-induced adhesion to VCAM1 in MPN pathophysiology. Such studies must involve more physiological models of adhesion including *ex vivo* flow chambers, *in vivo* microscopy, and animal models of arterial and venous thrombosis. Further potential connections between enhanced integrin-mediated adhesion to VCAM1 and MPN pathophysiology are discussed in the following outlook.

4.3. Outlook

In this final chapter, we discuss important questions that emerge from the results of this dissertation with respect to the existing scientific evidence and provide educated guesses that should be considered in future research.

4.3.1. Could integrin-mediated adhesion be induced by other MPN driver mutations?

Based on the phenotypic similarities of MPN patients, it is principally possible that all MPN driver mutants behave similarly with respect to the signaling consequences induced and that mutant MPL

and mutant CALR lead to similar alterations in adhesion to VCAM1 as JAK2-V617F. The fact that the signaling effects of all three mutations involve activation of JAK2 supports this notion. Furthermore, activation of MPL by TPO stimulation was shown to increase β_1-integrin-adhesion in hematopoietic cells (Cui et al., 1997; Gotoh et al., 1997). Moreover, direct interaction with integrins has been described for CALR. It has been shown to associate the cytoplasmic domain of α-integrins and to be essential for integrin-mediated adhesion of mouse embryonic stem cells and fibroblasts (Coppolino et al., 1997). Whether it plays a similar role for leukocyte integrins remains unclear.

However, MPN patients with different driver mutations differ significantly with respect to their clinical phenotype, including thrombotic risk, indicating differential signaling consequences with functional relevance, as we have discussed in the introduction. Moreover, even different mutations of the same protein, JAK2, showed different signaling effects and phenotypes in MPN mouse models mediated by differential binding to cytokine receptors (Yao et al., 2017). When considering the plethora of signaling events required for *in vivo* adhesion, it therefore seems likely that it is differentially affected by the driver mutations.

4.3.2. Could increased leukocyte adhesion to VCAM1 contribute to high cardiovascular risk of MPN patients?

More than from venous thrombosis cardiovascular morbidity and mortality in MPN patients result from arterial occlusion in the form of stroke and myocardial infarction (Carobbio et al., 2011; Hultcrantz et al., 2015). It has thus been speculated that atherosclerotic processes are accelerated in MPN patients due to sustained systemic inflammation (Hasselbalch, 2012).

Early atherosclerotic lesions of the arterial intima are maintained and promoted by immune cells recruited from the peripheral blood, most prominently foam cells which are inflammatory transformed monocytes. In line with increased leukocyte recruitment, animal models of atherosclerosis showed upregulation of integrin ligands MAdCAM1, VCAM1 and ICAM1 in atherosclerotic lesions (Iiyama et al., 1999; Nakashima, Raines, Plump, Breslow, & Ross, 1998; Zhi et al., 2014). VCAM1 was shown to be particularly involved in early atherosclerotic lesions (Cybulsky et al., 2001) and to be largely expressed with restriction to such lesions, whereas ICAM1 was expressed more broadly and even in lesion-protected regions (Iiyama et al., 1999). Apart from this upregulation of integrin ligands on atherosclerotic endothelium, the expression of leukocyte integrins themselves has shown to be enhanced in murine models of atherosclerosis. In APO-E-deficient mice, surface expression of $\alpha_4\beta_7$-integrin was increased on peripheral blood

lymphocytes (Zhi et al., 2014), as was surface expression of β_2-integrin on hematopoietic stem and progenitor cells (HSPCs) in LDL-R-deficient mice (X. Wang et al., 2015).

Moreover, this increased cell surface decoration with adhesion molecules has been shown to be accompanied by enhanced interaction of leukocytes and endothelium. (Ramos et al., 1999) showed that monocytic cell lines rolled and attached to endothelium of isolated carotid arteries of mice with atherosclerosis, but not control mice, and that rolling velocity increased upon antibody blockade of VCAM1. (Zhi et al., 2014) suggested that $\alpha_4\beta_7$-integrin-mediated leukocyte-plaque interaction contributed to plaque growth based on their observation that APO-E/β_7-integrin double-knockout mice developed a markedly decreased atherosclerotic plaque area induced by a Western diet compared to APO-E-knockout mice. (X. Wang et al., 2015) showed increased, β_2-integrin-dependent homing of HSPCs from atherosclerotic mice (LDL-R-deficient and high fat diet compared to LDL-R-deficient and normal chow diet) to injured arteries resulting in more severe inflammation *in vivo*.

Taken together, these studies suggest that integrin-mediated recruitment of leukocytes to early atherosclerotic lesions occurs in animal models of systemic atherosclerosis and contributes to plaque expansion[21]. Increased adhesion of JAK2-V617F-positive leukocytes to VCAM1 expressed on early atherosclerotic lesions could thus increase leukocyte recruitment and accelerate plaque expansion.

As a first step to evaluate this hypothesis, it is required to define which cell types show increased integrin-mediated adhesion to VCAM1 in MPN patients beyond the involvement of neutrophils that our group has already demonstrated (Gupta et al., 2017). As mentioned above, the involvement of monocytes would be particularly interesting in the context of foam cell formation in atherosclerotic processes. Circulating HSPCs have been connected to neointima formation after vessel injury, but their role remains controversial (Hagensen, Shim, Thim, Falk, & Bentzon, 2010; X. Wang et al., 2015). Increased adhesion of JAK2-V617F-positive neutrophils are of particular importance in the context of venous thrombosis in MPN patients, because neutrophils have been shown to be indispensable for thrombus propagation in a physiological model of murine deep vein thrombosis (von Brühl et al., 2012).

More than only assessing changes in adhesion to VCAM1, it will also be crucial to find out whether JAK2-V617F affects further integrin-ligand interactions. For example, lymphocyte adhesion and homing mediated by LFA1 ($\alpha_L\beta_2$-integrin)-binding to ICAM1 has also been shown to be JAK2-dependent (Montresor et al., 2013). Accordingly, overexpression of JAK2-V617F in

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21 This recruitment is of particular importance for early atherosclerotic lesions (Robbins et al., 2012) and for exacerbation following certain stimuli as myocardial infarction (Dutta et al., 2012). In contrast, in more advanced atherosclerosis, local proliferation of macrophages in atheromata has been shown to outweigh the recruitment of blood-borne monocytes (Robbins et al., 2013).

32D cells led to increased static adhesion to ICAM1 in our laboratory (unpublished data). Thus, JAK2-V617F can trigger increased adhesion mediated by multiple different integrin heterodimers at the same time. To resolve which integrins are involved in JAK2-V617F-induced adhesion in a particular cell type *in vivo* is not only of importance in the context of thrombus formation, but especially when considering that increased adhesion to a particular integrin ligand could lead to differential homing (Sun et al., 2014).

A second step must involve *in vivo* models of adhesion and of thrombosis, as already mentioned above.

4.3.3. Could enhanced adhesion to VCAM1 link extramedullary hematopoiesis to vascular events in MPNs?

Extramedullary hematopoiesis is a core hallmark of MPNs that adversely affects patients depending on its localization, most frequently in the form marked splenomegaly leading to abdominal discomfort, pain, and early satiety (Anderson et al., 2015; Harrison et al., 2017). The ability of HSPCs to egress from the bone marrow niche and to cycle in the peripheral blood has been well described, for example in the context of physiological circadian oscillations (Méndez-Ferrer, Lucas, Battista, & Frenette, 2008) and release following a number of myeloproliferative stimuli, most prominently G-CSF (Chiu et al., 2015). Together with the frequent finding of concordant JAK2-V617F mutation status of HSPCs in bone marrow and spleen of MPN patients (Hsieh et al., 2007; Konoplev, Hsieh, Chang, Medeiros, & Lin, 2007) it stands to reason that, in MPNs, mutant HSCPs are released from the bone marrow and settle in the spleen and other extramedullary organs, although to our knowledge, this has not been directly demonstrated.

HSPC homing to and release from the bone marrow critically depends on integrin-mediated adhesion, particularly involving the interaction of $\alpha_4\beta_1$- and $\alpha_4\beta_7$-integrin with VCAM1 and MAdCAM1 (Hartz et al., 2011; Katayama et al., 2004; Murakami et al., 2016; Papayannopoulou, Priestley, Nakamoto, Zafiropoulos, & Scott, 2001). These processes are regulated by CXCL12 expression in the bone marrow, a potent activator of integrin inside-out signaling that increases integrin affinity (Katayama et al., 2006; Méndez-Ferrer et al., 2008), to which JAK2-V617F could possibly confer hypersensitivity (see above). Thus, if JAK2-V617F also enhanced adhesion of HSPCs in MPN patients, this could critically affect homing. Nevertheless, several factors would have to act together to explain that HSPCs are not simply retained in the bone marrow due to enhanced adhesion to VCAM1 (Papayannopoulou, Craddock, Nakamoto, Priestley, & Wolf, 1995), but that, conversely, they are released and preferentially home to the spleen instead. One potential explanation is explored by (Hart et al., 2016), who showed that VCAM1 expression was reduced

in the bone marrow of MF patients prior to allogeneic HSCT, compared to AML patients, concomitant with poor engraftment. This study indicates that the expression of adhesion molecules in a particular niche can be altered in MPN patients thereby possibly contributing to changes in HSPC homing.

Interestingly, splenic myelopoiesis has recently been demonstrated to play an important role in atherosclerosis. (Robbins et al., 2012) found that HSCs progressively relocated from the bone marrow to the spleen in mouse models of atherosclerosis. In the spleen, they expanded and differentiated to neutrophils and monocytes which were released to the peripheral blood. Importantly, spleen-born monocytes homed to atherosclerotic lesion where they gave rise to fully functional foam cells being at least as, if not more, inflammatory as the bone marrow-derived foam cells contributing to the lesions. Moreover, the authors showed that a very similar splenic production of neutrophils and monocytes could be induced by peritoneal injection of lipopolysaccharide, a different inducer of systemic inflammation. Based on this observations, it is tempting to speculate as to whether cells produced by extramedullary hematopoiesis in MPN patients, who are known to suffer severe systemic inflammation, also contribute to atheromata thereby possibly accelerating systemic atherosclerosis.

Intriguingly, the group went on to show that splenic myelopoiesis was critically dependent on VCAM1 because splenic macrophages retained HSCs in the spleen by VCAM1-mediated adhesion (Dutta et al., 2015). When they silenced VCAM1 *in vivo*, myeloid cell numbers in atheromata of APO-E-deficient mice decreased and local monocyte-mediated inflammation following myocardial infarction was mitigated. Increased adhesion to VCAM1 of HSCs in MPN patients, that remains to be demonstrated, could thus possibly enhance splenic monocytopoiesis and foster inflammatory processes of the cardiovascular system.

5. Summary

This dissertation is written in English as an exemption following paragraph 6, subparagraph 4 of the doctoral regulations of the Medical Faculty, Otto von Guericke University. The necessary permission is attached in the supplements. In accordance with the paragraph mentioned, we provide a German summary in addition to the English version. Please note that both texts have been written independently, i.e. not as direct translations.

5.1. English summary

In this study, we investigated the effects of JAK2-V617F – a constitutively active mutant of the intracellular tyrosine kinase JAK2 – on integrin-mediated adhesion and affinity to VCAM1 in hematopoietic cell lines. In the first part, we employed an overexpression model of JAK2-V617F and JAK2-WT, respectively, in murine BaF3 cells. In the second part, we used HEL cells expressing the mutant endogenously and abrogated aberrant signaling activity by treatment with small molecular inhibitors of JAK2 or with JAK2-specific shRNA.

Our main finding is that JAK2-V617F activity was consistently associated with higher levels of cell adhesion to VCAM1 – compared to overexpression of JAK2-WT in BaF3 cells or to sham-treated HEL cells. With regard to integrin affinity, the models featured different results. On the one hand, in BaF3 cells, overexpression of JAK2-V617F was associated with increased affinity to VCAM1 as assed by soluble ligand binding. This change was not due to increased surface expression of β_1-integrin. When 32D JAK2-V617F cells – a myeloid cell line that exhibited increased adhesion and affinity to VCAM1 upon overexpression of JAK2-V617F similar to BaF3 cells – where cocultured together with 32D JAK2-WT cells, the latter showed no change in soluble VCAM1 binding compared to cells from separate culture. This indicated that enhancement of integrin adhesion and affinity in JAK2-V617F-positive cells was not mediated by secretion of extracellular mediators, but by an intracellular mechanism. In HEL cells, on the other hand, neither total integrin affinity to VCAM1 nor β_1-integrin activity (by HUTS-21 epitope exposure) were consistently reduced when JAK2-V617F signaling was disrupted by pharmacological or genetic inhibition. Since β_1-integrin surface expression was slightly reduced in some, but not all conditions, it might have contributed to impaired adhesion.

In summary, we report elevated integrin-mediated adhesion to be a feature of JAK2-V617F-positive cells. Because high thrombotic risk and extramedullary hematopoiesis of MPN patients could in part be mediated by this effect, it is desirable to investigate as to whether our findings translate to more physiological models.

5.2. German summary

In der vorliegenden Dissertation untersuchten wir den Einfluss von JAK2-V617F – einer konstitutiv aktiven Mutante der intrazellulären Tyrosinkinase JAK2 − auf die durch Integrine vermittelte Zelladhäsion zu VCAM1 in hämatopoetischen Zelllinien. Im ersten Teil der Arbeit nutzen wir dafür ein JAK2-V617F-Überexpressionsmodell in murinen BaF3-zellen. Im zweiten Teil untersuchten wir humane HEL-zellen, die die Mutation von sich aus tragen, indem wir die gestörte Signalaktivität des JAK2-V617F-proteins durch spezifische Inhibitoren oder durch shRNA-vermittelten Knockdown blockierten.

Das Hauptergebnis unserer Studie ist die Feststellung von erhöhter Zelladhäsion zu VCAM1 in JAK2-V617F positiven Zellen; in BaF3-zellen gegenüber einer JAK2-WT-überexpression und in HEL-zellen gegenüber einer entsprechenden Kontrollbehandlung. Die Affinität von Integrinen zu VCAM1 war in beiden Modellen unterschiedlich beeinflusst. Auf der einen Seite zeigten BaF3-zellen mit JAK2-V617F-Mutation eine erhöhte Affinität zu VCAM1, die nicht durch eine erhöhte Oberflächenexpression von β_1-integrin verursacht wurde. Ein Kokulturmodell von 32D-Zellen mit JAK2-V617F- und JAK2-WT-überexpression, das sich in Bezug auf Adhäsion und Affinität zu VCAM1 ganz ähnlich dem BaF3-modell verhielt, zeigte, dass die Integrinaffinität zu VCAM1 in Wildtypzellen durch die Kokultur mit Mutanten nicht beeinflusst wurde. Somit schien es wahrscheinlich, dass die Erhöhung von Adhäsion und Affinität zu VCAM1 in JAK2-V617F-zellen nicht durch die Sekretion von extrazellulären Mediatoren, sondern durch intrazelluläre Effekte vermittelt wurde. Auf der anderen Seite waren in der Zusammenschau der pharmakologisch oder genetisch JAK2-inhibierten HEL-zellen weder die Gesamtaffinität gegenüber VCAM1 noch die an der HUTS-21-Exposition gemessene Aktivität von β_1-integrin erniedrigt. Da die Oberflächenexpression von β_1-integrin in Experimenten mit einigen shRNAs und Inhibitoren vermindert war, jedoch nicht konsistent in allen, könnte dies möglicherweise zum Effekt reduzierter Adhäsion beigetragen haben.

Zusammenfassend konnten wir zuvorderst die erhöhte Adhäsion von JAK2-V617F-positiven Zellen zu VCAM1 feststellen. Da diese, wie wir in der Diskussion dargelegt haben, für zentrale Aspekte der MPN, nämlich erhöhtes kardiovaskuläres Risiko und extramedulläre Hämatopoese, mitverantwortlich sein könnte, halten wir es für lohnenswert, unsere Erkenntnisse durch physiologischere Modelle zu validieren und zu erweitern.

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Declaration

I make the following declaration in accordance with the doctoral regulations of the Medical Faculty of the Otto von Guericke University Magdeburg (§5(2)/Anlage 6).

Ehrenerklärung

Ich erkläre, dass ich die der Medizinischen Fakultät der Otto-von-Guericke-Universität zur Promotion eingereichte Dissertation mit dem Titel
Influence of the JAK2-V617F mutation on integrin-mediated adhesion to VCAM1 in murine and human cell lines in the context of classical Philadelphia-negative myeloproliferative neoplasms

in der
Universitätsklinik für Hämatologie und Onkologie,
Medizinische Fakultät der Otto-von-Guericke-Universität Magdeburg

mit Unterstützung durch
Herrn Prof. Dr. med. Thomas Fischer (Direktor der Klinik)

ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Ich übertrage der Medizinischen Fakultät das Recht, weitere Kopien meiner Dissertation herzustellen und zu vertreiben.

Dresden, 30.05.2018

Felix Carl Saalfeld

Curriculum vitae

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Education

2010 – 2017 Medicine, Otto-von-Guericke University (OvGU), Magdeburg, Germany
- First state examination 1.0 (excellent) – 2012[22]
- Second state examination 1.0 (excellent) – 2016
- Third state examination 1.0 (excellent) – 2017

2002 – 2010 Norbertusgymnasium, Magdeburg, Germany
- A levels – Overall score 1.2 (excellent) – 2010

Publications

Gupta, N., Edelmann, B., Schnoeder, T. M., **Saalfeld, F. C.**, Wolleschak, D., Kliche, S., … Fischer, T. (2017). JAK2-V617F activates β1-integrin-mediated adhesion of granulocytes to vascular cell adhesion molecule 1. *Leukemia*, *15*(January), 1–3. http://doi.org/10.1038/leu.2017.26

Scholarships

2011 – 2017 Scholar of the Konrad Adenauer Foundation
- Seminars on socially relevant topics from politics, economics, science, arts
- Monthly funding

2014 Scholar of the DFG Research Training Group 1167 during doctoral research stay

Relevant experience

2017 Tutor, German Student Academy[23], Braunschweig
- Scientific summer school for 15 outstanding students (age 16) by 2 tutors
- Designed and held a three-week course on radiation oncology with physicist

2016 Volunteer, Social-pastoral Center St. Peter, Duisburg-Marxloh (4 weeks)
- Diagnosed and treated patients without health insurance (under supervision)
- Managed drug donations and organized special treatment in private practices

2010 – 2014 Active member of student council of OvGU Medical Faculty Magdeburg
- Organized several information events on scientific training and scholarships
- Member of administrative committees (e.g. for filling of professorial chairs)

since 2005 Subsidiary office as organist in several church parishes (catholic and protestant)

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22 German grading system: excellent (1), above average (2), average (3), below average (4), poor (5)

23 Deutsche Schülerakademie

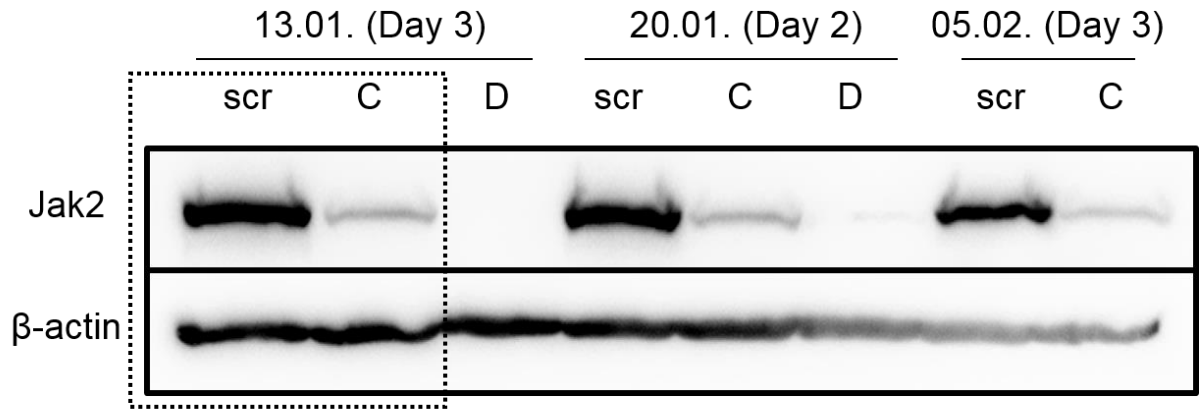
Herewith I sign my Curriculum vitae following the doctoral regulations of the Medical Faculty of the Otto von Guericke University Magdeburg.

Dresden, 30.05.2018

Felix Carl Saalfeld

Supplements

Supplemental figures



Supplemental figure 1. In the process of establishing the JAK2 knockdown experiments, we infected HEL cells with lentiviral particles and assessed knockdown efficiency and viability after 2, 3, and 4 days. Knockdown efficiency was determined by Western blotting comparing the results from different days after infection on one blot as shown here. The marked bands have are depicted in *Figure 14. Western blots showing shRNA-mediated knockdown of JAK2 in HEL cells.*

Supplemental documents (Permission to use the English language)

Sehr geehrter Herr Saalfeld,

am 10. Oktober 2014 erhielt Herr Prof. Dr. Mawrin, Vorsitzender der Promotionskommission, Ihren Antrag auf Erlaubnis zur Einreichung einer englischsprachigen Dissertation zur Verleihung des akademischen Grades „Dr. med." (doctor medicinae).

Im Auftrag von Herrn Prof. Mawrin möchte ich Ihnen mitteilen, dass Sie das Einverständnis haben, Ihre Dissertation in englischer Sprache einzureichen. Wie in der Promotionsordnung der Medizinischen Fakultät der Otto-von-Guericke Universität Magdeburg nach § 6 Abs. 4 gefordert,
soll die Dissertation zusätzlich eine deutschsprachige Zusammenfassung beinhalten. Die Verteidigung sollte in deutscher Sprache erfolgen.

Für weitere Rückfragen stehen wir Ihnen gern zur Verfügung.

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